Transient anti-angiogenic treatment improves delivery of cytotoxic compounds and therapeutic outcome in lung cancer

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Running title: Improving drug delivery by tumor vessel normalization

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Abstract:

Extensive oncologic experience argues that the most efficacious applications of anti-angiogenic agents rely upon a combination with cytotoxic drugs. Yet there remains a lack of clarity about how to optimize scheduling for such drug combinations. Prudent anti-angiogenic therapy might transiently normalize blood vessels to improve tumor oxygenation and drug exposure. Using [15O]H2O Positron Emission Tomography (PET) imaging in a preclinical mouse model of non-small cell lung cancer, we observed that short-term treatment with the VEGFR/PDGFR inhibitor PTK787 licensed a transient window of improved tumor blood flow. The improvement observed was associated a reduced leakiness from tumor vessels, consistent with induction of a vascular normalization process. Initiation of a cytotoxic treatment in this window of tumor vessel normalization resulted in increased efficacy, as illustrated by improved outcomes of erlotinib administration after initial PTK787 treatment. Notably, intermittent PTK787 treatment also facilitated long-term tumor regression. In summary, our findings offer strong evidence that short-term anti-angiogenic therapy can promote a transient vessel normalization process that improves the delivery and efficacy of a targeted cytotoxic drug.
Introduction:

Solid tumors cannot grow without access to and recruitment of blood vessels (1), (2). Tumor vessels are characterized by a leaky, disorganized and abnormal phenotype (3). This leakiness leads to extravasation of plasma proteins resulting in a high interstitial fluid pressure within tumors that interferes with the delivery of drugs (4). Moreover, this abnormal phenotype of tumor vasculature supports tumor progression and resistance to treatment. The goal of anti-angiogenic treatment is to inhibit tumor vessel growth, thus abrogating the delivery of nutrients and oxygen to the tumor. Anti-angiogenic compounds either target angiogenic growth factors as bevacizumab (anti-vascular endothelial growth factor (VEGF)) or receptor kinases that are known to regulate tumor angiogenesis as e.g. VEGFR (vascular endothelial growth factor receptor) and PDGFR (platelet derived growth factor receptor). However, recent data indicate that a reduction in tumor vessels induced by anti-angiogenic treatment using an antibody against VEGFR2 (DC101) results in an inhibition of tumor growth but on the other hand permits tumor invasiveness (5). This increase in invasiveness is most probably due to elevated hypoxia within the tumor during anti-angiogenic therapy (6).

Nevertheless there is strong evidence that transient application of anti-angiogenic agents can "normalize" the abnormal tumor microvessels (7), (8). This prudent application of anti-angiogenic therapy might result in effective uptake of drugs and oxygen in the tumor enhancing cytotoxic therapeutic outcome in cancer therapy (4), (9). Moreover, structurally and functionally abnormal blood vessels impair blood flow into the tumor contributing to an
aggressive hypoxic microenvironment rendering the tumor unresponsive to traditional cytotoxic treatment regimes (10). Using MR imaging we could recently demonstrate that short-term anti-angiogenic treatment with a small molecule protein kinase inhibitor Vatalanib (PTK787) results in a reduction of vessel leakiness (11). Based on these findings we speculate that anti-angiogenic treatment induced normalization of tumor vessels might increase tumor blood flow and reduce interstitial fluid pressure (IFP). This reduction of the IFP and increase of tumor blood flow during anti-angiogenic treatment might improve anti-cancer drug delivery and, thus, treatment efficacy (12).

PTK787 (ZK 222584) and ZD6474 are potent inhibitors of VEGFR tyrosine kinases, PDFGR beta tyrosine kinase and c-Kit whereas ZD6474 in addition targets the epidermal growth factor receptor (EGFR) and RET (13, 14). We here sought to apply a multimodal imaging approach to monitor tumor blood flow in vivo in order to assess the normalization window during prudent anti-angiogenic treatment using the tyrosine kinase inhibitors PTK787 and ZD6474. Imaging tumor vessel normalization induced by prudent anti-angiogenic treatment was used to improve drug delivery of targeted compounds as Erlotinib and GDC0941.

