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Phenotypic characterization of circulating tumor cells (CTCs) in triple negative breast cancer patients

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Background: Molecular classification of breast cancer revealed that there are five different subtypes related to clinical outcome: luminal A, luminal B, normal-like, ERBB2-positive and basal-like. Triple negative patients (ER-negative, PR-negative, HER2-negative) belong to the basal-like subtype. Their tumors commonly express EGFR and are associated with poor prognosis. Circulating Tumor Cells (CTCs) have been proposed as a "real time liquid biopsy" in breast cancer patients. CTCs are associated with disease relapse and could serve as a target for molecular cancer therapies. The aim of the present study was, for the first time, the phenotypic characterization of CTCs in triple negative breast cancer patients.
Methods: We evaluated peripheral blood mononuclear cells (PBMC) cytospins from 40 triple negative patients and found CTCs in thirty one of them (15 early and 16 metastatic). The expression of Cytokeratins (CK), Estrogens Receptor (ER), Progesterone Receptor (PR), Epidermal Growth Factor Receptor (EGFR) and Human Epidermal Growth Factor Receptor (HER2) in CTCs was assessed using double immunofluorescent staining, confocal laser scanning microscopy and the Ariol system. PBMCs were stained with a monoclonal A45-B/B3 pancytokeratin antibody in combination with ER, PR, EGFR or HER2 antibodies, respectively.
Results: Our results demonstrated that ER, PR, EGFR and HER2 were expressed in 58%, 45%, 55% and 58% of the examined CK-positive patients. The respective proportions for early versus (vs) metastatic patients were 67% vs 50% for ER, 53% vs 38% for PR, 60% vs 44% for EGFR and 60% vs 56% for HER2. In addition ER, PR, EGFR and HER2 were expressed in 5.6%, 79.7%, 80.7% and 13.4%, respectively, of the total examined CTCs. Triple staining experiments with the Ariol system revealed no co-expression of CK/EGFR/ER in CTCs; however, there was only one patient with CTCs co-expressing CK/HER2/ER.
Conclusions: ER is expressed but in a minority of CTCs in triple negative breast cancer patients though the majority of CTCs revealed expression of PR and EGFR. However the clinical significance of these findings remain unknown and need further evaluation.

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The axon guidance molecule Slit2 regulates the motility of neuroendocrine cancer cells

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Background: Early blood-borne metastasis and a dense vascularization are characteristics for neuroendocrine tumors of the gastroenteropancreatic system (GEP-NET). Both events imply a central role for the interaction of blood vessels and cancer cells for the tumor biology of GEP-NET. Recently, a new function of the axon guidance molecule Slit2 and its receptors Robo1 and Robo4 has been established as a guidance factor for tumor and endothelial cells. Here we evaluated the role of the Slit2/Robo system in GEP-NET metastasis and angiogenesis.
Methods: Expression of Slit2, Robo1 and Robo4 on human GEP-NET tissue and cancer cell lines was determined by immunohistochemistry, RT-PCR and immunoblotting. We further analyzed the effect of vector-based overexpression of Slit2 on migration and agar colony formation of human GEP-NET cells using transwell and HTCA assays. HUVECs were incubated with tumor cell conditioned media to characterize the effect of Slit2 on endothelial cell migration and lamellipodia formation.
Results: Tissues from human GEP-NET as well as the corresponding neuroendocrine tumor cell lines BON and QGP showed variable expression of Slit2. However, in line with a consistent histological detection of Robo1 in epithelial cells of GEP-NET specimens, Robo1 was found abundantly expressed in BON and QGP cancer cell lines. In contrast, Robo4 was specifically expressed in endothelial cells of the tumor vasculature. Stable transfection of Slit2-deficient BON cells with a constitutive Slit2-pCMV vector substantially inhibited directed tumor cell migration and colony formation, while leaving cell proliferation unaffected. The effects of Slit2 were mediated in an auto-/paracrine manner, since Slit2 conditioned media also inhibited the migration of wild-type BON and QGP cells. Moreover, Slit2-mediated suppression of tumor cell motility involved restored E-cadherin expression and loss of vimentin expression in BON cells, indicating that Slit2 induced a mesenchymal-to-epithelial transition phenotype. Finally, tumor cell derived Slit2 repelled migrating primary endothelial cells by inhibiting VEGF-induced endothelial lamellipodia formation.
Conclusion: These data provide evidence for an intrinsic auto-/paracrine function of the Slit2/Robo system for the migration of GEP-NET cells as well as for their angio-regulatory interaction with endothelial cells *in vitro*. The differential expression of the Slit2 receptors Robo1 and Robo4 on tumor cells and the vascular compartment, respectively, thus imply a dual role of Slit2 in the process of both metastasis and angiogenesis of human GEP-NETs.

