Tumor distribution and efficacy of antiangiogenic receptor tyrosine kinase inhibitors

Ph.D. Thesis

Szilvia Török

Semmelweis University
Clinical Medicine Ph.D. School

Consultants: Dr. Balázs Döme, MD, PhD, head of department
Dr. György Marko-Varga, PhD, professor

Official reviewers:
Dr. Anna Sebestyén, PhD, senior research fellow
Dr. Zsolt Horváth, MD, PhD, associate professor

Head of the final Examination Committee:
Dr. András Matolcsy, DSc, professor
Members of the Final Examination Committee:
Dr. Béla Szende, DSc, professor emeritus
Dr. Krisztina Vellainé Takács, PhD, associate professor

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1. INTRODUCTION

Under physiological conditions, angiogenesis is activated in response to low oxygen level. The process is regulated by the HIF (hypoxia-inducible factor) complex. In case of hypoxia, the HIF alpha and beta subunits form a complex, recruits its co-activator, p300/CBP (CREBB Binding Protein) and binds to the Hypoxia Responsive Element of the target genes, thus modifying their transcription. Several of these target genes are responsible for inducing angiogenesis in order to increase the oxygen delivery of the tissue. VEGF (vascular endothelial growth factor) is considered to be the key hypoxia dependent cytokine for endothelial sprouting. Besides, many other factors are also able to positively influence the process of angiogenesis such as platelet-derived growth factors (PDGFs), fibroblast growth factors (FGFs), placental growth factor, angiopoietins, Jagged, epidermal growth factor, hepatocyte growth factor and interleukin-8.

These ligands bind to their receptors on the surface of endothelial cells. Binding the growth factor leads to the activation of signaling cascades, influencing the survival, proliferation and migration of ECs and thus the maintenance of existing- and development of new vessels. Moreover, these pathways regulate further processes involved in angiogenesis, such as secretion of additional growth factors, upregulation of angiogenic receptors, alterations in cell-cell and cell-matrix interactions by matrix metalloproteinases, and activation of the members of endothelial cell adhesion molecule family.

The healthy body controls angiogenesis by balancing a series of angiogenesis stimulating and inhibiting factors. By overexpressing the angiogenesis stimulating factors, blood vessel growth becomes highly intensive in some pathological conditions, including cancer.

In 1971, Judah Folkman assumed that malignant cells secrete proangiogenic factors in order to ensure that tumors reaching about 2 mm diameter access the necessary amount of oxygen and nutrients by developing their own blood supply. Moreover, by regulating the expression of HIF and/or other proangiogenic factors, the presence of different oncogenes or loss of function of tumor suppressor genes can also facilitate tumor induced angiogenesis.

The most important molecules regulating neovascularization are RTKs, such as VEGFRs, PDGFRs, FGFRs, and the TIE receptors. Besides, serine-threonine kinases, such as the members of TGF receptor family also regulate the process of angiogenesis. Other receptors exert angiogenesis stimulation not by phosphorylating partner molecules, but by enhancing the effects of potential transcriptional activators, such as the Notch receptor family.

Beside endothelial sprouting driven by the VEGF-VEGFR axis, alternative vascularization mechanisms, which cannot necessarily be regulated by the main angiogenic molecules also exist. These processes include intussusceptive angiogenesis, vessel incorporation, glomeruloid angiogenesis, postnatal vasculogenesis and vasculogenic mimicry.

Because of the dominance of the proangiogenic factors in malignant tissues, tumor blood vessels differ from the normal ones both in function and structure. In a healthy adult the normal vasculature is static, the ECs have long half-life, they are located on a well defined basal membrane (BM), linked with cell junctions and stabilized by pericytes and vascular smooth muscle cells (VSMC). Maintenance of the vessel is secured by autocrin signals and the presence of signals from oxygen sensors provide appropriate blood flow. In contrast, tumor blood vessels usually have deficient pericyte and VSMC coverage. The BM is often degraded, the morphology of ECs are changed, and cell-cell junctions are often lost. As a result, vessels get dilated negatively impacting the ratio of vessel surface, which supplies the tumor tissue with oxygen and nutrients. The loosening of the signals of oxygen sensors, eventually leads to abnormal blood flow in the tumor vasculature. The degraded BM not only leads to enhanced metastatization capacity and the possibility of the creation of mosaic blood vessels, but vessels get hyperpermeabilized as well, causing an increase in the interstitial fluid pressure, which cannot be restored because of the decreased lymphatic function. Thus, the oxygen supply of the tumor decreases, resulting in an increased glycolytic activity to provide energy and the necessary building material for the growing tumor. The altered acidic microenvironment selects for hypoxia resistant, aggressive tumor cells, and further supports the invasion and metastatization ability of malignus cells, creating a vicious circle in

