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Hypoxia induced CA9 inhibitory targeting by two different sulfonamide derivatives including Acetazolamide in human Glioblastoma

Harun M. Said [a,*], Carsten Hagemann [b], Fabrizio Carta [c], Astrid Katzer [a], Bülent Polat [a], Adrian Staab [d], Andrea Scozzafava [c], Jelena Anacker [e], Giles H. Vince [b,f], Michael Flentje [a], Claudiu T. Supuran [c]

[a] Dept. of Radiation Oncology, University of Würzburg, Germany
[b] University of Würzburg, Dept. of Neurosurgery, Tumorbiology Laboratory, Germany
[c] Università degli Studi di Firenze, Dipartimento di Chimica, Laboratorio di Chimica Bioinorganica, Via della Lastruccia, 3, Rm. 188, I – 50019 Sesto Fiorentino (Firenze), Italy
[d] Dept. of Radiation Oncology, University of Tubingen, Tubingen, Germany
[e] Dept. of Gynaecology and Obstetrics, University of Würzburg, Germany
[f] Allgemein öffentliches Klinikum – Klagenfurt am Wörthersee (KABEG), (LKH – Klagenfurt), Abteilung Neurochirurgie, Feschnigstrasse 11, Klagenfurt, Austria

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ABSTRACT

HIF-1α regulated genes are mainly responsible for tumour resistance to radiation- and chemo-therapy. Among these genes, carbonic anhydrase isoform IX (CA9) is highly over expressed in many types of cancer especially in high grade brain cancer like Glioblastoma (GBM). Inhibition of the enzymatic activity by application of specific chemical CA9 inhibitor sulphonamides (CAI) like Acetazolamide (Aza.), the new sulfonamide derivative carbonic anhydrase inhibitor (SU.D2) or indirect inhibitors like the HIF-1α inhibitor Chetomin or molecular inhibitors like CA9-siRNA are leading to an inhibition of the functional role of CA9 during tumorigenesis. Human GBM cells were treated with in vitro hypoxia (1, 6, or 24 h at 0.1%, O_2). Aza. application was at a range between 250 and 8000 nM and the HIF-1α inhibitor Chetomin at a concentration range of 150–500 nM. Cell culture plates were incubated for 24 h under hypoxia (0.1% O_2). Further, CA9-siRNA constructs were transiently transfected into GBM cells exposed to extreme hypoxic aeration conditions. CA9 protein expression level was detectable in a cell-type specific manner under normoxic conditions. Whereas U87-MG exhibited a strong aerobic expression, U251 and U373 displayed moderate and GaMG very weak normoxic CA9 protein bands. Aza. as well as SU.D2 displayed inhibitory characteristics to hypoxia induced CA9 expression in the four GBM cell lines for 24 h of hypoxia (0.1% O_2) at concentrations between 3500 and 8000 nM, on both the protein and mRNA level. Parallel experiments using CA9-siRNA confirmed these results. Application of 150–500 nM of the glycolysis inhibitor Chetomin under similar oxygenation conditions led to a sharply reduced expression of both CA IX protein and CA9 mRNA levels, indicating a clear glucose availability involvement for the hypoxic HIF-1α and CA9 expression in GBM cells. Hypoxia significantly influences the behaviour of human tumour cells by activation of genes involved in the adaptation to hypoxic stress. The main objective in malignant GBM therapy is either to eradicate the tumour or to convert it into a controlled, quiescent chronic disease. Aza., SU.D2, Chetomin or CA9-siRNA possesses functional CA9 inhibitory characteristics when applied against human cancers with hypoxic regions like GBM. They may be used as alternative or in conjunction with other direct inhibitors possessing similar functionality, thereby rendering them as potential optimal tools for the development of an optimized therapy in human brain cancer treatment.

1. Introduction

Carbonic anhydrases (CAs) are widespread metalloenzymes in higher vertebrates including humans.[1,2] Regulated by the HIF-1 cascade, hypoxia leads to strong overexpression of Carbonic anhydrase isoform IX (CA IX) in many tumors with the overall consequence that the imbalance in pH in the tissue is increased. In contrast to normal tissues (pH 7.4) most hypoxic tumors are acidic (pH 6). The role of CA IX in the hypoxic tumor acidification processes has been demonstrated by our group[3] and by Pastorekova's group.[4] The upregulation of CA IX expression by hypoxia is accomplished by the HIF-1 activation cascade. CA IX is down regulated by the wild-type of the von Hippel–Lindau tumor suppressor pro-

* Corresponding author. Address: Dept of Radiation Oncology, Medical Faculty, University of Würzburg, Josef Schneider Str. 11, D-97080 Würzburg, Germany. Tel.: +49 16 37531174.
E-mail addresses: said_h@klinik.uni-wuerzburg.de, said@scientist.com (H.M. Said).

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tein.[5,6] CA IX distribution in human tissues is unusual: it is expressed in only a few normal tissues with decrease or loss of expression during carcinogenesis[7]; however, there is an ectopically CA IX expression in numerous tumors, predominantly carcinomas, mostly derived from tissues that are not expressing CA IX under normal conditions. Modulation of CA9 expression is possible via different options so that many functional (and sometimes selective).