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Cytoplasmic mislocalization of RUNX3 by activated Shh is correlated with the development of TGF-β resistance in gastric cancer

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RUNX3 that belongs to the RUNX family of transcription factors acts as a tumor suppressor in gastric cancer. The inactivated RUNX3 is associated with transforming growth factor-β (TGF-β)-resistant cell types, and TGF-β has been implicated in Sonic hedgehog (Shh)-induced cellular signaling in gastric cancer. We hypothesized that the relationship between Shh signaling and RUNX3 may be involved in the development of TGF-β resistance.
Cells transfected with control vector only or Shh were treated with control vehicle, TGF-β, or TGF-β plus cyclopamine (a specific inhibitor of Smo) and then growth inhibitory effect of TGF-β was assessed by MTT assay. The effects of N-Shh or cyclopamine on expression of RUNX3 mRNA were observed by RT-PCR analysis. Contribution of N-Shh or cyclopamine to RUNX3 stabilization was monitored by Western blotting for RUNX3 in cells treated with cyclohexamide or MG132 (a specific inhibitor of proteasomes). The localization of Runx3 was monitored by immunofluorescence staining and nuclear fractionation assay.
Treatment with TGF-β of control vector- and Shh-overexpressing cells produced different results; control vector-overexpressing cells exhibited a significant decrease in cell growth, whereas almost no decrease was observed in Shh-overexpressing cells. RT-PCR analysis showed that there was no significant difference in the expression levels of RUNX3 mRNA between cells treated with either Shh, cyclopamine, or control vehicle. Importantly, treatment with MG132 led to reduction of RUNX3 proteins in Shh-overexpressing cells, whereas blockade of the Shh pathway by cyclopamine resulted in accumulation of RUNX3 after MG132 addition. Confocal microscopy and nuclear fractional experiment showed that overexpression of Shh blocked nuclear translocation of RUNX3 by TGF-β. Moreover, RUNX3 sequestered in the cytoplasm by Shh overexpression is rapidly degraded through a proteasome-mediated pathway. On the contrary, treatment of Shh-overexpressing cells with cyclopamine induced TGF-β-mediated growth inhibition via the stabilization of RUNX3 and the induction of RUNX3 translocation to the nucleus.
These results indicate that suppression of TGF-β-RUNX3 signaling by activated Shh is correlated with the development of TGF-β resistance in gastric cancer.

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LC06, a novel angiopoietin-2 selective human antibody with potent anti-tumoral and anti-angiogenic efficacy in different xenograft models

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Background: The Angiopoietins (Ang-1 and Ang-2) have been identified as agonistic and antagonistic ligands of the endothelial RTK Tie2, respectively. Ang-1/Tie2 signaling transduces survival signals, regulates mural cell recruitment, and controls the quiescent endothelial cell (EC) phenotype. In turn, Ang-2 destabilizes the vascular endothelium and primes EC responsiveness to cytokines and promotes tumor growth. Ang-2 is strongly expressed in the remodeling vasculature and almost not present in the quiescent vasculature making it a very attractive target for anti-tumor therapy. To investigate the functional role of blocking Angiopoietins, we generated Ang-2 selective antibodies that neutralize the binding of Ang-2 to its receptor Tie2 and Ang-1/Ang-2 crossreactive antibodies.
Material and Methods: Human antibodies against Ang-2 were generated by phage display. Affinity for human and murine Ang-2 and Ang-1 was determined by Biacore. The inhibition of the Ang-2-Tie2 and Ang-1-Tie2

