

International CA-IX Symposium

Functional & Clinical Aspects

Abstract Booklet



The Discovery, Distribution and Function of CA-IX

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G250/MN/CAIX: historical and future perspectives

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In 1986 we described monoclonal antibody (mAb) G250, recognizing a molecule expressed in Renal Cell Carcinoma (RCC) with a very restricted expression pattern in normal tissues. Additionally, cross-reactivity with non-RCC carcinomas was observed. Because normal kidney tissue did not express the target molecule we suggested that the mAbG250 antigen expression in RCC might be activated by an onc-gene or by viral infection. In 2000 the target molecule was characterized and shown to be identical to MN/CAIX, described in 1994 by Pastorek as a human tumor-associated protein. This molecular characterization linked CAIX expression with HIF transcription factors, as shown earlier by Ratcliffe and Harris, nicely explaining the expression pattern in RCC and non-RCC carcinomas.

Although the mAbG250 target remained unknown, many preclinical and clinical studies were performed in view of the exquisite tumor-specificity. The first (biopsy-based) clinical trial performed under the guidance of Old at MSKCC, described in 1993, demonstrated several pivotal aspects: most notably virtually no uptake in other tissues resulting in excellent tumor visualization, and very high tumor uptake. Various (non-randomized) clinical trials have now been completed with therapeutic efforts focussed on radioimmunotherapy and passive immunotherapy in metastatic RCC patients. The collective results suggest that mAbG250 treatment might extend survival time.

In another line of research CAIX was described as prognostic marker and predictor of survival in advanced RCC patients by Belldegrun and Atkins showed that CAIX expression was predictive for high dose IL-2 responders. Parallel studies have demonstrated the value of CAIX expression as prognostic and survival marker for non-RCC

tumors as well. Finally, CAIX PET imaging might become a valuable diagnostic tool as recently illustrated by Divgi. During this symposium several aspects of this molecule that appears to combine an extraordinary number of highly desirable properties will be discussed, including a number of new developments.

Molecular mechanisms of CA IX-mediated pH regulation under hypoxia

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Hypoxia is a crucial factor of tumour physiology that leads to massive molecular and phenotypic changes associated with cancer progression and resistance to treatment. These changes include shift to oncogenic metabolism, which produces extracellular acidosis, a typical feature of tumour microenvironment. Acidosis has been traditionally attributed to accumulation of lactate and protons excessively produced by glycolysis, extruded from cells and poorly removed by tumour vasculature. However, experiments with glycolysis-deficient cells indicate that carbon dioxide is a significant source of acidity in tumours and suggest a role for carbonic anhydrase (CA), a zinc metalloenzyme catalyzing the reversible conversion of carbon dioxide to bicarbonate and proton. There are 15 human CA isoforms that regulate diverse physiological processes based on ion transport and pH balance.

CA IX isoform is predisposed to act in control of tumour pH. It is an active enzyme with an extracellular catalytic site, it is tightly regulated by hypoxia, its expression is associated with many tumours and serves as a marker of hypoxia and prognostic indicator. Recent studies demonstrated that CA IX cooperates with bicarbonate transporters, participates in extracellular acidification in response to hypoxia, and its function can be inhibited by CA IX-selective sulphonamides. CA IX can also diminish intracellular pH gradient in hypoxic core of 3D tumour spheroids. Reduced CA IX expression in tumour cells perturbs their in vitro survival under hypoxia. Deletion of the catalytic domain from CA IX delays tumour growth in vivo. Interestingly, mutations in intracellular tail of CA IX perturb its pH-regulating function exerted by the extracellular catalytic domain.

Altogether, these data show a direct functional implication of CA IX in regulation of pH in tumour microenvironment and enable to propose novel strategies based on selective CA IX inhibitors as tools for in vivo imaging of hypoxic tumours and promising anti-cancer drugs.

Significance of pH regulation in tumour progression and implications for therapeutic approaches

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Carbonic anhydrases (CAs) are involved in cellular pH regulation and have been implicated in some pathogenic processes including tumour progression.

We found two articles very attractive as we started to search functional connections between CA activity and tumorigenesis. First, Martinez-Zaguilan et al. showed that human melanoma cells cultured at acidic pH gained a more aggressive behaviour than the control cells. Second, Teicher et al. demonstrated that inhibition of CA activity by acetazolamide may be beneficial as an adjunct to cancer chemotherapy, because it produced additive tumor growth delays with anticancer drugs in vivo. In 2000, we showed that a CA inhibitor, acetazolamide, can inhibit the invasive potential of renal cancer cells in vitro. Soon after that report Supuran et al. showed that sulfonamides can inhibit growth of several cancer cell lines.

Recently, there have been a number of studies demonstrating different CA isozymes in tumors. Among the isozymes CA II, IX and XII have clearly become the most attractive targets for potential cancer therapy approaches. Although CA II is downregulated in most cancers, it is ectopically expressed in the neovessel endothelium in melanomas and esophageal, renal, lung and brain cancers. Recently, an anti-CA II autoantibody response by dendritic cell therapy was reported to be associated with a more favourable clinical outcome.