tumor progression. Moreover, as a result of the inadequate blood flow, the resulting hypoxia and/or the tightened BM, efficacy of conventional anticancer therapies are inadequate due to limited penetration. These abnormalities of tumor blood vessels and the resulting aggressiveness of cancers, accompanied by decreased efficacy of conventional treatment led Rakes Jain to the elaboration of the vessel normalization theory. Accordingly, the inhibition of the signal of proangiogenic factors and the subsequent normalization of tumor blood vessels has become a critical step in cancer therapy.

The following molecules are identified as antivascular agents:

Conventional therapeutic drugs have been found to have antiangiogenic functions as "side effect". Vascular disrupting agents selectively target tumor vessels, causing fast and dynamic effects. They destroy rapidly dividing ECs in the tumor tissue by targeting the colchicine binding site of tubulins or induce vascular collapse through TNF-α. As a result, the vascular supply shuts down, causing necrotization as a response of insufficient oxygen and nutrient delivery. Vasoactive agents combinatorially block existing vessels and suppress the formation of new ones. Moreover, they do not seem to preferentially target the larger vessels in the tumor center, but also the small ones in the periphery, causing hyperabnormalization and hyperpermeability of the vessels. This allows chemotherapeutic agents better access to the tumor. However, these drugs are highly toxic when administered systemically, thus local, small dose and metronomic application is preferable.

Angiogenesis inhibitors block the formation of new vessels from preexisting ones, but do not affect already established vasculature. They are thought to mainly act on small vessels of the tumor edge, mostly at the early stage of tumorigenesis or metastatization. In the absence of selective HIF inhibitors and the presence of a number of factors that also regulate the expression of angiogenic cytokines in tumors, the common way is to target molecules downstream of HIF by the blockade of receptor - growth factor communication.

Growth factors can be targeted with either monoclonal antibodies (mABs) or soluble „trap/decoy” receptors. Receptors on ECs can be targeted at their extracellular domain by mABs, thus blocking ligand binding, or by RTKIs at the tyrosine kinase domain of the proteins,

which serve as docking sites for molecules mediating the angiogenic signal.

Initially, no resistance to antiangiogenic tyrosine kinase inhibitors was expected, because they target ECs which are genetically stable and therefore unlikely to develop mutations. In spite of that, drug resistance in patients treated with antiangiogenic therapies is an important clinical problem. Both primary (no initial response is shown to therapy) and secondary (after a short regression period, the tumor recovers) resistances have been documented. There is a substantial volume of literature on the potential mechanisms that can lead to tumor resistance against antiangiogenic RTKIs. These include hypoxia-driven mechanisms, the activation of compensatory angiogenic molecular pathways, the mobilization of myeloid or endothelial progenitor cell populations, the downregulation of target receptors in endothelial or tumor cells and also a switch to an alternative vascularization mechanism. Although suboptimal pharmacokinetics have also been described, so far most studies dealing with antiangiogenic RTKIs and drug resistance in solid tumors have focused on the above mechanisms.

Although ADME can fundamentally influence the therapeutic benefit of different drugs, until recently, extensive ADME studies were conducted rather late in the process of drug development, mainly in phase I clinical studies. This may be one of the key factors behind the low, 11%, overall first-in-man to registration rate of novel drug candidates in the 1990s. This proportion was especially poor (5%) among drugs in the field of oncology.

