Antitumor efficacy of a monoclonal antibody that inhibits the activity of cancer-associated carbonic anhydrase XII

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Carbonic anhydrase XII (CA XII) is a membrane-tethered cell surface enzyme that is highly expressed on many human tumor cells. CA members in this class of exofacial molecules facilitate tumor metabolism by facilitating CO2 venting and intracellular pH regulation. Accordingly, inhibition of exofacial CAs has been proposed as a general therapeutic strategy to target cancer. The recent characterization of 6A10, the first CA XII-specific inhibitory monoclonal antibody, offered an opportunity to evaluate this strategy with regard to CA XII-mediated catalysis. Using functional assays, we showed that 6A10 inhibited exofacial CA activity in CA XII-expressing cancer cells. 6A10 reduced spheroid growth in vitro, under culture conditions where CA XII was active (i.e. alkaline pH) and where its catalytic activity was likely rate-limiting (i.e. restricted extracellular HCO3− supply). These in vitro results argued that the antibody exerted its growth-retarding effect by acting on the catalytic process, rather than on antigen binding per se. Notably, when administered in a mouse xenograft model of human cancer, 6A10 exerted a significant delay on tumor outgrowth. These results corroborate the notion that exofacial CA is critical for cancer cell physiology, and they establish the immunotherapeutic efficacy of targeting CA XII using an inhibitory antibody.

Precis

This study offers a preclinical proof-of-concept for immune targeting a cell surface carbonic anhydrase that is widely expressed in human cancer, as a general therapeutic strategy.
Introduction

Carbonic anhydrase XII (CA XII) is, alongside a related isoform IX, a cancer-associated, membrane-tethered exo-facial enzyme. Early evidence has suggested that CA XII expression is up-regulated under hypoxic conditions (1, 2), akin to CAIX. In contrast to CA IX, CAXII is upregulated by estrogen and therefore present in many breast cancers (3). More generally, the enzyme is overexpressed in many forms of human cancer, including renal, pancreatic, gut, oral, brain, lung and ovarian (2, 4-11), but is also present in some normal tissues, such as kidney, gut and endometrium (4, 6, 12), expressed in combination with other CA isoforms. CA XII has also been found to be overexpressed in glaucoma (13) and in advanced atherosclerotic plaques (14). The enzyme is successfully used as a sero-diagnostic biomarker for lung cancer (15) and as a molecular marker for the detection of breast cancer lymph node metastasis (16).

Despite considerable progress made, the molecular biology and physiology of CA XII is less well understood than that of CA IX. Like CA IX, CA XII is a catalyst for CO₂ hydration (17, 18). Cancer cells produce vast quantities of this metabolic end-product by mitochondrial and pentose shunt decarboxylation, and by titration reactions between acids (e.g. lactic) and bicarbonate (HCO₃⁻) (19, 20). The catalytic activity of exo-facial CAs has been shown to facilitate CO₂ venting from cells (21, 22), optimize extracellular buffering power to reduce extracellular pH (pHₑ) transients during acid/base membrane transport (23), and to supply extracellular HCO₃⁻ (24) for HCO₃⁻-dependent pH-regulating transporter proteins (e.g. Na⁺-HCO₃⁻ cotransport). These CA-dependent processes are important for controlling intracellular pH (pHᵢ), which must be regulated to within narrow limits that are permissive for biological functions. The challenge of regulating pHᵢ is particularly apparent in tumors, which are characterized by an elevated metabolic rate and aberrant capillary perfusion (20). It has therefore been hypothesized that the cancer-associated CAs (isoforms IX and XII) play an important role in disease progression. In agreement with the notion that cancer progression selects for phenotype, not genotype (25), pH may play a critical role as a selection pressure driving stable adaptive changes (26, 27). Aside from impairing pHᵢ regulation, inhibition or knock-down of CA IX or CA XII have been demonstrated to suppress tumor growth and migration (17, 28-30). Consequently, the targeting of cancer-associated exo-facial CAs is now being considered as a novel anti-cancer therapeutic strategy.

The recent characterization of an anti-CA XII monoclonal antibody (6A10) with full inhibitory potency against CA XII (31) has made it possible to study the effects of CA XII-specific inhibition on tumor growth. Functional assays have confirmed that 6A10 inhibits recombinant CA XII at low nanomolar concentrations and that blocking of CA XII interferes with the growth of multicellular
tumor spheroids \textit{in vitro} (32). The present study confirms the inhibitory effect of 6A10 on native CA XII expressed in intact cells, and shows that inhibition of CA XII activity slows the growth rate of cancer cells in culture and in in xenograft mouse models. Our findings confirm the antibody's significant anti-tumor activity and highlight the potential of immunotherapy in the treatment of CAXII-expressing cancers.

