Biochemical and Biophysical Research Communications 440 (2013) 701–706

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The dual mTORC1 and mTORC2 inhibitor AZD8055 inhibits head and neck squamous cell carcinoma cell growth *in vivo* and *in vitro*

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ARTICLE INFO

Article history:
Received 27 September 2013
Available online 5 October 2013

Keywords:
Head and neck squamous cell carcinoma
AZD8055
mTOR
JNK
Cancer cell death

ABSTRACT

The serine/threonine kinase mammalian target of rapamycin (mTOR) promotes cell survival and proliferation, and is constitutively activated in head and neck squamous cell carcinoma (HNSCC). Thus mTOR is an important target for drug development in this disease. Here we tested the anti-tumor ability of AZD8055, the novel mTOR inhibitor, in HNSCC cells. AZD8055 induced dramatic cell death of HNSCC lines (Hep-2 and SCC-9) through autophagy. AZD8055 blocked both mTOR complex (mTORC) 1 and mTORC2 activation without affecting Erk in cultured HNSCC cells. Meanwhile, AZD8055 induced significant c-Jun N-terminal kinase (JNK) activation, which was also required for cancer cell death. JNK inhibition by its inhibitors (SP 600125 and JNK-IN-8), or by RNA interference (RNAi) alleviated AZD8055-induced cell death. Finally, AZD8055 markedly increased the survival of Hep-2 transplanted mice through a significant reduction of tumor growth, without apparent toxicity, and its anti-tumor ability was more potent than rapamycin. Meanwhile, AZD8055 administration activated JNK while blocking mTORC1/2 in Hep-2 tumor engrafts. Our current results strongly suggest that AZD8055 may be further investigated for HNSCC treatment in clinical trials.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) refers to a large heterogeneous group of carcinomas of face, nasopharynx, oral cavity, oropharynx, hypopharynx, and/or larynx [1,2]. HNSCC represents about 5% of all cancers diagnosed every year in United States [1], and is an important contributor of cancer related deaths [1,3]. Despite recent progresses in basic and clinical researches, the standard treatments for HNSCC (surgery, radiotherapy or chemotherapy [4,5]) have not able to significantly improve the patients' survival [1,6–8]. Meanwhile, 30–50% of patients will develop local or regional recurrence, and distant metastases after these treatments [4,5]. Thus, the search of potential alternative therapies for HNSCC is encouraging and urgent.

The serine/threonine kinase mammalian target of rapamycin (mTOR) promotes cell survival and proliferation, and is constitutively activated in head and neck squamous cell carcinoma (HNSCC) [9,10], therefore it represents a major drug target for this disease. mTOR kinase forms two distinct multiprotein complexes, namely mTOR complex 1 (mTORC1), a complex composed of mTOR, Raptor and PRAS40 [11], as well as mTOR complex 2 (mTORC2), which contains mTOR, Rictor and Protor [12–14]. mTORC1, or the rapamycin sensitive mTOR complex, phosphorylates downstream targets including p70S6K (S6K) and 4E-BP1, and is important for protein translation and cell growth [11]. While mTORC2 phosphorylates AKT at Ser 473, and increases its enzymatic activity [15]. AZD8055 is a novel and potent ATP-competitive mTOR inhibitor, which blocks both mTORC1 and mTORC2 activation [16–18]. Here we investigated the role of AZD8055 on HNSCC cell growth and survival, and focused on its mechanism.

2. Materials and methods

2.1. Chemicals and reagents

Antibodies of rabbit IgG-HRP, mouse IgG-HRP and tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho(p)-AKT (Ser 473), p-AKT (Thr 308), p-S6K (Thr 389), p-S6 (Ser 235/236), AKT1, S6, p-Erk1/2 (Thr202/Tyr204), p-mTOR (Ser 2481), mTOR, Rictor, Raptor, mSin1, p-JNK1/2 (Thr183/Tyr185) and JNK1 antibodies were obtained from Cell Signaling Technology (Bevery, MA). SP 600125, JNK-IN-8, LY 294002 and RAD001 were obtained from Selleck (Shanghai, China). AZD8055, AZD2014 and INK-128 were purchased from selleck.cn (Shanghai, China). 3-methyladenine (3-MA) and z-VAD-fmk were obtained from Calbiochem (CA, USA).

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http://dx.doi.org/10.1016/j.bbrc.2013.09.130

2.2. Cell culture

Cell lines (Hep-2, SCC-9 and HEK-293) were cultured as monolayer in RPMI 1640 or DMEM supplemented with 10% (v/v) FBS, 2 mmol/L L-glutamine and 1% antibiotic.

