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SELDI-TOF Proteomic patterns of human and experimental brain tumors: potential for new biomarkers and for pharmaco-proteomic endpoints in anti-angiogenic therapy

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In the postgenome era, proteomics provides a powerful approach for the analysis of neoplastic tissues, and for uncovering novel endpoints for the evaluation of drug efficacy and toxicity. As an alternative to the 2-dimensional gel electrophoresis, a new technique was employed to generate protein expression patterns from whole tissue extracts. Surface-enhanced laser desorption/ionization (SELDI) allows the retention of proteins on a solid-phase chromatographic surface (ProteinChip Array) with direct detection of retained proteins by the highly sensitive time of flight-mass spectrometry (TOF-MS). Using this system, we conducted a study on human brain tumor samples including gliomas and meningioma. We present data showing that SELDI analysis is rapid, reproducible, and capable of identifying protein signatures that appear to differentiate the different histological types with specific pattern characteristics of specific clinical outcomes. We also investigated the potential of this technique for pharmaco-proteomic study of new therapeutical approaches in preclinical model of glioblastoma. The effect of the antiangiogenic agent Neovastat on serum protein patterns was thus investigated. Neovastat is a complex mixture of naturally occurring antiangiogenic agent exhibiting multifunctional mechanisms of action. It is currently in phase III study in patients with kidney and lung carcinoma and phase II in patient with refractory multiple myeloma. Preclinical efficacy of Neovastat has been documented in glioblastoma models. We observed that Neovastat induces specific modification of tissues and serum protein patterns thus indicating that with this fast and powerful ProteinChip array technology, it becomes possible to investigate complex changes at the protein level in cancer associated with tumor progression. This pharmaco-proteomic technique provides potential endpoint that can be related to specific approaches such as the use of antiangiogenic agents in preclinical and human trials.

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Halofuginone activity in relation to collagen type I, VEGF and MMP-2 expression in human tumor cell lines and xenografts *in vitro* and *in vivo*

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Halofuginone (HF) is a low molecular weight quinazolinone alkaloid which inhibits insult induced excessive transcription of collagen type I *in vitro* and *in vivo*. It has further been shown that HF can reduce metastasis formation and angiogenesis related to its effects on homeostasis of stromal proteins such as matrix metalloprotease 2 (MMP-2). Halofuginone is currently being investigated in phase I clinical trials in collaboration with the EORTC. This study was designed to correlate drug response to expression of possible surrogate markers such as collagen type I and MMP-2 in tumor cells. Collagen type I, VEGF and MMP-2 protein levels were determined in 60 human tumor xenografts and cell lines by ELISA assay of lysates and by immunohistochemistry (IHC) on tissue microarrays. Six cell lines with high and low target expression were selected for *in vitro* evaluation. HF was potently active in cells with a mean IC_{50} of 56 nM, the most sensitive line was the MEXF 462NL melanoma (IC_{50} = 2 nM), followed by the renal cancer RXF 944L (IC_{50} = 5 nM). More responsive cells showed either high VEGF and high collagen I expression or high collagen I and MMP-2 levels. More resistant models had only one elevated target protein. *In vivo* activity of HF was evaluated in the orthotopically (o.t.) growing renal cancer xenograft RXF 944LX, the soft tissue sarcoma SXF 463 and the melanoma MEXF 276 in nude mice. HF was administered i.p. at 2.5 μg/mouse daily for 3 weeks. This dose and schedule had been determined as optimal. Treatment was initiated 3 days after transplantation. Tumor growth was followed by serial caliper measurements over 2 months. Significant tumor growth inhibition was seen with T/Cs of 36 % in SXF 463 and 33 % in MEXF 276 xenografts. Animals bearing o.t. RXF 944LX were sacrificed 14 d after initiation of treatment and tumors, kidneys, lungs and livers were examined macroscopically and microscopically. HF produced a 69% reduction of primary kidney tumor growth and prevented metastasis to liver and lung. However, HF treated RXF 944LX mice had large abdominal tumor masses. Whilst IHC of control and treated RXF 944LX tissue revealed little effects on VEGF or MMP-2 expression, collagen I was markedly down-regulated by HF ($p < 0.004$). The latter might explain the inhibited invasion and adhesion of renal tumor cells after injection in the kidney. Our data suggest that collagen type I might be a useful surrogate marker for patient selection in clinical trials.

