Synergistic effects of imatinib and carboplatin on VEGF, PDGF and PDGF-Rα/ß expression in squamous cell carcinoma of the head and neck *in vitro*

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Abstract. Head and neck squamous cell carcinoma (HNSCC) is an aggressive epithelial malignancy. The development of new treatment modalities in order to improve long-term survival of patients with HNSCC is imperative. Numerous studies have demonstrated that carcinogenesis and tumor cell dissemination is influenced by the tumor microenvironment. The protein-kinase-receptors (PTKs) are essential elements of the intracellular signal transduction pathway and regulate cell growth, development and apoptosis. Cell proliferation, migration, induction of tumor vascularization and carcinogenesis, invasion is regulated by a variety of angiogenic factors, such as PDGF (platelet-derived growth factor), VEGF (vascular endothelial growth factor) and their respective tyrosine kinase receptors (PDGF-R and VEGF-R). They present promising targets for anti-cancer therapy through abrogation of impaired signaling pathways. Indeed, imatinib, a small molecule drug targeting these protein kinases, has antiproliferative effects in several cancer types. The purpose of this study was to investigate the potential synergism of imatinib and carboplatin on the expression of PDGF, PDGF-R α/ß and VEGF in different HNSCC cell lines. Several tumor cell lines were subjected to increasing concentrations of carboplatin (3 and 7.5 μmol/l) and imatinib (18 and 30 μmol/l) and ELISA, immunohistochemical methods and RQ-PRC after 48, 72, 120 and 240 h were used to assess their expression levels. While PDGF-Rα/ß expression was unimpaired at lower imatinib concentrations (18 μmol/l), PDGF-Rα/ß expression was suppressed at 30 μmol/l, and suppression was enhanced by the presence of carboplatin. By RQ-PCR, a significant reduction of PDGF-Rα/ß expression was detected ($p<0.5$). We observed explicit significant reduction in VEGF levels with increasing concentrations of imatinib and with the combination of the two chemotherapeutic drugs ($p<0.5$). We report for the first time evidence of synergism of imatinib and carboplatin in suppressing VEGF, PDGF and PDGF-Rα/ß expression in HNSCC.

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is an aggressive epithelial malignancy known to be the most common neoplasm arising in the upper aerodigestiv tract. With a global annual incidence of approximately 644,000 cases and 352,000 HNSCC-associated deaths, squamous cell carcinoma of the head and neck (HNSCC) is the fifth most common cancer worldwide (1,2). The poor five-year survival rate has remained unchanged in the last decades, despite improved techniques in surgery, and the radiation and chemotherapy established (3). Thus, the development of new treatment modalities in order to improve long-term survival of patients with HNSCC is imperative.

Promotion of tumor growth, angiogenesis and invasion of HNSCC is attributed to different cell types populating the tumor stroma, such as endothelial cells, cancer-associated fibroblasts (CAFs), pericytes and infiltrating inflammatory cells. These cell types are implicated as functionally important for carcinogenesis by providing proliferative and anti-apoptotic regulatory factors leading to tumor angiogenesis, and facilitating invasion (4-9). Various studies could demonstrate the fundamental role of a concerted performance of genetically altered tumor cells interacting with ostensibly normal cell types in carcinogenesis. These factors and their interaction constitute the tumor microenvironment (10).

A variety of angiogenic peptides, such as VEGF (vascular endothelial growth factor), PDGF (platelet-derived growth factor), released from tumor cells and macrophages regulate the induction of the tumor vascularization. Various functions, including endothelial cell migration, proliferation and capillary tubule formation are performed by these factors.

Platelet-derived growth factor (PDGF). One of the detected factors providing this cellular interaction and phenotypic character of the CAFs is platelet-derived growth factor (PDGF) (6,8,11). The involvement of the different isoforms of PDGF plays a decisive role in regulating cell proliferation, chemotaxis and survival in normal cells as well as in the process of tumorigenesis by autocrine and paracrine stimulation (12-14). The biological activity of the different compounds of the PDGF family, characterized by the polypeptide chains A, B, C, D, forming the homodimer AA, BB, CC, DD and heterodimers AB, is based on the association with the tyrosine kinase receptors, PDGF-α and -ß receptors (15). Upon binding of PDGF to its cognate receptor, receptor tyrosine kinase activity is induced through receptor dimerisation and subsequent autophosphorylation. Active PDGF-R induces signaling molecules that conduct to the different biological effects of PDGF mentioned above (16,17), such as MAP kinase (mitogen-activated protein kinases) or ras/raf (family of three serine/threonine-specific protein kinases)/MEK/ERK (extracellular-signal-regulated kinases) signaling.

Previous studies demonstrated the upregulated expression of PDGF and the associated receptors in various malignant human tumors such as non-small cell lung (18) and prostate cancer (14). PDGF promotes vascularization of stroma and therefore tumor proliferation (19,20) in melanoma (21), fibrosarcoma (22), breast (23) and squamous carcinoma (24). Many additional angiogenic factors have been identified including vascular endothelial growth factor (VEGF) (25).

Vascular endothelial growth factor (VEGF). VEGF is a potent endothelial mitogen factor that also promotes angiogenesis and enhances vascular permeability (26). Its overexpression is associated with enhanced tumor growth and angiogenesis in a mouse model (27) as well as in various malignant human tumors including HNSCC (28-30). Furthermore, Riedel *et al* demonstrated raised VEGF serum levels in HNSCC patients compared with healthy subjects (31). Angiogenesis induced by VEGF overexpression in HNSCC tumors is inversely correlated with inhibition of apoptosis (32,33), illustrating the anti-apoptotic potential of VEGF. Inhibition of angiogenesis by blocking these angiogenic cytokines or their pathways has become a major target in experimental cancer therapies.

Protein-tyrosine-kinase-receptors (PTKs) are essential elements of the intracellular signal transduction pathway, which regulate cell growth, development and apoptosis, which in case of mutations in different types of cancer often cause disruption of normal cell signaling pathways and might lead to malignant transformation (34). For example mutations in PTKs lead to a constitutively activition ot the downstream signaling.

Imatinib (STI571). The tyrosine kinase inhibitor imatinib (Gleevec®), also known as STI571, manufactured by Novartis, Basel, Switzerland), is a small molecule drug of the 2-phenylaminopyrimidine class. It is a potent inhibitor of bcr-abl (abelson murine leukemia viral oncogene homolog), c-kit (CD117) and PGDF-R, initially used in the treatment of chronic myeloid leukemia (CML) (35-38).

The ability of imatinib to target specific tyrosine kinases, which are implicated in tumorigenesis and metastasis, makes it an ideal therapeutic agent with few associated side-effects. The drug is currently used in a limited number of cancers such as gastrointestinal stromal tumors, melanomas and various forms of lung cancer (39,40). Indeed several studies have demonstrated the proapoptotic effects of imatinib, and thus it was subsequently evaluated in concert with chemotherapeutics acting through distinct mechanism, such as those derived from cisplatin family.

