Abstract. Background: Vinorelbine has been identified as an effective cytostatic drug in head and neck squamous cell carcinoma (HNSCC). To predict the individual chemoresponse, the application of an ex vivo assay to quantify the response of HNSCC to vinorelbine is presented. Patients and Methods: Twelve biopsies from primary HNSCC sites and five biopsies from metastatic lesions were analyzed (n=17). The specimens were investigated ex vivo for the overall, epithelial and stromal chemoresponse to vinorelbine. Results: By selective evaluation of the epithelial chemoresponse, the applied assay identified three specimens as vinorelbine-sensitive (18%), including one de novo sensitivity in a metastatic lesion of a vinorelbine-resistant hypopharyngeal carcinoma. Conclusion: Applying flavin-protecting culture methods and with careful correction for the stromal cell effect, the assay generated reliable data for the assessment of the individual chemoresponse of HNSCC specimens to vinorelbine. The assay may, therefore, facilitate the implementation of individualized chemotherapy protocols.

Patients and Methods

Patients and HNSCC specimens. After obtaining individual informed consent, twelve biopsy specimens from primary HNSCC sites and five biopsy specimens from cervical lymph node metastases (n=17) were taken from 13 patients with a histologically-confirmed diagnosis of primary HNSCC. The biopsies were taken either during definitive surgical treatment procedures, or during diagnostic pan endoscopies under general anesthesia. The specimens were collected in ice-cold culture medium and processed immediately.

Drugs, enzymes and reagents. Nystatin, G-penicillin-Na, gentamycin and streptomycin were purchased from Sigma (München, Germany). Trypsin and collagenase (EC 3.4.24.3, type IV) were obtained from Difco (Detroit, MI, USA) and Sigma, respectively. Vinorelbine (Pierre Fabre, Freiburg, Germany) was purchased as a pharmaceutical preparation. All other reagents were of analytical grade.

Cells and cell culture methods. KB cells (ATCC, Bethesda, MD, USA) were propagated under standard conditions as described previously (11). All experimental steps were carried out under exclusive illumination with sodium-discharge lamps, emitting monochromatically at λ=589 nm (Philips, Marburg, Germany). This specific illumination was used to avoid the known adverse, flavin-mediated, photo-oxidative effects in cell culture systems, especially in chemosensitivity testing comprising vinca alkaloids (12, 13).
Control experiments with KB cells. Control experiments with KB cells served as an internal standard for each colony formation assay. The same drug solutions and culture media as in the latter were used. These experiments were performed as described previously (11). In the present study, cytotoxicity tests in KB cells revealed adequate pharmacological assay conditions for the assessment of vinorelbine (data not shown).

**Colony formation assay.** The handling of the specimens, as well as the colony formation assay, were performed as described previously (11). Briefly, following harvest, the specimens were minced and transferred into 30 ml of pre-warmed, flavin-free RPMI 1640 medium containing 10% (v/v) FBS, nystatin, G-penicillin, gentamicin and streptomycin. For enzymatic disintegration, 300 IU/ml collagenase type IV were added and the suspension was incubated for 24 h under standard conditions. After centrifugation, the pellets were carefully resuspended in fresh culture medium supplemented as described above (w/o collagenase). Three hundred-µl aliquots of the suspension were transferred to microwells coated with extracellular matrix (Pesasel & Lorey, Hanau, Germany). After a short sedimentation period, diluted VRL solutions (3 µl/well) were added to establish concentration gradients. The applied VRL concentration gradient (1.5 nM, 3.0 nM, 6.0 nM, 24.0 nM, 96.0 nM and 384.0 nM) started with the IC50 values for KB cells (13) and covered the clinically achievable steady state concentration of VRL for a dosage of 30 mg/m² (11.1 nM) (14). After 72 h of incubation under standard conditions, the cells were washed (phosphate-buffered saline, pH 7.8) and the adherent cells and cell colonies were fixed with methanol before Giemsa staining.

**Microscopic evaluation of the drug response.** The microscopic examination was performed using Zeiss ICM 405 and Zeiss Axiocvert 200M inverted microscopes equipped with phase optics and polarization-optical differential interference contrast (PlasDIC, Zeiss, Jena, Germany). The microphotographs were taken using a micropublisher digital CCD-camera (Q-Imaging, Burnaby BC, Canada) and were documented by Volocity imaging software (Improvision, Tübingen, Germany). Giemsa staining allowed for microscopic identification of stromal and epithelial cell colonies (>16 cells) in all the specimens tested. To determine the overall drug response (OR) of a specimen to VRL, the drug concentration which caused a complete suppression of any ex vivo colony formation (C100) was determined. In terms of the clinical steady state concentration, the specimens were classified as sensitive, if the C100 was below or equal to the steady state concentration of VRL and as resistant, if the C100 exceeded the steady state concentration of VRL. If colony formation was found for all the concentrations tested, the results are referred to as C100>384 nM vinorelbine.

As with the microscopic identification of the C100, the drug concentrations which caused a complete suppression of epithelial (Ce100) or stromal (Cs100) colony formation ex vivo were identified. The specimen’s epithelial drug response (ER) and the stromal drug response (SR) were classified in the same manner as described for the OR.