Mass spectrometry is a powerful technique, enabling the parallel determination of label-free drugs and their metabolites from different tissue compartments, that gives researchers the opportunity to analyze the adsorption, distribution and elimination of the native drug and its active/toxic metabolites as well.

Although the role of VEGF and its receptor in the growth, vascularization, and metastatization of colorectal cancer is already known since the 1990s, and a number of antiangiogenic molecules are approved to treat these conditions, no classical antiangiogenic RTKI is effective in that kind of cancers.

2. AIMS

The clinical experiences with antiangiogenic RTKIs are controversial, despite their predicted inhibitory and normalizing effects on vessel growth. No biomarker of tumor response has yet been linked to the effect of these agents. Taken into consideration that ADME can fundamentally influence the therapeutic benefit of different drugs, the following specific aims have been defined:

1. to develop a method for the detection of antiangiogenic RTKIs and their metabolites in different tissues.
2. to analyse the distribution and levels of different antiangiogenic receptor tyrosine kinase inhibitors (RTKIs) inside subcutaneous murine tumors growing in mice by using MALDI-MSI.
3. to evaluate the potential associations between intratumoral antiangiogenic drug levels and distributions and 3.1./ tumor growth inhibition; 3.2./ blood vessel density and area; 3.3./ blood vessel integrity (basement membrane, pericyte and α-smooth muscle actin (SMA) coverage 3.4./ the size and localization of hypoxic areas of the tumor tissue; 3.5./ the expression and distribution of receptors targeted by the RTKIs.

3. METHODS

Tumor models

Two different mouse colon adenocarcinoma models, C26 and C38 were used for our experiments.

The C26 cell line was cultured in RPMI 1640 medium with 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified atmosphere at 37°C, 5% CO_2. Groups of six female Balb/C mice were inoculated subcutaneously (*s.c.*) with $2x10^6$ C26 cells.

The C38 tumors were maintained by serial s.c. transplantations of tumor cubes measuring 5×5×5 mm, and at last transplanted into female C57black/6 mice for our experiments.

Drugs

For drug treatment of C26 bearing mice, 5 antiangiogenic RTKIs either approved by both the FDA and the EMEA (pazopanib, sorafenib, sunitinib) or investigated in Phase III trials (motesanib, vatalanib) were selected. In the C38 model a vatalanib-treated and a control group was generated. These RTKIs are known to have significant IC50 values of the main angiogenic receptor, VEGFR2, and may also inhibit PDGF- and FGF receptors.

Drugs were suspended in 2% carboxymethylcellulose with 2 mg/mL methyl-4-hydroxibenzoate. Control animals received the suspending medium only.

Treatments

RTKI treatments began 2 weeks after tumor cell injection in case of the C26 model and 9 days after C38 tumor implantation and were performed once daily at a dose of 100 mg/kg *per os* with a feeding tube 5 times a week for two weeks.

Tumor size was measured three times a week with a caliper and expressed in mm^3 by the formula for the volume of a prolate ellipsoid (length x width2 $\pi/6$) in case of both models.

To assess intratumoral hypoxia, a bolus of i.p. pimonidazole (60 mg/kg) was administered 2 hours before the mice were sacrificed. Two hours after the last RTKI treatment, blood was drawn from the

canthus and the animals were sacrificed. Tumors were removed and snap frozen.

Analysis of vascular parameters and target receptors

For the analysis of RTKI distributions, vascular parameters and target receptor expressions, 10 serial frozen sections were cut from each tumor. Sections #5 and #7 were used to analyze the distribution and levels of the given RTKI by MALDI-MSI and for subsequent haematoxylin&eosin (HE) staining. Sections #1-4 were labeled with either of the following primary antibodies: anti-FGFR1, anti-PDGFRα, anti-PDGFRβ and anti-VEGFR2. For hypoxia detection (section #6), we used the Hypoxyprobe-1 Plus Kit. Sections #8-10 were labeled with either of the following primary antibodies: anti-laminin (for endothelial basement membrane labeling), anti-desmin (for pericyte labeling) and anti-α-smooth muscle actin (anti-αSMA). All of the above primary antibodies were developed with an appropriate fluorescent secondary antibody. For intratumoral microvessel density and area measurements, sections #1-4, #6 and #8-10 were co-stained with anti-mouse CD31 antibody, followed by a counterstain with Hoechst 33342 before mounting under glass coverslips in ProlongGold Antifade Reagent.