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Antiangiogenic and antitumoral activity of novel heparin derivatives

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We have synthesized novel heparin derivatives with the aim to potentiate the antiangiogenic/antitumor properties and to abolish the anticoagulant effects of the original heparin. Among several compounds tested *in vitro*, ST1514 and its low molecular weight derivative ST2184, were selected to be studied *in vivo*. In the mouse model of B16/BL6 melanoma experimental lung metastases, treatment with ST1514 and ST2184 (25mg/kg coinjected i.v. with 1.5x104 cells/mouse) decreased of 63% ($p<0.01$) and 60% ($p<0.05$) the number of lung colonies, respectively. In the same animal model, subcutaneous treatment with ST2184 (100mg/kg) gave 61% and 46% metastasis inhibition, when administered within 30 min or 1 h before cell inoculation, respectively ($p<0.01$ at all time points). Moreover, all animals treated with ST2184 at 100mg/kg survived and did not show side effects. In contrast, animals treated with the same dose of heparin had pronounced bleeding and edema formation at the injection site and 25% died soon after treatment. In addition, the antitumor/antiangiogenic activity of ST 2184 was tested on a human tumor xenograft model. MeWo human melanoma cells stably expressing enhanced green fluorescent protein (EGFP) were injected intradermally in nude mice and treatment with ST2184 started 3 days later. In a first set of experiments, tumor-induced angiogenesis assessed at day 15 in the skin around the tumors, was significantly reduced ($p<0.01$) in animals treated with ST2184 (25mg/kg s.c twice daily for 10 days). In another set of experiments, tumor growth, assessed by imaging tumor cell fluorescence, was significantly decreased (53% of inhibition vs vehicle; $p<0.05$) starting from day 24 of treatment with ST2184 (50mg/kg kg s.c twice daily). In the same experiment, the administration of ST2184 (25mg/kg or 50mg/kg s.c twice daily) in combination with the camptothecin derivative ST1481 (0.25mg/kg per os qdx6/wx2w) caused further significant reduction of tumor volume compared to ST1481 alone starting from day 9 of treatment. We conclude that ST1514 and ST2184 have powerful antiangiogenic, antimetastatic and antitumor activity and could be used for the treatment of angiogenesis-related diseases. Their use in combination with chemotherapy is recommended.

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Phase I trial of recombinant human endostatin (rHu-endo) administered by continuous infusion (CI) intravenously (IV) in patients with solid tumors: a preliminary report

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Endostatin is a 20-Kb protein that has been shown in preclinical models to inhibit tumor growth. Phase I trials of rHu-Endo administered by daily short infusions did not show any significant toxicities while suggesting a biological effect. Preclinical data suggests that a continuous exposure to rHu-Endo enhanced its efficacy. A dose escalation phase I trial was conducted to determine the pharmacokinetics, toxicities, and assessment of tumor blood flow and glucose metabolism. To improve our ability to correlate dose with pharmacokinetic (PK) and biological endpoints, six patients were entered onto each dose level. Planned dose levels were 30, 60, 120, 180 and 300 mg/m^2/d of rHu-Endo IV administered by CI. To date, 18 (M=10/8=F) patients have been enrolled onto the first 3 dose levels. Median age=56.5 yrs; Median PS=1; Tumor types included: melanoma(4), lung cancer(5), hemangiopericytoma(2), sarcoma(2), and others(5); Median number of prior therapies=2. Nine patients also received XRT prior to study entry. To date, no significant drug-related toxic effects have been observed. No response have been observed to date, but 11 patients had stable disease >2 months. Preliminary PK parameter estimates are shown in the table below. With the first 3 dose levels completed, the estimated total clearance of rHu-Endo administered by CI in this trial was approximately 1/3 of that estimated when rHu-Endo was administered by short infusion. This decrease in rHu-Endo

