Anti-tumor Effect of Temsirolimus against Oral Squamous Cell Carcinoma Associated with Bone Destruction

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Abbreviations list: PBS: phosphate buffered saline; TRAP: tartrate-resistant acid phosphatase; RANKL: receptor activator for nuclear factor kB ligand; OPG: osteoprotegerin

Footnotes

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Conflict of Interest
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Abstract

The mammalian target of rapamycin (mTOR) is engaged in the molecular pathogenesis of oral squamous cell carcinoma which frequently invades the maxilla or the mandible. However, the effects of an mTOR inhibitor on bone destruction associated with oral squamous cell carcinoma are still unclear. In this study, we investigated the anti-tumor effect of temsirolimus mediated mTOR inhibition against advanced oral squamous cell carcinoma. Temsirolimus inhibited the proliferation and migration of HSC-2 oral squamous cell carcinoma cells \textit{in vitro} and suppressed the growth of oral squamous cell carcinoma xenografts \textit{in vivo}. Significantly, we have clearly shown that temsirolimus inhibited osteoclast formation both \textit{in vitro} and \textit{in vivo}. RT-PCR analysis showed that temsirolimus decreased the mRNA expression of receptor activator for nuclear factor \(\kappa\)B ligand (RANKL), known as an osteoclast differentiation factor in bone stromal ST2 cells. Moreover, temsirolimus normalized blood free calcium concentration in mouse models for humoral hypercalcemia. These findings suggest that the mTOR signaling is a potential target of oral squamous cell carcinoma associated with bone destruction and hence we describe the efficacy of temsirolimus for the treatment of advanced oral squamous carcinoma.
Introduction

The invasion of oral squamous cell carcinoma cells into the maxilla or the mandible is a common clinical problem (1, 2). However, bone resection for the treatment leads to physical damage and has a major influence on the quality of life because of the postoperative disruption of speech and swallowing function. Other approaches for the treatment of advanced oral squamous carcinoma are being investigated. Recently, single treatment of mammalian target of rapamycin (mTOR) inhibitors or combination treatment of the inhibitors with other anti-cancer agents demonstrated anti-tumor activity in numerous tumors, such as metastatic pancreatic tumors (3), recurrent/metastatic breast cancer (4), advanced renal cell carcinoma (5-7), and advanced gastric cancer (8). It has been reported that tumors with continuous Akt activation are more sensitive to the inhibition of mTOR (9-11) and Akt is frequently activated in oral squamous cell carcinoma (12). However, it is still unclear as to how mTOR signaling participates in osteoclastic bone resorption by oral squamous cell carcinoma. In the present study, we have analyzed the anti-tumor effect of temsirolimus against advanced oral squamous cell carcinoma and investigated how mTOR signaling involves in osteoclastic bone resorption associated with tumor invasion.
Materials and Methods

Cell lines and culture conditions
The human oral squamous cell carcinoma cells HSC-2 and SAS, newly obtained from the Human Science Research Resources Bank (Osaka, Japan), and the murine bone marrow stromal cells ST2 and the murine macrophage cells RAW264.7, newly obtained from the RIKEN BioResource center Cell Bank (Tsukuba, Japan) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). All these cell lines were characterized by genotyping in the cell banks. The primary CD11b+ bone marrow cells were cultured in a Modified Eagle Medium (αMEM). All cell lines were cultured in 10% CO₂ at 37 °C.

Purification of osteoclast progenitors
Bone marrow cells were washed twice by centrifugation in 20 ml of cold buffer containing sterile PBS supplemented with 0.5% bovine serum albumin (Sigma, St. Louis, MO, USA) and 2 mM EDTA (Sigma). The cell pellet was resuspended in 80 μl buffer per 10⁷ cells, and the cells were magnetically labeled by adding 20 μl of anti-CD11b microbeads per 10⁷ cells. The cells were next incubated for 30 min on ice and then washed by centrifugation with a volume of buffer 10-fold that of the labeling volume and resuspended in 500 μl of buffer per 10⁸ cells. CD11b+ cells were depleted using an MD depletion column (Miltenyi Biotec Inc. Bergisch Gladbach, Germany) placed in the magnetic field of a MidiMACS separation unit (Miltenyi Biotec Inc.).
Histochemical and immunohistochemical analysis of surgically resected samples