Slides were scanned by TissueFAXS and analyzed by ImageJ and TissueGnostics 4.0.0140 softwares.

Microvessel densities and areas were then calculated by counting the number of blood vessels or the CD31-positive pixels in the total area of ten intratumoral regions from the living part of the tissue. The percentages of microvessels that were positive for laminin, desmin or αSMA were also calculated. For quantification of hypoxia and PDGFRα, PDGFRβ and FGFR1 expressions, the percentages of hypoxic regions and positively labeled cells were determined across the entire area of section. VEGFR2 density was calculated by counting the VEGFR2 positive tumor cells and blood vessels, and for quantification of VEGFR2 area, the pixel number of tumor images that were occupied by VEGFR2 positive endothelial or tumor cells were calculated on 10 viable tumor regions.

Compound characterization

For compound characterization drugs were dissolved in 50% methanol at 0.5 mg/mL concentration. The matrix, 7.5 mg/mL α-cyano-4-hydroxycinnamic acid was dissolved in 50% acetonitrile and 0.1% trifluoroacetic acid. 1μL of the compound solution was applied with 1μL matrix solution to the MALDI plate. Full mass spectra were obtained by using a MALDI LTQ Orbitrap XL mass spectrometer at 60,000 resolution in positive polarity mode. The spots were sampled in survey mode collecting 20 experiments for a single run. The nitrogen laser was set to 10 μJ. The detected precursor ion was fragmentized by using 40% normalized collision energy (NCE) during a 30 ms activation time, while activation *Q* of 0.25 was applied. The precursor ions were isolated with *m/z* 2.0 width and MS/MS spectra were collected at normal scan rate in centroid mode.

Tissue imaging of antiangiogenic RTKIs

10-μm frozen sections were cut using a cryotome and placed onto glass slides. After drying of the tissue, 0.5 mL matrix solution was applied stepwise to avoid wetting of the sections by using an airbrush, while its position was kept constant. Full mass spectra were collected by using the Orbitrap mass analyzer at 60,000 resolution (at *m/z* 400) in positive mode with a 150−800 Da mass range with activated automatic gain control mode. Tissue sections were sampled with 100 μm raster size. The nitrogen laser was operated at 10.0 μJ. For obtaining MS/MS data, the observed peaks of the precursor antiangiogenic drugs were isolated with *m/z* 2.0 width isolation window and fragmentized, using 40% NCE, 30 ms activation time and 0.25 activation *Q*. For MS/MS spectra generation, the minimal signal required was 500 counts. The fragment ions were analyzed in the linear ion trap at normal scan rate. Evaluation of the spectra was performed with Xcalibur v 2.0.7. software, while the visualization of drugs, metabolites and fragment ion distribution was implemented with the ImageQuest™ software.

Quantification of the precursor compounds

For tissue quantification of intratumoral drug concentration, calibration curves of each compound were established on tissue

sections of untreated control C26 and C38 tumors. Drugs were dissolved and diluted in 50% methanol (concentration range: 0.001–0.5 μmol·mL−1) and 0.5 μL from each concentration was applied on the tissue section. Spraying and detection conditions were the same as those during the tissue section analysis of in vivo treated tumors. Average signal intensities of the applied concentrations were measured and normalized to total ion current by using Xcalibur v 2.0.7. and ImageQuest™ softwares. Calibration curves were created and then used to estimate the tissue drug concentrations of in vivo-treated tumor sections.

Compound detection in the blood

20μL plasma samples were collected and proteins were removed by acetonitrile precipitation. Pierce C18 Tips were used to concentrate the RTKIs from the precipitated plasma samples following the manufacturer's instructions. 1μL of the sample was applied on the MALDI plate with 1μL matrix solution using the same instrument settings as those for the compound characterization.

Throughout our MS experiments drugs were considered to be identified if both the precursor molecule and at least one fragment ion were discovered in the spectra.