CA IX is an attractive cancer-associated enzyme which has become a major focus in CA research during the past decade. It is highly expressed in malignancies including renal, ovarian, colorectal, lung and brain cancers. CA IX research has already produced promising therapeutic molecules which

are in clinical trials, and CA IX-specific inhibitors are also in the pipeline. CA XII is another plasma membrane-bound enzyme which is overexpressed in cancer. Although the studies on CA XII are in a quite preliminary phase, it represents another potential target for therapeutic applications.

Hypoxia Pathways Regulating CAIX

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The gene encoding carbon anhydrase IX is subject to high amplitude regulation by the hypoxia inducible factor (HIF) system. Thus expression of carbonic anhydrase IX potentially provides a marker of the activation of this system by hypoxia and certain oncogenic mechanisms.

Hypoxia inducible factor (HIF) is an α/β heterodimeric transcriptional complex that mediates a broad range of responses to hypoxia, extending directly or indirectly to the regulation of hundreds of genes. Analysis of the regulatory HIF- α subunits has demonstrated that activity is controlled by a series of oxygen dependent enzymatic hydroxylations at specific residues.

In human HIF-1 α hydroxylation sites identified to date are Pro402 and 564, and Asn803. Combined structural/genetics approaches have identified the relevant enzymes as members of the 2-oxoglutarate dependent dioxygenase super-family. HIF prolyl hydroxylation is performed by a closely related set of isoenzymes (PHD1-3) that differ in abundance and sub-cellular localization.

Hydroxylation of either HIF-1 α Pro402 or Pro564 promotes interaction with a specific site on the β -domain of the von Hippel-Lindau tumour suppressor protein (pVHL). In oxygenated cells this process targets HIF- α for rapid proteasomal destruction, with pVHL acting as part of a multi-component E3 ubiquitin ligase. HIF asparaginyl hydroxylation is performed by a protein termed FIH (Factor Inhibiting HIF).

In oxygenated cells hydroxylation of HIF-1 α Asn 803 prevents interaction with the p300 transcriptional co-activator. These reactions have an absolute requirement for dioxygen and limitation of activity in hypoxia allows HIF- α to escape

destruction, recruit co-activators and activate the HIF transcriptional cascade.

The majority of both sporadic and hereditary renal cell carcinomata manifest bi-allelic inactivation of VHL and consequent upregulation of hypoxia pathways irrespective of oxygen availability. In this setting expression of carbonic anhydrase IX therefore marks inactivation of VHL irrespective of tumour hypoxia. The implications of activating these pathways for tumour development will be discussed.

The use of PET/CT in the diagnosis of tumour phenotype

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The increasing detection of renal masses incidentally by imaging for other conditions and the increasing awareness that renal function may be irreversibly impaired by surgery, has led to the search for improved methods for pre-operative characterization.

Pre-operative identification of tumor type may have important implications in the choice of treatment for renal neoplasms. While both dynamic contrast-enhanced CT and ultrasound using Doppler flow measurements have shown promise, these techniques rely on increased tumor vascularity and thus suffer from a lack of specificity.

Antibody cG250 against carbonic anhydrase-IX is over-expressed in nearly all clear cell renal carcinomas, the most malignant renal tumors. We carried out a clinical trial to assess antibody PET with ^{124}I -cG250 in the prediction of clear cell renal carcinoma.

Twenty-six patients scheduled to undergo resection of their renal masses were imaged with ^{124}I -cG250 pre-operatively, and PET/CT findings were correlated with histology. Fifteen of 16 clear cell carcinomas were positive on antibody PET, and all nine non-clear cell masses negative, with a sensitivity of 94%, and the specificity and negative predictive accuracy each being 100%. The one clear cell carcinoma negative on antibody PET did not express CA-IX.

Antibody PET with ^{124}I -cG250 has potential in the identification of clear cell renal carcinoma, with a negative scan being highly predictive of a less aggressive tumor. A large multi-center trial is planned in the USA. Antibody PET imaging may also have a role in the identification of CA-IX expression, and thus of hypoxia, in other tumor types.

CAIX and prognosis kidney cancer

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CAIX expression is observed in a wide variety of tumors including cervical, lung, brain, and breast. Higher CAIX is associated with poor histologic features and is an independent predictor of poor disease-specific survival in different malignancies.

We confirmed the diffuse pattern of CAIX in clear cell renal cell carcinoma (RCC) in 321 specimens from the primary tumor. A total of 94% of tumors demonstrated diffuse membrane expression of CAIX. Decreased CAIX staining is not associated with Fuhrman grade and T classification though it is associated with worse performance status. We determined that >85% membrane staining was the ideal cutoff for determining patient survival.

Using this threshold, 79% of patients demonstrate high CAIX expression. We found that in patients with metastatic disease (n=150), disease-specific survival was 25 months vs 5.5 months for high and low CAIX expression, respectively. Low CAIX expression in metastatic patients is a strong independent predictor of poor outcome (HR 3.10). For patients with localized disease, low CAIX expression was not found to influence survival. Separate analysis of high-risk M0 patients demonstrated low CAIX expression is associated with poor survival. Response to IL-2 based systemic therapy demonstrated an association with CAIX expression. All complete responders had high CAIX expression. The response rate was 27% and 14% for patients demonstrating high and low CAIX expression respectively. These results were later confirmed by work published by Dr Atkins from the Harvard group who demonstrated a higher frequency of CAIX expression among responders.