Materials and Methods:

Cell lines and reagents

NSCLC cell line H1975 was purchased from the American Type Culture Collection (ATCC). PC9 was purchased from European Collection of Cell Cultures.
Both cell lines were maintained in RPMI-1640 medium enriched with 10% FCS and 1% Penicillin+Streptomycin. ZD6474, PTK787, Erlotinib were purchased from LC labs and GDC0941 from Axon Medchem. Compound stocks were stored at -20°C and dissolved in DMSO in vitro. For in vivo studies Erlotinib was dissolved in 6% Captisol® (CyDex Inc., USA) at a concentration of 9mg/ml. GDC0941 was dissolved in MCT (0.5% methylcellulose with 0.2% Tween-80 in distilled water) at concentrations of 22.5mg/ml (monotherapy) and 15mg/ml (in combination with anti-angiogenic therapy). ZD6474 and PTK787 were dissolved in sterilized, deionized water with 1% Tween 80 at a concentration of 10mg/ml. All solutions were stored on a rotating device at 4°C for animal therapy.

**Western blotting**

Western blotting was performed as described previously (15). For Western blotting the following antibodies were used: β-actin (clone C4) (MPBiomedicals LLC, USA), pEGFR, pAKT (S473), pERK (Cell Signaling Technology, USA), anti-rabbit-HRP- and anti-mouse-HRP-antibody (Millipore, Germany).

**Immunofluorescence**

Vascular leakage was assessed by i.v. injection of 0.1 mL 10 mg/mL FITC-dextran (200,000 kDa) from Sigma. After 30 min mice were anesthetized followed by perfusion with 4% paraformaldehyde injected into the aorta via an incision in the left ventricle and washed one time with PBS. Blood and fixative passaged out via the right atrium. Tumor sections were collected and immersed in 30% sucrose solution until samples dropped to the bottom of the vials. A cold bath
was prepared with dry ice and methanol. Tissue Tek wells were labeled and filled up with Jung tissue freezing medium (Leica Biosystems, Germany). Excess sucrose was removed from tissues and placed in the centre of wells and frozen by floating them on the methanol bath. Blocks were stored at -20°C and sliced at 10 to 20μM on cryostat. Slides were dried at room temperature for at least 2 hours and stained with anti-mouse CD31 (1:25, BD Pharmingen, Germany), anti-pVEGFR-2 (1:300, Cell Signaling Technology, USA), fixed and processed for analysis in a Biorevo (Keyence) BZ-9000 microscope.

**Tumor samples and immunohistochemistry**

All tumors were collected after perfusion, stored in 4% paraformaldehyde overnight and transferred to PBS. Tissues were embedded in paraffin following standard protocol and stained with primary antibodies as follows: mouse CD31 (1:25, BD Pharmingen), cleaved Caspase3 (1:750, Cell Signaling), pAKT (1:25, Cell Signaling) and alpha smooth muscle actin α-SMA (1:50) (Abcam) for marking pericytes. Corresponding secondary antibody detection kits for reduced background on murine tissue were used (Histofine Simple Stain Mouse MAX PO, medac) and stained on an automated stainer (LabVision Autostainer 480S, Thermo Scientific).

**Xenograft experiments**

All animal procedures were approved by the local animal protection committee and the local authorities (Bezirksregierung Köln). 8 weeks old healthy *nu/nu*
athymic male mice weighing 30g in an average were purchased from Janvier, Europe. Tumors were generated by s.c. injection of PC9 and H1975 tumor cells (5x10^5 cells/tumor). Tumor-bearing mice were treated by oral gavage with the following set ups: PTK787 (n=8 mice with 3 tumors/mouse) or ZD6474 (n=8 mice with 3 tumors/mouse) 75mg/kg daily as monotherapy, Erlotinib 30mg/kg daily as monotherapy (n=8 mice with 3 tumors/mouse), GDC0941 75mg/kg daily as monotherapy (n=8 mice with 3 tumors/mouse), vehicle (n=5 mice with 3 tumors/mouse), Erlotinib 30mg/kg (n=14 mice with 3 tumors/mouse) or GDC0941 50mg/kg (n=8 mice with 3 tumors/mouse) pretreated with PTK787 and continued as monotherapy during indicated timespan. The size of tumors ranged between 70 mm^3 and 125 mm^3. Monotherapy and vehicle of each drug was used as control. Tumor volume was recorded accordingly.

