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Ex Vivo Responsiveness of Head and Neck Squamous Cell Carcinoma to Glufosfamide, a Novel Alkylating Agent

RALPH DOLLNER[1], ANDREAS DIETZ[1], MARIJANA KOPUN[2], MATTHIAS HELBIG[3], FRANK WALLNER[3] and CHRISTOF GRANZOW[2]

[1]*Department of Otorhinolaryngology, Head and Neck Surgery, University of Leipzig, Leipzig;*
[2]*German Cancer Research Center, Department of Molecular Toxicology, Heidelberg;*
[3]*Department of Otorhinolaryngology, Head and Neck Surgery, University of Heidelberg, Heidelberg, Germany*

Abstract. *Background: Glufosfamide is a novel alkylating agent in which the active metabolite of isophosphoramide mustard is glycosidically linked to ß-D-glucose. Targeting the elevated glucose uptake of tumor cells expressing the SAAT1 glucose transporter, glufosfamide represents an attractive new drug for cancer chemotherapy. The present study investigates the ex vivo responsiveness of Head and Neck Squamous Cell Carcinoma (HNSCC) specimens to glufosfamide. Patients and Methods: Twenty-one unselected HNSCC specimens were investigated using a novel ex vivo colony formation assay to determine the epithelial drug response. The individual responsiveness to glufosfamide and to cis-platinum was determined. Results: Five out of 21 evaluable HNSCC specimens were sensitive to glufosfamide. There was a tendency for glufosfamide sensitivity in platinum-resistant specimens and vice versa. Conclusion: The effectiveness of glufosfamide observed in the present ex vivo study suggests at least an equipotentiality of glufosfamide in comparison to cis-platinum. The potential clinical usefulness of glufosfamide in HNSCC warrants further evaluation.*

β-D-Glucosyl-ifosfamide mustard (D 19575, glc-IPM, INN = glufosfamide, Figure 1) is a novel alkylating agent in which the active metabolite of isophosphoramide mustard is glycosidically linked to ß-D-glucose (1). The cellular uptake of glufosfamide is mediated by the Na^+-D-glucose cotransporter SAAT1 (SGLT3) (2), and possibly also by other transporter proteins. Considering the elevated glucose utilization by tumor cells (3), this novel targeting mechanism may be particularly promising against malignant tumor cells (1,4,5). Preclinical studies have shown that glc-IPM has a lower myelotoxicity and a higher anti-tumor activity than ifosfamide (4) and therapeutic responses observed during the phase I study (6) have prompted further clinical evaluation of glc-IPM. A recently published phase II study on glufosfamide as first-line treatment for advanced pancreatic carcinoma revealed a rather modest activity for this cancer entity (7). Nevertheless, promising preclinical data exist for other malignant diseases, *e.g.* in childhood acute leukemia (8), and warrant further evaluation. Since ifosfamide is a known active drug in the treatment of head and neck squamous cell carcinoma (HNSCC) (9-11), glufosfamide represents an interesting drug candidate particularly for this cancer entity. However, the activity of glufosfamide as a potentially new drug in the treatment of head and neck squamous cell carcinoma has not been evaluated so far.

The objective of the present *ex vivo* study was to investigate the activity of glc-IPM in individual HNSCC specimens using an *ex vivo* colony formation assay which allows for the identification of the specific drug response of epithelial elements from HNSCC biopsies (12). In addition, this study compares the *ex vivo* activity of glc-IPM with that of *cis*-platinum to establish an estimate of the potential clinical effectiveness of glufosfamide.

Patients and Methods

Patients and HNSCC specimens. After obtaining individual informed consent, 19 patients with histologically confirmed diagnosis of primary HNSCC were enrolled in this study. The primary tumors were located in the larynx (4 patients), in the hypopharynx (7 patients) and in the oropharynx (8 patients). A total of 24 biopsies was taken from primary tumors (n=18), or from cervical lymph node metastases (n=6). The mean wet weight of the harvested specimens was 89.2 mg (range: 56.7-145.3 mg) for the primary tumor biopsies and 104.2 mg (range: 48.0-176.0 mg) for the biopsies from metastasis.

