

Antitumor effects of IDN5109 on head and neck squamous cell carcinoma

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Abstract. Taxanes, a new class of antitumor drugs, are effective against a large number of human tumors, although there are problems with drug resistance. The novel taxane, IDN5109, is characterized by its high tolerability, antitumor efficacy, ability to overcome multidrug resistance, and oral bioavailability. We investigated the cellular response of IDN5109 to head and neck squamous cell carcinoma (HNSCC), and compared the antitumor activity of IDN5109 with that of paclitaxel. This is the first demonstration of antitumor effects of IDN5109 on HNSCC. In *in vitro* experiments, IDN5109 showed antiproliferative effects against HNSCC cell lines. After treatment with IDN5109, Bcl-2 and Bcl-XL were down-regulated, Bax was up-regulated, and caspase-3 was activated. After treatment with IDN5109, concentrations of both VEGF and IL-8 in the culture supernatant of HNSCC cells decreased. In *in vivo* experiments, the oral administration of IDN5109 showed antitumor effects against HNSCC tumor xenografts. Immunohistochemistry showed that IDN5109 inhibited tumor angiogenesis and induced apoptosis in HNSCC cells, producing a decreased blood vessel density and increased apoptosis index. On the basis of these results, IDN5109 is useful as a chemotherapeutic agent against HNSCC.

Introduction

Taxanes are a new class of antitumor drugs that inhibit microtubule disassembly (1). They are potent inhibitors of cell growth and cell cycle progression, inducing apoptotic cell death, and are endowed with antiangiogenic properties. The current clinical taxanes, paclitaxel and docetaxel, are

effective against a large number of human tumors with broad activity in solid tumors including ovarian, breast, and non-small cell lung carcinoma, as well as head and neck carcinoma (2,3).

Although paclitaxel and docetaxel are the drugs of choice for many chemotherapeutic regimens, some patients are resistant to taxanes. IDN5109, also a taxane, was originally selected for its ability to overcome drug resistance (4). IDN5109 was derived from the diterpene 14- β -hydroxy-10-deacetylbaicatin III extracted from the needles of *Taxus wallichiana* (5). The therapeutic benefit of IDN5109 was confirmed in preclinical studies. IDN5109 showed an improved pharmacological profile compared with paclitaxel, had antitumor activity after i.v. administration (4), and maintained its antitumor activity after oral administration (6,7).

Taxane-based therapies have shown significant activity in clinical trials in patients with head and neck squamous cell carcinoma (HNSCC) (8-12). Therefore, more effective taxanes may improve the therapeutic options for HNSCC.

The antitumor effect of IDN5109 on HNSCC has not been reported. The purpose of this study was to investigate the cellular response of HNSCC to IDN5109 and evaluate its antitumor efficacy on HNSCC xenografts. We examined cell cycle perturbations, modulation of apoptotic and angiogenic factors, cell growth inhibition, and apoptosis. The efficacy of IDN5109 was compared to that of paclitaxel, the most frequently used taxane in clinical practice (13).

Materials and methods

Drugs. Paclitaxel was provided by Bristol-Myers/Squibb (Wallingford, CT, USA) and IDN5109 was provided by Bayer (Leverkusen, Germany). The chemical structure of IDN5109 is shown in Fig. 1. For the antiproliferative assay, the two taxanes were dissolved in ethanol at 1 mg/ml, then diluted in culture medium. For the antitumor activity study, paclitaxel was dissolved in absolute ethanol, Cremophor ELP and cold 0.9% NaCl solution (5%, 5% and 90% of the final volume, respectively), and IDN5109 was dissolved in Tween-80, absolute ethanol, and 0.9% NaCl solution (10%, 10% and 80% of the final volume, respectively) according to a previously reported procedure (4,14).