interaction was investigated by ELISA and measuring the inhibition of Tie2 phosphorylation on HEK293-Tie2 cells. Antitumoral efficacy was assessed in established s.c. Colo205 and orthotopic i.m.f.p. KPL-4 xenografts in SCID beige mice. Tumors were explanted for histological analysis. Inhibition of angiogenesis was assessed in the cornea micropocket assay.
Results: Two lead antibodies, LC06 and LC08, were selected by biochemical and cellular assays: LC06 is selective for Ang-2; and LC08 shows cross-reactivity for Ang-1. Selectively blocking Ang-2 by LC06 resulted in a very potent tumor growth inhibition in subcutaneous and orthotopic tumor models that was at least comparable to the tumor growth inhibition mediated by the Ang-1/Ang-2 cross-reactive antibody LC08. However, selectively blocking Ang-2 by LC06 appeared to result in larger necrotic areas compared to blocking both cytokines. These effects were attended with a reduction of intratumoral microvessel density indicating an anti-angiogenic mechanism. Remaining vessels were better perfused hypothesizing a normalization phenotype. Further more, Ang-2 neutralizing antibodies potently inhibited VEGF-induced angiogenesis in the mouse corneal angiogenesis assay.
Conclusions: Taken together, these data provide strong support for the application of Ang-2 selective antibodies for the treatment of cancer patients by affecting neovascularization as well as survival of tumor cells.

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MMP-9 as a stromal target in cancer

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Matrix metalloproteinase 9 (MMP-9) is a secreted zinc metalloprotease which influences tumor recurrence and invasiveness, and is associated with angiogenesis. MMP-9 is one of two major gelatinases in the MMP family, which in addition to efficiently and rapidly cleaving unfolded collagen has been reported to cleave other matrix and nonmatrix components. Some of the reported extracellular catalytic actions of MMP-9 include generation of tumstatin from type IV collagen and release of soluble Kit ligand or bioactive VEGF, the latter being a major contributor to tumor angiogenesis.
Utilizing our phage display technology, we have identified DX-2802, a selective human monoclonal antibody targeting MMP-9. DX-2802 (IgG1 Lambda) potently inhibits human and mouse MMP-9 (IC_{50} = 2−3 nM) but does not inhibit a panel of other metalloproteinases tested. This antibody displays potent anti-invasive activity *in vitro* and significantly attenuated outgrowth of metastatic foci in the MC38 experimental intra-splenic mouse model in part by reducing tumor angiogenesis. Interestingly, DX-2802 did not affect the number of lesions in the livers or primary tumor growth in the cecum demonstrating that the effect of the antibody is not on the tumor cells themselves but on the tumor microenvironment. Our results are consistent with those previously published in *mmp-9* knockout mice crossed to the MMTV- PyVMT model of breast cancer (Martin et al, Cancer Res, *68:* 6251–6259, 2008) and show that MMP-9 may be considered as a stromal target in cancer.

Cell-cycle-interactive agents

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NCIC CTG IND.177: Phase I study of AT7519M given as a short infusion twice weekly

