Antitumor Efficacy of a Monoclonal Antibody That Inhibits the Activity of Cancer-Associated Carbonic Anhydrase XII

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Introduction
Carbonic anhydrase XII (CA XII) is, alongside a related isoform IX, a cancer-associated, membrane-tethered exofacial enzyme. Early evidence has suggested that CA XII expression is upregulated under hypoxic conditions (1, 2), akin to CA IX. In contrast to CA IX, CA XII 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 carbonic anhydrase 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 serodiagnostic biomarker for lung cancer (15) and as a molecular marker for the detection of breast cancer lymph node metastasis (16).

Abstract
Carbonic anhydrase XII (CA XII) is a membrane-tethered cell surface enzyme that is highly expressed on many human tumor cells. Carbonic anhydrase 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. Cancer Res; 73(21); 6494–503. ©2013 AACR.
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 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 xenograft mouse models. Our findings confirm the antibody's significant antitumor activity and highlight the potential of immunotherapy in the treatment of CA XII-expressing cancers.

Materials and Methods

Antibodies, cells 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 in vitro 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 obtained from ATCC and were identified in detail elsewhere (28). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM; Sigma) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Sigma), Glutamine–Penicillin–Streptomycin mixture (Sigma) and incubated at 37°C with 5% CO2-enriched air. Cell lines were obtained from ATCC and were identified April 2013 by STR DNA testing conducted at Eurofins.

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

Immunofluorescence

Cells were plated on a cover slip in a 35 mm Petri dish, grown to 100% confluence, then fixed using 1 mL of pre-cooled methanol (−20°C) for 10 minutes at 4°C. Antigen blocking was conducted with a 1% bovine serum albumin and PBS solution for 30 minutes. Subsequently, cells were incubated with the primary rat monoclonal antibody 6A10 for 1 hour at 37°C. The sample was then washed with PBS and 0.2% Tween-20 four times, for 10 minutes each time. Thereafter, cells were incubated with the secondary goat anti-rat Alexa Fluor 488 antibody (green), diluted by 1:1,000 in blocking solution for 1 hour at 37°C. To simulate hypoxia cells were treated with 1 mmol/L DMOG for 48 hours. Control cells were treated with DMSO for 48 hours to simulate dense normoxic cells. Cells were imaged with a Zeiss Confocal Laser Scanning Microscope (LSM) 700 system.

Immunoprecipitation of CA XII

Eight micrograms of the anti-CA XII antibody 6A10 was mixed with 200 μL of protein-L agarose beads in 1 mL PBS and incubated for 1 hour at room temperature. The beads were washed with PBS thrice, centrifuged for 2 minutes each, then halved. The anti-6A10 rat monoclonal antibody was added to either DMSO or DMOG A549 cell lysates, each 500 μL, and the bead–antibody mixtures were incubated on a shaker overnight at 4°C. Both samples were then centrifuged at 15,000 rpm for 20 seconds. The lysates were removed, and each sample washed thrice with PBS and centrifuged for 2 minutes each time. Forty-ﬁve microliters of Laemmli loading buffer containing β-mercaptoethanol were added and samples boiled at 100°C for 5 minutes. The samples were then centrifuged and loaded into lanes to conduct polyacrylamide electrophoresis and Western blot analysis.

Western blotting

A549 and T47D cells were lysed at 4°C with buffer containing in (mmol/L): 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 13,000 rpm for 20 minutes at 4°C. Proteins were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Antigen blocking was conducted 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-CA XII (R&D Biosystems) antibody; monoclonal mouse M75 antibody raised against human CA IX; and polyclonal rabbit antibody raised against CA II (both kind gifts from Prof. S. Pastorekova, Slovak Academy of Sciences, Bratislava, Slovakia), polyclonal goat antibody raised against actin (Santa Cruz). The primary incubation of membranes was followed by incubation with horse-radish peroxidase-conjugated secondary antibodies; both for 1 hour on a shaker at room temperature. Enhanced chemiluminescence (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

Carbonic anhydrase activity was determined from membrane fragments extracted from A549 and T47D cells grown to 90% to 100% confluency. Cells were washed with ice-cold PBS and scraped down into ice-cold buffer containing in (mmol/L): 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 9,000 rpm at 4°C to pellet the membrane fraction, which was then resuspended in fresh buffer. The centrifugation and buffer-replacement process was conducted twice to prevent contamination of cell membrane solution with cytosolic carbonic anhydrase isoforms.

Measurement of carbonic anhydrase activity in the membrane fraction of lysed cells

A 0.67 mL aliquot of membrane-fragment suspension was added to a 2 mL vessel, well stirred, and cooled to 2°C.
pH electrode (Biotrode) was inserted to monitor solution pH at 1 Hz. To start the carbonic anhydrase-catalyzed reaction, a 0.33 mL 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 conducted on membrane-free “blank buffer” samples and also in samples treated with 100 μmol/L acetazolamide. The increase in CO2 hydration rate above the spontaneous rate is a measure of carbonic anhydrase catalysis (22).

