

## Head and Neck Squamous Cell Carcinoma Cell Lines are Highly Sensitive to the New Taxanes, BMS-184476, BMS-188797, *In Vitro*

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**Abstract.** *Background:* The development of synthetic taxanes targets at increasing solubility of the drug, improved clinical efficacy and reduced side-effects. We evaluated the sensitivity of head and neck squamous cell carcinoma cell lines to BMS-184476 and BMS-188797 *in vitro*. *Materials and Methods:* The effects of paclitaxel and the synthetic taxanes were tested on six recently established cell lines (3 oral cavity SCC, 3 laryngeal SCC) using the 96-well plate clonogenic assay. The IC<sub>50</sub> values, corresponding to the mean inactivation dose, were obtained from the dose response curves. *Results:* All tested cell lines were considerably more sensitive to both synthetic taxanes compared to paclitaxel. As a rule, the IC<sub>50</sub> concentration for paclitaxel was 4-5.5 times higher than that of BMS-184476 or BMS-188797. The sensitivity to these drugs varied from cell line to cell line, and time lapse video microscopy showed a mitotic arrest with subsequent apoptosis after four hours with BMS-188797. *Conclusion:* These results indicate that the synthetic taxanes could be useful clinically and warrant *in vitro* testing in combined modality models, like concurrent chemoradiation.

Surgery is the standard treatment in head and neck squamous cell carcinoma (HNSCC). Pre- or postoperative radiation is often combined with the treatment. Combined modality therapies have been used to an increasing extent with paclitaxel being successfully used in these combinations. Concurrent chemoradiation trials have given promising results in locoregionally advanced HNSCC (1-3).

Paclitaxel has established efficacy in a number of different tumors (4). It has been successfully used in the

treatment of several solid tumors during the past decade. It has been incorporated in the regimen in the treatment of breast, ovarian, lung and head and neck cancers (5-8). It has also been successfully used in chemoradiation protocols (5). Side-effects like hypersensitivity reactions (HSR), neutropenia and neurotoxicity are known problems during the treatment and, therefore, proper premedication is required (4). The development of new taxanes has been extensive in order to diminish the side-effects, increase the solubility of the drug, hopefully increase the efficacy or widen the antitumor spectra, or even conquer the resistance to taxanes.

New taxanes have given promising results in both preclinical and preliminary clinical settings (9,10). The new taxane analogues BMS-184476 and BMS-188797 have proven to be effective and the side-effects have been fewer than with paclitaxel (11-14). Antitumor activity in taxane-refractory solid tumors has also been demonstrated with these new taxanes (15).

The purpose of this study was to test the effects of two new taxanes, BMS-184476 and BMS-188797, on head and neck squamous cell carcinoma (SCC) cell lines *in vitro*. Comparison with the effects of paclitaxel on these cell lines was done simultaneously.

### Materials and Methods

*Cell lines.* Six head and neck SCC cell lines were used in the study. All cell lines used were established in the University of Turku, Finland. UT-SCC-1A and -33 are SCCs from the oral cavity and UT-SCC-9, -11 and -34 are SCCs of laryngeal origin. UT-SCC-2, -33 and -34 were established from primary tumors, UT-SCC-1A and -11 from local recurrence and UT-SCC-9 from a neck metastasis (16,17). Characteristics of the cell lines are listed in Table I.

*Cell culture.* Prior to the experiments, the cells were grown in Dulbecco's modified Eagle's minimal essential medium containing 2 nM glutamine, 1% nonessential amino acids, 100 U/ml penicillin, 100 U/ml streptomycin and 10% fetal bovine serum (FBS). The cells were kept in logarithmic growth by passing them weekly or bi-weekly.

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Table I. Characteristics of the cell lines.

