Original Paper

The mTOR Kinase Inhibitor CZ415 Inhibits Human Papillary Thyroid Carcinoma Cell Growth

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Key Words

Papillary thyroid carcinoma (PTC) • mTOR • CZ415 • Autophagy

Abstract

Background/Aim: Mammalian target of rapamycin (mTOR) plays an important role in papillary thyroid carcinoma (PTC) cell progression. CZ415 is a novel, highly-efficient and specific mTOR kinase inhibitor. The current study tested the potential anti-tumor activity of CZ415 in human PTC cells. **Methods:** The established (TPC-1 cell line) and primary human PTC cells were treated with CZ415. Cell survival and growth were tested by Cell Counting Kit-8 assay and BrdU ELISA assay, respectively. Cell apoptosis was tested by caspase-3/-9 activity assay, Hoechst-33342 staining assay and single-stranded DNA ELISA assay. Cell cycle progression was tested by propidium iodide-FACS assay. The mTOR signaling was tested by Western blotting assay and co-immunoprecipitation assay. The mouse xenograft tumor model was applied to study the effect of CZ415 *in vivo*. **Results:** In cultured human PTC cells, treatment with CZ415 at nM concentrations significantly inhibited cell survival and growth. CZ415 induced apoptosis activation and cell cycle arrest in human PTC cells. CZ415 disrupted assembling of mTORC1 (mTOR-Raptor association) and mTORC2 (mTOR-Rictor-GβL association) in TPC-1 cells, which led to de-phosphorylation of the mTORC1 substrates (S6K1 and 4E-BP1) and the mTORC2 substrate AKT (Ser-473). Further studies show that the autophagy inhibitor 3-methyladenine (3-MA) or Beclin-1 shRNA aggravated CZ415-induced cytotoxicity against PTC cells. *In vivo*, CZ415 oral administration inhibited TPC-1 xenograft tumor growth in mice. **Conclusion:** Our results show that mTOR blockage by CZ415 inhibits PTC cell growth *in vitro* and *in vivo*.

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Introduction

Thyroid cancer is a main health threat in China [1, 2] and around the world [3, 4]. Its incidence has been tripled in the past decades [3-5]. Over three-quarter of thyroid cancers are papillary thyroid carcinomas (PTC) [1, 2]. PTC's pathogenesis is still not fully understood [1, 2]. Existing studies suggest that radiation and genetic susceptibility contribute to the

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initiation and progression of PTC [6-11]. The molecularly-targeted therapy is important for better and efficient treatment of PTC [8-13].

Over-activation of mammalian target of rapamycin (mTOR) is important for a number of pro-cancerous cell behaviors, including cell survival, proliferation and cell cycle progression, as well as apoptosis escape, chemo-resistance and angiogenesis [14]. Dysregulation of mTOR signaling is often detected in PTC and other thyroid cancers [15-18]. Furthermore, mTOR inhibition can inhibit PTC cell progression [15-18]. mTOR has two multi-protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is composed of mTOR, Raptor and PRAS40, which phosphorylates the downstream substrate proteins, p70S6K1 (S6K1) and eIF4E-binding protein 1 (4E-BP1) [14]. The function of mTORC1 can be inhibited by rapamycin and its analogs, RAD001 and CCI-779. mTORC2 is mainly composed of mTOR, Rictor, Sin1 and GβL (also known as mLST8) [14]. mTORC2 can function as the upstream kinase of AKT (at Ser-473) and several other AGC kinases [14]. The activity of mTORC2 can't be directly inhibited by rapamycin, yet prolonged rapamycin treatment could also inhibit mTORC2 [19].

Recent studies have developed CZ415 as a novel mTOR kinase inhibitor [20-22]. It can interfere with the mTOR kinase domain to block mTORC1 and mTORC2 activation simultaneously [20-22]. Our study tested its activity in human PTC cells.

Materials and Methods

Chemicals and reagents

CZ415 was a gift from Dr. Wang [20]. All the antibodies were provided by Cell Signaling Tech (Shanghai, China). The autophagy inhibitor 3-methyladenine (3-MA) was purchased from Sigma-Aldrich (Shanghai, China). The cell culture reagents were provided by Hyclone (Shanghai, China). Puromycin was purchased from Biyuntian (Wuxi, China).

