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ORIGINAL PAPER

Differential effects of cetuximab and AEE 788 on epidermal growth factor receptor (EGF-R) and vascular endothelial growth factor receptor (VEGF-R) in thyroid cancer cell lines

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Abstract This study evaluated the role of EGF and the effects of EGF-targeting drugs (Cetuximab, AEE 788) on growth, apoptosis, and autocrine VEGF-secretion of thyroid cancer (TC) cells. Autocrine activation of the epidermal growth factor receptor (EGF-R) is commonly regarded to contribute to the malignant phenotype of TC cells and may therefore represent a rational therapeutic target. Out of a number of TC cell lines two anaplastic (Hth74, C643), one follicular (FTC133), and one papillary thyroid cancer cell line (TPC1) were analyzed in depth for VEGF-R-and EGF-R-expression, basal and EGF-stimulated (1–100 ng/ml) VEGF protein secretion and proliferation. Subsequently the antiprolifereative and antiangiogenic effect of cetuximab (Erbitux®), a monoclonal antibody that blocks the EGF-R and AEE 788, a novel dual-kinase inhibitor of EGF-R and VEGF-R were assessed, and the downstream EGF-R signal transduction was analyzed by means of detecting phosphorylated pEGF-R, pVEGF-R, pAkt, and p-MAPK. EGF stimulated VEGF-mRNA expression and protein secretion in all TC cell lines. The EGF-R antagonist Cetuximab consistently decreased VEGF secretion in all TC cell lines (min. 15%, n.s. in C643 cells and max. 90% in Hth74 cells, $P < 0.05$), but did not affect tumor cell proliferation in vitro. In contrast, the EGF-R- and VEGF-R-kinase inhibitor AEE 788 not only reduced VEGF secretion (min. 55%, $P < 0.05$ in C643 and max. 75%, $P < 0.05$, in FTC133), but also exhibited a dose-dependent inhibition of tumor cell proliferation (min. 75%, $P < 0.05$ in C643 and max. 95%, $P < 0.05$ in Hth74) and was a potent inductor of apoptosis in two of four TC cell lines. These effects were always accompanied by reduced levels of pEGF-R, pVEGF-R, pAkt, and pMAPK. Although inhibition of the EGF-receptor by Cetuximab potently disrupts autocrine secretion of VEGF, only the concurrent inhibition of the VEGF- and EGF receptor, e.g., by AEE 788 induces reduced proliferation and apoptosis in vitro. This suggests a particular rationale for the use of tyrosine kinase inhibitors with dual modes of action such as AEE 788 in thyroid cancer.

Keywords Thyroid cancer · EGF · EGF-R · Angiogenesis · Tyrosine kinase inhibitor

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Introduction

Thyroid cancer is the most common endocrine malignancy and its prevalence is increasing considerably [1]. When treated by surgery and radioiodine long-term survival can be achieved for the majority of well differentiated thyroid cancers [2]. However, anaplastic (ATC) and poorly differentiated carcinomas, which are unable to trap iodine, exhibit an extremely aggressive behavior and represent some of the most lethal malignant diseases. Their median survival rarely exceeds 3–6 months [3–5]. So far, no

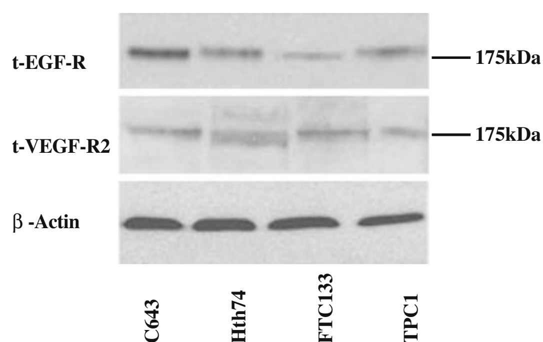


Fig. 1 Detection of t-EGF-R (total EGF-R) and t-VEGF-R2 in thyroid cancer cell lines by Western Blot. The figure shows EGF-R and VEGF-R2 to be expressed by all of the thyroid cancer cell lines that were used for blocking experiments at different individual levels

effective treatment can be offered to patients suffering from ATC.

