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CC-223 inhibits human head and neck squamous cell carcinoma cell growth

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ABSTRACT

mTOR over-activation is associated with the progression of head and neck squamous cell carcinoma (HNSCC). CC-223 is a novel and potent mTOR kinase inhibitor. Its activity against human HNSCC cells is studied here. In established SCC-9 cells and primary human oral cavity carcinoma (OCC) cells, CC-223 treatment at only nM concentrations significantly inhibited survival, proliferation and cell cycle progression. Furthermore, CC-223 provoked apoptosis activation in human HNSCC cells. CC-223 is more efficient in killing HNSCC cells than other known Akt-mTOR inhibitors: RAD001, MK-2206 and AZD-2014. CC-223 was however non-cytotoxic to the primary human oral epithelial cells. Further studies demonstrate that CC-223 almost completely blocked mTOR complex 1 (mTORC1) and mTORC2 activation in SCC-9 cells and primary OCC cells. *In vivo*, oral administration of CC-223 at well-tolerated doses potently inhibited SCC-9 xenograft tumor growth in severe combined immunodeficient mice. mTORC1 and mTORC2 activation was largely inhibited in CC-223-treated tumor tissues. Overall, targeting the mTOR kinase by CC-223 inhibits human HNSCC cell growth *in vitro* and *in vivo*. CC-223 might have a translational value for the treatment of HNSCC.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is a heterogeneous family of carcinomas of face, nasopharynx, oral cavity, and larynx [1–3]. HNSCC is commonly diagnosed at late/advanced stages, possibly due to the absence of specific symptoms [1–4]. The lack of effective treatment options will further cause HNSCC progression [1–4]. The patients' prognosis and five-year overall survival are far from satisfactory [1–4], and the novel treatments against this disease are urgently needed [1–4].

Molecule-targeted therapy is extremely important for HNSCC [1–4]. Multiple lines of evidence have confirmed that dysregulation and sustained-activation of mammalian target of rapamycin (mTOR) is pivotal for HNSCC progression [5–7]. mTOR activation is critical for multiple cancerous behaviors, including cell survival, proliferation, and metabolism, as well as metastases and cancer-associated angiogenesis [8]. Therefore, mTOR has become a key therapeutic target of HNSCC [5–7]. mTOR inhibitors have displayed promising anti-cancer efficiency in preclinical HNSCC studies [9–11].

mTOR is in at least two multiple-protein complex, including the mTOR complex 1 (mTORC1) and the mTOR complex 2 (mTORC2) [8]. mTORC1 is formed by mTOR, PRAS40, Raptor, and mLST8 [12,13], whose activation will phosphorylate of two main substrates: p70S6K1 and eIF4E-binding protein 1 (4E-BP1) [12,13]. Rapamycin and its analogs (*i.e.* RAD001) can only partly inhibit mTORC1 [8]. mTORC2 is composed of mTOR, Rictor and mSin1, and possible others [8,12,13], and it phosphorylates Akt (at Ser-473) and other AGC kinases [8,12,13]. The recent study by Mortensen et al., has developed CC-223 as a novel and extremely efficient small-molecule mTOR kinase inhibitor [14]. The activity of CC-223 against human HNSCC cells is tested in this study.

2. Methods

2.1. Chemicals, reagents and antibodies

CC-223 was obtained from Selleck (Shanghai, China). The

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antibodies utilized in this study were described previously [15], and were purchased from Cell Signaling Tech (Shanghai, China). The cell culture reagents were provided by Gibco (Suzhou, China). The caspase-3 specific inhibitor (z-DEVD-fmk) and the pan caspase inhibitor (z-VAD-fmk) were purchased from Biyuntian (Wuxi, China).

2.2. Cell culture

SCC-9 HNSCC cells (an established human cell line) were provided by the Cell Bank of Shanghai Institute of Biological Science (Shanghai, China). SCC-9 cells were maintained in FBS-containing DMEM medium [10]. Two lines of primary human oral cavity carcinoma (OCC) cells and one line of the oral (cavity) epithelial cells were provided by Dr. Xie [16], and cells were cultured as described [16]. The study of the primary human cells was approved by the Ethics Committee of all authors' institutions. Experiments were conducted according to Declaration of Helsinki. Written-informed consent was obtained from each participant.

2.3. Cell viability assay

Cell counting kit-8 (CCK-8, Sigma, Shanghai, China) assay was performed to test the viability of HNSCC cells/epithelial cells after the applied CC-223 treatment. The CCK-8 absorbance optical density (OD) at 450 nm was recorded.