Cell culture. Cell lines derived from human HNSCC were used in this study. The origins of these cell lines were oral

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Key words: IDN5109, taxane, head and neck squamous cell carcinoma, oral administration, anti-angiogenesis

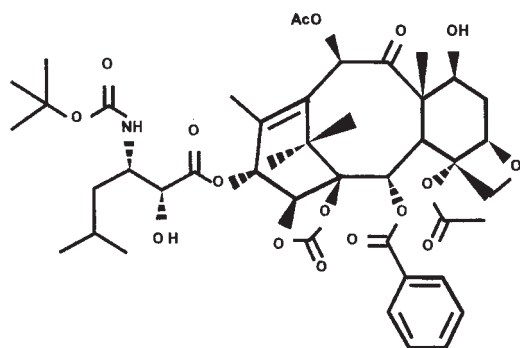


Figure 1. The chemical structure of IDN5109.

floor (YCU OR891), hypopharynx (YCU H891), mesopharynx (YCU M862, KCC M871 and YCU M911), larynx (KCC L871 and YCU L891), tongue (KCC T871 and YCU T873), and maxillary sinus (KCC MS871 and YCU MS861). These cell lines were established in the Department of Otolaryngology, Yokohama City University School of Medicine and the Research Institute, Kanagawa Cancer Center.

All cell lines were maintained in RPMI-1640 medium (Life Technologies Inc., Tokyo, Japan) supplemented with 10% fetal calf serum, 2 mM glutamin, 100 units/ml penicillin, and 100 μ g/ml streptomycin at 37°C in a 5% CO₂ atmosphere.

In vitro antiproliferative assays. The HNSCC cell lines were grown as suspensions, plated in 96-well plates (Falcon; Becton Dickinson Labware, Lincoln Park, NJ, USA) and treated with drugs 24 h after seeding at 5×10^3 cells/well. After 48 h exposure to drugs, the MTT assay was carried out with Tetra Color One (Seigaku Co., Ltd. Tokyo, Japan). Relative growth inhibition was calculated compared to vehicle-treated control cells, and IC₅₀ values were determined as the drug concentrations showing 50% survival.

Cell cycle analysis. For cell cycle analysis, YCU H891 cells were trypsinized and fixed in 70% ethanol after 24 h of treatment with taxanes. Cell cycle perturbations were measured on propidium iodide-stained cells using a FACScan flow cytometer.

Western blot analysis. YCU H891 tumor cells were grown in a 100-mm plastic dish (Falcon), then exposed to IDN5109 (0.005 μ g/ml) for 48 h. After removal of media, cells were washed twice with PBS and lysed with lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 1 mM EGTA, 0.5% CHAPS, 10% glycerol, 5 mM mercaptoethanol, and 0.1 mM phenylmethylsulphonyl fluoride). After the removal of cell debris by centrifugation, protein concentration was determined. Equal amounts of proteins were separated by SDS-PAGE, then transferred onto nitrocellulose membranes. Membranes were reacted with (1:1000 diluted) primary antibodies to Bcl-2, Bcl-XL and Bax (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The reaction was developed using the ECL detection kit (Amersham Biosciences, UK) and exposed to photographic film.

Evaluation of caspase-3. YCU H891 tumor cells (1×10^6) were grown in a 100-mm plastic dish (Falcon), then exposed

Table I. Antiproliferative activity of taxanes.

HNSCC cell line	Primary sites	IC ₅₀ (μ g/ml)	
		IDN5109	Paclitaxel
YCU H891	Hypopharynx	0.012	0.02
KCC L871	Larynx	0.050	0.70
KCC T871	Tongue	0.350	3.90
KCC MS871	Maxillary sinus	0.050	0.10
YCU M862	Mesopharynx	0.070	0.40
KCC M871	Mesopharynx	0.030	2.40
YCU MS861	Maxillary sinus	0.038	0.78
YCU L891	Larynx	0.090	7.60
YCU M911	Mesopharynx	0.070	0.74
YCU OR891	Floor of the mouth	0.018	0.45
YCU T873	Tongue	0.060	3.90

In the proliferation assay described in Materials and methods, tumor cells were exposed to taxanes for 48 h. Data are expressed as IC₅₀, the drug concentration (in μ g/ml) that causes 50% inhibition of cell proliferation.

Table II. Cell cycle distribution in YCU H891 cells with IDN5109 and paclitaxel.

