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Amphiregulin contributes to tumor progression in lung adenocarcinoma

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Background: Amphiregulin(AREG), a ligand of EGFR, is associated with shortened survival of patients with NSCLC and poor prognosis. However, the ability of amphiregulin to mediate distinct function in lung metastasis remains unknown.
Material and Methods: PC9 cells (human pulmonary adenocarcinoma cell line harboring EGFR exon 19 deletion) were stably transfected with a vector harboring AREG cDNA and colonies were selected. AREG protein was measured by ELISA analysis. Anoikis was induced in P03 (cell transfected with AREG-expressing clones) and PA3 cell lines(cell transfected with the empty vector) with the use of poly-HEMA. Apoptosis assay was performed by Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. The cells were cultured in soft agar to evaluate anchorage-independent colony formation. P03 or PA3 cell lines were injected intravenously into BALB/c nude mice. 6 weeks later, lungs were excised, and the number of nodules formed on all lobe surfaces was counted to assess the importance of AREG as mediator of lung colonization. Immunohistochemistry for COX-2, CD31, VEGF-D, MMP-1, $P21^{WAF1/Cipl}$ were performed on lung cancer tissues. Vessel number and vessel size were quantified with the computer program ImageJ. Five to six independent tumors were analysed and at least three sections per tumor were quantified.
Results: Greater anoikis resistance was associated with higher levels of AREG expression.PA3 cell line resulted in a larger and more colonies in a cologenic assay. The expressions of CD31, VEGF-D, MMP-1, $P21^{WAF1/Cipl}$ were increased in AREG transfected lung cancer tissues. Not only the tumour size was increased, but also the vessel density and the vessel size were increased.
Conclusions: AREG may contribute to its metastatic capacity and tumor angiogenesis, providing a possible explanation for the aggressive nature of lung cancer that overexpress AREG.

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Design of improved calixarene-based anti-tumor agents

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Galectin-1 (gal-1), which binds β-galactoside groups on various cell surface receptors, is crucial to cell adhesion and migration, and is found to be elevated on endothelial cells and stroma in several cancers. Previously, we identified gal-1 as the target for the designer peptide anginex, a potent antiangiogenic and anti-tumor agent. Here, we report on the development of new agents that target gal-1 and thereby inhibit tumor growth in mice. Since small molecules have pharmacological advantages over therapeutic peptides, we designed non-peptide mimetics to approximate the molecular dimensions, amphipathicity, and cationic topology of the β-sheet-folded anginex peptide. Since calix[4]arene mimics the overall backbone dimensions of the active entity within anginex, we chose calix[4]arene as the scaffold. Earlier, we reported on calixarene-based compounds 0118 and 1097 that are potent anti-angiogenic and anti-tumor agents (Dings RPM, et al. JNCI 2006;98(13):932−6). Using a structure-based design approach, we chemically modified the hydrophobic and hydrophilic faces of these amphipathic anginex mimetics, which resulted in the discovery of new compounds that possess *in vitro* and *in vivo* activities that are greatly improved over 0118 or 1097. In the syngeneic B16F10 melanoma tumor mouse model, the best new compound inhibits tumor growth about 20-fold better than e.g. 0118. Overall, this research contributes to the design of peptidomimetics as novel therapeutics that inhibit tumor growth, potentially in the clinical setting.
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Serum levels of placental growth factor correlate with survival in patients with neuroendocrine tumors of the pancreas