2.4. BrdU ELISA assay of cell proliferation

Cells with the applied CC-223 treatment were simultaneously incubated with BrdU (10 μM, Cellular Signaling). BrdU incorporation was tested via an enzyme-linked immunosorbent assay (ELISA) kit. BrdU ELISA OD at 450 nm was recorded.

2.5. Colony formation assay

Following the applied CC-223 treatment, SSC-9 cells (10^4 cells per dish) were re-suspended in 0.5% agar-containing DMEM, which were plated onto a pre-solidified10-cm diameter dish. Cells were further cultured in CC-223-containing medium (renewed every two days) for a total of 10 days. Afterwards, the colonies were counted manually [17].

2.6. Assay of caspase activity

After the indicated CC-223 treatment, 10 μg of cytosolic extracts per treatment were mixed with the described caspase assay buffer [18] and the specific 7-amido-4-(trifluoromethyl)-coumarin (AFC)-conjugated caspase-3/-9 substrate [18]. After 30 min incubation at the room temperate, the release of AFC was examined by the Fluoroskan fluorescence machine [18]. The caspase-3/-9 activity intensity of treatment group was always normalized to that of the control group.

2.7. Histone DNA ELISA assay of cell apoptosis

In the apoptotic cells, the amount of Histone-bound broken DNA will be increased due to caspase-dependent cleavage. The histone DNA ELISA plus kit (Roche, Shanghai, China) was utilized to quantify the broken DNA. The ELISA OD at 450 nm was recorded to reflect cell apoptosis intensity.

2.8. TUNEL nuclei staining of cell apoptosis

After the indicated CC-223 treatment, cells were further stained with TUNEL fluorescein dye (10 μM, Sigma) for 10 min at the room temperature under the dark. Cells with intensified or fragmented TUNEL staining in the nuclei was labeled as the apoptotic cells. At least 200 cells of five random views were included to calculate the TUNEL percentage (vs. total cells).

2.9. FACS assay

After the applied CC-223 treatment, SSC-9 cells were stained with Annexin V and/or propidium iodide (PI) (10 μg/mL each, Biyuntian, Wuxi, China). We utilized the Beckman Coulter fluorescence-activated cell sorting (FACS) to sort both early apoptotic cells (Annexin V^+/PI^-) and late apoptotic cells (Annexin V/PI^+) cells. Annexin V percentage was recorded. The PI distribution was also tested for measuring cell cycle distribution.

2.10. Western blotting assay

The cell lysis buffer was purchased from Biyuntian (Wuxi, China), which was added to cultured cells and fresh SCC-9 tumor tissues to achieve total lysates. Thirty μg protein lysates per treatment were separated by 10–12% of SDS-PAGE gels, and proteins were transferred to the polyvinylidene difluoride (PVDF) blot (Sigma, Nanjing, China). The blot was blocked, and was incubated with applied primary and corresponding secondary antibodies. The enhanced chemiluminescence (ECL) detection kit was utilized to visualize the targeted protein band based on the molecular weight. Quantification of each band was performed via the ImageJ software (NIH).

2.11. Tumor xenograft assay

SCC-9 cells were inoculated *s.c.* to the left flanks of the female severe combined immunodeficient (SCID) mice (4–6 week old). After three weeks, the xenograft SCC-9 tumors were established, with the volume of each tumor close to 100 mm^3. The SCID mice were then randomly assigned into three groups (10 mice per group) with the indicated treatment. The tumor volume was measured once every 5 days using the described method [16]. The animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Medical University and comply with the National Institutes of Health guide for the care and use of laboratory animals.

2.12. Statistical analysis

All values were expressed as the mean ± standard deviation (SD). A *p*-value, calculated by ANOVA, of less than 0.05 was considered statistically significant. Data of *in vitro* experiments were summarizing one set of experiment. The whole set of experiments were always repeated 3–5 times, and similar results were obtained.