CA IX inhibitors have been developed in the past few years. They are based on the idea that inhibition of tumor acidification processes and re-establishment of a more normal pH might lead to tumor regression, especially when used in combination with classical anticancer drugs. This establishes CA IX as a diagnostic tool and novel drug target for the development of therapeutic agents.[8–16] It is well known that CA IX is highly overexpressed in many types of cancer. CA9 expression in tumor cells which is regulated by the HIF-1α transcription factor,[17–19] is induced by hypoxia and correlates with a poor response to classical anti-cancer therapeutic approaches like chemo- and radiotherapies.[17,18] Such chemo- and radio resistance, which is induced by the chemical CA IX contribution to the acidification of the tumor environment by efficiently catalyzing the hydration of carbon dioxide to bicarbonate and protons. This leads to chemo-resistance to weakly basic anticancer drugs and the acquisition of metastatic phenotypes.[20,21] Most anticancer drugs are transported into the cell by either active transport or passive diffusion followed by further metabolisation.[22] Among them are potent CA IX-selective inhibitors derived from Acetazolamide (Aza.), benzenesulfonamides and ethoxzolamide which have been shown to inhibit the growth of several tumor cells in vitro and in vivo.[23,24] These drugs are pH sensitive. Therefore, it is suggested that their cytotoxic activity depends on both intracellular pH (pHi) and extracellular pH (pHe).[25] Targeting CA IX with such specific CA IX inhibitors,[26] or also antibodies[27] should on one hand contribute to enhance the effect of weakly basic drugs and on the other hand, reduce the acquisition of metastatic phenotypes by controlling of the pH imbalance in the tumor cells.[28] Practically, CA inhibitors have been previously shown to elicit synergistic effects when used in combination with other chemotherapeutic agents in animal models.[29] Aza. is a clinically used Sulphonamide. It is a promiscuous Carbonic anhydrase inhibitor (CAI), inhibiting CA II, IX and XII in the low nanomolar range, but being less effective against Carbonic Anhydrase I.[30–32] See also Table 1. These selective CA IX inhibitors could prove useful for elucidating the role of CA IX in hypoxic cancers, for controlling the pH imbalance in tumor cells and for developing diagnostic or therapeutic applications for tumor management.[28]

The function of these inhibitors is as follows: The key interaction occurs between 1 and CA II. Involved is the primary Sulfonamide group which, forms an adduct with a slightly distorted tetrahedral geometry by displacement of the hydroxide ion bound to the tri-coordinated hystine zinc in the native enzyme. Moreover the proton residue on the sulfonamide group and one of its oxygen's interacts with the OH (3.0 Å) and the NH (2.9 Å) of Thr199, respectively. In addition to the well established van der Waals contacts between the thiadiazole ring and the amino acids Leu 198 and Val 121, the interaction of both the nitrogen atoms of the heterocycle with the OH of Thr 200 is noteworthy. Finally, an intramolecular coordination between the sulphur atom of the ring with the carbonyl oxygen of the acetamido function locks the carbonyl functionality in a favourable conformation to further interact by means of hydrogen bonding with Gln 92 and the methyl group with Phe 131 (Fig. 1).[31–34]

The aim of this study is to specifically inhibit CA9 expression induced by hypoxia in vitro both on protein and mRNA level in order to assign the potential alternative modalities that might be suitable for the treatment of human cancer patients, and especially brain cancer patients.

Table 1
Measurement of inhibitory capacity of Acetazolamide against the different Carbonic anhydrase classes

Inhibitor	hCA I	K_i (nM)[a]		hCA IX	hCA XII
		hCA II			
(Acetazolamide)	250	12		25	5.7
SU.D2	265	420		81	15

[a] The inhibition has been measured by a stopped flow CO_2 hydrase assay, cf. Khalifah, J. Biol. Chem. 1971,[33].

2. Methods

2.1. Cell culture, hypoxia treatment, preparation and immunoblotting of human tumor cell protein extracts

Early-passage human malignant glioma cell lines U251 and U373 from the American Type Culture Collection (ATCC, Rockville, MD) and GaMG, a cell line that was established from a patient with Glioblastoma multiforme (Gade Institut of the University Bergen, Norway),[35] were grown on glass Petri dishes in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (FBS) and non-essential amino acids. Additionally, all culture media were supplemented with penicillin (100 IU/ml)/streptomycin (100 μg/ml) and 2 mM L-glutamine. Cells were treated with in-vitro hypoxia for 1, 6 or 24 h at 0.1%, 1% and 5% O_2, respectively, in a Ruskinn (Cincinnatti, OH, USA) Invivo2 hypoxic workstation as previously described.[36] For reoxygenation experiments, dishes were returned to the incubator after 24-hour hypoxia treatment. Whole-cell lysates were prepared with 0.1 ml RIPA buffer (1XTBS, 1% Nonidet P-40 (Amresco, Vienna, Austria), 0.5% sodium deoxycholate, 0.1% SDS; protease inhibitors pepstatin A 1.4 μM, aprotinin 0.15 μM and leupeptin 2.3 μM and 100 μM PMSF (all from Sigma, St. Louis, MO, USA). To inhibit protein dephosphorylation, phosphatase inhibitor mix (Sigma) was added. Using a syringe fitted with a 21 gauge needle to shear DNA, lysates were transferred to a micro centrifuge tube, followed by 30 min incubation on ice. Subsequently, cell extracts were cleared by centrifugation at 15,000*g* for 12 min at 4 °C. 20 μg of protein extracts were separated by 8% SDS–polyacrylamide gel electrophoresis and transferred to a 0.45 μm nitrocellulose membrane (Protran BA 85; Schleicher & Schuell, Dassel, Germany). Non-specific binding was blocked by 5% non-fat milk powder in TBS overnight at 4 °C. Protein detection was performed in nuclear extracts by incubation with the M75 mouse monoclonal antibody directed against CA IX (Bayer Healthcare Co., diluted 1:7200) and HIF-1α antibody (610959 BD Biosciences, dilution 1:500) with nuclear extracts in 2.5% non fat milk powder in TBS for 1 h at room temperature. Secondary antibody for both was a goat anti-mouse immunoglobulin/HRP, dilution 1:2000, (P0447, stock solution 400 μg/ml; Dako Cytomation, Denmark) with which samples were incubated for one additional hour at room temperature followed by five washes as described above. Membranes were also probed with anti-β-actin antibody (A 5316, 1:10,000, Sigma–Aldrich, Germany) or anti-β-tubulin mouse monoclonal antibody (Sigma, 1:2000). For re-probing, membranes were stripped with stripping buffer (100 mM β-mercaptoethanol, 2% Sodium Dodecyl Sulphate (SDS), 62.5 mM Tris–HCl pH 6.7) at 60 °C for 30 min. Bound antibodies were detected by developing the membrane with the ECL Plus Western Blotting detection system (Amersham Biosciences, Cambridge, UK) for 5 min with subsequent development of the Hyperfilm ECL (Amersham).