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Background: Genetic and pharmacological studies have recently established the proangiogenic role of placental growth factor (PlGF), a VEGF homolog, and its receptor Flt1 in tumor angiogenesis. Dense vascularization is a characteristic of neuroendocrine tumors of the pancreas (pNET) and correlates with an unfavorable course of disease. Here, we evaluated the role of PlGF and its soluble receptor sFlt1 as prognostic parameters and potential therapeutic targets in pNET.
Methods: PlGF and sFlt1 serum levels in 101 patients with histological proof of pNET and 96 healthy control subjects were determined using the Roche Elecsys® assay and correlated with clinicopathologic parameters, including tumor grading and patients' survival. Immunhistochemical studies further allowed to determine the expression of PlGF in tumor specimens of 30 patients with pNET compared to healthy pancreatic control tissues.
Results: As compared to healthy controls, serum levels of PlGF and sFlt1 were found to be significantly elevated in patients with pNET (19,3 vs 12,9 pg/ml and 83,6 vs 72,4 pg/ml; $P < 0{,}0001$). Moreover, compared to patients with PlGF levels below median, PlGF levels above median were associated with poor prognosis and reduction of tumor related survival (log-rank test, $P = 0{,}041$), thus indicating that PlGF serum levels represent a prognostic marker in pNET disease. Accordingly, we observed a significant correlation of PlGF serum levels with the grade of tumor differentiation based on Ki-67 labeling. Finally, in line with elevated PlGF serum levels, immunhistochemical studies revealed an increased tumor PlGF expression in human specimens of pNET as compared to healthy pancreatic control tissues (28% vs. 3%; $P < 0{,}05$).
Conclusions: These data provide first evidence that PlGF represents an independent prognostic parameter in patients with pNET and most probably plays a pivotal role in the tumor biology of pNET. Thus, further studies are warranted to evaluate the potential of PlGF as a target molecule to optimize therapeutic strategies and to improve the prognosis of this tumor entity.

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INK128, a potent and selective TORC1/2 inhibitor, demonstrates anti-tumor activity in preclinical models of renal cell carcinoma by a distinct mechanism

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Background: Renal cell carcinoma (RCC) is characterized by mutations or silencing of the von Hippel–Lindau gene leading to an accumulation of HIF1alpha (HIF1a), a key mediator of hypoxia-triggered neo-angiogenesis. The mammalian target of rapamycin (mTOR) is upstream of HIF1a and downstream of the VEGF pathway. mTOR kinase operates in two distinct intra-cellular multi-protein complexes, TORC1 and TORC2, that together regulate growth, metabolism, angiogenesis and survival by integrating nutrient and hormonal environmental signals. Pharmaceutical derivatives of rapamycin, a partial allosteric inhibitor of TORC1, provide clinical proof of concept for the therapeutic value of targeting mTOR in RCC, but also provided insights into how mechanistically novel TORC1/2 inhibitors might demonstrate greater efficacy. Through rational drug design we have discovered INK128, a potent and selective TORC1/2 inhibitor with excellent drug-like properties. We investigated the mechanism of action, pharamcokinetc pharmacodynamic (PK/PD) correlation and efficacy of INK128 in preclinical *in vitro* and *in vivo* models of RCC.
Methods: mTOR and PI3K-isoform kinase IC_{50}'s were generated in a homogeneous time-resolved fluorescence (FRET) screen using commercially available reagents from Invitrogen (mTOR) and Millipore (PI3K isoforms). A range of tumor cells were treated with various concentrations of INK128 for 2 hours and subsequently lysed in cell lysis buffer. Lysates were subjected to SDS-PAGE followed by Western blot analysis to detect downstream signaling markers. Cell proliferation was performed using the CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega). Anti-tumor activity of INK128 was assessed in mouse xenograft models. INK128 was administered orally as a solution at doses of 0.3 mg/kg (QD), 1 mg/kg (QD), and 3 mg/kg (Q2D). Tumors were extracted at 2 hours post last dose (n = 3/time point). Pathway inhibition was determined by Western blot analysis of tumor lysates and immunohistochemistry (IHC) analysis of formalin fixed paraffin embedded tumor tissue.
Results: *In vitro*, INK128 inhibits phosphorylation of S6 and 4EBP1, downstream substrates of TORC1, as well as NDRG1 and AKT,