Solutions
Solutions contained (in mmol/L): 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 2 mL), the surface of which was pretreated with 0.01% poly-L-lysine to facilitate cell/spheroid adhesion. Solutions were heated to 37°C and delivered at a constant rate of 2 mL/min. Downstream suction was adjusted to maintain a steady-state solution volume of approximately 0.5 mL. Cells were exposed for 3 minutes to medium containing 50 μmol/L WGA-fluorescein (Invitrogen), a membrane-tagging dye that reports pH at the extracellular surface of the cell, near the site of CA XII catalysis (21). The dye was excited alternately at 405 and 488 nm 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 mmol/L HEPES, 10 mmol/L MES, 120 mmol/L NaCl, 4.5 mmol/L KCl, 10 mmol/L glucose, 1 mmol/L MgCl2, 1 mmol/L CaCl2) at pH 6.8 and 7.4.

Statistical analysis of data
SE 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. Eight thousand cells in 20 μL DMEM with the 6A10 or an isotype antibody at a final concentration of 20 μg/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 descissions and incubated at 37°C. Cell proliferation was calculated with MTT assays.

Immunocytochemistry
Excised xenografts were cryopreserved and cut into 4 μm sections, stained with a Cy3- or fluorescein isothiocyanate-coupled goat anti-rat secondary antibody and imaged using a Zeiss Axioscience Z1 microscope.

Xenograft studies
NSG mice (NOD/SCID/IL2 receptor gamma chain knock-out) were obtained from Jackson Laboratory 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 Hank’s balanced salt solution (HBSS), and 100,000 cells per mouse in a final volume of 200 μL 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 (10–500 μg) and 100 μg/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 100 μg 6A10 or an anti-GST isotype antibody once per week or left untreated (n = 8 for each group). No antibody-related toxicity was observed, that is, 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) as described (33). Briefly, mice were anesthetized with isoflurane and fixed in the imaging chamber. One hundred micrometers of coelenterazine (Synchem) was then injected into the tail vein and animals were immediately imaged for 15 seconds using a field of view of 12.5 cm with open emission filter settings, binning at 8 and f/stop at 1. Quantification was conducted 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 shown an inhibitory effect of 6A10 on recombinant CA XII (inhibitory constant IC50 = 5.7 mmol/L; 31), the first anti-CA XII monoclonal antibody with biologic 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 showed that both cell lines express CA XII protein under normoxic incubation and following 48-hour treatment with the hypoxia-inducible factor (HIF)-stabilizing drug dimethylxalyl glycine (DMOG; 1 mmol/L) to induce hypoxic responses (Fig. 1A). In addition, CA IX expression was also detected in A549 cells (Fig. 1A). Immunoprecipitation of CA XII protein from A549 whole-cell lysates...
using 6A10 confirmed selective antibody binding to CA XII (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 pretreated with DMOG (data not shown).

Figure 1. Measuring CA XII expression, activity, and inhibition by 6A10. A, Western blot analysis 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 mmol/L DMOG to evoke hypoxic signaling. Alternative splice variants of CA XII 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 nuclei with Hoechst 33342 (blue). D, assay for carbonic anhydrase activity. Representative time courses recorded from suspensions containing T47D membrane fragments (i). Spontaneous CO₂ hydration kinetics were measured in the presence of the broad-spectrum carbonic anhydrase inhibitor acetazolamide (ATZ; 100 μmol/L). Dose-response curve determined in A549 membrane fragments (ii). Summary of carbonic anhydrase activity data for the soluble and membrane fractions obtained from A549 lysates (iii). Data for T47D lysates (iv). Dose: acetazolamide (ATZ), 100 μmol/L; MSC8, 10 μg/mL; 6A10, 10 μg/mL. E, measuring carbonic anhydrase activity at the external surface of intact A549 cells (n = 10), tagged with WGA conjugate of fluorescein. Superfusion with 5% CO₂/12 mmol/L HCO₃⁻ buffered solutions. pH₄ transients were measured upon adding and then removing 20 mmol/L NH₄⁺-containing solution. Exofacial carbonic anhydrase activity decreases the size of these pH₄ transients due to faster buffering by CO₂/HCO₃⁻. 20 μg/mL 6A10 inhibited exofacial carbonic anhydrase activity completely (to levels recorded with 100 μmol/L acetazolamide). F, experiments repeated on T47D cells (n = 10).