We reported preliminary results from a prospective study in metastatic clear cell RCC. High CAIX expression was observed in 62.5% of patients. The

one-year disease-specific survival was 83% vs. 63% for those with high and low CAIX expression (p=0.01). The response rate to IL-2 in patients displaying high CAIX expression was 37.5% (3/8), which included 2 complete responders.

With multiple therapeutic options now available to oncologists, we believe that patients with clinicopathologic predictors of response and high CAIX expression be offered first line IL-2 therapy. Given the available expression data we believe that at the present time CAIX is the best and most powerful diagnostic and predictor marker for clear cell RCC. Its role as a therapeutic target awaits confirmation from ongoing clinical trials.

Carbonic Anhydrase IX (CAIX) is a Diagnostic, Prognostic and Therapeutic Molecular Marker in Bladder Cancer

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Hypoxia is a common consequence of the rapid growth of many vascular tumors, and is an important regulator of a network of gene expression. Carbonic anhydrase IX (CAIX) protein, an hypoxia-inducible member of the carbonic anhydrase family that regulates intracellular pH during periods of hypoxia, is thought to play a role in the regulation of cell proliferation, oncogenesis and tumor progression.

The role of CAIX in the biology of transitional cell carcinoma of the bladder (TCC) has now been evaluated by immunohistochemical analysis of both paraffin embedded tumor tissues and voided urine samples. A tissue microarray containing 522 primary TCCs, 38 metastatic, and 135 normal urothelial tissue specimens, each in triplicate, and 128 urinary cytology samples from voided urine specimens were selected randomly from healthy subjects and TCC patients. Immunohistochemical staining of both tissue and urine was performed using the mouse monoclonal antibody MN-75. The percentage of positive cells was evaluated for its association with tumor stage, grade, and survival using standard statistical methods.

All normal urothelial tissue samples were negative for CAIX expression, while 71% of the TCCs expressed CAIX, showing primarily membranous staining of luminal tumor cells. CAIX expression was higher in non-invasive (Ta) vs. invasive (T1-T4) TCC, low grade vs. high grade TCC, and metastases vs. the corresponding primary tumor. In urinary cytology specimens 100% of low-grade lesions were CAIX positive, while specificity was 88% in normal urothelial cells. Staining of urine samples showed the same trend as in the TMA, with high-grade and invasive tumors less frequently positive than low-grade tumors.

For patients with Ta TCC undergoing TUR, higher CAIX expression was associated with poorer recurrence-free survival. In patients with T1 tumors undergoing TUR, higher CAIX expression also conveyed a worse prognosis with respect to recurrence-free and progression-free survival. In patients who underwent cystectomy, higher CAIX expression was associated with worse overall, and was a strong, independent prognostic factor in multivariate analysis (HR 1.75).

Conclusions:

- CAIX is differentially expressed in normal urothelium, non-invasive vs. invasive TCC, low grade vs. high grade TCC, and in primary tumor vs. metastasis.
- CAIX is an important predictor of TCC recurrence, progression, and survival.
- CAIX is absent from normal urothelium, and may be a useful adjunct to diagnostic cytology for the diagnosis of low-grade lesions.
- Since CAIX expression is absent in normal urothelial cells while being highly expressed in non-invasive low grade TCC, intravesical CAIX targeted therapy might be effective in these high CAIX-expressing tumors.
- Metastatic tumors express high CAIX, and evaluation of systemic CAIX targeted therapy as a therapeutic approach is warranted.

CAIX Expression as a Biomarker in Renal Cancer

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Clear cell RCC (cRCC) is characterized by inactivation of VHL resulting in failure to degrade HIF and thus overexpression of various hypoxia inducible proteins, including CAIX.

In contrast to many tumors, CAIX overexpression in cRCC appears to be an early event and is associated with a better prognosis. We have investigated the relationship of CAIX expression and response to various therapies, its relationship to VHL mutations and the concordance of CAIX immunostaining in histologic sections and TMA.

Response to IL-2 and durable survival was correlated with high (>85% of cells) CAIX expression. Survival greater than five years was only seen in the high CAIX expressing group. Furthermore, the combination of histologic features and CAIX expression divided patients into two roughly equivalent populations with one containing 96% of responding patients. This latter model is currently being confirmed in a prospective trial (The IL-2 Select Trial).

A preliminary analysis suggests that the benefit of sorafenib on both median tumor shrinkage and PFS is also greatest in patients with high CAIX expressing tumors. In contrast, high CAIX expression was not associated with response to temsirolimus, although there was a trend toward improved survival in this population.

In a validation study, good concordance was found when CAIX staining was compared in tissue sections and TMA cores. Only 17% cases were discordant and the TMA analysis produced similar results with regard to IL-2 response as the whole sections analysis.

