Antitumor Effect of Novel HSP90 Inhibitor NVP-AUY922 against Oral Squamous Cell Carcinoma

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Abstract. Heat-shock protein 90 (HSP90) is a major cellular chaperone protein. HSP90 supports the correct conformation, stabilization, activation, and localization of ‘client’ oncoproteins, many of which are involved in tumor progression. Therefore, the use of HSP90 inhibitors has become a new strategy in antitumor therapy. However, the effects of an HSP90 inhibitor on oral squamous cell carcinoma are still unclear. NVP-AUY922 (Novartis) is a novel 4,5-diaryloxazole adenosine triphosphate-binding site HSP90 inhibitor. In this study, we investigated the antitumor effect of novel HSP90 inhibitor NVP-AUY922 against oral squamous cell carcinoma. NVP-AUY922 inhibited the proliferation of oral squamous cell carcinoma cells in vitro. NVP-AUY922 caused degradation of client protein inducing ErbB2, p-Akt, p-S6, hypoxia-inducible factor 1-α (HIF1-α) and vascular endothelial growth factor (VEGF) and up-regulation of HSP70 in HSC-2 oral squamous cell carcinoma. NVP-AUY922 increased the expression of cleaved caspase-3 and induced apoptosis in HSC-2 cells. Treatment of NVP-AUY922 induced a robust antitumor response and suppressed p-Akt and VEGF expression in an HSC-2 xenograft model. In summary, NVP-AUY922 exhibits in vitro and in vivo efficiency against oral squamous cell carcinoma, representing a promising therapeutic approach for oral squamous cell carcinoma.

Key Words: HSP90 inhibitor, NVP-AUY922, oral squamous cell carcinoma.
Histochemical and immunohistochemical analysis of surgically resected samples. From 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 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 or radiation therapy before surgery was performed. All tumor samples were obtained after consulting the patients. The sections were sequentially dewaxed through a series of xylene, graded ethanol, and water immersion steps. After autoclaving in 0.2% citrate buffer for 15 minutes, the sections were incubated with 3% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity. Sections were incubated with a 1:200 dilution of antibodies against HSP90 (rabbit IgG) and ErbB2 (rabbit IgG) (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C followed by 3 washes with Tris-buffered saline (TBS). The slides were then treated with a streptavidin-biotin complex (Envision System Labeled polymer, horseradish peroxidase (HRP); Dako, Carpinteria, CA, USA) for 60 minutes at a dilution of 1:100. The immunoreaction was visualized by using 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 then mounted for examination.

Immunoblot analysis. HSC-2 and SAS 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 15 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 by electroblotting to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in 0.1% triton X-100 and incubated with the primary antibodies for 2 hours at room temperature. The membranes were washed 3 times with buffer containing 0.1% triton X-100 and incubated with horseradish peroxidase-conjugated goat anti-rabbit antibodies 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.

Detection of HSP70 and HSP90. After growth on culture dishes, HSC-2 cells were fixed with 100% formaldehyde at room temperature (RT) for 30 minutes, permeabilized, and then incubated at RT for 1 hour with HSP70 or HSP90a primary antibody according to the manufacturer’s procedure (HSP70 and HSP90a Detection kit; Thermo Fisher Scientific K.K., Yokohama, Japan). Dishes were the viewed and analyzed by cellomics ArrayScan VTI HCS Reader (Thermo Fisher Scientific K. K.) using Compartmental Analysis BioApplication Software (Thermo Fisher Scientific K. K.).

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 8×10^6 HSC-2 cells into the dorsal flank as described previously (15). The mice were randomly assigned into two groups (n=8 per group). Each group of the mice was treated with intraperitoneal injection of 100 μl solution containing NVP-AUY922 (20 mg/kg) vehicle only twice a week for 26 days. 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 (r1/2 + r 2/2)^3, where r1=longitudinal radius, and r2=transverse radius, as described previously (16). These experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Okayama University Graduate School of Medicine and Dentistry.

Apoptosis assay. HSC-2 cells were stained with annexin V and propidium iodide using the Annexin V Apoptosis Detection Kit from Pharmingen (San Jose, CA, USA) according to the manufacturer’s protocol. Annexin V-positive cells were detected using flow cytometry with a FACS Caliber system and the Cell Quest Pro software program from Becton Dickinson (San Jose, CA, USA). All experiments were performed in triplicate.

Statistical analysis. Data were analyzed using, posthoc, Bonferroni and Dunnett’s test for the analysis of multiple group comparisons using SPSS statistical software (version 10). Results are expressed as the mean±S.D. P<0.05 were considered statistically significant.