\textbf{Materials and methods}

\textbf{Antibodies, cell lines and cell culture methods:} 6A10 is a rat IgG2a monoclonal antibody that has been described elsewhere (31). The isotype control antibody used in the \textit{in vivo} studies is a rat IgG2a antibody specific for Glutathione-S-transferase. A549 (ATCC CCL-185), Kato III (ATCC HTB-103), T47D and HT29 (ATCC HTB-38) are cell lines established from a human lung, gastric, ductal breast and colon cancer, respectively. HT29-shCA9 and HT29-shEV were a kind gift from A. McIntyre (Oxford, UK) and were described in detail elsewhere (28). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; \textit{Sigma}) supplemented with 10\% (v/v) heat-inactivated foetal calf serum (\textit{Sigma}), Glutamine-Penicillin-Streptomycin mixture (\textit{Sigma}) and incubated at 37°C with 5\% CO$_2$ enriched air. Cell lines were obtained from ATCC and were identified April 2013 by STR DNA testing performed at Eurofins (Ebersberg, Germany).

To simulate hypoxia prior to immunofluorescence, cells were incubated with 1mM dimethyloxaloylglycine (DMOG), a cell permeable, competitive inhibitor of HIF-prolyl hydroxylases, for 48h. Control cells were treated with the drug-vehicle dimethyl sulfoxide (DMSO) only. Before pH$_e$-imaging experiments, intact A549 and T47D cells were incubated for 24h with 20µg/ml 6A10 to block extracellular-facing CAXII activity or with PBS (control conditions); before pH$_i$-imaging experiments, spheroids were grown for up to 6 days in the presence of 20µg/ml 6A10.

\textbf{Immunofluorescence:} Cells were plated on a cover slip in a 35mm Petri dish, grown to 100\% confluency, then fixed using 1ml of pre-cooled methanol (-20°C) for 10min at 4°C. Antigen blocking was performed with a 1\% Bovine Serum Albumin (BSA) and PBS solution for 30min. Subsequently, cells were incubated with the primary rat monoclonal antibody 6A10 for 1h at 37°C. The sample was then washed with PBS and 0.2\% Tween-20 four times, for 10min each time. Thereafter, cells were incubated with the secondary goat anti-rat Alexa Fluor 488 antibody (green), diluted by 1:1000 in blocking solution for 1h at 37°C. To simulate hypoxia cells were treated with 1mM DMOG for 48h. Control cells were treated with DMSO for 48h to simulate dense normoxic cells. Cells were imaged with a Zeiss Confocal Laser Scanning Microscope (LSM) 700 system.
Immunoprecipitation of CAXII: 8µg of the anti-CAXII antibody 6A10 was mixed with 200µl of protein-L agarose beads in 1ml PBS and incubated for 1h at room temperature. The beads were washed with PBS thrice, centrifuged for 2 min each time, then halved. The anti-6A10 rat monoclonal antibody was added to either DMSO or DMOG A549 cell lysates, each 500µl, and the bead-antibody mixtures were incubated on a shaker overnight at 4°C. Both samples were then centrifuged at 15,000 rpm for 20s. The lysates were removed, and each sample washed thrice with PBS and centrifuged for 2min each time. 45µl of Laemmli loading buffer containing β-mercaptoethanol were added and samples boiled at 100°C for 5min. The samples were then centrifuged and loaded into lanes in order to conduct polyacrylamide electrophoresis and Western Blot.

Western blotting: A549 and T47D cells were lysed at 4°C with buffer containing in (mM): 1% Triton X-100, 0.5% Nonidet P-40 Substitute, NaCl (150), NaF (50), Tris-HCl (50), at pH 7.5, and Complete protease inhibitors (Roche). Lysed cell fragments were pelleted at 13,000rpm for 20min at 4°C. Proteins were resolved by SDS poly-acrylamide gel electrophoresis and transferred to a PVDF membrane. Antigen blocking was performed overnight at 4°C with 5% skimmed milk in PBS and 0.2% Nonidet P-40.

The relevant proteins were detected using the following primary antibodies: with goat polyclonal anti-CAXII (R&D biosystems) antibody; monoclonal mouse M75 antibody raised against human CAIX; and polyclonal rabbit antibody raised against CAII (both kind gifts from Prof. S. Pastorekova), polyclonal goat antibody raised against actin (Santa Cruz). The primary incubation of membranes was followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies; both for 1h on a shaker at room temperature. Enhanced chemiluminesence (ECL Pierce) was used to visualize protein expression, and the membranes developed on X-ray film (Fuji). Acquired data were normalized to actin.