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Background: AT7519M is a small molecule inhibitor of multiple cdks (1, 2, 4, 5, 9) with lower potency against 3, 6 and 7. A recent phase I trial examined a daily short infusion for 5 days every three weeks. Dose dependent QTc prolongation was noted on this schedule. This study examines safety and tolerability of AT7519 delivered on an alternative schedule.
Material and Methods: Patients with refractory solid tumours or lymphoma were eligible and received escalating doses of AT7519M on days 1,4,8,11 every 3 weeks. A protocol amendment in 2007 excluded patients at risk of QTc prolongation and instituted serial EKG evaluation. Pharmacokinetics (PK) were planned for all patients. Patients at the recommended phase II dose level (RP2D) were planned for Holter monitoring and serial tumour and tissue acquisition to examine pharmacodynamic (PD) effects.
Results: 29 patients were treated at 4 dose levels from 14.4 mg/m^2 to 32.4 mg/m^2. RP2D was 27 mg/m^2. Dose limiting toxicity included mucositis, rash, fatigue and muscle weakness, renal dysfunction and febrile neutropenia. The most common toxicities were fatigue (46%), mucositis (50%), nausea or vomiting (36%). Hematologic toxicity was mild other than 1 patient who had grade 4 neutropenia documented. There was no evidence of QTc prolongation, including in external review of EKGs. Nine patients have had stable disease (2.5–11.1 months). PK are dose proportional. Accrual continues to the expanded RP2D level and patients are undergoing Holter testing (QTc) and PDs.
Conclusions: AT7519M given in a short infusion appears to be tolerable and is not associated with QTc prolongation noted with other schedules. NCIC CTG plans phase II trials in mantle cell lymphoma and CLL.

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GNE-900, an orally bioavailable selective CHK1 inhibitor, illustrates that optimal chemosensitization is schedule and tumor type dependent

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Checkpoint kinase 1 (CHK1) is a serine/threonine kinase, which functions as a central mediator of the S-phase checkpoint, blocking the G2/M transition to allow for repair of DNA damage. Inhibition of CHK1 is a strategy for selectively potentiating the efficacy of chemotherapeutic agents in G1 checkpoint defective tumor cells while minimizing toxicity to normal, checkpoint competent cells. Here, we show that GNE-900 is an ATP-competitive, selective, and orally bioavailable CHK1 inhibitor optimized from an HTS lead using structure-based drug design. In combination with chemotherapeutic agents, GNE-900 sustains ATR signaling, enhances DNA damage and induces apoptotic cell death. Checkpoint abrogation correlates with defects in the p53 G1 checkpoint gene and results in premature mitotic entry and induction of cell death. Importantly, we demonstrate that this class of CHK1 inhibitor has little single agent activity in the absence of chemotherapy and does not strongly potentiate the cytotoxicity of chemotherapeutic agents in normal bone marrow cells. In vivo scheduling studies using BrdU incorporation demonstrate that optimal timing for administration of CHK1 inhibitors following treatment with gemcitabine is coincident with release from S-phase arrest. With this schedule, gemcitabine antitumor activity is significantly enhanced in combination with GNE-900 in both gemcitabine-sensitive and resistant tumors. In summary, we demonstrate that in vivo potentiation of gemcitabine activity is mechanism-based, with optimal efficacy observed when S-phase arrest is induced first, followed by checkpoint abrogation with CHK1 inhibitor. Evaluation of alternate dosing schedules following administration of chemotherapy will be critical to the clinical development of this class of kinase inhibitors.

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AS703569/R763, a pan Aurora kinase inhibitor, shows strong antitumor activity in vitro and in vivo in a panel of triple-negative breast cancer cell lines and xenografts

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Background: Triple-negative breast cancers (TNBC) represent of breast cancers (BrCa) that has a particularly aggressive phenotype and poor clinical outcomes. Although there are agents in development for this indication, to date, there is no approved targeted therapy for TNBC. Because Aurora kinases (AKs) play critical roles in chromosome segregation and cell division, we investigated, both *in vitro* and *in vivo*, the effects of AS703569, a small molecule inhibitor of AK, in TNBC relative to other types of breast cancers.
Material and Methods: AS703569 was evaluated for activity in proliferation and cell cycle assays of a panel of breast cancer cell lines. The efficacy of AS703569 was also determined *in vivo* in one TNBC cell line, both alone and in combination with standard of care (SoC) agents, as well as in 10 xenograft models of patient-derived primary human breast cancer. Immunohistochemical analysis of phospho histone H3 (pHH3) expression, the biological indicator of Aurora kinase B activity, was perfomed in 3 of the primary xenograft models.
Results: TNBC cell lines were more sensitive to AS703569 than were other types in a BrCa cell line panel. Cell cycle analyses showed a dose