Cell culture

TPC-1 human thyroid cancer cell line was purchased from the Cell Bank of Shanghai Institute of Biological Science (Shanghai, China). Cells were propagated in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS). Tumor tissues from a total of four written-informed primary PTC patients (47/54/52/62 years old, all male) were obtained at the time of thyroidectomy. Tumor tissues and surrounding normal thyroid tissues were separated carefully. The tissues were minced, followed by digestion as described [23]. The resulting primary cells were filtered under a 50-μm nylon cell strainer. Primary cells were maintained in described complete DMEM/F12 medium [23]. The fibroblasts and blood vessels were removed carefully [23]. A total of four lines of primary PTC cells ("Line1/2/3/4") and two lines of thyroid follicular epithelial cells ("Epi-1/2") were established. Thyroid cancer and epithelial cell markers were validated as described [23]. The protocols using human samples/cells were in accordance with the principles expressed in the Declaration of Helsinki, and were approved by the Institutional Review Board (IRB) and Ethics Board of all authors' institutions.

BrdU ELISA assay

Cells were plated onto ninety-six-well tissue culture plates (Corning, Shanghai, China). Cells with the applied CZ415 treatment were incubated with BrdU (10 μM). Afterwards, BrdU incorporation was determined via a commercial available ELISA kit (Cell Signaling Tech). BrdU ELISA optic density (OD) at 405 nm was recorded.

CCK-8 assay

Cell Counting Kit (CCK-8) (Beyotime, Shanghai, China) assay was performed to test cell viability. Briefly, cells were plated onto ninety six-well tissue culture plates at 1000 cells per well. Following the treatment, 10 μL of CCK-8 reagent was added to each well. After one hour, CCK-8's OD at 450 nm was measured.

Colony formation assay

Cells were seeded onto six-well tissue culture plates at 500 cells per well, which were treated with/out CZ415 every two days for a total of ten days. Afterwards, colonies were washed twice using PBS, fixed with methanol/acetic acid, and stained in 1% crystal violet. The number of colonies was counted.

Cell cycle assay

The cell cycle distribution was assessed by the propidium iodide (PI, Invitrogen, Shanghai, China) flow cytometry assay. Cells were plated onto six-well tissue culture plates at 3×10^5 per well. Following the applied treatment, cells were washed, harvested and fixed. Cells were then incubated with DNase-free RNase and stained with PI, and were tested via a FACSCalibur (BD Biosciences, Shanghai, China).

Caspase-3/-9 activity assay

After the following CZ415 treatment, 20 μ g protein lysates per treatment were incubated with the caspase assay buffer (Biyuntian, Wuxi, China) together with the caspase-3 substrate Ac-DEVD-pNA (Biyuntian) or the caspase-9 substrate Ac-LEHD-pNA (Biyuntian) for 45 min. The release of pNA, reflecting the relative caspase activity, was detected at 450 nm.

Apoptosis assay

Following the treatment, cells were stained with nuclei dye Hoechst-33342 (Sigma). Normal nuclei show faint delicate chromatin staining, nuclei showing intensified Hoechst-33342 condensation/brightness (early apoptotic cells) or fragmentation (late apoptotic cells) were labeled as apoptotic cells.

ssDNA ELISA assay

The production of denatured single-stranded DNA (ssDNA) is an established marker of cell apoptosis, which was examined by a nucleosomal monoclonal antibody using the ELISA format as described previously [24]. The ssDNA ELISA OD value at 450 nm was tested.

Western blotting assay

The quantified lysate proteins (40 μ g per treatment of each lane) were separated by 10-12% of SDS-PAGE gels, and were electrophoretically transferred to the PVDF membrane (Millipore, Shanghai, China). The blot was blocked (in 10% milk), followed by probing with applied primary and secondary antibodies. Enhanced chemiluminescence (ECL) reagents were used to analyze targeted protein expression, and β -Tubulin ("Tubulin") was always tested as the protein loading control.

mTOR co-Immunoprecipitation (Co-IP)

The protein lysates (1000 μ g per treatment) were pre-cleared. The pre-cleared lysate samples were incubated with anti-mTOR antibody (Cell Signaling Tech) overnight. Protein G Sepharose (30 μ L per treatment, Sigma) was then added again to the lysates. mTOR complexes were then washed and subjected to Western blotting assay.

shRNA

The lentiviral particles with Beclin-1 shRNA (sc-29797-V), Rictor shRNA (sc-61478-V) as well as the scramble control shRNA were purchased from Santa Cruz Biotech (Shanghai, China). The shRNA-containing lentivirus was added to cultured TPC-1 cells for 12 hours. After infection, puromycin (3.0 μ g/mL) was added to select stable cells if necessary. Knockdown of targeted protein (Beclin-1 or Rictor) was confirmed by the Western blotting assay.

