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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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