clearance resulted in a dose proportional area under the concentration-time curve (AUC) 3× higher in the CI infusion schedule versus the short infusion studies. Sequential CT and FDG PET scans were acquired to assess the effects of rHu-Endo on tumor blood flow and tumor glucose metabolism, respectively. At the 60 and 120 mg/m^2/d dose levels, a substantial decrease in FDG metabolism was observed; while blood flow estimated by first pass metabolism did not significantly change over a 28 day period. Prior to starting rHu-Endo, one patient had two FDG PET scans over a 28 day period. During this drug-free period, blood flow to analyze metastatic lesions increased by 41% from baseline. Following two cycles of rHu-Endo, blood flow decreased by 47% from baseline. This trial is currently accruing patients at the higher dose levels.

Dose Level (mg/m^2/day)	Cycle 1 Mean CI (ml/min/m^2)	Single Day Mean AUC (mg/ml·min)	Cycle 1 Mean AUC (mg/ml·min)	Cycle 1 Mean Est. Css (mcg/ml)
30(n=6)	135.9 ± 77.1	0.26 ± 0.10	7.11 ± 2.63	0.16 ± 0.07
60(n=5)	131.1 ± 12.8	0.46 ± 0.04	12.45 ± 1.19	0.32 ± 0.03
120(n=5)	114.0 ± 25.9	1.10 ± 0.24	29.57 ± 6.43	0.76 ± 0.17

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Structure-growth regulatory potency relationship investigation of TIMP-1 (tissue inhibitor of metalloproteinases) C-terminal domain fragments

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Although TIMP-1 is widely known as a common matrix metalloproteinase (MMP) inhibitor, originally it was identified as a growth factor and is able to stimulate the growth of certain cell lines. It is noteworthy that TIMP-1 looses its growth stimulatory activity upon complex formation through its C-terminal domain with proMMP-9, but reduction and alkylation does not affect it. Assuming the importance of the C-terminal domain sequences in the growth stimulatory activity, peptide fragments related to this domain were synthesized and subjected for studies on SAR. In resting MCF-7 cell cultures TIMP peptides at the early treatment period induced higher DNA content without augmenting cell population, and at later non-apoptotic type of cell death was observed. The abolishment of DNA content elevation in the presence of EGF may indicate the participation of a cell surface receptor in the action of the peptides. TIMP peptides increased MMP-2, but reduced MMP-9 production in the HT-1080 cell cultures. The above data indicate that in growth factor deprived circumstances C-terminal fragments of TIMP-1 cause cell death and modulate the equilibrium between MMP-2 and MMP-9. No conclusion can be drawn from the SAR investigations for the presence of a well defined active center in the TIMP-1 C-terminal domain, it may rather be supposed that pharmacophores at different positions of the molecule are involved in the growth modulating activity of TIMP-1.

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Radiation and the endothelium: the importance and the modulatory effects of VEGF, bFGF, alphavbeta3 and the extracellular matrix components on ionizing radiation-induced endothelial cell damage

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In recent decades, radiation research primarily concentrated on the cancer cell compartment. Much less is known about the effect of ionizing radiation on the endothelial cell compartment and the complex interaction between the tumor and its microenvironment, which includes the ECM, cytokines, integrins and endothelial cells. Here we report that ionizing radiation is a potent antiangiogenic agent that inhibits endothelial cell survival, proliferation, tube formation and invasion. VEGF and bFGF were able to reduce the sensitivity of endothelial cells to radiation-induced damage, and this radioresistance could be reversed by the receptor tyrosine kinase inhibitors SU5416 and SU6668. Endothelial cells were found to be more sensitive to ionizing radiation than PC3 prostate cancer cells. IR upregulated VEGF and bFGF in PC3 cells and, interestingly, VEGFR2 and the integrin alphavbeta3 in endothelial cells. In a co-culture system, irradiation of the prostate cancer cells enhanced endothelial cell invasiveness through a Matrigel matrix. Because of the observed upregulation of alphaVβ3, we explored the modulatory role of ECM components on endothelial cell proliferation, plating efficiency and clonogenic survival. We observed that fibronectin and collagen I increased endothelial cell proliferation and survival without significantly affecting radiosensitivity. In contrast, laminin enhanced intrinsic radiosensitivity. Together these findings form the basis of a complex model of multifactorial communication between the tumor and its microenvironment that is modulated by ionizing radiation. This model may help us to better understand how tumors protect their microvasculature from radiation-induced damage. Simultaneously, our results rationalize concurrent administration of angiogenesis inhibitors and radiotherapy in cancer treatment.