Preparation of A549/T47D membrane fragments: CA activity was determined from membrane fragments extracted from A549 and T47D cells grown to 90–100% confluency. Cells were washed with ice cold PBS and scraped down into ice cold buffer containing in (mM): NaCl (15), KCl (35), potassium gluconate (105), HEPES (20), MES (20), Complete protease inhibitors (Roche), pH adjusted to 7.8 at room temperature. Cells in suspension were then disrupted by freeze-thaw cycle and centrifuged at 9000rpm at 4°C to pellet the membrane fraction which was then re-suspended in fresh buffer. The centrifugation and buffer-replacement process was performed twice to prevent contamination of cell membrane solution with cytosolic CA isoforms.

Measurement of CA activity in the membrane fraction of lysed cells: A 0.67ml aliquot of membrane-fragment suspension was added to a 2ml vessel, well-stirred and cooled to 2°C. A
narrow pH electrode (Biotrode, Hamilton, UK) was inserted to monitor solution pH at 1 Hz. To start the CA-catalyzed reaction, a 0.33ml aliquot of 100% CO₂-saturated water (2°C) was added to the vessel. CO₂ hydration yielded H⁺-ions, which could be measured with the pH electrode. Cooling was necessary to bring the reaction kinetics within the resolving power of the pH electrode. The hydration rate constant was derived from the pH time-course using a fitting algorithm developed previously (22). To determine the spontaneous CO₂ hydration rate, measurements were performed on membrane-free “blank buffer” samples and also in samples treated with 100µM ATZ. The increase in CO₂ hydration rate above the spontaneous rate is a measure of CA-catalysis (22).

**Solutions:** Solutions contained (in mM): CO₂/HCO₃⁻ buffered normal Tyrode (NT): NaCl (130), KCl (4.5), MgCl₂ (1), CaCl₂ (1), NaHCO₃ (12), glucose (11). For ammonium-containing NT, NaCl was substituted with NH₄Cl. Solutions were bubbled with 5% CO₂/95% air to attain a pH of 7.2.

**Live cell imaging of pH:** Cells were imaged using a Zeiss LSM 700 with a transparent superfusion chamber (capacity 2ml), the surface of which was pre-treated with 0.01% poly-L-lysine to facilitate cell/spheroid adhesion. Solutions were heated to 37°C and delivered at a constant rate of 2ml/min. Downstream suction was adjusted to maintain a steady-state solution volume of approximately 0.5ml. Cells were exposed for 3 min to medium containing 50µM WGA-fluorescein (Invitrogen, UK), a membrane-tagging dye that reports pH at the extracellular surface of the cell, near the site of CAXII catalysis (21). The dye was excited alternately at 405 and 488nm and fluorescent emission detected at 520 ± 20 nm. The ratio of fluorescence excited at 405 and 488 nm was later calibrated using highly buffered solutions (10 mM Hepes, 10 mM Mes, 120 mM NaCl, 4.5 mM KCl, 10 mM glucose, 1 mM MgCl₂, 1mM CaCl₂) at pH 6.8 and 7.4.

**Statistical analysis of data:** Standard error bars are shown for all data. Data from the measurement of spontaneous CO₂ hydration rates in membrane fraction were assessed using a two-tailed T-test and considered to be significantly different when P<0.05. In vitro data were assessed using paired two-tailed T-tests and considered to be significant when P<0.05.

**Multicellular spheroids:** Spheroids were cultivated as hanging drops. 8,000 cells in 20µl DMEM with the 6A10 or an isotype antibody at a final concentration of 20µg/ml were pipetted onto the inner side of the lid of a cell culture dish. The lid was quickly reversed and placed onto the dish containing PBS to avoid desiccations and incubated at 37°C. Cell proliferation was calculated with MTT assays.

**Immunocytochemistry:** Excised xenografts were cryopreserved and cut into 4µM sections, stained with a Cy3- or FITC-coupled goat anti-rat secondary antibody and imaged using a Zeiss AxioImager Z1 microscope.
**CA XII inhibiting antibody**