Cell line	Clinical stage <sup>a</sup>	Primary tumor location	Specimen site	Type of lesion
UT-SCC-1A	T2N1M0	gingiva	gingiva	recurrence
UT-SCC-2	T4N1M0	floor of mouth	floor of mouth	primary
UT-SCC-9	T2N1M0	glottic larynx	neck	metastasis
UT-SCC-11	T1N0M0	glottic larynx	larynx	recurrence
UT-SCC-33	T2N0M0	gingiva of mandible	gingiva of mandible	primary
UT-SCC-34	T4N0M0	supraglottic larynx	supraglottic larynx	primary

<sup>a</sup>TNM status of primary tumors according to AJC (American Joint Commission of staging)

Table II. The plating efficiencies, passages used and the previously evaluated intrinsic radiosensitivities of the six HNSCC cell lines.

Cell line	Plating efficiencies	Passages used	Intrinsic radiosensitivities (Gy)
UT-SCC-1A	0.18-0.37	17-28	1.7 ± 0.3
UT-SCC-2	0.22-0.79	12-33	1.8 ± 0.2
UT-SCC-9	0.13-0.84	3-28	1.4 ± 0.1
UT-SCC-11	0.31-0.77	19-33	2.0 ± 0.2
UT-SCC-33	0.25-0.63	17-32	2.0 ± 0.2
UT-SCC-34	0.38-0.65	19-30	2.0 ± 0.1

Table III. Taxane sensitivities of the HNSCC cell lines.

Cell line	Paclitaxel	BMS-184476	BMS-188797
UT-SCC-1A	2.1±0.1 nM	0.41±0.15 nM	0.42±0.18 nM
UT-SCC-2	1.2±0.1 nM	0.080±0.025 nM	0.32±0.12 nM
UT-SCC-9	1.5±0.1 nM	0.26±0.12 nM	0.35±0.19 nM
UT-SCC-11	1.6±0.1 nM	0.50±0.10 nM	0.53±0.15 nM
UT-SCC-33	1.3±0.1 nM	0.24±0.11 nM	0.34±0.08 nM
UT-SCC-34	1.5±0.1 nM	0.11±0.03 nM	0.25±0.02 nM

**Drug preparation.** Paclitaxel (Taxol®, Bristol-Myers Squibb, Espoo, Finland) was received as an infusion concentrate 6 mg/ml. BMS-184476 and BMS-188797 (Bristol-Myers Squibb) were received in powder form and dissolved initially in ethanol. All three drugs were dissolved in 0.9% sodium chloride for solutions of 0.1 mM. Stock solutions of 100 nM were prepared in Ham's F-12 medium, kept at -18°C and thawed immediately before the experiments. Final dilutions of 0.05-1.0 nM were used.

**Clonogenic assay.** The cells were grown in T25 culture flasks in the midlogarithmic phase (40-60% confluency) and fed with fresh medium on the day before plating for the experiments. The clonogenic assay was performed as described earlier (18). In brief, the cells were harvested by trypsin/EDTA, counted and diluted to a stock solution of 4167 cells/ml. Further dilutions of this single cell suspension, with or without the drug, were made in 50 ml of Ham's F-12 medium containing 15% FBS. The cells in that suspension were plated in 96-well culture plates by applying 200 µl/well using an octapipette. The number of cells plated per well was adjusted by the plating efficiency (PE) of each cell line. The plates were incubated in a water vapour saturated atmosphere containing 5% CO<sub>2</sub> at 37°C. After four weeks, the number of positive wells was counted using an inverted phase-contrast microscope. Wells with colonies consisting at least 32 cells were considered positive. The drugs were allowed to remain in the plates during the whole incubation period.

**Data analysis.** The PE was calculated using the formula  $-\ln(\text{number of negative wells} / \text{total number of wells}) / \text{number of cells plated per well}$ . Fraction survival data as a function of the drug dose were found to be fitted in the linear quadratic equation. A

microcomputer program was used to fit data to  $F = A * \exp[-(\alpha D + \beta D^2)]$ . IC<sub>50</sub> values, corresponding to the 50% inhibition of surviving fraction, were measured to assess the *in vitro* sensitivity of the cell lines to the drugs. IC<sub>50</sub> values were obtained from the dose response curves after fitting the data to the LQ model.