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 carbonic anhydrase isoforms. The carbonic anhydrase-catalyzed...
reaction was initiated by adding 100% CO$_2$-saturated water to buffered suspensions (20 mmol/L HEPES + 20 mmol/L MES; pH 8.0 at 4°C) containing cell membrane fragments (12 v/v). The rate of the H$^+$-yielding CO$_2$ hydration reaction (monitored by measuring medium pH with an electrode) is related to carbonic anhydrase activity (Fig. 1D, i). 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. 1D, ii) indicate that the membrane-bound carbonic anhydrase activity is pH-insensitive over the range 6.7–7.7. The addition of 6A10 reduced carbonic anhydrase catalysis in a dose-dependent manner (Fig. 1D, iii) towards the spontaneous rate (confirmed by experiments in the presence of the broad-spectrum carbonic anhydrase inhibitor acetazolamide, 100 μmol/L). The IC$_{50}$ of 6A10 measured in A549 membrane fragments was 0.96 μg/mL, and therefore 10 μg/mL was deemed to inhibit CA XII fully (Fig. 1D, iii). 6A10 (30 μg/mL) did not affect measured carbonic anhydrase activity in the strongly CA IX-positive breast line MDA-MB-468 (97 ± 13% of control activity; ref. 23), establishing that 6A10 does not cross-react with this other exofacial carbonic anhydrase. The near-full inhibition of carbonic anhydrase 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 carbonic anhydrase catalysis, despite CA IX immunoreactivity (Fig. 1A). This was confirmed by the absence of any inhibitory effect of the anti-CA IX antibody M5866 (which blocks up to 57% of CA IX activity in CA IX-overexpressing cell lines; Fig. 1D, iv; ref. 34). Results of the assay are summarized in Fig. 1D, iv (A549 cells) and Fig. 1D, v (T47D cells). Per gram of total protein, membrane fragments had higher carbonic anhydrase activity than the soluble fraction in both cell lines and 10 μg/mL 6A10 potently inhibited membrane carbonic anhydrase activity.

Further characterization of 6A10 was conducted 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% CO$_2$/12 mmol/L HCO$_3^-$ at pH = 7.2. Rapid exposure to and subsequent withdrawal of 20 mmol/L ammonium-containing solution by means of solution-switching evoked surface-pH$_e$ transients: exposure to ammonium evoked transmembrane NH$_4^+$ entry and extracellular NH$_4^+$ deprotonation at the extracellular membrane surface (producing a fall in pH$_e$), whereas ammonium removal evoked the reverse reaction (rise in pH$_e$). The ability of extracellular CO$_2$/HCO$_3^-$ to buffer these surface-pH$_e$ transients depends on exofacial carbonic anhydrase activity. Out-of-equilibrium surface-pH$_e$ transients were significantly larger in the presence of acetazolamide (Fig. 1E and F) in both T47D and A549 cells, confirming that both cell lines have considerable extracellular-facing carbonic anhydrase activity. Experiments were repeated on cells pretreated with 20 μg/mL 6A10 for 24 hours. Surface-pH$_e$ transients were larger and no longer affected by treatment with acetazolamide, indicating that 6A10 had inhibited exofacial carbonic anhydrase activity (Fig. 1E and F). Overall, these data show that the 6A10 antibody is a highly potent, full inhibitor of exofacial CA XII catalytic activity in A549 and T47D cells natively expressing the protein. In A549 cells, which express both CA IX and CA XII protein, the latter is the major contributor to total extracellular-facing carbonic anhydrase activity.

**6A10 inhibits cancer cell growth**

The effect of CA XII inhibition on the growth of CA XII-positive cells (A549 and Kato III) was measured using the MTT assay. The starting concentration of HCO$_3^-$ was varied, at constant CO$_2$ partial pressure, to alter the time-course of medium [HCO$_3^-$] and pH over a 3-day growth period (whereby higher medium [HCO$_3^-$] 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 [HCO$_3^-$], i.e., under conditions were HCO$_3^-$ availability is reduced throughout the growth period, relative to high-starting [HCO$_3^-$] controls. The HCO$_3^-$-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 [HCO$_3^-$].

Many cell lines and cancers in vivo coexpress CA XII along with the exofacial 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 carried out on the HT29 colon cancer cell line, which expresses both carbonic anhydrase 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; ref. 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 II expression. HT29-shCA9 and HT29-shEV cells were grown under hypoxia (1% O$_2$) for 3 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 mmol/L 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 carbonic anhydrase activity conferred by CA XII in HT29 cells is adequate to maintain normal growth under hypoxia. This observation supports the notion of a critical role of CA XII 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 HCO$_3^-$-free medium titrated to
different starting pH levels. Over the course of growth, these media are expected to acidify further due to metabolism. After 3 days of growth, cell proliferation was measured in an MTT assay at 595 nm. C, CA IX was induced in HT29-shEV cells growing in hypoxia (1% O2), whereas the gene was 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 was expressed at comparable levels on the surface of HT29-shCA9 and HT29-shEV cells. Expression was enhanced modestly under hypoxia (bold line) as compared with normoxia (dotted line). Tinted histogram shows the isotype control. HT29-shCA9 cells (E) and HT29-shEV cells (F) were seeded in the media described in A and proliferation was measured in an MTT assay. *, P < 0.05.