From the surgically resected lower gingival squamous cell carcinoma mandible samples, decalcified, hematoxylin-eosin (HE)-stained specimens were prepared. Sections from the deepest part of the invasion and the boundary between the tumor and the bone were evaluated primarily by light microscopic observation. All the patients were examined and treated at Okayama University Hospital (Okayama, Japan) between 2000 and 2010, and the diagnosis was clinicopathologically confirmed. No patient had received chemotherapy and/or radiation therapy before surgery was performed. All tumor samples were obtained with the consent of the patients. The sections were sequentially dewaxed through a series of xylene, graded ethanol, and water immersion steps. After having been autoclaved in 0.2% citrate buffer for 15 minutes, the sections were incubated with 3% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity. A primary anti-mTOR (rabbit IgG), p-mTOR Ser²⁴⁴⁸ (rabbit IgG) (Cell Signaling Technology, Danvers, MA, USA) was used for the immunohistochemical analysis. The specimens were incubated with a 1:200 dilution of the antibody overnight at 4°C, followed by 3 washes with TBS. The slides were then treated with a streptavidin-biotin complex (Envision System labelled polymer, horse radish peroxidase (HRP); Dako, Carpinteria, CA, USA) for 60 minutes at a dilution of 1:100. The immunoreaction was visualized using a 3,3'-diaminobenzidine (DAB) substrate-chromogen solution (Dako Cytomation Liquid DAB Substrate Chromogen System, Dako), and counterstaining was performed with hematoxylin. Finally, the sections were immersed in an ethanol and xylene bath and mounted for examination.

Cell proliferation assay
The HSC-2 cells were plated in a 96-well plate at 1 x 10⁴ cells per well in the presence of temsirolimus (Supplementary Figure S1), which was obtained from Wyeth (Madison, NJ, USA). A 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay was done to obtain a relative cell number after 72 h of incubation under the experimental procedure (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega Co, Madison, WI, USA).

**Immunoblot analysis**

HSC-2, SAS, RAW264.7 or ST2 cells were rinsed once with ice-cold phosphate buffered saline (PBS) and lysed in an ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1% Triton X-100, 1% NP-40, 10 mM NaF, 100 mM leupeptin, 2 mg/ml aprotinin, and 1 mM phenylmethyl sulfonyl fluoride). Cell lysates containing 10 μg of total protein in a lysis buffer were electrophoresed in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and the proteins were transferred to nylon membranes (Immobilon-P; Millipore Co.). The membrane was incubated with primary and secondary antibodies according to the ECL chemiluminescence protocol (RPN2109, Amersham Biosciences, Buckinghamshire, UK) to detect secondary antibody binding. Antibodies against mTOR (rabbit IgG), p-mTOR Ser²⁴⁴⁸ (rabbit IgG), S6 (mouse IgG), p-S6 Ser²³⁵/²³⁶, Akt (rabbit IgG), p-Akt Ser⁴⁷³ (mouse IgG), IκBα (rabbit IgG), and p-IκBα Ser³²/³⁶ (mouse IgG) were purchased from Cell Signaling Technology and used at a 1:200 dilution. Horseradish peroxidase-conjugated goat anti-rabbit antibodies or goat anti-mouse IgG were used as the secondary antibodies at a 1:1000 dilution.
RNA extraction and reverse transcription-PCR