Prior studies suggested a potential synergism upon the co-administration of imatinib and cisplatin, through their abilities of targeting distinct cell-proliferative functions and were shown to enhance the therapeutic ratio (41), our study is the first to our knowledge evaluating the potential synergism of imatinib and carboplatin for HNSCC.

Synergism with platin-based chemotherapy. Imatinib shows synergistic effects concerning growth inhibition with the established chemotherapeutic substances in leukemia and in adenocarcinomas (42,43). Zhang *et al* showed synergistic effects leading to an increased growth inhibition of the simultaneous treatment of NSCLS (non-small-cell lung cancer) with cisplatin and imatinib (44). Iain *et al* allocate this statistically significant interaction between these two drugs concerning the growth inhibitory effects for HNSCC (45). The hypothesis of the mechanism of the synergy postulates that imatinib delays DNA (deoxyribonucleic acid) cross-link repair induced by cisplatin. Furthermore, imatinib has been reported to alter the balance between pro- and antiapoptotic factors by upregulating pro-apoptotic BCL-2 (B-cell lymphoma), one member of apoptosis regulators (46,47). The observed synergy in the literature may be caused by the effect of imatinib on the repair of DNA damage and consequently maintaining or enhancing the molecular triggers that initiate cell death pathways. The explicit underlying mechanism of the synergism still remains unclear.

Carboplatin, cis-diammine [cyclobutane-1,1-dicarboxylate-O,O')platinum(II)], is one of the most important drugs for the treatment of head and neck cancer. The molecular mechanism of carboplatin in inhibiting cell growth is through the formation of intrastrand crosslinked adducts of DNA. DNA crosslinking causes abrogation of DNA synthesis, suppression of RNA (ribonucleic acid) synthesis, leading to cell cycle perturbation and induction of tumor suppressor genes such as p53 (tumor protein 53) and p73 (tumor protein 73) (49). Thus, widespread DNA damage caused by carboplatin ultimately initiates various apoptotic signaling pathways, leading to cell death and reduction of tumor growth (48). This cytotoxic profile has made carboplatin one of the most successful chemotherapeutic agents for a number of cancers.

The purpose of this study was to evaluate synergistic effects of imatinib, both alone and in combination with carboplatin, as an established chemotherapeutic agent in HNSCC treatment, on the expression of VEGF, PDGFα and the corresponding receptors, PDGF-Rα/ß *in vitro*. To our knowledge, this is the first report of a chemotherapeutic study treating HNSCC with imatinib and carboplatin.

Materials and methods

Cell lines. The HNSCC cell lines 11B, 14C and 22B were obtained from Dr T.E. Carey (University of Michigan, MI, USA). They originate from human HNSCC of the oro- and hypopharynx and larynx. Cell cultures were grown at 37˚C in

a fully humidified atmosphere with 5% CO_2 using Dulbecco's modified minimum essential medium (DMEM) (Fisher Scientific Co., Pittsburgh, PA, USA) containing 10% fetal calf serum (FCS) and antibiotics (Life Technologies Inc., Gainthersburg, MD, USA). Imatinib was kindly provided by the manufacturer (Novartis). Imatinib and carboplatin were stored at 4˚C and dissolved in sterile water before use. The HNSCC cell lines were subjected to different concentrations of imatinib (18 and 30 μmol/l) and/or carboplatin (3 and 7.5 μmol) and grown for 2, 3, 5 and 10 days. Drug concentrations were chosen based on AlamarBlue (AbD Serotec Oxford, UK) cell proliferation assay, which quantitatively measures proliferation of HNSCC tumor cell lines in response of the chemotherapeutic drug and its concentration. After the incubation period the supernatants were collected and stored at -20˚C until further analysis. After harvesting the cells through centrifugation of the suspension cell lines, cells were stored at -80˚C for PCR (polymerase chain reaction) analysis.

Immunohistochemistry of PDGF-A/B ligand. Immunohistochemical studies were performed using a monoclonal rabbit antibody directed against human PDGF-A/B (ACRIS Antibodies, Herford, Germany). The cells were cultured overnight on glass coverslips (Nunc, Wiesbaden, Germany) before immunohistochemistry was done. When 50% confluent, cells were exposed to the chemotherapeutics for different time periods as described above and subsequently fixed with acetone and alcohol (2:1). After washing the cells with PBS (phosphate-buffered saline) the samples were incubated with the peroxidase block (Dako Hamburg, Germany) for 30 min. Following further PBS washes (3x5 min). Cells were incubated with 10% normal sheep serum for 30 min. Cells were exposed to the rabbit monoclonal antibody against PDGF-A/B for 30 min at room temperature 1:100 (ACRIS Antibodies). The incubated cell lines were cooled overnight at 4˚C. Afterwards the cells were washed with PBS as before and incubated with secondary antibody (1:100) (anti-rabbit, Amersham, Freiburg, Germany) for 45 min at room temperature. The incubation was followed by a three-times washing procedure. Strepavidin HRP (horseradish peroxidase)-conjugated secondary antibody was detected by AEC (aminoehtylcarbazole red) or DAB (diaminobenzidine brown) as chromogen and incubated for 5-15 min. After blocking of endogenous peroxidase, cells were washed various times. Finally sections received a counterstaining with Harris' hematoxylin for 30 sec. This procedure was followed by coverslipping.

The results of the observed immunohistochemical rates of PDGF expression were determined semi-quantitatively. The stain intensity was as follows: strong reactivity, >80% of the cells were positive; moderate reactivity, 50-80% of the cells stained positive; weak reactivity, <50% of the cells were positive; and negative immunostained cells (0% reactivity).

ELISA total PDGF-Rα/ß (receptor) and VEGF (ligand). Cells having been exposed to the different chemotherapeutics at their respective concentrations as described above were also rinsed with PBS and then lysed using lysis buffer under gently vortexing at 2-8˚C for 30 min. Cell debris was separated by centrifugation (14,000 x g for 5 min) and supernatant was kept for further analysis.

VEGF concentrations were determined by ELISA (enzyme-linked immunosorbent assay) (R&D Systems, Wiesbaden, Germany). The system used a solid-phase monoclonal antibody directed against VEGF and an enzyme-linked polyclonal antibody, raised against recombinant VEGF165. The specificity of anti-human VEGF antibodies used in the ELISA kit was examined by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting. According to the manufacturer's directions, each ELISA assay measured 100 μl of supernatant. All analyses and calibrations were carried out in duplicate. The calibrations on each microtiter plate included recombinant human VEGF standards provided in the kit. Optical density was determined using a micro-plate reader at a wavelength of 450 nm. Wavelength correction was set to 540 nm and concentrations were reported as pg/ml. The variability between assays as reported by the manufacturer ranged from 6.2 to 8.8% when VEGF concentrations were between 50 and 1000 pg/ml.

The DouSet IC ELISA (R&D Systems) measures human PDGF-Rα/ß by development of a sandwich ELISA with an immobilized capture antibody specific for human PDGF-R, which binds both tyrosin-phosphorylated and unphosphorylated PDGF-R. After washing away the unbound material, a biotinylated detection antibody specific for total human PDGF-R is used to detect tyrosin-phosphorylated and unphosphorylated PDGF-R, utilizing a standard strepavidin-HRP format.