[¹⁵O]H₂O / [¹⁸F]FLT Positron Emission Tomography (PET) imaging:

Animals bearing macroscopic tumors were investigated on day 0 followed by start of treatment with PTK787 75mg/kg or ZD6474 75mg/kg daily, day 4, day 8 and day 18 using a FOCUS microPET scanner (Siemens Microsystems, Inc., Knoxville, TN, max. transaxial resolution 1.3mm). In total, 25 animals underwent [¹⁵O]H₂O and [¹⁸F]FLT imaging, each animal carried 3 tumors. The PTK787-treated group contained 15 animals, the vehicle treated group 10 mice. All animals underwent PET imaging at 4 different time points. Initially 32 animals were included in the study, 7/32 mice died during PET imaging. We calculated percentage changes in tracer uptake with day 0 as baseline for each time point and tumor. [¹⁵O]H₂O PET imaging was performed before [¹⁸F]FLT PET. [¹⁸F]FLT
PET was measured one hour after $[^{15}O]H_2O$ PET. $[^{15}O]H_2O$ was injected dynamically via tail vein and PET images were acquired for 2 min. after injection of 400 μCi/mouse. $[^{18}F]FLT$ was administered i.v. (200 μCi/mouse). PET imaging was performed 60 min. after injection (16). Data evaluation was performed using in-house VINCI software. Data evaluation was based on a region of interest (ROI) analysis. For data analysis we used the maximal and the mean voxel radioactivity of the defined ROI within the tumors. The size of tumors ranged between 70 mm$^3$ and 125 mm$^3$. The mediastinum was chosen as a reference for determination of uptake ratio, since we observed constant uptake for $[^{18}F]FLT$ in this region. The heart was used as reference for calculation of the $[^{15}O]H_2O$. All data were decay corrected.

**Mass spectrometry**

For absolute quantification of Erlotinib and OSI-420 in positive ESI MRM (multi reaction monitoring) mode a Acquity UPLC / Xevo™ TQ (Waters) with MassLynx and absolute quantification TargetLynx (Waters) were used. An Acquity UPLC BEH C18 1.7 μm, 2.1 x 50 mm column was used at 25°C. Solvent A was 0.1% formic acid (Biosolve) and B acetonitrile (Biosolve). A linear gradient from 95% A to 5% in 4.10 min at a flow rate of 0.4 ml/min was used. The following MRM transitions were used for Erlotinib m/z 394.03 (M+H$^+$) to 277.95 (quantifier), m/z 394.03 to 303.95 (qualifier), m/z 394.03 to 335.94 (qualifier), for OSI-420 m/z 380.03 to 277.85 (quantifier), m/z 380.03 to 249.89 (qualifier), m/z 380.03 to 321.93 (qualifier). All compounds were fresh prepared during 2 months and dissolved in 0.1% Formic acid (Bisolove) prepared with
0.22 μm MilliQ-Water. With Erlotinib eluting at 2.94 min a standard calibration curve was calculated using following concentrations: 0.2, 0.5, 1, 5, 20, 50, 150, 300, 500, 750 ng/mL (prepared individually from stock solutions 100 μg/ml). With OSI-420 eluting at 2.51 min a standard calibration curve was calculated using following concentrations: 0.1, 0.5, 1, 2, 4, 6, 8, 10 ng/mL (prepared individually from stock solutions 100 μg/ml). Correlation coefficient: r < 0.990; response type: external standard, area; curve type linear; weighting 1/x. The peak integrations were corrected manually, if necessary. Quality control standards of each standard were used during sample analysis and showed between 0.5% and 40% deviation respectively. Blanks after the standards, quality control and sample batch proved to be sufficient. No carry over was detected.

Statistics

Fisher’s exact tests were performed using R version 2.7.1 (http://www.r-project.org). Data are presented as mean ±SD in all figure panels where error bars are shown. A level of significance of p< 0.05 was chosen (where mentioned).
Results:

Short-term anti-VEGFR/PDGFR treatment induces a time window of improved blood flow into the tumor

