Molecular radiobiology

Specific inhibition of carbonic anhydrase IX activity enhances the *in vivo* therapeutic effect of tumor irradiation

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ABSTRACT

Background and purpose: Carbonic anhydrase (CA) IX expression is increased upon hypoxia and has been proposed as a therapeutic target since it has been associated with poor prognosis, tumor progression and pH regulation. The aim of this study was to evaluate the antitumor activity of a high CAIX-affinity indane-sulfonamide (11c) combined with irradiation, compared with the general CA inhibitor acetazolamide (AZA).

Material and methods: HT-29 carcinoma cells with or without (genetic knockdown, KD) CAIX expression were incubated with 11c/AZA under different oxygen levels and proliferation, apoptosis and radiosensitivity were evaluated. 11c/AZA was administered intravenously ($1\times$/day; 5 days) to tumor-bearing mice and tumor irradiation (10 Gy) was performed at day 3 of the injection period. Tumor growth and potential treatment toxicity were monitored ($3\times$/week).

Results: Treatment with 11c/AZA alone resulted in tumor regression, which was further increased in CAIX expressing cells by combining 11c with irradiation. AZA demonstrated also an additional effect in the KD tumors when combined with irradiation. CAIX inhibition *in vitro* significantly reduced proliferation and increased apoptosis upon hypoxia exposure without affecting intrinsic radiosensitivity.

Conclusions: Specific inhibition of CAIX activity enhanced the effect of tumor irradiation and might, therefore, be an attractive strategy to improve overall cancer treatment.

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High tumoral carbonic anhydrase (CA) IX expression has been associated with poor prognosis, tumor progression and aggressiveness [1,2]. CAIX is a dimeric glycoprotein, contains an HRE element essential for its transcriptional activation upon hypoxia by HIF-1α and regulates tumor pH by catalyzing the reversible hydration of carbon dioxide to bicarbonate and a proton [2,3]. Since the active site of CAIX resides in the extracellular space, it contributes to the acidification of the extracellular environment during hypoxia [4]. Furthermore, it has been shown that CAIX plays an important role in maintaining a neutral intracellular pH within tumors. The bicarbonate ion resulting from the CAIX catalyzed reaction can be imported into the cytoplasm by exchange with a chloride anion to serve as substrate for intracellular carbonic anhydrases such as CAII in order to neutralize intracellular protons [5]. Since CAIX is implicated in both extra – and intracellular pH regulation, it has been proposed as a potential therapeutic target.

A possible approach to target CAIX would be via inhibiting its enzymatic activity with specific pharmacological inhibitors [6]. Previously, we have demonstrated that binding of sulfonamides to CAIX requires both its expression and its activation and this occurs only during hypoxia [7]. Also in animal models, sulfonamide accumulation was proven to be dependent on the tumor oxygenation [8]. Administration of membrane-impermeable derivatives of acetazolamide, a general carbonic anhydrase inhibitor, resulted in tumor growth inhibition [9]. Aromatic sulfonamides were able to reduce tumor cell proliferation and intracellular pH, accompanied with an increase in ceramide-mediated apoptotis [10]. Recently, treatment of mammary tumor-bearing mice with novel CAIX-specific (ureido)-sulfonamide and glycosylcoumarins inhibitors resulted in a significant inhibition of tumor growth and metastasis formation [1].

These reports emphasize that CAIX-specific sulfonamides are promising to pursue for their tumor-specific therapeutic properties with irradiation. The aim of this study was to explore in genetic

models the antitumor activity of an indanesulfonamide (11c) with high affinity for CAIX, compared with the general clinically approved CA inhibitor acetazolamide (AZA). First we investigated the binding property of a fluorescent labeled sulfamate (FC11) to validate the genetic models. Furthermore, we hypothesize that specific inhibition of CAIX activity only targets CAIX under hypoxia resulting in decreased tumor cell proliferation, induction of apoptosis and an enhanced effect of irradiation *in vivo* in a CAIX dependent manner.