The capture antibody was diluted to the concentration 1:180 (4 μg/ml). The diluted capture antibody was carried out 100 μl per well and the plate was sealed and incubated overnight. Afterwards three times aspiration and washing of each well with 400 μl tween wash buffer. Adjacent plates were blocked by adding 300 μl of block buffer to each well and incubated at room temperature for 1-2 h. According to the manufacturer's directions, each ELISA assay measured 100 μl of supernatant of the sample. Washing was repeated with tween buffer as described. After having diluted the detection antibody to a concentration of 500 ng/ml, 100 μl of this was added to each well with an incubation period of 2 h. Again cells were washed and 100 μg streptavidin-HRP were added to each well and incubated for 20 min at room temperature. Afterwards 100 μl substrate solution was added for 20 min followed by 50 μl stop solution to each well. Optical density was determined using a micro-plate reader at a wavelength of 450 nm. Wavelength correction was set to 540 nm and concentrations were reported as pg/ml. All analyses and calibrations were carried out in duplicate.

RNA extraction and cDNA synthesis. After distributing the tumor cell lines in 6-well chambers and after reaching 50% confluence, the cells were exposed to the different drug concentrations for up to 10 days.

Total RNA was extracted after homogenization of 15-30 mg tissue with the Ultra Turrax Tube Drive (Ika, Staufen, Germany) using TRIzol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. A sample of 5 mg of total RNA was reverse transcribed using random hexamer priming and Moloney murine leukemia virus reverse transcriptase (Invitrogen). After that RNA extraction of all frozen samples and reverse transcription to cDNA was performed.

Table I. PDGF-Rα/ß oligonucleotides used for RQ-PCR assays and cloning of the plasmid standard.

Assay		Name	Sequences 5'-3'
PDGF-Rα quantification	LightCycler primer	PDA12	CCAAGAGATGGACTAGTGCTTG
PDGF-Rα quantification	LightCycler primer	PDA15	TAGCTCCGTGTGCTTTCATCAG
PDGF-Rα quantification	LightCycler hybridisation probe	PDA15FL	GAATAGGGATAGCTTCCTGAGCCACCA-Fluorescein
PDGF-Rα quantification	LightCycler hybridisation probe	PDA15LC	LCRED640-CCAGAGAAGCCAAAGAAAGAGCTGGA
PDGF-Rß quantification	LightCycler primer	PDB13F	CGTCAAGATGCTTAAATCCACAGC
PDGF-Rß quantification	LightCycler primer	PDB15R	TGATGATATAGATGGGTCCTCCTTTG
PDGF-Rß quantification	LightCycler hybridisation probe	PDB14FL	GCTGAAGATCATGAGTCACCTTGGGC-Fluorescein
PDGF-Rß quantification	LightCycler hybridisation probe	PDB14LC	LCRED640-CCACCTGAACGTGGTCAACCTGTTG
PDGF-Rß quantification	LightCycler primer	PDB9F	GAGACCTCAAAAGGTGTCCACG

Real-time quantitative reverse transcriptase polymerase chain reaction (RQ-PCR). Expression analysis of PDGF-Rα, PDGF-Rß was performed as described (51). VEGF-A and VEGF-B expression analysis was executed using the LightCycler instrument 1.5 (Roche Diagnostics, Mannheim, Germany). Each 20-μl reaction mix contained 4 μl LightCycler Faststart DNA Master^{plus} Hyb Probes master mix (Roche Diagnostics), 2 μl cDNA template or plasmid dilution, 0.5 μM forward primers and 0.5 μM reverse primer 0.25 μM anchor probe and 0.25 μM sensor probe (TIB Molbiol, Berlin, Germany). The primer and probe sequences are shown in Table I. Cycler conditions were the following: 10 min denaturation at 95˚C, 50 cycles of 10 sec at 59/60˚C (annealing PDGFRA and PDGFRB/VEGFR, VEGF) and 26 sec at 72˚C (elongation). A 5-log series of plasmid dilutions (see below) was amplified within the PCR runs for quantification of PDGFRA, PDGFRB. ß-glucuronidase (GUS) mRNA (messenger ribonucleic acid) was quantified as internal control as previously described (51). The LightCycler software prepares standard curves using linear regression analysis of the plasmid dilutions and calculates copy numbers of the unknown sample (51).

Cloning of quantification standards. For plasmid preparation, nested or single-step RT-PCR (real-time polymerase chain reaction) products from sequences (PDGFRA, PDGFRB), were amplified from cell lines (HL-60, or SW480) (primers Table I). The Expand high fidelity plus PCR system™ (Roche Diagnostics) was used. PCR transcripts were cloned into the PCR2.1-TOPO vector and introduced into *E. coli* TOP10F' according to the manufacturers' instructions (Invitrogen). Plasmid DNA containing the desired construct was isolated using the Plasmid Midi and Maxi Kit™ (Qiagen, Hilden, Germany) and inserts were confirmed by bidirectional direct sequencing. The resulting plasmid was linearised by *Xba*I digestion at 37˚C for 2 h followed by heat inactivation at 65˚C for 20 min. GUS mRNA transcripts were measured as internal control using a standard plasmid (pME-2) containing BCR-ABL (breakpoint cluster region), ABL (abelson murine leukemia viral oncogene homolog), and GUS sequences (56). Dilutions of the linearised plasmid were prepared in 10 mM Tris-HCl pH 8.0; 1 mM EDTA containing 20 μg/ml tRNA (transfer ribonucleic acid) (Roche Diagnostics).

Statistical analysis. Statistical analysis was performed in collaboration with Dr Chr. Weiss, Institute of Biomathematics, Faculty of Medicine, Mannheim. All data were subjected to the Means procedure. $p \leq 0.05$ was considered statistically significant. The statistical test was the two coefficient variance analysis and the Dunnett's-test.

Results

In order to analyse the effect of carboplatin and imatinib on HNSCC cell lines, we added two concentrations of imatinib (18 and 30 μmol/l) and carboplatin (3 and 7.5 μmol/l). PDGF, PDGF-Rα/ß and VEGF expression in the supernatants of HNSCC the cell lines were assessed by ELISA, immunohistochemical methods and RQ-PCR after 2, 3, 5 and 10 days post addition of the chemotherapeutic.