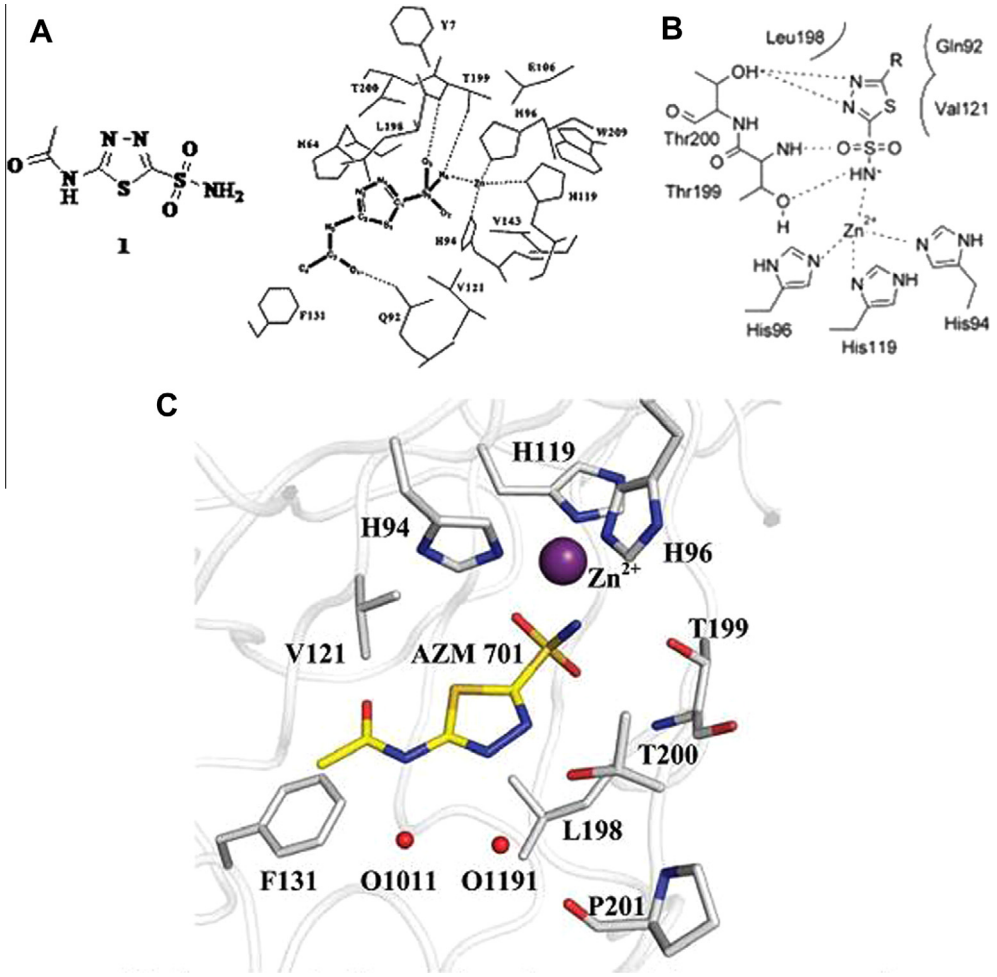


Figure 1. (A and B). Schematic presentation of themain Azetazolamide interactions in adduct with CAII (after Vidgren J et al. 1990 and Alterio, V et al. 2012). (C) High-resolution structure of human carbonic anhydrase II complexed with Acetazolamide, see; Sippel KH et al 2009, [34]. The PDB data code is 3HS4.

2.2. Total RNA isolation of from Glioblastoma (GBM) tumor cell lines

Total RNA was isolated from cultured tumor cells as reported previously[37] and described in,[38] including the digestion of contaminating DNA with the provided DNase.

2.3. Analysis of mRNA expression level in human GBM cell lines by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

To analyse the inhibitory effect of a sulphonamide derivative Aza. (MW: 426.59) in a concentration range of 250–8000 nM on CA9 expression, RT-PCR was performed using primers designed using published information on mRNA sequences in GenBank (sequence accession numbers: CA9 NM_001216, β-actin NM_001101 and HIF-1α NM_001530.2). An aliquot of 1–5 μg of total mRNA from human Glioblastoma cell lines was transcribed at 42 °C for 1 h in a 20 μl reaction mixture using 200 U RevertAid™ M-MuLV reverse transcriptase (RT), oligo(dT)18 primer and 40 U Ribonuclease inhibitor (all from Fermentas, Ontario, Canada). For PCR-reactions primers were designed in flanking exons with Primer3 software (available online http://frodo.wi.mit.edu/cgibin/primer3/primer3_www.cgi), based on information indicated above in order to amplify a 342 bp CA9 product (forward primer 5′-ACCCTCTCTGACACCCTGTG-3′, reverse primer 5′-GGCTGGCTTCTCACATTCTC-3′) a 668 bp β-actin product (forward primer (F1) 5′-CGTGCGTGACATTAAGGAGA-3′, reverse primer (R1) 5′-CACCTTCACCGTTCCAGTTT-3′) a 233 bp HIF-1α product (forward primer (F1) 5′-TTACAGCAGCCAGACGATCA-3′, reverse primer (R1) 5′-CCCTGCAGTAGGTTTCTGCT-3′). The PCR reactions were performed with 25–32 cycles with increments of 5 cycles using PCR systems and reagents acquired from Promega™ (Promega GmbH, Mannheim, Germany) and applied according to the manufacturer's instructions. PCR product separation was on 1% agarose gels (Sigma–Aldrich, Steinheim, Germany) and visualized by ethidium bromide staining (0.07 μg/ml ethidium-bromide; Biorad, Munich, Germany).