3. Results

3.1. CC-223 inhibits survival, proliferation and cell cycle progression in human HNSCC cells

SCC-9 is an established human HNSCC cell line [9,11]. SCC-9 cells, cultured in FBS-containing complete medium, were treated with CC-223 (from 1 to 1000 nM). CCK-8 assay was performed to test cell viability after 72 h. Results show that CC-223 dose-dependently inhibited SCC-9 cell survival (reflected by CCK-8 OD reduction, Fig. 1A). The IC-50 of CC-223 was close to 10–100 nM (Fig. 1A). It was yet ineffective at the lowest concentration (1 nM)

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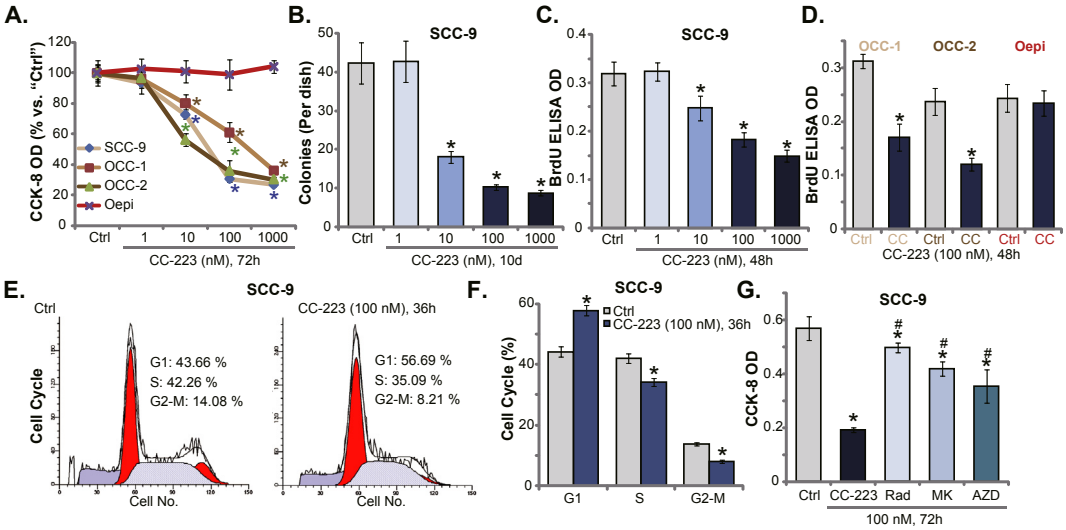

Fig. 1. CC-223 inhibits survival, proliferation and cell cycle progression in human HNSCC cells. SCC-9 cells (**A-C**, **E-G**), the primary human oral cavity carcinoma (OCC) cells ("OCC1/2 lines") (**A** and **D**) or the primary human oral epithelial cells ("Oepi") (**A** and **D**) were treated with designated concentration of CC-223, RAD001 ("Rad"), MK-2206 ("MK") or AZD-2014 ("AZD"), cells were further cultured for indicated time, cell survival, proliferation and cell cycle progression were tested by assays mentioned in the text. "Ctrl" stands for untreated control group (Same for all Figures). For each assay, n = 5. Bars stand for mean ± standard deviation (S.D., Same for all Figures). $^{*}p < 0.05$ vs. "Ctrl" cells. $^{\#}p < 0.05$ vs. CC-223 treatment (**G**). Experiments in this figure were repeated four times, and similar results were always obtained.

(Fig. 1A). To test the effect of CC-223 in the primary human cells, two lines of the primary human oral cavity carcinoma (OCC) cells ("OCC1/2", from Dr. Xie [16]) were treated with CC-223. CCK-8 assay results demonstrate that CC-223, at 10–1000 nM, similarly inhibited the viability of the primary human cancer cells (Fig. 1A). Conversely, the very same CC-223 treatment was yet non-cytotoxic to the primary human oral epithelial cells ("Oepi", also from Dr. Xie [16]) (Fig. 1A), indicating a unique activity of the mTOR kinase inhibitor [14,15,19,20] against the cancerous cells.

To test cell proliferation, the colony formation assay and the BrdU ELISA assay were performed. Results show that CC-223 decreased the number of colonies (Fig. 1B) and BrdU incorporation (Fig. 1C) in SCC-9 cells, indicating proliferation inhibition. The anti-proliferative activity of CC-223 was again dose-dependent (Fig. 1B and C). By performing the same BrdU ELISA assay, we show that CC-223 (100 nM) suppressed proliferation of the primary human OCC cells ("OCC1/2") (Fig. 1D). It was again in-effective to the proliferation of the primary epithelial cells (Fig. 1D). Cell cycle arrest could be an important reason of proliferation inhibition in human cancer cells. Activation of mTOR is vital for cell cycle progression [12,21]. Thus, cell cycle distribution in CC-223-treated cells was analyzed next. The PI FACS assay results demonstrate that treatment with CC-223 (100 nM, 36 h) led to increase of G1-phase SCC-9 cells, but a significant decrease in S- and G2-*M*-phase cells (Fig. 1E and F). These results indicate that CC-223 induced G1-S arrest in SCC-9 cells.

