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Effect of Small-molecule Tyrosine Kinase Inhibitors on PDGF-AA/BB and PDGFRα/β Expression in SCC According to HPV16 Status

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Abstract. *Background: Despite extensive research into new treatment options, the prognosis for head and neck squamous cell carcinoma remains poor. Platelet-derived growth factor (PDGF) is up-regulated in HNSCC and expression levels decrease after surgery, suggesting its role in tumour development. The influence of HPV on the PDGF/PDGF receptor (PDGFR) pathway remains unclear. In this study, we investigated the effect of small-molecule tyrosine kinase inhibitors (TKIs) on the expression of PDGF and its receptor in vitro using squamous cancer cell lines with different human papillomavirus 16 (HPV16) status. Materials and Methods: Two human HPV16-negative cell lines (UMSCC-11A/-14C) and one HPV16-positive cell line (CERV196) were used. Tumour cells were incubated with 20 μmol/l of TKIs nilotinib, dasatinib, afatinib, gefitinib and erlotinib for 24-96 h. Cell proliferation was assessed via proliferation assay and protein concentrations of PDGF-AA and BB and PDGFRα and -β via sandwich enzyme-linked immunosorbent assay. For statistical analysis, the results were compared with those from an untreated negative control. Results: PDGF-AA/BB and PDGFRα/-β were detected in all three tested cell lines. The addition of TKI led to a significant ($p<0.05$) decrease of PDGF/PDGFR at different time points and cell lines. The strongest effects were seen for the expression of PDGF-AA, which was consistently inhibited by most drugs. The effects of the TKI were independent of the HPV status. Conclusion: Proteins of this pathway can effectively be inhibited by small molecule TKIs. PDGF-AA seems to be a promising target for future studies with selective TKIs.*

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Head and neck squamous cell carcinoma (HNSCC) describes a heterogeneous group of epithelial malignancies of the upper aerodigestive tract. The worldwide annual incidence of HNSCC is at approximately 800,000 cases with a mortality rate of 430,000 cases per year, making HNSCC one of the most frequent cancer-associated causes of deaths (1, 2). Standard treatment options for HNSCC consist of different combinations of surgery, radiochemotherapy and new therapy approaches such as checkpoint inhibitors or antibodies to epidermal growth factor receptor (EGFR). Despite the extensive research and the rising development of targeted therapies, the prognosis remains poor (2). Abuse of tobacco and alcohol are the main risk factors for the development of HNSCC, known to have a synergistic effect (3). Even though substance abuse in patients with HNSCC is becoming rarer, a rise of incidence rates has been reported, particularly of oral cavity and oropharyngeal tumours. This finding has primarily been linked to infection with the human papillomavirus (HPV). A prevalence of over 20% can be seen in patients with oral squamous cell carcinoma (4, 5). HPV is divided into low-risk and high-risk types according to their risk of facilitating cancer formation. Most HPV infections are rapidly dealt with by an immune response. Infections with the high-risk types take longer to overcome and present a higher risk of viral DNA integration into the host genome. This leads to an overexpression of the viral oncogenes E6 and E7 and subsequently to a stimulation of cell proliferation and genomic instability (6). Among the high-risk HPV-types, HPV16 and -18 are most commonly found, with HPV16

being detected in almost 90% of HPV-associated oropharyngeal tumours (7). Patients with HPV-associated HNSCC tend to be younger, less frequently substance abusers and are often diagnosed with advanced stages because of early lymph nodal metastases at a small primary tumour size (8). HPV-positive HNSCC is associated with a better prognosis, but recent studies did not show promising results regarding the de-escalation of treatment (9).

Cancer is caused by a broad variety of molecular factors and the modulation of involved signalling pathways. In the present study, we examined the effect of small-molecule tyrosine kinase inhibitors (TKIs) on the platelet-derived growth factor (PDGF)/PDGF receptor (PDGFR) pathway in HPV-positive and HPV-negative squamous cell carcinoma cells. PDGF was first identified as a mitogen for mesenchymal cells (10, 11). Today there are five known isoforms: PDGF-AA, -AB, -BB, -CC and -DD. PDGF-AA, -AB and -BB consist of the A and B polypeptide chains, connected either as homodimeric or heterodimeric molecules by a disulphide bond (10). PDGF-CC and -DD were only discovered at the beginning of this century and are therefore less well known (12). The growth factors simultaneously bind to two receptors (PDGFRα and -β) leading to receptor autophosphorylation and the subsequent activation of different signalling pathways such as the mitogen-activated protein-kinase (MAPK) and phosphoinositide 3-kinase pathway. Through signal transduction via these pathways, the PDGF family plays an important role in cell proliferation, chemotaxis and cell migration, as well as angiogenesis and wound healing. By stimulating cell growth and angiogenesis, it is not only involved in physiological processes but also in the development of atherosclerosis and the formation of malignant tumours (13-16).