Material and methods

Cell culture and model

Exponentially growing colorectal (HT-29, ATCC HTB-38) carcinoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. To knockdown *CA9*, the HuSH-29 shRNA targeting *CA9* (TR314250) and empty vector (R20003) were purchased from Origene. Cell lines were transfected using FuGENE 6 (Roche, Germany) according to the manufacturer's instructions. Cells were then grown under selective pressure (300 ng/ml puromycin) until no mock-transfected cells remained. Two individual clones were selected using cloning cylinders (Sigma) and maintained under selection. Data of the independent clone KD2 are presented in Supplementary Fig. 1. Low oxygen conditions were acquired in a MACS VA500 micro-aerophilic workstation (Don Whitley Scientific, Shipley, UK). The atmosphere in the chamber consisted of 0.2% (hypoxia) or <0.02% (anoxia, 0%) O_2, 5% CO_2 nd residual N_2 [11]. In parallel, normoxic (20% O_2) dishes were incubated in air with 5% CO_2.

Inhibition CAIX activity

The fluorescent labeled sulfamate (FC11) directed against CAIX has been prepared by reaction of sulfamyl chloride with the residue obtained from the reaction of fluorescein isothiocyanate (FITC) with 4-aminophenol (see Supplementary data). 11c was synthesized as previously described [12] and acetazolamide (AZA) was obtained from Sigma. The structure and K_i value of the different compounds are depicted in Fig. 1A. Compounds were dissolved in culture medium containing 1% DMSO at a final concentration of 100 μM just before addition to the cells. For the animal experiments, 11c and AZA were dissolved in NaCl 0.9% containing 1% DMSO to a final concentration of 45 mg/kg and injected intravenously via a lateral tail vein.

Immunoblotting and quantitative real-time PCR

Experiments were performed as previously described [8,13]. Antibodies used were M75 (kindly provided by Silvia Pastorekova, Institute of Virology, Slovak Academy of Science, Bratislava, Slovak Republic) against CAIX or β-actin (Cell Signaling) as loading control. CAIX (F-CATCCTAGCCCTGGTTTTTGG, R-GCTCACACCCCCTT TGGTT) and VEGF (F- GACTCCGGCGGAAGCAT, R- TCCGGGCTC GGTGATTTA) gene abundance was detected with power SYBR® Green I (Applied Biosystems) and normalized to the expression levels of 18S RNA.

FACS analysis

Cells were incubated with FC11 30 min before fixation in freshly prepared 2% paraformaldehyde. Apoptosis was detected using Alexa Fluor® 488 annexin V (Molecular Probes) and propidium iodide (PI; Sigma) according to the manufacturer's guidelines. Fluorescence was analyzed using flow cytometry (FacsSort, BD Biosciences) with FITC and TRITC settings and data were normalized

to the signal intensity of normoxic exposed cells. Apoptotic cells were scored as annexin V positive and PI negative.

Immunofluorescence

Cells were grown in culture slides (BD Biosciences), incubated with FC11, fixed with 2% paraformaldehyde and fluorescence was visualized using a Zeiss Axioskop. Cells for apoptosis staining were permeabilized using 0.2% Triton X-100 (Sigma). Non-specific binding was blocked using 5% normal goat serum (RT, 2 h). Cells were incubated (4 °C, ON) with cleaved Caspase-3 monoclonal antibody (Cell Signaling) followed by incubation (RT, 2 h) goat anti-rabbit Alexa Fluor® 488 conjugate. Before mounting with Shandon Immunomount (Thermo Fisher), cells were incubated with DAPI (rt, 10 min).

CA activity assay

Carbonic anhydrase activity was determined as described previously [14]. Cells were washed with cold isotonic HBS buffer and incubated (20 min) with sulfonamide. pH was monitored over time before and after addition of cold CO_2 saturated solution.