We also compared the activity of CC-223 with other known Akt-mTOR inhibitors, including the mTORC1 inhibitor RAD001 [22], the Akt specific inhibitor MK-2206 [23] and the known mTOR kinase inhibitor AZD-2014 [18]. CCK-8 assay results show that CC-223 was significantly more potent in killing SCC-9 cells than the same concentration (100 nM) of RAD001, MK-2206 and AZD-2014 (Fig. 1G). Collectively, these results show that CC-223 efficiently and specifically inhibits survival, proliferation and cell cycle progression of human HNSCC cells.

3.2. CC-223 induces apoptosis activation in human HNSCC cells

The effect of CC-223 on cell apoptosis was tested next. Results in Fig. 2A show that CC-223 dose-dependently increased the activity of caspase-3 and caspase-9 in SCC-9 cells. Further, the amount of Histone-bound DNA was also increased in CC-223 (10–1000 nM)-treated SCC-9 cells. Additionally, the percentage of SCC-9 cells with positive TUNEL nuclei staining was increased after CC-223 treatment (Fig. 2C). Annexin V FACS assay was also performed to further confirm cell apoptosis. Representative images in Fig. 2D show that CC-223 (10–1000 nM) treatment significantly increased the percentage of SCC-9 cells with the positive Annexin V staining. Quantitative results integrating five sets of repeat Annexin V FACS results confirm that CC-223 (10–1000 nM)-induced effect was significant (Fig. 2E).

To study the role of apoptosis in CC-223-induced cytotoxicity against SCC-9 cells, the caspase inhibitors were utilized. As shown in Fig. 2F, pre-treatment with the caspase-3 specific inhibitor (z-DEVD-fmk) or the pan caspase inhibitor (z-VAD-fmk) largely attenuated CC-223 (100 nM)-induced CCK-8 viability reduction in SCC-9 cells. The results indicate that apoptosis activation mediates CC-223-induced SCC-9 cell death. The TUNEL staining assay results in Fig. 2G show that CC-223 (100 nM) also provoked apoptosis in the two lines of the primary human OCC cells ("OCC1/2"), but not in the primary human oral epithelial cells ("Oepi"). Importantly, CC-223-induced apoptosis in SCC-9 cells was significantly more potent than the same concentration of RAD001, MK-2206 and AZD-2014 (Fig. 2H). Collectively, these results show that CC-223 efficiently induces apoptosis in human HNSCC cells.

3.3. CC-223 blocks mTORC1 and mTORC2 activation in human HNSCC cells

The potential effect of CC-223 on mTOR activation was evaluated. As demonstrated in Fig. 3A, treatment with CC-223 (100 nM, for 4 h) significantly inhibited phosphorylation of p70S6K1 (Ser-389) and Akt (Ser-473) in SCC-9 cells, indicating mTORC1 and mTORC2 inhibition [8,13,24]. On the other hand, *p*-Erk1/2 was unaffected by CC-223 in SCC-9 cells (Fig. 3A). Total p70S6K1, Akt1/2 and Erk1/2 were unchanged after CC-223 treatment (Fig. 3A). The similar results were also obtained in the two lines of primary human OCC cells ("OCC1/2"), where CC-223 almost blocked p70S6K1