**Xenograft studies:** NSG mice (NOD/SCID/IL2 receptor gamma chain knockout) mice were obtained from Jackson Laboratory (Lund, Sweden) and housed at the animal facility of the Helmholtz Center Munich and experiments were carried out under a license from the State of Bavaria. A549 human lung cancer cells were transduced with a vesicular stomatitis virus G protein-pseudotyped lentivirus encoding GFP and Gaussia luciferase (LUC) essentially as described elsewhere (33). Cells expressing the highest GFP levels were isolated using a Becton Dickinson Aria III cell sorter and amplified, yielding a homogenous population with respect to GFP expression (Supplementary Fig. S1). A549/GFP/LUC cells were counted, resuspended in HBSS and 100,000 cells/mouse in a final volume of 200µl HBSS were injected intraperitoneally. The data shown here are representative of three independent experiments. In an earlier experiment, mice were injected with different doses of antibodies (10 µg -500 µg) and, 100µg/ml 6A10 was determined of the lowest dose showing an antitumor effect. For the experiments described herein and starting from day 0 of injection, mice were treated with either 100µg 6A10 or an anti-GST isotype antibody once per week or left untreated (n=8 for each group). No antibody-related toxicity was observed, i.e. body weight, habitus and behavior were normal and identical to animals of the untreated group. Also macroscopic post mortem inspection did not reveal any indication for antibody-related tissue and organ damage. Tumor growth rates were monitored by longitudinal measurements of whole body bioluminescence signals (photons/second) using an IVIS Lumina II Imaging System (Caliper Life Sciences, Mainz, Germany) as described (33). Briefly, mice were anesthetized with isofluorane and fixed in the imaging chamber. 100µg coelenterazine (Synchem, Felsberg, Germany) were then injected into the tail vein and animals were immediately imaged for 15 sec using a field of view of 12.5 cm with open emission filter settings, binning at 8 and f/stop at 1. Quantification was performed with the Living Image software 4.2 (Caliper). Survival curves were calculated using Mantel-Cox tests.
Results

6A10 inhibits CA activity at the membrane of CA XII expressing A549 and T47D cells

Previous studies have demonstrated an inhibitory effect of 6A10 on recombinant CA XII (inhibitory constant IC_{50}=5.7 nM) (31), the first anti CAXII monoclonal antibody with biological activity on its target enzyme. Confirmation of this inhibitory effect was tested on CA XII expressed natively by A549 and T47D cell lines. Western blotting demonstrated that both cell lines express CA XII protein under normoxic incubation and following 48 hour treatment with the hypoxia-inducible factor (HIF)-stabilizing drug dimethyloxalyl glycine (DMOG; 1mM) to induce hypoxic responses (Fig. 1A). Additionally, CA IX expression was also detected in A549 cells (Fig. 1A). Immunoprecipitation of CAXII protein from A549 whole-cell lysates using 6A10 confirmed selective antibody binding to CAXII (Fig. 1B). Immunocytochemistry detected CA XII protein at the cell surface membrane in monolayers of either cell line (Fig. 1C). A similar expression pattern was also observed in cells pre-treated DMOG (data not shown).

The effect of 6A10 on the catalytic activity of CA XII was first measured in membrane fractions prepared from T47D and A549 cells (Fig. 1D). Samples were prepared by centrifugation and three washing steps to remove soluble intracellular CA isoforms. The CA-catalyzed reaction was initiated by adding 100% CO_{2}-saturated water to buffered suspensions (20 mM Hepes+20mM MES; pH 8.0 at 4°C) containing cell-membrane fragments (1:2 v/v). The rate of the H^{+}-yielding CO_{2} hydration reaction (monitored by measuring medium pH with an electrode) is related to CA activity (Fig 1Di). The pH-dependence of the hydration rate constant (k_{h}) was determined by piecewise fitting to the recorded pH time course. Results of the best-fit (Fig 1Dii) indicate that the membrane-bound CA activity is pH-insensitive over the range 6.7-7.7. The addition of 6A10 reduced CA catalysis in a dose-dependent manner (Fig 1Diii) towards the spontaneous rate (confirmed by experiments in the presence of the broad-spectrum CA inhibitor acetazolamide, ATZ, 100 µM). The IC_{50} of 6A10 measured in A549 membrane fragments was 0.96 µg/ml, and therefore 10 µg/ml was deemed to inhibit CAXII fully (Fig. 1Diii). 6A10 (30 µg/ml) did not affect measured CA activity in the strongly CAIX-positive breast line MDA-MB-468 (97±13% of control activity) (23), establishing that 6A10 does not cross-react with this other exo-facial CA. The near-full inhibition of CA activity in A549 membrane fragments with 6A10 indicates that endogenous CA IX activity in this cell line does not contribute significantly towards total membrane-associated CA catalysis, despite CAIX immunoreactivity (Fig 1A). This was confirmed by the absence of any inhibitory effect of the anti-CA IX antibody MSC8 (which blocks up to 57% of CA IX activity in CAIX over-expressing cell lines, (34) (Fig 1Div). Results of the assay are summarized in Fig 1Div (A549 cells) and Fig 1Dv (T47D cells). Per
gram of total protein, membrane fragments had higher CA activity than the soluble fraction in both cell lines and 10 µg/ml 6A10 potently inhibited membrane CA activity.