Correspondence to: Ralph Dollner, MD, Dept. Otorhinolaryngology, Head and Neck Surgery, University of Leipzig, Liebigstrasse 18a, D-04103 Leipzig, Germany. Tel: ++49-341-9721700, Fax: ++49-341-9721709, e-mail: Ralph.Dollner@medizin.uni-leipzig.de

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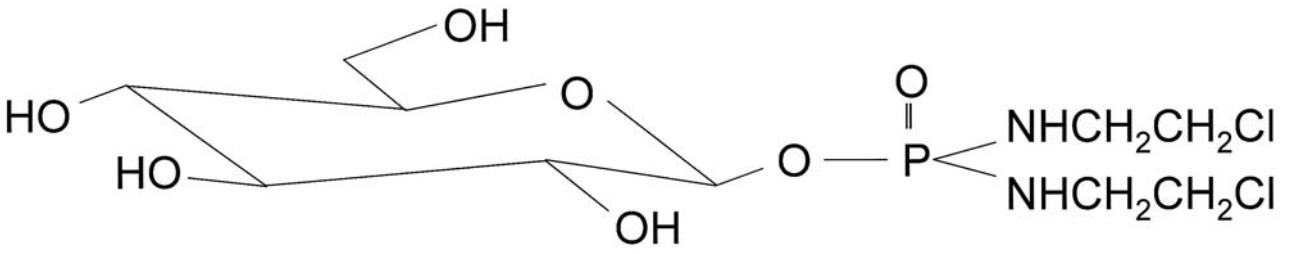

Figure 1. *Structure of glufosfamide (D-19575, glc-IPM). Molecular weight: 383.1. Chemical description: $C_{10}H_{21}Cl_2N_2O_7P$ β-D-glucopyranosyl-N,N'-bis-(2-chloroethyl)-phosphoro-diamidic acid*

Evaluability rate. Following the previously described criteria for considering a HNSCC specimen evaluable in the colony formation assay (12), 3 out of the 24 specimens (2 from primary tumors and 1 from a lymph node metastasis) were excluded from further investigation due to insufficient growth of epithelial cells in the control wells. Accordingly, 21 specimens (evaluabilty rate: 87.5 %) from 17 patients were evaluable and form the basis of this study.

Cytostatic drugs. Cis-platinum (*cis*-DDP) was purchased as a pharmaceutical preparation from Medac (Hamburg, Germany). Glufosfamide (1,13) was a gift from Dr. Manfred Wießler, German Cancer Research Center. Frozen aqueous stock solutions of *cis*-DDP (6.17 mM) and methanolic stock solutions of glc-IPM (100 mM) were stored at -20˚C until use.

Colony formation assay. The handling of the specimens as well as the colony formation assay has been described previously (12). Briefly, the specimens were minced prior to resuspension in flavin-free RPMI 1640 medium (Biochrom, Berlin, Germany) and enzymatic digestion (collagenase type IV, Sigma, Munich, Germany). The medium was buffered with 1.134 g/L sodium bicarbonate and 1.072 g/L HEPES, and supplemented with 10 percent (v/v) of fetal bovine serum (FBS, Integro, Zaandam, Holland), 100 IU/mL penicillin G (Hoechst, Frankfurt, Germany), 100 μg/mL amikacin (Bristol, Munich, Germany), 100 μg/mL streptomycin (Grünenthal, Stolberg, Germany) and 240 IU/mL nystatin (Sigma). Aliquots of the tissue digest were transferred to microwells coated with extracellular matrix (Paesel & Lorey, Hanau, Germany). Diluted solutions of *cis*-DDP and glc-IPM were added to establish drug concentration gradients calibrated using two pharmacological indices: i) the respective IC_{50} values for KB cells and ii) the clinically achievable plasma concentration of the cytostatic drugs (12). The IC_{50} values for *cis*-DDP and glc-IPM in KB cells were 0.2 μM and 8.0 μM, respectively (14). Starting from the IC_{50} values for KB cells, the concentration gradient included and exceeded the clinically tolerable maximal plasma level (CP) of *cis*-DDP (6.7 μM (15)) and glc-IPM (330.0 - 370.0 μM (6)). Accordingly, the concentration gradients applied ranged from 0.2 to 51.2 μM for *cis*-DDP and from 8 μM to 1 mM for glc-IPM. A minimum of eight drug-free control wells were obligatory in each test. After 72 hours of incubation of the test plates (36.5˚C, 2.5 % CO_2, in humidified air), the formed, adherent cell colonies were fixed with methanol prior to Giemsa staining (12). Consistently, KB cells were used as an internal standard in parallel tests employing the same drug solutions and culture media as in the colony formation assays (12).