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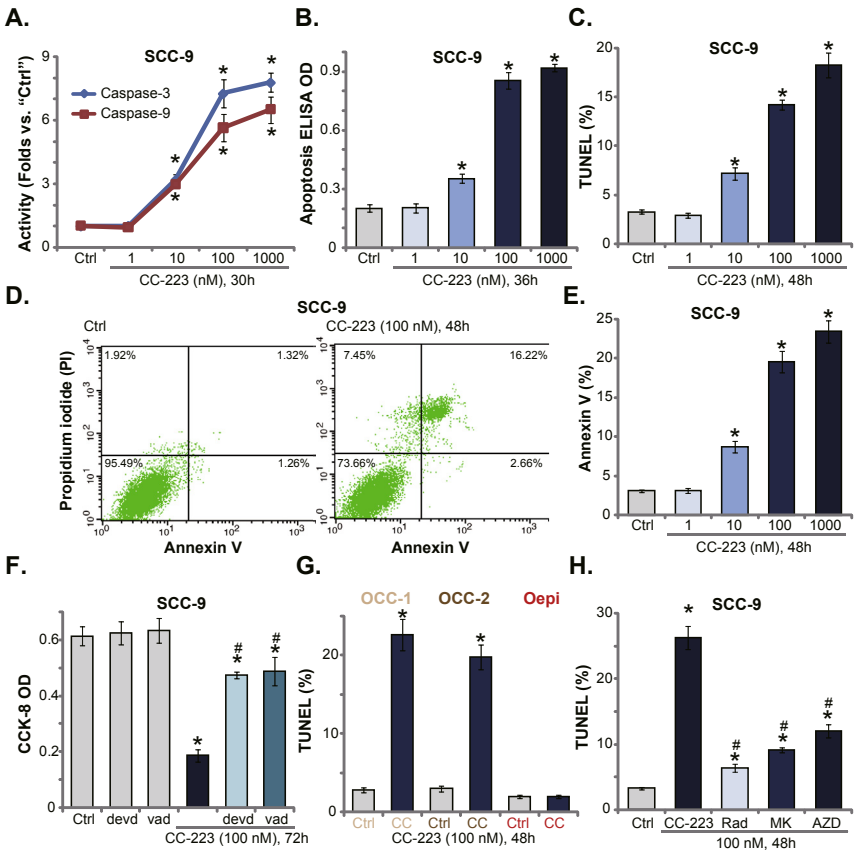

Fig. 2. CC-223 induces apoptosis activation in human HNSCC cells. SCC-9 cells (**A-F**, and **H**), the primary human oral cavity carcinoma (OCC) cells ("OCC1/2 lines") (**G**) or the oral epithelial cells ("Oepi") (**G**) were treated with designated concentration of CC-223, RAD001 ("Rad"), MK-2206 ("MK") or AZD-2014 ("AZD"), cells were further cultured for indicated time, cell apoptosis was tested by assays mentioned in the text (**A-E**, **G** and **H**). To test viability by the CCK-8 assay, SCC-9 cells were also pretreated for 30 min with 50 μM the caspase-3 specific inhibitor z-DEVD-fmk ("devd") or the pan caspase inhibitor z-VAD-fmk ("devd") (**F**). For each assay, n = 5. $^{*}p < 0.05$ vs. "Ctrl" cells. $^{\#}p < 0.05$ vs. CC-223 treatment (**F** and **H**). Experiments in this figure were repeated five times, and similar results were always obtained.

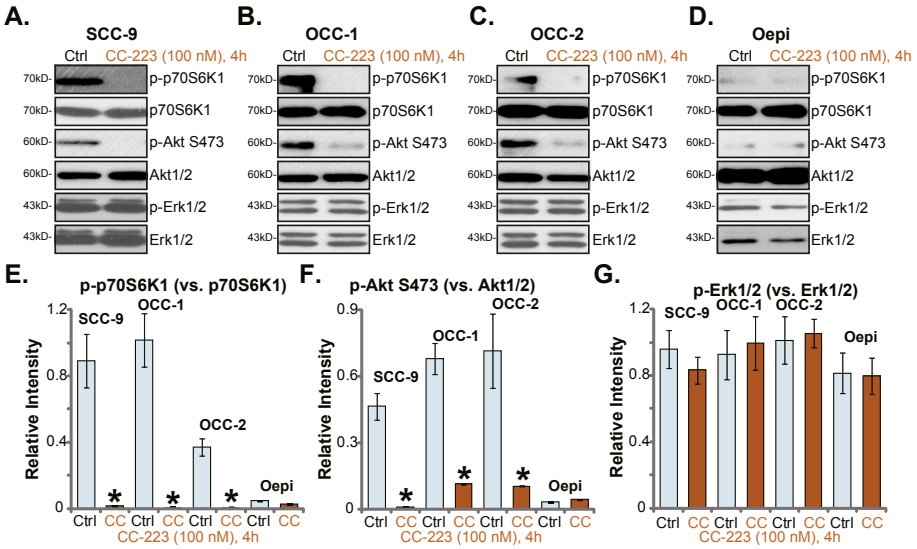

Fig. 3. CC-223 blocks mTORC1 and mTORC2 activation in human HNSCC cells. SCC-9 cells (**A**), the primary human oral cavity carcinoma (OCC) cells ("OCC1/2 lines") (**B** and **C**) or the oral epithelial cells ("Oepi") (**D**) were treated with 100 nM of CC-223 for 4 h, total cell lysates were obtained, and expression of listed proteins were shown. Five sets of repeated blot data were quantified, relative intensity (vs. total kinase) of phosphorylated-p70S6K1 (**E**), phosphorylated-Akt (**F**) and phosphorylated-Erk1/2 (**G**) were shown. $^{*}p < 0.05$ vs. "Ctrl" cells. Experiments in this figure were repeated five times, and similar results were always obtained.

