Pretherapeutic drug evaluation by tumor xenografting in anaplastic thyroid cancer

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ABSTRACT

Background: Despite various attempts at modifying usual treatment modalities, anaplastic thyroid cancer (ATC) is still associated with unfavorable prognosis. Results of preclinical investigations are often of limited transferability to clinical tumor biology. Individualized multimodal treatment regimens, including novel growth-inhibiting drugs, might be a future option.

Methods: Tumor tissue, freshly prepared from a patient operated for ATC, was xenotransplanted to nude mice. While the patient obtained a hyperfractionated external beam radiation, mice carrying xenotransplanted tumors were randomized (n = 6) and treated by multikinase inhibitors (sorafenib [S]: vascular endothelial growth factor receptor [VEGF-R], platelet derived growth factor receptor, RET; vandetanib [V]: VEGF-R, endothelial growth factor receptor [EGF-R]; and MLN8054 [M]: Aurora kinases [AK]). Antiproliferative, anti-angiogenic, and proapoptotic effects were evaluated.

Results: Treatment of successfully xenotransplanted fresh ATC tumor tissue by multikinase inhibitors and aurora kinase inhibitor reduced the tumor volume up to 61% depending on the drug and time of application (3 wk of treatment: 46% [M], 34% [V], 30% [S]; 5 wk of treatment: 61% [S]). Tumor cell proliferation (BrdU) was reduced between 34% and 58% [S] and [V]. Reduction of tumor vascularity was between 67% [V] and 33% [S] and was accompanied by decreased EGF-R/VEGF-R2 receptor activity [V/V,S]. Tumor cell apoptosis (caspase 3 activity) increased up to 2.4-fold [S].

Conclusions: Successful in vivo evaluation of novel drugs in xenotransplanted fresh tumor tissue allows in-time (while patient receives standard treatment) prospective analysis for possible additional clinical application. However, technical specifications have to be taken into account to obtain stable in vivo tumor growth. Based on the individual results, a tailored clinical drug application seems possible.

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1. Introduction

Anaplastic thyroid carcinoma (ATC) is a rare tumor that makes up less than 2% of thyroid carcinomas (TC); however, it accounts for up to 40% of the tumor-related deaths from TC. TC incidence is increasing in several countries [1], but it is unclear whether the incidence of ATC that may appear de novo or from a pre-existing differentiated carcinoma will rise accordingly as well. ATC represents one of the most lethal tumor diseases. Less than 20% of patients survive 1 y from diagnosis, and median survival still does not exceed 3–8 mo, since the established treatment of TC by surgery and radioiodine is insufficient. Despite various attempts to improve the outcome of ATC, the clinical course did not change within the last decades [2,3]. However, single reports document benefits from an aggressive multimodal treatment in small series, reaching a mean survival of up to 60 mo [4]. Based on the analysis of ATC patients’ clinical data, a prognostic score, identifying patients that may profit from a more aggressive approach, was suggested [5].

New insights into the biological behavior of ATC focusing on genetic alterations and dysfunction of signaling pathways offer the possibility of targeted therapies [6]. A large number of new drugs apart from “classical” chemotherapeutics have been shown to successfully inhibit tumor-promoting pathways preclinically even in ATC cells [6,7]. However, only a few of them entered clinical trials, generally focusing on advanced differentiated or medullary thyroid cancer. The few phase II trials that included ATC patients generally showed only minor effectiveness of monotherapeutic approaches. The application of imatinib or fosfretabulin resulted in a 6-mo overall survival rate of 23%–45% [8,9]. Therefore, rising evidence advocates individualized targeted combination therapy to improve the clinical effect of innovative compounds. Based on literature reports and on the results of our own investigations, three compounds (Aurora kinase inhibitor MLN8054, multikinase inhibitors vandetanib and sorafenib) were selected to be evaluated in this setting.

Aurora kinases (A–C) are key regulators of mitosis and have been shown to be overexpressed in many human tumors including ATC, where they account for aberrant cell proliferation [10–12]. Tumor-inhibiting effects were demonstrated preclinically by in vivo siRNA experiments and by blocking Aurora kinase activity in ATC cell lines using VX-680 as an inhibitor [11,13]. By using MLN8054 [14], profound antitumor activity was demonstrated on ATC cells in vitro and in vivo [15].