In vivo tumorigenesis assay

The female severe combined immunodeficient (SCID) mice (4-5 weeks of age, 17.0-18.0g of weight) were purchased from the Experimental Animal Center of Suzhou University (Suzhou, China). TPC-1 cells (5×10^6 cells of each mouse) were injected to the dorsal flank of the SCID mice. Within three weeks, the volume of each tumor was around 100 mm³, and the tumor-bearing mice were randomly assigned into two groups (10 mice per group), receiving CZ415 or the vehicle control. Tumor size was measured every 6 days. The tumor volume = $(D \times d^2)/2$ was applied to evaluate tumor volume, where "D" is the longest diameter and "d" is the shortest diameter. All experiments on animal were performed according to the Animal Experimental Ethics Committee of authors' institution.

Statistics

The results were expressed as the mean $\pm$ standard deviation (SD). Statistical significance ($p < 0.05$) was evaluated by one-way ANOVA followed by Bonferroni post hoc test (SPSS 18.0, Chicago, IL).

Results

CZ415 inhibits human papillary thyroid carcinoma cell survival and growth

The structure and molecular weight of CZ415 were presented in Fig. 1A. TPC-1 is an established human PTC cell line [25, 26]. TPC-1 cells were cultured in FBS-containing complete medium, and were treated with different concentrations (10-1000 nM) of CZ415. The cell growth curve results in Fig. 1B show that treatment with CZ415 at 100/1000 nM significantly inhibited TPC-1 cell growth. The anti-growth activity of CZ415 was dose-dependent, and 10 nM of CZ415 was ineffective (Fig. 1B). CZ415 (100/1000 nM) also inhibited BrdU incorporation in TPC-1 cells (Fig. 1C). Cell Counting Kit-8 (CCK-8) assay was applied to test TPC-1 cell viability. The results demonstrate that CZ415 dose-dependently decreased TPC-1 cell CCK-8 viability optical density ("OD", Fig. 1D). The clonogenicity assay results show that the number of viable TPC-1 colonies was significantly decreased following 100/1000 nM of CZ415 treatment (Fig. 1E).

Then we tested the potential effect of the novel mTOR kinase inhibitor [20-22] on the primary human PTC cells. Four lines of primary human PTC cells were established, and named as "Line1/2/3/4". Line1 and Line2 primary human PTC cells were PTEN deficient with high basal AKT activation (Fig. 1F). Line3 and Line4 cells were PTEN positive with weak AKT activation (Fig. 1F). Treatment with CZ415 (1000 nM) inhibited viability (CCK-8 OD) of all four lines of primary human PTC cells (Fig. 1G). PTEN-depleted cells (Line1 and Line2) were more sensitive to CZ415 (Fig. 1G).

In order to test the activity of CZ415 in the non-cancerous cells, two lines of primary human thyroid epithelial cells were cultured, named as "Epi-1" and "Epi-2" (See Methods). Notably, treatment with CZ415 at a high concentration (1000 nM) failed to inhibit the viability of these epithelial cells (Fig. 1H). These results demonstrate a unique response of this mTOR kinase inhibitor against cancerous cells.