Lactic acid assay

Lactic acid concentration in the culture medium of cells exposed to sulfonamide treatment was determined using the Lactic Acid kit (Biosentec) following the manufacturer's instructions.

Animal experiments

Cells were resuspended in Basement Membrane Matrix (Matrigel™ BD Biosciences) and injected subcutaneously into the lateral flank of adult NMRI-*nu* (nu/nu) mice (28–32 g). Animals were treated with sulfonamides at a tumor volume of 165 ± 58 mm³ for 5 days (45 mg/kg daily) and irradiated with a single dose (10 Gy, [15]) in the middle of the treatment. Tumor growth was monitored until reaching 4× the volume at irradiation time and treatment toxicity was scored by body weight measurements. Animal facilities and experiments were in accordance with local institutional guidelines for animal welfare and were approved by the Animal Ethical Committee of the University.

Cell growth

Cells were seeded at 5000 cells/well in 24-well plates and exposed for normoxia or hypoxia for 1–3 days. After incubation, cells were fixed with 4% paraformaldehyde (10 min) and stained with 0.1% Crystal violet solution (30 min). Extraction was done with 10% acetic acid (15 min) and absorbance at 590 nm was measured (FLUOstar Omega).

Clonogenic survival

Cells were exposed to normoxia or anoxia for 24 h, subsequently irradiated (N: 2, 4, 6 and 8 Gy; A: 4, 8, 12 and 16 Gy; 225 kV Philips X-ray tube), trypsinized and plated in triplicate for clonogenic survival. Cells were allowed to form colonies during 14 days which were fixed and stained with 4% methylene blue in 70% ethanol. Afterward, colonies were counted in an automated way (Oxford Optronix).

Statistics

All statistical analyses were performed with GraphPad Prism version 5.01 for Windows (GraphPad Software, 2007, California, USA).
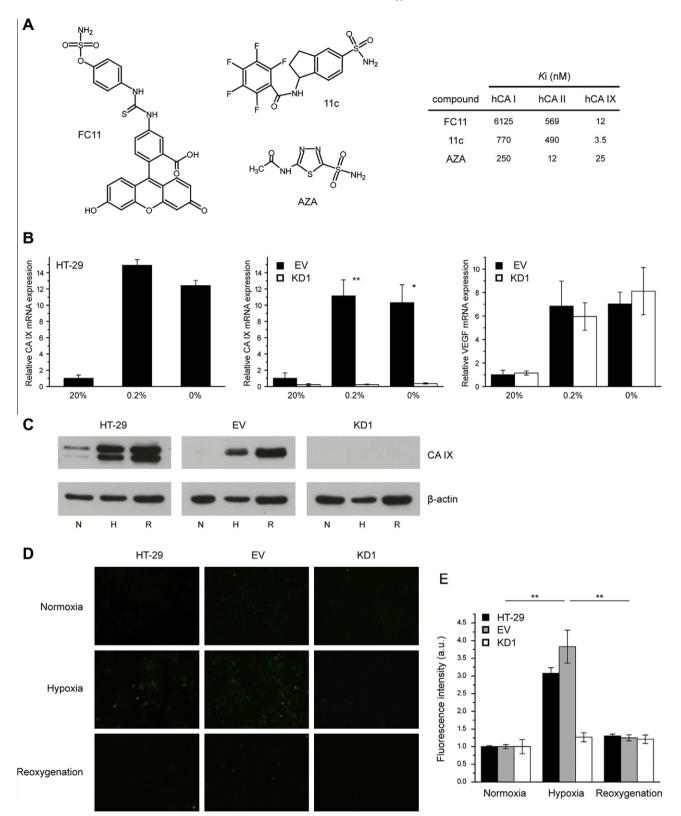
Fig. 1. (A) Chemical structure and affinity (K_i) for CAI, CAII and CAIX of the investigated CA inhibitors. (B) CAIX and VEGF mRNA expression under different oxygen concentrations (%) relative to normoxia for HT-29 parental, EV and KD1 cells. (C) Western blot analysis of CAIX expression in normoxic (N), hypoxic (H) or reoxygenated (R: 0.2% O_2, 24 h followed by 1 h 20% O_2) conditions for the different cell lines. β-Actin was used as the loading control. (D) Representative fluorescence analysis of cells treated with 100 μM FC11 under the respective oxygen conditions. (E) Quantitative FACS analysis of FC11 binding relative to normoxia. Data represent the mean ± SD of at least three independent experiments. Asterisk indicates significant difference (*$P < 0.05$, **$P < 0.01$). Data for an independent CAIX KD (KD2) are shown in Supplementary Fig. 1.