Treatment	G ₀ -G ₁ (%)	S (%)	G ₂ -M (%)
Control	53.7	27.7	18.6
Paclitaxel	20.0	27.1	53.0
IDN5109	3.8	42.9	53.2

Cell cycle distribution in YCU H891 cells treated with IDN5109 (0.01 μ g/ml) and paclitaxel (0.02 μ g/ml). After 24 h treatment, cells were harvested, fixed, stained with propidium iodide, and analyzed by flow cytometry.

to IDN5109 (0.001, 0.01, and 0.1 μ g/ml) for 48 h. After the removal of media, cells were lysed with lysis buffer. After the removal of cell debris by centrifugation, the protein concentration was determined, and caspase-3 activity was measured with an Apocpyto Colorimetric Assay kit (MBL, Nagoya, Japan).

Evaluation of VEGF and IL-8 secretion. YCU H891 cells (1×10^6) were seeded in a 100-mm plastic dish (Falcon). The supernatant of the cells was treated with 0.005 μ g/ml of IDN5109. After 48 h, concentrations of vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) were determined using the Quantine ELISA kit (R&D System, Minneapolis, MN, USA). The protein concentration of these factors was measured by absorbance compared with a standard curve and analyzed by NJ-2100.

In vivo antitumor activity studies. All *in vivo* experiments were carried out using 6-week-old female athymic nude (nu/nu) mice weighing 20-25 g (Oriental Yeast Co., Ltd., Tokyo, Japan). Mice were maintained in laminar flow rooms with constant temperature and humidity. The animals were

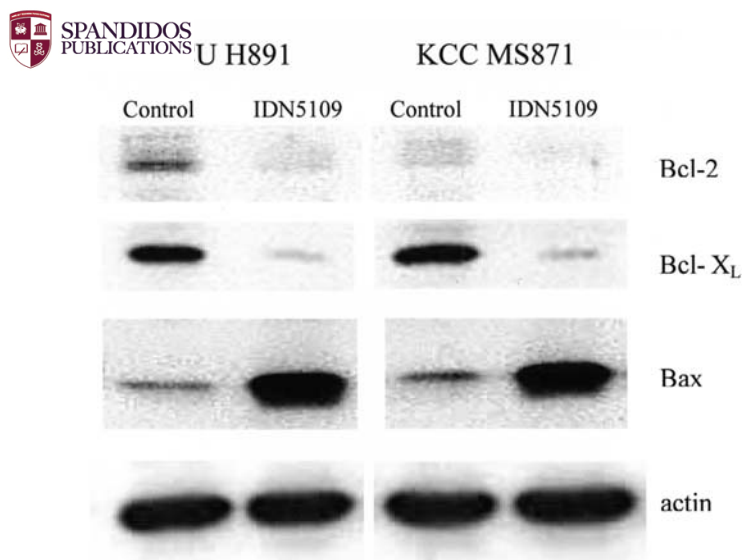


Figure 2. Effect of taxanes on Bcl-2, Bcl- XL, and Bax expression in YCU H891 and KCC MS871 cells. Cells were treated for 48 h. Whole-cell extracts were processed for immunoblotting with anti-Bcl-2, anti-Bcl-XL, or anti-Bax antibodies. After treatment with IDN5109, Bcl-2 and Bcl-XL were downregulated and Bax was upregulated.

maintained and the experiments conducted at the Yokohoma City University School of Medicine Laboratory Animal Facility according to the guidelines for animal experiments set by the Animal Experiment Committee of Yokohoma City University School of Medicine.

YCU H891 cells from *in vitro* cell culture were inoculated subcutaneously into the right flank of athymic mice (1×10^7 cells/mice). Each control or drug-treated group included five mice bearing lateral subcutaneous tumors. Tumors were implanted on day 0, and tumor growth was followed by weekly measurements of tumor diameters with a Vernier caliper. Tumor weight (TW) was calculated according to the formula: $TW \text{ (mg)} = \text{tumor volume (mm}^3\text{)} = d^2 \times D/2$, where d and D are the shortest and longest diameter, respectively. Drug treatment was started when mean TW was 50-100 mm³. IDN5109 and paclitaxel were administered p.o. or i.v. every 4th day, 3 times at a dose of 54 mg/kg. Control mice were given the solvent solution.