Further characterization of 6A10 was performed on intact cells, loaded with the wheat germ agglutinin (WGA) conjugate of fluorescein, a pH-sensitive fluorescent dye. WGA-fluorescein reported extracellular pH, close to the site of CA XII activity. Cells were superfused with solutions buffered by 5% CO₂/12 mM HCO₃⁻ at pH=7.2. Rapid exposure to and subsequent withdrawal of 20 mM ammonium-containing solution by means of solution-switching evoked surface-pHₑ transients: exposure to ammonium evoked trans-membrane NH₃ entry and extracellular NH₄⁺ deprotonation at the extracellular membrane surface (producing a fall in pHₑ), whereas ammonium removal evoked the reverse reaction (rise in pHₑ). The ability of extracellular CO₂/HCO₃⁻ to buffer these surface-pHₑ transients depends on exo-facial CA activity. Out-of-equilibrium surface-pHₑ transients were significantly larger in the presence of ATZ (Fig. 1E-F) in both T47D and A549 cells, confirming that both cell lines have considerable extracellular-facing CA activity. Experiments were repeated on cells pre-treated with 20 µg/ml 6A10 for 24 hours. Surface-pHₑ transients were larger and no longer affected by treatment with ATZ, indicating that 6A10 had inhibited exo-facial CA activity (Fig. 1E-F). Overall, these data demonstrate that the 6A10 antibody is a highly potent, full inhibitor of exo-facial CA XII catalytic activity in A549 and T47D cells natively expressing the protein. In A549 cells, which express both CAIX and CAXII protein, the latter is the major contributor to total extracellular-facing CA activity.

### 6A10 inhibits cancer cell growth

The effect of CAXII inhibition on the growth of CAXII-positive cells (A549 and Kato III) was measured using the MTT assay. The starting concentration of HCO₃⁻ was varied, at constant CO₂ partial pressure, to alter the time-course of medium [HCO₃⁻] and pH over a 3-day growth period (whereby higher medium [HCO₃⁻] is associated with more alkaline pH, under the constraints of the Henderson-Hasselbalch equation). Growth of A549 cells was reduced in the presence of 6A10 (Fig 2A). This effect was greater with lower starting [HCO₃⁻], i.e. under conditions were HCO₃⁻ availability is reduced throughout the growth period, relative to high-starting [HCO₃⁻] controls. The HCO₃⁻-dependence of the 6A10 effect was more pronounced in Kato III cells (Fig 2B). These results indicate that 6A10 has a significant inhibitory effect on growth in vitro, which is more pronounced when cells are grown in media with lower starting [HCO₃⁻].
CA XII inhibiting antibody

Many cell lines and cancers in vivo co-express CA XII along with the exo-facial isoform CA IX, raising the possibility that CA XII-specific inhibition could be compensated by intact CA IX activity. This was tested by comparing the effect of CA XII inhibition with 6A10 on CA IX-positive and CA IX-negative cells. Experiments were performed on the HT29 colon cancer cell line, which expresses both CA isozymes. The effects of 6A10 were studied on subclones transduced with either an expression vector carrying a short-hairpin RNA against CA IX (HT29-shCA9) or an empty control vector (HT29-shEV) (30). Cells transduced with shCA9 RNA displayed no detectable CA IX expression even under hypoxia, whereas the enzyme was evident in HT29-shEV cells (Fig. 2C). Immunoblotting (Fig. 2C) and flow cytometry (Fig. 2D) revealed that knockdown of CA IX had no effect on CA XII expression. HT29-shCA9 and HT29-shEV cells were grown under hypoxia (1% O2) for three days in the presence of 6A10 or an isotype control antibody. Inhibition of CA XII activity with 6A10 significantly reduced the growth of CA IX-negative HT29-shCA9 in media containing 44 mM bicarbonate and in bicarbonate-free HEPES-buffered medium (Fig. 2E). In contrast, no inhibitory effects were observed with HT29-shEV cells (Fig. 2F), indicating that the CA activity conferred by CAXII in HT29 cells is adequate to maintain normal growth under hypoxia. This observation supports the notion of a critical role of CAXII catalysis in facilitating growth, which in turn could be targeted efficaciously with 6A10.

