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Identification of target tissue for glioma gene therapy by multi-tracer PET imaging

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Objective: To identify viable target tissue in heterogenous gliomas amenable for biological treatment strategies such as gene therapy.
Methods: Rat F98 and RG2 glioma as well as human Gli36dEGFR and U87dEGFR glioma cells were grown as subcutaneous tumors in nude rats (n=12) and nude mice (n=8). After tumors had grown to at least 200 mm3, DNA-, protein- and glucose-metabolism were determined by means of 3-deoxy-3-[18F]-fluoro-L-thymidine (FLT), methyl-[11C]-L-methionine (MET) and [18F]-2-fluoro-2-deoxy-D-glucose (FDG) PET after intravenous administration of FLT (250-300 μCi/rat; 50-100 μCi/mouse), MET (600 μCi/rat; 200 μCi/mouse) and FDG (250-300 μCi/rat; 50-100 μCi/mouse) using new generation ECAT HRRT (Siemens, CTI) and microPET scanners (Concord).
Results: In small tumors (< 500 mm3), homogenous uptake of all three tracers indicated actively proliferating tumor tissue as potential target tissue for gene therapy. In larger tumors (> 500 mm3), heterogenous tracer uptake was observed with a rim of high FLT-, MET- and FDG-uptake immediately adjacent to metabolically inactive or necrotic tumor, where no specific tracer accumulation occurred. The metabolically active tumor tissue presented as a narrow band indicating that application and targeting of gene therapy vectors into the active proliferating tissue compartment in large tumors might be difficult.
Conclusions: Identification of target tissue for gene therapy is possible by multi-tracer PET in experimental animals at high spatial resolution. Multi-tracer PET imaging of tumor metabolism and gene expression shall contribute to the development of standardized gene therapy protocols and of efficient and safe vector applications in humans.

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Antitumor activity of g3139 (Genasense™) plus dacarbazine against human melanoma is superior to G3139 alone

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Bcl-2 inhibits apoptosis and is expressed in major tumor types, including melanoma. Bcl-2 maintains viability and contributes to chemo- and radiotherapy resistance of cancer cells. Dacarbazine (DTIC) is the standard single chemotherapeutic agent for melanoma; however, response rates and duration are low. Previously, we reported that Bcl-2 antisense oligonucleotide G3139 (oblimersen sodium, Genasense™, Genta Inc.) dramatically enhances DTIC effectiveness in a human melanoma xenograft model (Nat. Med. 4:232-4, 1998). In this study, we compare the antitumor activity of G3139 as a single agent versus the combination treatment with DTIC against human melanoma cells in a xenograft model. Spleen weights and IL-6 levels were also measured. METHODS: SCID mice were inoculated s.c. into the left lower flank with 518A2 cells. After developing palpable tumors (day 9), mice were randomized into 3 groups (n=8), implanted with miniosmotic pumps filled with saline or G3139 in saline. Mice were treated for 14 days with saline, G3139 (5mg/kg/d), or G3139 (5mg/kg/d) plus DTIC (80 mg/kg/d i.p. for 5 days on days 5-9 after pump implantation). Blood samples were collected for plasma IL-6 and IL-12 analysis. After 14 days of treatment, mice were sacrificed and tumors and spleens were evaluated by excision and weighing. RESULTS: The tumor weights were analyzed for between-group comparisons using an analysis of variance (ANOVA) model. G3139 as a single agent resulted in moderate reduction in tumor weights relative to saline-treated controls (P=0.08), whereas tumor weight reduction by G3139 + DTIC combined treatment was highly significant relative to either saline or G3139 only groups (P<0.001). Spleen weights and IL-12 levels were elevated 2-3 fold in both the G3139 and G3139 + DTIC groups versus saline controls, while IL-6 values were in the normal range and similar among the 3 treatment groups. CONCLUSION: These data confirm that G3139 markedly enhances the efficacy of dacarbazine in human melanoma xenograft, while the antitumor effects of G3139 as a single agent are minimal and support the continuing evaluation of G3139 combined with dacarbazine in patients with malignant melanoma. Further analysis by gene array and proteomics is ongoing.