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and Akt phosphorylation, without affecting Erk1/2 phosphorylation (Fig. 3B and C). Notably, the basal level of phosphorylated-p70S6K1 and phosphorylated-Akt was extremely low in the human oral epithelial cells (Fig. 3D), which could be the primary reason of ineffectiveness of the mTOR kinase inhibition in the epithelial cells (Figs. 1 and 2). Five sets of repeated Western blotting data were quantified, and results confirm that CC-223-mediated inhibition of phosphorylated-p70S6K1 (Fig. 3E) and phosphorylated-Akt (Fig. 3F) was significant in HNSCC cells. Erk1/2 phosphorylation was not significantly affected by CC-223 treatment (Fig. 3G).

3.4. CC-223 oral administration inhibits SCC-9 xenograft tumor growth in mice

The potential activity of CC-223 *in vivo* was examined. We utilized a SCID mice xenograft tumor model. As described, SCC-9 cells (8×10^6 per mouse) were injected *s.c.* to the left flanks of the SCID mice. When the tumors were about 100 mm^3 in volume (about three weeks), CC-223 administration was started. As compared to the vehicle control (aqueous 0.5% carboxymethyl cellulose plus 0.25% Tween-80) administration, CC-223 administration (10/25 mg/kg body weight, gavage, daily for 21 consecutive days) [14,15] largely inhibited SCC-9 xenograft tumor growth in the SCID mice (Fig. 4A). The volume of tumors with CC-223 treatment was significantly lower than the vehicle control tumors (Fig. 4A). When analyzing daily tumor growth, which was calculated by: (tumor volume at Day-30 — tumor volume at Day-0)/30, we show that SCC-9 tumor growth was significantly inhibited by CC-223 administration (Fig. 4B). The weight of SCC-9 tumors (at Day-30) was also significantly lower after CC-223 administration (Fig. 4C). In line with the previous findings [15], we didn't observe a significant difference in animal body weight between the three groups (Fig. 4D), neither did we observe any apparent toxicities. Therefore, the tested animals were well-tolerated to the CC-223 administration regimens. At treatment Day-3, four hours after initial CC-223 administration, one tumor of each group was isolated, and tumor tissue lysates were subjected to the Western blotting assay. Results in Fig. 4E show that phosphorylated-p70S6K1 and phosphorylated-Akt were largely inhibited in CC-223-treated tumors (as compared to the vehicle control tumor). For each tumor, five random tissue parts were tested for signaling. Quantified results in Fig. 4F show that CC-223-mediated p70S6K1/Akt inhibition was significant. These results demonstrate that CC-223 inhibits mTORC1/2 activation *in vivo*.

4. Discussion

mTOR over-activation is an important driving factor of HNSCC progression [5–7,9–11]. Sustained mTOR activation in human cancer cells is linked to cancer progression and poor prognosis [25]. Thus, mTOR is a key molecule target protein for HNSCC [5–7,9–11]. The anti-cancer activity of the traditional mTORC1 inhibitors, including rapamycin and its analogs (*i.e.* RAD001, CCI-779, AP23573) [26], is generally weak. This is possibly due to the fact that rapalogs only partially inhibit 4E-BP1 phosphorylation and mTORC1 [27,28]. Furthermore, mTORC1 inhibitors are unable to direct inhibit mTORC2, the latter is also vital for HNSCC progression [8,24]. Inhibition of mTORC1 is also shown to activate key oncogenic signaling (*i.e.* Akt and Erk-MAPK) as negative feedback regulation [29–31]. Recent cancer studies show that the mTOR kinase inhibitors, also known as "the second-generation of mTOR inhibitors", simantanuously block both mTORC1 and mTORC2, which might be more efficient in suppressing mTOR-driven cancers [27,28].