PDGF is found in the majority of HNSCCs (17). HPV positivity does not change the expression level of PDGF in HNSCC (18, 19). Previous studies of our group investigated the effect of imatinib, 5-fluorouracil, docetaxel and imatinib in combination with carboplatin on the expression of PDGF and vascular endothelial growth factor (VEGF) (18-20). Research on the inhibition of tumour angiogenesis in HNSCC is focused mainly on inhibiting VEGF (21, 22). PDGF is an independent angiogenic factor and is also able to stimulate VEGF expression (23). Therefore, it is a suitable target for antiangiogenic therapies in HNSCC, possibly in combination with VEGF inhibitors (24). Moreover, PDGF-AA has been hypothesized to play a role in the chemotaxis of stromal cells in HNSCC (25).

The PDGFRs are tyrosine kinases located at the cell membrane of various cells. Deregulated tyrosine kinases are known to play a major role in tumour development due to their multiple functions in signalling cascades. Small molecules have been discovered that are able to selectively inhibit specific tyrosine kinase activity by binding to the enzyme site competing with ATP (26). Imatinib, a TKI with activity against Abelson murine leukemia viral oncogene homolog 1 (ABL), breakpoint cluster region (BCR)–ABL, PDGFRβ, and tyrosine-protein kinase KIT (KIT), was approved as a treatment for chronic myeloid leukaemia in 2001 (27). Since then, new TKIs have been developed and approved to be used in the treatment of other tumour entities such as non-small cell lung cancer, pancreatic cancer and myeloproliferative diseases (28, 29). Nilotinib is a BCR–ABL inhibitor which is even more effective in the treatment of chronic myeloid leukaemia than imatinib (30), similar to dasatinib, which is also able to inhibit sarcoma tyrosine kinases (31). They both also inhibit PDGFR and KIT (31). Other small-molecule TKIs such as gefitinib, afatinib and erlotinib are used in the treatment of non-small cell lung cancer by selectively inhibiting EGFR (29, 32, 33). Cetuximab is an antibody to EGFR and is already approved for the treatment of recurrent and metastatic HNSCC (34), but small-molecule targeted therapies with TKIs have yet to be established. Dasatinib showed promising results against HNSCC in *in vitro* studies (35) but did not show an advantage *in vivo* when compared to established drugs such as methotrexate or cetuximab (36-39). However, treating patients with afatinib prolonged progression-free survival when compared to methotrexate (40). The molecular effects of small-molecule TKIs are not fully understood. A better understanding of the signalling pathways involved might lead to a better selection of patients who are suitable for treatment with TKIs, or to the discovery of new drug combinations.

In this study, we investigated the effects of selective TKIs erlotinib, gefitinib, afatinib, nilotinib and dasatinib on the expression of PDGF and PDGFR *in vitro* using HPV16-positive and HPV16-negative squamous cancer cell lines.

Materials and Methods

Cell lines, drugs and study design. Two human HPV16-negative cell lines [University of Michigan Squamous Cell Carcinoma (UMSCC) given by T.E. Carey, Ph.D., University of Michigan, Ann Arbor, MI, USA] and one human HPV16-positive cell line (CERV196; Cell Lines Service GmbH, Eppelheim, Germany) were used. The HPV16-negative cell lines originated from a primary squamous cell carcinoma (SCC) of the epiglottis (UMSCC-11A) and a skin metastasis of a floor of mouth SCC after surgery and radiochemotherapy (UMSCC-14C); the HPV16-positive cell line originated from a cervical SCC. UMSCC-11A and UMSCC-14C cells were cultured in Eagle's minimum essential medium (Gibco, Life Technologies, Carlsbad, CA, USA), containing 2 mM of L-glutamine and 10% fetal calf serum and antibiotics/antimycotics according to the instruction manual (Gibco, Life Technologies). CERV196 tumour cells were cultured in Eagle's minimum essential medium (Gibco, Life Technologies), supplemented with 2 mM L-glutamine, 1.0 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 g/l sodium pyruvate and 10% of fetal bovine serum (Gibco, Life Technologies). The cells were incubated under

standardised conditions at 37˚C, with 5% CO_2 and 95% humidity. New passages of the cells were generated by adding a phosphate-buffered saline solution supplemented with a combination of 0.05% trypsin and 0.02% EDTA (Sigma Aldrich; Merck KGaA, Darmstadt, Germany) at 37˚C for 5 min. Nilotinib, afatinib, dasatinib, gefitinib and erlotinib were provided by Professor Dr. Hofheinz, Oncological Department, University Hospital Mannheim, Medical Faculty Mannheim, University of Heidelberg, Germany. They were stored at room temperature and dissolved in dimethylsulfoxide when needed. Tumour cells were then incubated with 20 μmol/l of each drug at 37˚C for 24, 48, 72 and 96 h. Untreated cells served as a negative control.

Proliferation assay and enzyme-linked immunosorbent assay (ELISA) for PDGF-AA/BB and PDGFR-α/β. All experiments were repeated at least three times (n=3). To assess the proliferation of HNSCC tumour cells the alamarBlue (AbD Serotec, Raleigh, NC, USA) cell proliferation assay was used according to the manufacturer's protocol.