**Time-lapse video microscopy.** One day after plating, prior to starting the experiments, the cells were fed with fresh medium or a medium containing 5 nM paclitaxel, 5 nM BMS-184476 or 5 nM BMS-188797. The medium was equilibrated with 5% CO<sub>2</sub> at 37°C in a incubator for 10 minutes. The culture flask was then capped and transferred to a 37°C heated stage of an inverted microscope (Nikon, Diaphot, Nikon Corp., Tokyo, Japan) and the filming was started. Cells were viewed using phase-contrast optics at 20x objective magnification coupled to a JVC 3CDD KY-F30 video camera (Victor Company, Tokyo, Japan). An edge of a representative colony was selected for the field to be analysed containing 27 - 60 cells (median 40 cells). The time-lapse video recording was performed so that two successive pictures were taken at 30-second intervals (Panasonic AG-6720A). The video recorder and the microscope were coupled to a timer (LIBT2, Red Lion, USA) which lit the microscope lamp for 5 seconds in every 30 seconds in synchrony with the recorder. The culture flask was shielded from ambient room light.

Filming was continued for 72 hours. Subsequently the film was viewed, frame by frame, on a video monitor. The cumulative numbers of premitoses, mitoses, apoptoses, multinucleated cells and necroses per field were counted at 24-hour intervals. Premitosis was considered to have begun when a cell became round and condensed. Mitosis was considered to have ended with the appearance of cell division. The cells were considered to be

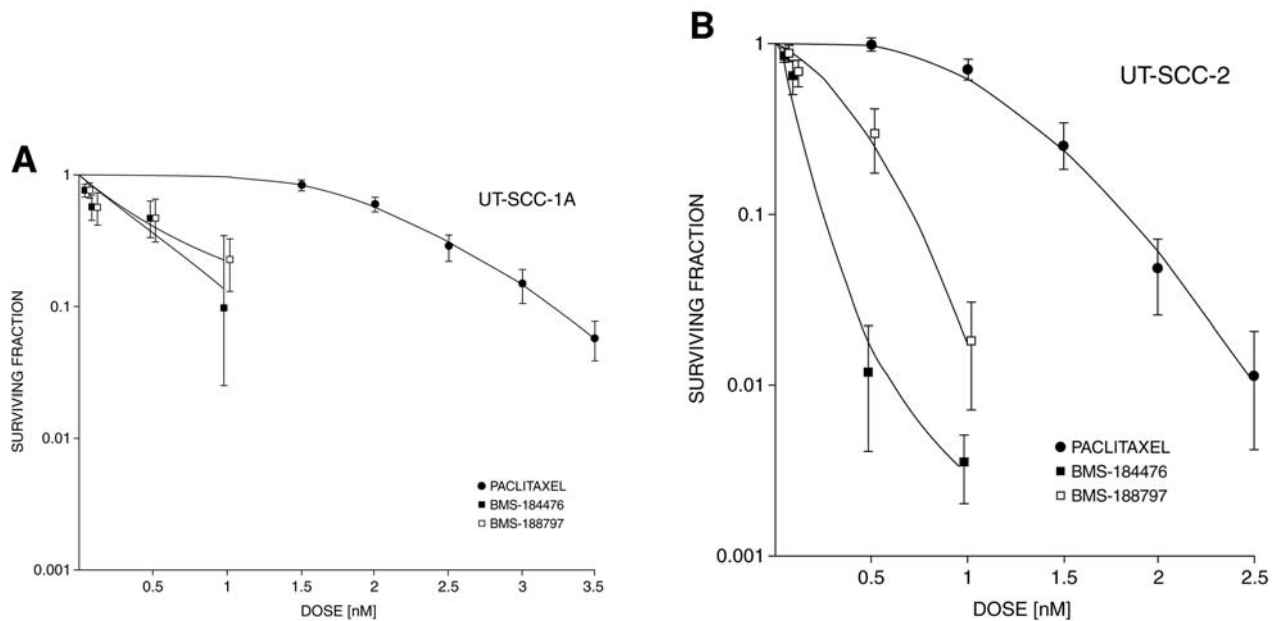


Figure 1. Sensitivity of HNSCC cell lines, UT-SCC-1A and -2, to taxanes *in vitro*. The figure shows the fraction survival curves as function of taxane dose. The results are given as the average of the actual data points, and the bars represent one SD. The data were fitted by the linear quadratic equation to produce the fraction survival curves.