An unpaired student's *t*-test and non-parametric Mann–Whitney U test for small groups were used to determine the statistical signifi- cance of differences between two independent groups of variables. For all tests, a $P < 0.05$ was considered significant.

Results

HT-29 colorectal carcinoma cells showed elevated *CA9* mRNA and CAIX protein levels in response to reduced oxygen concentrations, which remained high upon reoxygenation (Fig. 1B and C). To investigate the role of CAIX in extracellular acidosis and to facilitate the establishment of a causal relationship between CAIX expression and its therapeutic effect in more detail, *CA9* was silenced genetically in a constitutive manner. A significant reduction in *CA9* mRNA levels was observed for the KD cell line at 0.2% (hypoxia; $P = 0.002$) and 0% (anoxia; $P = 0.029$) O_2 as compared with a scrambled control (EV) cell line, which demonstrated similar expression levels as the HT-29 parental cell line. Additionally, CAIX protein levels were abolished in the KD cells (Fig. 1C). Furthermore, other HIF responsive genes were not affected as demonstrated by the increased *VEGF* expression levels upon reduced oxygen concentrations for both cell lines (Fig. 1B).

To define the oxygen conditions required for inhibitor binding, accumulation of the fluorescent sulfamate FC11 with high affinity for CAIX ($K_i = 12$ nM) was investigated. Significant higher ($P = 0.009$) FC11 binding was observed at HT-29 and EV cells exposed to hypoxia (0.2%) as compared with their normoxic counterparts (Fig. 1D and E), corresponding with elevated CAIX expression. Binding was dramatically reduced ($P = 0.002$) compared with hypoxic conditions when FC11 was added upon reoxygenation, although CAIX expression levels remained high. No FC11 accumulation was observed at KD cells, corresponding with the absence of CAIX expression.

Next we examined whether CAIX depletion resulted in a decreased capacity to acidify the extracellular environment upon
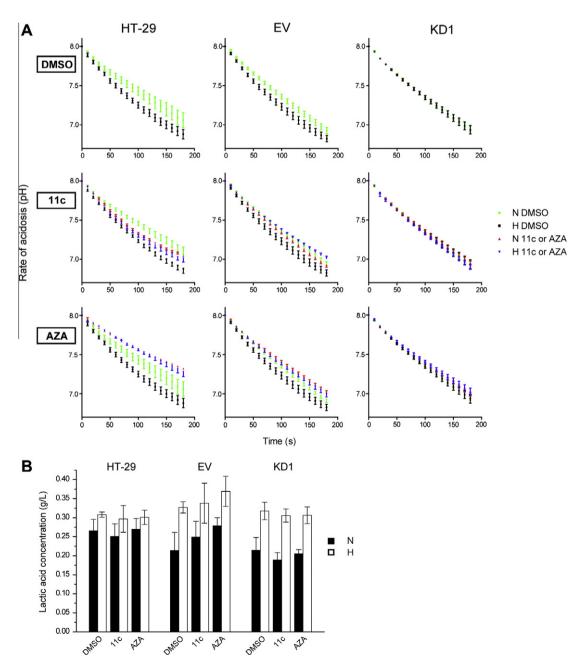