Results

The expression of HSP90 and ErbB2 in mandibular squamous cell carcinoma. Figure 1A illustrates a representative histological pattern in a patient with oral squamous cell carcinoma in the mandibular region. HSP90 and ErbB2 were highly expressed in tumor cells. All cases showed strong intensity of HSP90 and ErbB2 immunoreactivity.

Inhibition of HSP90 by NVP-AUY922 suppressed cell growth of oral squamous cell carcinoma cells. To analyze the antitumor effect of NVP-AUY-922 against oral squamous cell carcinoma in vitro, trypan blue exclusion assay was performed. As shown in Figure 1B, NVP-AUY-922 significantly reduced the number of viable HSC-2, HSC-3, HSC-4 and SAS cells proportionately with increasing dose up to 72 hours after treatment.

NVP-AUY922 suppressed the expression of its client oncoproteins. We analyzed the HSP90 and its client oncoprotein after NVP-AUY922 treatment in HSC-2 cells. Immunoblot analysis demonstrated that levels of ErbB2, p-Akt Ser473 p-S6 Ser235/236, caspase-3, HIF1-α and VEGF were dose-dependently reduced 12 hours after NVP-AUY922 treatment. On the other hand, HSP70 and cleaved caspase-3 were up-regulated 12 hours with increasing amounts of NVP-AUY922. However, NVP-AUY922 did not change the expression of HSP90 and β-actin (Figure 2A). Immunofluorescence analysis revealed that treatment of NVP-AUY922 results in increased HSP70 expression in dose-dependent
manner (Figure 2B and C). These results suggested that NVP-AUY922 caused degradation of client protein including ErbB2, p-Akt, p-S6, HIF1-α and VEGF and induced apoptosis, suppressing cell viability of oral squamous cell carcinoma cells.

NVP-AUY-922 induces annexin V-positive and PI-negative population in HSC-2 cells. We analyzed the apoptotic effect of NVP-AUY-922 against HSC-2 cells using an annexin V and PI assay. We observed an increase in apoptosis of HSC-2 cells after treatment with NVP-AUY-922 for 12 h (Figure 2C). The percentage of apoptotic cells among those treated with NVP-AUY-922 at 10 nM was 18.6%, whereas it was 0.8% in untreated cells.

NVP-AUY922 suppressed oral squamous cell carcinoma xenografts in mice. In order to analyze the antitumor effect of NVP-AUY-922 in vivo, we established oral squamous cell carcinoma xenografts in mice and treated with NVP-AUY-922 for 21 days. The tumor volume was significantly suppressed by NVP-AUY-922 treatment compared to the control group.
Figure 2. Effect of NVP-AUY922 on the expression of HSP90 client proteins, and apoptosis in oral squamous cell carcinoma. A: Detection of HSP90, ErbB2, HSP70, p-Akt, p-S6, caspase-3, cleaved caspase-3, VEGF, and HIF1-α expression by immunoblot analysis after the indicated NVP-AUY922 treatment for 12 h in HSC-2 cells. C and D: Detection of HSP70 and HSP90α after 10 nM NVP-AUY922 treatment for 12 h in HSC-2 cells. Cells were imaged and analysed by using Cellomics Array Scan VTI HSC-reader. D: Flow cytometric analysis of FITC annexin V staining with propidium iodide (PI) after 10 nM NVP-AUY922 treatment for 12 h in HSC-2 cells. Significant differences between the indicated groups are shown as *p<0.05, **p<0.01.
Figure 3. The effect of NVP-AUY922 on oral squamous cell carcinoma cells grown in nu/nu mice. A: Macroscopic appearance of HSC-2 tumors in nu/nu mice 26 days after tumor inoculation. B: Volume of tumor was monitored over time (days) after inoculation of tumor. Eight mice were used for each group. Tumor growth was expressed as mean tumor volume±SD. Statistical significance was defined as *p<0.05, **p<0.01. C: Immunohistochemical analysis of pAkt and VEGF in xenograft tumors.
carcinoma xenograft tumors derived from HSC-2 cells in nude mice. As shown in Figure 3A and B, intraperitoneal injection of NVP-AUY-922 significantly suppressed tumor growth compared to vehicle ($p<0.01$). Further histological examination of tumor indicated that p-Akt and VEGF were highly expressed in tumor cells in the control group. However, the NVP-AUY922-treated group showed weak expression of p-Akt and VEGF (Figure 3C). There were no deaths of mice during the experiments.