Vandetanib targets epidermal growth factor receptor (EGF-R), vascular endothelial growth factor receptor 2 (VEGF-R2), and the proto-oncogene RET, a key regulator in TC development. The antiproliferative and antiangiogenic properties of vandetanib have been investigated in several in vitro and preclinical studies (for review see [16]), leading to various clinical phase I/II trials in solid tumors including TC. Vandetanib was approved by the US Food and Drug Administration for the treatment of medullary TC.

The multikinase inhibitor sorafenib acts mainly by blocking the ERK1/2 pathway, VEGF-R [1–3], platelet derived growth factor-beta, Flt-3, and c-Kit, which are known to be involved in proliferation and angiogenesis [17]. Its antitumoral activity was documented for many human cancers including TC (for review see [18]). For advanced TC—including ATC—a phase II trial was performed documenting an overall clinical benefit rate of 77% and a progression-free survival of 79 wk [19].

Preclinical drug evaluation using patient-derived tumor tissue (PDTT) xenografting was proven to be an effective model that allows investigation of the effectiveness of treatment concepts by closely mirroring the real clinical situation [20]. So far, neither PDTT xenografting nor real-time investigation of the individual tumor biology was evaluated in ATC patients. Our impression is that the concept of a personalized course of treatment, based on the individual tumor biology, may represent a step forward towards a more effective treatment for patients with ATC.

2. Materials and methods

2.1. Case report

A 49-year-old man, operated in an external hospital for a rapidly growing thyroid nodule, was confirmed to have ATC by histopathologic examination. Since a total thyroidectomy and a central lymph node dissection were performed during primary operation and postoperative histology outlined a pT4pN1B (4/12),V1,R1,G3 situation with persisting lymph node metastasis in the compartments II–IV according to Robbins classification, the patient was transferred to our hospital for further treatment. After different options were discussed by our tumor board, we decided to perform a multimodal therapeutic strategy, including lymphadenectomy, hyperfractionated external beam radiation (EBR), and chemotherapy.

2.2. Operation and preparation of patient-derived human tumor tissue

Cervical re-exploration 1 wk after the primary procedure displayed huge lymph node metastasis in the compartments II–V and a modified radical lymph node dissection was performed. After confirming anaplastic tumor tissue in the dissected lymph nodes by frozen section, fresh tumor tissue was carefully prepared. After removal of fat and connective tissue and avoiding the use of necrotic tumor material, tumor tissue was rinsed and transferred to fetal calf serum–free culture media, put on ice, and immediately transferred to the animal laboratory.

2.3. Drugs

MLN8054, a benzoic acid derivative, was kindly provided by Millennium Pharmaceuticals (Cambridge, MA) and dissolved in 20% hydroxypropyl-β-cyclodextrin and 3.5% sodium bicarbonate (50:50).

Vandetanib, a hetero-aromatic-substituted anilinoquinazoline, was kindly provided by Astra Zeneca (London, UK) and formulated in phosphate-buffered saline containing 0.5% Tween 80. Sorafenib, a bi-aryl urea, was purchased from LC
Labs (Woburn, MA) and dissolved in CremophorEL (Sigma, Munich, Germany) and 95% ETOH (50:50).

2.4. In vivo animal experiment

Six-week-old athymic nude mice (nu/nu) were purchased (Harlan Winkelmann, Borchern, Germany) and allowed to adapt to the laboratory environment for 1 wk.

Immediately before implantation, patient-derived human tumor tissue was cut in pieces of 3 × 3 mm and transplanted on the right flank of the nude mice under local anesthesia. Procedures were finished within 120 min after explantation of tumor tissue in the operating theater. After 2 wk, when tumors with a tumor volume (TV) in the range of 20–600 cc had developed, some of the mice were randomized (n = 6 each) and treated with sorafenib [S] (20 mg/kg, 5 d/wk, orally) or the corresponding vehicle. The tumor tissue of the other mice was removed, cut in small pieces, and implanted to further nude mice (“passaging”) for future treatment with vandetanib [V] (50 mg/kg, 5 d/wk, orally) and MLN8054 [M] (10 mg/kg, 5 d/wk, orally) or the corresponding vehicles. The tumor take rate was between 75% and 85% within the first passages (1–4) and displayed lower values (25%–50%) after repeated passaging (>5). Tumor size, animal weight, and side effects were monitored weekly and tumor volumes were calculated as TV = L × W² × 0.5 (L = length, W = width). After 3 [V, M] or 5 [S] wk, depending on the tumor growth of the control group, mice were sacrificed and tumor tissue removed and processed for immunohistochemistry.