We employed the human lung cancer cell line PC9. PTK787 treatment improved tumor blood flow after four days of treatment by 12% (day 0: SD = 7.47 Range = 22.42%; day 4: SD = 9.15 Range = 35.04%), as determined by changes of maximal voxel activity in $^{[15]O}$H$_2$O PET ($^{[15]O}$H$_2$O; Figure 1A, right panel, Figure 1B). We could measure a steady and significant increase in tumor blood flow by 33.58% (day 8 SD = 5.52 Range = 34.57%) until day 8 of treatment with PTK787 (p-value<0.001) probably mediated by a transient normalization of vessels followed by a sharp decrease of 17.23% (day18: SD = 12.63 Range = 48.66%) till day 18. In contrast, blood flow decreased consistently from day 4 to day 8 by 20.42% (day 0: SD = 4.52 Range = 9.39%; day 4: SD = 4.67 Range = 8.60%; day 8: SD = 5.58 Range = 12.89%) and by 30.75% until day 18 (day 18: SD = 0.63, Range = 10.53%) in the vehicle-treated tumors (Figure 1A, left panel, Figure 1B). Simultaneously, uptake of 3'-deoxy-3'-$^{[18]F}$-fluoro-L-thymidine [$^{[18]F}$FLT), a marker of proliferation (17), was increased by 51.08% from day 0 to day 4 (day 4: SD = 26.81, Range = 527.31%) and by 76% form day 0 to day 8 (day 8: SD = 44.99, Range = 532.67%) (Supplementary Figure 1A,B,C) suggesting that the cells continued to progress through the cell cycle. In concordance with the [$^{[18]F}$FLT PET data, treatment of H1975 and PC9 cells with 10µM or 20µM PTK787 did not reduce tumor cell proliferation in vitro (Supplementary Figure 1D,E).
To investigate if improvement in blood flow can be also achieved by using other anti-angiogenic agents we used ZD6474 a tyrosine kinase inhibitor that targets VEGFR2 and EGFR with additional activity against VEGFR3, VEGFR1, PDGFRβ and the RET-tyrosine kinase. We treated H1975 xenografts with ZD6474 which are resistant to EGFR inhibition due the presence of T790M gatekeeper mutation of EGFR (15). There was an increase in blood flow by 21.39% (day 0: SD =0.91 Range = 10.12%; day 4: SD = 9.23, Range = 38.58%, day 8: SD = 5.82, Range= 30.15%) from day 0 to day 8 of ZD6474 treatment followed by a drop of 20.95% from day 0 to day 18. Vehicle-treated tumors displayed a stable decrease in blood flow by 8.95% from day 0 to day 8 and by 14.78% on day 18 (SD = 4.32, Range = 12.11%) (Supplementary Figure 2A,B). Proliferation remained unaffected as measured by an increase in [18F]FLT uptake by 67.1% from day 0 to day 4 (day 4: SD =3.17, Range = 193.5%) and by 78.02% form day 0 to day 8 (day 8: SD = 20.49, Range = 675.8%) (Supplementary Figure 3A,B).

These data indicate that prudent anti-VEGFR/PDGFR treatment produces a short-lived time window of about 7 days when tumor vessels are transiently normalized which can be monitored by an increase in blood flow by up to 50% into the tumor.

**Short-time anti-angiogenic treatment reduces leakiness and improves pericyte coverage in tumor blood vessels in xenografts.**

To elucidate if the improved blood flow into the tumors was indeed due to vessel normalization, permeability of the blood vessels were examined by fluorescence microscopy after tumor-bearing animals were perfused with FITC-dextran.
Blood vessels of vehicle-treated tumors were dilated with aberrant morphological pattern and displayed extensive leakiness associated with massive extravasation of FITC-dextran (Figure 2A, left panel, Supplementary Figure 3C) and correlated with high expression of CD31 and p-VEGFR2 (Figure 2A, left panel). However, in tumors that were treated with PTK787 for four days blood vessels showed strikingly reduced leakiness with almost no extravasation of FITC-dextran (Figure 2A, right panel, Supplementary Figure 3C) supported by eight fold reduction in signal intensity (Figure 2B) accompanied by diminished CD31 and p-VEGFR2 expression. (Figure 2A, right panel). Tumor vasculature was characterized by abnormal and discontinuous pericyte lining of vessels as indicated by arrows on day 0 (Figure 2C). Anti-angiogenic treatment for 4 days transiently improved pericyte coverage in contrast to vehicle-treated tumors which still exhibited incoherent pericyte coverage (Figure 2C, Supplementary Figure 4A). PTK787 treatment also corrected the morphological aberrations of the vessels with reduced tortuosity and improved maturity (Figure 2C).