2.3. CCK-8 cell viability assay

Cell viability was measured by Cell Counting Kit-8 (CCK-8) (Dojindo, Japan) assay according to manufacturer's protocol [19]. The OD value of group received the indicated drug was normalized to OD value of vehicle control group.

2.4. Apoptosis assay

Assays were performed according to the manufacturer's guidelines (BD Pharmingen). Briefly, after treatment, both floating and attached cells were collected for analysis. Cells were washed with cold PBS and resuspended in binding buffer containing 5 μl of Annexin V (BioVision.com) and fixed, followed by flow cytometry analysis (Beckman Coulter).

2.5. Western blots

After treatment, cells were lysed on ice with lysis buffer containing 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EDTA, 1% Triton X-100, 50 mmol/L NaF, 20 mmol/L Tris–HCl (pH 7.6), 0.1% SDS, protease, and phosphatase inhibitors. Total protein (30 μg) was separated on a 10% SDS–PAGE gel and analyzed by immunoblotting. Membranes were blocked in 10% nonfat milk in 1× TBS/0.1% Tween 20 (TBST) and then probed with the respective primary antibodies overnight at 4 °C. After washing and incubation with secondary antibodies, the immunoblotted proteins were visualized using the horseradish peroxidase SuperSignal West Pico Chemiluminescence Substrate according to the manufacturer's instructions.

2.6. JNK RNA interference (RNAi) in HEK-293 cells

Two siRNAs for SAPK/JNK were purchased from Cellular Signaling Tech (SignalSilence® SAPK/JNK siRNA I #6232) (JNK RNAi-1) and Santa Cruz (JNK1 siRNA, sc-29380) (JNK RNAi-2) respectively. HEK-293 cells were cultured in regular growth medium, and were