Angiogenesis is a prerequisite for tumor growth as well as metastatic spread and describes the recruitment of blood vessels by a growing primary tumor or metastasis. It is initiated by factors intrinsic to the tumor cells that induce migration and proliferation of endothelial cells [6, 7]. Pro-angiogenic factors, particularly VEGF, are essential for thyroid tumor development [8]. VEGF has been documented to be the main stimulator of angiogenesis in the thyroid gland and was found to be regulated by TSH and EGF [9, 10]. Its complex receptor and gene regulation was analyzed in thyroid cancer cell lines in vitro and its level of expression was found to be a negative prognostic factor in clinical series of papillary thyroid cancer [11–14]. Anti-angiogenetic strategies, e.g., neutralizing antibodies directed against VEGF, have already been proven successful in experimental thyroid cancer [15–18]. Within the framework of tumor growth and tumor angiogenesis EGF is associated to various steps of thyroid cancer development. Activation of the EGF-R, leads to activation of several downstream signal transduction pathways, including mitogen-activated protein kinase (MAPK) and (Akt) that promote cell proliferation and survival [19–21]. In anaplastic thyroid cancer, loss of TSH receptor and overexpression of EGF receptor (EGF-R) were suggested as markers of de-differentiation [22–24]. EGF-R has been documented to be associated with poor outcome in papillary thyroid cancers and EGF-R was found to be overexpressed in anaplastic thyroid carcinoma cells [25, 26]. Moreover, EGF-administration promoted proliferation and invasion of follicular thyroid cancer cells [27]. We have recently shown that EGF enhances autocrine VEGF secretion of thyroid cancer cells, particularly in the absence of functioning TSH receptor [10]. In line with this, administration of neutralizing anti EGF/ anti TGFα antibodies resulted in inhibition of thyroid cancer cell proliferation [28]. Recently an inhibition of thyroid tumor cell growth by the EGF-R-inhibitor Gefinitib (Iressa®) was demonstrated in vitro and in vivo, suggesting that inhibitors of EGF-R may be worthwhile to be explored for their anti-tumor activity in anaplastic and poorly differentiated thyroid cancers [26].

Cetuximab is a monoclonal antibody that specifically binds to EGF-R, thereby inhibiting downstream signal transduction pathways. It has been shown to enhance radiosensitivity, to inhibit cell proliferation, radiation-induced damage repair, and tumor angiogenesis in vivo and in vitro [29]. Based on its clinical activity Cetuximab has recently been approved for application in colorectal cancer [30].

AEE 788, a pyrimidine derivate, is a novel ''dual specific'' tyrosine kinase inhibitor targeting EGF-R (ErbB-1) and VEGF-R. It has been demonstrated to have anti-tumoractivity against breast-, lung-, bladder-, and squamous cell carcinoma of the oral cavity in vitro and in vivo [31, 32]. A previous report also suggested antiproliferative and antiangiogenic activity of this compound in follicular thyroid cancer in vitro and in vivo [33]. In the current study, the tumorsuppressing and antiangiogenic activity of Cetuximab a neutralizing antibody directed against EGF-R and AEE788, a dual tyrosine kinase inhibitor directed against EGF-R/VEGF-R was evaluated in a number of thyroid carcinoma cell lines. To this end proliferation of tumor cells, apoptosis and in vitro VEGF secretion as well as the effect of the substances on post EGF-R (downstream) signal transduction were analyzed. The overall aim of this study was to assess the potential of neutralizing antibodies against EGF-R (Cetuximab) or combined inhibition of EGF-R and VEGF-R by application of a dual tyrosine kinase inhibitor (AEE 788) to target the EGF/EGF-R system in thyroid cancer cell lines from various histological background for tumor suppression and anti-angiogenic therapy.

Results

EGF-dependent VEGF secretion in thyroid cancer cell lines

Autocrine stimulation and activation of oncogenic receptors provide a rationale for therapeutic inhibition. Therefore the expression levels of t-VEGF-R and t-EGF-R in thyroid cancer cell lines were first analyzed. Both receptors were expressed by all thyroid cancer cell lines, albeit at variable levels. Highest expression of t-EGF-R was determined in anaplastic C643:3 cells. The β-Actin referenced

expression of t-VEGF-R did not display remarkable differences among the presented thyroid cancer cell lines (Fig. 1).

Basal VEGF secretion differed distinctly among the various cell lines (100+/–40 pg/ml to 2200+/–450 pg/ml). EGF was confirmed as a major stimulator of VEGF-secretion. Here we demonstrated an up to 20-fold increase in EGF-stimulated VEGF secretion in Hth74 cells (1,800 pg/ml versus 100 pg/ml basal secretion), and a 3 and 2 fold increase of VEGF secretion in FTC133- (4,500 pg/ml versus 1,500 pg/ml), and TPC1-cells (1,800 pg/ml versus 900 pg/ml), respectively (Fig. 2a). In addition to the anaplastic thyroid cancer cell lines, one papillary (TPC1) and one follicular (FTC133) of high EGF driven VEGF secretion were chosen for further experiments. Of note, cell lines known to express a functional TSH receptor (e.g., HTC TSHr and C643), had only minor changes of VEGF-expression and -secretion in response to EGF, despite confirmed expression of the EGF-receptor. EGF-stimulated VEGF mRNA expression occurred in a dose-dependent manner in all of the thyroid cancer cell lines, as representatively shown for Hth 74 cells respectively (Fig. 2b). We did not observe relevant changes in VEGF mRNA levels due to FGM.