Immunohistochemistry for PDGF-α (ligand). The results of the immunohistochemically observed rates of expression were determined semi-quantitatively. The immunohistochemical studies against PDGF-α showed decreased reactivity with

Table II. Grading of immunostaining for PDGF-α.[a]

Immunostaining index	48 h	72 h	120 h	240 h
Control group	++ (3/3)	++ (2/3) +++ (1/3)	++ (2/3) +++ (1/3)	+++ (3/3)
Carboplatin 3 μmol	+ (1/3) ++ (2/3)	++ (2/3) ++ (1/3) +++ (1/3)	++ (2/3) +++ (1/3)	++ (1/3) +++ (2/3)
Carboplatin 7.5 μmol	++ (2/3) ++ (2/3)	+ (2/3) +++ (2/3)	+ (2/3) ++ (1/3)	+++ (2/3) +++ (1/3)
Imatinib 18 μmol	+ (2/3) ++ (1/3)	+ (2/3) ++ (1/3)	++ (1/3) ++ (1/3) ++ (1/3)	++ (1/3) ++ (2/3)
Imatinib 30 μmol	+ (1/3) + (2/3)	0 (1/3) + (2/3)	0 (1/3) + (2/3)	0 (1/3) + (1/3) +++ (1/3)
Carboplatin 3 μmol + imatinib 18 μmol	+ (2/3) + (1/3)	+ (2/3) + (0/3)	+ (1/3) ++ (2/3)	+ (2/3) ++ (1/3)
Carboplatin 7.5 μmol + imatinib 30 μmol	+ (2/3)	+ (2/3) + (1/3)	0 (1/3)	0 (1/3)

[a]0, no positive cells; 1, weak immunostaining; 2, moderate immunostaining; 3, strong immunostaining. (x/3), number of positive cell lines out of the UMSCC lines 14C, 11B and 22B.

rising concentrations of carboplatin or imatinib, respectively and reactivity decreased the longer the incubation period was. By contrast the controls showed high reactivity against PDGF-α independent to the length of incubation time. The immunostaining was localized to the cytoplasm of the cells (Fig. 1, Table II).

ELISA for PDGF-R α/β of HNSCC 11B, 14C, 22B. To determine the effect of carboplatin and imatinib, as well as the combination of these chemotherapeutic agents on PDGF-R of HNSCC 11B, 14C and 22B cells, we added different concentrations of imatinib (18 and 30 μmol/l) and carboplatin (3 and 7.5 μmol/l). To quantify cytosolic expression of PDGF-Rα/ß as well as VEGF (ligand) secretion of the supernatant of HNSCC, cell lines were treated with either non-containing (control group) or imatinib and/or carboplatin containing medium.

PDGF-Rα/ß levels showed a consistent trend towards a decreased expression according to the treatment with rising drug concentration and extension of incubation period, as well as the type of the agent (Table III).

ELISA of PDGF-Rα. For HNSCC 11B a maximal reduction of PDGF-Rα expression at 7.5 μmol/l carboplatin after 240 h (172 pg/ml; negative control 308 pg/ml) and imatinib (30 μmol/l) after 240 h (107.46 pg/ml; negative control 308 pg/ml) was detected. The combination of carboplatin and imatinib induced no further reduction of PDGF-Rα level (after 240 h, 218 pg/ml; 181 pg/ml, respectively). The level of significance was p=0.56.

HNSCC 14C cell lines proved to be less vulnerable towards the drug in that a noticeable reduction of PDGF-α could only be proven for carboplatin/imatinib (3 μmol/l + 18 μmol/l after 72, 120, 240 h) ($cPDGF_{72h}$ = 13 pg/ml/$cPDGF_{control72h}$ = 226 pg/ml; $cPDGF_{120h}$ = 27 pg/ml/$cPDGF_{control120h}$ = 85 pg/ml; $cPDGF_{240h}$ = 49 pg/ml/$cPDGF_{control240h}$ = 122 pg/ml).

The maximal concentration of 7.5 μmol/l carboplatin + 30 μmol/l imatinib showed the following expression rates after 72 and 120 h ($cPDGF_{72h}$ = 6 pg/ml/$cPDGF_{control72h}$ = 226 pg/ml; $cPDGF_{120h}$ = 27 pg/ml/$cPDGF_{control120h}$ = 85 pg/ml), not statistically significant (p=0.77). Single-drug exposure showed less consequence on the PDGF-Rα level.

For HNSCC 22B there was no significant reduction in PDGF-Rα expression detectable (p=0.25). There was evidence of decreasing PDGF-Rα levels with increasing concentrations of imatinib (single-drug application of 18 μmol/) ($cPDGF_{72h}$ = 293 pg/ml; $cPDGF_{120h}$ = 136 pg/ml and for 30 μmol/l $cPDGF_{72h}$ = 339 pg/ml; $cPDGF_{120h}$ = 34 pg/ml; $cPDGF_{240h}$ = 20 pg/ml) and the combination of imatinib/carboplatin (18+3.5 μmol/l and 30+7.5 μmol/l) after 72, 120 and 240 h. Whereas carboplatin as single-active drug did not show influence on PDGF-Rα level.

ELISA of PDGF-Rß. Application of carboplatin 3 μmol/l and increase of the dosage to 7.5 μmol/l leads to a non-significant reduction (p=0.28) of PDGF-Rß level after 24 h for HNSCC 11B. Conversely, exposure to imatinib caused a consistent reduction even after 120 h. The combination of both agents confirms this consistent trend of decreased PDGF-Rß (7.5+30 μmol/l) ($cPDGF\text{-}R\beta_{120h}$ = 14 pg/ml/$cPDGF\text{-}R\beta_{control120h}$ = 28 pg/ml; $cPDGF\text{-}R\beta_{240h}$ = 12 pg/ml/$cPDGF\text{-}R\beta_{control240h}$ = 34 pg/ml).

Within the timeframe of 48-78 h, there was evidence for a reduction of an PDGF-Rß expression in HNSCC 14C by