All procedures were monitored and approved by the local ethics committee and federal authorities and conducted in accordance to the guidelines for the welfare of animals in experimental neoplasia.

2.5. Immunohistochemical studies

Paraffin sections measuring 3 μm were used for immunohistochemical analysis. For 5-bromo-2’-deoxyuridine (BrdU) labeling, animals were treated with BrdU (1 mg/animal, intraperitoneally) 1 h before sacrificing and tumor slides were stained with an anti-BrdU antibody as described in detail in the Supplementary Appendix.

Morphometric analysis was performed using a Leica microscope (DMLB) and the Leica imaging system QWin (Leica, Wetzlar, Germany). BrdU, caspase 3, and phosphorylated histone H3 (pHisH3) staining were evaluated by determining the positively stained area per high-power field (×10 or ×40) in 20–30 random areas within the maximal tumor cell load. Tumor angiogenesis was quantified following CD31 staining by assessment of the vascular surface density (VSD, 1 mm⁻²) as described earlier [21]. Phospho (p)-EGFR and pVEGF-R2 staining was evaluated semi-quantitatively using the following score: 0 = negative (no membrane staining); + = weak (weak membrane staining); ++ = moderate (weak membrane staining plus some areas more strongly stained); and +++ = strong (strong membrane staining plus some areas more strongly stained). For comparison, the number of specimens displaying a specific score were determined for each group and expressed as percentage.

2.6. Statistical analysis

An extension [22] of the nonparametric Kruskal-Wallis test to time-series data was used to calculate exact P values testing for differences in the tumor volume growth pattern. Since the test is a rank-based procedure, we were able to incorporate growth curves that had some missing values at later time points in our analysis in the most conservative way possible by assuming missing tumor volume measurements in the control group as lower, and missing volume measurements in the test group as higher, than all other volume measurements at the time. The Wilcoxon rank-sum test was used to compare the BrdU, caspase 3, and pHisH3 staining as well as VSD.

3. Results

3.1. Effects on tumor development caused by treatment with MLN8054, vandetanib, and sorafenib

Animal weight remained nearly constant and no side effects were observed. Application of MLN8054 and vandetanib for 3 wk reduced tumor volume, assessed by continuous monitoring, by 46% versus 34% in the vehicle-treated control group. Using sorafenib, TV was reduced by 30% after 3 wk and by 61% after 5 wk (Fig. 1A–C). The tumor masses at the time of explantation paralleled the results of the calculated TV. Altogether, tumor growth inhibition can be stated for all treatment groups; however, due to inhomogeneous spontaneous tumor growth in the control groups, they did not reach statistical significance (P = 0.054 [M], P = 0175 [V], and P = 0.081 [S]).

3.2. Immunohistochemical analysis

3.2.1. Morphologic analysis of the tumor specimen and the tumor xenotransplants

Histologically, an undifferentiated, highly invasive solid tumor with lots of mitosis and apoptosis, vascular invasions, and large areas of necrosis as well as focal desmoplasia was seen within the remaining normal thyroid tissue, matching a squamous-type anaplastic thyroid cancer. The immunohistochemical analysis, however, displayed expression of cytokeratins (CK 5/6, 7, 8, 14, 19) and TTF-1 and no expression of thyroglobulin and calcitonin. So the expression pattern of tumor markers is not necessarily typical for ATC, making the differential diagnosis of an end-differentiated primary squamous cell carcinoma of the thyroid possible. The xenotransplants of the tumor tissue showed an equally histomorphologic structure when compared with the primary tumor.