Fig. 2. (A) Extracellular rate of acidification and (B) medium lactic acid concentrations of cells exposed to normoxia (N) or hypoxia (H) after pre-treatment with 100 μM 11c, AZA or vehicle (DMSO). Data represent the mean ± SD of at least four independent experiments. Data for an independent CAIX KD (KD2) are shown in Supplementary Fig. 1.

hypoxia. The faster rate of acidification upon hypoxia was abolished in the KD cells relative to the HT-29 parental and EV cells (Fig. 2A top). To define the necessity for CAIX activity for extracellular acidification in hypoxic conditions, the effect of a specific CAIX (11c) and a general CA (AZA) sulfonamide inhibitor on hypoxia-induced changes in extracellular pH was tested in these cells. The hypoxic acidification rate could be inhibited by the addition of 11c to HT-29 and EV cells, while no effect was seen in the KD cells (Fig. 2A middle). The effect of AZA was even more pronounced, resulting in an acidification rate lower than seen under normoxic conditions (Fig. 2A bottom) and was also observed for the KD cells, indicating the lack of specificity of AZA for CAIX. Production of lactic acid was significantly higher upon hypoxia and no differences could be observed in cultures of CAIX-positive and CAIX-negative cells or upon CAIX activity inhibition (Fig. 2B), indicating that the lower hypoxic rate of acidification upon CAIX activity inhibition was not caused by interfering with the lactic acid production pathway.

To investigate in this exploratory study the consequences of CAIX activity inhibition on tumor growth and radiotherapy response, HT-29 tumors were established and mice were treated with 11c at a volume of 165 mm^3 for 5 days and irradiated with a single dose (10 Gy) at the middle of the treatment. 11c treatment resulted in a significant slower tumor growth ($P = 0.024$) compared to vehicle controls. An increased specific doubling time was observed when treated with irradiation as a single treatment, which was further increased ($P = 0.016$) by combining 11c with irradiation (Fig. 3A top). To exclude non-specific activity of 11c, experiments were repeated in the EV and KD models. A first observation was that the KD tumors grew slower compared with the EV tumors, indicating the importance of CAIX expression for tumor progression. Therefore, animals were treated with sulfonamides at a similar volume for both the EV and the KD tumors. No effect of 11c ($P = 0.530$) and no enhanced tumor effect upon combination treatment ($P = 0.724$) were observed relative to the vehicle control and irradiation alone, respectively, for the KD tumors as compared with the EV tumor model (Fig. 3A bottom), demonstrating the CAIX specificity of 11c treatment. Next, we examined if the clinically approved general CA inhibitor AZA exerted similar effects on therapy response. AZA treatment resulted in an increased specific doubling time only in the EV group ($P = 0.009$). Although no effect was found for the KD tumors ($P = 0.202$), AZA demonstrated additional effects on both groups when combined with irradiation (Fig. 3B), confirming its broader spectrum of action. Additionally, none of the treatment schedules caused any observable toxicity as monitored by body weight loss (data not shown).

To exclude that CA inhibition has an effect on intrinsic radiosensitivity, the response of the EV and KD cells to a combination of sulfonamides and a range of radiation doses *in vitro* was measured under normoxia and anoxia (Fig. 4A). Both cell lines demonstrated a more radioresistant phenotype under anoxic conditions. However, incubation with 11c or AZA before irradiation did not result in enhancement of the irradiation effect. Therefore, the increased growth delay could not be explained by changes in radiosensitivity.

