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Hedgehog Targeting by Cyclopamine Suppresses Head and Neck Squamous Cell Carcinoma and Enhances Chemotherapeutic Effects

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Abstract. *Background: The hedgehog signaling pathway (HH) is involved in tumorigenesis in a variety of human malignancies. In head and neck squamous cell carcinomas (HNSCC), Hh overexpression was associated with poor prognosis. Therefore, we analyzed the effect of Hh signaling blockade with cyclopamine on colony formation of cells from HNSCC samples. Patients and Methods: HNSCC biopsies were cultured alone for reference or with serial dilutions of cyclopamine (5-5,000 nM), docetaxel (137.5-550 nM), or cisplatin (1,667-6,667 nM) and their binary combinations. Cytokeratin-positive colonies were counted after fluorescent staining. Results: Cyclopamine concentration-dependently inhibited HNSCC ex vivo [(IC_{50}) at about 500 nM]. In binary combinations, cyclopamine additively enhanced the suppressive effects of cisplatin and docetaxel on HNSCC colony formation. Conclusion: Our findings define SMO – a Hh component- as a potential target in HNSCC and suggest the utility of Hh targeting in future multimodal treatment regimens for HNSCC.*

Head and neck squamous cell carcinoma (HNSCC) is a highly aggressive cancer with poor prognosis and low overall survival (1). Worldwide incidence rates are at approximately 650,000 new cases every year, primarily diagnosed in advanced stages (Union for International Cancer Control (UICC III/IVA/IVB) with about 50% five-year survival; the prognosis is worse for those with metastatic disease (2). Besides known risk factors (*e.g.* alcohol and smoking) and occupational exposure to other poorly understood factors, the entirety of the relevant tumor biology is still unclear. Mortality rates of HNSCC have remained almost unchanged for decades, even though the detection of tumor-specific pathways has developed rapidly and offers a number of new targets in tumor therapy regimes (*e.g.* the epidermal growth factor-receptor, EGFR, and its tyrosine kinases). Proven standardized concepts of tumor therapy in first- or second-line protocols are combinations of well-established cytostatic drugs such as platinum-derivates like cisplatin, taxanes like docetaxel or others (*e.g.* 5-fluorouracil, 5-FU), and their binary or tertiary combinations. These are also recommended as treatment options together with targeted therapy. Agents successfully used in targeted therapy studies in HNSCC, so far, are mainly those targeting EGFR or interfering with its intrinsic tyrosine-kinase activity (by tyrosine-kinase inhibitors; TKI). To date, only cetuximab (Erbitux®), a monoclonal antibody acting as an EGFR inhibitor, enhancer of EGFR degradation and trigger of antibody-dependent cell-mediated cytotoxicity, has been approved for HNSCC treatment. Data regarding its value in combination with radiotherapy or single-agent treatment have been published (3, 4). This first monoclonal antibody for HNSCC therapy aroused the vision of more efficient targeted treatment in the near future because the EGFR pathway is only one of numerous molecular pathogenic factors which seem to be responsible for HNSCC development and spread. These factors include numerous pathways which are either dysregulated due to the presence of oncogenic proteins of human papillomavirus (HPV) subtypes and involved in cell-cycle dysregulation (*e.g.* CDKN2A [p16INK4A] or increased p53 degradation), but also altered expression and signaling of pathways involved in angiogenesis and vascularization [vascular endothelial growth factor (VEGF) and its receptors (VEGF -R-1,2, and 3)], degradation of the extracellular matrix [*via* activated matrix metalloproteinases (MMP)], and are mostly caused by genomic instability and somatic

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Key Words: Head and neck squamous cell carcinoma (HNSCC), hedgehog pathway, cyclopamine, cisplatin, docetaxel, synergism.