CZ415 induces apoptosis activation in human papillary thyroid carcinoma cells

Apoptosis activation can be a main reason of growth inhibition and cytotoxicity in cancer cells [27, 28]. Western blotting assay results in Fig. 2A show that CZ415 (100/1000 nM) treatment induced cleavage of both caspase-3 and caspase-9 in TPC-1 cells. The activity of caspase-3 and caspase-9 were increased significantly in CZ415 (100/1000 nM)-treated cells (Fig. 2B). Cleaved-caspase-3 and cleaved-caspase-9 will travel into cell nuclei, causing DNA cleavage. The results show that the content of single strand DNA ("ssDNA") was increased after CZ415 (100/1000 nM) treatment (Fig. 2C). Hoechst-33342 staining assay was also performed to test cell apoptosis. The non-apoptotic normal nuclei showed faint delicate

Fig. 1. CZ415 inhibits human papillary thyroid carcinoma cell survival and growth. The structure and molecular weight of CZ415 are presented (A). The established TPC-1 cells (B-E), the primary PTC cells ("Line1/2/3/4", F and G) or the primary human thyroid epithelial cells ("Epi-1" and "Epi-2", H) were either left untreated ("C", same for all Figs.) or treated with applied concentration of CZ415 (10/100/1000 nM), cells were further cultured in the conditional medium for indicated time; Cell growth (B and C) and cell survival (D, E, G and H) were tested by the assays mentioned in the text. Expression of listed proteins in the primary PTC cells ("Line1/2/3/4") were shown (F). Data were presented as mean $\pm$ standard deviation (SD, n=5). *p<0.05 vs. "C" group. The experiments were repeated three times, and similar results were obtained.

chromatin staining, the nuclei with increased Hoechst33342 condensation/brightness (early apoptotic cells) or fragmentation (late apoptotic cells) were labeled as apoptotic nuclei. CZ415 (100/1000 nM) significantly increased the percentage of TPC-1 cells with apoptotic nuclei (Fig. 2D). These results suggest that CZ415 induced apoptosis activation in TPC-1 cells.

Hoechst-33342 apoptotic nuclei staining assay results in Fig. 2E show that CZ415 (1000 nM, 48 hours) induced apoptosis activation of all four lines of primary human PTC cells. Its pro-apoptotic activity is more significant in two lines of PTEN-depleted human PTC cells ("Line1/2") (Fig. 2E). Conversely, CZ415 failed to induce significant apoptosis in primary thyroid epithelial cells (Fig. 2F).

CZ415 disrupts PTC cell cycle progression

We also analyzed cell cycle distribution in CZ415-treated PTC cells. Quantified results in Fig. 3A confirmed that treatment with CZ415 (1000 nM, 24 hours) disrupted TPC-1 cell cycle progression. Following the CZ415 treatment, G0-1 phase TPC-1 cells were increased, and the G2-M phase cells were decreased (Fig. 3A). These results suggest that CZ415 induced G1-S arrest in TPC-1 cells. It has been previously shown that mTOR is required for the expression of several cell cycle-related proteins, including Cyclin D1 [29] and Cyclin E1 [30]. Here, our results show that Cyclin D1 and Cyclin E1 were downregulated by CZ415 (1000 nM, 12 hours) in TPC-1 cells (Fig. 3B-C).

CZ415 blocks mTORC1 and mTORC2 activation in PTC cells

mTOR is in two multiple protein complexes (mTORC1/2). The mTOR-Raptor complex ("mTORC1") can phosphorylate S6K1 and 4E-BP1. mTOR-Rictor-GβL complex ("mTORC2") phosphorylates AKT at Ser-473 [14]. The co-immunoprecipitation ("Co-IP") assay was performed to test the assembly of these two mTOR complexes. Results in Fig. 4A show that the mTOR-Raptor complex and mTOR-Rictor-GβL complex were disrupted by CZ415 (1000 nM, 2 hours) in TPC-1 cells. Expression of mTOR complex proteins, including mTOR, Raptor, Rictor and GβL, were unchanged after CZ415 treatment (Fig. 4A, "INPUT"). Treatment with

Fig. 2. CZ415 induces apoptosis activation in human papillary thyroid carcinoma cells. TPC-1 cells (A-D), the primary human PTC cells ("Line1/2/3/4", E) or the primary human thyroid epithelial cells ("Epi-1" and "Epi-2", F) were treated with applied concentration of CZ415 (10/100/1000 nM), cells were cultured in the conditional medium for indicated time; Cell apoptosis was tested by assays mentioned in the text. Data were presented as mean $\pm$ standard deviation (SD, n=5). *p<0.05 vs. "C" group. The experiments were repeated three times, and similar results were obtained.