EGF-R-blockage by Cetuximab inhibits VEGF secretion, but does not affect cell proliferation nor apoptosis

The data above indicated that blocking EGF-R should overcome basal and EGF-stimulated autocrine VEGF-secretion. Indeed, cetuximab significantly inhibited VEGF secretion in three of four tested thyroid cancer cell lines, particularly in those that were highly responsive to EGF-mediated VEGF secretion (Fig. 3). For example, cetuximab almost completely inhibited VEGF secretion in Hth74 cells (95%, $P < 0.05$), but only slightly (10–15%, n.s.) in C643 cells. Unexpectedly however, with the exception of the high EGF responsive Hth74 cells, cetuximab did not affect tumor cell proliferation of TC cell lines in vitro, irrespective of the presence of supplemented EGF (example: Cetuximab 10 μg/ml vs. control, incubation time 5 days: Mean OD in C643 0.71 ± 0.15 vs. 0.65 ± 0.09, in Hth74 0.88 ± 0.2 vs. 0.8 ± 0.12, in FTC133 0.44 ± 0.11 vs. 0.39 ± 0.08 and in TPC1 0.48 ± 0.17 vs. 0.51 ± 0.22, all n.s.). Likewise, cetuximab did not cause apoptosis in any of the thyroid cancer cell lines. Hence, inhibition of EGF-mediated VEGF secretion by cetuximab at concentrations of up to 10 ng/ml, had per se no effect on apoptosis or proliferation of thyroid cancer cells in vitro.

AEE 788 inhibits VEGF-secretion, cell proliferation, and causes apoptosis of thyroid cancer cell lines

AEE 788 blocks the kinase activity of the EGF-R [31]. We therefore evaluated if this compound—in analogy to the EGF-R-blocker cetuximab—reduces VEGF-secretion. As depicted in Fig. 4a, AEE 788 reduced VEGF secretion in all tested cell lines. Asked, whether dual inhibition of the EGF-R and VEGF-R has also additional inhibitory effects, we could demonstrate, that in contrast to cetuximab, AEE 788 also caused a dose-dependent inhibition of tumor cell

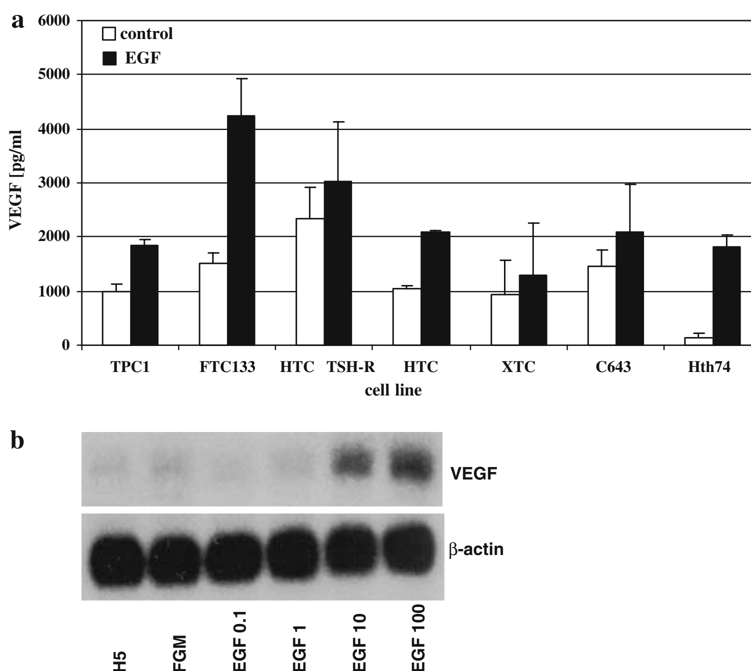


Fig. 2 (**a**) Synopsis of EGF (epidermal growth factor) stimulated VEGF secretion of thyroid cancer cell lines (TPC1: Papillary-, FTC 133, HTC, HTC TSH-R: Follicular-, XTC: Huerthle cell-, C 643, Hth 74: Anaplastic-thyroid cancer). Stimulation employed 100 ng/ml EGF (values are given as mean, cell number adjusted VEGF [pg/ml] ± SD). (**b**) Northern Blot analysis of EGF stimulated VEGF gene expression (steady-state levels) of Hth74 cells. VEGF gene expression increased following a single pulse of EGF at the indicated concentrations (ng/ml respectively)