mutations (5). Some of the aberrant pathways might offer additional potential targets for tumor therapy in the near future. In this context of potential biomarkers and key molecules in tumor development, a growing number of studies have now also focused on the hedgehog signaling pathway (HH) and suggested its pivotal role in carcinogenesis. It is one of the most interesting and fundamental pathways in embryo development, and stem cell regulation, and even carcinogenesis. In the recent past, (re)activated Hh pathway and overexpression of its signaling components have been detected in a variety of solid human malignancies, and it was held responsible for tumorigenesis, spread, and poor prognosis in tumors of the pancreas (6), prostate (7), breast (8), and stomach (9). Promising anti-tumoral results in animal xenograft models by blocking the Hh signaling pathway were published. In brain and skin tumors, phase II studies with Hh pathway inhibitors (*e.g.* GDC-0449) have been successfully performed, and the primary data have aroused the hope of clinical researchers (10). However, the relevance of the Hh pathway in HNSCC is still unclear. The first hints of the importance of Hh expression in HNSCC were published by Chung *et al.* (11), who observed an association of high GLI1-expression, the transcription factor of the Hh pathway, with poor survival of patients with head and neck cancer treated with radiation therapy. The first data about Hh protein (over)expression in HNSCC were published recently by Schneider *et al.* (12) and Wang *et al.* (13), but they did not consider all components. Our own quantitative analysis of Hh expression in HNSCC underlines the meaning of SHH, GLI2 and in particular GLI1 (14). Assuming that Hh overexpression in HNSCC influences tumor growth and spread, Hh inhibitory agents might have beneficial effects, and may possibly add to the tumor-inhibiting effects of cytostatic drugs. The aim of this study was to determine the effects of the steroid alkaloid cyclopamine, a smoothened (SMO) and subsequent Hh signaling inhibitor, on tumor growth and colony formation of epithelial cells from HNSCC *ex vivo*. We initially investigated the impact of cyclopamine on growth of the cell-line KB, but, however, mainly focused on the impact of cyclopamine on colony formation of primary epithelial cells derived from biopsy samples of HNSCC. We were interested in its ability to affect the latter if applied alone or in combination with the guideline-conforming cytostatic drugs cisplatin and docetaxel and analyzed the mode of action exerted by cyclopamine in binary mixtures. To the best of our knowledge, this is the first study on the effect of Hh blockade in HNSCC.

Patients and Methods

After receiving informed consent from patients, tumor biopsies were taken under general anesthesia from 49 patients suffering from HNSCC during panendoscopy or definitive surgery of either primary tumors or recurrent disease (Table I). After resection, probes were immediately placed into tubes containing tumor medium (TM; see below) and immediately transferred to the laboratory, weighed, and studied by means of a quality controlled colony formation assay (FLAVINO assay) under flavin-protecting conditions as described previously (15-18). Since colony formation of the tumors *ex vivo* was not always sufficient for analysis of dose-response curves, not all of them could be investigated. Included in this study were 18 HNSCC samples fulfiling the inclusion criteria of pathophysiologically confirmed HNSCC plus sufficient colony formation of epithelial cells [≥4 colonies consisting of ≥3 vital cells, (CF_{EC})] in untreated controls. The stages of the 18 further investigated HNSCC following the criteria of the American Joint Committee on Cancer (AJCC) (19) are shown in Table I. The investigational study was approved by the Ethics Committee of the Medical Faculty of the University Leipzig (no. 201-10-12072010 and no. 202-10-1207210).

Table I. *Description of samples from patients with head and neck squamous cell carcinoma.*

Number	Gender	Localization	TNM	UICC	Grading
1	M	Paranasal sinus	T4aN0M0	IVA	2
2	M	Larynx	T3N3M0	IVB	2
3	M	Tonsil	T2N1M0	III	3
4	M	Larynx	T2N2bM0	IVA	3
5	M	Tonsil	T3N2cM0	IVA	3
6	M	Oral cavity	T4aN2cM0	IVA	2
7	M	Supraglottic	T2N2bM0	IVA	3
8	F	Metastasis cervical	TxN3M0	IVB	3
9	M	Tonsil	T3N2bM0	IVA	Not defined
10	M	Metastasis cervical	T0NxM1	IVC	2
11	M	Metastasis cervical	TxNxM1	IVC	2
12	M	Nasopharynx	T4aN2bM0	IVA	Not defined
13	M	Oral cavity	T4aN0M0	IVA	2
14	F	Larynx	T4aN1M0	IVA	G2
15	M	Oral	T4bN3M0	IVB	2
16	M	Oral	T2N0M0	II	3
17	M	Metastasis cervical	TxN2bM0	IVB	3
18	M	Paranasal sinus	T4aN0M0	IVA	3

Localization, stage [TNM, Union of International Cancer Control (UICC)], and grading of biopsies analyzed in the FLAVINO assay according to the Cancer Staging Manual (Sixth Edition) published by the American Joint Committee on Cancer, 2009 (19).

Materials. TM was used as the cell culture medium for chemoresponse testing of the HNSCC samples as well as of the KB cells for quality control. TM consisted of phenol red and flavin-free RPMI-1640 (Bio&Sell, Feucht, Germany) supplemented with 10% (v/v) fetal calf serum (FCS; Invitrogen®, Darmstadt, Germany), amikacin, nystatin, penicillin, and streptomycin (all from Sigma, Munich, Germany). Following the addition of FCS, TM contained 20 nM of riboflavin. The complete TM underwent sterile filtration (0.22 μM) before use. All experimental steps were carried out utilizing flavin-protecting conditions (avoidance of adverse flavin-mediated photo-induced reactions by illumination without short-wavelength light either using low pressure sodium discharge lamps (SOX-18W; Philips, Eindhoven, the Netherlands) with discrete

excitation wavelengths of λ=589 nm and λ=589.6 nm), or neon light illumination in the yellow spectrum above λ=550 nm (OSRAM L 36W/62; OSRAM AG Munich, Germany). Cisplatin was purchased from Sigma. Docetaxel (Taxotere®) was purchased as a pharmaceutical preparation from Sanofi-Aventis Deutschland GmbH (Berlin, Germany).