**Discussion**

Previous studies have shown that inhibition of Hsp90 has emerged as a promising therapeutic strategy to produce antitumor effects in a wide range of neoplastic malignancies. These effects are believed to reside in cancer cells’ exquisite requirement to maintain a host of deregulated signaling pathways that allow them to survive propagate and spread even under the adverse conditions that transformation, growth deregulation and host defenses normally impose. As many of these pathways rely on proteins whose stability is dependent on HSP90, blockade of this chaperone has a particularly debilitating effect on the multiple signaling networks that maintain cancer cell survival (17, 18). However, the role of HSP90 involvement in oral squamous cell carcinoma is not well understood. In this report, we evaluated preclinical evidence of the potent anti tumor activity of NVP-AUY922 in oral squamous cell carcinoma cell. The targeting of HSP90 has been seized on as an important strategy for developing novel oral squamous cell carcinoma therapeutics.

NVP-AUY922 inhibited oral squamous cell proliferation in vitro with a range from 1 to 10 nM, which was consistent with the concentration described to data (9, 10, 19). The growth inhibition effect has been described previously with other HSP90 inhibitors, including 17-AAG and NVP-BEP800 (6, 20), and likely reflects the dependence of cancer cell on growth factor signaling for their proliferation. The distinct features of HSP90 inhibition include up-regulation of HSP70 and destabilization followed by proteasomal degradation of client proteins involved in oncogenic signaling. Previously, it was shown that NVP-AUY922 treatment induced HSP70 and reduced c-Raf and cyclin-dependent kinase 4 in A2780 cells, indicating sufficient sensitivity of this cell line to HSP90 inhibition (9). One of the client proteins of HSP90 is the ErbB2 protein, which is frequently expressed in oral squamous cell carcinoma (21, 22). In this study, NVP-AUY922 induced the degradation of key client proteins, including ErbB2 and Akt in human oral squamous cell carcinoma cells at a concentration causing growth arrest. The key deregulated signaling cascade, including the ErbB2, Akt and mTOR pathways, contributes to the development of head and neck oral squamous cell carcinoma (23). AZD8931, a reversible inhibitor of signaling by ErbB2 and ErbB3, shows great antitumor activity in head and neck squamous cell carcinoma xenograft and on the downstream signaling pathways, proliferation and apoptosis (24). Inhibition of key downstream signaling pathways in the present study was also detectable through the dose-dependent inhibition of p-Akt, p-S6 and cleaved caspase-3. Our results suggest that Akt and S6 play a key role in the NVP-AUY922 induced apoptosis seen in oral squamous cell carcinoma.

Administration of NVP-AUY922 exhibited efficacy in the oral squamous cell carcinoma xenograft model used here. Inhibition of tumor growth was associated with clear down-regulation of HSP90 client proteins, including Akt inhibition in the HSC-2 xenografts, as we also observed in the in vitro study. The definite in vivo molecular effects also correlated with another important effect of NVP-AUY922, the anti-angiogenic effect. Progression of oral squamous cell carcinoma requires activation of angiogenesis, which depends on HIF1-α-regulated VEGF expression through the Akt signaling pathway (23). Eccles et al. reported that NVP-AUY922 inhibited in vitro endothelial cell function associated with angiogenesis and reduced VEGF in human tumor xenografts (9). Gsapor et al. observed that NVP-AUY922 inhibited both p-Akt and HIF1-α and reduced microvessel density in human tumor xenografts (25). These reports suggest that the anti-angiogenic effect of NVP-AUY922 is dependent on VEGF depletion as shown in the HSC-2 xenograft model. Suppression of HIF1-α expression by NVP-AUY922 might be the consequence of HSP90 client protein proteasomal degradation or reflect inhibition of Akt which stabilizes HIF1-α protein and enhances HIF1-α transcriptional activity (26). In addition, the HIF1-α-VEGF angiogenic pathway is highly reliant on HSP90 and its inhibition results in oxygen-independent proteasomal degradation of HIF1-α (27).

These data provide a preclinical rationale to support further clinical investigation of NVP-AUY922, either as a single agent or in combination, for patients with oral squamous cell carcinoma. Because of the wide array of HSP90 client proteins, translational development of HSP90 inhibitors has gone in many directions (12, 28).

In conclusion, our results indicate that NVP-AUY922 had potent in vitro growth-inhibitory and apoptosis-inducing activity against oral squamous cell carcinoma cells. This effect was mediated through the down-regulation of proteins involved in survival and angiogenesis of cancer cells. Further studies of this agent in patients with oral squamous cell carcinoma are warranted.

**Conflict of Interest**

The Authors have no conflicts of interest to declare.
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