2.4. Down-regulation of CA9 expression by small interfering RNA, the CA9 inhibitory sulphonamide derivative Acetazolamide and Chetomin

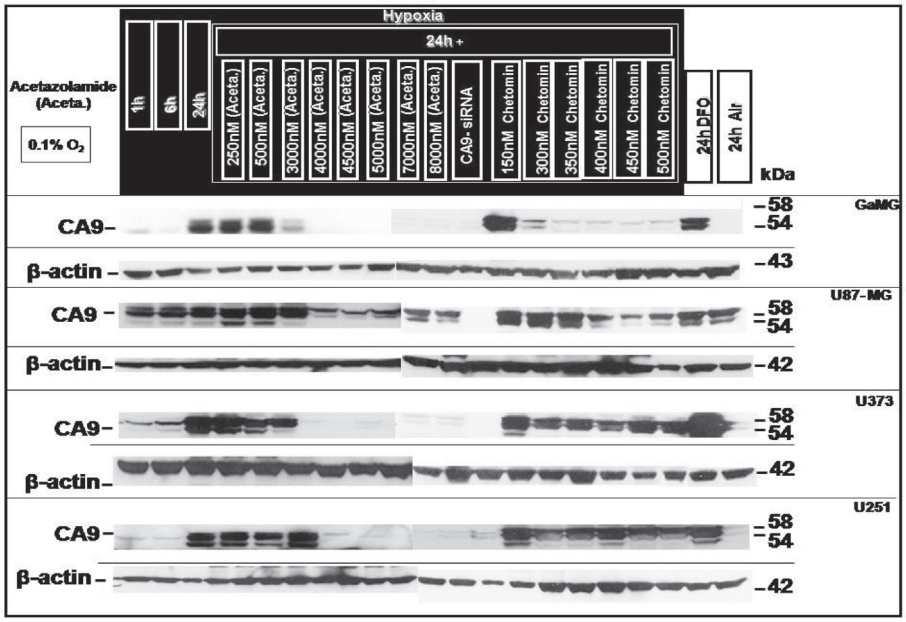
Human GBM cell lines U373, U251, U87-MG and GaMG were grown up to 50% confluence on 10 cm plates in complete medium (RPMI 1640 medium or DMEM depending on the cell line) supplemented with 10% foetal calf serum, 100 μg/ml streptomycin, and 100 units/ml penicillin. The siRNA were designed and supplied by Santa Cruz Biotechnology. Detection of reduced CA9 mRNA and protein levels was performed by RT-PCR as well as immunoblotting. The latter has been performed as described above. Chetomin (150–500 μM) was used as a glycolysis inhibitor and was added

to the growth medium shortly before the respective hypoxia treatment. Aza. or SU.D2 were added to the growth medium in the mentioned concentration at the beginning of the controlled hypoxic exposure and an addition for second time 12 h after the first addition and in the hypoxic chamber.

2.5. Densitometry and statistical analysis

Protein expression pattern intensity on Western blots and mRNA level expression signal intensity from RT-PCR experiments were determined with 1D Kodak Image Analysis Software. The signals were measured in Kodak light units (KLU) and divided by the corresponding signals of the loading controls β-tubulin and β-actin, as previously described.[37–42] The relative changes in protein expression resulting from submission to hypoxic conditions or hypoxic conditions with subsequent re-oxygenation were analysed in relation to the 24 h normoxic value. Three to four individual experiments were performed. The Mann–Whitney *U* test for independent samples was used to analyse these data. The Student's *t* test for unpaired samples was used to analyse overall cell numbers. In both tests $p \leqslant 0.05$ was considered to be statistically significant. All tests were carried out using the statistical package SPSS, release 12.0.1 for Windows (SPSS Inc., Chicago, Ill., USA).

A - Inhibition under Hypoxia by via non chemical and chemical inhibitors including the sulphonamide derivative compound Acetazolamide on the CA9 expression on protein level.



B - Inhibition under Hypoxia by via non chemical and chemical inhibitors including a new sulphonamide derivative compound.

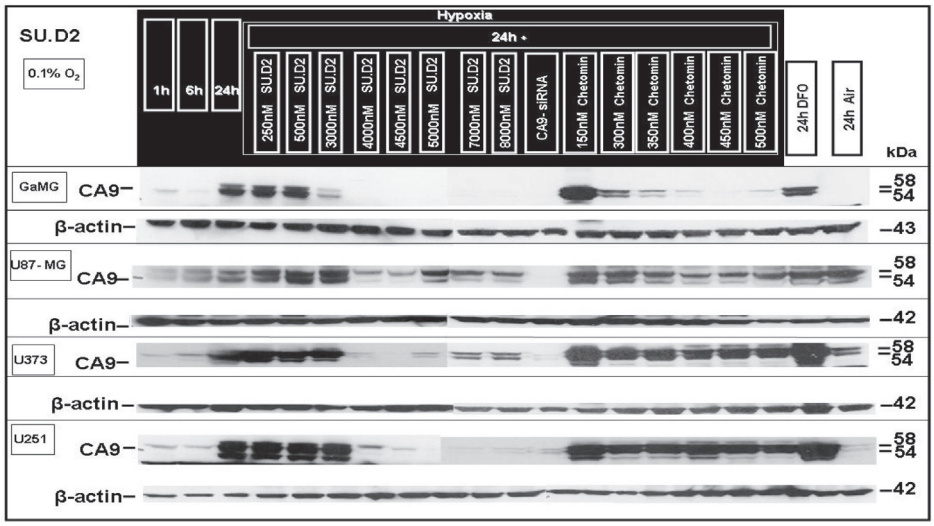


Figure 2. Comparative in vitro analysis of CA IX protein expression under hypoxia including hypoxia induced *CA9* inhibition by chemical or non chemical alternatives. (A) Inhibition under Hypoxia by via non chemical and chemical inhibitors including the sulphonamide derivative compound Acetazolamide on the CA9 expression on protein level. (B) Inhibition under Hypoxia by via non chemical and chemical inhibitors including a new sulphonamide derivative compound.