Finally, high CAIX expression correlated with presence of VHL mutations: 15/20 (75%) VHL mutant tumors expressed high CAIX compared to 5/16 (31%) VHL wild-type tumors ($p=0.0172$). Taken together, these results suggest that CAIX expression may serve as a surrogate marker for VHL mutant cRCC and its high expression is associated with good prognosis as well as response to both immunotherapy and VEGFR targeted therapy.

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ABSTRACT 1: CAIX-specific functionalized nanoparticles

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Introduction:

Antigen specific nanoparticles may serve as targeted drug delivery units and in this way reduce the side effects that accompany many current cancer therapies. Clinical trials have demonstrated that tumor accumulation of chimeric Mab cG250, identifying carbonic anhydrase IX (CAIX), abundantly overexpressed in RCC, is the highest of any antibody in a solid tumor.

In this study we explored the possibility of introducing CAIX specificity via Mab cG250 to two types of nanoparticles: polymersomes, prepared from PEGylated polystyrene (PS-PEG) and lyophilisomes, prepared from protein (elastin)

Material and Methods:

Polymersomes were synthesised from PS-PEG/PS-PEG-maleimide. For detection BSA/ICG was encapsulated. The maleimide anchor enabled coupling of SATA modified cG250. Protein based lyophilisomes were prepared from a mixture of elastin and cG250 through a lyophilisation procedure. The reactivity of both immunoparticles was studied by scanning electron microscopy (SEM), confocal microscope imaging, flowcytometry and cell binding assays.

Results:

CAIX-specific binding was clearly visualised by SEM analysis and confocal microscope imaging. Results from flowcytometry and cell binding assays supported these findings.

Conclusion:

By the introduction of CAIX-specificity to two very distinct types of nanoparticles we show feasibility of targeted nanoparticles, which may lead to specific controlled-release imaging and therapy.

ABSTRACT 2:

CAIX and HIF-1a in metastatic clear cell renal cell carcinoma at baseline and during IL-2 based therapy

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Purpose:

High Carbonic Anhydrase IX (CAIX) is associated with good prognosis in metastatic renal cell carcinoma (RCC), whereas the impact of Hypoxia Inducible Factor 1a (HIF-1a) is less well characterized. The purpose of this study was to evaluate the prognostic impact of both baseline CAIX and HIF-1a and explore the prognostic information of both markers in combination and during immunotherapy.

Materials and Methods:

Pretreatment core-needle tumor biopsies were available from 66 patients with metastatic clear cell RCC all treated by IL-2 based immunotherapy. Forty-six patients had additional biopsies taken at week 3, 5 or 8. Immunohistochemical expression was evaluated using MN75 (CAIX) and clone 54, BD Biosciences (HIF-1a). Both parameters were scored in each biopsy and were mutually compared and related with clinical parameters and overall survival.

Results:

In all 66 patients we confirmed that high expression of CAIX before immunotherapy is related with favorable overall survival ($p=0.04$) whereas baseline expression of HIF-1a did not correlate with survival ($p=0.64$). The correlation between CAIX and HIF-1a was statistically significant (Spearman's $\rho=0.56$; $p<0.001$). Among the 46 patients with repeated tumor biopsies, CAIX was stable in 30 patients (65%), increased, $n=6$ (13%), and decreased, $n=10$ (22%), over time, whereas HIF-1a had stable values in only six patients (13%), increasing in 26 (57%), and decreasing in 14 (30%). Using a 20% increase as cut point, 17 patients had

increasing HIF-1a and 29 had stable or decreasing values. A 'Low Risk Group' ($n=24$) was defined as CAIX above 85% AND stable HIF-1a, and a 'High Risk Group' ($n=22$) as CAIX<85% OR increasing HIF-1a. All seven long-term survivors were in the low risk group and all patients in the high risk group had died within two years ($p<0.001$).

Conclusions:

These results suggest that adding HIF-1a to CAIX in metastatic renal cell carcinoma may improve selection of patients benefiting from immunotherapy. Further exploration of these and other markers in the hypoxic pathway may help in prospective tailoring of immunotherapy in advanced RCC.

ABSTRACT 3: Characterization of G250 monoclonal antibody binding to human cancer-associated carbonic anhydrase IX

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G250 monoclonal antibody is utilized in different immunotherapeutic approaches to target renal cell carcinoma cells via CA IX antigen, a cancer-associated carbonic anhydrase isoform regulated by hypoxia.

CA IX protein is composed of a large extracellular domain (ECD), a single-pass transmembrane region and a short intracellular tail. ECD consists of an N-terminal proteoglycan-like region (PG) and a well-conserved, catalytically active carbonic anhydrase (CA) domain. So far, binding site of the G250 MAb on the CA IX molecule and its binding under hypoxia or in presence of CA inhibitors have remained unexplored. However, recent availability of several CA IX-specific MAbs, CA IX protein variants, and transfected cell lines allowed us to investigate these issues.

Different immunoassays with CA IX deletion variants revealed that G250 MAb recognizes a non-denatured form of CA IX with intact catalytic domain. In accord, G250 can bind to a soluble CA IX shed to extracellular space, but not to the alternatively spliced variant of CA IX lacking the C-terminal part of the CA domain. Furthermore, G250 MAb does not cross-react with other tested CA isoforms including CA I, II, and XII.