Statistical analysis

Differences in parametric and non-parametric variables between multiple groups were analyzed using ANOVA and Dunnett's posthoc test or using the Kruskal–Wallis test followed by a posthoc Dunn's multiple comparison test, respectively. For comparing two groups, t- or Mann-Whitney U tests were applied. Differences were considered statistically significant when $p<0.05$. All statistical analyses were carried out using GraphPad Prism 5.0 software.

4. RESULTS

Tumor growth inhibition

A significant relative tumor growth inhibitor effect was shown after two weeks of RTKI treatment, when comparing the % change in tumor volume in the C26 model. Sunitinib was the only drug affecting tumor growth in this experimental setting.

In the C38 model, two weeks of vatalanib treatment significantly reduced tumor burden.

Immunohistochemical analysis

The growth of *s.c.* tumors in mice is known to be angiogenesis-dependent. Thus, in the next step of the experiment we tested, if the differences in tumor growth are in line with the effects of drugs on vascular parameters and the expression of target angiogenic receptors of the RTKIs. Therefore, we stained tissue sections for these parameters.

The expression level of antiangiogenic RTKs may significantly influence treatment response, and in turn, successful therapy can also regulate target receptor localization and function. In the C26 model expression of PDGFRα, -β and FGFR1 was observed not only in mural cells, but also on tumor cells. Expression patterns of PDGFRα, -β and FGFR1 did not change in response to treatment with any of the compounds in the C26 model. C38 tumor cells did not express the aforementioned receptors, but a definite cell population expressing PDGFRα, -β and FGFR1 was detectable on the mural cells. Similarly to the C26 model, no change in the expression of PDGFRα, -β and FGFR1 was seen.

VEGFR2 expression was detected both on tumor and endothelial cells in the C26 model. Significant differences were shown both when counting the VEGFR2 signal and when measuring the area of VEGFR2+ cells in C26 tumors. The post-hoc test showed that VEGFR2 expression was altered only in the sunitinib treated group.

C38 tumors were characterized by a weak VEGFR2 expression of the CD31+ endothelial layer, but no signal of the receptor on tumor cells was observed. No difference in the expression of VEGFR2 in the treated vs. non treated groups of C38 was detected.

We found a significant difference between the microvessel density (MVD) of the C26 groups. The post-hoc test showed a suppressed MVD by sunitinib, motesanib and minimally vatalanib in that model. Microvessel area was also decreased in the sunitinib, motesanib and less intensively in the sorafenib treated group. In the C38 model however, no difference in the vessel density, but in vessel area was detected. It is also important to mention, that major differences in the vasculature of the two groups were observed. While C26 tumors had lots of small vessels, C38 tumors were characterized by only a few, but large and complex vascular structures.
MVD clearly correlated with tumor oxygenation. Hypoxia was located in the less vascularized areas of the tumor. Accordingly, a significant increase in the intratumoral hypoxic areas was observed in the sunitinib treated group. The ratio of hypoxic areas did not differ in the C38 model.
Besides the inhibition of EC proliferation, multi-target antiangiogenic RTKIs also influence PDGFR and FGFR positive pericyte and VMSC recruitment to tumor blood vessels. Therefore, the inhibition of these receptors may result in not only decreased MVD and consequently lower blood flow rate of tumors, but could also facilitate cancer cell metastatization. To observe the structural changes of the vasculature in response to treatment, we examined the expression of laminin, desmin and αSMA of tumor sections. While all vessels remained underlaid with a definite layer of laminin and covered with αSMA, desmin expression has decreased in response to sunitinib and motesanib treatment in the C26 model. Both laminin and αSMA expression were definite and did not change in response to treatment in the C38 group, but unlike in C26, no difference in desmin expression was observed either.

Mass spectrometric analysis

Compound characterization

To determine if these differences in the biological effects of the compounds arise from the differences in the intratumoral drug concentrations, we have developed a method to characterize the antiangiogenic RTKIs in tissue samples.