Measurement of protein concentrations was achieved using sandwich ELISA according to the manufacturer's instructions. DuoSet ELISA's (R&D Systems, Inc., Minneapolis, MN, USA) were used for PDGF-AA/BB (DY 221/DY 220) and PDGFRα/β (DYC 322/DYC 285). Optical density was measured with an MRX Microplate Reader (DYNEX Technologies, Chantilly, VA, USA) at a wavelength of 450 nm with a wavelength correction of 540 nm. The detection range was 15.6-1,000 pg/ml for PDGF-AA, 31.2-2,000 pg/ml for PDGF-BB, 312-20,000 pg/ml pg/ml for PDGFRα and 250-16,000 pg/ml for PDGFβ. The interassay coefficient of variation given by the manufacturer was below 10%.

Statistical analysis. Mean values were used for statistical analysis and are presented±standard deviation. The two coefficient variance test (SAS Statistics software, version 9.3; SAS Institute, Inc., Cary, NC, USA) and Dunnett's test were performed. Values of $p \leq 0.05$ were considered to be statistically significant. Statistical analysis was performed in cooperation with Professor Dr. C. Weiss, Institute of Biomathematics, Medical Faculty Mannheim, University of Heidelberg, Germany.

Results

PDGF-AA. PDGF-AA was detected in all three tested cell lines, with the expression levels rising in untreated cells over the course of 96 h. The levels of PDGF-AA were the highest in comparison to the other tested proteins. The protein level of PDGF-AA increased with the duration of exposure in all tested cell lines. The concentration of PDGF-AA was highest in HPV16-negative UMSCC-14C cells, although not significantly. The addition of erlotinib, nilotinib, dasatinib, gefitinib or afatinib led to a significant decrease (all $p<0.05$) at all time points in the UMSCC-14C cell line. The results for the other tested HPV16-negative cell line, UMSCC-11A, were not uniform: Afatinib and nilotinib significantly down-regulated the expression of PDGF-AA at all time points (all $p<0.05$), whereas gefitinib had little effect on PDGF-AA expression. Gefitinib led to a reduction of PDGF-AA at 24, 48 and 72 h but the effect was not significant. Dasatinib significantly reduced the expression level of PDGF-AA after 48 h ($p<0.02$), 72 h ($p<0.001$) and 96 h ($p<0.002$), while erlotinib only significantly down-regulated the expression of PDGF-AA within the first 72 h (all $p<0.05$). In the HPV16-positive cell line CERV196, the effect of the drugs resulted in an even more significant suppression of PDGF-AA (all $p<0.01$). The only exception was seen after incubation with nilotinib, for which a significant decrease in expression level was only seen at 48 h ($p<0.008$). The data for PDGF-AA are shown in Table I.

PDGF-BB. PDGF-BB was detected in all three tested cell lines, with the expression levels rising over the course of 96 h in untreated HPV16-negative cells. In the untreated HPV16-positive cell line, the expression started to decline after 72 h. All tested drugs except for nilotinib led to a decrease in PDGF-BB level in the UMSCC-11A cell line; afatinib and dasatinib significantly reduced PDGF-BB at all tested time points (all $p<0.05$), erlotinib reduced it after 48 h, whereas gefitinib did so after 72 h with a significant effect. After addition of nilotinib, the expression of PDGF-BB increased, reaching significance after 48 h (all $p<0.05$). In the UMSCC-14C cell line, none of the tested drugs led to a significant difference in expression level of PDGF-BB in comparison to the negative control within the first 48 h. After 48 h, afatinib, erlotinib, gefitinib and nilotinib up-regulated the expression level with a significant difference after 72 h ($p<0.05$). After 96 h of incubation, with afatinib the expression level of PDGF-BB increased slightly, however, in comparison to the negative control, the expression of PDGF-BB was significantly lower ($p<0.03$). Nilotinib continued to increase expression of PDGF-BB significantly ($p<0.01$ at 72 h and 96 h).

For the HPV16-positive cell line CERV96, addition of all drugs except nilotinib led to a significant increase in expression of PDGF-BB after 24 h (all $p<0.001$). At the subsequent time points, we did not observe a clear pattern of PDGF-BB expression after incubation with the selective TKIs except for nilotinib. Nilotinib led to a time-dependent increase in PDGF-BB level, with a significant difference from the negative control starting at 48 h (all $p<0.05$). A slight increase of PDGF-BB expression after incubation with erlotinib was seen, although without statistical significance. Afatinib, gefitinib and dasatinib led to unstable expression patterns of PDGF-BB. Afatinib led to a decrease in expression levels after 48 and 72 h ($p<0.001$) and increased expression after 96 h, without statistical significance. When treated with dasatinib and gefitinib, the expression level of PDGF-BB was significantly up-regulated at first (both $p<0.001$ after 24 h), then started to decrease (48 and 72 h) and then increase again, with a significant difference from the negative control at 96 h (both $p<0.01$). The data for PDGF-BB are shown in Table II.