FLAVINO assay. KB cell chemoresponse assay. For quality control purposes and examination of reproducible test conditions alongside each HNSCC colony formation assay, a previously described KB cell chemoresponse assay (21) was carried out simultaneously. Briefly, KB cells from stock cultures adapted to RPMI-1640 free of phenol red and containing only 20 nM riboflavin were harvested and 10,000 viable cells were added to each well of a microtiter plate containing dilutions of either cyclopamine, cisplatin, or docetaxel derived from exactly those dilutions created for the *ex vivo* test of the individual HNSCC samples and were adjusted for the indicated concentrations. KB cells were incubated for 72 h (36.5˚C, 3.5% CO_2, humidified air) and thereafter analyzed using a modified version of the 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) bioassay. The inhibitory concentrations (IC) of cytostatic drugs leading to diminished proliferation or even cytotoxicity were calculated, *i.e.* the concentration reducing the metabolic capacity of the cells [that is reflected by the optical density (OD) at λ=570 nm which is closely related to the number of viable cells] to 50% of the OD in control wells receiving only medium (IC_{50}). The IC_{50} values were compared to the previously published IC_{50} values for KB cells (20, 21).

HNSCC colony formation assay. A tissue sample from each patient with HNSCC was minced into pieces of about 1 mm^3, transferred into pre-warmed TM and disintegrated using collagenase type IV (230 mU/ml; Sigma). After 16 h of incubation, the digests were washed. The pellet of disintegrated HNSCC was resuspended and pipetted into wells of fibronectin-coated 48-well microtiter plates (Greiner Bio-One, Nürtingen, Germany) containing serial dilutions of cyclopamine, cisplatin or docetaxel in duplicate, and also binary combinations of four cyclopamine and three cisplatin, or four cyclopamine and three docetaxel concentrations, and a quadruplicate treated only with solvent for reference (control). As a result of the sometimes limited sample size, and despite the aim of starting the chemoresponse test by adding 4 mg of collagenase digest, a median of 2 mg/well (interquartile range 1.7-2.0 mg) of HNSCC digests were seeded per well. The applied concentrations of cisplatin and docetaxel were chosen based on data specifying the tolerable plasma level (TPL) in humans [cisplatin: 6.67 μM (22), docetaxel: 0.55 μM (23)]. After 74 h at 36.5˚C and 3.5% CO_2 in humidified air, supernatants were withdrawn and the wells gently washed twice using phosphate-buffered saline. Adherent cells and cell colonies were fixed with 90% (v/v) ethanol and air-dried. Staining of epithelial cells facilitating differentiation of epithelial and stromal cell colonies was performed using a murine monoclonal antibody reacting with seemingly all cytokeratins (Santa Cruz Technologies, Santa Cruz, CA, USA). This antibody was applied at a dilution of 1:400 and cells were incubated for at least 90 min, but mostly overnight to achieve proper staining. Bound anti-cytokeratin antibodies were visualized using a Cy2-labeled polyclonal goat-anti-mouse IgG (Jackson Immune Research, Suffolk, UK), also applied at a 1:400 dilution and incubated in the dark for at least 1 h at room temperature or refrigerated overnight. Counting of colonies was performed within two days of staining.

Analyses of dose response curves and statistical investigations. Colonies were counted by one trained investigator using a Zeiss Axiovert 200M inverted microscope (Zeiss, Jena, Germany). All analyses were performed only if sufficient colony formation of epithelial cells (CF_{EC}) was detected in all four control wells receiving 0.1% (v/v) ethanol. In the case of ≥4 colonies per each control well, the IC_{50} was calculated for the pure compound and the compound in the binary mixture. The IC_{50} values for the formation of epithelial cell colonies with the individual treatments were compared by Student's *t*-test for paired samples regarding the correlation and the significance of differences using SPSS Statistics 18.0 for Windows version 18.0.1 (SPSS Inc., Chicago, IL, USA). A *p*-value below 0.05 was regarded as significant.

Evaluation of drug synergism. A modified probability sum test according to Jin (24) was used to evaluate the mode of action of two drugs in binary mixture (synergism, antagonism, or additivity). The formula used is: q=P(A+B)/(PA+PB-PAxPB). Synergism (efficacy above additivity) is judged to be present only in case of $q>1.15$, antagonism by $q<0.85$, while $1.15>q>0.85$ indicates additivity.