6A10 inhibits tumor cell growth in a pH-dependent manner

The catalytic process mediated by CA XII is strongly inhibited by acidic pH substantially lower than 7.0 (35). If the catalytic process were critical for the effects of 6A10 on growth, the efficacy of the antibody would show pH-dependence. To assess this in vitro, CA XII-expressing A549 cells were grown as spheroids in HEPES-buffered HCO3−-free medium titrated to different starting pH levels. Over the course of growth, these media are expected to acidify further due to metabolism. After three days of culture, cell growth was assessed by the MTT assay (36). As shown in Fig. 3, overall growth rates of A549 cells were strongly pH-dependent, with an optimum under conditions where starting pH was ~7.2. Of interest, cells did not survive pH values higher than 7.3 in nominally HCO3−-free media (data not shown). The inhibitory effect of 6A10 on cell growth was observed when cells were grown from a starting pH of 7.1 and 7.3. In contrast, no such effect was observed in cells growth from a more acidic starting pH (between 6.2 and 6.8), that is, under which CA XII catalysis is expected to be inhibited from the start of the experiment. In other words, antitumoral efficacy was not seen under conditions where the enzyme had been inhibited by H+ ions from the start of incubation. These data show that CA XII enzyme activity is mandatory for optimal growth of A549
cells and that the inhibitory effects of 6A10 correlate directly with CA XII catalytic activity. It is noteworthy that the relationship between proliferation and ambient pH will be acid-shifted relative to the plot of proliferation vs starting pH because of on-going medium acidification over three days of culture. Overall, the data indicate that anti-CA XII antibody growth-retarding efficacy relies critically on the antibody’s ability to block CA catalytic activity.

6A10 shows antitumor activity in a xenograft tumor model

The effects of CA XII inhibition with 6A10 on tumor growth in vivo were studied in a human xenograft model using immunocompromised NSG mice. 100,000 A549/GFP/LUC cells were injected intraperitoneally and xenograft-bearing mice were treated with 6A10 (100 µg/mouse), and compared with isotype-treated and untreated control mice. 6A10 was well tolerated and no side effects were observed. Firstly, CA XII expression and 6A10 binding was assessed in established 6A10-treated tumors. As shown in Fig. 4A, xenografted A549 cells expressed high levels of membrane CA XII. Staining of tumors from 6A10-treated animals with a rat-specific secondary antibody alone confirmed 6A10 binding to tumor cells. Interestingly, binding was most prominent at the tumor margins but was also evident in intratumoral regions (Fig. 4B). Binding of 6A10 to tumors was also detected in vivo using 6A10 labeled with the infrared dye 800CW (Supplementary Fig. S2). Representative hematoxylin/eosin stains of excised tumors are shown in the Supplementary Fig. S4.

In the next series of experiments, mice were injected with A549/GFP/LUC cells as described above and xenograft-bearing animals were treated with 6A10 or an isotype control antibody once per week (100 µg/mouse), or left untreated. The xenograft growth rate was monitored weekly by longitudinal measurement of whole body bioluminescence. The bioluminescence data of day 81 post inoculation are shown exemplarily in Fig. 5A and the calculated data are presented in Supplementary Fig. S3. These measurements revealed that 6A10 treatment significantly reduced the xenograft growth rate over time, as compared with the isotype antibody which had no protective effect (Fig. 5B). Consequently, 6A10-treated animals reached a tumor burden of 2x10⁸ light units approximately 20 days later (Fig. 5C) and had a significantly extended overall survival time (Fig. 5D). Postmortem inspections (not shown) demonstrated that tumors had spread mainly to the kidney, the liver and the spleen. Since immune effector activities are essentially absent in NSG mice (37), the observed antitumor activity of 6A10 is solely attributable to the direct inhibition of CA XII activity. Taken together, inhibition of CA XII with 6A10 had a significant antitumor effect in vivo.
Discussion

A characteristic feature of the elevated metabolic activity of tumor cells is the generation of ATP by constitutive glycolysis, intracellular accumulation of lactic acid, and, consequently, the ensuing challenge of protecting cells from excessive intracellular acidification. The aforementioned pH-challenge is exacerbated by typically inadequate blood perfusion delivered by an aberrant vasculature, which is also responsible for the hypoxic conditions present in rapidly growing solid tumor tissues. Cancer cells have evolved a sophisticated and efficacious homeostatic system to regulate pH, involving acid/base transporters in collaboration with buffers and carbonic anhydrase (CA) enzymes. The membrane-bound, exo-facial CA isoforms provide catalysis for facilitating CO₂ diffusion, extracellular H⁺ buffering and supplying extracellular HCO₃⁻ for membrane transporters. Collectively, isoforms such as CA IX and CA XII play an important role in pH control, and inhibition of these exo-facial CAs would disturb the pH balance in expressing tumors. As pH is a key phenotypic variable of the tumor chemical milieu, believed to exert selection pressure and drive disease progression, any manipulation of pH may change the trajectory of cancer disease (25). Consequently, targeting cancer metabolism by interfering with pH regulation is nowadays considered a promising therapeutic approach (38).