Table I. *Platelet-derived growth factor AA (PDGF-AA) expression (pg/ml) in human papillomavirus 16 (HPV16)-negative UMSCC-11A and UMSCC-14C and HPV16-positive CERV196 cell lines after incubation with nilotinib, dasatinib, gefitinib, erlotinib or afatinib compared to untreated control cells. Statistically significant differences (p<0.05) are shown in bold.*

Cell line	Control	Nilotinib		Dasatinib		Gefitinib		Erlotinib		Afatinib	
	Mean±SD	Mean±SD	*p*-Value	Mean±SD	*p*-Value	Mean±SD	*p*-Value	Mean±SD	*p*-Value	Mean±SD	*p*-Value
UMSCC-11A											
24 h	158.7±15.0	73.0±19.0	**<0.001**	136.7±23.3	0.522	134.0±47.2	0.606	106.3±25.5	**0.040**	64.3±5.0	**<0.001**
48 h	672.0±118.0	162.3±30.6	**<0.001**	405.7±58.0	**0.013**	538.3±100.4	0.664	430.3±56.1	**0.034**	148.3±23.7	**<0.001**
72 h	1006.0±153.1	519.3±97.9	**0.001**	506.3±36.1	**<0.001**	819.7±24.7	0.091	704.0±127.4	**0.024**	250.0±28.4	**<0.001**
96 h	1054.0±6.2	672.0±45.1	**<0.001**	666.3±119.7	**0.001**	1140.0±103.9	0.324	1004.0±15.1	0.164	524.0±137.0	**0.019**
UMSCC-14C											
24 h	574.0±28.2	269.0±32.4	**<0.001**	284.3±46.1	**<0.001**	319.0±28.2	**0.013**	310.7±34.3	**0.007**	124.7±8.5	**<0.001**
48 h	1412.7±123.0	866.3±28.2	**<0.001**	702.0±19.1	**<0.001**	698.7±1.5	**<0.001**	714.3±92.7	**<0.001**	410.3±25.7	**<0.001**
72 h	1488.0±4.3	1345.0±53.1	**0.006**	867.3±35.2	**<0.001**	1060.7±46.4	**<0.001**	1096.7±13.9	**<0.001**	830.3±32.3	**<0.001**
96 h	2359.3±93.1	2122.3±131.5	**0.024**	1568.7±106.9	**0.001**	1778.0±138.1	**0.002**	1556.0±244.7	**<0.001**	1156.0±122.3	**<0.001**
CERV196											
24 h	125.0±14.8	114.3±4.2	0.507	82.3±8.7	**0.001**	80.3±0.6	**0.001**	60.3±2.5	**<0.001**	42.3±1.2	**<0.001**
48 h	455.0±33.8	345.3±20.0	**0.007**	185.3±26.0	**<0.001**	282.0±34.5	**0.003**	128.7±12.1	**<0.001**	130.0±7.2	**<0.001**
72 h	854.3±35.8	730.7±17.2	0.064	337.0±17.4	**<0.001**	341.0±39.5	**<0.001**	276.0±18.1	**<0.001**	270.0±49.4	**<0.001**
96 h	1026.7±25.7	1022.7±52.0	0.997	458.0±10.2	**<0.001**	528.3±16	**<0.001**	524.3±100.8	**<0.001**	385.3±48.2	**<0.001**

Table II. *Platelet-derived growth factor BB (PDGF-BB) expression (pg/ml) in human papillomavirus 16 (HPV16)-negative UMSCC-11A and UMSCC-14C and HPV16-positive CERV196 cell lines after incubation with nilotinib, dasatinib, gefitinib, erlotinib or afatinib compared to untreated control cells. Statistically significant differences (p<0.05) are shown in bold.*

Cell line	Control	Nilotinib		Dasatinib		Gefitinib		Erlotinib		Afatinib	
	Mean±SD	Mean±SD	*p*-Value	Mean±SD	*p*-Value	Mean±SD	*p*-Value	Mean±SD	*p*-Value	Mean±SD	*p*-Value
UMSCC-11A											
24 h	23.4±0.8	31.7±5.8	0.086	18.4±0.9	**0.005**	121.9±165.5	0.384	26.1±1.6	0.076	7.8±1.5	**<0.001**
48 h	93.6±11.2	142.4±17.5	**0.004**	38.5±3.4	**<0.001**	89.5±7.3	0.855	68±14.6	**0.028**	30.1±1.2	**<0.001**
72 h	181.3±27	273.9±12.9	**0.002**	40.6±2.7	**<0.001**	106.9±9.9	**0.001**	107.6±6.5	**0.001**	44.9±1.7	**<0.001**
96 h	229.1±4.4	342.5±34.2	**0.022**	40.7±7.2	**<0.001**	111.1±1.6	**<0.001**	125.1±4.9	**<0.001**	47.6±7.6	**<0.001**
UMSCC-14C											
24 h	4.8±16	4.8±1.8	>0.99	4.4±0.3	0.943	5.4±1.0	0.965	7..1±0.6	**0.050**	3.4±0.3	0.285
48 h	9.4±0.3	11.2±2.8	0.393	9.8±0.9	0.841	6.8±1.4	0.076	5.9±3.8	0.393	9.4±2.4	>0.99
72 h	10.4±1.8	36.4±2.2	**<0.001**	7.3±1.6	0.058	36.7±2.7	**<0.001**	35.7±0.9	**0.003**	19.3±2.7	**0.015**
96 h	39.6±8	117.9±27.6	**0.001**	33.3±3.3	0.263	38.0±2.4	0.950	32.2±1.4	0.263	22.4±2.8	**0.026**
CERV196											
24 h	1.7±0.8	5.5±0.6	0.248	9.3±0.3	**<0.001**	11.5±0.7	**<0.001**	9.2±0.2	**<0.001**	7.6±1.9	**<0.001**
48 h	7.7±0.6	11.2±0.4	**0.017**	7.3±0.7	0.939	9.4±1.1	0.789	10.5±1.4	0.297	5.8±0.5	0.341
72 h	10.4±0.1	22.9±0.6	**<0.001**	11±0.8	0.527	11.6±1.2	0.650	10.4±0.6	>0.99	7.6±0.7	**<0.001**
96 h	9.9±0.3	33.6±2.2	**<0.001**	7.1±0.9	**0.007**	16.1±0.8	**0.001**	12.7±1.1	0.068	9.5±0.9	0.641