Results

Effect of cyclopamine on KB cells and colony formation of HNSCC ex vivo. KB cells were used as a reference. We observed significant suppressive effects at a cyclopamine concentration of 500 nM ($p<0.05$) (Figure 1A). Increasing cyclopamine concentrations (1,500 nM-15,000 nM) led to further suppression of KB cells compared with controls ($p<0.001$) (Figure 1). Overall, higher concentrations of cyclopamine were necessary to achieve similar suppressive effects in KB cells compared to HNSCC (Figure 1 A and B). The cyclopamine concentration of 500 nM suppressed proliferation of KB cells about 20%, but lower cyclopamine concentrations had no significant suppressive effects. In HNSCC, we observed a dose-dependent inhibition of colony formation of CF_{EC} with increasing concentrations of cyclopamine (Figure 1B). When tumor cell growth in controls (medium plus 0.1% ethanol, cyclopamine concentration 0 nM) was defined as 100%, incubation with as low as 5 nM cyclopamine already suppressed colony formation by about 25% ($p<0.001$). Increasing concentrations of cyclopamine led to further significant suppression. Colony formation suppression of 50% was reached at a cyclopamine concentration of 500 nM (IC_{50}), and 70% colony formation suppression was seen at 5 μM (Figure 1B). However, the stepwise 10-fold increased cyclopamine concentrations failed to increase the suppressive effect on CF_{EC} significantly, when only pure cyclopamine was applied.

Cisplatin and docetaxel reduce colony formation in a dose-dependent manner. We next investigated the inhibitory effects of cisplatin and docetaxel as cytostatic drugs. Tumor cell incubation with 137.5 nM docetaxel reduced colony formation by more than 50%, and 550 nM docetaxel enhanced this effect up to 90% colony formation suppression in comparison with colony formation in medium-alone

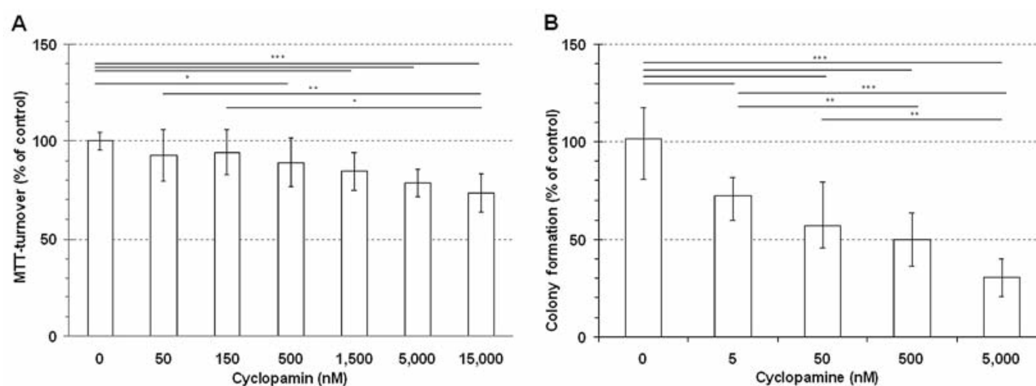

Figure 1. *Effects of cyclopamine on KB cells (A) and head and neck squamous cell carcinomas (B). A: Turnover of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to its blue formazan form was used as measure of viability and proliferation of KB cells after six days and indicates cytostatic effects of cyclopamine (n=5). B: Cyclopamine dose-dependently suppressed colony formation (CFEC) of HNSCC in the FLAVINO assay (n=18). *p<0.05, **p<0.01; ***p<0.001.*

(Figure 2; Table II; all $p<10^{-8}$). Doubling of docetaxel concentration increased the suppressive activity significantly. The same effects were also observed after 72-h incubation of HNSCC with cisplatin (Figure 2).

Cyclopamine promotes inhibitory effects of cisplatin and docetaxel. The combination of cyclopamine with docetaxel or cisplatin increased the efficacy compared to cyclopamine- alone and led to significant suppression of colony formation (Figure 2, Tables II and III). All inhibitory effects on CF_{EC} in HNSCC of the tested agents were enhanced if a binary mixture, including cyclopamine, was applied. When cisplatin was combined with higher cyclopamine concentrations near the maximum tolerable plasma level, we observed a concordant and significantly more strongly suppressed CF_{EC} almost without exception. Similar tendencies were obvious when cyclopamine and docetaxel were combined. Compared to single compounds and at serial concentrations, significant suppressive effects on colony formation of HNSCC were observed. As shown in Table II, increasing doses of cyclopamine not only increased suppressive activity on CF_{EC} of HNSCC, but also the suppressive activity of cisplatin and docetaxel. However, significant increases in efficacy in binary cyclopamine combinations were found, even at a low concentration, of cyclopamine, in particular at higher concentrations of cisplatin or docetaxel (Figure 2, Tables II and III).