Evaluation of vessel density. To evaluate vessel density, an immunohistochemical technique was used. The vessels in the tumor tissues were stained using an antibody to CD31, an endothelial marker (1:200; BD Biosciences, Tokyo, Japan). YCU H891 tumors were harvested and immediately frozen in OCT medium for cryosection. Tumor sections at 6 μ m were prepared and fixed in cold acetone. The sections were blocked with 2% goat serum and 1% bovine serum albumin in PBS, then stained with the antibody. Slides were developed using 3,3'-diaminobenzidine substrate biotinylated peroxidase reagent (Vector Laboratories, Inc., Burlingame, CA). The number of vessels and subsets were scored from a minimum of five microscopic fields from five independent tumors treated with IDN5109 or control groups. The average number of vessels per field was determined under a microscope at a x20 magnification.

Evaluation of apoptosis. Terminal deoxynucleotidyl transferase-mediated cUDP nick-end labeling (TUNEL) was done for evaluation of apoptosis. This was done with an Apoptosis In Situ Detection kit (Wako Chemical, Osaka, Japan) for frozen sections. The number of immunoreactive cells based on the distribution of apoptotic cells was scored from a minimum of five microscopic fields of each section treated with IDN5109 or the control groups. The apoptotic index was shown as a percentage of TUNEL-positive cells relative to the total number of cells. For statistical comparison, TW of treated versus control mice was compared using the Student's t-test (two-tailed), and $p < 0.05$ was considered significant.

Results

Antiproliferative effect of IDN5109 on HNSCC cells. We first determined the antiproliferative effect of IDN5109 on HNSCC cells. Cells were cultured with media alone (control) or media containing IDN5109 (0-1.0 μ g/ml). As shown in Table I, HNSCC cells were more sensitive to IDN5109 than paclitaxel. The IC_{50} of IDN5109 ranged between 0.012 and 0.090 μ g/ml. On the other hand, the IC_{50} of paclitaxel ranged between 0.02 and 3.90 μ g/ml.

Cell cycle distribution. We next examined the mechanism by which IDN5109 caused cell death in HNSCC cell lines. Cell cycle analysis with flow cytometry indicated that exposure to IDN5109 for 24 h induced a significant arrest in the G₂/M phase of the cell cycle, similar to exposure to paclitaxel (Table II). The antitumor effects of paclitaxel are generally believed to result mainly from interference with the normal function of microtubules and blockage of cell cycle progression in the G₂/M phase (15,16). This indicates that IDN5109 has a cell death mechanism similar to that of paclitaxel.

Induction of apoptosis regulators by IDN5109. To further explore mechanisms of molecular cell death induced by IDN5109, we examined the expression of apoptosis regulators Bcl-2 (anti-apoptotic protein), Bcl-XL (anti-apoptotic protein), and Bax (pro-apoptotic protein) after treatment with IDN5109. YCU H891 and KCC MS871 cells were treated with control medium or IDN5109 (0.005 μ g/ml) for 48 h, and Western blot analysis was performed. The effects of IDN5109 treatment are illustrated in Fig. 2. After treatment with IDN5109, Bcl-2 and Bcl-XL were down-regulated and Bax was up-regulated. This shows that IDN5109 has the ability to induce apoptosis.

Activation of caspase-3 by IDN5109. To demonstrate the activity of caspase-3 in apoptosis caused by taxanes, YCU H891 cells were incubated in media alone (control) or media containing IDN5109 (0.001-1.0 μ g/ml) or paclitaxel (0.001-1.000 μ g/ml). Caspase-3 activity was examined using a Colorimetric Assay kit. As shown in Fig. 3, caspase-3 activation was observed in YCU H891 cells treated with IDN5109 with a significant difference between the group treated with IDN5109 and the control. The activation induced by IDN5109 was significantly superior to that by paclitaxel. This indicates that IDN5109 has a higher ability to induce apoptosis than paclitaxel.