PDGFRα. PDGFRα was detected in all three tested cell lines, with the expression levels rising over the course of 96 h in untreated HPV16-negative cells. In the untreated HPV16-positive cell line, the expression initially increased and then started to decline after 72 h. In the UMSCC-11A cell line, the effect of the different drugs was inconsistent, showing significant up-regulation of expression at first but then expression started to decline, reaching a significant decrease for some of the tested drugs. Gefitinib first increased the PDGFRα level until 48 h but then led to a continuous decline in expression with a significant effect after 96 h ($p<0.001$). Afatinib also led to a significant decrease of the expression of PDGFRα in UMSCC-11A cells after 96 h ($p<0.001$), without a continuous decline at earlier

Table III. *platelet-derived growth factor receptor α (PDGFRα) expression (pg/ml). in human papillomavirus 16 (HPV16)-negative UMSCC-11A and UMSCC-14C and HPV16-positive CERV196 cell lines after incubation with nilotinib, dasatinib, gefitinib, erlotinib or afatinib compared to untreated control cells. Statistically significant differences (p<0.05) are shown in bold.*

Cell line	Control	Nilotinib		Dasatinib		Gefitinib		Erlotinib		Afatinib	
	Mean±SD	Mean±SD	*p*-Value	Mean±SD	*p*-Value	Mean±SD	*p*-Value	Mean±SD	*p*-Value	Mean±SD	*p*-Value
UMSCC-11A											
24 h	120.0±5.3	168.3±26.1	**0.015**	159.3±9.9	**0.002**	134.3±37.0	0.699	163.0±7.0	**0.032**	105.7±45.7	0.794
48 h	140.0±9.5	151.7±9.5	0.249	199.0±15.5	**0.001**	159.3±12.9	0.354	167.0±88.6	0.827	144.3±4.6	0.957
72 h	185.7±14.6	179.7±14.4	0.879	147.7±20.4	0.136	149.3±35.2	0.142	136.3±28.1	**0.028**	173.0±12.5	0.534
96 h	225.0±5.2	162.0±12.2	0.257	148.3±16.2	**0.011**	153.7±10.3	**<0.001**	153.0±20.8	**<0.001**	131.7±9.0	**<0.001**
UMSCC-14C											
24 h	141.7±40.5	107.3±32.7	0.336	116.7±12.7	0.534	94.0±8.7	0.297	137.0±15.6	0.988	120.7±18.5	0.575
48 h	142.0±18.0	146.3±19.7	0.969	167.7±8.0	**0.039**	136.3±1.2	0.986	158.0±6.2	0.492	119.0±7.6	0.146
72 h	169.3±25.5	150.7±6.4	0.405	112.7±17.6	**0.009**	167.3±11.7	0.998	123.0±9.6	0.124	153.3±13.0	0.596
96 h	199.3±17.1	171.3±49.1	0.527	132.0±17.1	**0.002**	147.3±9.1	0.051	135.7±19.4	0.076	168.3±20.0	0.254
CERV196											
24 h	97.0±20.7	126.3±4.2	0.345	166.3±21.1	**0.018**	204.3±52.6	**0.009**	178.0±9.2	**0.003**	180.3±41.5	**0.008**
48 h	158.3±5.9	157.7±32.5	>0.99	150.7±14.4	0.953	189.0±19.7	0.712	19>0.99±24.4	0.390	169.7±39.0	0.930
72 h	190.3±8.3	189.3±23.6	>0.99	197.7±5.0	0.912	192.3±21.1	>0.99	174.7±22.1	0.743	167.7±25.4	0.457
96 h	168.0±5.2	226.7±47.6	0.116	170.0±55.1	>0.99	175.0±16.5	>0.99	126.0±22.9	0.484	165.3±17.0	0.970

time points. Nilotinib up-regulated expression after 24 h ($p<0.02$) with inconsistent but mainly decreasing protein levels at later time points. Erlotinib and dasatinib induced a significant increase in expression after 24 h (erlotinib, $p<0.04$) and at 24 and 48 h (dasatinib, both $p<0.01$), then started to induce a decrease, reaching a significant difference after 96 h (both $p<0.05$). In UMSCC-14C, only dasatinib led to a significant decrease in protein level of PDGFRα, starting after 72 h ($p<0.01$). At earlier time points it actually induced an increase in expression, reaching a significant difference after 48 h ($p<0.04$). The addition of gefitinib, nilotinib, erlotinib and afatinib to the UMSCC-14C cell line did not have a significant effect on PDGFRα expression at any time point. In CERV196 cells, all tested TKIs except for nilotinib caused a significant increase in PDGFR-α expression after 24 h (all $p<0.05$). At later time points, this effect disappeared. No significant difference from the negative control was observed. The data for PDGFR-α are shown in Table III.