3. Results

3.1. CA IX expression regulation in GBM cells on protein level in response to the inhibitory effect of CA9-siRNA, Chetomin and the Sulfonamide derivative compounds including Acetazolamide and SU.D2, in vitro

Analysis of expressed CA IX protein by Western blot under extreme hypoxic conditions (0.1% O_2) and the inhibitory effect of Aza., SU.D2 Chetomin and CA9 siRNA on the CA9 mRNA expression showed a clear Aza. concentration-dependent CA IX expression under hypoxic and normoxic conditions in the four cell lines U87-MG, U373, U251 and GaMG.

Inhibition of CA9 mRNA that is expressed under extreme hypoxic aeration conditions (0.1% O_2) by Aza. occurred in a concentration dependent manner. In GaMG 4000 nM was sufficient to inhibit the CA9 mRNA expression as induced by 24 h extreme hypoxia (0.1% O_2). A similar inhibitory effect was present upon application of 500 nM of the HIF-1α inhibitor Chetomin (Fig. 2A). In parallel these cells were incubated under hypoxia with addition of 250–8000 nM of the high molecular carbonic anhydrase inhibitory sulphonamide derivative or with Chetomin (150–500 nM). Aza.

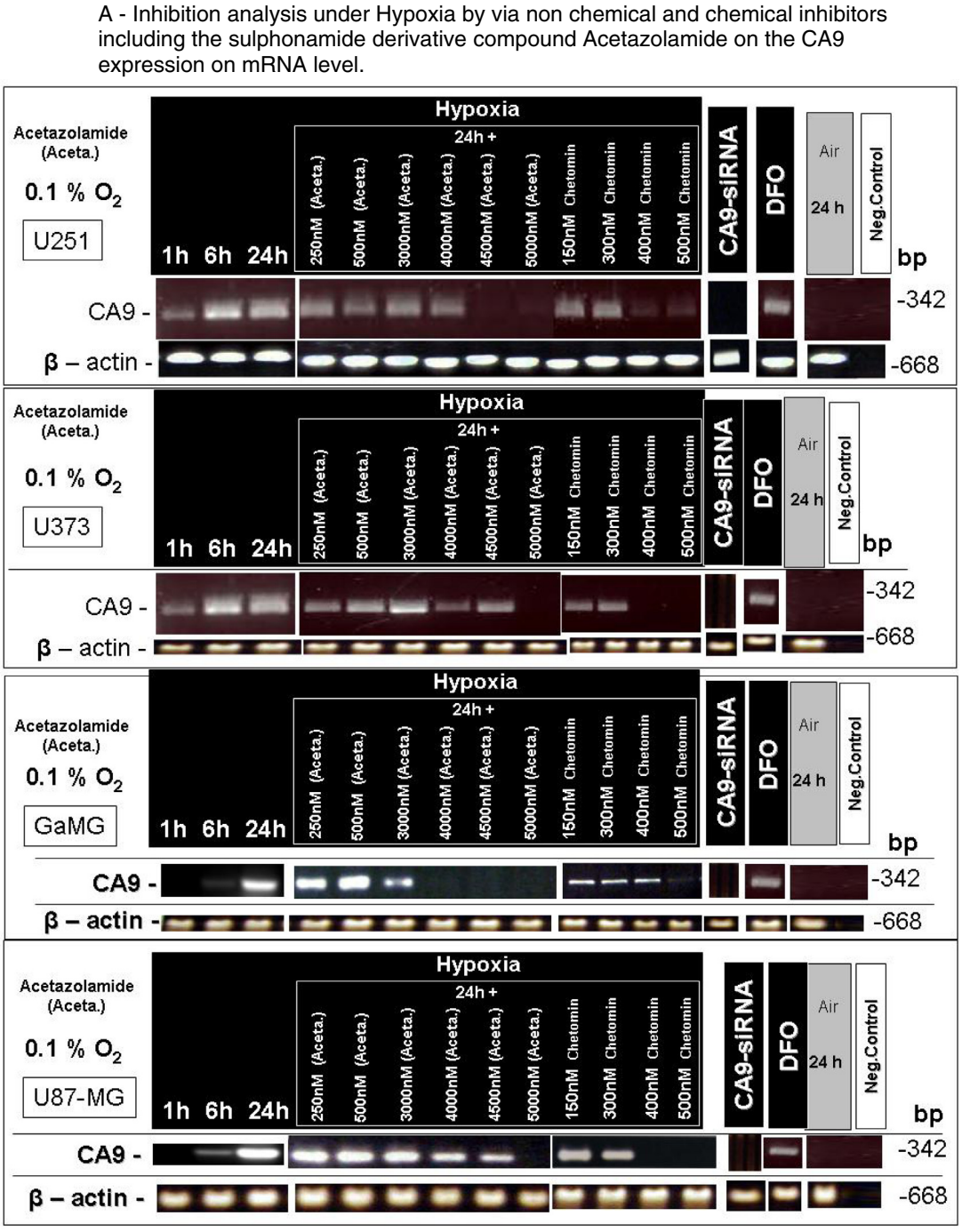
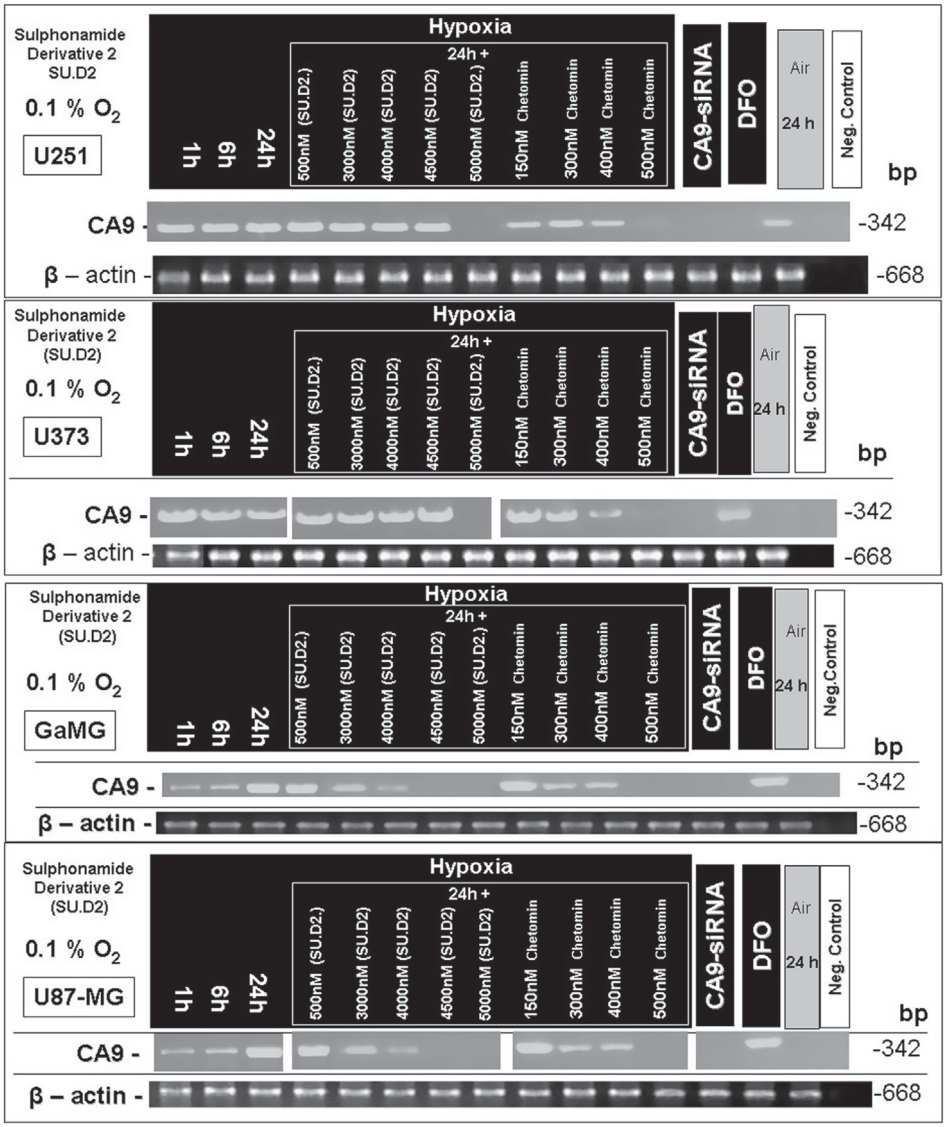