Pretreatment with anti-angiogenic agents improves cytotoxic therapeutic outcome in NSCLC with enhanced delivery of Erlotinib into the tumor

In the next step, we investigated if augmented blood flow induced by short-term anti-angiogenic treatment had any improved therapeutic efficacy in NSCLC. Mice bearing macroscopic PC9 tumors were treated by an oral gavage of PTK787 (75mg/kg daily) for 1 week. Since [15O]H2O PET data indicated that tumor blood flow improves within a time window of 7 days of anti-angiogenic therapy,
Erlotinib treatment was started within this ‘Normalization window’ from day 4 onwards and continued as monotherapy for 13 days. Mice receiving Erlotinib therapy pretreated with PTK787 had a sharp initial increase in tumor volume from 100% on day 1 to 221.28% (SD = 27.09, Range = 162%) on day 4 followed by a massive reduction to 45.63% (SD = 7.11, Range = 6.9%) on day 7 and finally almost complete shrinkage of tumor after 16 days of treatment (9.14% (SD = 3.2, Range = 7.2%) of original mass left) (Figure 3A). Erlotinib as monotherapy restricted tumor proliferation resulting in a slow reduction (up to 50% of tumor mass) but not as strong as with intermittent PTK787 treatment (p-value<0.001) (Figure 3A). PTK787 monotherapy had similar effects like vehicle treatment (with an increase from 100% on day 1 to 245% and 220% (SD = 17.9, Range = 38.8%) respectively on day 4) (Figure 3A).

To check if the normalized blood vessels were effectively delivering drugs into the tumors, we measured Erlotinib concentration within the tumor via mass-spectrometric analysis. Under monotherapy with Erlotinib, there was a slight improvement of the drug uptake into the tumor from day 1 (start of treatment) to day 4 by 20% (SD = 5.2, Range = 34.16%) (Figure 3B). In contrast, tumors pre-treated with PTK787 for 4 days displayed an increased Erlotinib uptake by 140% (SD = 10.56, Range = 35.64%) on the first day of Erlotinib treatment (day 4), which improved up to 160% (SD = 11.04, Range = 25.64%) on day 8 (Figure 3B). Monotherapy sets showed no further improvement in drug uptake which was reduced by 42% (SD = 4.32, Range = 23.72) on day 8 (Figure 3B).

Western blot analysis of lysates from tumors treated with PTK787 and Erlotinib showed an overtime decrease in pEGFR signal from day 1 to day 4 of treatment corresponding to pAKT and pERK levels (Figure 3C). There was no change in
signal intensity of pERGR, pAKT or pERK in the vehicle or monotherapy sets
(PTK787 alone or Erlotinib alone) (Figure 3C). Western blot results correlated
with histology where Ki 67 positive cells were dramatically reduced in PTK787
pretreated tumors receiving Erlotinib on day 1 compared to tumors receiving
Erlotinib as monotherapy on day 1 (Supplementary Figure 4B). Ki 67 positive
cells were reduced further in number from day 0 to day 4 until only a few Ki 67
positive cells were left on day 8 in the tumors receiving Erlotinib pretreated with
PTK787 (Supplementary Figure 4B). Histology results also showed complete
inhibition of pAKT from day 0 to day 4 in PTK787 pretreated tumors receiving
Erlotinib (Figure 3D). pAKT levels remain inhibited on day 8 with induction of
necrosis (Figure 3D). Even though tumor cells were healthy in both sets on day
0 (Figure 3E), heavy induction of apoptosis (cleaved caspase 3) was detected in
tumors receiving Erlotinib pretreated with PTK787 on day 4 (Figure 3E, right
panel) which remained consistent on day 8 (Figure 3E, right panel). However,
in Erlotinib monotherapy sets there was only moderate induction of apoptosis
ovetime (Figure 3E, left panel).
To confirm that this effect of tumor shrinkage was only due to better drug
delivery facilitated by prudent anti-angiogenic treatment, macroscopic H1975
tumor bearing mice pretreated with PTK787 were treated with PI3K kinase
inhibitor GDC0941. Tumors receiving GDC0941 therapy pre-treated with
PTK787 receded by 50% (SD = 4.7, Range = 10.07) over 28 days compared to
just a mild growth-inhibition observed in GDC0941 monotherapy sets which
tumor volumes surpassed by 250% (SD = 10.41, Range = 22.10) on day 22
(Figure 4A).
Intermittent anti-angiogenic treatment facilitates long-term tumor regression

A long-term xenograft study with subcutaneous PC9 tumors was performed where mice were treated with a continuous dose of Erlotinib combined with a short PTK787 treatment every ten days. Tumors remained regressed in this combination model over the entire time span of 65 days (Figure 4B). However, in mice treated with Erlotinib only, there was an initial tumor regression up to 40% of original tumor volume until day 22 followed by a stable disease (Figure 4B).