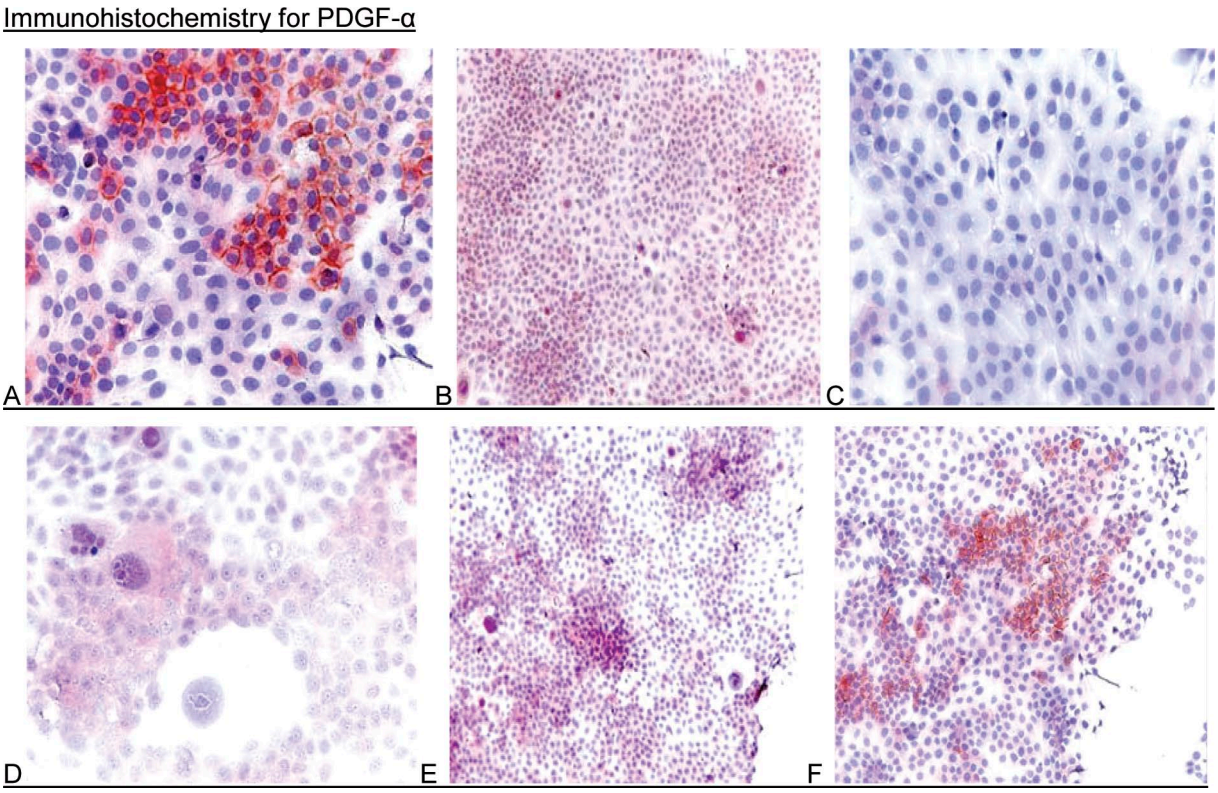

Figure 1. Immunohistochemistry for PDGF-α expression; (A and B) Positive immunohistochemical reactivity against PDGF-α (x20) after incubation with carboplatin and imatinib (3+18 μmol) after 48 h. (C and D) Positive immunohistochemical reactivity against PDGF-α (x20-40) with carboplatin and imatinib (7.5+30 μmol) after 120 h. (E-F) Control group with strong immunoreactivity.

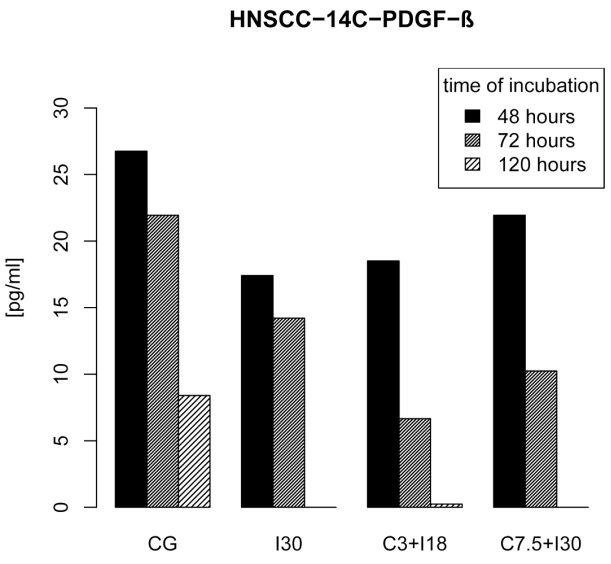

Figure 2. ELISA methods; PDGF-Rß expression in HNSCC 14C. Decreased expression of PDGF-Rß with rising concentrations of the applicated drugs (C, carboplatin; I, imatinib; CG, control group) after incubation periods of 48-120 h (p=0.2).

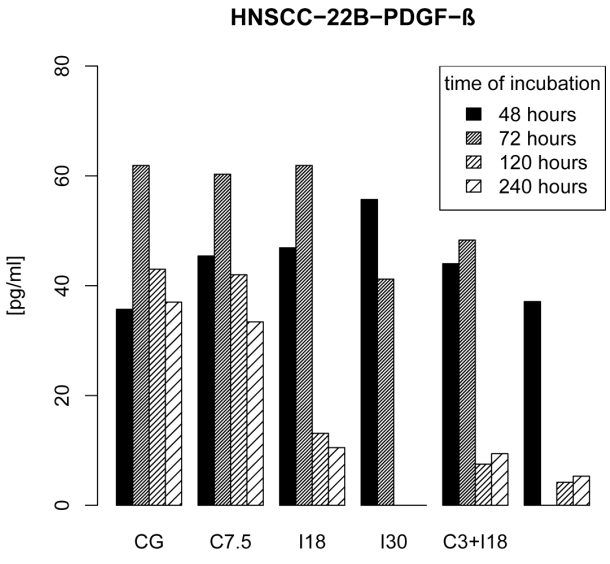

Figure 3. ELISA methods for PDGF-Rß expression in HNSCC 22B. Decreased expression of PDGF-Rß with rising concentrations of the applicated drugs (C, carboplatin; I, imatinib; CG, control group) after incubation periods of 48-240 h (p=0.32).

imatinib compared to the controls (imatinib 30 μmol/l) (cPDGF-R$ß_{48h}$ = 17/cPDGF-R$ß_{control48h}$ = 26 pg/ml; cPDGF-R$ß_{72h}$ = 14 pg/ml/cPDGF-R$ß_{control72h}$ = 21 pg/ml; cPDGF-R$ß_{120h}$ = 0.5 pg/ml/cPDGF-R$ß_{control120h}$ = 8 pg/ml). In contrast, carboplatin had no influence on the PDGF-Rß level. The simultaneous incubation with imatinib and carboplatin seemed to be much more efficient in lowering the measured PDGF-Rß concentration measured as imatinib alone (imatinib 30 μmol/l + carboplatin 7.5 μmol/l) (cPDGF-R$ß_{78h}$ = 10 pg/ml/cPDGF-R$ß_{control78h}$ = 21 pg/ml; cPDGF-R$ß_{120h}$ = 0.3 pg/ml/cPDGF-R$ß_{control120h}$ = 8 p/ml) (p=0.2) (Fig. 2). For HNSCC 22B only the maximum dose of imatinib and its combination with carboplatin induced a tendency for a decreased cPDGF-Rß level in an irrelevant manner compared to controls (p=0.32) (Fig. 3).

Table III. A, PDGF-Rα expression in ELISA in HNSCC cell lines (pg/ml) after application of carboplatin alone and in combination with imatinib after 48 to 240 h.

Time of incubation (h)	Control group	Carboplatin 7.5 μmol/l	Imatinib 18 μmol/l	Imatinib 30 μmol/l	C3 + I18	C7.5 + I30
HNSCC 11B						
48	100.12	151.75		136.94	256.23	174.02
72	241.22	241.22		188.9	278.77	308.91
120	361.81	196.36		166.58	151.75	196.36
240	308.91	174.02		107.46	181.46	218.76
HNSCC 14C						
48	324.00	13.61		813.71	188.90	301.37
72	226.24	414.91		20.63	13.61	6.69
120	85.48	181.46		0	27.71	27.71
240	122.18	114.82		159.16	49.18	151.75
HNSCC 22B						
48	159.16	308.91	331.55	339.11	354.24	
72	0	316.45	293.83	339.11	384.55	
120	0	0	136.94	34.83	136.94	
240	178.16	20.93	178.16	20.93	120.00	

B, PDGF-Rß expression in ELISA in HNSCC cell lines (pg/ml).