Total RNA was isolated using TRIzol reagent (Life Technologies Inc.) according to the manufacturer's instructions. Complementary DNA was generated from 1 μg of total RNA using a first strand cDNA synthesis kit (Invitrogen) in a final volume of 20 μl, then amplified for 30 cycles using two oligonucleotide primers: 5’-ACACCTCACCACCAATGC-3’ and 5’-GTACGCTTCCCGATGTTT-3’ for RANKL, 5’-ACCAAGTGAGGCAGGAG-3’, 5’-TCTGTTGGAGGTTCGAGTG-3’ for OPG, and 5’-TGAACGGGAAGCTCACTGG-3’ and 5’-TCCACCACCTGTGCTGTA-3’ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each PCR cycle was carried out for 30 seconds at 94°C, 30 seconds at 55°C, and 1 minute at 68°C. The PCR products were then separated with 2% agarose gels containing ethidium bromide and visualized under ultra violet light. The expected sizes of PCR products were 197 bp for RANKL, 191 bp for OPG, and 307 bp for GAPDH.

TRAP staining and osteoclast activity assay

RAW264.7 murine macrophage cells were treated with 50 ng/ml of recombinant mouse RANKL (PEPROTECH EC, London, UK) for 5 days. The cells were then fixed and stained for TRAP (Sigma) and the number of TRAP-positive multinucleated cells (nuclear number >3) in each well was counted. For osteoclast activity assay, murine CD11b+ bone marrow cells were plated into a calcium phosphate apatite-coated substrate (OAAS) plate (Osteogenic Core Technologies, Seoul, Korea) at a density of 1 x 10^6 cells/well. The cells were treated with RANKL (50 ng/ml) for 5 days and then incubated with both temsirolimus and RANKL (50 ng/ml) for more than 7 days. The remaining cells in the plate were lysed using 1 N NaOH with a
6% sodium hypochlorite solution. Five images per well were obtained using an inverted microscope (200%), and the resorbed area was measured using image software (Lumina Vision/OL).

**Scratch assay**

HSC-2 cells were grown to confluence on six-well tissue culture dishes and a single scratch was made in the confluent monolayer using a sterile 200 μl pipette tip. The monolayer was washed with PBS and then complete medium containing 1, 10 or 50 nM temsirolimus or vehicle alone was added. Serial photographs of the same scratched section were taken after 24 hours. The number of cells that had migrated over the margins of the wounds was counted after 24 hours of temsirolimus treatment.

**Animal experiments**

Human oral squamous cell carcinoma xenografts were established in 5-week-old female BALB/c nude mice (Clea Japan, Inc.) by s.c. inoculation of 8 x 10^6 HSC-2 cells into the dorsal flank as described previously (13, 14). The mice were randomly assigned into two groups (n=10 per group). Each group of the mice was treated with intraperitoneal injection of 200 μL solution containing temsirolimus (20 mg/kg) (15, 16) or vehicle only twice a week for 14 days. Body weights and the volume of tumors were measured from 14 days after tumor inoculation. The tumor volume (cubic mm) was calculated from the equation 4π/3 x (r_1/2 + r_2/2)^3, where r_1 = longitudinal radius, and r_2 = transverse radius, as described previously (14). All of the mice were sacrificed and the weight of tumors was measured 38 days after tumor inoculation.
Mouse models of bone invasion by human oral squamous cell carcinoma were established in 5-week-old female BALB/c nude mice (Clea Japan, Inc.) by inoculation of $1 \times 10^5$ HSC-2 cells into the bone marrow space of the left tibial metaphysis as described previously (17). Radiographs of the left hind limb were obtained 14 days after tumor inoculation.

Mouse models for humoral hypercalcemia of malignancy was established in 5-week-old male C57 black/6J mice (Clea Japan, Inc.) by subcutaneous implantation on their back of osmotic pumps (Alzet miniosmotic pump, model 1007D; Alzet Osmic Pumps Company, Palo Alto, CA, USA) filled with human PTHrP-(1-34) (Sigma) or vehicle (physiological saline containing 2% L-cysteine). Based on the pumping rate of 0.5 ml/h, the weekly PTHrP intake was calculated to be 3 mg/mouse. Blood calcium concentration was measured using a calcium analyzer (634 Ca/pH Analyzer, Chiron Diagnostics Corp, UK). These experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Okayama University Graduate School of Medicine and Dentistry.