PDGFRβ. PDGFRβ was detected in all three tested cell lines with the expression levels rising over the course of 96 h in untreated HPV16-negative cells. In the untreated HPV16-positive cell line, the expression started to decline after 48 h. Afatinib is the only selective TKI that led to a significant decrease in protein expression of PDGFRβ in either of the tested HPV16-negative cell lines at all time points (all $p<0.05$). In the HPV16-positive cell line, it only induced a significant decrease of PDGFRβ expression after 48 h ($p<0.01$) and 72 h ($p<0.002$). Treatment with dasatinib resulted in a significant increase of PDGFRβ over the first 48h in UMSCC-11A (both $p<0.05$). After 72 and 96 h, no significant effect was observed. In UMSCC-14C, a significant afatinib-induced decrease of PDGFRβ expression was observed at 48h, 72 and 96 h (all $p<0.05$). In the HPV16-positive cell line CERV96, afatinib led to an increase of PDGFRβ expression at all time points but a statistically significant increase of expression was only seen after 24 h ($p<0.001$). Treatment with nilotinib led to a significant down-regulation of PDGFRβ after 24 h ($p<0.04$) and with erlotinib after 96 h ($p<0.005$) in the UMSCC-11A cell line. Gefitinib led to a significant increase of PDGFRβ expression after 48 h in UMSCC-11A cells ($p<0.013$) and a significant decrease at 48 h in UMSCC-14C cells ($p<0.025$). In the HPV16-positive cell line CERV96, afatinib was the only drug that led to a significant decrease in expression. dasatinib, erlotinib, gefitinib and nilotinib even induced a significant increase in expression at different time points: Dasatinib and gefitinib after 24 h, erlotinib and gefitinib after 48 h, and nilotinib after 96 h (all $p<0.05$). The data for PDGFRβ are shown in Table IV.

Discussion

HNSCCs are treated with multiple different regimens. Surgery, radiotherapy, chemotherapy and targeted therapies such as checkpoint inhibitors (nivolumab, pembrolizumab) and monoclonal antibodies (cetuximab) are established agents but have so far failed to improve the poor prognosis of HNSCC significantly. Recent research projects have

Table IV. *platelet-derived growth factor receptor β (PDGFRβ) expression (pg/ml) in human papillomavirus 16 (HPV16)-negative UMSCC-11A and UMSCC-14C and HPV16-positive CERV196 cell lines after incubation with nilotinib, dasatinib, gefitinib, erlotinib or Afatinib compared to untreated control cells. Statistically significant differences (p<0.05) are shown in bold.*

Cell line	Control	Nilotinib		Dasatinib		Gefitinib		Erlotinib		Afatinib	
	Mean±SD	Mean±SD	*p*-Value	Mean±SD	*p*-Value	Mean±SD	*p*-Value	Mean±SD	*p*-Value	Mean±SD	*p*-Value
UMSCC-11A											
24 h	396.7±12.1	356.3±26.3	**0.039**	525.7±30.2	**0.006**	424.7±54.3	0.654	457.3±64.7	0.276	275.3±14.4	**<0.001**
48 h	433.7±25.4	481.3±54.9	0.280	539.7±47.3	**0.009**	554.0±40.6	**0.012**	472.0±165.7	0.940	351.3±2.3	**0.001**
72 h	530.0±14.0	554.0±39.9	0.598	505.3±21.9	0.924	520.0±77.6	0.985	407.7±96.2	0.101	308.0±37.0	**<0.001**
96 h	590.0±43.7	427.33±78.3	0.124	501.3±37.0	0.483	610.7±20.0	0.894	462.3±35.5	**0.004**	364.3±139.6	**0.017**
UMSCC-14C											
24 h	327.0±80.2	225.33±72.15	0.148	233.3±4.0	0.093	279.3±61.78	0.727	356.7±36.3	0.782	215.0±6.2	**0.026**
48 h	398.3±29.7	338.33±73.0	0.258	319.7±26.4	**0.016**	293.7±7.5	**0.024**	326.7±57.4	0.160	190.7±17.7	**<0.001**
72 h	400.0±37.2	388.7±51.9	0.949	276.7±7.5	**0.014**	488.3±40.1	0.105	432.0±22	0.851	299.3±4.0	**0.007**
96 h	566.3±113.9	601.33±53.4	0.918	238.0±29.8	**0.001**	422.7±53.3	0.153	409.3±39.3	0.315	325.0±27.1	**0.004**
CERV196											
24 h	529.7±57.7	736.0±38.9	0.055	927.7±101.24	**<0.001**	961.7±202.82	**0.005**	753.0±311.5	0.370	690.0±146.7	0.180
48 h	850.7±125.7	830.3±59.0	0.994	948.0±44.0	0.362	1337.00±120.42	**0.002**	1065.0±84.04	**0.048**	565.3±52.5	**0.003**
72 h	704.0±44.2	759.0±58.1	0.687	736.0±42.5	0.853	1234.3±761.9	0.283	629.0±47.3	0.398	499.3±54.2	**0.001**
96 h	796.0±73.6	1138.67±46.9	**0.001**	898.7±40.2	0.476	1265.0±109.5	**0.004**	789.0±84.3	>0.99	669.5±23.3	0.061

focused on targeted therapies individually chosen for each patient. For this approach, reliable tumour markers are needed that can be analysed even before treatment starts. If stable predictive tumour markers were established, it would be possible to assess a potential response before choosing a treatment option. To date, pembrolizumab is the first drug to be established in the treatment of HNSCC for which the elevation of a predictive biomarker is tested before starting the treatment.