HNSCC 11B						
48	0.63	10.24	3.56	3.56	11.20	4.30
72	15.25	31.81	26.75	17.41	23.11	18.51
120	27.99	30.52	71.48	12.18	21.94	14.21
240	34.43	17.41	18.51	15.25	18.51	12.18
HNSCC 14C						
48	26.75			17.41	18.51	21.94
72	21.94			14.21	6.66	10.24
120	8.40			0	0.24	0
HNSCC 22B						
48	35.70	45.40	46.90	55.70	44.00	37.10
72	61.90	60.30	61.90	41.20	48.30	0
120	43.00	42.00	13.10	0	7.50	4.20
240	37.00	33.40	10.50	0	9.40	5.30

C

HNSCC 14C						
0	20.78	20.78	20.78	20.78	20.78	20.78
48	26.75	19.64	17.41	17.41	18.51	21.94
72	21.94	29.25	0.24	14.21	6.66	10.24
120	8.40	18.51	12.18	0	0.24	0
240	2.24	6.66	8.40	9.31	12.18	0
HNSCC 22B						
0	5.84	5.84	5.84	5.84	5.84	5.84
48	35.76	45.46	46.90	55.78	44.04	37.11
72	61.93	60.37	61.93	41.23	48.35	0
120	0	0	13.18	0	7.52	4.29
240	0.79	4.47	10.52	0	9.42	5.37

C, carboplatin; I, imatinib. Concentration: 3, 7.5 and 18 μmol/l.

Table IV. Expression of PDGF-Rα and PDGF-Rß in HNSCC in RQ-PCR in relation ß-glucuronidase (GUS) and ABL (abelson murine leukemia viral oncogene homolog).

Time of incubation (h)	Control group	Carboplatin 7.5 μmol/l	Imatinib 18 μmol/l	Imatinib 30 μmol/l	C3 + I18	C7.5 + I30
HNSCC 11B						
PDGF-Rα/GUS						
0	0.05323	0.05323		0.05323		0.05323
120	0.01438	0.03823		0.14815		0.00000
240	0.01530	0.06886		0.00000		0.00000
PDGF-Rß/ABL						
0	0.522054		0.522054	0.522054	0.522054	0.522054
72	0.362418		0.258181	0.106766	0.254031	0.110540
120	0.446373		0.217097	0.000000	0.140436	0.000000
UMSCC 14C						
PDGF-Rα/GUS						
0	0.00417		0.00417	0.00417	0.00417	0.00417
48	0.00318		0.00098	0.00168	0.00141	0.00089
240	0.00342		0.00096	0.00096	0.00159	0.00169
PDGF-Rß/GUS						
0	0.039		0.039	0.039	0.039	0.039
120	0.133		0.132	0.038	0.113	0.139
UMSCC 22B						
PDGF-Rα/GUS						
0	0.00039			0.00039		0.00039
48	0.00056			0.00038		0.00058
240	0.00055			0.00000		0.00000
PDGF-Rß/ABL						
0	0.082381		0.082381	0.082381	0.082381	0.082381
72	0.056009		0.031163	0.019423	0.033968	0.035104
120	0.088427		0.021664	0.000000	0.073617	0.000000
240	0.103772		0.045745	0.000000	0.024313	0.000000

mRNA expression of cPDGF-Rα/ß in HNSCC tumor cell lines. One further aim of this study was to measure the effect of the chemotherapeutic agents, as a single-active drug or in combination on PDGF-Rα/ß mRNA expression in HNSCC tumor cell lines 11B, 14C, 22B (in relation to the housekeeping proteins such as GUS/ABL) (Table IV).

For PDGF-Rα of HNSCC 11B only the maximum dose of imatinib (30 μmol/l) and the addition of carboplatin (7.5 μmol/l) illustrated a verifiable effect on reduced mRNA expression after 120 and 240 h ($PDGF\text{-}R\alpha/GUS_{240h} = 0.0005$, $PDGF\text{-}R\alpha/GUS_{control240h} = 0.02$) (p=0.34). Whereas PDGF-Rß level showed a more explicit reduction when exposed to 30 μmol/l imatinib (single-drug application) and when carboplatin 7.5 μmol/l is added after 120 and 240 h, but they were not statistically significant (30 μmol/l: $PDGF\text{-}R\beta/GUS_{120h} = 0.03$, $PDGF\text{-}R\beta/GUS_{control120h} = 3.2$; $PDGF\text{-}R\beta/GUS_{240h} = 0.0008$, $PDGF\text{-}R\beta/GUS_{control240h} = 1.3$; +7.5 μmol/l carboplatin: $PDGF\text{-}R\beta/GUS_{240h} = 0.0005$, $PDGF\text{-}R\beta/GUS_{control240h} = 1,3$) (p=0.1). In relation to the housekeeping protein ABL there was a significant reduction of PDGF-Rß level after the tumor cell lines were exposed to 30 μmol/l imatinib and 7.5 μmol/l carboplatin (p=0.001) (Fig. 4).

Similarly to the before mentioned ELISA, HNSCC 14C seemed to be less susceptible for a reduced PDGF-Rß level when treated with the agents, so that there was no noticeable trend in mRNA expression of PDGF-Rß versus the controls (p=0.34). We detected a significant depression of PDGF-Rα when incubated with imatinib 18 μmol/l alone (p=0.001) and 30 μmol/l (p=0.001) as well as the additional exposure with carboplatin 3 and 7.5 μmol/l (p=0.001) (Fig. 5). This fact could also be proven in relation to ABL (p=0.014). Carboplatin as single-drug application had no effect in suppressing PDGF-Rα level. HNSCC 22B complemented this hypothesis of downregulation of mRNA expression of PDGF-Rα and -Rß/GUS by exposure of imatinib 30 μmol/l and in synergism with carboplatin 7.5 μmol/l, but not statistically significant (p=0.68; p=0.58). Again carboplatin showed no influence on the mRNA expression of PDGF-R when applied alone. In context to the housekeeping protein ABL we detected a significant suppressed PDGF-Rß level after the incubation with

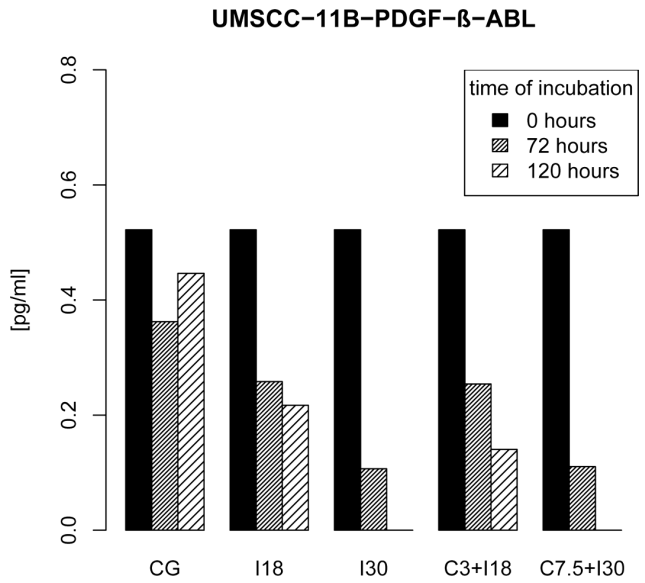

Figure 4. RQ-PCR, mRNA expression of PDGF-Rß/ABL in HNSCC 11B; (C, carboplatin; I, imatinib; CG, control group). Incubation period 0-120 h (p=0.001).