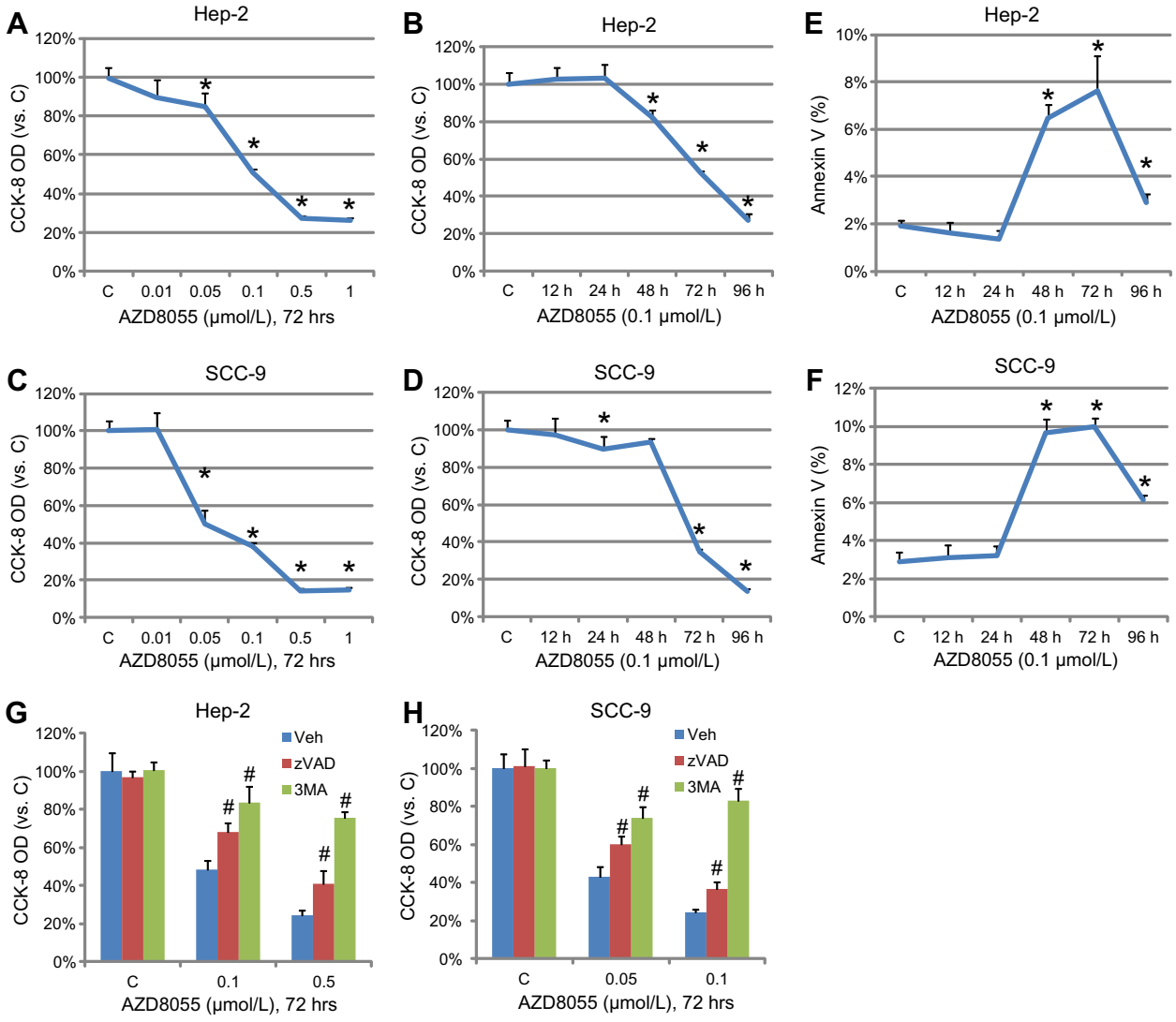

Fig. 1. Cytotoxic effects of AZD8055 in cultured HNSCC cells, HNSCC cell lines Hep-2 and SCC-9 were exposed to AZD8055 at indicated concentrations for 72 h (A and C), or exposed to 0.1 μmol/L of AZD8055 for indicated time (B and D), cell survival was measured by CCK-8 assay, and cell apoptosis was tested through Annexin V assay (E and F). Hep-2 and SCC-9 cells were pre-treated with z-VAD-fmk (50 μmol/L) or 3-methyladenine (3-MA, 0.5 mmol/L) for 1 h, followed by indicated concentration of AZD8055, cells were further cultured for 72 h, and cell survival was tested by CCK-8 assay (G and H). The mean of three independent experiments performed in triplicate was shown. Statistical significance was analyzed by ANOVA. $^{*}p < 0.01$ vs Control. $^{\#}p < 0.01$ vs AZD8055 group.

seeded in a six-well plate with 60% of confluence. JNK SiRNA (100 nmol/L) was transfected to cultured cells through Lipofectamine 2000 TM protocol (Invitrogen, Carlsbad, CA), control cells were transfected with same concentration (100 nM) of scramble siRNA (Santa Cruz). After transfection, cells were further cultured for 48 h. The expression of JNK in transfected cells were checked by Western blots.

2.7. Hep-2 xenograft

The research protocol was approved, and mice were housed and maintained under specific pathogen-free conditions in the Animal Care Facility of the authors' institution (Jinshan Hospital Affiliated to Fudan University), according with Health Animal Care and Use Committee. Based on the procedure reported [20], female BALB/c nude mice, 8 weeks of age, weighing 20–22 g, were acclimatized for 1 week before being injected s.c. with 3×10^6 Hep-2 cells that had been re-suspended in 100 μl of growth medium. After 6–7 days, established tumors around 0.2 cm^3 in diameter were detected, mice were randomized and divided in three groups. Ten mice per group were treated with AZD8055 (5 mg/kg, bid, oral) or rapamycin (5 mg/kg, bid, i.p.) twice daily (bid) for 4 weeks. AZD8055 was formulated in 30% (w/v) captisol (pH 3.0) [18], rapamycin was formulated as reported [10]. The control group received the vehicle only. Tumor size were measured weekly by the modified ellipsoid formula: $(\pi/6) \times AB^2$, where A is the longest and B is the shortest perpendicular axis of an assumed ellipsoid corresponding to tumor mass [21,22]. Body weight was measured weekly as control for treatment toxicity. Mice survival was also recorded.

2.8. Statistical analysis

In each experiment, a minimum of three wells/dishes was used. Each experiment was repeated a minimum of three sets. Data were presented as mean ± SD. The differences were determined by one-way ANOVA in appropriate experiments followed by Newman–Keuls post hoc test. A probability value of $p < 0.01$ was taken to be statistically significant.