In the current study, we show that CC-223, the novel small-molecule mTOR kinase inhibitor [14,15,19], is extremely efficient in killing established (SCC-9) and primary human HNSCC cells. At only nM concentrations, the mTOR kinase inhibitor induced significant cytotoxicity, proliferation inhibition and apoptosis in human HNSCC cells. Importantly, CC-223 simantanuously blocked both mTORC1 and mTORC2, but didn't induce feedback activation of pro-cancerous cascades (Akt and Erk) as seen by mTORC1 inhibitors [29–31]. Significantly, CC-223 was significant more potent in killing HNSCC cells than the known mTORC1 inhibitor RAD001 and the Akt specific inhibitor MK-2206.

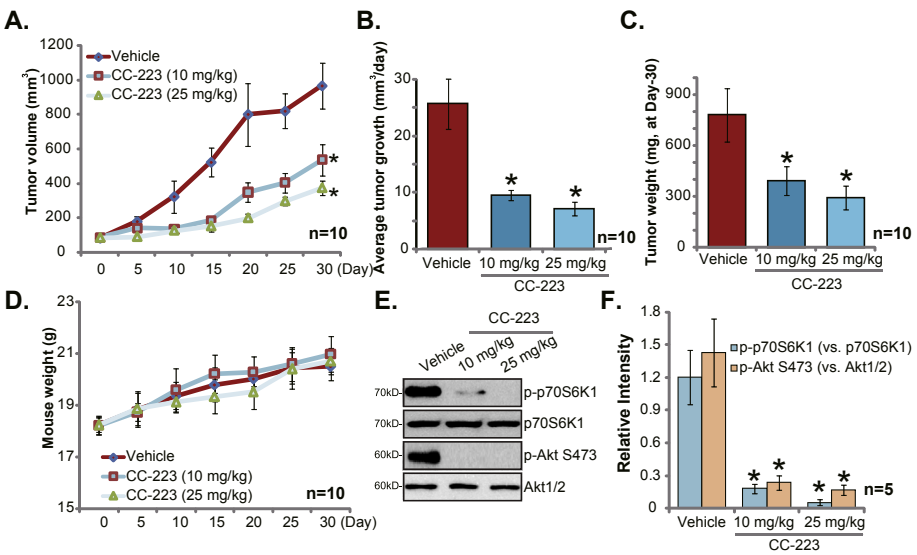

Fig. 4. CC-223 oral administration inhibits SCC-9 xenograft tumor growth in mice. SCC-9 xenograft tumor-bearing SCID mice (n = 10 mice per group) were treated with CC-223 (10/25 mg/kg body weight, gavage, daily for 21 consecutive days) or the vehicle control ("Vehicle"), the tumor volume (**A**) and the body weight of the mice (**D**) were recorded every 5 days for a total of 30 days; The estimated daily tumor growth was calculated as described (**B**); At day-30, each tumor was removed and freshly weighted (**C**). At treatment Day-3, four hours after initial CC-223 administration, one tumor of each group was isolated, and five random tissue parts of each tumor were tested for signaling, representative Western blotting images (**E**) and quantified results (**F**) were shown. $^*p < 0.05$ vs. "Vehicle" group.

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It is intriguing that CC-223 is even more efficient against HNSCC cells than AZD-2014, which is the other known mTOR kinase inhibitor [10,18,32]. It could be due to the more potent inhibition of mTOR kinase by CC-223 [14] than AZD2014. It is also possible that CC-223 might provoke mTOR-independent mechanisms to help kill HNSCC cells. Indeed, a very recent study by Xie et al., has shown that CC-223 disrupts mitochondrial function, leading to mitochondrial permeability transition pore (mPTP) opening and reactive oxygen species (ROS) production, which also participates in hepatocellular carcinoma cell death.

It is worth noting that CC-223 is orally-bioavailable. Our *in vivo* studies demonstrate that CC-223 oral administration in SCID mice largely inhibited SCC-9 xenograft tumor growth. Significantly, the experimental mice were well-tolerated to the tested CC-223 regimens, showing no apparent toxicities. A phase-I study of CC-223 in patients with advanced cancer has concluded that CC-223 was tolerable to the cancer patients, with manageable toxicities [20]. These results suggest that this novel mTOR kinase inhibitor might have important translational value for the treatment of HNSCC, and may warrant further investigations as a promising *anti*-HNSCC agent.

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Author contributions

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

Conflicts of interest

The listed authors have no conflict of interests.

Transparency document

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