**In vivo radiography and measurement of osteolytic lesion areas**

Osteolytic bone destruction was assessed on radiographs as previously described (13, 14). The mice were anesthetized with an intraperitoneal injection of pentobarbital (0.05 mg/g body weight), placed laterally in a prone position against the films (22 x 27 cm; Fuji industrial film FR: Fuji Photo Film Co. Ltd., Tokyo, Japan), and exposed to soft X-rays at 35 kV for 20 seconds by the use of a Sofron apparatus (Sofron, Tokyo, Japan). The radiolucent bone lesions in the hind limbs were observed microscopically (IX81, Olympus Corporation, Tokyo, Japan) and the areas were quantified with Lumina Vision/OL (Mitani Corporation, Tokyo, Japan).
Histochemical and histomorphometric analysis of the mouse model

The tibia of the mouse model of bone invasion by human oral squamous cell carcinoma was excised, fixed in 10% neutral-buffered formalin, decalcified, and then embedded in paraffin. Serial sections (5 μm) were cut longitudinally and the sections were stained with Mayer’s hematoxylin-eosin solution. Images of the growth plate and proximal tibia were photographed using an Olympus IX81 microscope. For the measurement of the osteoclast number, the sections were stained with TRAP. The total tumor area and osteoclast number/mm of tumor/bone interface were measured in the midsections of the tibiae and femora. Histomorphometric analysis was performed using Lumina Vision/OL-analyzing software.

Statistical analysis

Data were analyzed using the unpaired Student’s t-test for the analysis of two groups, one-way ANOVA, post hoc, Bonferroni and Dunnett’s test for the analysis of multiple group comparisons using SPSS statistical software (version 10). Results were expressed as the mean ± S.D. *, P < 0.05, and ** P < 0.01 were considered statistically significant.
Results

The expression of mTOR in osteolytic mandibular squamous cell carcinoma

Figure 1A illustrates a representative histological pattern of invasive bone destruction observed in a patient with oral squamous cell carcinoma in the mandibular region. mTOR and phosphorylated mTOR (p-mTOR) were highly expressed in tumor cells that had invaded the bone matrix. Ten out of ten cases of aggressive invasive phenotype showed a strong intensity of mTOR and p-mTOR immunoreactivity.

Inhibition of mTOR by temsirolimus suppressed cell growth and cell migration of HSC-2 oral squamous cell carcinoma cells

To analyze the anti-tumor effect of temsirolimus against oral squamous cell carcinoma in vitro, trypan blue exclusion and migration assays were performed. As shown in Fig. 1B, temsirolimus significantly decreased the number of HSC-2 cells proportionately with an increasing dose in 72 hours after treatment. The IC₅₀ of an HSC-2 cell was 20 nM (data not shown). The migration assay showed that the number of migrated HSC-2 cells was dose dependently inhibited after temsirolimus treatment (P < 0.01) (Fig. 1C). Subsequently, we analyzed the phosphorylation of mTOR and S6 after temsirolimus treatment in HSC-2 cells. Immunoblot analysis demonstrated that the phosphorylation of mTOR Ser²⁴⁴⁸, S6 Ser²³⁵/²³⁶ and Akt Ser⁴⁷₃ were decreased 15 minutes after temsirolimus treatment. However, temsirolimus did not change the expression of total mTOR, S6, Akt, or β-actin in both HSC-2 (left panel) and SAS (right panel) cells (Fig. 1D).

These results suggested that temsirolimus decreases the phosphorylation of mTOR, S6 and Akt,
and suppresses the cell viability and migration activity of oral squamous cell carcinoma cells.