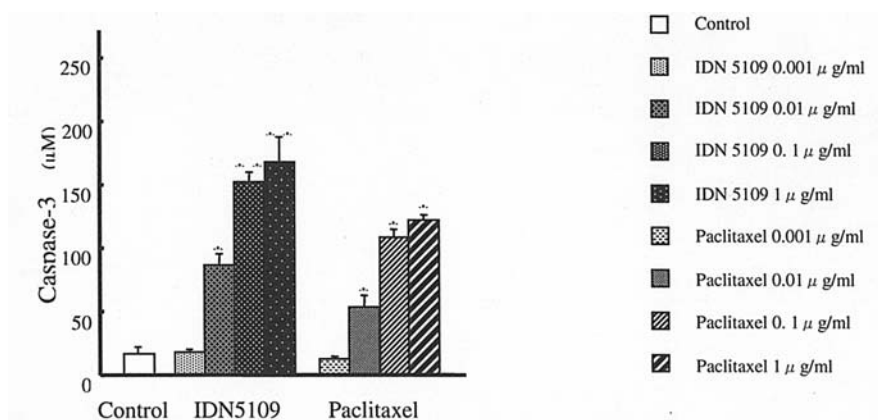


Figure 3. Caspase-3 activity measured with an Apocyto Colorimetric Assay kit was significantly increased with treatment of IDN5109. * $p < 0.05$ versus control. ** $p < 0.01$ versus control.

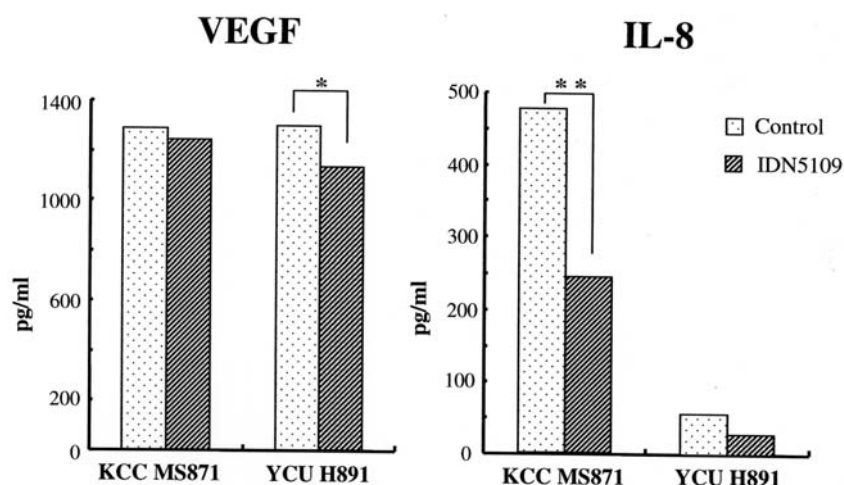


Figure 4. The concentrations of angiogenic factors in supernatant of HNSCC cells, as detected with ELISA. VEGF concentration was significantly decreased in YCU H891, and the IL-8 concentration was significantly decreased in KCC MS871. * $p < 0.05$ versus control. ** $p < 0.01$ versus control.

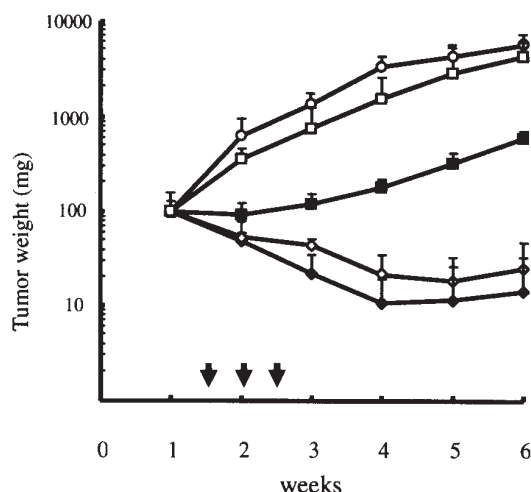


Figure 5. Growth curve of HNSCC YCU H891, xenografted in nude mice after taxane treatment (every 4th day, 3 times). Control (○); 54 mg/kg p.o. paclitaxel (□); 54 mg/kg p.o. IDN5109 (■); 54 mg/kg i.v. paclitaxel (◐); and 54 mg/kg i.v. IDN5109 (◑). Arrows, days of treatment

Suppression of VEGF and IL-8 by IDN5109. The suppression of VEGF and IL-8 was examined by ELISA using YCU H891 and KCC MS871 cells. As shown in Fig. 4, both

factors decreased after exposure to IDN5109 (0.005 $\mu\text{g/ml}$, 48 h). This shows that IDN5109 has the ability to reduce the suppression of these two angiogenic factors.