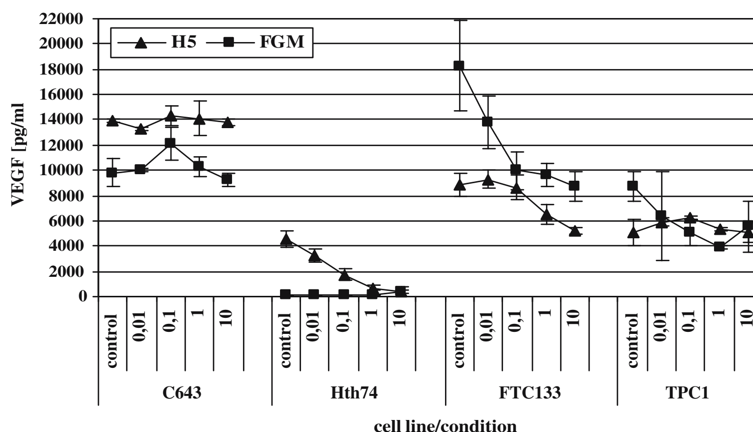
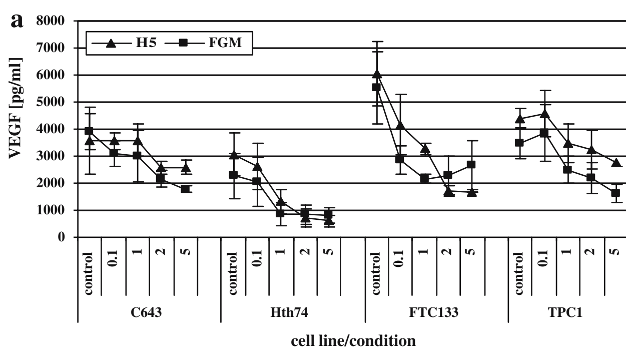
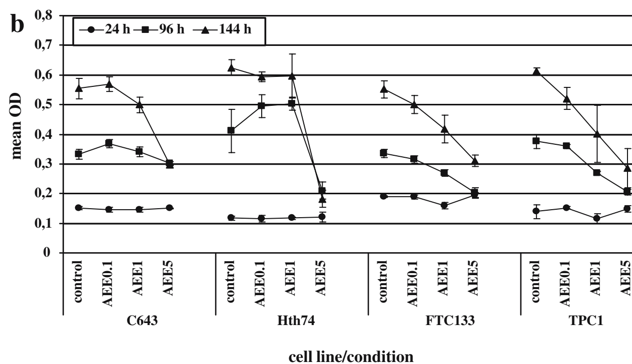


Fig. 3 Effect of a neutralizing anti-EGF antibody (Cetuximab) on the in vitro VEGF secretion of thyroid cancer cell lines. Experiments employed 0.01–10 μg/ml Cetuximab and EGF (100 ng/ml), values are given as mean, cell number adjusted VEGF [pg/ml] ± SD





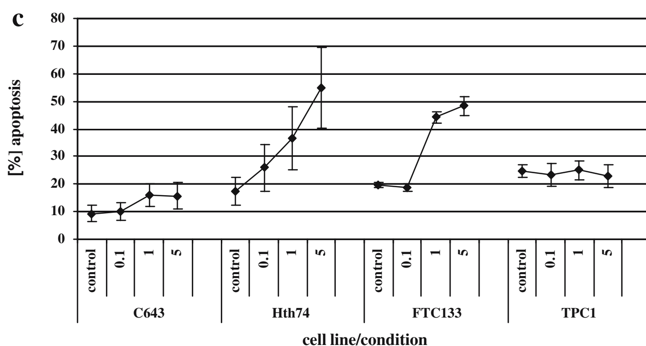


Fig. 4 (**a**) Effect of a dual tyrosine kinase inhibitor (AEE 788®) to in vitro VEGF secretion of thyroid cancer cell lines. Experiments employed 0.1–5 μM AEE 788® and EGF (100 ng/ml), values are given as mean, cell number adjusted VEGF [pg/ml]±SD. (**b**) Synopsis of the effect of a dual tyrosine kinase inhibitor (AEE 788®) to proliferation of thyroid cancer cell lines in vitro at different times of incubation (24, 96, 144 h). Experiments employed 0.1–5 μM AEE 788®, values are given as mean optical density (OD) ± SD. (c) Effect of the dual tyrosine kinase inhibitor (AEE 788®) on apoptosis of thyroid cancer cell lines. Experiments employed 0.1–5 μM AEE 788, values are given as [%] apoptotic cells ± SD

proliferation. The strongest inhibition of proliferation as measured by means of MTT colorimetric assay was seen in C643 and Hth74 (75% and 95%, respectively, $P < 0.05$) (Fig. 4b). The inhibitory concentration (IC 50) at 48 h was calculated with 4 μM in C643, 3 μM in Hth74, 3.5 μM in FTC133 and 4.5 μM in TPC1 cells, respectively (Fig. 4b).