Fig. 3. CZ415 disrupts PTC cell cycle progression. TPC-1 cells were treated with CZ415 (1000 nM), cells were cultured in the conditional medium for applied time, cell cycle distribution was analyzed by PI FACS assay (A); Expression of listed proteins were shown (B), and results were quantified (C). Data were presented as mean $\pm$ standard deviation (SD, n=5). *p<0.05 vs. "C" group.

Fig. 4. CZ415 blocks mTORC1 and mTORC2 activation in PTC cells. TPC-1 cells (A, B, G and H), the primary PTC cells ("Line1", C) or the primary human thyroid epithelial cells ("Epi-1", D) were treated with CZ415 (1000 nM) or PP242 (1000 nM), cells were cultured for 2 hours; the mTOR-Raptor association and the mTOR-Rictor-GβL association were tested by co-immunoprecipitation ("Co-IP") assay (A and G, left panel); Expression of the listed proteins were shown [A and G, right panel ("INPUT"), B-D and H]; TPC-1 cells (E and I) or the primary PTC cells ("Line1", F and J) were treated with CZ415 (1000 nM), P242 (1000 nM), MK-2206 (1000 nM), RAD001 (1000 nM), or infected with Rictor shRNA ("shRictor") lentivirus; Cells were further cultured for 72 hours, and cell viability was tested by CCK-8 assay (E, F, I and J). Rictor expression was also shown (E and F, the upper panel). Data were presented as mean $\pm$ standard deviation (SD, n=5). *p<0.05 vs. "C" group. # p<0.05 vs. "CZ415" only group. The experiments were repeated three times, and similar results were obtained.

CZ415 almost completely blocked phosphorylation of mTORC1 substrates S6K1 (Thr-389) and 4E-BP1 (Ser-65), as well as the mTORC2 substrate AKT (Ser-473) (Fig. 4B). Total S6K1, 4E-BP1 and AKT1 were unchanged (Fig. 4B). Furthermore, the activation or phosphorylation of ERK1/2 was also not affected by CZ415 (Fig. 4B).

Western blotting assay results in Fig. 4C show that CZ415 treatment blocked phosphorylation of S6K1 (Thr-389) and AKT (Ser-473) in the primary human PTC cells ("Line-1"). Ad-

ditionally, the basal phosphorylation of S6K1 (Thr-389) and AKT (Ser-473) were extremely low in the primary thyroid epithelial cells ("Epi-1") (Fig. 4D). This might explain why the epithelial cells were not killed by the mTOR kinase inhibitor (Fig. 1 and 2). ERK1/2 phosphorylation and expression were unchanged after CZ415 treatment (Fig. 4C and D). These results demonstrate that CZ415 blocked mTORC1 and mTORC2 activation in human PTC cells.

We compared CZ415 activity with other known AKT-mTOR inhibitors, including the AKT specific inhibitor MK-2206 [31-33] and the mTORC1 inhibitor RAD001 [34, 35]. Results in Fig. 4E show that CZ415 was more potent than MK-2206 and RAD001 in inhibiting TPC-1 cell survival. Meanwhile, CZ415-induced cytotoxicity was more significant than mTORC2 inhibition by Rictor shRNA (Fig. 4E). The similar results were also observed in the primary human PTC cells ("Line-1"), and CZ415's cytotoxicity was more robust than MK-2206, RAD001 and Rictor shRNA (Fig. 4F). Thus, mTORC1/2 dual inhibition by CZ415 is more efficient in killing PTC cells than blockage of either mTORC1 or mTORC2.

Importantly, treatment with PP242, another known mTOR kinase inhibitor [36], had no significant effect on the assembling of mTORC1 and mTORC2 in TPC-1 cells (Fig. 4G). The expression of mTOR complex proteins, mTOR, Raptor, Rictor and GβL, were not affected by PP242 (Fig. 4G, "Input"). PP242 treatment only resulted in partial

Fig. 5. Autophagy inhibition sensitizes CZ415-induced cytotoxicity in PTC cells. TPC-1 cells with Beclin shRNA ("shBeclin-1") or scramble control shRNA ("shC") were treated with/without CZ415 (100 and 1000 nM), cells were cultured in the conditional medium for applied time; Beclin-1 expression was tested by Western blotting assay (A, the upper panel); Cell survival and apoptosis were tested by CCK-8 assay (A, the lower panel) and Hoechst-33342 staining assay (B), respectively. Data were presented as mean $\pm$ standard deviation (SD, n=5). *p<0.05 vs. "C" group. # p<0.05 vs. "shC" group. The experiments were repeated three times, and similar results were obtained.