Based on the competitive binding assay, antigenic site of G250 appears to overlap with the antigenic sites of other CA domain-specific MAbs VII/20, V/12 and V/10. Binding experiments with the normoxic and hypoxic cells that constitutively express CA IX showed that the G250 MAb binding is not affected by hypoxia. Moreover, G250 MAb can bind to CA IX in the presence of CA IX-selective enzyme inhibitor.

Altogether, our data show that the G250 MAb binds to a conformational epitope localized in the CA domain, that it is suitable for specific detection of shed CA IX in body fluids and can be potentially used in combination therapy with CA-IX selective inhibitors.

Supported by WILEX.

**ABSTRACT 4: Monoclonal antibody VII/20 binds to
 catalytic domain and induces internalization of
 cancer-related carbonic anhydrase IX independently of hypoxia**

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Receptor internalization is an important process regulating the abundance of and signal transduction via cell surface proteins. It has a particular impact on the outcome of immunotherapy with specific monoclonal antibodies (MAbs) against the cancer-related antigens. The internalizing MAbs can be exploited either to block the receptor-mediated transmission of the growth/survival signals or to deliver therapeutic drugs selectively to cancer cells. Carbonic anhydrase IX (CA IX) is a suitable target for cancer immunotherapy. It is a transmembrane protein expressed in various human tumours, but not in the corresponding normal tissues. Expression of CA IX is increased by hypoxia and correlates with cancer prognosis.

We have generated several MAbs against the extracellular part of CA IX. MAb VII/20 binds to the catalytic domain, as shown by CA IX deletion variants. Confocal analysis revealed that this MAb is also capable to induce internalization of CA IX. The internalization was not affected by hypoxia and low extracellular pH that are typical for tumour microenvironment. VII/20-mediated internalization also led to down-regulation of CA IX at the cell surface. Our results suggest that CA IX behaves as a signalling molecule and that properties of VII/20 are promising for its therapeutic application.

Supported by VEGA (2/6113/26) and EUROXY (LSHC-CT-2003-502932).

ABSTRACT 5: Expression of Carbonic Anhydrase IX (CA9) in Renal Neoplasms: Implications for Use as A Diagnostic Marker

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Background:

CA9 is a tumor associated antigen found on the cell surface of a number of human cancers. Recently, CA9 has been shown to be a useful diagnostic and prognostic biomarker for clear cell renal cell carcinoma. Its expression in other renal neoplasms, and its utility as a differential diagnostic marker, however, is not well documented.

Methods:

A tissue microarrays (TMA) was constructed from 60 normal kidneys, 23 clear cell renal cell carcinoma (CCRCC), 20 papillary renal cell carcinomas (PRCC), 16 chromophobe renal cell carcinomas (ChRCC), and 19 oncocytomas (ONC), 14 pelvic urothelial carcinoma (TCC) and 20 angiomyolipoma (AML). The TMA was immunostained for CA9. Membranous CA9 expression was scored as negative, weak and strong. The percentage of positive cells was also recorded.

Results:

CA9 expression was absent in normal renal tissues. Strong and weak positive staining was present in 83% and 4% of CCRCC, respectively, with 47.6% of tumor cells positive for CA9 expression. Thirteen % of CCRCC was negative. Ten % of PRCC was also positive for CA9. One such case had papillary structures lined with clear cells. All pelvic TCCs were positive for CA9, with strong expression in 86% and weak expression in 14% of cases. However, only 11.3% of tumor cells were positive for CA9. All ChRCC, ONC and AML were negative for CA9.

Conclusion:

Although expressed by the majority of CCRCC, CA9 is also found in all the renal pelvic TCC and in a minority of PRCC, but is absent in ChRCC and oncocytoma. CA 9 may potentially be useful in the differential diagnosis of selected renal tumors, such as CCRCC vs ChRCC, and TCC vs ChRCC.

ABSTRACT 6:

Sutent® treatment leads to decreased uptake of mAb cG250 in RCC

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Introduction:

Sunitinib (Sutent®) is a tyrosine kinase inhibitor that targets the vascular endothelial growth factor receptor and platelet-derived growth factor receptor. Sutent® has recently been registered as first-line treatment for patients with mRCC. However, complete responses have been rare, and most patient progress eventually. Thus, Sutent® represents the best, but still suboptimal treatment for mRCC and combination therapy might be beneficial. Chimeric mAb cG250 identifies carbonic anhydrase IX, abundantly over-expressed in RCC. Tumor accumulation of cG250 is the highest of any antibody in a solid tumor. Combination of Sutent® with mAb cG250 might lead to improved tumor responses and survival in patients with mRCC. The aim of this study was to explore the effect of Sutent® on mAb cG250 biodistribution.

Since Sutent® treatment leads to drastically decreased antibody uptake, combination treatment strategies with such agents may have a small window of opportunity and should be carefully designed.