Discussion

Using in vivo PET imaging we demonstrate that transient anti-angiogenic treatment using PTK787 improves tumor blood flow in vivo. This transient tumor vessel normalization results in an improved delivery of targeted compounds as Erlotinib into the tumor. Most strikingly, enhanced availability and distribution of Erlotinib within the tumor induced by transient PTK787 treatment was followed by a significant increase in tumor shrinkage. This improvement in tumor response is consistent with recent findings demonstrating that high-dose EGFR-targeted drug exposure results in more efficient target inhibition (18).
Several mechanisms have been described as potential targets to improve the
function of tumor vessels. Recently, Chakroborty et al. described Dopamine as a
potential drug to improve the function of tumor vessels. The normalization effect
was mainly mediated by an up-regulation of angiopoietin-1 and the Krüppel-like
factor 2 (19). Similarly, treatment with the Cox-2 inhibitor apricoxib increased
the maturity of tumor vessel in vivo (20). In line with our findings this vascular
normalization effect was associated with a significantly enhanced efficacy of
gemcitabine plus Erlotinib (21). Tumor vessel normalization upon apricoxib
treatment was primarily induced by a transient reduction in VEGF secretion
within the tumor in vivo. Of note, the tumor vessel normalization effect was time
and dose dependent (20). Rakesh Jain recently reported that the tumor vessel
normalization effect induced by inhibition of the VEGF-VEGFR2 axis is time and
dose dependent (22, 23). We applied [15O]H2O PET imaging to decipher this time
dependent tumor vessel normalization effect. In line with the tumor vessel
normalization hypothesis tumor blood flow increased within the first 8 days of
PTK787 treatment but again declined till day 18 of treatment accompanied by a
decrease in pericyte coverage (19, 24). Thus, our data strongly indicate that
tumor vessel normalization induced by PTK787 is time dependent. We found
similar results with ZD6474, a tyrosine kinase inhibitor that primarily targets
EGFR and VEGFR2 (13). As we applied H1975 xenografts that are resistant to
EGFR treatment the ZD6474 induced effect on the tumor vasculature seems to be
mainly driven by inhibition of VEGFR2. Similarly, Huang et al. found that low-
dose treatment with an anti-VEGFR2-antibody (DC101) results in an increase in
pericyte coverage in a breast cancer in vivo model (25). These and our data
indicate that the primary target to induce tumor vessel normalization is most
probably VEGFR2. However, further investigations are required to decipher in
detail the responsible tyrosine kinases that drive tumor vessel normalization.
In our study the implication of H\textsubscript{2}O-PET-guided use of PTK787 and ZD6474
treatment significantly improved the delivery of the cytotoxic compounds
Erlotinib and GDC0941. [\textsuperscript{15}O]H\textsubscript{2}O PET has been already successfully applied in
human lung cancer patients (26). Thus, [\textsuperscript{15}O]H\textsubscript{2}O PET reflects a highly accurate
method that can easily be translated into clinical application. The
implementation of [\textsuperscript{15}O]H\textsubscript{2}O PET enables to establish combined anti-angiogenic
and cytotoxic PET guided treatment protocols in individual patients to improve
the delivery of cytotoxic compounds.
Cytotoxic agents combined with anti-angiogenic therapy has shown only little
efficacy in advanced stage NSCLC patients. In a recent Phase III trial the addition
of bevacizumab to chemotherapy for newly diagnosed GBM did not improve
overall survival (27). In a recent human study docetaxel uptake was reduced in
NSCLC after patients were administered with bevacizumab (28). These data
indicate that inhibition of VEGF potentially bears an anti-vascular than tumor
vessel normalization effect supporting the notion that scheduling and dosing of
the anti-angiogenic treatment is essential to induce and maintain tumor vessel
normalization. This is also confirmed by our PET data as the vascular
normalization effect seems to be transient as continuous treatment with PTK787
or ZD6474 result in a reduction in tumor flow after more than 8 days of
treatment. Our data strengthen the use of H\textsubscript{2}O PET in clinical studies to define
the optimal dose and schedule of anti-angiogenic drugs as bevacizumab and
PTK787 to improve the delivery of cytotoxic drugs in to the tumor.
In summary, our findings are consistent with the vascular normalization hypothesis and are indicative of the fact that prudent anti-angiogenic therapy leads to evanescent vessel normalization resulting in better cytotoxic therapeutic outcome. However, optimal designs of drug scheduling and efficient imaging techniques are absolutely indispensable to achieve maximal clinical outcome.