Evaluation of the drug response. Microscopical identification of Giemsa-stained epithelial cell colonies (> 16 cells) was performed to determine the epithelial drug response (ER) to a certain drug. Therefore, the drug concentration which caused a complete suppression of epithelial *ex vivo* colony formation (Ce_{100}) was determined. Considering the CP, the specimens were classified as *sensitive*, if the Ce_{100} was below or equal to the respective CP and as *resistant*, if the Ce_{100} exceeded the respective CP.

Correction for stromal cell contamination. According to our recently published study regarding the impact of stromal cell contamination on chemosensitivity tests (12), the results of the present investigation explicitly describe the drug response of epithelial colonies grown from tumor tissue digests. Since stromal cell chemoresistance to *cis*-DDP as well as to glc-IPM was evident in some cases of the present study (data not shown), correction for stromal cell colonies was crucial to obtain reliable results in terms of the epithelial drug response.

Results

Ex vivo response of HNSCC to cis-platinum. Suppression of the *ex vivo* Ce_{100} was investigated in 21 HNSCC specimens by exposure to *cis*-platinum. Individual Ce_{100}-values for *cis*-DDP varied widely from 3.2 to more than 51.2 μM. The latter value indicates that full suppression of epithelial colony formation could not be achieved by the highest concentration tested (51.2 μM). Such *cis*-DDP resistance was observed in 10 out of a total of 21 tested HNSCC specimens.

The responsiveness of the 16 biopsy specimens from primary tumors revealed that only in one single case was the determined Ce_{100}-value lower than the clinically tolerable maximal plasma level. The Ce_{100}-value of this tumor for *cis*-DDP was 3.2 μM, clearly suggesting sensitivity to *cis*-DDP (patient# 7, hypopharyngeal carcinoma, $T_3N_0M_0$, see Table I).

The Ce_{100}-values of all other specimens tested were found to be higher than the achievable plasma level of *cis*-DDP. Thus, 15 out of 16 specimens tested were classified as resistant to *cis*-DDP according to the criteria applied for the determination of ER.

Table I. *Ex vivo drug response of HNSCC specimens to cis-platinum and glufosfamide. The concentrations of cis-DDP and glc-IPM which completely suppress epithelial colony formation (Ce_{100}) in the colony formation assay are shown for 21 individual HNSCC specimens (16 primary tumors, 5 metastases). Accordingly, the individual Ce_{100}-values indicate a resistant (●) or sensitive (○) epithelial drug response (ER). N.d.: not determined.*

HNSCC characterization			Specific suppression of epithelial colony formation*							
			Primary Tumor				Metastasis			
Patient	Localization and TNM-Stage (UICC, 1997)		*cis*-DDP [μM]		glc-IPM [μM]		*cis*-DDP [μM]		glc-IPM [μM]	
			Ce100	ER	Ce100	ER	Ce_{100}	ER	Ce_{100}	ER
1	Larynx	T3N0M0	> 51.2	●	33.6	○	n.d.	-	n.d.	-
2	Larynx	T2N0M0	12.8	●	537.6	●	n.d.	-	n.d.	-
3	Larynx	T3N0M0	51.2	●	537.6	●	n.d.	-	n.d.	-
4	Larynx	T4N2cM0	51.2	●	1000.0	●	> 51.2	●	1000.0	●
5	Hypopharynx	T4N2cM0	> 51.2	●	16.8	○	n.d.	-	n.d.	-
6	Hypopharynx	T4N2cM1	51.2	●	134.4	○	n.d.	-	n.d.	-
7	Hypopharynx	T3N0M0	3.2	○	537.6	●	n.d.	-	n.d.	-
8	Hypopharynx	T2N2cM0	> 51.2	●	537.6	●	> 51.2	●	1000.0	●
9	Hypopharynx	T4N3M0	n.d.	-	n.d.	-	> 51.2	●	1000.0	●
10	Hypopharynx	T3N2bM0	> 51.2	●	537.6	●	> 51.2	●	537.6	●
11	Hypopharynx	T4N0M0	12.8	●	537.6	●	n.d.	-	n.d.	-
12	Oropharynx	T3N2bM0	12.8	●	33.6	○	n.d.	-	n.d.	-
13	Oropharynx	T3N2bM0	> 51.2	●	134.4	○	n.d.	-	n.d.	-
14	Oropharynx	T2N2cM0	12.8	●	537.6	●	> 51.2	●	1000.0	●
15	Oropharynx	T2N0M0	12.8	●	1000.0	●	n.d.	-	n.d.	-
16	Oropharynx	T2N2bM0	12.8	●	537.6	●	n.d.	-	n.d.	-
17	Oropharynx	T4N2bM0	12.8	●	537.6	●	n.d.	-	n.d.	-