**Temsirolimus suppressed osteoclast formation and activity**

Further, we studied the effect of temsirolimus on osteoclastogenesis. To elucidate the effect of temsirolimus in RANKL-mediated osteoclast formation, RAW264.7 murine macrophage cells, known as osteoclast precursor cells, were treated with RANKL (50 ng/ml) in the presence of temsirolimus for 5 days. Temsirolimus inhibited the number of TRAP-positive multinucleated osteoclast formation induced by RANKL in a dose dependent fashion (Fig. 2A). To define the molecular mechanisms of the inhibitory effects of temsirolimus on osteoclast formation, we examined the effects of temsirolimus on signaling pathways induced by RANKL in RAW264.7 cells. Immunoblot analysis demonstrated that mTOR and IκBα were transiently activated 5 minutes after RANKL treatment (50 ng/ml) in RAW264.7 cells (Fig. 2B). However, temsirolimus significantly blocked the RANKL induced phosphorylation of mTOR and IκBα at a concentration of more than 10 nM (Fig. 2C). Temsirolimus did not change the expression of total mTOR, Akt, and β-actin (Fig. 2B and 2C). Then, we analyzed the effect of temsirolimus on the bone resorptive activity of mature osteoclasts. Osteoclast activity assay using CD11b+ bone marrow cells demonstrated that 50 and 100 nM of temsirolimus significantly inhibited the bone resorptive activity of osteoclasts ($P < 0.05$ and $0.01$, respectively) (Fig. 2D). These results indicate that temsirolimus suppresses RANKL inducing phosphorylation of mTOR and IκBα and inhibits osteoclast formation and function.

**Temsirolimus suppressed oral squamous cell carcinoma xenografts in mice**
In order to analyze the anti-tumor effect of temsirolimus \textit{in vivo}, we established oral squamous cell carcinoma xenograft tumors derived from HSC-2 cells in nude mice. As shown in Fig. 3A and 3B, intraperitoneal injection of temsirolimus significantly suppressed tumor growth compared to vehicle ($P < 0.01$). An important observation to note is that the temsirolimus treatment did not cause body weight reduction, whereas significant body weight loss was seen in control mice (Fig. 3C). Temsirolimus did not affect body weight in non-tumor bearing mice. There were no deaths during the experiments on mice.

**Temsirolimus inhibited osteolytic bone destruction associated with invasion of oral squamous cell carcinoma \textit{in vivo}**

Smink et al. (18) suggested that mTOR signaling is important in the regulation of autophagy, a process proposed to be involved in osteoclast function, and we determined the effect of temsirolimus on osteolytic bone destruction induced by oral squamous carcinoma \textit{in vivo}. As shown in Fig. 4A, the osteolytic lesions were clearly visible in the hind limbs of the mouse models of bone invasion by human oral squamous cell carcinoma treated with vehicle only. Surprisingly, few destructive lesions were detected in the hind limbs of the mouse group treated with temsirolimus. The total area of radiographic osteolytic lesions from all tibias was significantly suppressed by temsirolimus treatment compared to control ($P < 0.05$). Histological analysis revealed that the tumor cells invaded the bone marrow space and destroyed both trabecular and cortical bone in control mice. In contrast, the bone remained intact after temsirolimus treatment. Histomorphometric analysis confirmed that tumor area in the hind limbs after temsirolimus treatment was significantly suppressed compared to the control (Fig. 4B). In
addition, the number of TRAP positive osteoclasts was significantly less in the hind limbs of the mouse group treated with temsirolimus than those in the control mice \( (P < 0.05) \) (Fig. 4C). These results suggested that temsirolimus significantly suppressed the oral squamous cell carcinoma tumorigenicity, as well as osteoclastogenesis in osteolytic bone destruction associated with invasion of oral squamous cell carcinoma.