Finally, growth inhibition by AEE 788 was accompanied by a dose-dependent induction of apoptosis in two of the four cell lines studied (Fig. 4c). Low micromolar concentrations of AEE 788 caused a distinct apoptotic response in Hth74 and FTC133 cells, but much less pronounced in C643 cells. No increase in apoptotic cells was observed in TPC1 at concentrations of up to 5 μM (Fig. 4c), however at higher concentrations of up to 10 μM we also observed tumor cell death in TPC1 (data not shown). Hence, AEE 788 mediated EGF-R/VEGR-inhibition had a significantly superior antiproliferative efficacy than blockage of EGF-R with cetuximab.

AEE 788 blocks EGF-R/VEGF-R-dependent signal transduction

As representatively shown for C643 cells, AEE 788 did not affect receptor expression levels of VEGF-R and EGF-R, nor its downstream signaling cascade partners Akt and MAPK, but caused a dose-dependent inhibition of the EGF-/VEGF-receptor phosphorylation in all of the TC cell lines (Fig. 5). AEE 788 completely inhibited VEGF-receptor phosphorylation at 100 nM in all cell lines. AEE 788 also completely blocked EGF-receptor phosphorylation at 50 nM in all cell lines. This was associated by an inhibition of phosphorylation/activation of the downstream-targets of the EGF-R p-Akt, and p-MAPK. Of note, the concentrations of AEE 788 needed to completely block downstream p-Akt, and p-MAPK were well above those required to block the upstream receptors. This may indicate additional and EGF-R/VEGF-R-independent signaling input on MAPK and Akt which are inhibited by AEE 788 at higher concentrations.

Discussion

The common failure of established therapeutic strategies in poorly differentiated and anaplastic thyroid cancer urgently mandates new options of treatment. Ample evidence exists that overexpression and -activation of EGF and EGF-R contribute to de-differentiation of anaplastic and poorly differentiated thyroid cancer and cause poor prognostic outcome [23–25]. It has recently been demonstrated that the serum-level of EGF and VEGF may be used as a prognostic marker in thyroid cancer [34].

In this work, we were able to demonstrate EGF-R and at the same time VEGF-R to be expressed by transformed thyroid cells. Their presence was confirmed on the protein as well as on mRNA level and included the phosphorylated, i.e., activated forms of both receptors in all of the tumor cell lines. The fact that VEGF-R is expressed not only by tumor-associated endothelial cells, where it causes activation of tumor angiogenesis, but also by thyroid tumor cells is a novel finding that may implicate autocrine stimulation of angiogenesis or cell growth. Indeed autocrine activation of the VEGF/VEGF-R system had previously been suggested to function as an autocrine loop in thyroid carcinogenesis [35, 36]. VEGF secretion was demonstrated to be regulated by activation of EGF-R by its specific ligand EGF in undifferentiated thyroid cancer cells, a finding we had previously reported especially to occur in the absence of a TSH receptor [10]. It is well known that undifferentiated thyroid carcinoma cells are capable of de novo synthesis and secretion of EGF and up-regulation of EGF-R [23, 24]. Taken together, this evidence would strongly support the concept of an autocrine loop resulting in VEGF-secretion as a consequence of EGF synthesis and activation of EGF-R.

Previous work had already shown the efficacy of VEGF-targeting strategies to inhibit growth of experimental thyroid cancer [15–18], and blocking EGF-R would be critical to fully interrupt such an autocrine EGF/EGF-R/VEGF loop. Moreover, it is currently unclear, whether simultaneous blocking of EGF-R and VEGF-R further adds to interrupt this autocrine loop. It is therefore that we addressed this issue in a panel of undifferentiated thyroid cancer cell lines by comparing two EGF-R-blocking drugs: cetuximab, a monoclonal anti-EGF-R antibody that blocks its activation, and AEE 788 a dual-kinase inhibitor that blocks both, EGF-R and VEGF-R.