Our investigation demonstrates the efficacy of the CA XII-inhibiting monoclonal antibody, 6A10, in reducing growth of cancer cells in vitro and in xenograft tumor models in vivo. Our results provide evidence that CA XII optimizes tumor cell growth (Fig 2), but only under conditions where the enzyme is expected to be catalytically-active, that is, when the pH at the start of incubation was sufficiently alkaline to allow the CA catalytic process to persist during the growth-period when media gradually acidify (Fig 3). As evident particularly in CAXII-positive Kato III cells, the growth-retarding effect of 6A10 was particularly strong when starting growth conditions had low HCO₃⁻ and hence low pH, i.e. characteristic of the acidic tumor milieu (23). The catalytic activity of CAXII appears to be more resistant to inhibition at low pH compared to CAIX, as CAXII activity was unaffected over the range 6.7 to 7.7, whereas CAIX is half-inhibited when pH falls to 6.8 (28). Thus the relative contribution of CAIX and CAXII to total membrane-associated CA catalysis will depend strongly on ambient pH. Under conditions of limiting HCO₃⁻ supply and low extracellular pH, exo-facial CA activity may be essential for tumor physiology and growth. Inhibition of the membrane-tethered CAs IX and/or XII may result in an antitumor effect arising from inadequate control of intracellular pH caused by insufficient HCO₃⁻ provision and slow pH-buffering by CO₂/HCO₃⁻. Support for this hypothesis comes from our xenograft model of lung cancer grown in NSG mice. In this experiment, 6A10 had a substantial growth-inhibiting effect in vivo (Fig 5). The growth-delaying effect of CAXII
inhibition is consistent with the role that exo-facial CA isoforms play in facilitating the venting of cellular acid. Intact CAXII activity normally allows for facilitated CO\textsubscript{2} diffusion, resulting in apparently smaller diffusional delays for CO\textsubscript{2} removal, allowing tumors to attain larger volumes. NSG mice are heavily immunocompromised and show no detectable activities of the adaptive and innate immune system. Consequently, it is unlikely that effector functions like ADCC or CDC would have contributed towards the observed antitumor activity of 6A10. Instead, the effect of the antibody on xenograft growth is most likely linked to CA XII inhibition.

Although a significant antitumor effect of 6A10 was observed \textit{in vivo}, our \textit{in vitro} obtained with HT29 cells, endogenously expressing both CA IX and CA XII, argue for a degree of redundancy between exo-facial CA isoforms. Since CA IX and XII co-expression is found in at least some tumor cells (39-43), and in line with results obtained by Chiche et al. (17), it is tempting to speculate that the concurrent and selective inhibition of both enzymes would result in an improved and additive antitumor effect. Since hypoxia is already evident in non-vascularized micrometastases, there would also be mileage in investigating whether CA XII inhibition could also impair metastasis formation.

The results presented herein demonstrate that inhibition of CA XII catalysis can substantially interfere with tumor growth, highlighting this enzyme as a promising target for novel immunotherapeutic approaches, also in conjunction with CA IX targeted therapy.

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References


Figure Legends

Figure 1: Measuring CA XII expression, activity and inhibition by 6A10. (A) Western blot for CA XII, CA IX and actin (loading control) in A549 and T47D cell lysates, prepared from cells incubated under control normoxic conditions (DMSO) and in the presence of 1 mM dimethylxalyl glycine (DMOG) to evoke hypoxic signaling. Alternative splice variants of CAXII underlie the presence of several bands. (B) Immunoprecipitation of CA XII from A549 lysates with the 6A10 antibody. (C) Immunofluorescence of A549 and T47D monolayers stained for CA XII with 6A10 (green) and for nuclei with Hoechst 33342 (blue). (D) Assay for CA activity. (i) Representative time courses recorded from suspensions containing T47D membrane fragments. Spontaneous CO₂ hydration kinetics were measured in the presence of the broad-spectrum CA inhibitor acetazolamide (ATZ; 100 µM). (ii) Dose-response curve determined in A549 membrane fragments (n=5 per dose). (iii) Summary of CA activity data for the soluble and membrane fractions obtained from A549 lysates. (iv) Data for T47D lysates. Doses: ATZ, 100 µM; MSC8, 10 µg/ml; 6A10, 10 µg/ml. (E) Measuring CA activity at the external surface of intact A549 cells (n=10), tagged with wheat germ agglutinin (WGA) conjugate of fluorescein. Superfusion with 5% CO₂/12 mM HCO₃⁻ buffered solutions. pH₆ transients were measured upon adding and then removing 20mM NH₄⁺-containing solution. Exo-facial CA activity decreases the size of these pH₆ transients due to faster buffering by CO₂/HCO₃⁻. 20 µg/ml 6A10 inhibited exo-facial CA activity completely (to levels recorded with 100 µM ATZ). (F) Experiments repeated on T47D cells (n=10).