**Temsirolimus improved hypercalcemia and osteolysis induced by PTHrP**

To confirm the effects of temsirolimus on PTHrP induced osteolysis *in vivo*, we used mouse models for humoral hypercalcemia of malignancy induced by PTHrP. As shown in Fig. 5A, blood calcium concentration increased and reached a maximum of 2.73 mM for 6 days after PTHrP treatment. However, temsirolimus treatment improved the hypercalcemia induced by PTHrP and calcium concentration remained at normal level \( (P < 0.05) \). Administration of vehicle did not effect the blood free calcium concentration. We then measured the body weight of each mouse group after treatments. The body weight decreased and reached a minimum of 10.95 g for 8 days after PTHrP treatment. However, temsirolimus significantly prevented the body weight loss by PTHrP injection \( (P < 0.05) \). The control vehicle treated group had no effect on the mouse body weight (Fig. 5B). To analyze the effects of temsirolimus on hypercalcemia, we analyzed osteoclast formation in the coculturing system of ST2 cells as osteoblast lineage cells and CD11b<sup>+</sup> bone marrow cells as osteoclast precursor cells in the presence of PTHrP with or without of temsirolimus. As shown in Fig. 5C, temsirolimus significantly inhibited TRAP-positive multinucleated osteoclast formation in a dose dependent manner and completely suppressed osteoclast formation at a concentration of 50 nM. Next, we examined RANKL and OPG mRNA
expression after PTHrP treatment in bone marrow stromal ST2 cells. RT-PCR and immunoblot analysis showed that RANKL mRNA and protein expression mildly increased 24 hours after PTHrP treatment. Significantly, temsirolimus inhibited the upregulation of RANKL mRNA and protein expression after PTHrP treatment in a dose dependent manner and 100 nM temsirolimus strongly reduced the RANKL mRNA and protein expression in ST2 cells. On the other hand, OPG mRNA and protein expression were reduced 24 hours after PTHrP treatment; however, temsirolimus reversed this effect at a dose of 1 nM and significantly upregulated the OPG mRNA and protein expression at a dose of 100 nM (Fig. 5D). These results suggest that downregulation of RANKL mRNA and protein expression and upregulation of OPG mRNA by temsirolimus in ST2 cells inhibited the differentiation of CD11b+ bone marrow cells into mature osteoclasts in the coculturing system and the inhibition of osteoclastogenesis by temsirolimus treatment improved hypercalcemia after PTHrP treatment in vivo.
Discussion

Previous studies have implicated a role for mTOR in oral squamous cell carcinoma and other malignancies. However, the role of mTOR involvement in oral squamous cell carcinoma induced bone destruction is not well understood. In the present study, the inhibition of mTOR signaling with temsirolimus inhibited bone destruction associated with oral squamous cell carcinoma invasion by not only suppressing osteoclastogenesis and osteoclast function, but also by inhibiting tumor proliferation and migration. These results indicate that mTOR is a critical mediator of osteolytic bone destruction in the bone tumor microenvironment.

Here, we have shown that both HSC-2 cells and osteoclasts were potently and efficaciously inhibited by temsirolimus at the level of proliferation and osteoclast formation and activation (Fig. 1-3). Temsirolimus inhibited HSC-2 cell growth with an IC$_{50}$ of 20 nM, whereas it inhibited RAW264.7 cell growth with an IC$_{50}$ of 54 nM. The discrepancy of IC$_{50}$ could be due to differences in the cell systems and HSC-2 cells might be more sensitive to temsirolimus than RAW264.7 cells. HSC-2 cells might overexpress mTOR in the bone destructive region more significantly than in the primary site. Osteoclasts also might activate the mTOR signaling by RANKL signaling and binding to the bone matrix (19) and the osteolytic destruction site would be expected to be more sensitive to mTOR inhibition than normal organs.

To investigate the molecular mechanism of anti-osteoclastic effects, we investigated RANKL-induced signaling pathways in RAW264.7 murine macrophage cells after temsirolimus treatment. Our data showed that temsirolimus inhibited IkB$\alpha$ activation induced by RANKL in RAW264.7 murine macrophage cells (Fig. 2B and 2C). Our previous study demonstrated that the inhibition
of Akt suppressed phosphorylation of IκBα and NFκB DNA-binding activity, and blocked RANKL induced osteoclastogenesis in mouse bone marrow macrophages (BMMs) (20). RANKL is also reported to lead to the amplification of nuclear factor of activated T cells (NFATc1), known as the master transcription factor for osteoclast differentiation. These findings imply that the DNA binding of NFκB, IκBα and NFATc1 have strongly taken part in the RANKL inducing osteoclastic effect. We also found that temsirolimus markedly inhibited and diminished the capacity to excavate apatite pits. Our data on osteoclast functional inhibition by temsirolimus is in agreement with a report that everolimus suppresses bone resorption and cathepsin K expression by osteoclasts (21).