Table IV. Time-lapse videomicroscopy. The mitotic and apoptotic frequency of the cell lines after 5 nM of taxane. The values used are cumulative and ratios to initial cell number. Initial cell numbers are given in parenthesis.

	Mitoses/initial cell no.	Apoptosis/initial cell no.
Control	1.18 (27) 1.46 (50)	0.18 (27) 0.12 (50)
Paclitaxel 5 nM	0.7 (60)	0.13 (60)
BMS-188797 5 nM	0 (30)	0.5 (30)
BMS-184476 5nM	0.5 (52) 0.7 (28)	0.1 (52) 0.14 (28)

multinucleated when the condensed cell again became flat without producing a daughter cell (morphologically multinucleated). An apoptotic cell death was recorded either when a flat cell condensed rapidly or an already condensed cell (considered as premitotic) died after violent cytoplasmic pulsation and blebbing. Necrosis was characterised by a non-cycling cell dying after a rapid swelling and rupture of cell membranes. The time in premitosis was calculated by following the individual cells from the beginning of premitosis to the beginning of apoptosis (19).

## Results

Six SCC cell lines were evaluated in this study. The plating efficiencies, passages used and the previously evaluated intrinsic radiosensitivities (expressed as area under the survival curve in the 96-well plate clonogenic assay) of the cell lines (16,17) are listed in Table II.

The sensitivities of the cell lines to paclitaxel, BMS-184476 and BMS-188797 expressed as  $IC_{50}$  values are listed in Table III. The dose-response curves in Figure 1 represent the effects of these three drugs on clonogenic growth on individual HNSCC cell lines.  $IC_{50}$  values for paclitaxel varied between 1.2-2.1 nM, mean 1.5 nM. The  $IC_{50}$  values of the two taxane analogues were significantly lower: the  $IC_{50}$  values of BMS-184476 varied between 0.080- 0.50 nM, mean 0.27 nM, and those of BMS-188797 ranged from 0.25 nM to 0.53 nM, mean 0.37 nM. The difference between the  $IC_{50}$  values of original paclitaxel and the analogues was 4-5.5 fold. Furthermore, with three of the cell lines, UT-SCC-2,-33 and -34, the effect of BMS-184476 was even more potent than with BMS-188797.

Time-lapse video microscopy was used to analyse the morphological changes induced by 5 nM paclitaxel, BMS-184476 and BMS-188797 in the cultured laryngeal SCC cells. In Table IV the results are shown as the ratio of events to the initial cell number. As expected on the basis of our previous experiments, the control cultures of all cell lines showed frequent mitoses (19). Apoptoses were also seen in the control cultures; they represented 12% and 18% of the initial

cell number. In the cultures treated with 5 nM paclitaxel the amount of mitoses decreased slightly but the amount of apoptoses was unchanged. Five nM BMS-184476 had the same effect as 5nM paclitaxel but 5 nM BMS-188797 could prevent all mitoses and induced apoptosis. These cells stayed mitotically arrested for a median 4 hours (range 1h 40min to 24h), after which the cells died morphologically by apoptosis *i.e.* by cellular shrinkage, violent pulsation and blebbing of the plasma membrane during subsequent hours. The apoptoses represented 50% of the initial cell number while the rest of the cells remained morphologically in interphase (Table IV).

## Discussion

Paclitaxel has already become a standard treatment in several cancers such as breast, ovarian, lung and head and neck cancers (5-8). Currently, it has also been incorporated in many chemoradiation protocols (3). The optimal dosage of paclitaxel has been under investigation and it has been presumed that better antitumor effects could be reached with shorter and more intense schedules (20). Effective peak plasma concentrations could be reached using 3-hour weekly administration, but then again the side-effects may limit the optimal dosage. On the other hand, the efficacy of paclitaxel may depend on the exposure duration rather than peak concentration; with continuous infusion, highest cumulative paclitaxel doses could be reached (21). It may be presumed that with the new taxanes, with greater potency, a smaller quantity of the analogue may be required to produce the cytotoxic effects than with paclitaxel. However, there are also other properties that influence the final therapeutic index of the new drugs.