Materials and Methods:

Nude mice bearing human RCC xenografts were treated with Sutent® or solvent for 7 or 14 days. At day 7, mice were injected i.v. with ¹²⁵I-cG250. Animals were sacrificed at predetermined days and cG250 biodistribution determined. Tumors were analyzed by immunohistochemistry for the presence of endothelial cells, activated endothelial cells, laminin, smooth muscle actin and uptake of mAb cG250.

Results and Discussion:

While on Sutent® treatment tumor uptake of cG250 decreased dramatically, tumor growth was slightly inhibited and vascular density decreased considerably as judged by various markers. When treatment was stopped at day 7, neovascularization recovered immediately, mainly at the tumor periphery.

ABSTRACT 7: Treatment of metastatic renal cell cancer with autologous T-cells genetically retargeted against carbonic anhydrase IX - first clinical experience and continuation

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Adoptive transfer of autologous T-lymphocytes that are gene-transduced to express antigen-specific receptors ('genetic retargeting') represents an experimental therapy to provide tumor-specific immunity to cancer patients. We studied safety and proof of this concept in patients with metastatic renal cell carcinoma (RCC).

A single-chain antibody-type (scFv) receptor based on the mAb G250 was constructed. This mAb recognizes an epitope on carboxy-anhydrase IX (CAIX). Three patients with CAIX+ metastatic RCC were treated with a maximum of 8 infusions of 2×10^7 to 2×10^9 gene modified T-cells.

We successfully generated functional scFv(G250)+ T-cells *ex vivo*. Infusions of the gene-modified T-cells were initially well tolerated. However, following 4-5 T cell infusions patients developed liver enzyme disturbances reaching toxicity grades 2-4, necessitating cessation of treatment in two patients. Examination of liver tissue from one patient revealed T cell infiltration around the bile ducts and CAIX expression on bile duct epithelium. Circulating scFv(G250)+ T-cells and scFv(G250) DNA copies were transiently detectable in all patients. In addition, we showed that after infusion the scFv(G250) gene-modified T cells maintained their transgene-specific immune functions *in vivo*. All three patients developed anti-scFv(G250) antibodies and showed progressive disease between 36 and 106 days after start of treatment. The scFv(G250)-retargeted T-cells exerted receptor-specific function *in vivo*. However, reactivity of these cells most likely with CAIX expressed on bile duct epithelium hinders administration of numbers expected to yield anti-tumor activity.

We have developed a strategy to attenuate the anti-CAIX activity of retargeted T-cells against normal tissues expressing target antigen to circumvent the observed adverse events. The Dutch regulatory authorities have approved this amended protocol and accrual of patients is currently ongoing.

Lamers et al., Hum Gene Ther 16: 1452-1462 (2005);

Lamers et al., J Clin Onc 24:e20-222 (2006);

Lamers et al., Cancer Gene Ther 13: 503-509 (2006);

Lamers et al. Cytotherapy 8: 542-553 (2006);

Lamers et al., Cancer Immunol Immunother 2007, doi 10.1007/s00262-007-0330-3.

ABSTRACT 8: Lack of prognostic and predictive value of CA IX in radiotherapy of squamous cell carcinoma of the head and neck with known modifiable hypoxia: An evaluation of the DAHANCA 5 study

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Background and Purpose:

CA IX is suggested to be an endogenous marker of hypoxia in tumours like squamous cell carcinomas of the head and neck (HNSCC). The aim of the present study was to investigate whether CA IX served as a prognostic factor for outcome in a large population of HNSCC and if CA IX was able to discriminate the tumours that did benefit from hypoxic modification with nimorazole.

Material and Methods:

Paraffin-embedded formalin-fixed pre-treatment tumour tissue was available from 320 of the 414 patients treated in the randomized DAHANCA 5 protocol with primary radiotherapy +/- the hypoxic radiosensitizer nimorazole. CA IX was measured using immunohistochemistry and results were divided into four groups of CA IX expression: <1%, 1-10%, 10-30% and >30% of the tumour area with positive membrane staining. Locoregional control and disease-specific survival was used as endpoints.

Results:

Expression of CA IX was not correlated to any of the tumour or patient characteristics investigated. Furthermore, CA IX did not serve as a prognostic marker in the total cohort as well as in the group of 150 patients treated without nimorazole. Finally, no relation was found between the different expression levels of CA IX and the influence of nimorazole when locoregional control or disease-specific survival was used as endpoints.

Conclusions:

This is to date one of the largest studies of CA IX in HNSCC. The data suggest that CA IX have no prognostic or predictive potential in patients with cancer in the head and neck treated with conventional radiotherapy and concomitant nimorazole.

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ABSTRACT 9: Tumor-associated extracellular carbonic anhydrase 9 unifies spatial intracellular pH regulation in three-dimensional growth

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CA9 is a membrane-tethered, extracellular-facing carbonic anhydrase (CA) isoform. Expression of CA9 is increased in cancer and is regulated by hypoxia-inducible factor 1. The physiological advantage of this enzyme is not well-established.