It has been reported that various bone resorbing factors are produced and secreted from malignant tumor cells; particularly PTHrP is involved in the pathogenesis of hypercalcemia associated with malignant tumors including oral squamous cell carcinoma (22). In this study, temsirolimus improved hypercalcemia induced by PTHrP in vivo and this is the first report describing a role for mTOR in regulating hypercalcemia through the PTHrP signaling pathway in stromal cells. Our results are consistent with reports demonstrating that everolimus suppresses the OVX-induced loss of cancellous bone (21), the lack of effect of rapamycin on bone (23), and other immunosuppressive drugs, such as corticosteroid and cyclosporin exerting negative bone effects (24, 25). To better understand the mechanisms of mTOR signaling on PTHrP induced hypercalcemia, we used a coculturing system of bone marrow stromal ST2 cells and murine CD11b+ bone marrow cells as osteoclast precursor cells in the presence of PTHrP with or without of temsirolimus. Stromal cells are essential for osteoclastogenesis through a cell-to-cell interaction with osteoclast progenitor cells (26). Temsirolimus increased OPG mRNA expression
and reduced RANKL mRNA expression in murine bone stromal ST2 cells after PTHrP treatment and resulted in suppressing differentiation of murine CD11b⁺ bone marrow cells into osteoclasts in the coculturing system. These results suggest that temsirolimus inhibits osteoclast formation by suppressing osteoblast function.

In summary, this study is, to the best of our knowledge, the first one to show that mTOR should be considered a target for the treatment of oral squamous cell carcinoma induced bone osteolysis and our findings strongly suggest that single use of temsirolimus or combination treatment of temsirolimus with other agents might be considered as an attractive approach to treat advanced oral squamous cell carcinoma.
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Figure Legends

Figure 1. Expression of mTOR in osteolytic mandibular squamous cell carcinoma and the effect of temsirolimus on cell viability and migration activity in oral squamous cells.

A. Histomorphometric and immunohistochemical analysis of oral squamous cell carcinoma of the mandibular region. HE-stained sections and immunohistochemical staining of mTOR and p-mTOR in a section of resected mandible. Bn : bone, Tm : tumor tissue. Bar, 200 mm.

B. Growth-inhibitory effects of temsirolimus on HSC-2 cells. An MTS assay was conducted to obtain a relative cell number as reflected by the absorbance at 490 nm in the presence of the indicated amounts of temsirolimus. Significant differences between the indicated groups were defined as *P< 0.05, **P< 0.01.

C. The ability of cell migration under temsirolimus treatment was determined by scratch assay as described in “Materials and Methods”. The number of cells that migrated into the scratched area were counted and displayed in a histogram. **Statistical significance was defined as P < 0.01.

D. Detection of p-mTOR, mTOR, p-S6, S6, p-Akt and Akt expression by immunoblot analysis after temsirolimus (10 nM) treatment in HSC-2 (left panel) or SAS (right panel) cells.
Figure 2. The effects of temsirolimus on osteoclast formation and bone resorption.

A. Temsirolimus suppressed differentiation of RAW264.7 cells into osteoclast induced by RANKL. RAW264.7 cells were treated with RANKL (50 ng/ml) and indicated concentration of temsirolimus in 96 well plates. Cells were fixed and stained for TRAP 5 days after treatment and the number of TRAP-positive multinucleated cells (nuclear number > 3) in each well was counted.

B. Detection of p-mTOR, mTOR, p-IκBα, and Akt expression by immunoblot analysis after RANKL (50 ng/ml) treatment in murine macrophage RAW264.7 cells. β-actin was shown as the control.

C. Temsirolimus suppressed phosphorylation of mTOR and IκBα induced by RANKL in RAW264.7 cells. RAW264.7 cells were treated with RANKL (50 ng/ml) and indicated concentration of temsirolimus for 5 minutes. Immunoblot analysis was performed after the treatment. β-actin was shown as the control.