Evaluation of drug interactions. The assessments of the mode of action according to Jin (24) revealed predominantly additive effects of either cisplatin or docetaxel with increasing concentrations of cyclopamine (Figure 3; Table III). Whereas the drug combination of cyclopamine (5-5,000 nM) and a low docetaxel concentration (137.5 nM) resulted in antagonistic effects in some HNSCC due to the occasionally observed hormetic effect of lower cisplatin concentrations, combined suppressive effects of cyclopamine and docetaxel at higher concentrations were found to be almost exclusively additive, *e.g.* when docetaxel was used at a concentration of 550 nM. Similar effects were found when cisplatin and cyclopamine were combined. The combination of cisplatin (6,667 nM) and cyclopamine (5-5,000 nM) had predominantly additive (83-94%) or even synergistic effects (5-16%; Table III). Figure 3 visualizes the findings regarding the q values in binary cyclopamine combinations. As expected from Table III, the median and interquartile range as well the mean and 95% confidence of q, as assessed in 18 HNSCCs *ex vivo* demonstrate that the predominant mode of action of cyclopaminelopamine in the binary mixture with either cisplatin or docetaxel is additivity. The dotted line reflects the interval of additivity ($1.15 \geq q \geq 0.85$; shown in percent).

Discussion

To the best of our knowledge, this is the first study showing that targeting SMO in HNSCC is able to suppress growth and in particular colony formation of HNSCC and that SMO-targeting is able to additively increase the anti-tumoral activity of treatment by cisplatin or docetaxel. This is of particular importance, since overexpressed or mutated proteins in the HH pathway or activation of the related transcription factors were demonstrated in a variety of human malignancies, including HNSCC, and are associated with poor prognosis (5-11). In mammals, the most relevant ligand is sonic hedgehog (SHH), besides the two additional existing homologs indian hedgehog (IHH) and desert hedgehog (DHH). The cellular response is controlled by two transmembrane receptor proteins [(PTCH) 1 and 2], which both are able to inhibit the downstream protein smoothened (SMO) whenever the ligand (SHH, DHH,

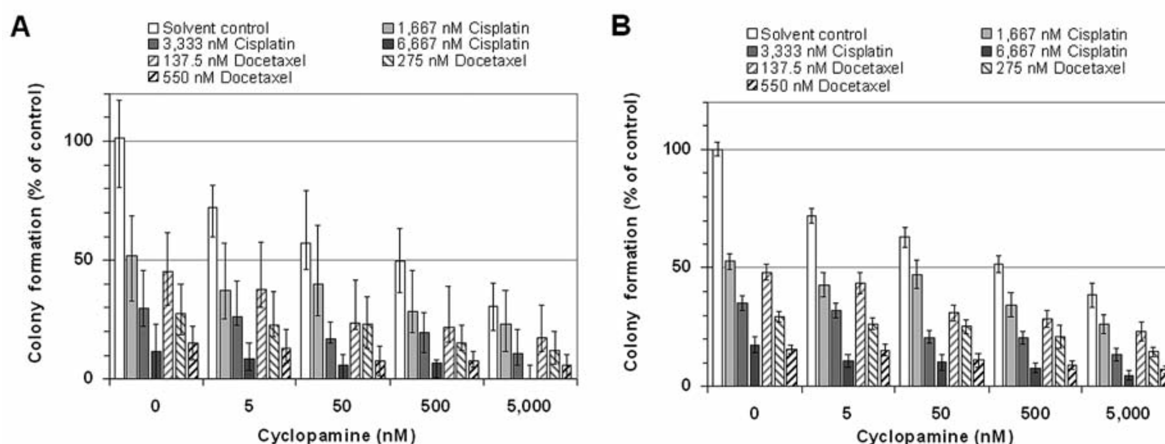

Figure 2. *Combined effects of cyclopamine with two approved chemotherapeutics, cisplatin (grey-scale) and docetaxel (hatched) on colony formation (CFEC) of 18 head and neck squamous cell carcinomas in the FLAVINO assay. A: Median and interquartile range; B: mean and 95% confidence interval.*