**Results**

**Overall response (OR) to VRL.** The complete suppression of any ex vivo colony formation was investigated in 17 HNSCC specimens by graded exposure to VRL. The OR is shown in

<table>
<thead>
<tr>
<th>No.</th>
<th>Biopsy site</th>
<th>HNSCC localization, TNM*</th>
<th>Quantitative response</th>
<th>VRL (nM)</th>
<th>Response indices</th>
<th>VRL</th>
</tr>
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<tbody>
<tr>
<td>1</td>
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<td>OR</td>
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</tr>
<tr>
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<td>Ce100</td>
<td>384.0</td>
<td>ER</td>
<td>●</td>
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<tr>
<td>3</td>
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<td>Hypopharynx, T3N0M0</td>
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<td>384.0</td>
<td>OR</td>
<td>●</td>
</tr>
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<td>384.0</td>
<td>ER</td>
<td>●</td>
</tr>
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<td>OR</td>
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</tr>
<tr>
<td>6</td>
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</tr>
<tr>
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<tr>
<td>9</td>
<td>Tumor</td>
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<td>ER</td>
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</tr>
<tr>
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<td>Tumor</td>
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<td>384.0</td>
<td>OR</td>
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</tr>
<tr>
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</tr>
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<td>13</td>
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<tr>
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<td>384.0</td>
<td>ER</td>
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</table>

*NSCC: head and neck squamous cell carcinoma; VRL: vinorelbine; Metast.: metastasis. Full circles (●) represent resistant and open circles (○) sensitive response patterns according to the indices: overall response (OR), epithelial response (ER) and stromal response (SR). *TNM classification according to the UICC (2002). do.: the same. For C50- Ce100, Cs100 definitions see Patients and Methods.
Table I. The evaluation of the OR revealed resistant response patterns to VRL in all the specimens investigated. It is noteworthy that the OR in all but one specimen (transglottic laryngeal carcinoma T3N0M0, specimen #4) was dominated by stromal cell resistance.

Epithelial response (ER) to VRL. Three specimens showed a sensitive ER to VRL (specimens #2, #3 and #11, Table I). The remaining 14 specimens were found to be resistant to VRL according to the ER definition used. The VRL-sensitive specimen #11 originated from a cervical lymph node metastasis of a primary hypopharyngeal carcinoma (T2N2cM0), which was also biopsied and analyzed (specimen #10). Interestingly, the selective sensitivity to VRL of the metastatic lesion was not found in the primary tumor (VRL-resistant specimen #10, see Table I and Figure 1) and, therefore, indicates a de novo sensitivity to VRL in the metastatic lesion.

Stromal response (SR) to VRL. The drug response patterns of the stromal elements showed, in all but one specimen (transglottic laryngeal carcinoma T3N0M0, specimen #4), resistance to VRL according to the SR definition used. A comparison of the corresponding Cs100 and Ce100 for VRL showed in five out of 17 specimens (29%) a complete suppression of stromal colonies by drug concentrations higher than those required for the suppression of epithelial colony formation (Table I).

Discussion

Since multimodal therapy regimens in the first-line treatment of HNSCC have proven to represent equipollent treatment alternatives to primary surgery, especially in laryngeal and hypopharyngeal carcinoma (15-18), attempts to predict individual chemoresponses have renewed the interest in...
reliable chemosensitivity testing (10). The systematic elimination of adverse flavin-mediated photoreactions which distort quantitative chemosensitivity determination (12, 13), as well as the correction for stromal cell contamination in testing solid tumors (11), form the basis for the detection of the quantitative chemoresponse of HNSCC specimens \textit{ex vivo}. Applying these principles, the present study aimed to investigate the response of HNSCC specimens to VRL.

Seventeen biopsy specimens from 13 patients suffering from HNSCC were analyzed. The results for the individual epithelial chemoresponse tests identified three VRL-sensitive specimens: one specimen from a T2N0M0 laryngeal carcinoma (#2, see Table I), and two specimens from cervical lymph node metastasis of advanced hypopharyngeal carcinomas (T4N2M0 specimen #3, T2N2cM0 specimen #11; see Table I). These selective epithelial sensitivities to VRL would not have been detected by evaluating the overall cellular sensitivity of the specimens only since, in all three cases, stromal cell chemoresistance dominated the OR evaluation. This finding underlines the impact of correcting for stromal cell contamination in evaluating the chemoresponse of HNSCC \textit{ex vivo}.

From the clinical point of view, the sensitivity of specimen #11 was of particular interest. This VRL-sensitive specimen originated from a cervical lymph node metastasis of a hypopharyngeal carcinoma (T4N2M0), which was also analyzed (specimen #10) and found to be VRL-resistant (see Table I). Such selective sensitivity to VRL in a metastatic lesion could form the basis for applying an individualized chemotherapy schedule, including VRL, in a postoperative adjuvant or, in the case of metastatic spread, in a multimodal therapy concept with curative intent (19). An attractive alternative to the intravenous administration of VRL, especially in the palliative setting, was found with oral VRL (20). According to the pharmacological indices used in this study to distinguish sensitive and resistant response patterns, an equipollent activity to the intravenous used in this study to distinguish sensitive and resistant response patterns, an equipollent activity to the intravenous administration of VRL in the treatment of cisplatin-resistant nasopharyngeal carcinoma. Head Neck 28: 74-80, 2006.


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References


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