In the present work, we show that CA9 plays an important role in spatial pH regulation in three-dimensional tissue-structures but not in superfused, single cells. We have transfected human bladder carcinoma RT112 cells with the CA9 gene, and grown these into multi-cellular spheroids. These were imaged confocally for intracellular pH (pHi) with carboxy-SNARF-1. Due to large diffusion distances ($>200\text{ }\mu\text{m}$), spheroids are predisposed to pHi-gradients (up to 0.25 pHi units) and core-acidosis. These gradients were over two-fold smaller in spheroids expressing CA9, compared with size-matched non CA-expressing spheroids. Incubation with the membrane-impermeant CA inhibitor, 14v (500nM), doubled the size of standing pHi gradients in CA9-expressing spheroids. The drug had no effect on standing gradients in non CA9-expressing spheroids.

In spheroids lacking CA9, addition/removal of superfusate CO₂ induced pHi-changes that were delayed in the spheroid core by up to 10s, relative to the periphery. In the presence of CA9, these delays were collapsed. Inhibition with 14v increased core-periphery temporal delays. Our results demonstrate that CA9 plays a key role in unifying pHi regulation spatially, minimizing pHi gradients and maintaining a high pHi across a tissue for optimal growth. CA9 exerts its effect by facilitating CO₂ diffusion between respiring cells and the well-stirred extracellular fluid.

We speculate that CA9 expression may be of great importance to tissue survival during periods of inadequate blood perfusion. This would explain the rationale for its regulation by HIF and the observation that aggressive tumors are associated with CA9 expression. An appreciation of the role of CA9 in cancer biology will help our understanding of the therapeutic value of CA inhibitors.

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Carbonic anhydrase (CA, EC 4.2.1.1) isoform CA IX is highly overexpressed in many cancer types being present in few normal tissues. It constitutes an interesting target for the anticancer therapy due to its overexpression in many cancer tissues and not in their normal counterparts. Its expression is strongly induced by hypoxia present in many tumors, being regulated by the HIF transcription factor present in several tumor cells and correlated with a bad response to classical chemo- and radiotherapies.

CA IX was recently shown to contribute to acidification of the extratumoral tumor medium environment, by efficiently catalyzing the hydration of carbon dioxide to bicarbonate and protons with its extracellularly situated active site, leading both to the acquisition of metastatic phenotypes and to a chemoresistance with weakly basic anticancer drugs. Inhibition of this enzymatic activity by specific and potent inhibitors was on the other hand shown to revert these processes, establishing a clear-cut role of CA IX in tumorigenesis. Indisulam, a sulfonamide anticancer drug in clinical trials, acts as a low nanomolar CA IX inhibitor.

The development of a wide range of potent and selective CA IX inhibitors could thus provides useful tools to for highlighting the exact role of CA IX in the hypoxic cancers, and to control the pH (im)balance of the tumor cells, and to develop novel diagnostic or therapeutic applications for the management of hypoxic tumors . Indeed, both fluorescent inhibitors or positively-charged, membrane impermeant sulfonamides have been recently developed as CA IX inhibitors and used as proof-of-concept tools for demonstrating that CA IX constitutes a novel and interesting target for the anticancer drug development (1-4).

1 *Alterio et al., J. Am. Chem. Soc.* 2006, 128, 8329-35;

2 *Thiry et al., Trends Pharm. Sci.* 2006, 27, 566-573;

3 *Supuran C.T. Curr. Top. Med. Chem.* 2007, 7, 825-833,

4 *Supuran C.T. & Scozzafava A. Bioorg. Med. Chem.* 2007, 15, 4336-50.

**ABSTRACT 11: Chalcedony (a crystalline variety of silica)
formation in human glioblastoma from aged patients:
Possible CA-IX role**

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Chalcedony is a microcrystalline fibrous form of silica. It consists of nanoscale intergrowths of quartz and the optically length-slow fibrous silica polymorph moganite. Chalcedony formation is due to the relationship between pH and oxidation potential (Eh) (Krumbein and Garrels, 1952).

A biogenically produced crystalline mineral phase (i.e. chalcedony) has been observed in electric organs (cholinergic nerves and electrocytes) from living electric fish (Prado Figueroa et al., 2005; 2007). Chalcedony (SiO₂) was also detected in human brain from aged patients (Prado Figueroa et al., 2006).

Glioblastoma is the most aggressive type of primary brain tumor and it involves overexpression of the enzyme carbonic anhidrase IX (CA-IX). From these evidences, we decided to document the presence of chalcedony in human astrocytoma II and glioblastoma multiforme from autopsy derived and also biopsy.

In thoses sections (H-E) naturally occurring fluorescent mineral was observed by confocal laser scanning microscope Leica TCS SP2. Our data showed that there is a green fluorescent chalcedony in astrocytoma II, it was about 1 - 20 micron in size. In glioblastoma, green fluorescent chalcedony appeared infiltrating all the tissue. CA-IX is involved in physiological pH regulation and may be in the polymerization - depolymerization of chalcedony in the human brain. Moreover, silicase (a carbonic anhidrase enzyme) is implicated in amorphous silica depolymerization from sponges.