3. Results

3.1. Cytotoxic effects of AZD8055 in cultured HNSCC cells

In cultured Hep-2 cells, AZD8055 administration induced significant loss of cell survival (cell death), and the effect of AZD8055 was both dose- (Fig. 1A) and time-dependent (Fig. 1B). Similar results were also observed in another HNSCC cell line SCC-9 (Fig. 1C and D). Annexin V FACS apoptosis assay was applied to test cell apoptosis in AZD8055-treated HNSCC cells, and we only observed a moderate cell apoptosis (less than 10%) by AZD8055 in both Hep-2 and SCC-9 cells (Fig. 1E and F). Meanwhile, the apoptosis inhibitor (z-VAD-fmk) only slightly inhibited AZD8055′ cytotoxicity in both Hep-2 (Fig. 1G) and SCC-9 (Fig. 1H) cell lines. On the other hand, autophagy inhibitor 3-MA rescued a significant proportion of cancer cells from AZD8055-induced death (Fig. 1G and H). These results suggested that although AZD8055 induced some apoptosis, the cytotoxic effect by AZD8055 in HNSCC cells was mainly due to autophagy, our results are consistent with previous observations showing autophagy mediates AZD8055′s cytotoxicity [16,17].

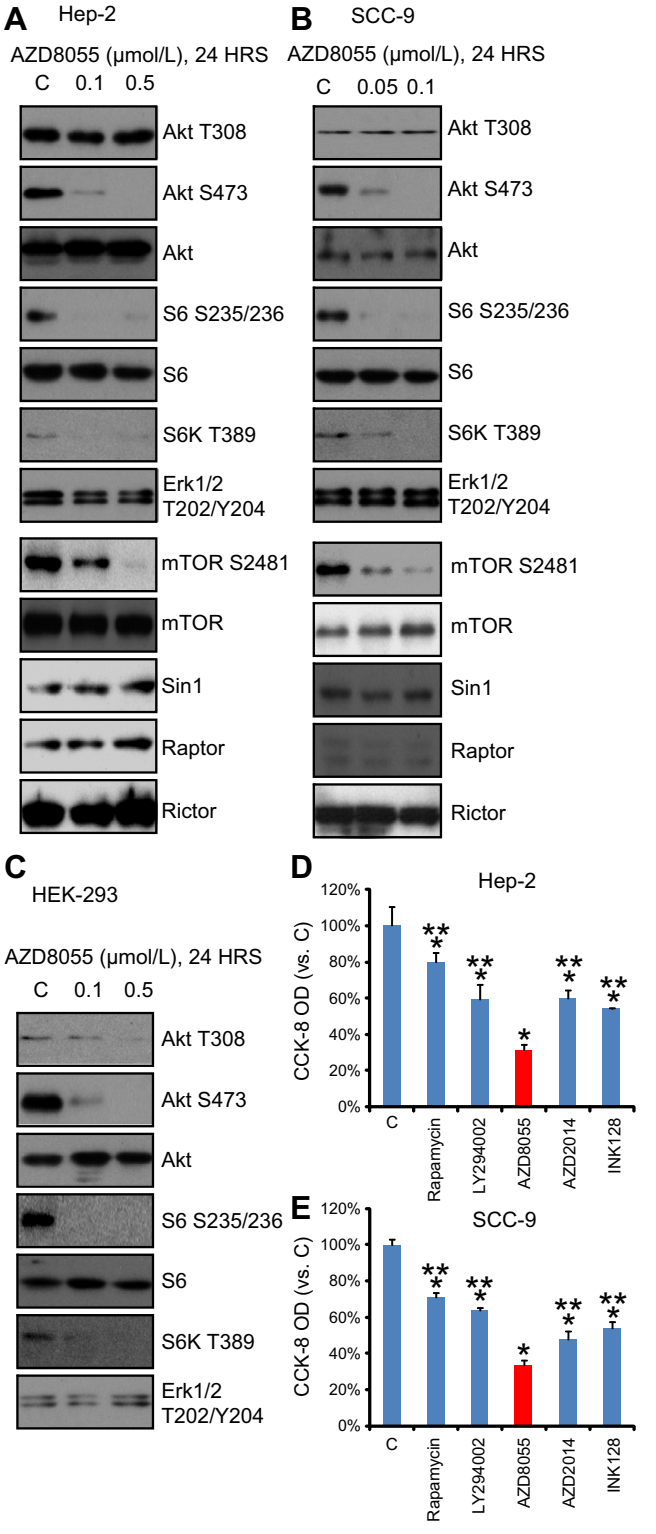

Fig. 2. AZD8055 inhibits mTORC1 and mTORC2 activation in cultured HNSCC cells Hep-2 (A), SCC-9 (B) and HEK-293 (C) cells were treated with AZD8055 at indicated concentration for 24 h, regular and/or phospho levels of Akt, S6, S6K, mTOR, Raptor, Rictor, Sin1 and Erk1/2 were measured by western blot. Hep-2 (D) or SCC-9 (E) cells were treated with PI3K/Akt and mTOR dual inhibitor LY 294002 (LY, 1 μmol/L), mTORC1 inhibitor rapamycin (0.5 μmol/L), mTORC1/2 dual inhibitor AZD2014 (0.1 μM), INK-128 (0.1 μM) or AZD8055 (0.1 μM) for 72 h, cell viability was analyzed. The mean of three independent experiments performed in triplicate was shown. Statistical significance was analyzed by ANOVA. $^{*}p < 0.01$ vs Control. $^{**}p < 0.01$ vs. AZD8055 group.

3.2. AZD8055 blocks mTORC1 and mTORC2 activation in cultured HNSCC cells