Figure 3. Hypoxia induced *CA9* mRNA gene expression analysis in vitro under hypoxia including the hypoxia induced *CA9* inhibition by chemical or non chemical alternatives. (A) Inhibition analysis under Hypoxia by via non chemical and chemical inhibitors including the sulphonamide derivative compound Acetazolamide on the CA9 expression on mRNA level. (B) Inhibition under Hypoxia via non chemical and chemical inhibitors including a new sulphonamide derivative compound (SU.D2) on mRNA.

possessed the highest inhibitory effect on CA9 at 4000 nM, followed by U373 and U251, while a complete CA IX inhibition was not possible in the PTEN mutated U87-MG cells. SU.D2 induced a CA9 inhibition at nearly similar concentrations in a similar manner (Fig. 2B). The HIF-1α inhibitor Chetomin only was able to down-regulate CA9 protein expression in GaMG to a basic expression level and did not substantially change the expression in the other three tumor cells analysed. Experiments were repeated for three times and the figure represents one representative experiment out of three. Treatment with 100 μM DFO under aerobic conditions served as a positive control while cells incubated under aerobic conditions as negative control and β-actin as a loading control.

3.2. CA9-mRNA expression in GBM cells, in vitro, in response to the inhibitory effect of CA9-siRNA, Chetomin and the Sulfonamide derivative CA inhibitor compounds including Acetazolamide

Clear inhibition of CA9 mRNA expression could be shown by semi-quantitative RT-PCR analysis, in the four cell lines U87-MG, U373, U251 and GaMG under extreme hypoxic conditions (0.1% O_2) in dependency of the Aza. concentration. These cells were incubated under hypoxia with addition of 250–5000 nM of the high molecular CAI Aza., SU.D2 or with Chetomin (150–500 nM). In GaMG cells the CA9 inhibition occurred at comparatively low concentrations of Aza. (4000 nM) followed by the other cell lines U373, U251 and U87-MG at 4500 nM and 5000 nM, respectively (Fig. 3A). On the other hand, when applying SU.D2, U251 and U373 could inhibit the CA9-mRNA RNA expression at 4500 nM, while in GaMG and interestingly in U87-MG 4000 nM where enough to provoke a similar effect (Fig. 3B). CA9-siRNA inhibited CA9-mRNA expression completely and Chetomin in all cell lines examined started to reduce the CA9 expression at 400 nM and CA9-mRNA was completely inhibited at a concentration of 500 nM (Fig. 3B). On the other hand, in the experimental approach where Aza. was used as an CA9 inhibitor, the HIF-1α inhibitor Chetomin down-regulated CA9-mRNA expression in GaMG at 400 nM while in the other three tumor cells analysed only 300 nM Chetomin was necessary (Fig. 3A). Both experimental series were repeated for three times and the figure represents one representative experiment out of



B - Inhibition under Hypoxia via non chemical and chemical inhibitors including a new sulphonamide derivative compound (SU.D2) on mRNA.

Fig. 3. (*continued*)

three. Treatment with 100 μM DFO under aerobic conditions served as a positive control while cells incubated for 24 h under aerobic conditions as negative control and β-actin as a loading control.