Fig. 6. CZ415 inhibits TPC-1 tumor growth in mice. TPC-1 tumor-bearing SCID mice were administrated with CZ415 (20 mg/kg body weight, gavage, daily for 24 days) or vehicle control (Saline), tumor volumes (A, in mm³) and mice body weights (D, in gram) were recorded every 6 days for a total of 42 days; Estimated daily tumor growth (in mm³ per day) was calculated as described (B); At day-42, each single tumor of the two groups was isolated and weighted (C). Data were presented as mean $\pm$ standard deviation (SD, n=10). At Day-3 (E) and Day-6 (F), 2 hours after initial CZ415/Vehicle treatment, one tumor per group was isolated, the tumor tissues were lysed, and expression of listed proteins in tumor lysates were tested by the Western blotting assay.*p<0.05 vs. "Vehicle" group.

inhibition of phosphorylation of AKT, S6K1 and 4E-BP1 in TPC-1 cells (Fig. 4H). ERK1/2 activation, as expected, was not affected by PP242 (Fig. 4H). Consequently, PP242 was less effective than CZ415 in inhibiting the viability of PTC-1 cells (Fig. 4I) and the primary human PTC cells ("Line-1") (Fig. 4J).

Autophagy inhibition sensitizes CZ415-induced cytotoxicity in PTC cells

It has been shown that mTOR blockage can induce feedback autophagy activation, which might serve as a pro-survival factor to inhibit cancer cell death [37-39]. To block autophagy, pharmacological and genetic strategies were utilized. Three-methyladenine (3-MA) is a well-known autophagy inhibitor [40]. Beclin-1 is a key protein of autophagy initiation and progression [41, 42]. Beclin-1 knockdown has been utilized to block autophagy in cancer cells [43]. The applied lentiviral Beclin-1 shRNA induced efficient knockdown of Beclin-1 in TPC-1 cells (Fig. 5A, the upper panel). Significantly, CZ415 (100/1000 nM)-induced TPC-1 cell viability reduction (Fig. 5A, the lower panel) and apoptosis activation (Fig. 5B) were potentiated by 3-MA and Beclin-1 shRNA. These results imply that autophagy inhibition could increase CZ415's sensitivity, and autophagy could be a key resistance factor for CZ415 treatment in PTC cells.

CZ415 inhibits TPC-1 tumor growth in mice

At last, the potential anti-tumor activity of CZ415 *in vivo* was tested. TPC-1 cells were inoculated via s.c. injection to the severe combined immunodeficient (SCID) mice. When the volume of each tumor was around 100 mm³, tumor-bearing mice were randomly separated into two groups, which were treated with CZ415 or the vehicle control (n=10 for each group). Tumor growth curve results in Fig. 6A demonstrated that oral administration of CZ415 (20 mg/kg body weight [22], daily for 24 days) significantly inhibited TPC-1 xenograft tumor growth in mice. The daily tumor growth was calculated using the formula: [Tumor volume at day-42 (mm³) - Tumor volume at day-0 (mm³)]/42. Results in Fig. 6B show that estimated daily tumor growth was 24.70 ± 3.05 mm³ per day in the vehicle mice, and it reduced to 8.09 ± 2.01 mm³ per day after CZ415 treatment (Fig. 6B). At day-42, tumors of each group were isolated and weighted, and CZ415-treated tumors weighted dramatically lower than the vehicle control tumors (Fig. 6C). The mice body weight was not significantly different between the two groups (Fig. 6D).

At Day-3 and Day-6, two hours after CZ415/Vehicle treatment, one tumor per group was isolated, the tumor tissues were lysed for mTOR signaling analysis. When compared to the vehicle control tumors, results show that phosphorylation of AKT, S6K1 and 4E-BP1 were largely inhibited in the tumor tissues after CZ415 treatment (Fig. 6E and F). These results suggest that CZ415 possibly also blocks mTORC1 and mTORC2 activation *in vivo*. Collectively, our results show that CZ415 could inhibit TPC-1 xenograft tumor growth *in vivo*.