Published studies demonstrate that AZD8055 is a novel and potent inhibitor of both mTORC1 and mTORC2 [23,24]. Considering that constantly activated mTOR signaling contributes to HNSCC progression [25], we then examined AZD8055′s effect on Akt/mTOR signaling. Western blots results demonstrated that AZD8055 almost blocked mTORC1 and mTORC2 activation in Hep-2 (Fig. 2A) and SCC-9 (Fig. 2B) cells, as well as in HEK-293 cells (Fig. 2C). The activation of mTORC1 was reflected by phospho-S6K (Thr 389) and phospho-S6 (Ser 235/236), while activation of mTORC2 was reflected by phospho-Akt (Ser 473) (Fig. 2A–C). Note that Akt Thr 308 phosphorylation and Erk1/2 phosphorylation were not affected by AZD8055 (Fig. 2A–C). Meanwhile, AZD8055 inhibited mTOR Ser 2481 phosphorylation. However, the expressions of regular mTORC1/2 components including mTOR, SIN1, Raptor and Rictor as well as regular S6 and Akt were not affected by AZD8055 (Fig. 2A–C). We found that AZD8055 was more potent than mTORC1 inhibitor rapamycin and Akt/mTOR dual inhibitor LY 294002 in killing of Hep-2 (Fig. 2D) and SCC-9 (Fig. 2E) cells. Further, AZD2014 and INK-128, two other mTORC1/mTORC2 dual inhibitors [26,27], were also shown to inhibit the survival of Hep-2 and SCC-9 cells (Fig. 2D and E). However, AZD8055 was more efficient than these two (Fig. 2D and E). Above results suggested that other possible mechanisms beside mTOR inhibition could also contribute to AZD8055′s effect.

3.3. JNK activation is required for AZD8055-induced HNSCC cell death

JNK activation mediates cell death in response to a number of stresses [28]. Next, we examined whether JNK could be activated by AZD8055, and if so, what the role JNK played in mediating cancer cell death. Western blot results in Fig. 3A–C showed that AZD8055 induced a significant JNK activation in HNSCC lines Hep-2 and SCC-9 lines, as well as in HEK-293 cells. In response to AZD8055, level of phospho-JNK1/2 (Thr 183/Tyr 185) increased in above cells (Fig. 3A–C). Significantly, two JNK inhibitors (SP 600125 and JNK-IN-8) rescued HNSCC cells (Hep-2 and SCC-9) from AZD8055-induced cytotoxicity (Fig. 3D and E). Meanwhile,