Next we investigated whether inhibition of CAIX activity could affect proliferation and apoptosis as possible explanation for the *in vivo* enhanced effect when combining irradiation and sulfonamides. In general, proliferation was higher for the EV and KD cells compared with the HT-29 parental cell line. Under normoxic conditions, neither 11c nor AZA inhibited tumor cell proliferation (Fig. 4B). Although cell growth decreased for all cell lines under hypoxic conditions, proliferation was more reduced by 11c and AZA administration only in CAIX expressing cells. However, the antiproliferative capacity of AZA was lower than that of 11c. In the KD cells, the sulfonamides could not decrease cell proliferation (Fig 4B). AZA and more efficiently, 11c significantly induced early

apoptotic events in HT-29 parental and EV cells under hypoxia as monitored by cleaved caspase-3 fluorescence (Fig. 4C), while no effect was found for the KD cells. Flow cytometry detection of annexin V showed an increased hypoxia to normoxia apoptotic ratio for 11c and to a lesser extent for AZA only in HT-29 and EV cells (Fig. 4D), confirming our findings on caspase-3 activation.

Discussion

CAIX has been associated with tumorigenic transformation, tumor cell migration and invasion through its capacity to acidify the extracellular microenvironment of hypoxic tumors [16] and is, therefore, a potential therapeutic target. Recently, sulfonamide-based CAIX inhibitors have been proposed as potential antitumor agents [6]. Accumulation of a novel fluorescent labeled sulfamate FC11 (K_i = 12 nM) correlated with CAIX expression in HT-29 and EV cells exposed to hypoxia. Although CAIX expression levels remained high upon reoxygenation in these cells, in agreement with the known half-life of 38 h [17], sulfamate accumulation was markedly reduced. Additionally, no accumulation was observed in CAIX KD cells. Previously, we obtained similar results with a fluorescent labeled sulfonamide [7,8] and together these data indicate that sulfonamides only accumulate on CAIX expressing cells during hypoxia.

Hypoxia is known to cause an acidification of the extracellular environment and it has been reported that CAIX is the main contributor for this process [4,14]. We obtained similar data showing that CAIX depletion slows the faster acidification rate under hypoxia. Furthermore, acetazolamide and indanesulfonamide 11c were also able to prevent extracellular acidification only during hypoxia in a CAIX-dependent manner, with a more CAIX-specific effect for 11c compared to the general CA inhibitor acetazolamide, in agreement with previous reports [14]. It has been reported that lactic acid production is increased upon hypoxia and could predict treatment outcome by its possible contribution to the tumor acidification [18]. Our data indicate that CAIX inhibition had no influence on lactic acid production either under normoxia or hypoxia. Similar results were obtained upon elimination of the CAIX catalytic active domain [4], indicating that the extracellular acidity upon hypoxia better is explained by CAIX activation than by lactic acid production.