First, the quasimolecular ion and fragmentation pattern of each compound was defined on a MALDI stainless steel target plate.
The quasimolecular ion of motesanib was detected at *m/z* 374.199. Fragmentation of the molecule resulted in ions at *m/z* 212.1, 189.1 and 163.1.
Pazopanib was detected at *m/z* 438.17. Subsequent MS/MS fragmentation of the precursor ions led to fragment ions at *m/z* 421.1, 357.1, 342.1.
Sorafenib was found with a quasimolecular ion at *m/z* 465.093. Fragment ions at *m/z* 447.1, 425.1, 270.2 and 252.2 were detected.
Sunitinib was identified at *m/z* 399.218. Subsequent MS/MS fragmentation of the precursor ions led to fragment ions at *m/z* 326.1 and 283.1.
The quasimolecular ion of vatalanib was seen at *m/z* 347.105. Fragment ions at *m/z* 320.2, 311.2, 294.2, 268.1, 254.1 and 220.2 were generated.
When applied to the tissue surface, drug molecules showed similar ionization and fragmentation properties to those generated on the MALDI plate.

Precursor compound and metabolite detection in the blood
Adsorption of the drugs was examined in the peripheral blood, drawn just before sacrificing the animals. In both models all applied drugs absorbed successfully with notable signal intensities being observed in the peripheral blood. Besides, different metabolites of the drugs were also traceable.
No difference in the signal intensities and metabolization pattern of vatalanib in the blood samples taken from the Balb/C and the C57black/6 mice was detected.

Tissue imaging of antiangiogenic RTKIs
Calibration of the drug molecules resulted in linear correlation between concentration and normalized average signal intensity for all compounds in the examined concentration range. Based on the calibration curves, average signal intensities were translated into drug concentration (μmol/mL) data of C26 and C38 tumors. While intratumoral sorafenib and vatalanib levels did not differ between

drug-treated and control C26 tumors, the concentrations of motesanib, pazopanib and sunitinib were significantly elevated (vs. control), with the highest values detected in the sunitinib-treated animals.

Importantly, the above described drug concentrations refer to the entire tumor section and striking differences in the drug distribution were observable within the in vivo-treated C26 tumors. As for sunitinib, the drug was quite homogeneously distributed within the viable C26 tumor areas and apoptotic regions showed notably lower signal intensities. In contrast, the distribution of motesanib and pazopanib was inhomogeneous with the highest signal intensities observed in non-viable areas. Only traces of sorafenib and vatalanib were detected in the C26 model.

In a previously published study, the members of our group found significantly decreased C38 tumor burdens in C57Bl/6 mice treated with vatalanib. Accordingly, in order to determine why mice bearing C26 tumors respond notably poorer to vatalanib than those with C38 tumors, we also utilized MALDI-MSI of C38 tumors and addressed whether there are animal model-specific variations in the tumor tissue penetration and distribution of antiangiogenic RTKIs. In contrast to the C26 model, vatalanib was well-distributed with notable signal intensities in the C38 tumors**.** In line with this, in vatalanib-treated mice bearing C38 tumors, the intratumoral drug concentration was significantly higher than that in the group of untreated controls. It is also important to mention that we found significantly higher vatalanib concentrations in C38 than in C26 tumors.

No correlation between drug signal intensities in the blood and in the corresponding tumor tissue was detected.

We also identified intratumoral metabolites of sunitinib, motesanib and vatalanib, however, only in tumors, where the precursor molecules were also detectable.

The precursor compound, its fragment ions and all the measured metabolites showed an overlapping tissue pattern. This co-localization in case of the fragment ions can be interpreted as a molecular fingerprint that confirms the identity of RTKIs.

5. CONCLUSIONS

1. Our results provide the first evidence that MALDI-MSI can be used to conduct ADME studies on low molecular weight antiangiogenic drugs.
2. Limited tumor tissue drug penetration contributes to primary resistance against angiogenesis inhibitors.
3. Drug concentration detected in the viable regions of the tumor is related to the antitumor and antivascular effects of the applied compounds.
4. The effects of antiangiogenic RTKIs are dependent on the tumor model used.
5. Effective treatment in the C26 model, but not the C38 model resulted in a decreased expression of VEGFR2 and desmin, and an increase in the intratumoral hypoxia.

6. PUBLICATIONS

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