One of the pathways involved in the tumorigenesis and angiogenesis of varied cancer types is the PDGF pathway. Previous studies of this group investigated the impact of imatinib (also in combination with cisplatin), 5-fluorouracil and docetaxel on the PDGF pathway in HNSCC but also focused on the involvement of other signalling molecules such as VEGF, β-catenin, E-cadherin and matrix metallopeptidase 9 (18-20, 41, 42). Therapies targeting PDGFR are successfully administered for lung cancer, pancreatic cancer and other, non-malignant diseases. It has already been shown that PDGF is up-regulated in HNSCC and that the expression level decreases after surgery. This suggests that PDGF plays a role in the tumour development of HNSCC and might be used as a possible biomarker for prospective targeted therapies (16, 43, 44).

TKIs have been studied in trials as a second-line treatment for recurrent and metastatic HNSCC. The addition of gefitinib to docetaxel did not improve survival rates in comparison to docetaxel alone, neither did gefitinib in comparison to methotrexate alone (36, 37). Afatinib, however, was able to prolong progression-free survival in comparison to methotrexate (40) and led to tumour shrinkage similar to that induced by cetuximab in another trial (45). Dasatinib inhibited migration and invasion by HNSCC cell lines (35), but was not able to induce an objective response when given as monotherapy in a phase II study (38).

In this study, we analysed the effects of five different TKIs on PGDF-AA/BB and PDGFRα/β expression levels. All tested tumour cell lines expressed PDGF-AA/BB, PDGFRα and -β with increasing levels over exposure time.

PDGFR is mostly found on mesenchymal cells, but in malignant tumours epithelial cells can undergo a process known as epithelial–mesenchymal transition and then begin to express PDGFR and become sensitive to PDGF stimulation (46). Out of these four tested proteins, PDGF-AA was expressed at the highest level, whereas the other tested target proteins showed lower expression levels which started to decline in the HPV16-positive cell line at late time points. These results are in line with previous studies (25, 47) and make PDGF-AA the most promising target of this study. In our study, PDGF-AA was consistently inhibited by most drugs and the inhibition of PDGF-AA showed the best response in comparison to the inhibition of PDGF-BB or PDGFR.

Most TKIs target more than one kinase activity (48). A small-molecule kinase inhibitor is typically not specific because the ATP binding site of tyrosine kinases is similar between the different types of kinases (49). For nilotinib (50, 51) and dasatinib (52) a direct influence on PDGFRα/β and PDGF-BB has already been described (53). Therefore, we expected these two drugs to have the greatest impact on

PDGF and PDGFRs, as was previously shown for imatinib (18). In our study, dasatinib successfully reduced expression of PDGF/PDGFR in some cell lines, with the best results seen in the reduction of PDGF-AA level over all cell lines. Nilotinib was able to induce a decrease in PDGF-AA in the HPV16-negative cell lines, but in fact had a contrary effect on PDGF-BB, with an increase of expression, and had little effect on PDGFR. In a previous study, dasatinib was shown to have a stronger impact than nilotinib on PDGFRβ, while both drugs were able to induce a decrease of expression (53). In our study, dasatinib reduced PDGFRβ expression in the UMSCC-14C cell line and nilotinib had a smaller effect only after 24 h on the UMSCC-11A cell line. It has already been discussed that better results in the UMSCC-11A cell line might be due to its origin from a primary tumour contrary to the UMSCC-14C cell line, which was derived from a skin metastasis which already had been treated and therefore might possibly be genetically altered (41). In our study, the drugs did not consistently show better results against the UMSCC-11A cell line, but dasatinib induced a decline in expression of PDGF-BB in the UMSCC-11A cell line that was not seen in the UMSCC-14C cell line. When aiming to inhibit PDGFRβ, only dasatinib led to a significant decrease in expression in the UMSCC-14C cell line but an increase in the UMSCC-11A cell line.

Gefitinib, erlotinib and afatinib are inhibitors of the EGFR and are approved for the treatment of non-small cell lung cancer. They have no direct effect on the PDGF/PDGFR mediated signalling pathways (53). However, it has been shown that PDGFRβ and EGFR form active heterodimers and through this mechanism a transactivation of EGFR signalling occurs when cells are stimulated with PDGF. Inhibition of PDGF/PDGFR signalling was therefore also mediated by direct inhibitors of EGFR (54). In our study, all of the tested TKIs showed a significant effect on PDGF-AA expression except for gefitinib against the UMSCC-11A cell line. Similarly to the previously described results with dasatinib, the EGFR inhibitors gefitinib, erlotinib and afatinib had the greatest effect on the PDGF-BB level in the UMSCC-11A cell line, sometimes even inducing an increase in expression level in the other cell lines, without leading to consistently better results on the other tested proteins in this cell line. The difference in PDGFR expression after treating the cells with gefitinib and erlotinib was not significant in most cell lines, but afatinib induced a significant decline in PDGFRβ in all cell lines. Our results are in line with previous studies testing gefitinib and erlotinib as they showed only a small impact on PDGFR level (53).