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Heat shock protein 27 prevents gamma radiation-induced apoptosis

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The ability of heat shock proteins (HSP) to protect cells from stressful stimuli, including hyperthermia, oxidative stress or cytotoxic drugs and their unusually high levels in a wide range of tumours, suggest that these proteins could limit the efficiency of radiation therapy. Among them, HSP27 acts as molecular chaperones, modulates redox parameters and prevents the activation of the apoptosome-dependent apoptotic pathway. To our knowledge, no previous study has considered its role in g-radiation induced apoptosis. This work focuses on the mechanisms by which HSP27 could modulate the three levels of radiation-induced apoptosis: the induction phase (ceramide release), the regulation phase (mitochondrial events), and the executive phase (apoptosome assembly and caspases activation). The radiosensitive human leukemic Jurkat cell line, which does not express the protein HSP27 at the basal state or in response to g-ray, was transfected with a NeoPCI plasmid containing either the full-length human HSP27 cDNA or an insertless plasmid. The kinetics (4 to 48h) and sequence of apoptotic events triggered in response to 10 Gy irradiation were investigated in both HSP27-transfected and insertless control cell lines. HSP27 did not interfere with the inductive phase of apoptosis since the increasing release of ceramide observed in Jurkat control cells from 4 to 48h post-irradiation, was not prevented by HSP27 overexpression. At the mitochondrial level, HSP27 overexpression did not influence the progressive fall in mitochondrial transmembrane potential. In contrast, the generation of reactive oxygen species appeared significantly lower 24h after irradiation in the HSP27 transfected cells. Moreover, the cytochrome c release was also significantly delayed by HSP27 overexpression. In the course of the executive phase, caspase-3 and caspase-9 activations were significantly decreased 24h and 48h post-irradiation in transfected cells. Finally, the delayed cytochrome c release combined to the decrease of caspases activation led to a significant decrease of apoptotic death in HSP27 overexpressing cells (18% of transfected cells in the sub-G1 phase 48h post-irradiation, compared to 43% in the time-matched control cells). We conclude that HSP27 prevents gamma radiation induced apoptosis by delaying cytochrome c release and reducing effector caspase activation. Thus, it could be implicated in the mechanisms of radioresistance in tumours overexpressing HSP27.

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Biomarkers of *in vivo* response to BCL-2 antisense in human prostate cancer xenografts

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Bcl-2 anti-apoptotic gene expression is targeted with antisense oligonucleotide G3139 (Genesense‰, oblimersen, Genta Inc.), which currently is in clinical trials. To test a hypothesis that G3139 can decrease bcl-2 expression in androgen-refractory prostate cancer *in vivo*, PC-3 cells were implanted in 15 mice, allowed to establish for 10 days, then continuous infusions with G3139 at 10 mg/kg/day was started on Day 0. A control mouse (no drug) was sacrificed on Day 0, followed by one G3139-treated mouse sacrificed daily through Day 14. Global gene expression in xenografts at Day 1 and Day 14 was compared with the control using Operon microarray with 1,154 human cancer genes. The expression of anti-apoptotic [BCL-2 and BCL-X(L)] and pro-apoptotic (BAX) proteins was evaluated in all tumor homogenates (n=15) by western blots, quantified by densitometry and normalized for b-actin levels. Microarray analysis revealed that G3139 significantly (>10-fold) downregulated 19 genes, including gravin and plasminogen activator inhibitor, and >5-fold upregulated 28 genes, including mitochondrial aminotransferase and cyclin-dependent kinase inhibitor. The levels of bcl-2-like mRNA (D87461) decreased from 1.41+0.16 at Day 1 to 0.57+0.05 at Day 14. The corresponding values for bax (U66879) were 0.85+0.02 and 1.68+0.019, respectively. The*bcl-2/bax* ratios predict therapeutic response in patients with prostate cancer (Urology 1998, 52:1085). In this study, *bcl-2-like/bax* decreased from 1.65 at Day 1 to 0.34 at Day 14. BCL-2 increased expression by 80% from Day 0 to Day 14 in comparison with the control. BCL-X(L) and BAX levels increased by 30% and 100%, respectively, however, BCL-2/BAX decreased about two-fold by Day14. Net increase in BCL-2 could be due to downregulation of low bcl-2 expressing