downstream substrates of TORC2. Expression levels of cell cycle regulating proteins and activation status of related pathways were also characterized. In mouse xenograft model, INK128, rapamycin and Nexavar show strong activity in inhibiting tumor growth, however, differ in the mechanisms underlying their anti-tumor activity. INK128 inhibits phosphorylation of AKT, NDRG1, S6 and 4EBP1; rapamycin only inhibits S6 phosphorylation and induces AKT phosphorylation whereas Nexavar has little effect on the PI3K/AKT/mTOR pathway. INK128 induced autophagy and decreased cyclin D1 expression. INK128 and rapamycin both inhibit expression of HIF-1a and VEGF which contributes to their anti-angiogenesis activity. We conclude that rapamycin and Nexavar exert anti-tumor activity mainly by attacking the tumor microenvironment while the anti-tumor effect of INK128 is derived from both cell-autonomous (direct inhibition of tumor cell growth) and non-autonomous (anti-angiogenesis) activities. Combination of INK128 with Nexavar displayed enhanced activity in RCC tumor model. In preclinical tumor model, INK128 induced dose-dependent pharmacodynamic inhibition of phosphorylation of S6, AKT and 4EBP1 in PBMCs, skin and tumor tissue at plasma concentrations leading to tumor growth inhibition.

Conclusion: In summary, INK128, a potent, selective, and orally active dual TORC1/2 inhibitor demonstrated anti-tumor activity in preclinical models of RCC by a distinct mechanism. INK128 is currently being studied in a phase I clinical trial.

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Pleiotropic stromal effects of VEGFR2 antibody therapy in renal cell carcinoma models

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The benefits of targeting vascular endothelial growth factor A (VEGF) signaling in cancer patients are frequently attributed to effects on tumor endothelial cells. Targeting non-endothelial cell stromal components to further impact tumor cell growth and survival is being pursued through combination strategies or through cross-reactivity of VEGF receptor targeting small molecules with other growth factor receptors important for the survival and/or proliferation of non-endothelial stromal cells. However recent data points to the potential for targeting lymphatic vessels and pericytes, in addition to blood vessels, with VEGF specific targeting agents. Here in fact we demonstrate the ability of 7 days treatment with an antibody specific to one of the VEGF receptors, VEGFR2 (DC101, 40 mg/kg IP, 3×/wk), to significantly reduce CD31 expressing blood vessels, α-smooth muscle actin (αSMA) positive pericytes and LYVE-1 expressing lymphatic vessels in the tumor stroma of subcutaneous (SKRC-29) and orthotopic (786-O-LP) models of renal cell carcinoma (RCC), a cancer for which VEGF targeted therapy has known efficacy. DC101 decreased CD-31 positive blood vessel density by 72% in the SKRC-29 model and 78% in the 786-O model ($p < 0.001$ for all comparisons versus control). LYVE-1 and αSMA positive vessel like structures were reduced by 58% and 37%, respectively, in the SKRC-29 model, and 73% and 60% in the 786-O-LP model ($p < 0.05$ for all). Sunitinib (40 mg/kg PO, daily), a tyrosine kinase inhibitor targeting VEGFR2 and several other growth factor receptors, also caused a significant loss of tumor blood vessels in the SKRC-29 and 786-O-LP models (63% and 61% decrease, respectively) after 7 days of therapy, but had a weaker effect than DC101 on LYVE-1 and αSMA staining density at the doses utilized; 24 and 9% reduction in the SKRC-29 model, respectively, and 45 and 23% reduction in the 786-O-LP model. Our data have important implications for combination therapy design, supporting the conclusion that targeting VEGFR2 alone in renal cell carcinoma has the potential to have pleiotropic anti-cancer effects on the tumor stroma.

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Angiogenesis-related gene profiles with predictive value in advanced ovarian carcinoma (AOC)

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Background: Multimodal therapy with cytoreductive surgery plus chemotherapy is the standard of care in AOC. Parameters such as age, extent of residual disease after surgery, and the histopathological subtypes are imperfect predictors of response. Several genes involved in angiogenesis proved to have prognosis capacity in advanced ovarian carcinoma. The aim of this study is to build profiles with a predictive value of response to treatment in patients of AOC derived from genes involved in angiogenesis.