The responsiveness of all the 5 biopsy specimens from metastatic lesions was classified as resistant to *cis*-DDP throughout. In 4 specimens from metastases, the response to *cis*-DDP could be compared to that of the corresponding primary tumor. In 2 of these cases, the metastatic specimen was more resistant to *cis*-DDP than the primary tumor specimen (patients #4 and #14). In 2 further patients with hypopharyngeal carcinoma (patients #8 and #10), the response of the metastases to *cis*-DDP was identical to that of the primary tumors. All the data are given in Table I.

Ex vivo response of HNSCC to glufosfamide. As for *cis*-DDP, suppression of Ce_{100} by glufosfamide was investigated in 21 HNSCC specimens. Again, the individual Ce_{100}-values for glc-IPM varied strongly from 33.6 μM to 1.0 mM. Full suppression of Ce_{100} was achieved in all tested specimens (concentration range tested: 8.0 μM – 1.0 mM).

In 5 out of 16 biopsy specimens from primary tumors, the individual Ce_{100}-value was below the clinically tolerable maximum plasma level for glc-IPM. The Ce_{100}-values of these tumors ranged from 16.8 to 134.4 μM glc-IPM, suggesting sensitivity to this drug. Interestingly, all of the 5 glc-IPM-sensitive HNSCC specimens were resistant to *cis*-DDP (see Table I).

In agreement with the results for *cis*-DDP, the responsiveness of the 5 biopsy specimens from metastases was classified as resistant to glc-IPM throughout and, if comparable, at least as resistant to glc-IPM as the primary tumors.

Discussion

Glufosfamide is a new alkylating drug which showed promising effects during preclinical evaluation (16), and has recently undergone clinical evaluation by the EORTC in

pancreatic cancer (7), glioblastoma (17) and in the second-line treatment for advanced non-small cell lung cancer (18). The effectiveness of glufosfamide in HNSCC has not been addressed so far.

The aim of the present study was to undertake a first evaluation of the activity of glufosfamide in HNSCC. Therefore, we investigated the responsiveness of unselected HNSCC biopsy specimens using a recently developed *ex vivo* colony formation assay, which allows us to determine the specific epithelial drug response (12). Since most current chemotherapy regimes in HNSCC are platinum-based (9,11), *cis*-platinum was chosen as a reference drug for estimating the possible clinical value of glufosfamide.

Among the 16 unselected HNSCC specimens from primary tumors tested for *ex vivo* response in the present study, only one specimen (patient #7, Table I) was sensitive to *cis*-platinum (6.3%). This patient was primarily treated with surgery followed by adjuvant radiotherapy. Accordingly, the clinical predictive value of the *ex vivo* test result could not be judged in this case. Nevertheless, the overall results to *cis*-platinum would be in agreement with the clinical response rate of 5% complete remissions for *cis*-platinum in combination with 5-fluorouracil in advanced HNSCC (19). In contrast to our results for *cis*-platinum, 5 out of 16 HNSCC specimens of primary tumors (31.3%) were sensitive to glc-IPM. Thus, glc-IPM was found to be more effective than *cis*-platinum in suppressing the *ex vivo* epithelial colony formation of specimens from the primary tumor. However, in all of the five tested metastatic specimens, neither glc-IPM, nor *cis*-platinum were effective in suppressing the *ex vivo* epithelial colony formation. Although the number of the metastatic specimens tested in this study is limited, our results point to the crucial role of chemoresistant metastases which clearly determine the clinical success of HNSCC chemotherapy.

Since the sensitivity to glc-IPM of the tested HNSCC specimens was consistently accompanied by resistance to *cis*-platinum (and *vice versa*), one could speculate that combining glc-IPM and *cis*-platinum might increase the efficacy of HNSCC chemotherapy. Recent studies on ifosfamide-platinoid combinations in HNSCC chemotherapy have demonstrated encouraging results for this combination (10). Considering these studies and the results of the present investigation, combining platinum compounds and glufosfamide would appear reasonable. Although the toxicity profiles of platinum compounds (20) and glufosfamide (6) are different, the nephrotoxicity of glufosfamide will certainly require special attention.

In conclusion, the present *ex vivo* study identifies glc-IPM as an attractive novel drug candidate for HNSCC chemotherapy, suggesting further studies on the role of glc-IPM in HNSCC.

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