cells that leads to cell death, enrichment of the remaining population of high bcl-2 expressing cells, and/or increased stability of BCL-2. In conclusion, G3139 treatment of PC-3 xenografts affected the *bcl-2-like/bax* and BCL-2/BAX at the mRNA and protein level. These *in vivo* effects observed at drug doses comparable with those used in the clinical trials (7 mg/kg/day) suggest that G3139 might be efficacious in androgen-refractory prostate cancer.

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Quantitative analysis of p53 targeted gene expression and visualization of p53 transcriptional activity following intratumoral administration of adenoviral p53 *in vivo*

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Objective: To analyze the mechanism of antitumor effects of adenoviral p53 (Ad-p53), we quantitatively assessed p53 targeted gene expression following intratumoral administration of Ad-p53 in nude mice.
Materials and Methods: 1) Human lung cancer (H1299) xenografts were established in nude mice, and treated by intratumoral administration of Ad-p53. We quantitatively assessed expression of p53 targeted gene including p21, MDM2, Noxa, and p53AIP1 by real-time quantitative RT-PCR and analyzed induction of apoptosis by TUNEL method 1, 2, 3, 7, and 14 days after the treatment. 2) We established H1299 cells expressing the GFP reporter gene under the control of p21 promoter (H1299/p53R-GFP). H1299/p53R-GFP tumors were treated with Ad-p53 as same. The transcriptional activity of exogenous p53 was visualized as intratumoral GFP expression in real-time manner by using 3CCD camera.
Results: 1) mRNA expression of p53 targeted gene (except p53AIP1) was maximum 1 day after Ad-p53 treatment, and then gradually decreased. Apoptosis was evident in situ 2 to 3 days after Ad-p53 treatment. 2) Maximal expression of GFP was achieved 3 days after the treatment, and it remarkably decreased on day 7.
Conclusions: We demonstrated that Ad-p53 treatment rapidly induced p53 targeted gene in tumors. We also succeeded to visualize p53 transcriptional activity *in vivo*. Quantitative analysis of p53 targeted gene expression and visualization of p53 transcriptional activity may be used to assess new therapeutic approaches.

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Ribozyme-PEI complex technology allows efficient down-regulation of gene expression *in vitro* and *in vivo*

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Ribozymes catalyze the sequence-specific cleavage of RNA molecules which is particularly attractive since this allows the effective abrogation of the expression of any selected protein. Therefore, ribozymes represent potentially interesting therapeutic agents in tumour therapy. Moreover, they offer powerful strategies in proteomics and target validation applications to analyse the functions of potential genes of interest, e.g. in tumour biology. So far, however, use of enzymatically active RNA molecules like hammerhead ribozymes has without chemical modification been severly hampered by ribozyme instability and poor cellular uptake. In this work, we introduce the, ribozyme-PEI complex technology' as a method for protection and cellular delivery of bioactive ribozymes. This is achieved by complexation fo ribozymes with polyethylenimines, i.e. synthetic branched polymers with high cationic charge density which form non-covalent complexes with nucleic acids and have been used so far only as DNA transfection reagents. We show that the ribozyme-PEI complex technology allows complete stabilization of ribozymes or any RNA against degradation. Upon their highly efficient cellular uptake, non-toxic PEI-complexed ribozymes display intracellular bioactivity already at low concentrations as demonstrated in tissue culture by down-regulation of two different genes in different cell lines. In particular, we describe a $\sim$ 60 % depletion of an FGF-binding protein (FGF-BP) which has been described previously as rate-limiting for tumor growth and metastasis in squamous cell carcinomas and serves as an, angiogenic switch molecule'. Likewise, the growth factor pleiotrophin (PTN) which is overexpressed in several tumors and which can be rate-limiting for tumor growth and angiogenesis, is efficiently downregulated in tissue culture upon targeting with PEI-complexed PTN ribozymes. In a mouse xenograft model, we show that upon i.p.-injection intact ribozymes are taken up by the tumor. This *in vivo* delivery of PEI-complexed anti-PTN ribozymes results in marked reduction of tumor growth and of intratumoral PTN levels. We describe the ribozyme-PEI complex technology as a novel, widely applicable method for exogenous delivery of any bioactive RNA ribozyme *in vitro* and *in vivo* without chemical modification.