In vivo studies of the antitumor effects of taxanes on YCU H891. Fig. 5 shows the antitumor effects of IDN5109 and paclitaxel against YCU H891 xenografts. We compared the effects of the two taxanes at the maximum tolerated dose of paclitaxel (4). We also compared the efficacy of i.v. and oral administration of IDN5109, and the efficacy of oral administration of IDN5109 and paclitaxel. The best antitumor efficacy was achieved by IDN5109 given i.v. Oral administration of IDN5109 was less effective than i.v., but there was still a significant difference between the group given IDN5109 p.o. and the control group. Paclitaxel given p.o. did not show any activity.

Inhibition of angiogenesis and induction of apoptosis by IDN5109. To investigate the effect of IDN5109 on tumor neovascularization, an effect that has been reported to contribute to the *in vivo* efficacy of taxanes (17,18), we analyzed the vessel density of the tumors using antibodies against CD31. Fig. 6 shows that treatment with IDN5109 had significant antiangiogenic activity compared with the control.

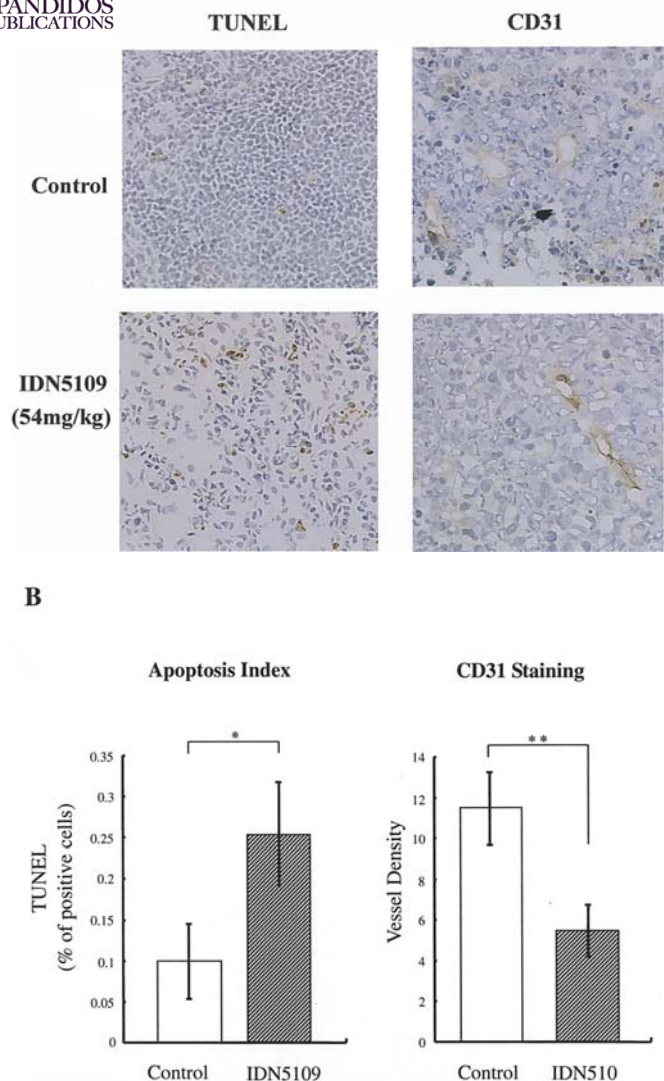


Figure 6. Tissue-based studies of YCU H891 tumor xenografts treated with IDN5109 (54 mg/kg/d). (A) Immunohistochemical analysis of tumor cells stained with an antibody to CD31 and apoptosis by TUNEL (original magnification, x200). (B) Apoptosis index (TUNEL) and CD31 vessel staining. Results are expressed as a percentage of positive cells for each marker (Student's t-test; * $p < 0.05$ and ** $p < 0.01$).

We then examined apoptotic cell death induced by IDN5109 *in vivo*. The treatment with IDN5109 resulted in an increase of TUNEL-positive cells. Quantitative data are summarized in Fig. 6B. The apoptotic index was significantly higher in the group treated with 54 mg/kg of IDN5109 than the control group.