The specific and often multiple effects of small-molecule TKIs are not yet fully understood. As previously mentioned, they are able to inhibit several tyrosine kinases, leading to both specific effects and side-effects for each drug. The partly unexpected results of our study with an increase of PDGF-BB expression after treatment with nilotinib might be due to a response to the inhibition of tyrosine kinases that has not been explored yet. It has been shown that inhibition of one tyrosine kinase can favour the heterodimerization of other tyrosine kinases, leading to the activation of parallel pathways (55). It is possible that in HNSCC, nilotinib favours a pathway that leads to a compensatory overexpression of PDGF-BB.

The effects of the small-molecule TKIs were independent of the HPV16 status of the tested cell lines. A similar study of our group showed the same results for imatinib (18). In HPV-positive cells, the oncoproteins E6 and E7 disable tumour-suppressor genes P53 and retinoblastoma and subsequently contribute to carcinogenesis (56). A link between the papillomavirus transmembrane protein E5 and the PDGFRβ has been found, but activation of PDGFR by one of the main oncoproteins E6/E7 in HPV16-positive cells has not been discovered yet (57, 58). Gu and Matlashewski stated that HPV-related oncoproteins E6 and E7 have no influence on MAPK activity which is mediated by PDGF, but a significant influence on other PDGF-related signalling pathways cannot be excluded (59). Recent studies contradict this finding and show that the MAPK and nuclear factor kappa-light-chain-enhancer of activated B-cells pathways are important for E6- and E7-mediated carcinogenesis (60, 61). E5 activates PDGFRβ (59), which could explain the increased expression of the receptor in the HPV16-positive cells. E6/E7 are responsible for most of the mechanisms involved in cancer genesis such as inhibition of apoptosis or genomic instability, but E5 is nevertheless an oncoprotein involved in tumour cell proliferation (62). Although we did not find a significant difference in the inhibition of the PDGF/PGDFR pathway between HPV16-positive and -negative squamous cancer cells by small-molecule TKIs, previous studies suggest that the pathway is influenced by HPV-related oncoprotein E5 (57, 58).

In our study, the small TKIs had a stronger impact on PDGF expression than on PDGFR expression even though they should act only through direct inhibition of the receptors. Other groups have also confirmed that inhibition of the receptors leads to a decrease of expression of the downstream ligands (63). In healthy tissue, PDGF acts mostly as a ligand for nearby cells in a paracrine manner (64). The PDGFR-expressing cells of different malignant tumour entities are able to produce PDGF themselves, leading to an autocrine PDGF–PDGFR loop. The establishment of this autocrine loop seems to play a role in self-sufficient tumour growth and is even proposed to be an essential factor in the development of metastasis in breast cancer (64, 65).

The best overall results were seen in the suppression of PDGF-AA in the HPV16-negative cell line UMSCC-14C and the HPV16-positive cell line CERV196. The up-regulation of PDGF-AA is correlated with a worse prognosis in tumours such as gastric carcinoma, and pancreatic , lung

and prostate cancer (25, 66, 67). Moreover, PDGF-AA has been hypothesized to be one of the driving factors for chemotaxis of stromal cells in HNSCC (25). The neutralization of PDGF-AA and the inhibition of PDGFRα lead to a significant reduction of chemotaxis *in vitro* (25). Inhibition of PDGFRβ did not have the same effect. The tumour microenvironment is the focus of intensive research because it plays an important role in supporting tumour growth and resistance to radiochemotherapy (68).

Because PDGF-AA expression levels was the highest in all tumour cell lines and it was most effectively inhibited by the tested small-molecule TKIs, it seems to be the most promising target for the development of a biomarker and for further research for treatment options in HNSCC. It has already been discussed that malignant tumours are able to evade the effect of targeted therapy strategies which have only one single receptor as target. Therefore, the formation of receptor heterodimers composed of EGFR and PDGFR might be an effective approach to increasing the effectiveness of a targeted therapy strategy (69). Future research projects with small-molecule TKIs should include approaches combining EGFR- and PDGFR-specific inhibitors.

In conclusion, our results show that proteins of the PDGF/PDGFR pathway can be effectively inhibited by small-molecule tyrosine TKIs and therefore warrant further research for development of targeted therapies in HNSCC.

Conflicts of Interest

All Authors declare that they have no conflicts of interest.

Authors' Contributions

Lena Huber, Benedikt Kramer and Christoph Aderhold conceived and planned the experimental design. Benedikt Kramer and Christoph Aderhold carried out the experiments. Lena Huber, Anne Lammert, Christoph Aderhold and Benedikt Kramer contributed to the interpretation of the results. Lena Huber and Benedikt Kramer took lead in writing the article. All Authors provided critical feedback, discussed the results and commented on the article.

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