Discussion

The traditional mTORC1 inhibitors, *i.e.* rapamycin and its analogs ("rapalogs"), have displayed promising anti-tumor activity [44, 45]. Whereas, the application of these inhibitors had several drawbacks [44, 45]. First, rapalogs bind to FKBP12, leading to only partial inhibition of mTORC1 [44, 45]. Second, these mTORC1 inhibitor can provoke feedback activation of multiple key oncogenic pathways, including ERK and AKT cascades [46]. Third, rapalogs have direct inhibition on mTORC2. In fact, rapamycin treatment could provoke AKT activation via the IRS-1-mediated feedback pathway [47, 48]. Fourth, the clinical administration of these mTORC1 inhibitor are usually limited due to poor solubility [44, 45]. Due to these limitations, mTOR kinase inhibitors, also known as the "second generation of mTOR inhibitors", were recently developed [44, 45].

CZ415 is a novel, highly-efficient and specific mTOR kinase inhibitor [20-22]. It displayed an extremely superior K_d (nM ranges) and applicable pharmacokinetic/pharmacodynamic

properties [22, 49]. In the current study, our results show that CZ415 disrupted the assembling of both mTORC1 (mTOR-Raptor association) and mTORC2 (mTOR-Rictor-GβL), causing de-phosphorylation of the mTORC1 substrate S6K1 and 4E-BP1 as well as the mTORC2 substrate AKT (Ser-473). CZ415 had no significant effect on ERK activation in human PTC cells. Treatment with CZ415 at nM concentrations significantly inhibited human PTC cell survival and growth, and also induced apoptosis activation and disrupted cell cycle progression. Notably, CZ415's cytotoxicity was more potent than MK-2206, RAD001 and Rictor shRNA.

One interesting finding of our research was that CZ415 disrupted the assembling of both mTORC1 and mTORC2, which resulted in almost complete inhibition of mTORC1 and mTORC2 in human PTC cells. Whereas, the other known mTOR kinase inhibitor PP242 had no direct effect on mTORC1/2 assembling, and it only caused partial inhibition of mTORC1/2 in human PTC cells. Therefore, it is possible that CZ415 binding to mTOR may induce mTOR conformational change, which leads to the disassembling of both mTORC1 and mTORC2. These results can also explain the superior activity of CZ415 against human PTC cells.

In vivo, oral administration of CZ415 at a well-tolerated dose (20 mg/kg body weight) significantly suppressed TPC-1 tumor growth in mice. Importantly, CZ415 was non-cytotoxic to the non-cancerous thyroid epithelial cells, and the results of *in vivo* administration show that there were no apparent toxicities to the experimental mice. Therefore, the current pre-clinical study displayed a superior anti-tumor activity of CZ415 against human PTC cells.

Although sustained and profound autophagy could promote cell death ("autophagic cell death"), drug-induced feedback autophagy activation is mostly gentle and has pro-survival ability especially in cancer cells [28, 50, 51]. In the process of autophagy, cell degrades its own components via lysosomal machineries, which provides energy and nutrients for cell to survive [52]. mTOR is a key kinase regulating cell autophagy [53, 54]. Activation of mTOR is shown to suppress cell autophagy through directly inhibiting Ulk1, which is the upstream kinase initiating cell autophagy [55]. mTOR blockage, on the other hand, could lead to feedback autophagy activation [53, 54]. Indeed, a number of mTOR inhibitors were shown to active autophagy in cancer cells, which counteracted cell death [37-39, 56]. Conversely, autophagy inhibition can increase the sensitivity and the anti-cancer efficiency of mTOR inhibitors [37-39]. In the current study, our results show that the autophagy inhibitor 3-MA or Beclin-1 shRNA potentiated CZ415-induced cytotoxicity in PTC cells. Therefore, cell autophagy could also be a key resistance factor of CZ415 in PTC cells.

Conclusion

Together, our results demonstrate that mTOR blockage by CZ415 inhibits PTC cell growth *in vitro* and *in vivo*. CZ415 could be further studied as a promising anti-PTC agent.

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All the listed authors in the study carried out the experiments, participated in the design of the study and performed the statistical analysis, conceived of the study, and helped to draft the manuscript.

Disclosure Statement

The authors declare no Disclosure Statements.

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