Cetuximab reduced in vitro VEGF secretion up to 90%, when administered to thyroid cancer cells. It eliminated the stimulating effect of exogenously administered EGF on VEGF-secretion. This effect is most likely mediated by reduced levels of hypoxia-inducable-factor 1 alpha (HIF-1 alpha), as was recently demonstrated in A431 epidermoid carcinoma cells [37]. Besides from down-regulating HIF-1

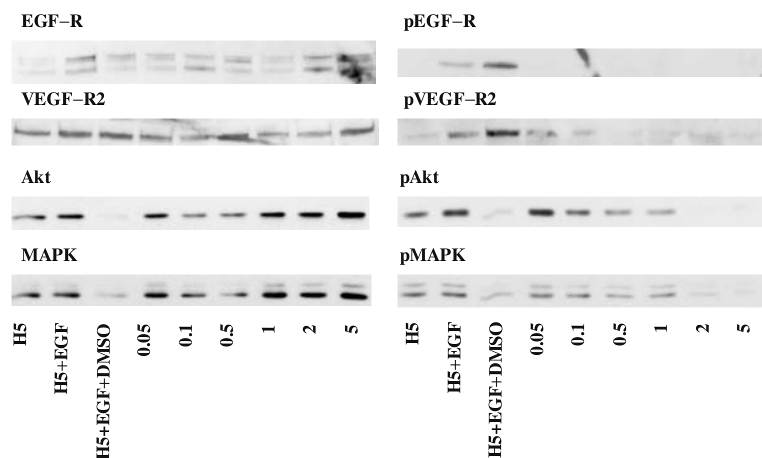


Fig. 5 Effect of the dual tyrosine kinase inhibitor AEE 788 on EGF/VEGF related signal transduction in thyroid cancer cell lines in vitro. Western Blot scans of total EGF-R, VEGF-R2, Akt and MAPK as well as the phosphorylated forms in cell lysat of an anaplastic thyroid cancer cell line (C643) are depicted. AEE 788 was employed at concentrations of 0.05–5 [μM]

alpha, other inhibitors such as gefitinib, erlotinib were demonstrated to decrease VEGF expression by decreasing Sp1 binding to the proximal core VEGF promoter [38]. Importantly, Cetuximab did not inhibit proliferation of thyroid cancer cells, which was convincingly demonstrable in three out of four cell lines, suggesting a quite specific anti-angiogenic effect of the Cetuximab. This observation is well in line with reports from thyroid, pancreatic, and prostate cancer, were administration of Cetuximab had only little effect on in vitro proliferation [39–41]. Conflicting data exist with regard to the in vivo effects of Cetuximab on tumor proliferation in experimental cancer [39, 42]. However, when combined with chemotherapy, Cetuximab was demonstrated to enhance the effect of irinotecan on xenotransplanted anaplastic thyroid cancer cells [41]. Taken together, it would appear that the responsible mechanism of Cetuximab's anti-tumor effect might primarily be a highly specific down-regulation of EGF-driven VEGF-secretion contributing to anti-angiogenesis, rather than inhibition of thyroid carcinoma cell growth.

In contrast, AEE 788, a substance known to block both EGF-R and VEGF-R, not only inhibited VEGF-secretion, but also caused inhibition of proliferation and induced apoptosis of tumor cells. Thus, AEE 788 was not as selective with regard to anti-angiogenetic action as observed for Cetuximab. However, with regard to inhibition of tumor cell growth AEE 788 may have more potential, because of the additional effect on tumor cell proliferation and induction of apoptosis. This may suggest that disrupting the autocrine loop at the level of EGF-R may be a less effective anti-tumor strategy than simultaneous inhibition of EGF-R and VEGF-R by means of a dual-kinase inhibitor. A possible explanation for the increased efficacy of dual-kinase inhibition may be that constitutive activation of VEGF-R has mitogenic activity independent from EGF-R mediated VEGF-secretion. Such mitogenic activity of VEGF-R was recently hypothesized for thyroid carcinoma, but awaits confirmation [36]. For breast carcinoma cells, it was demonstrated that specific VEGF receptors are expressed by the tumor cells and that these receptors respond to autocrine VEGF, resulting in the activation of signaling pathways that impede apoptosis and promote cell migration [43]. Alternatively, AEE 788 may block other kinases or downstream signaling pathways, independent from EGF-R or VEGF-R, that are not affected by cetuximab—as was just recently shown in anaplastic thyroid cancer cells, where imatinib blocked PDGFR [44]. Finally, we have shown, that AEE 788 potently inhibits downstream signaling pathways such as Akt- and Ras/MAPK-signaling, known to be important for proliferation and survival of thyroid carcinoma cells as well as associated to the metastatic phenotype [45–49]. In follicular thyroid cancer cells AEE788 alone showed enhanced growth inhibition compared to other TKI (PKI166 and PTK787) and all three TKI displayed individual potential to induce cell apoptosis [33]. This may be attributed to the fact that AEE788, in addition to its primary inhibition of EGFR and VEGFR pathways, can also inhibit c-Src, ckit, and RET pathways at higher doses [31]. Given its significant efficacy as single substance and its multiple modes of action, AEE 788 may be even more effective in combination with conventional chemotherapy. Such strategies have already been shown to be effective in experimental colon, prostate, ovarian, and anaplastic thyroid carcinoma [50–53].