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References


Figure legends:

Figure 1:

Multimodal imaging of tumor blood flow using $[^{15}O]$H$_2$O PET in xenografts (PC9).

(A) PET imaging was performed on nude mice with macroscopic subcutaneous tumors on day 0 (before start of therapy) and at the indicated time points after treatment with vehicle (A, left panel) and PTK787 (A, right panel). (B) Quantitative analysis of tumor blood perfusion before (day 0) and after 4, 8, 18 days of PTK787 treatment (B, blue line) compared to vehicle sets (B, red line).

Figure 2:

Blood vessel morphology and permeability.

(A) Vascular leakage was assessed by i.v. injection of 0.1 mL 10 mg/mL FITC-dextran (200,000 kDa). Perfused tumors were collected and 10 to 20μM thick slices were stained with anti-mouseCD31 and anti-pVEGFR2 antibody, fixed and processed for microscopy control set (A, left panel) and PTK787-treated tumors (A, right panel). (B) Signal intensity of the total area of green staining (FITC-dextran) was quantified (four fields per tumor in both control and PTK787 treated groups). (C) Histology of tumors stained for α-SMA (brown, pericytes) comparing untreated vasculature (C, left panel) with PTK787 sets (C, right panel).
Figure 3:

*Prudent anti-angiogenic treatment improves delivery of Erlotinib into the tumor and promotes therapeutic outcome.*

(A) Tumor volumes in nude mice were recorded over time under treatment with PTK787 (75mg/kg), Erlotinib (30mg/kg) and PTK787 (75mg/kg) + Erlotinib (30mg/kg) and vehicle control at indicated days.

(B) Quantification of Erlotinib uptake as measured by Mass-Spectrometry in PTK787 pre-treated tumors (blue column) between day 4 and 8 compared to uptake in the tumors receiving Erlotinib just as monotherapy (4 independent tumors from different mice per set up). (C) Tumor lysates were prepared from different therapy modules (as indicated) and immunoblotted with phosphospecific antibodies. Representative western blots are shown. (D) Histology of tumor samples from C comparing pAKT expression between Erlotinib monotherapy and Erlotinib pre-treated with PTK787 tumors on day 0 (before start of treatment) and on indicated days after therapy. (E) Induction of apoptosis (cleaved caspase 3) in Erlotinib monotherapy and Erlotinib pre-treated with PTK787 tumors on day 0 (before start of treatment) and on indicated days after therapy.

Figure 4:

*Short anti-angiogenic treatment improves therapeutic outcome of GDC0941; long term tumor regression with intermittent anti-angiogenic therapy.*

PC9 cells were engrafted s.c. in nude mice and tumor volumes were recorded over time for either (A) 28 days under treatment with PTK787 (75mg/kg), GDC0941 (75mg/kg) and PTK787 (75mg/kg) + GDC0941 (50mg/kg) at indicated days (n= 6 mice with 3 tumors/mouse for each set up) or (B) for 65 days under treatment with PTK787 (75mg/kg), Erlotinib (30mg/kg) and Erlotinib (30mg/kg) with intermittent PTK787 treatment from day 1 to day 7, day 17 to day 25, day 35 to day 43 and day 51 to day 58 (n= 6 mice with 3 tumors/mouse for each set up).
Figure 1

A

vehicle treated  PTK787 treated 75mg/kg daily

treatment started on day 0 after imaging

day 0

% injected dose/cc

0 0.7

day 4

% injected dose/cc

0 0.7

day 8

% injected dose/cc

0 0.7

day 18

% injected dose/cc

0 0.7

B

relative $[^{15}O]H_2O$ uptake

PTK787 treated  vehicle

days of treatment

0% 25% 50% 75%

0 4 8 18
Transient anti-angiogenic treatment improves delivery of cytotoxic compounds and therapeutic outcome in lung cancer

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