3.2.2. Effects of treatment with MLN8054, vandetanib, and sorafenib on tumor cell proliferation

The effect of the compounds on tumor cell proliferation was analyzed by BrdU immunohistochemistry (Fig. 2; Supplementary Fig. 1A,B). In the vandetanib group BrdU staining decreased significantly, by about 34% (3.7% ± 0.8% compared with 5.6% ± 1.2% in the control group, P = 0.016) and in the sorafenib group by about 58% (2.4% ± 1.2% compared...
with 5.7% ± 0.8%, P = 0.029). However, in the MLN8054 group, we found a nonsignificant trend towards increased BrdU labeling (P = 0.225).

3.2.3. Effects of treatment with MLN8054, vandetanib, and sorafenib on tumor angiogenesis (CD31)
Vascular surface density, assessed by CD31 staining (Fig. 3; Supplementary Fig. 1C,D), was significantly reduced by MLN8054 (VSD = 0.0027 ± 0.0005 [1/μm] compared with 0.0049 ± 0.0005 [1/μm] in the control group, P = 0.004) and vandetanib (VSD = 0.0016 ± 0.0002 [1/μm] compared with 0.0056 ± 0.0015 [1/μm], P = 0.008). But it was hardly affected in the sorafenib group (0.0025 ± 0.0014 [1/μm] compared with 0.0029 ± 0.0012 [1/μm]), and this trend was without statistical significance (P = 0.686). Expressed as percentage, VSD was reduced about 40% [M], 67% [V], and 33% [S], showing vandetanib as the most potent in inducing antiangiogenic effects in this experiment.

3.2.4. Effects of treatment with MLN8054, vandetanib, and sorafenib on tumor apoptosis (caspase 3 activity)
In all treatment groups a trend towards elevated caspase 3 activity was found (Fig. 4; Supplementary Fig. 1E,F). The effect was most pronounced in the sorafenib group. Here 0.339% ± 0.138% of the tumor surface stained positively for cleaved caspase 3 compared with 0.189% ± 0.028% in the control samples, indicating a 2.4-fold increase of caspase 3 activity. In the MLN8054 and vandetanib groups caspase 3 activity increased about 1.6-fold (0.300% ± 0.159% compared with 0.189% ± 0.055%) and 1.3-fold (0.181% ± 0.068% compared with 0.140% ± 0.047%), displaying nearly no proapoptotic effects. Statistical analysis resulted in significance levels of P = 0.329 [M], P = 0.421 [V], and P = 0.114 [S], documenting none of the effects as significant.

3.2.5. Effects of treatment with MLN8054 on histone H3 activity
Downregulation of pHisH3 has been noted to indicate Aurora B inhibition. But, analyzing tumor samples by pHisH3
immunohistochemistry (Supplementary Fig. 2A,B), only minor differences were found between the MLN8054-treated group and the corresponding control group. So, about 0.374% ± 0.182% and 0.316% ± 0.074% of the tumor area stained positive in the treated and control groups, respectively, but this difference was without statistical significance ($P = 0.537$).

### 3.2.6. Effects of treatment with vandetanib and sorafenib on EGF-R and VEGF-R2 activity

To test for specific inactivation of known targets, the sorafenib-treated tumors were analyzed for pEGF-R and the vandetanib-treated for pEGF-R and pVEGF-R2 by immunohistochemistry (Fig. 5A–C; Supplementary Fig. 2C–F). Generally, the receptors were found in the tumor cells themselves, primarily in the outer regions of tumor cell nests. Vandetanib potently inhibits EGF-R and the VEGF-R activity (Fig. 5A and B; Supplementary Fig. 2C–F). So, neither moderate nor strong staining intensity for pEGF-R was found in the vandetanib group, whereas in the control group about 60% of the tumor samples displayed these staining intensities. Instead, the number of negatively stained samples increased to about 65%, compared with 7% in the control group, and the percentage of weakly stained samples was similar in both control groups (Fig. 5A).

Comparable results were found for pVEGF-R2. Staining graded as strong or moderate was nearly absent in the vandetanib group, whereas the percentage of weakly stained samples distinctly increased to 53% versus 37%, and that of negatively stained samples to 47% versus 13%, in the control group (Fig. 5B).