IHH) is absent. Otherwise, ligand binding to PTCH leads to SMO activation and the activation of the transcription factors GLI1-3. GLI proteins mediate a nuclear transcriptional response of HH target genes (25). Inappropriate activation of HH signaling by overexpression or mutation was found to increase snail protein expression, with a loss of cellular adhesion (26), activate anti-apoptotic genes and angiogenic factors (27), reduce apoptotic gene activation (28), and interfere in the cell-cycle sequence (29). These HH-mediated properties may contribute to tumor development and facilitate tumor growth and spread, with consequent poor prognosis for different tumor entities. In healthy adult tissues, the HH pathway regulates stem cells during regeneration and tissue repair (30), but disturbances within this system may easily lead to cancer cell formation. In fact, stem cells and cancer cells share some properties such as asymmetric cell division, expression of multidrug-resistance proteins, lack of apoptotic regulation, and differentiation into different tissues. Tumor repopulation after chemotherapy might be promoted by HH signaling, with the consequences of early recurrence, treatment resistance, and poor prognosis (11, 31-34). The stem cell-regulating properties of HH might be a key to its pivotal role in tumorigenesis and therefore seems worth investigating. Our findings suggest a central role of the HH pathway in HNSCC. Cyclopamine, the first described inhibitor of the HH pathway, was able to suppress growth and in particular CF_{EC} of HNSCC *ex vivo*, and increased the cytostatic efficacy of cisplatin and docetaxel, and mainly acted in this respect in an additive or even synergistic way. However, consolidated findings about the role of the HH pathway in HNSCC are very rare. Its importance was first mentioned in 2010 by Chung *et al*. (11), who observed a worse prognosis in patients with high levels of GLI1 expression after radiation therapy. In 2010, Keysar and Jimeno (35) reported HH pathway overexpression (SMO and GLI1) in putative HNSCC cancer stem cell subpopulations [(CD)24, (CD)44 and (CD)24 (ALDH)], although there is currently no agreed consensus about stem cell markers in HNSCC. Meanwhile, Schneider *et al*. (12) and Wang *et al*. (13) published data about HH protein expression in HNSCC, but the samples sizes were small, not all components were considered, and results concerning expression patterns were partially controversial. In a recent study, we demonstrated increased expression of most HH pathway proteins, and in particular of the SMO-activated transcription factor GLI1 (14). Nevertheless, as highlighted by our results presented here, the plant-derived inhibitor cyclopamine has indeed tumor-inhibiting effects. This provides evidence for the importance and activity of the HH pathway in HNSCC, but also indicates the potential of SMO targeting regarding augmentation of the anti-tumoral effects of classical chemotherapies. However, it is not clear whether HH activation is a result of ligand (SHH) overexpression; pathway mutations, as in Gorlin's syndrome; or aberrant activation.

Cyclopamine is a steroid alkaloid from *Veratrum californicum* with teratogenic effects, attributed to inhibition of the HH pathway during embryogenesis (36). It is one of the first small molecule inhibitors of the HH pathway (37), with useful properties blocking SHH-mediated processes. It was later established that cyclopamine achieves this inhibition by direct binding to the seven-transmembrane α-helical bundle of the SMO receptor (41). Most HH inhibitors, to date, target SMO, and several have advanced into human clinical trials. SMO inhibition by cyclopamine (or other small molecule inhibitors, such as IPI-269609) lead to down-regulation of HH