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Gelsolin gene therapy using adenovirus vector for orthotopically transplanted human urinary bladder carcinoma in nude mice

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Gelsolin is an actin-regulatory protein that is undetectable or reduced in human urinary bladder tumors compared to normal epithelial cells (Tanaka, M. et al: Cancer Res, 55: 3228, 1995). Whether the over- expression of gelsolin could inhibit tumor growth was investigated in an orthotopic bladder cancer nude mouse model using recombinant adenovirus encoding wild-type gelsolin (Ad-GSN). Growth of the bladder cancer cells, KU-7 and UMUC-2 was inhibited when these cells were transduced with Ad-GSN *in vitro*. Flow cytometric analysis was conducted to examine the cell cycle after transducing the adenovirus. Over- expression of gelsolin caused these cells to arrest or delay at the G2/M phase of the cell cycle. *In vivo* cell growth was compared to control groups of these cells transduced with adenovirus containing the E. coli β-galactosidase gene (Ad-βgal). KU-7 cells were transplanted into the bladder of nude mice (Day 0) followed by three injections into the urethra (Day 2, Day 3, and Day 4) with either Ad-GSN or Ad-βgal at $1 \times 10(9)$ PFU. Eight days after initial adenovirus exposure (Day 10), each bladder was sectioned and stained then tumor mass digitally determined. In the orthotopic bladder cancer model, Ad-GSN treated animals had approximately 90 % less tumor mass compared to controls. Ad-GSN provided a significant tumor suppressive effect on human bladder cancer cells in this orthotopic nude mouse model. Adenovirus-mediated over- expression of gelsolin may be useful therapy for human bladder cancer.

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Inhibition of oncogenic RET signaling by Ad vector-mediated expression of a dominant-negative RET-mutant: implications for medullary thyroid cancer treatment

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Mutations in the RET proto-oncogene which lead to constitutive tyrosine kinase activity are the underlying cause for the development of medullary thyroid cancer (MTC). To date, treatment of MTC is primarily restricted to surgical removal of neoplastic tissue, which is often inefficient and enhances the risk of developing metastasis. To investigate an alternative strategy in MTC treatment, we took advantage of a dominant-negative RET (dn-RET) mutant, Ret51Flag, which inhibits oncogenic signal transduction by retaining the oncogenic RET protein in the endoplasmic reticulum (ER), thereby reducing the amount of oncogenic RET protein from the cell surface. We generated an adenoviral (Ad) vector expressing dn-RET51Flag protein under control of the C-cell specific synthetic calcitonin promoter (TSE2.CP1), shown to produce high levels of transgene only in thyroid C-cells (Ad-TSE2.CP1-dn-RET51Flag). Immunofluorescence microscopy in MTC-derived TT cells revealed restriction of the endogenous oncogenic RET in the ER combined with the lack of expression on the cell surface following virus infection, confirming the dominant-negative effect of dn-RET51Flag. Moreover, we observed a strong inhibition of cell viability which is partially dependent on cell growth inhibition and the induction of apoptosis. In nude mice, injection of Ad-TSE2.CP1-dn-RET51Flag treated thyroid cancer cells resulted in a significant suppression of tumor growth *in vivo*. In summary, our data indicate that succesful inhibition of oncogenic RET receptor tyrosine kinase expression by a dominant-negative RET-mutant might be a powerful approach for *in vivo* therapy of MTC. Supported by grant PU188/3-1/3-2 from the Deutsche Forschungsgemeinschaft.