Sorafenib treatment also reduced VEGF-R2 activity. Strong pVEGF-R2 staining was absent in the treatment group and moderate staining was reduced to 11% compared with 41% in the control group. In contrast, the percentage of samples displaying weak or negative staining increased to 49% and 41%, compared with, respectively, 34% and 5% in the control group (Fig. 5C).

Altogether, EGF-R and VEGF-R2 activity was profoundly downregulated by vandetanib and VEGF-R2 activity by sorafenib.

### 3.3. Clinical course of the patient

After cervical re-exploration and modified radical lymph node dissection with resection of huge lymph node metastasis, the patient underwent a multimodal therapeutic strategy including EBR and chemotherapy. Due to the histopathologic findings of the tumor specimen, a complete clinical work-up of the patient, including clinical and lab investigation, endoscopy, ultrasound, computed tomography, and magnetic resonance imaging scan, was conducted after the operation to exclude the possibility of distant metastasis of an undifferentiated carcinoma of nonthyroid origin to the thyroid gland.

While the patient completed the EBR, drug effects on xenotransplanted tumors were evaluated. According to an off-label protocol based on previous investigations, the patient was treated with 400 mg sorafenib twice a day for 3 cycles in an adjuvant setting paralleling the in vivo investigation that displayed antitumor activity in the individual xenotransplants. A clinical follow-up 1 y after surgery, including clinical and lab investigation, ultrasound, and magnetic resonance imaging, showed no recurrence.

### 4. Discussion

Fortunately, anaplastic thyroid cancer is an uncommon malignancy of the thyroid; however, it accounts for a large number of the tumor-related deaths among patients suffering from TC. The highly aggressive clinical course of the tumor

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**Fig. 3** — In vivo effects of MLN8054, vandetanib, and sorafenib on angiogenesis, as revealed by measurement of vascular surface density following CD31 immunostaining in 30 fields ($\times 40$) per slide, were analyzed. Means ± SD of VSD are depicted for the treatment and control group each. *$P \leq 0.05$.

**Fig. 4** — Effects of MLN8054, vandetanib, and sorafenib on caspase 3 activity in xenografted ATC tumor tissue. Means ± SD of caspase 3 positivity for the treated and control groups ($n = 6$) are depicted. Thirty fields ($\times 40$) per slide were analyzed. Significance level: MLN8054 $P = 0.329$, vandetanib $P = 0.421$, and sorafenib $P = 0.114$. 
biology often makes a tumor-extirpating therapy impossible, underlining a desperate need for an effective multimodal treatment. Although in the past years some groups reported encouraging results from preclinical protocols of novel chemotherapeutic agents, they often failed to confirm these results in a clinical setting. This again raises the question of the clinical applicability of results obtained from cell line-based in vitro or xenotransplant models. A promising new approach that possibly provides a more accurate reflection of human tumor biology is patient-derived human tumor tissue xenografting. Here, we report a successful PDTT xenograft model of ATC tissue to nude mice corresponding to other cancer entities arising from glands like pancreatic [23] or prostate cancer [24], which allows the real-time investigation of novel drugs intended for a personalized antitumor therapy. Major advantages of PDTT xenografting, such as displaying the real tumor biology of the individual xenograft; tumor-stromal relationships as an essential prerequisite for growth; or the availability of a continuous source of vital, transplantable tumor tissue, as detailed by Jin et al. [20], could be confirmed for ATC. Further, acceptable tumor take rates between 75% and 85% and successful “passaging” allowed evaluation of the antitumor activity of the three different drugs. Although tumor growth was potently inhibited in all treatment groups, inhomogenous tumor development in the control groups accounted for high standard deviations, precluding significant results when analyzing the individual compounds.

With an up to 46% reduced tumor growth, development of PDTT ATC xenografts was strongly inhibited by MLN8054, confirming the Aurora kinase pathway to be an interesting therapeutic approach for ATC, although not achieving the results of cell line-based xenografting [15]. MLN8054-mediated tumor growth inhibition was accompanied by increased BrdU labeling. Possibly this indicates Aurora A inhibition, since an increased mitotic index, caused by delayed mitotic progression following MLN8054 treatment of colon cancer xenografts, has been reported to be consistent with Aurora A inhibition, although we did not find significant differences in histone H3 activity [14]. Tumor growth inhibition was further accompanied by antiangiogenic effects and slightly increased caspase 3 activity, indicating proapoptotic effects. Both antiangiogenic and proapoptotic effects have been reported elsewhere [14,25], as well as by our group [15].