Figure 2. 6A10 interferes with the growth of tumor cells. (A) A549 cells and (B) Kato III cells were grown in standard medium with 44mM bicarbonate or in HEPES-buffered medium without bicarbonate (0mM) for three days at a constant CO₂ partial pressure of 5%. Over the course of the experiment, medium HCO₃⁻ and pH will change, but differences between experimental conditions will persist. After three days of growth, cell proliferation was measured in an MTT assay at 595nm. (C) CA IX is induced in HT29-shEV cells growing in hypoxia (1% O₂), whereas the gene is efficiently knocked-down in HT29-shCA9 cells. CA IX knockdown had no significant effect on CA XII expression levels. GAPDH = loading control. (D) CA XII is expressed at comparable levels on the surface of HT29-shCA9 and HT29-shEV cells. Expression is enhanced modestly under hypoxia (bold line) as compared to normoxia (dotted line). Tinted histogram shows the isotype control. (E) HT29-shCA9 cells and (F) HT29-shEV cells were seeded in the media described in (A) and proliferation was measured in an MTT assay. *p<0.05.
Figure 3. The inhibitor effect of 6A10 on cell growth depends on the starting pH of culture media. (A) A549 cells were seeded in HEPES-buffered, bicarbonate-free cell culture media adjusted to different pH values, and incubated in an atmosphere of 5% CO$_2$ over the course of 3 days, media will become gradually more acidic due to metabolism, and medium HCO$_3^-$ will be given by the Henderson-Hasselbalch equation (higher HCO$_3^-$ at higher pH). Proliferation was measured in an MTT assay three days later. *p = 0.026; **p = 0.004. (B) Data replotted from (A), showing extent of growth inhibition by 6A10. Proliferation in 6A10 was plotted against proliferation in isotype, matched for starting medium pH (indicated in brackets). Straight line is best-fit through the data-points at acid pH (6.2-6.8), at which 6A10 did not have a growth-inhibiting effect. Growth inhibition was significant (P<0.05) for starting pH >7.0.

Figure 4. CA XII is expressed on, and 6A10 binds to, A549 xenografts. (A) Cryosections of excised xenografts from a 6A10-treated animal were stained with a Cy3-labeled secondary antibody to detect infiltrated 6A10. (B) Single cell suspensions of excised xenografts were stained with 6A10 and a Cy5-labeled secondary antibody and analyzed by flow cytometry.

Figure 5. 6A10 inhibits the growth of xenografts in vivo. (A) NSG mice were inoculated with A549/GFP/LUC cells and the tumor growth rate was measured by whole body bioluminescence once per week as described in Materials and Methods. Shown is the measurement at day 81 after inoculation. Two animals of the isotype group and one animal of the untreated group did not develop tumors and were therefore excluded from the experiment. (B) Longitudinal growth of xenografts as measured by bioluminescence. (C) Calculation of the time post infection when tumor burden reached a light emission of 2x10$^8$ photons/seconds (p/s) as determined by BLI. ***p<0.001; ****p<0.0001. (D) Survival curves of tumor-bearing animals. Mantel-Cox tests revealed p=0.02 for the 6A10-treated group vs. the isotype-treated group and p=0.037 for the 6A10-treated group vs. the untreated group.
Figure 1

A. Western blot analysis of CA IX and CA II in cell lysates treated with DMOG (DM) or DMOG (DMSO) and IP with 6A10.

B. Immunofluorescence images of A549 and T47D cells stained for CA and actin. Scale bar: 30 μm.

Di. pH changes in medium upon exposure to control, 6A10, and +ATZ.

ii. CO₂ hydration rate as a function of pH for A549 membrane fragments.

iii. CA activity (normalized) in A549 cells treated with 6A10 (μg/ml).

iv. 10³ × Kₘ (s⁻¹) per mg/ml protein for A549 and T47D cells treated with ATZ, MSC8, 6A10, and +ATZ.

v. 10³ × Kₘ (s⁻¹) per mg/ml protein for A549 and T47D cells treated with ATZ, MSC8, 6A10, and +ATZ.

E. Surface pH of A549 cells (untreated) and A549 cells + 6A10 treated with 20 mM NH₄⁺ with and without ATZ.

F. Surface pH of T47D cells (untreated) and T47D cells + 6A10 treated with NH₄⁺ with and without ATZ.
Antitumor efficacy of a monoclonal antibody that inhibits the activity of cancer-associated carbonic anhydrase XII

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