Discussion

This is the first detailed study of the effects of IDN5109 on HNSCC *in vitro* and *in vivo*. In this study, IDN5109 proved to be an effective drug for suppressing the growth of HNSCC cells both *in vitro* and *in vivo*. IDN5109 displayed a higher efficacy than paclitaxel *in vitro* and comparable antitumor activity on nude mice bearing an established xenograft of YCU H891 cells.

Furthermore, IDN5109 given p.o. was active against HNSCC xenografts, whereas paclitaxel given p.o. showed a

complete lack of antitumor efficacy. The hypothesis that IDN5109 given p.o. had a high bioavailability is based on the fact that IDN5109 is a poorly sensitive substrate to P-gp (7), whereas paclitaxel is a highly sensitive substrate as documented in many studies (1,19). P-gp plays a role in drug excretion from the circulation into the intestinal lumen after i.v. treatment and limits oral absorption of the drug (20,21). It has been reported that IDN5109 is the first taxane with good oral bioavailability (7). Although the effects of IDN5109 achieved by oral delivery were lower than those achieved by i.v. treatment at the same dose level, IDN5109 given p.o. was able to suppress the growth of HNSCC cells.

We also examined the mechanism by which IDN5109 caused apoptosis in HNSCC cells. In apoptosis, as a result of triggering apoptotic signals, a cascade of specific cysteine proteases, the caspases (ICE/CED-3 protease family) are activated (22,23). In this study, in YCU H891, IDN5109 induced increased caspase-3 activation, which was involved in IDN5109-induced apoptosis of HNSCC cells.

We also demonstrated that apoptosis regulatory proteins, i.e. Bcl-2, Bcl-XL and Bax, were regulated in IDN5109 to induce apoptotic events in HNSCC cells. Bcl-2 and Bcl-XL proteins serve as suppressors of cell death. On the other hand, Bax proteins are considered inducers of cell death (24). IDN5109 increased Bax expression, while Bcl-2 and Bcl-XL were decreased by IDN5109.

To evaluate apoptotic events *in vivo*, the TUNEL method was used. After treatment with IDN5109, there was an increased number of TUNEL-positive tumor cells and the apoptotic index was significantly higher in tumors treated with IDN5109 than in the control. These results indicated that IDN5109 induced apoptotic events in HNSCC cells and had an antiproliferative effect on HNSCC cells.

Cell cycle analysis with flow cytometry indicated that exposure to IDN5109 induced a significant arrest in the G₂/M phase of the cell cycle, similar to exposure to paclitaxel. Because cells at the G₂/M phase are considered to be highly sensitive to radiation (25-27), many investigators believe that the combination of paclitaxel and radiotherapy may produce an additive or synergistic effect due to the ability of paclitaxel to induce cell cycle arrest at the G₂/M phase. On the basis of the cell cycle analysis, IDN5109 also appears to induce high sensitivity to radiation, similar to paclitaxel.

Tumor angiogenesis is the process leading to the formation of blood vessels within a tumor and plays a key role in cancer cell survival, local tumor growth, and the development of distant metastasis (28). Some chemotherapeutic drugs have been reported to have antiangiogenic activity (29,30). A variety of growth promoting factors, e.g. VEGF, IL-8, and other proteins and peptides, can induce angiogenesis (31). The overexpression of these factors has been positively correlated with lymph node metastasis and prognosis in HNSCC (32,33). It has been suggested that the down-regulation of VEGF can contribute to the angiogenic properties of paclitaxel (34), although the mechanisms underlying such an effect are unknown. In this study, we found that IDN5109 effectively reduced VEGF and IL-8 production in HNSCC cells *in vitro*. The suppressive effect of IDN5109 on VEGF production, as observed in *in vitro*-treated YCU H891 cells, may affect tumor growth *in vivo* both by

acting directly on tumor cells and indirectly suppressing tumor neovascularization as in Fig. 6.

In conclusion, we showed that IDN5109 was a highly active agent for HNSCC cells by inducing apoptosis and inhibiting angiogenesis. Even after p.o. administration, it appears to have good efficacy and is a useful chemotherapeutic agent against HNSCC cells.

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