In summary, this study describes the co-expression of functional EGF-R and VEGF-R in all histologic types of thyroid cancer cell lines and shows for the first time the regulation of VEGF by EGF through EGF-R. It demonstrates that disrupting the EGF/EGF-R/VEGF/VEGF-R autocrine loop by a dual-kinase inhibitor is more effective to induce apoptosis, to inhibit VEGF-secretion and tumor cell proliferation in thyroid carcinoma cell lines as compared to a monoclonal antibody that blocks EGF-R only. However, the antibody exhibited a highly selective anti-VEGF-effect and thus well suited to be used in multiple drug regimes. These data warrant the use of AEE 788 and cetuximab in clinical trials of undifferentiated thyroid cancers potentially in combination with anti-proliferative chemotherapeutics.

Materials and methods

Thyroid cancer cell lines and culture conditions

The following cell lines were evaluated for EGF regulated VEGF secretion: TPC1 [54], papillary-, FTC133 [55], HTC, HTC-TSH-R [56], follicular-, XTC [57] Huerthle cell-, C 643, Hth 74 [58], anaplastic thyroid cancer. Four cell lines (TPC1, FTC 133, Hth74, C643) of high EGF-mediated VEGF secretions were used for further investigations. The cell lines were maintained in Full Growth Medium (FGM = DMEM-h21/Ham's F12 1:1 (v/v), containing 25 mM Hepes, 0.055g/l sodium pyruvate and 0.365 g/l glutamine, 10% fetal bovine serum and 10,000 U/l penicillin, 100 mg/l streptomycin) at 37°C in a 100% humidified, 5% CO2 atmosphere as described [57].

For experiments, thyroid cancer cells in exponential growth were harvested by brief incubation with cold trypsin-EDTA (0.025%/M, Sigma) and vitality was assessed by tryphan blue exclusion. Depending on the experiment serum free H5 medium (DMEM-h21/Ham's F12 1:1 (v/v) with Glutamin. supplemented with bovine insulin (10 μg/ml), human transferrin (5 μg/ml), somatostatin (10 ng/ml), glycyl-L-histidyl-L-lysine acetate (2 ng/ml) and hydrocortisone (10^{-8} M)) was used instead of FGM

during experiments, generally functional experiments were conducted in both H5 and FGM conditions. Culture flasks and dishes were obtained from Corning (Corning, NY), cell culture medium was purchased from Biochrom (Berlin, Germany) and h-EGF from Sigma (Munich, Germany).

Northern blot analysis

Total RNA of thyroid cancer cells was isolated using the RNeasy-kit and QiaShredder (Qiagen, Hilden, Germany). About 5–10 μg of total RNA were separated on 1.5% (w/v) agarose/formaldehyde (2.2 M) gel and transferred to Hybond N+ nylon membranes (Amersham, Arlington Heights, IL) by capillary blotting. Random prime labeling and hybridization procedures were carried out as reported using a 320 bp 32P VEGF-probe hybridizing to three mRNA species of VEGF [59]. Control hybridizations with human ß-actin cDNA verified that equal amounts of RNA were loaded. Experiments were repeated three times with similar results.

Tyrosine kinase inhibitor and neutralizing antibody

Cetuximab (Erbitux®) was generously provided by Merck AG (Darmstadt, Germany) and was employed at concentrations of 0.01, 0.1, 1, and 10 μg/ml during experiments. Dilutions were prepared using H5 medium. AEE 788 was generously provided by Novartis Pharma AG (Basel, Switzerland) and was employed at concentrations of 0.1, 1, 2, and 5 μM during proliferation and VEGF secretion experiments. Prior to experiments AEE 788 was resolved in DMSO (WAK Chemicals, Steinbach, Germany), resulting in a stock solution of 10 mM. Further dilutions were prepared using H5 medium equivalent to a 10 mM DMSO stock.