4. Discussion

Hypoxia in tumor tissue cells is connected to CA IX expression with further selection of the appropriate tumor cells for metastasis.[43] Such CA IX expression is considered as an optimal target for human cancer treatment because of the specific expression of CA9 in tumor cells with restricted expression in normal living tissue.[18,19,43] The inhibition of CA IX expression in some experimental examination series showed its importance for the outbreak and physiological development of the different cancer diseases with displaying the effect of the hypoxic oxygenation conditions in tumor cells and CA9 expression regulation control. Lou et al. were able to show that silencing of CA-IX expression in 4T1 mouse metastatic breast cancer cells resulted in regression of orthotopic mammary tumors and inhibition of spontaneous formation of lung metastasis. In addition a stable CA IX depletion in MDA-MB-231 human breast cancer xenografts resulted in attenuation of primary tumor growth attenuation.[44] In another case depletion of CA IX together with the Carbonic Anhydrase

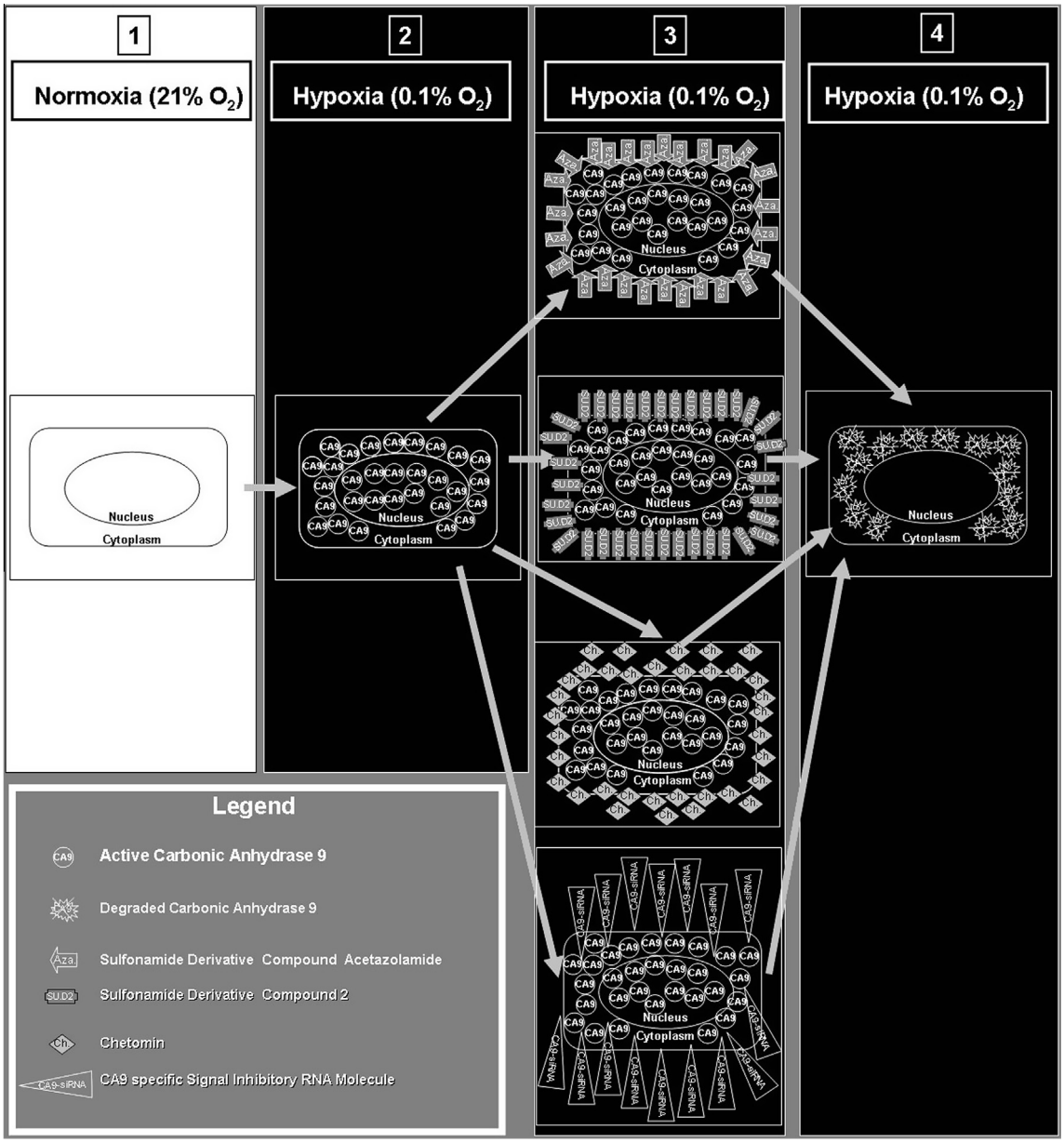


Figure 4. Inhibitory function of Sulfonamide derivative compounds like Acetazolamide and others when compared to other chemical or molecular inhibitory alternatives like Chetomin or CA9-siRNA, respectively, in human GBM cells under Hypoxic oxygenation conditions of the tumor cells microenvironment. The figure summarizes the different alternative CA9 inhibition approaches applied within the framework of the experimental series conducted. (1) Complete CA9 depletion by the regulatory activity of the hypoxic HIF-1α pathway under normoxic oxygenation conditions. (2) Activation of the Hypoxic HIF-1α pathway which leads to an increase of the CA9 mRNA expression and as a consequence to accumulation of CA IX protein in the cytoplasm of the cancer cells of the hypoxic tumor microenvironment. (3) Tumor cells are exposed to Acetazolamide (Aza.), or another sulfonamide derivative compound (SU. D2) or Chetomin, which are diffused into the cytoplasm of the cancer cell or CA9-siRNA which the cells were transfected with. (4) As a consequence, the CA9 mRNA and CA IX protein are degraded leading to an CA9 expression in the cytoplasm which is at its minimal essential concentrations for the tumor cells.