Recently, it has been demonstrated that the tumor response to fractionated irradiation is determined by the amount of extracellular acid production [18,19]. Since CAIX-specific sulfonamides counteract extracellular acidification, we hypothesize that these compounds are able to sensitize tumor to irradiation. First, we observed that CAIX silencing resulted in slower tumor growth relative to the scrambled controls. Previous reports from breast and colorectal xenograft models confirmed the requirement of hypoxia-induced CAIX expression in the regulation of tumor growth [1,20]. Single treatment with CAIX-specific sulfonamide 11c or general CAIX inhibitor acetazolamide resulted in a significant slower tumor growth compared to vehicle controls in a CAIX-dependent manner. Similar results were obtained using CAIX-specific (ureido)-sulfonamides and glycosylcoumarins or membrane-impermeable derivatives of acetazolamide in mammary or colorectal tumor-bearing mice [1,9]. Irradiation as a single treatment increased the specific doubling time independent of CAIX expression. Previously, we have demonstrated that a single dose of 10 Gy is sufficient to induce a growth delay in colorectal xenografts [21]. This is the first report showing that CAIX-specific sulfonamides could increase the effect of irradiation in a CAIX dependent manner, since an effect was observed only for the HT-29 parental and EV groups, while no enhancement was observed for the KD tumors, demonstrating CAIX specificity of the
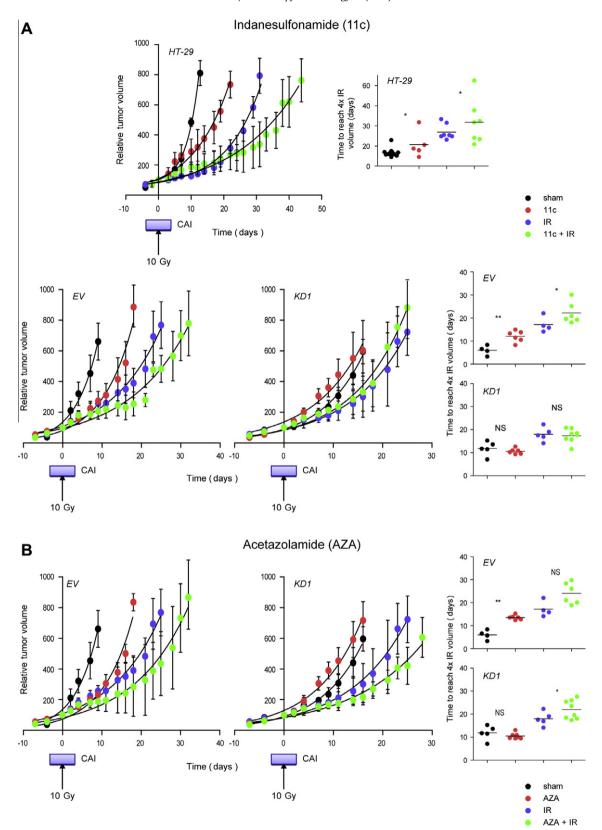
Fig. 3. 11c (A) or AZA (B) was administered when tumors reached an average size of 165 mm^3 (45 mg/kg, 5 days) and tumors were irradiated (10 Gy) at day 3 (set to 0) of this carbonic anhydrase activity inhibitor (CAI) treatment. Tumor growth was monitored until reaching 4× the volume at irradiation time and specific doubling time was calculated. Data represent the mean ± SD of four to eight independent animals and asterisks indicate statistical significance (*$P < 0.01$; **$P < 0.001$), NS = not significant.

therapy. The reduced hypoxic rate of extracellular acidification upon sulfonamide treatment might explain this increased response to irradiation. In contrast, AZA demonstrated an additional effect in

both groups when combined with irradiation, which could be explained by the additional inhibition of the intracellular CAII. Recently, it has been shown that CAII is critical for pH homeostasis in
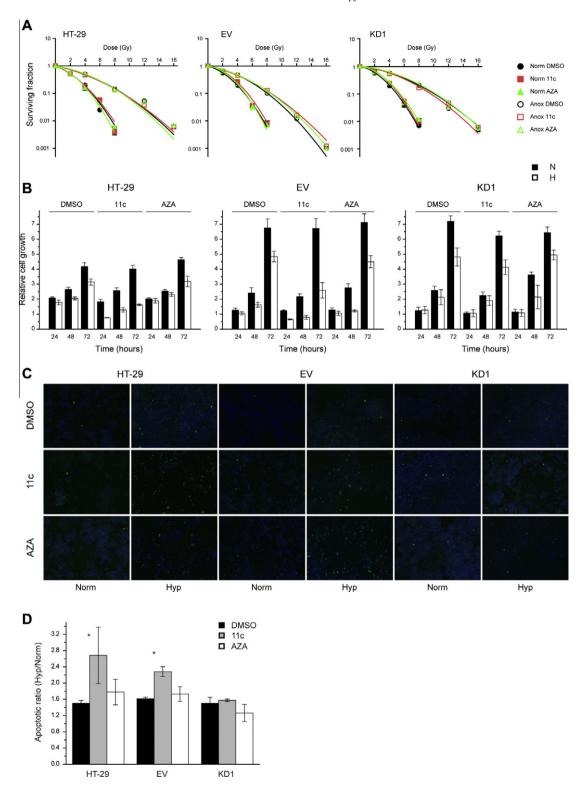
Fig. 4. (A) Clonogenic survival assay after exposure to different doses of irradiation upon normoxia (Norm: 0, 2, 4, 6 and 8 Gy) and anoxia (Anox: 0, 4, 8, 12 and 16 Gy) exposed cells after pre-treatment with 100 μM 11c or AZA. (B) Cell proliferation of cells exposed to 100 μM 11c or AZA was monitored under normoxic (N) or hypoxic (H) conditions by Crystal Violet staining after 1–3 days of growth. (C) Representative images of cleaved Caspase-3 staining (green) of normoxic (Norm) or hypoxic (Hyp) cells exposed to 100 μM 11c or AZA. Nuclear counterstaining is assessed with DAPI (blue). (D) Quantitative FACS analysis of the hypoxia to normoxia (Hyp/Norm) apoptotic ratio, as assessed by Alexa Fluor® annexin V fluorescence. Data represent the mean ± SD of three independent experiments. Asterisk indicates significant difference (*$P < 0.05$). Data for an independent CAIX KD (KD2) are shown in Supplementary Fig. 1.