Figure 2. 6A10 interferes with the growth of tumor cells. A549 cells (A) and Kato III (B) cells were grown in standard medium with 44 mmol/L bicarbonate or in HEPES-buffered medium without bicarbonate (0 mmol/L) for 3 days at a constant CO2 partial pressure of 5%. Over the course of the experiment, medium HCO3− and pH changed, but differences between experimental conditions persisted. After 3 days of growth, cell proliferation 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 approximately 7.2. Of interest, cells did not survive pH values higher than 7.3 in nominally HCO3−/C02− 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 growing 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 versus starting pH because of on-going medium acidification over 3 days of culture. Overall, the data indicate that anti-CA XII antibody growth-retarding efficacy relies critically on the antibody's ability to block carbonic anhydrase 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. A total of 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. First, 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 CA IX, whereas the gene was 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 was expressed at comparable levels on the surface of HT29-shCA9 and HT29-shEV cells. Expression was enhanced modestly under hypoxia (bold line) as compared with normoxia (dotted line). Tinted histogram shows the isotype control. HT29-shCA9 cells (E) and HT29-shEV cells (F) were seeded in the media described in A and proliferation was measured in an MTT assay. *, P < 0.05.
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 800 CW (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 postinoculation 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 $2 \times 10^8$ light units approximately 20 days later (Fig. 5C) and had a significantly extended overall survival time (Fig. 5D). Post mortem inspections (not shown) showed that tumors had spread mainly to the kidney, the liver, and the spleen. Because 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

![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% CO₂ over the course of 3 days; media became gradually more acidic due to metabolism, and medium HCO₃⁻ was given by the Henderson-Hasselbalch equation (higher HCO₃⁻ at higher pH). Proliferation was measured in an MTT assay 3 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 and matched for starting medium pH (indicated in brackets). Straight line was 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.](image)

![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.](image)
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 pHᵢ, involving acid/base transporters in collaboration with buffers and carbonic anhydrase enzymes. The membrane-bound, exofacial carbonic anhydrase isoforms provide catalysis for facilitating CO₂ diffusion, extracellular H⁺ buffering and supplying extracellular...
HCO$_3^-$ for membrane transporters. Collectively, isoforms such as CA IX and CA XII play an important role in pH control, and inhibition of these exofacial carbonic anhydrases 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 shows 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 carbonic anhydrase catalytic process to persist during the growth period when media gradually acidify (Fig. 3). As evident particularly in CA XII-positive Kato III cells, the growth-retarding effect of 6A10 was particularly strong when starting growth conditions had low HCO$_3^-$ and hence low pH, i.e., characteristic of the acidic tumor milieu (23). The catalytic activity of CA XII seems to be more resistant to inhibition at low pH compared with CA IX, as CA XII 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 CA IX and CA XII to total membrane-associated carbonic anhydrase catalysis will depend strongly on ambient pH. Under conditions of limiting HCO$_3^-$ supply and low extracellular pH, exofacial carbonic anhydrase activity may be essential for tumor physiology and growth. Inhibition of the membrane-tethered carbonic anhydrases IX and/or XII may result in an antitumor effect arising from inadequate control of intracellular pH caused by insufficient HCO$_3^-$ provision and slow pH-buffering by CO$_2$/HCO$_3^-$. 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-retarding effect of CA XII inhibition is consistent with the role that exofacial carbonic anhydrase isoforms play in facilitating the venting of cellular acid. Intact CA XII activity normally allows for facilitated CO$_2$ diffusion, resulting in apparently smaller diffusional delays for CO$_2$ 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 antibody-dependent cellular cytotoxicity or complement-dependent cellular cytotoxicity 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 in vivo, our in vitro obtained with HT29 cells, endogenously expressing both CA IX and CA XII, argue for a degree of redundancy between exofacial carbonic anhydrase isoforms. Because CA IX and XII coexpression is found in at least some tumor cells (2, 8, 10, 39, 40), and in line with results obtained by Chiche and colleagues (17), it is tempting to speculate that the concurrent and selective inhibition of both enzymes would result in an improved and additive antitumor effect. As 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 show 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.

Disclosure of Potential Conflicts of Interest
R. Zeidler has ownership interest (including patents) in patent application. No potential conflicts of interest were disclosed by the other authors.

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