The potency of vandetanib to inhibit tumor growth was shown for a broad spectrum of human cancer xenograft models [16,26]. In our study tumor growth was reduced by 34% after 3 wk. This effect was accompanied by decreased tumor cell proliferation and severely reduced microvessel density (VSD), whereas proapoptotic effects were marginal. Similar results were reported for ovarian and non–small cell lung cancer [26,27]. Antiangiogenic effects of vandetanib are mediated by targeting VEGF-R2 activity and indirectly by targeting EGF-R signaling, which adds to the regulation of VEGF secretion [28]. An inhibiting impact on both receptor systems, as documented elsewhere [26], was confirmed in our PDTT xenografts.

Antitumorigenic activity of sorafenib, including anti-proliferative, antiangiogenic, and proapoptotic effects, was shown in various preclinical models of human cancers based on xenografted tumor cell lines (for review see [18]) and also for orthotopic xenografts [29]. In our model, sorafenib treatment resulted in reduced tumor growth of 30% after 3 wk and 62% after 5 wk. For this drug, which was analyzed using the first passage of xenografted tumor tissue, prolonged treatment was possible due to the slow growth kinetics of the control group, which increased after “passaging” the xenotransplants. Tumor cell proliferation—documented by significantly reduced BrdU incorporation—was strongly affected, as was also shown for breast cancer [17]. Sorafenib was also shown to enhance tumor cell apoptosis by 2.4-fold increased caspase 3 activity; however, this result failed to reach significance. Further, we could confirm severely decreased VEGF-R2 activity, as was documented previously for xenotransplanted ATC and tumor biopsies of metastatic thyroid cancer [29,30]. However, the impact on angiogenesis remains unclear,
because we did not find distinct changes in the vascular surface density, which was demonstrated for various human tumors (for review see [18]).

Also, in our PDTT model, tumor development was distinctly affected by using MLN8054 as Aurora kinase inhibitor and targeting receptor tyrosine kinases with vandetanib and sorafenib. Furthermore, it can be assumed—that in this case—MLN8054 acted mainly by targeting Aurora A. Using vandetanib, tumor growth was predominantly affected by antiangiogenic mechanisms, whereas sorafenib acts primarily by affecting cell proliferation.

Since inhomogenous tumor growth of the PDTT xenotransplants resulted in high standard deviations so that differences between the groups failed to reach the criteria of significance, the protocol used in this setting needs technical modification. For future use of ATC PDTT xenotransplant models, it would be more suitable to stabilize growth the xenotransplants before starting drug application and parallelizing the treatment groups. This means that, in our opinion, it is necessary to passage the first xenotransplants to gain stable, growing tumors and to achieve enough tumor material, offering the possibility to run all examinations by parallel experiments within the second passage of the xenotransplants. Additional “passaging” of the tumor tissue more than four times should be avoided to exclude changes in the growth kinetics.

It can be assumed that in vivo testing of candidate drugs for a multimodal approach in ATC patients in addition to the clinical standard of operation and adjuvant radiochemotherapy offers the possibility of an individualized treatment. The positive clinical course of the patient in our study may have been promoted by the fact that treatment started—although extended lymph node metastases were present and thyroidectomy was performed as an R1 resection—at a moderate stage of the tumor, since it had not invaded the esophagus or trachea, and by the fact that the tumor—although displaying typical histology of ATC—did not present all of the “classic” immunohistochemical features of ATC. The impact of sorafenib treatment in this case is not clear; however, the principle of patient-derived human tumor tissue xenografting could be confirmed for de-differentiated thyroid cancer even though the results have to be proven by modified protocols as suggested to possibly gain significance. Pretherapeutic in vivo testing hopefully offers future options for tailored tumor therapy based on individual tumor-specific characteristics.

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Supplementary data

Supplementary data (2 supplementary figures and a supplementary appendix) related to this article can be found at 10.1016/j.jss.2013.06.017.

References