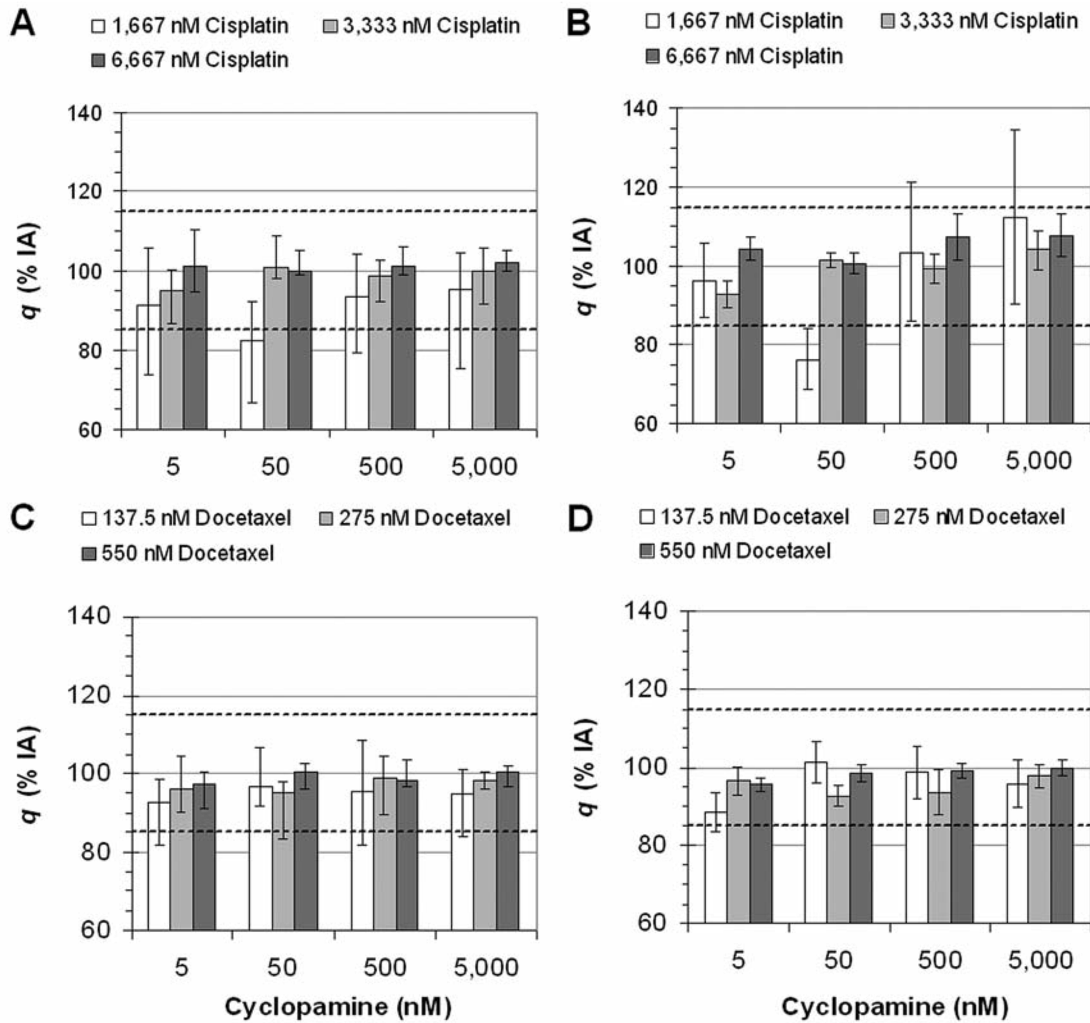

Figure 3. *Combination of cyclopamine and either cisplatin (A, B) or docetaxel (C, D) increased the suppressive effects on colony formation of 18 head and neck squamous cell carcinomas additively. The values for q obtained according to Jin (24) for 18 HNSCC (Table III) and presented. A, C: Median and interquartile range; B, D: mean and 95% confidence interval.*

target genes (38). HH inhibition has already demonstrated potential against tumor growth in tumor cell lines and in *in vivo* models of other tumor entities (6, 40-42). In cancer studies, cyclopamine treatment of human glioma and medulloblastoma cell lines resulted in inhibition of cell division and reduced tumor size. Additionally, HH inhibition with cyclopamine depleted stem-like glioma cells, a phenomenon that was not observed after radiation (43, 44). Systemic administration of cyclopamine reduced tumor size and tumor cell number in a mouse model of medulloblastoma (45). Similar effects have been observed in animals carrying xenografts of small cell lung cancer, when cyclopamine was injected subcutaneously (46). In pancreatic adenocarcinoma cell lines, growth-inhibiting effects were seen at doses between 5 μM and 20 μM (47). However, the need for these rather high concentrations may be related in some regard to inadequate experimental conditions, *i.e.* presence of artifacts attributable to photoactivation which are avoided using our patent experimental setting (16). In HNSCC, we observed 25% reduced colony formation at concentrations as low as 5 nM (IC_{50} of 500 nM) using our patented flavin-free *ex vivo* assay, which is suitable not only for evaluating the epithelial chemoresponse to single cytostatic drugs but also for drug combinations (48). However, for the epithelial cell line KB used as a reference, we needed even higher cyclopamine concentrations to achieve suppressive effects. In whole-embryo culture assays in mice, cyclopamine-induced teratogenic effects were observed at ≥2 μM, which in mice was reached in serum by *in vivo* cyclopamine infusion at 160 mg/kg/day (48), but other species seem to have different sensitivity to cyclopamine (50).

Table II. *Statistical results*.

		Cyclopamine (nM)				
Cis (nM)	Doc (nM)	0	5	50	500	5,000
0	0	-	2.97 E-08	5.66 E-10	8.21 E-12	1.23 E-11
1,667	0	2.01 E-11	2.88 E-12	9.26 E-10	7.46 E-14	1.37 E-18
3,333	0	4.96 E-18	2.48 E-20	5.09 E-25	4.09 E-25	1.06 E-27
6,667	0	1.53 E-19	1.55 E-27	7.24 E-24	6.99 E-31	1.59 E-32
0	137.5	1.71 E-13	8.97 E-14	3.29 E-20	3.90 E-20	1.19 E-19
0	275.0	1.85 E-21	6 .44 E-25	1.78 E-23	2.18 E-17	4.25 E-29
0	550.0	1.02 E-29	4.53 E-27	1.82 E-26	3.21 E-32	1.00 E-34

p-Values by Student's *t*-test for paired samples for differences in anti-neoplastic efficacy of cyclopamine, cisplatin (Cis), docetaxel (Doc), and binary combinations of cyclopamine with either cisplatin or docetaxel in respective to colony formation (CF_{EC}) in untreated controls as assessed in 18 evaluable head and neck squamous cell carcinomas in the FLAVINO assay.

Table III. *Analysis of drug interactions*.