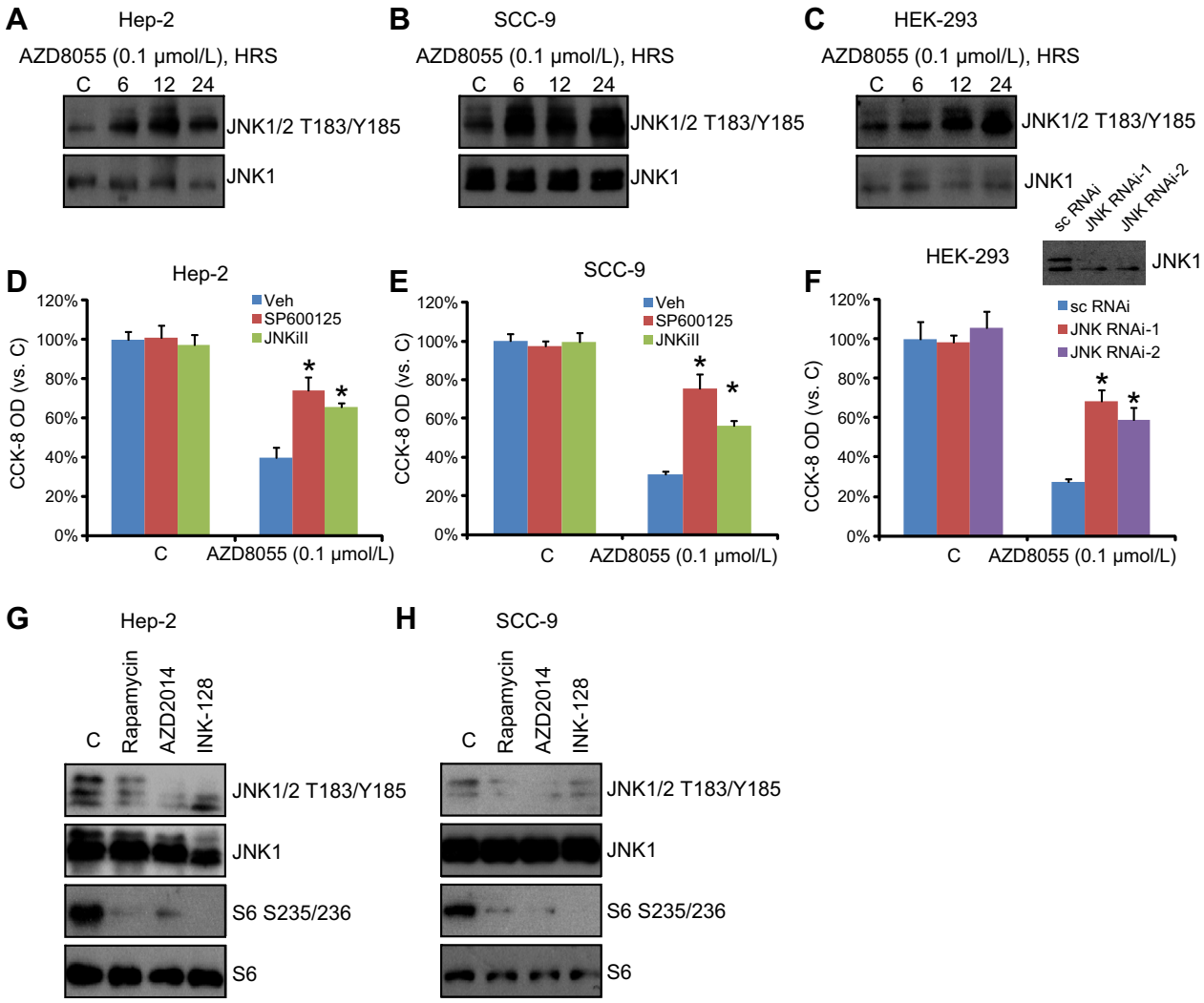

Fig. 3. JNK activation is required for AZD8055-induced HNSCC cell death Hep-2 (A), SCC-9 (B) and HEK-293 (C) cells were exposed to AZD8055 for indicated time, regular and phospho-JNK were tested. Hep-2 (D) and SCC-9 (E) cells were pre-treated with JNK inhibitor SP 600125 (1 μmol/L) or JNK-IN-8 (1 μmol/L) for 1 h, cells were also stimulated with indicated AZD8055 and cultured for 72 h, cell survival was analyzed. Scramble RNAi or JNK1/2 RNAi (JNK RNAi-1 or JNK RNAi-2, see methods) transfected HEK-293 cells were stimulated with AZD8055, cells were further cultured for 72 h before cell survival was tested (F), JNK1/2 expression in above cells was shown (F). Hep-2 (G) or SCC-9 (H) cells were treated with rapamycin (0.5 μmol/L), AZD2014 (0.1 μM) or INK-128 (0.1 μM) for 12 h, regular and phospho-levels of JNK and S6 were tested. The mean of at least three independent experiments performed in triplicate was shown. $^{*}p < 0.01$ vs AZD8055 only group. Statistical significance was analyzed by ANOVA.

JNK silencing by RNAis also suppressed AZD8055-induced HEK-293 cell death (Fig. 3F). These data suggested that JNK activation is required for AZD8055-induced HNSCC cell death. Notably, AZD2014 or INK-128 (mTORC1/2 dual inhibitors) failed to induce any significant JNK activation in above cells (Fig. 3G and H); Meanwhile, rapamycin also failed to activate JNK (Fig. 3G and H). Thus, AZD8055-induced JNK activation was unlikely due to Akt/mTOR inhibition.