ABSTRACT 12:

Imaging CA IX with fluorescent sulfonamide inhibitors distinguishes hypoxic and reoxygenated cells

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Background and Purpose:

Hypoxia is an important micro-environmental parameter influencing tumor progression and treatment efficacy. Guidance of hypoxia-directed treatments on an individual basis necessitates adequate measurements of hypoxia, at best in a non-invasive manner. Carbonic Anhydrase IX (CA IX) expression is associated with many frequently occurring tumours, where its levels are dramatically increased in response to hypoxia. Recently, sulfonamide based carbonic anhydrase inhibitors (CAI) showing specificity for CA IX have been designed. Aim of this study was to investigate the CAI binding properties under normoxia, hypoxia and reoxygenation.

Material and Methods:

Cervical (HeLa), colorectal (HT-29) and pVHL-deficient renal (RCC4) carcinoma cell lines demonstrating different CA IX expression levels were incubated with fluorescein labelled CAI (1mM) during normoxia, hypoxia (0.2%) and reoxygenation. CA IX expression levels were assessed using Western blotting. CAI binding was determined by immunostaining and flow cytometry.

Results:

CAI binding in hypoxic cells was significantly higher compared with normoxic cells ($P = 0.0007$) and correlated with upregulated CA IX levels. Binding occurred within 15 minutes of hypoxia,

but was gradually lost upon reoxygenation. Interestingly, although CA IX levels remained high upon reoxygenation, CAI binding was dramatically reduced ($P = 0.0015$) and no longer correlated with CA IX expression. Similarly, RCC4 cells, constitutively express CA IX, do not bind CAI under normoxic conditions.

Conclusions:

Our results confirm and extend previous results showing that CAI binding occurs only under hypoxia. The inability of CAI to bind CA IX in RCC4 cells and following reoxygenation in other cells demonstrates that formation of the active site not only depends on HIF-1-dependent gene activity, but also on the absence of oxygen per se. Our data also suggests an attractive possibility not only for imaging fluctuating hypoxia *in vivo*, but also for therapeutic targeting of CA IX resulting in individualized patient treatment.

ABSTRACT 13: Radioimmunotherapy with Lutetium-177 Labeled Monoclonal Antibody cG250 in Patients with Advanced Renal Cell Carcinoma

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Introduction

Chimeric monoclonal antibody cG250 recognizes carbonic anhydrase IX (CAIX), an antigen that is abundantly expressed in Renal Cell Carcinoma (RCC) and has limited expression in normal tissue. In previous clinical studies we have demonstrated that focal uptake of radiolabeled cG250 in RCC tissue may be extremely high (up to 0.52 %ID/g). Nevertheless, in a phase I/II study with ¹³¹I-labeled cG250 (¹³¹I-cG250) in mRCC patients only minor clinical responses were observed. cG250 labeled with the residualizing radiometal ¹¹¹In revealed superior tumor uptake as compared to ¹³¹I-cG250 in an intra-patient comparative study. In addition, radioimmunotherapy (RIT) studies in nude mice with s.c. RCC tumors demonstrated superior therapeutic efficacy of cG250 labeled with ¹⁷⁷Lu as compared to ¹³¹I therefore.

In the present study in RCC patients we aim to determine the maximum tolerated dose (MTD) and therapeutic efficacy of multiple infusions of ¹⁷⁷Lu-cG250.

Material & Methods

Patients with progressive metastatic clear cell RCC receive an i.v. scout dose of ¹¹¹In-cG250 to assess adequate tumor accumulation. If scintigraphic images show adequate cG250 accumulation, patients are eligible to receive a high activity dose of ¹⁷⁷Lu-cG250. In this activity dose escalation study, three patients are treated per dose level (initial dose level 1110 MBq/m², dose increments 370 MBq/m² until the MTD) is reached. Patients are evaluated for adverse events and tumor response. Whole-body scintigraphic images are recorded at 0, 2-4 and 5-7 days post injection to estimate tissue residence

times. When progressive disease and human-anti-chimeric-antibody responses are absent after 14 weeks, patients are eligible for re-treatment with 75% of the previous activity dose. In total patients can receive a maximum of three therapeutic doses.

Results

To date 13 patients have been treated (highest dose 2220 MBq/m²). At the 1850 MBq/m² dose level, one grade 4 hematological toxicity was observed, while other patients only showed grade 2 myelotoxicity. Besides the myelotoxicity, mild nausea and fatigue, no significant side effects were noted. Radioimmunoscintigraphy of ¹⁷⁷Lu-cG250 showed good to excellent targeting of the tumor lesions. ¹¹¹In-cG250/¹⁷⁷Lu-cG250 images were highly similar demonstrating that the ¹¹¹In-cG250 images could be used to estimate radiation doses to the tissues following injection of ¹⁷⁷Lu-cG250. Eight patients received a second and three patients received a third treatment cycle. Nine patients demonstrated stable disease after 12 weeks and one patient showed a partial response, which is ongoing after eight months.

Conclusions

¹⁷⁷Lu-cG250 showed excellent targeting of RCC lesions. 1850 MBq/m² can be safely administered to patients, and dose escalation is ongoing. These preliminary clinical observations at suboptimal dose levels indicate that ¹⁷⁷Lu-cG250 treatment can stabilise previously progressive mRCC.