Western blot analysis of EGF-R downstream signal transduction

Thyroid carcinoma cells were seeded at a density of 2–3 $\times$ 10^6 cells in 75cm^2 cell culture flasks and allowed to adhere for 24 h. After switching to serum free H5 medium cells were incubated with increasing concentrations of AEE 788 (0.01, 0.05, 0.1, 0.5, 1, 2, and 5 μM) for 1 hour followed by a 15 min stimulation with EGF (40ng/ml). Total cell lysates were prepared by using RIPA Buffer according to manufacturers instructions (Santa Cruz; Heidelberg, Germany). Protein concentrations were determined using a commercial kit (BCA-Protein Assay, Pierce, Rockford, Il, USA). Following 7.5% SDS-polyacrylamid gel electrophoresis (SDS-PAGE) under reducing conditions an immunoblot analysis was performed using anti-EGF-R (Santa Cruz, 1:200), anti-VEGF-R2 (Santa Cruz, 1:100–1:200), anti p-EGF-R (Cell signaling, 1:200–1:500), and anti-p-VEGF-R2 (Abcam, 1:1000). Total and activated Akt and MAPK were analyzed using anti-Akt (Cell Signaling, 1:750), anti-phospho-Akt (Cell Signaling, 1:750), anti-p44/42 MAPK antibody (Cell Signaling, 1:750) and anti-phospho-p44/42 MAPK (Cell Signaling, 1:750) as described [60]. Detection of signals was done using the ECL Western blotting detection reagent and analysis system (Amersham, Piscataway, NJ, USA).

In vitro proliferation assay

The evaluation of antiproliferative activity of Cetuximab and AEE 788 on thyroid cancer cells employed a MTT based assay as described elsewhere [59]. Prior to the experiments cell toxicity of compounds was determined to find optimal culture conditions. Concentrations of up to 10 μg/ml for Cetuximab and up to 5 μM of AEE 788 were found to be acceptable for experiments. Briefly TC cells were harvested, repeatedly washed with calcium- and magnesium-free PBS, resuspended in FGM and plated at a density of 1 $\times$ 10^4 vital cells into triplicate wells of flat bottom microtitration plates. Cells were allowed to adhere and resume full growth under optimized conditions (FGM) over 24 h. Then FGM was changed to specific culture conditions (H5, H5+EGF 10 ng/ml, FGM, FGM+EGF 10 ng/ml) and compounds were added in the following concentrations: Cetuximab 0.01, 0.1, 1, and 10 μg/ml; AEE 788 0.1, 1, and 5 μM. IC50 analysis employed additional concentrations (0.01, 0.033, 0.066, 0.1, 0.33, 0.66, 1, 3.3, 6.6, and 10 μM). Incubations were continued for up to ten generation times with refreshing the culture medium every third day. Cell numbers were determined at 24, 96, and 144 h. Optical densities were determined at 570 nm with a reference filter of 630 nm (Emax, Molecular Devices, Munich, Germany). Experiments were repeated three times in triplicates.

In vitro apoptosis assay

Drug-induced tumor cell apoptosis was evaluated by use of an apoptosis ELISA (Titer Tacs, R&D Systems, Minneapolis, MA, USA). Designed for in situ detection and quantification of apoptosis in monolayer cell cultures this assay is based on colorimetric detection of DNA fragments after adding biotinylated nucleotides to the 3′ ends of DNA fragments. TC cells, prepared as described above, were plated into triplicate wells of a flat bottom microtitration plate at a density of 2 $\times$ 10^4 cells/well. After 24 h FGM was changed to H5 medium and after another 24 h cells were incubated with increasing concentrations of Cetuximab (0.01, 0.1, 1, and 10 μg/ml) or AEE 788 (0.1, 1, and 5 μM) for 48 h. Then cells were fixed and the apoptosis assay was

carried out according to the instructions of the manufacturer. After termination of the colorimetric reaction absorbance was measured at 450 nm. Experiments were repeated three times and the extent of apoptosis was calculated as a function of total vital cell number referenced to additional, non-treated cells for each sample.

In vitro VEGF secretion

Drug dependent in vitro VEGF secretion of TC cells was determined by using a VEGF ELISA (R&D Systems; Minneapolis, MA, USA) as previously described [10]. Briefly, cells were seeded at a density of 1×10^5 cells into 24-well multiwell plates and allowed to resume growth for 24 h in FGM. Then cells were switched to serum free H5 for another 24 h. Thereafter H5 medium was changed to specific culture conditions (H5, H5+EGF and FGM, FGM+EGF) and Cetuximab (0.01, 1, and 10 μg/ml) or AEE 788 (0.1, 1, 2, and 5 μM) were added. After a 48-h incubation, conditioned medium (CM) was harvested, centrifuged (15,000 × g, 20 min, 4°C) to remove debris and aliquots were stored at –80°C until analyzed. ODs were measured at 450–570 nm (Emax, Molecular Devices) and VEGF was calculated as cell number referenced amount of VEGF (pgVEGF/ml). Experiments were repeated three times in triplicates.

Statistical analysis

Unless otherwise stated, values are expressed as the mean ± standard deviation. Student's paired *t*-test was used to evaluate differences of continuous variables from samples of interest and the respective controls during EGF-stimulation and evaluation of EGF-post receptor signal transduction or administration of compounds. All test were two-tailed. A *P*-value of <0.05 was considered to indicate significance.

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