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The alpha-v beta-3 antagonist S-247 inhibits the growth of primary renal tumor and spontaneous lung metastases in the RENCA model

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Tumor angiogenesis is a multistep process requiring migration, attachment and survival of endothelial cells. The integrin receptors play an essential role in tumor angiogenesis. Integrin receptor antagonists have been shown to inhibit tumor progression and metastases in preclinical models, and are currently in clinical development. In this study we assessed the effects of the alpha-v beta-3 (avb3) integrin antagonist S-247 in a murine model of renal tumor and spontaneous lung metastases. Murine syngeneic renal cell carcinoma cells (RENCA) were injected orthotopically into the renal capsule of Balb/c female mice. On day 4, animals were randomly assigned to control group and the experimental group. S-247 100 mg/kg in saline solution was administered by gavage twice a day. On day 22 all mice were sacrificed, and primary kidney tumors and lung metastases were analyzed. S-247 induced 49-68% inhibition of the primary tumor as compared to control ($p<0.01$). S-247 treated mice developed also significantly fewer spontaneous lung metastases than controls (up to 98% inhibition of microscopic lung colonies; controls 65; S-247 1.6; $p<0.01$). Preliminary immunohistochemical staining for CD31 and smooth muscle actin showed a reduction of microvessel and pericyte density in the in primary tumors of S-247 treated mice. Studies to evaluate the therapeutic effect of S-247 on established lung metastases in an "intervention" model are in progress. Imaging PET studies will be presented at the meeting. In conclusion, the avb3 integrin antagonist S-247 demonstrates significant anti-tumor and anti-metastatic activity in a murine model for renal cell cancer. Agents such as integrin receptor antagonists may represent an effective treatment in patients with renal cell carcinoma.

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A phase I study of the heparanase inhibitor PI-88 given subcutaneously (sq) in patients (pts) with advanced solid malignancies

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Heparan sulfates of the extracellular matrix (ECM) bind and sequester proangiogenic growth factors (GFs), such as bFGF and VEGF. Heparanase, which is overexpressed in many cancers, facilitates tissue remodeling and GF release from the ECM, and thereby promotes angiogenesis. PI-88 is a highly sulfated oligosaccharide that interferes with GF binding to heparan sulfates and inhibits heparanase. PI-88 inhibits both angiogenesis in the chick CAM assay, and tumor growth and metastasis in murine syngeneic and human xenograft tumor models. This ongoing phase I study is designed to evaluate the safety, pharmacokinetic (PK) behavior, and biological effects of PI-88 (80-250 mg) when given SQ on days 1-4 and 15-19 of a 28-day cycle to pts with advanced cancer. Dexamethasone 20 mg PO is given on days -1, 1, 14, and 15 for prophylaxis against immune-mediated thrombocytopenia. The rationale for this regimen included the convenience of SQ administration, as well as the possible identification of a distinct toxicity profile from that associated with prolonged intravenous administration. Thus far, 18 pts (median age 60 [range 19-77]; median PS 1) have received 40 courses (crs, range 1-11). Toxicities have included bruising at injection sites (gr 1, 36 crs), pain at known tumor sites (gr 1-2, 12 crs; gr 3, 2 crs), fatigue (gr 1-2, 13 crs), and peripheral neuropathy (gr 1-2, 4 crs). Two SAEs have occurred (pneumonia, second malignancy); neither is considered related to study drug. All