D. Temsirolimus inhibited bone resorptive activity of mature osteoclasts. Bone resorptive activity of mature osteoclasts differentiated from CD11b+ bone marrow cells were evaluated in...
an OAAS (osteoclast activity assay substrate) as described in “Materials and Methods”. Similar results were obtained in at least three independent experiments. Significant differences between the indicated groups were defined as $*P < 0.05$, $**P < 0.01$. 
Figure 3. The effect of temsirolimus on oral squamous cell carcinoma cells grown in nu/nu mice xenografts.

A. Macroscopic appearance of HSC-2 tumors in nu/nu mice 38 days after tumor inoculation. Upper panel: tumors from control mice. Lower panel: tumors from mice treated with temsirolimus.

B. Volume of tumor was monitored over time (days) after inoculation of tumor. Ten mice were used for each group. Tumor growth was expressed as mean tumor volume ±S.D. **Statistical significance was defined as $P < 0.01$.

C. Body weights of the tumor bearing mice treated with temsirolimus or vehicle only, and non-tumor bearing mice with temsirolimus as a control were monitored. All results were expressed as means ±S.D. *Statistical significance was defined as $P < 0.05$. 
Figure 4. Radiographic and histological analysis of tibial metaphysis bearing HSC-2 cells in mouse models of bone invasion by human oral squamous cell carcinoma after temsirolimus treatment.

A. Representative radiographs of the hind limb of the mouse model of bone invasion after temsirolimus or vehicle treatment. The arrowheads indicate osteolytic lesions. Osteolytic lesion area (mm$^2$) on the radiographs of the hind limb the mouse model of bone invasion treated with temsirolimus or vehicle.

B. Histological and histomorphometric analysis of the bone marrow in the mouse model of bone invasion. The tumor area (mm$^2$) from midtibial osteolytic lesions was calculated using Lumina Vision/OL-analyzing software. Scale bar: 0.5 mm.

C. Histological analysis of osteoclast in the bone marrow. Sections were stained with tartrate-resistant acid phosphatase (TRAP). The number of osteoclasts in the bone marrow was counted. Osteoclasts: OC. Tumor: Tm. Scale bar: 0.5 mm. *Statistical significance was defined as $P < 0.05$. 

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Figure 5. The effect of temsirolimus on PTHrP induced osteolysis and hypercalcemia.

A. The effect of temsirolimus in blood calcium concentration in mouse models for humoral hypercalcemia induced by PTHrP (3mg/week/mouse). Temsirolimus (20 mg/kg) or vehicle was administrated to the mice twice a week after PTHrP induction. Seven mice were used for each group. Results were expressed as means ±S.D. *Statistical significance was defined as $P < 0.05$.

B. The effect of temsirolimus on body weight in mouse models for humoral hypercalcemia. Body weights of the mice after vehicle or temsirolimus treatment were monitored. All results were expressed as means ±S.D. *Statistical significance was defined as $P < 0.05$.

C. Temsirolimus significantly inhibited osteoclast formation induced by PTHrP in murine bone stromal ST2 cells and murine CD11b$^+$ bone marrow cells. These two kinds of cells were cocultured with the indicated concentration of temsirolimus with or without 10 nM PTHrP. Cells were fixed and stained for TRAP 5 days after treatment and the number of TRAP-positive multinucleated cells (nuclear number > 3) in each well was counted. **Statistical significance was defined as $P < 0.01$.

D. Analysis of RANKL and OPG expression after PTHrP (10 nM) treatment with or without of temsirolimus by reverse transcription-PCR (left panel) or immunoblot analysis (right panel) in ST2 cells. The total RNA or protein was extracted 24 hours after treatment. The expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or actin is shown as a control.
Figure 1. Okui et al.
Figure 2. Okui et al.
Figure 4. Okui et al.
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Anti-tumor Effect of Temsirolimus against Oral Squamous Cell Carcinoma Associated with Bone Destruction

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