3.4. AZD8055. inhibits Hep-2 cell growth in vivo, and was more efficient than rapamycin

Finally, we tested the *in vivo* anti-tumor effect of AZD8055 in a Hep-2 xenograft model. In mice bearing Hep-2 cell xenograft, the group that received oral AZD8055 (5 mg/kg, bid, daily) treatment showed a great inhibition in tumor growth (Fig. 4A). Meanwhile, AZD8055 markedly increased the survival of Hep-2 transplanted mice (Fig. 4B).The effects of AZD8055 on mice body weight in the xenograft model were demonstrated in Fig. 4C, we did not see a significant toxicity of AZD8055, as reported by other studies [18]. Significantly, AZD8055 was more potent than rapamycin in inhibiting tumor growth (Fig. 4A) and improving mice survival (Fig. 4B). Thus, AZD8055 significantly inhibited Hep-2 cell growth *in vivo*, and was more efficient than rapamycin. We also tested the signal changes in engrafted Hep-2 tumor tissues isolated from mice administrated with vehicle or AZD8055. Western blots in Fig. 4D and E showed that AZD8055 activated JNK signaling while inhibiting Akt Ser 473 phosphorylation (the indicator of mTORC1 activation) and S6/S6K phosphorylation (the indicators of mTORC2 activation). Thus, similar as what we observed *in vitro*, AZD8055 also blocks mTORC1/2 signaling while activating JNK *in vivo*.

4. Discussions

Despite recent efforts in both basic and clinical research, HNSCC continues to have one of the worst 5-year survival among all cancers [1,5,29,30]. Cisplatin and 5-fluorouracil (5-FU) are the current standard chemotherapy for HNSCC patients [2]. Radiation is delivered either after or with chemotherapy [2]. Each of these options will cause significant toxicities [31]. Note to mention chemo/radiation resistance, recurrence and distant metastasis [31]. Remarkable progress has been made in our understanding of how the aberrant activation of signaling networks (oncogenes) contributes to HNSCC progression [2,32], which help us to identify novel mechanism-based anticancer treatments [2,32]. To this regard, dysregulated mTOR signaling among others is frequently observed in HNSCC [9,10,13], which has provided the molecular basis for current efforts exploring the clinical benefits of targeting mTOR in patients with advanced HNSCC [9,10,13].

Amornphimoltham et al. evaluated the status of phospho-S6, the downstream target of mTORC1 in HNSCC tissues, and found that mTORC1–p70S6K–S6 pathway was persistently activated in early dysplastic lesions, and that this over-activity of S6 persisted during HNSCC progression [10]. The authors used rapamycin (the mTORC1 inhibitor) to block mTORC1 activation *in vitro* and *in vivo*. Rapamycin inhibited tumor regression, along with a marked decrease in cancer cell proliferation, a enhanced cell apoptosis, and reduced tumor vascularity [10]. These results suggested that mTOR is a suitable target for pharmacologic intervention in HNSCC [10]. The limitation when using rapamycin and its analogues (RAD001 for example) clinically was thought to be largely due to the activation of the S6K-insulin receptor substrate (IRS) negative feedback loop, and lack of inhibition of mTORC2, and therefore increase in AKT activity [33]. Meanwhile, inhibition of