			Antagonism $q<0.850$		Additivity $q=1\pm0.15$		Synergism $q>1.15$	
Cisplatin (μM)	Docetaxel (nM)	Cyclopamine (nM)	n	%	n	%	n	%
1.667	-	5.00	7	38.89	7	38.89	4	22.22
1.667	-	50.0	10	55.56	7	38.89	1	5.56
1.667	-	500	6	33.33	11	61.11	1	5.56
1.667	-	5,000	6	33.33	10	55.56	2	11.11
3.333	-	5.00	4	22.22	12	66.67	2	11.11
3.333	-	50.0	1	5.56	16	88.89	1	5.56
3.333	-	500	2	11.11	14	77.78	2	11.11
3.333	-	5,000	2	11.11	14	77.78	2	11.11
6.667	-	5.00	0	0	15	83.33	3	16.67
6.667	-	50.0	2	11.11	15	83.33	1	5.56
6.667	-	500	0	0	17	94.44	1	5.56
6.667	-	5,000	1	5.56	15	83.33	2	11.11
-	137.5	5.00	5	27.78	12	66.67	1	5.56
-	137.5	50.0	3	16.67	13	72.22	2	11.11
-	137.5	500	6	33.33	8	44.44	4	22.22
-	137.5	5,000	5	27.78	12	66.67	1	5.56
-	275.0	5.00	4	22.22	12	66.67	2	11.11
-	275.0	50.0	6	33.33	11	61.11	1	5.56
-	275.0	500	2	11.11	15	83.33	1	5.56
-	275.0	5,000	4	22.22	13	72.22	1	5.56
-	550.0	5.00	1	5.56	17	94.44	0	0.00
-	550.0	50.0	1	5.56	17	94.44	0	0.00
-	550.0	500	1	5.56	17	94.44	0	0.00
-	550.0	5,000	1	5.56	16	88.89	1	5.56

Numbers (n) and percentage % of head and neck squamous cell carcinomas responding [as classified according to the modified probability sum test of Jin 2004 (24) at the binary combination indicated with either antagonism, additivity or synergism are shown.

Our results encourage further investigation concerning the HH pathway in HNSCC and the development of agents targeting this signaling cascade with less serious adverse effects than cyclopamine (49). Cyclopamine, has poor oral bioavailability and a short half-life, but as outlined above, novel inhibitors exist (*e.g.* IPI-269609) that might be more suitable in clinical practice (51). As reported here, combined treatment with the guideline cytostatic drugs cisplatin or docetaxel synergistically contributed to even greater inhibition of colony formation of HNSCC. Bahra *et al*. (47) reported on the synergistic effects of cyclopamine and gemcitabine in the reduction of tumor volume in pancreatic adenocarcinoma

xenografts and postulated a new therapeutic approach. Doubtless, this means that the direct blockade of an activated pathway that is responsible for or at least involved in tumor growth is a promising goal in cancer therapy, but the importance of the HH pathway in HNSCC was not addressed before and hence is not yet completely understood. Our results in HNSCC, highlighting additive or even synergistic suppression of CF_{EC} by the cytostatic drugs cisplatin and docetaxel if the treatment was combined with cyclopamine-triggered SMO inhibition, demonstrate a probably beneficial tumor-inhibiting effect compared to single-drug treatment. However, this might be attributable, at least in, part to the rather conservative interpretation of the quotient q according to Jin (24). This was done under the presumption of i) independent action of both treatments, and ii) u=0.05 for both types of errors, the two-sided α and the one-sided β. This leads to the definition of 0.85 and 1.15 as thresholds for the interpretation of the quotient q to consider the uncertainty of measurements. However, most of the HNSCCs responded *ex vivo* with a *q* value within this interval (Table III). If such HH blockade can in fact be translated into clinical practice, it is quite possible that combinations of drugs targeting SMO with guideline-conforming cytostatics will be superior to single-agent therapy, as reported for tyrosine kinase inhibitors (*e.g.* lapatinib) (48). The first phase II trials with GCD-0449 have been successfully performed for skin, brain, and breast tumors (see the listing of current clinical trials of the National Cancer Institute, www.cancer.gov), and other tumor entities are currently also under investigation. Our data form a rationale to extend this spectrum to HNSCC. We conclude that HH pathway inhibition with cyclopamine alone, and combined with cisplatin and docetaxel suppresses colony formation in HNSCC. This suggests a central role of this pathway in HNSCC and defines SMO as a potential target in HNSCC. Indeed, the first agents targeting SMO as a central HH component have entered phase II clinical trials for other solid tumor types. However, the results remain to be released, but according to our results, a promising benefit in HNSCC could also be expected. Our results make it very likely that simultaneous application of SMO inhibitors such as cyclopamine will results in augmentation of docetaxel- or cisplatin-based treatment regimens in HNSCC. This encourages both the investigation of currently available HH inhibitors in HNSCC clinical trials, but also the development of new agents with improved biophysiological properties for clinical use in the future.

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