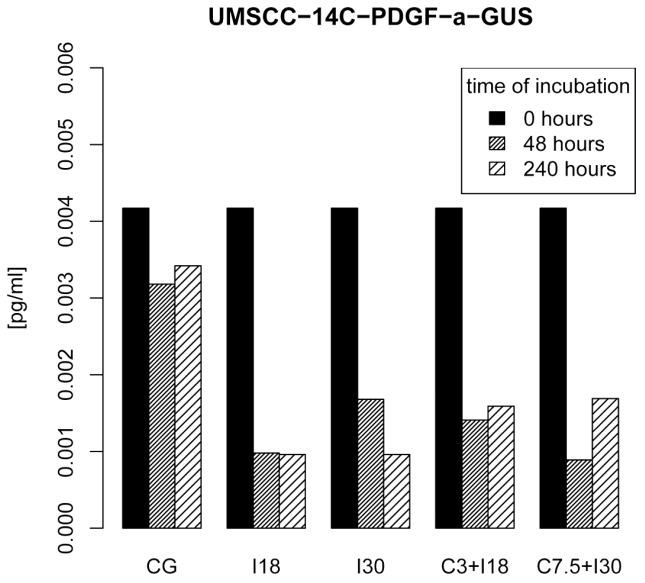

Figure 5. RQ-PCR, mRNA expression of PDGF-Rα/GUS in HNSCC 14C; (C, carboplatin; I, imatinib; CG, control group). Incubation period 0-240 h (p=0.001).

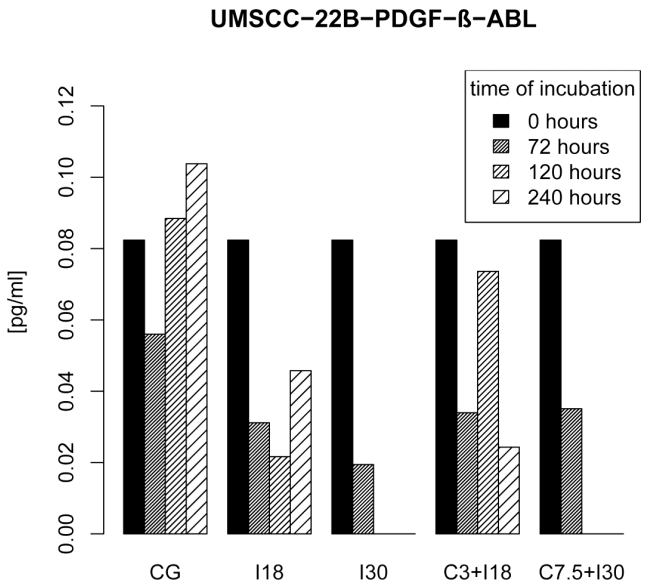

Figure 6. RQ-PCR, mRNA expression of PDGF-Rß/ABL in HNSCC 22B; (C, carboplatin; I, imatinib; CG, control group). Incubation period 0-240 h (p=0.001).

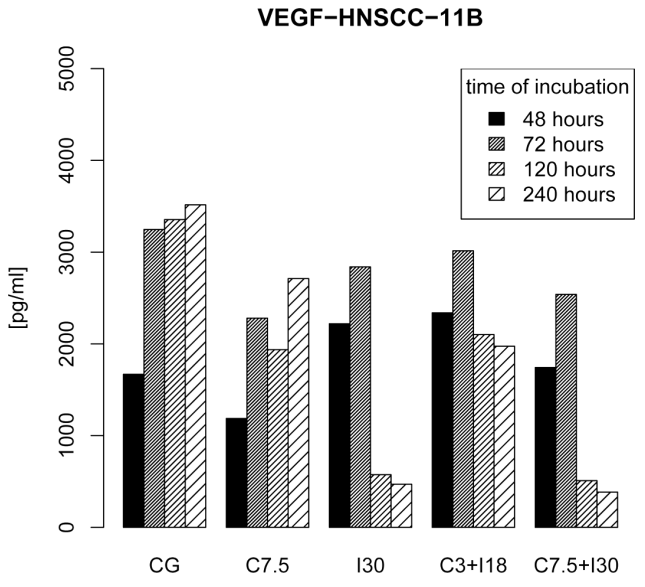

Figure 7. ELISA method; VEGF expression in HNSCC 11B. Decreased VEGF expression with rising concentrations of the applicated drugs (C, carboplatin; I, imatinib; CG, control group) after incubation periods of 0-240 h (p=0.026).

imatinib 18/30 μmol/l and in the combination with carboplatin 7.5 μmol/l (p=0.001) (Fig. 6).

ELISA of VEGF. VEGF expression in HNSCC 11B could be significantly reduced after being incubated with imatinib 30 μmol/l and after the incubation of the combination with carboplatin 7.5 μmol/l after 120 and 240 h (p=0.026) (after 120 h: $cVEGF_{imatinib30\mu mol/l}$ = 574 pg/ml; $cVEGF_{control120h}$ = 3355 pg/ml; after 240 h: $cVEGF_{imatinib30\mu mol/l}$ = 471 pg/ml; $cVEGF_{control240h}$ = 3515 pg/ml; after 120 h + 7.5 μmol/l carboplatin: $cVEGF_{imatinib30\mu mol/l}$ = 509 pg/ml; $cVEGF_{control120h}$ = 3355 pg/ml, after 240 h: $cVEGF_{imatinib30\mu mol/l}$ = 384 pg/ml, $cVEGF_{control240h}$ = 3515 pg/ml) (Fig. 7, Table V).

Effects of carboplatin, single agent, on VEGF expression was negligible. For the first time in HNSCC 14C carboplatin as a single drug at 7.5 μmol/l had a slight effect in suppressing the VEGF level. Whereas the tendency in distinct reduction of the VEGF expression was demonstrated when HNSCC 14C tumor cells were incubated with 30 μmol/l of imatinib (as single substance) and in combination with carboplatin

Table V. VEGF expression in HNSCC cell lines in ELISA (pg/ml) after application of carboplatin alone and in combination with imatinib after 48 to 240 h.

Time of incubation (h)	Control group 7.5 μmol/l	Carboplatin 30 μmol/l	Imatinib	C3 + I18	C7.5 + I30
HNSCC 11B					
48	1668	1187	2220	2338	1742
72	3247	2280	2839	3014	2539
120	3355	1937	574	2101	509
240	>3515	2711	471*	1975	384*
HNSCC 14C					
48	*	1465	1784	1747	1360
72	2373	2605	3124	2829	2402
120	2768	1860	2005	2717	817*
240	3495	2496	2791	3243	862*
HNSCC 22B					
48	2916	2613	3498	3540	3303
72	>3515	>3515	>3515	>3515	>3515
120	2930	2428	3269	1658	1402
240	3368	>3515	>3515	>3515	1933

C, carboplatin; I, imatinib. Concentration: 3, 7.5 and 18 μmol/l. *0 pg/ml.