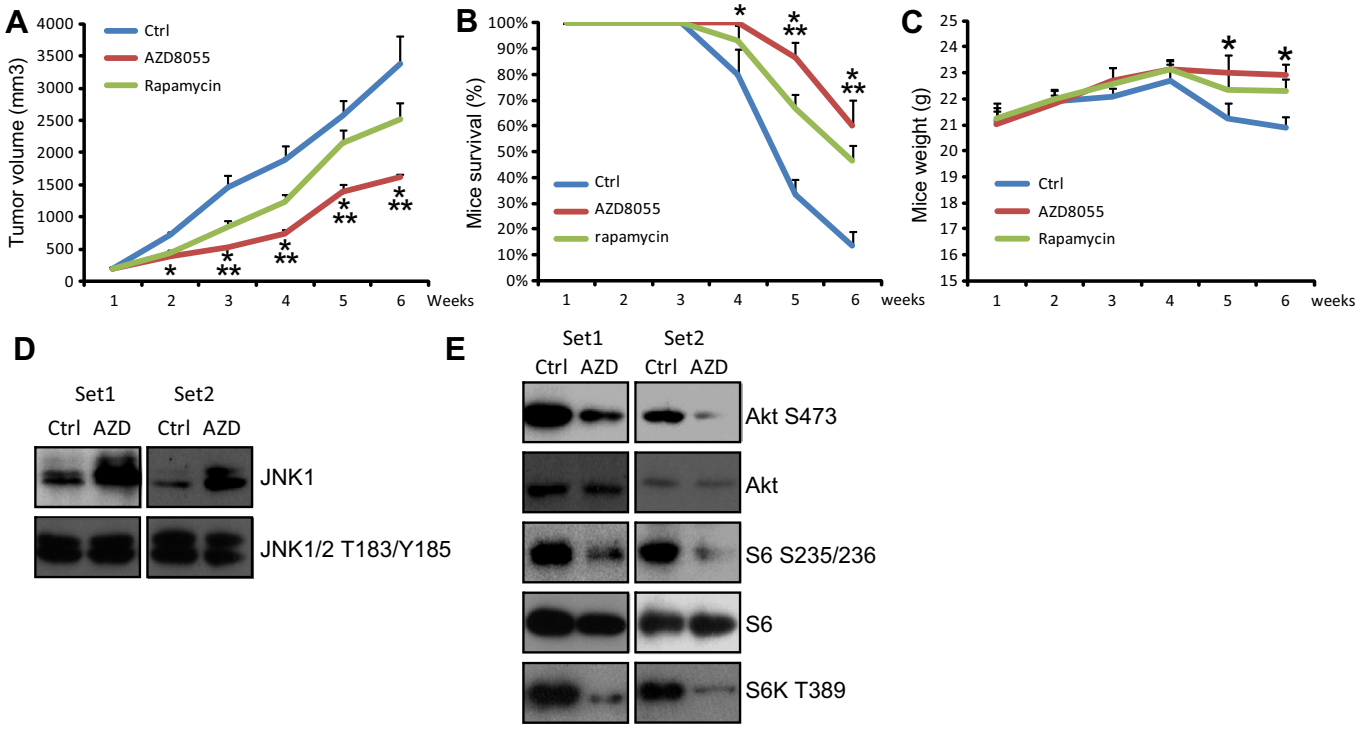

Fig. 4. AZD8055 inhibits Hep-2 cell growth *in vivo*, and was more efficient than rapamycin Mice bearing Hep-2 xenograft were treated with AZD8055 (5 mg/kg, bid, oral) or rapamycin (5 mg/kg, bid, i.p.). The control group received the vehicle only. Tumor size was measured weekly using method mentioned above (A). Mice survival rate were also recorded (B). Body weight was measured weekly to indicate treatment toxicity (C). 3 weeks after administration (vehicle or AZD8055), Hep-2 tumor engrafts were freshly removed, sliced into small pieces, solubilized and lysed using lysis buffer, followed by Western blot detecting with the indicated antibodies, data of two mice per treatment (vehicle or AZD8055) were presented (D and E). The mean of three independent experiments performed in triplicate is shown. $^{*}p < 0.01$ vs vehicle group. $^{**}p < 0.01$ vs rapamycin group. Statistical significance was analyzed by ANOVA.

mTORC1 by rapamycin was found to activate oncogenic Erk1/2 MAPK pathway through a PI3K-dependent feedback loop in human cancers [34]. To this regard, AZD8055 is a potent and selective ATP-competitive inhibitor of mTOR kinase, which blocks both mTORC1 and mTORC2 activation [18]. Thus it inhibits Akt activation by blocking phosphorylation at Ser 473 [16–18]. Here we found that AZD8055 blocked both mTORC1 and mTORC2 activation without affecting Erk signaling in cultured HNSCC cells. The anti-tumor effect of AZD8055 (both *in vivo* and *in vitro*) was significantly more potent than rapamycin.

Interestingly, we found dramatic JNK activation in AZD8055-treated cells, which was required for cancer cell death, as JNK inhibition suppressed cytotoxicity by AZD8055. Further studies will be needed to understand the mechanism by which AZD8055 activates JNK. Also, its role in mediating cancer cell death should also be explored. In conclusion, we here showed that AZD8055 significantly inhibits HNSCC cell growth *in vivo* and *in vitro*, our current results strongly suggest that AZD8055 may be further investigated for HNSCC treatment in clinical trials.

Acknowledgment

This research was supported by grants from the National Natural Science Foundation.

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