Materials and Methods: 61 patients with III/IV FIGO stage ovarian cancer who underwent surgical cytoreduction and received a carboplatin plus paclitaxel regimen were included. Clinical response was evaluated using CT after the completion of multimodal therapy. A second look laparotomy was performed in 34 of them, defining optimal debulking as $\leqslant 1$ cm (diameter) residual disease. RNAs were collected from formalin-fixed paraffin-embedded AOC samples. Expression levels of 82 angiogenesis related genes were measured using quantitative real time polymerase chain reaction. A logistic regression method was used to build multiple models based on the significant genes in the univariate analysis. The accuracy of the models was evaluated using Receiver Operating Characteristic (ROC) curves. The Akaike Information Criterion based selection was used to find the most accurate one. And Leave-one-out Cross Validation (LOOCV) method was applied to avoid overoptimistic predictions.

Results: All patients had advanced disease (FIGO stages III/IV). Most of them had FIGO stage III (51, 83.6%), grade 3 tumors (35, 57.4%), and serous histology (42, 68.9%). Two different predictive models were generated for clinical or pathological response to treatment. The first one, predictor of clinical response, comprises 8 genes with an AUC of 0.955 ($p < 0.001$). Leave-one-out cross validation was applied to avoid overfitting of the model, obtaining a corrected AUC of 0.880, 95%IC: 0,776−0,985. The second one, for pathological response, comprising 5 genes with an AUC of 0.950 ($p < 0.001$). When LOOCV was applied, a corrected AUC of 0.846, 95%IC: 0,717−0,976 was obtained.

Conclusions: It is feasible to identify gene expression profiles related to angiogenesis with predictive value for response in AOC. However, their predictive value should be further evaluated in prospective studies of patients with AOC.

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Restoration of paracrine signalling within the tumour microenvironment increases tumour growth and activation of c-Met

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Introduction: Paracrine signalling between tumour epithelial cells and the stroma in the tumour microenvironment is a pre-requisite for tumour growth and may determine responsiveness to therapeutic inhibition. Hepatocyte growth factor (HGF) and the HGF receptor, c-Met, are reported to be up-regulated in colorectal liver metastases (CRLMs) and considered to be markers of metastatic potential. They also involved promote epithelial–mesenchymal transition (EMT), an important developmental process which is frequently activated in carcinogenesis resulting in metastatic spread.

Methods: CRLMs ($n = 27$) were grown as sub-cutaneous xenografts in nude mice, and subsequently treated with c-Met inhibitors. Tissues were used for RNA extraction and quantitative PCR analysis, were enzymatically disaggregated to isolate epithelial and mesenchymal cells, and were used for immunohistochemistry.

Results: Analysis of primary human CRLMs showed that c-Met was overexpressed and accompanied by decreased E-cadherin, and increased EMT-related gene expression. Enrichment of individual cell types from primary CRLMs showed that HGF was mesenchymal, whereas c-Met was epithelial. Growth of tumours as sub-cutaneous xenografts revealed that human stroma, and thus paracrine signalling, was rapidly lost, and treatment of tumour-bearing mice with c-Met inhibitors had a generally poor response. Co-implantation of primary CRLM tumour epithelial cells with tumour-conditioned mesenchymal cells increased tumour growth, decreased necrosis, and increased c-Met activation. Implantation of tumour-associated fibroblasts into xenografts was also found to increase response to a c-Met inhibitor and to standard of care agents.

Conclusions: Paracrine HGF:c-Met signalling is rapidly lost in CRLM xenograft models indicating the need to restore human stroma, and its loss may reflect the poor response to inhibition. Recapitulation of the human tumour microenvironment in xenografts by implantation of human mesenchymal cells improves the therapeutic response, and therefore provide improved models to assess drug efficacy.