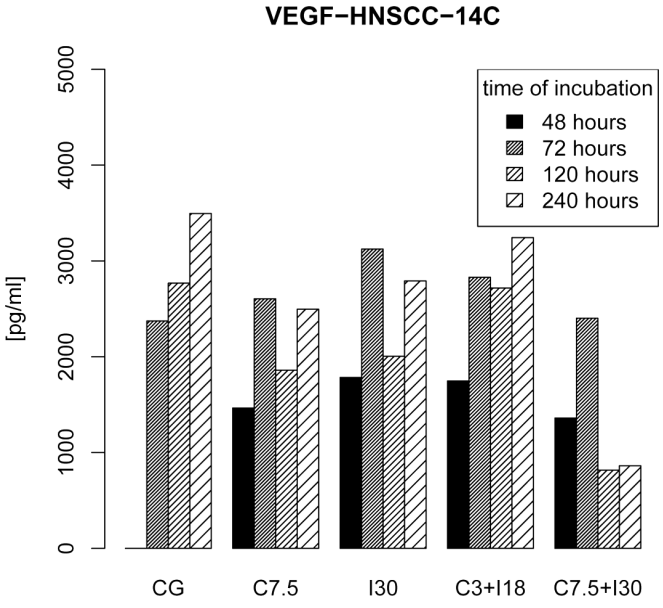

Figure 8. ELISA methods for VEGF expression in HNSCC 14C. Decreased VEGF expression with rising concentrations of the applicated drugs (C, carboplatin; I, imatinib; CG, control group) after incubation periods of 0-240 h (p=0.009).

7.5 μmol/l (p=0.009) (Fig. 8). The tendency of downregulated VEGF-expression continued for HNSCC 22B, which was already illustrated above in a more moderate manner (p=0.2).

Discussion

Despite the therapeutic options that have been established over the last decades, HNSCC tumors are still associated with poor prognosis, emphasizing the need for new and innovative therapeutic regimens. One strategy for example is the inhibition of angiogenesis of malignant tumors. Various studies have detected an induced angiogenic response *in vivo*. The correlation between high microvessel counts and recurrent or metastatic disease in HNSCC, suggested a concerted interaction of stromal factors, such as VEGF, PDGF and the tumor cells. The induction of tumor vascularization is regulated by a variety of angiogenic peptides released from tumor cells into the extracellular matrix. As one of these crucial factors PDGF and the corresponding receptor PDGF-Rα/ß are fundamental effectors in tumor cell growth, angiogenesis and survival of the tumor (6,8,11,13,14). Previous studies demonstrated the increased expression of cPDGF and the associated receptors in various malignant human tumors such as non-small cell lung (18) and prostate cancers (14). PDGF promotes the establishment of a well-vacularized stroma and hereby tumor proliferation by stimulating the angiogenesis process (19,20) in melanoma (21), fibrosarcoma (22), breast carcinoma (23) and squamous carcinoma (24). VEGF, as another fundamental stimulator of angiogenesis, enhances vascular permeability (26) and is associated with an increase of tumor growth and angiogenesis in a mouse model (27). Enhanced expression of VEGF has been detected in malignant human tumors including HNSCC (28-30).

Wang-Rodriguez *et al* showed PDGF-R, c-kit and c-Abl overexpression in HNSCC and with functional roles in HNSCC tumor biology, making them attractive as potential clinical targets (49). There is evidence of statistically significant synergistic effects in antiproliferative impact of imatinib in conjunction with cisplatin *in vitro* for HNSCC (27,41,45). Authors suggested that the greater sensitization to cisplatin through targeted inhibition of PTK by imatinib as an underlying mechanism, when illustrating the significantly growth inhibitory effect of the combined drug application

(41), when both drugs are administered simultaneously. It is postulated that imatinib does not alter the PTK protein expression levels but instead influences phosphorylation and activation of PTK (53,54). One explanation of synergism is the delayed DNA-crosslink adduct repair, induced by cisplatin, when exposed to imatinib; cells may undergo apoptosis due to their ability to replicate (41). Another mechanism could involve alteration of the apoptotic threshold by promoting the pro-apoptotic function of BCL-2 family regulators (46) and through this increasing the cytotoxic effect (45). However, to date the precise synergistic mechanism of imatinib in combination with cisplatin remains controversial.

The purpose of this study was to further investigate the potential synergism of imatinib and carboplatin on the expression of PDGF and PDGF-R as well as the expression of VEGF in HNSCC. We detected dose- and incubation time-dependent correlations in PDGF-Rα/ß level reduction in HNSCC 11B, 14C, 22B when exposed simultaneously to imatinib and carboplatin. Employing ELISA PDGF-Rα/ß in the different tumor cell lines showed different susceptibilities to the different chemotherapeutic drug as judged by altered PDGF-R expression levels. When imatinib was administered alone we could demonstrate a distinct trend for suppression of PDGF-receptor α/ß expression. Interestingly, expression of PDGF and PDGF-R was concomitant with neoplastic progression (12,20). The additional exposure of carboplatin enforces this effect in reduction of PDGF-R expression several fold but was not statistically significant in ELISA. This observation affirms the aforementioned synergism of these two different chemotherapeutic drugs, applicable for carboplatin as well as cisplatin. In contrast to Wang-Rodriguez, who described no altered PDGF-R expression, we determined an apparent reduction in PDGF-R concentration. A statistically significant correlation is proven for the reduction of normalized PDGF-Rα/ß mRNA expression for HNSCC 14C and 22B. Again there is evidence for the additional synergistic effect when imatinib is applied in conjunction with a platin-based chemotherapeutic drug. If administered alone, carboplatin had no influence in depressing PDGF-R expression. Restricted detection of PDGF with rising concentration of imatinib and carboplatin in immunohistochemistry and the significantly reduced VEGF-expression furthermore indicates the inhibition of the PDGF/PDGF-R autocrine tumor growth as well as the paracrine VEGF loop of imatinib (52). Comparable results were published for various other tumor entities such as neuroblastoma, where a reduction of PDGF and PDGF-R expression by imatinib leads to a suppressed VEGF concentration (55). This correlation is also being detected in ovarian cancer cells and xenograft small lung cell cancer, which illustrated that PDGF potently induce the VEGF expression, while imatinib reduced PDGF stimulated VEGF expression to basal levels (56,57).

In summary, we have demonstrated the potential of carboplatin and imatinib in suppressing PDGF-receptor expression in apparently synergistic manner. In RQ-PCR statistical significance in reduction of mRNA expression of PDGF-R was detected depending on the applied chemotherapeutics and incubation time. Furthermore, there is evidence for statistically significant reduction of the VEGF expression upon incubation with carboplatin and imatinib, which affirms once again the recently published synergism of imatinib and a platin-based chemotherapy. This effect of the synergism justifies an extension of this study, eventually to an animal model. The implementation of the combination of these chemotherapeutic drugs in clinical trials could possibly enhance the efficacy of a platin-based chemotherapy as a first line standard therapy without increased toxicity profile. Further trials are necessary to affirm these results and for a more complete understanding of the mechanism and interaction concerning cellular targets in signaling pathways being affected by these drugs.

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