CA XII in LS147Tr colorectal cancer xenografts resulted in a tumor growth reduction of 85%.[45] Results with similar tendency were obtained in other experimental series under a normoxic oxygenation status of the cancer cells or with constitutive or non constitutive CA IX expression status.[45–49] With its over-expression in many cancer tissues but not in their normal counterparts, CA IX constitutes an interesting target for novel approaches to anticancer therapy. CA IX has been shown to acidify the extra-tumoral medium, contributing to both the acquisition of metastatic phenotypes and chemo-resistance to weakly basic anticancer drugs. Consequently, further research needs to be done in the field of the tumor-associated CA IX isozyme to better understand its exact role in cancer. It was proven in previous experimental approaches that the Sulfonamide derivative Acetazolamide (Aza.) as well as (SU.D2) as chemical compound that possesses a relatively high degree of inhibitory specificity against Carbonic Anhydrase 9 (50) see also (Table 1). CA-IX-selective inhibitors constitute interesting tools to study the physiological or pathological effects of CA IX.[45–49] Several pathways contribute to tumor growth. Therefore, anti-tumor activity might be increased by agents that target multiple molecules, including CA IX, or by the combination of several agents to facilitate inhibition of several mechanisms. On the other hand, in another series of experiments published here for the first time, we were able to determine the physiological behaviour of hypoxia induced CA9 in response to the regulative effect of the CAI Aza., this, when compared to the inhibitory effect induced by SU. D2, in the GBM cells. Under normoxic oxygenation conditions the expression of the CA IX protein level was detectable in a cell-type specific manner. Whereas U87-MG exhibited a strong aerobic expression, U251 and U373 had moderate and GaMG very weak normoxic CA IX protein expression pattern (Fig. 2A and B). We could demonstrate that Aza., as well as SU. D2 displayed inhibitory characteristics against hypoxia induced CA9 expression in the four examined GBM cell lines under 24 h of hypoxia (0.1% O_2) at functional concentrations between 3500 nM and 8000 nM. Parallel experiments with the same tumour cell lines where CA9-siRNA was applied under identical conditions confirmed these results. Also, we could demonstrate the ability of the HIF-1α inhibitor Chetomin at concentrations between 150 nM and 500 nM under identical physiological conditions to regulate CA9 expression in GBM (Figs. 2A and B, 3A and B, respectively). CA9 inhibition, especially, that induced by Aza. and SU.D2 confirms the functional capability of such compounds to regulate the expression of this important molecule down to its complete inhibition, but, SU.D2 was more effective in the inhibition of CA9, suggesting a relatively higher degree of functionality in the expression regulation of hypoxia induced CA9 in brain cancer cells especially in GBM. This also causes inhibition of cell proliferation.[50] A parallel inhibition by other compounds like Chetomin or, alternatively, CA9-siRNA shows that it is possible to obtain such a hypoxia induced CA9 inhibitory function on a different inhibition level with all its accompanied consequences. Aza. as well as SU.D2 which are CAIs that represent functionally interesting candidates for the development of novel unconventional anticancer strategies targeting the hypoxic areas of tumors (Fig. 4), with respect to their minor differences in their functionality in their anti-CA9 activity in GBM. We have to mention that GBM is characterized by both, a very high CA IX expression and poor response or even unresponsiveness to radiotherapy as well as chemotherapy, rendering Aza. and SU.D2. both, to be a leading candidates of such novel anti cancer therapeutic approaches. The CA9 inhibitory capacity of Aza., as well as that of SU.D2, despite the previous discussion about the specificity of CA9 inhibition,[51] showed a similar tendency but with a different level of inhibition of CA9 expression (Fig. 4). In addition, this effect was tumor cell type specific, as it is the case for the hypoxia induced expression by the different cells.[25,52,53] Therefore, we can say that the CA9 inhibition reaches, at least in GBM, a point of higher specificity. Further experiments are necessary to determine the potential of each individual CAI sulfonamide derivative to specifically inhibit CA9. Thereby it has to be taken into account the difference in both their functional activity and their level of specificity depending on the tumor cell type examined. Such an approach, when compared to the glycolysis induced inhibition via IAA application, which has previously been shown to be HIF-1-inhibitory,[3,54–56] could represent a therapeutic strategy to target hypoxia-induced CA9 with respecting the differences of the CA9-inhibitio specificity of each compound when designing the potential applied anti-cancer therapeutic strategy prior its application (Fig. 4). Induction of CA9 sequence-specific posttranscriptional gene silencing in vitro in different GBM cell lines by RNA interference resulted in a strong inhibitory activity towards CA9 expression, both on mRNA and protein level. However, the success of such approaches still awaits the development of an efficient delivery system that can affect a large number of tumor cells.

5. Conclusions

CA IX is an interesting target for anticancer drug development. Many biochemical physiological and pharmacological data point to the potential use of inhibition of tumor-associated CA IX in the management of hypoxic tumors that do not respond to classical chemo- and radio therapy. CAIs provide the possibility of developing both diagnostic tools for the non-invasive imaging of these tumors and therapeutic agents that might perturb the extra-tumoral acidification process in which CA IX is involved. Many classes of highly effective in vitro CA IX inhibitors have been developed and the pharmacological evaluation of some of them is continuously ongoing. Much functional biological and pharmacological work is necessary to understand whether a successful new class of antitumor drugs might be developed from these encouraging observations. GBM therapy mainly aims to eradicate the tumour or convert it into a controlled, quiescent chronic disease. Application of glycolytic inhibitors at controlled doses under hypoxic oxygenation conditions leads to reduced HIF-1α accumulation and acts as indirect inhibitor of hypoxia regulated genes like CA9. The different CA9 modulating and inhibitory options studied here, including direct inhibitors like Aza., SU.D2, Chetomin, specific CA9-siRNA, or other chemical compounds possessing a similar function may be used as alternative or in conjunction with each other to optimize the human tumor treatment approaches applied nowadays and to enhance the quality of life of the patients.

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