GI-tract organs [22] and, therefore, treatment of these tissue-related tumors warrants precautions for additional toxicity.

We also examined the possible molecular mechanisms underlying this antitumor effect of CAIX inhibition. The increased growth delay of the combination treatment could not be explained by changes in intrinsic radiosensitivity, since the compounds had no effect on survival of cells exposed *in vitro* to a range of radiation doses. The current exploratory study suggests that the enhanced effect of sulfonamide treatment combined with irradiation is the result of changes in the tumor microenvironment which renders it more sen-

sitive to irradiation. On the other hand, *in vitro* inhibition of CAIX activity using sulfonamides also decreased cell proliferation and induced apoptosis only upon hypoxia and could, therefore, induce a significant antitumor effect, in agreement with previous reports [10]. Maintaining a neutral intracellular pH is a key cellular strategy to protect tumor cells against apoptotic death and to permit tumor cell proliferation. Our data suggest that the potential antitumor effect of CAIX inhibition may be linked to intracellular acidosis and the underlying molecular mechanisms are currently under investigation. Furthermore, the decreased cell proliferation and induced apoptosis were more efficient when inhibition is selective suggesting that the antitumor effect of CA inhibitors is mainly mediated by inhibition of CAIX. Furthermore, it has been shown that general CA inhibition using AZA can inhibit the invasive capacity of cells and this effect is attributed to inhibition of the CAII and CAXII isoforms, since only a low response was observed for CAIX-positive cells [23].

To exclude that the therapeutic effect of the combination of sulfonamide and irradiation results from normal tissue effects, it is necessary to investigate radiation-induced damage on intestinal (short-term toxicity) and lung (long-term toxicity) epithelium and to calculate the dose modifying factors (DMF) for both tumor and normal tissues. Recently, experts of an ESTRO committee aiming at writing guidelines for the development of targeted drugs associated with radiation, concluded that such normal tissue experiments are needed before new agents are tested with radiotherapy in a clinical setting (D. Hollywood, personal communication) [24]. We expect to find only an increased DMF for the tumor, since no signs for normal tissue toxicity were observed in the current study. Furthermore, we are currently designing stable carcinoma cells which can be induced to down-regulate gene expression in order to closely mimic the clinical situation, since tumors should be established with a wild type genotype and subsequently the CAIX gene can be switched off, allowing to investigate the effect of CAIX down-regulation.

In conclusion, our experiments demonstrate that specific inhibition of CAIX activity only targets CAIX under hypoxia resulting in a decreased hypoxic rate of extracellular acidification and tumor cell proliferation and induction of apoptosis without affecting the intrinsic radiosensitivity. *In vivo*, a therapeutic enhancement was observed only in CAIX expressing tumors when combined with irradiation, indicating a causal relationship between CAIX expression and its therapeutic effect. Furthermore, our data indicate the potential utility of CAIX inhibition as a new strategy for tumor-specific targeting to eventually improve the response to irradiation treatment.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.radonc.2011.05.045.

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