The development of synthetic taxanes has been focused on reducing side-effects. Cremophor EL is thought probably to be the main cause of the HSRs (22). By increasing the solubility of the drug, the amount of this harmful vehicle could be reduced, and the clinical efficacy of the drug may thereby be improved (10). Novel taxane analogues possess a single structural modification from paclitaxel and they seem to have improved solubility in aqueous cosolvents (9). Three-fold less Cremophor EL is needed to formulate BMS-184476 than to formulate paclitaxel (10). Interestingly, the new taxanes have presented not only lesser side-effects, but also somewhat superior efficacy in both *in vitro* and *in vivo* studies, as well as in clinical settings (11,12).

In the course of searching for novel taxane analogues, two C-7 paclitaxel ethers have been found to provide an efficacy advantage over paclitaxel (23). These two analogues, BMS-184476 and BMS-188797, have demonstrated improved efficacy compared to paclitaxel or docetaxel in a number of rodent solid tumors and human xenografts (11). The feasibility of BMS-184476 has been assessed in phase I studies (10,13,14,24) and has now proceeded to phase II (12). In the current study, the new taxane analogues BMS-184476 and BMS-188797 showed

a considerable efficacy on HNSCC *in vitro*. Six HNSCC cell lines were found to be sensitive to paclitaxel in nanomolar concentrations, but the influence of the two new taxanes on these cell lines was even more effective. The IC<sub>50</sub> values for paclitaxel varied between 1.2-2.1 nM, which is well below the achievable plasma concentrations. Previously, we found vulvar SCC and ovarian carcinoma cell lines also to be sensitive to paclitaxel in nanomolar concentrations; as a matter of fact, the mean IC<sub>50</sub> value of eleven vulvar SCC cell lines was exactly the same as the mean of 1.5 nM of the six HNSCCs tested in the current study (25,26). Remarkable sensitivity to the two new taxanes BMS-184476 and BMS-188797 was seen, mean IC<sub>50</sub> values being 0.27 and 0.37 nM, respectively. There was variance between the two analogues from cell line to cell line, BMS-184476 being the more potent of the two tested analogues, although both of the analogues were significantly and consistently more effective than paclitaxel. Over four-fold concentrations of paclitaxel were needed for equivalent cytotoxic effects when compared to the concentrations of the new taxane analogues. Kim *et al.* (27) reported similar differences between the efficacies of paclitaxel and the new taxanes with human lung cancer cells *in vitro*, as was found in this study. Additionally, in the current study we tested the effects of the three drugs at a concentration of 5 nM, with time-lapse video microscopy. With these considerably low doses the mitotic frequency of the cell lines treated with paclitaxel or BMS-184476 was slightly decreased, but the apoptotic frequency was unaffected. However, even with this low concentration a complete mitotic inhibition and subsequent induction of apoptosis after the mitotic arrest was seen with BMS-188797. Furthermore, no correlation between the effects of the tested taxanes and the intrinsic radiosensitivity of the cell lines could be seen. Nor has any correlation between these two properties been found earlier in our works with paclitaxel in SCC cell lines (19,26). This must be considered as an advantage in the concomitant chemoradiation setting.

Novel taxane analogues have also been demonstrated to have effect on paclitaxel-resistant cell lines (10,23). This could, along with other advanced properties, give the new drugs an advantage over the original cytotoxic agent. The increased efficacy with lower drug concentrations does not necessarily predict better therapeutic index, but when the amount of the solvent, Cremophor EL, could be decreased, leading to less side-effects, the benefit is obvious.

In the current study, remarkable efficacy of the two novel taxanes, BMS-184476 and BMS-188797, on HNSCC cell lines *in vitro* was seen. *In vitro* testing of the novel taxanes concurrently with radiation is warranted.

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