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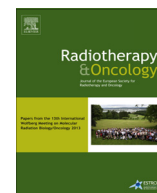
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Hypoxia

Targeting carbonic anhydrase IX by nitroimidazole based sulfamides enhances the therapeutic effect of tumor irradiation: A new concept of dual targeting drugs



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ABSTRACT

Background and purpose: Carbonic anhydrase IX (CAIX) plays an important role in pH regulation processes critical for tumor cell growth and metastasis. We hypothesize that a dual targeting bioreductive nitroimidazole based anti-CAIX sulfamide drug (DH348) will reduce tumor growth and sensitize tumors to irradiation in a CAIX dependent manner.

Material and methods: The effect of the dual targeting anti-CAIX (DH348) and its single targeting control drugs on extracellular acidification and radiosensitivity was examined in HT-29 colorectal carcinoma cells. Tumor growth and time to reach 4× start volume (T4×SV) was monitored for animals receiving DH348 (10 mg/kg) combined with tumor single dose irradiation (10 Gy).

Results: In vitro, DH348 reduced hypoxia-induced extracellular acidosis, but did not change hypoxic radiosensitivity. In vivo, DH348 monotherapy decreased tumor growth rate and sensitized tumors to radiation (enhancement ratio 1.50) without systemic toxicity only for CAIX expressing tumors.

Conclusions: A newly designed nitroimidazole and sulfamide dual targeting drug reduces hypoxic extracellular acidification, slows down tumor growth at nontoxic doses and sensitizes tumors to irradiation all in a CAIX dependent manner, suggesting no “off-target” effects. Our data therefore indicate the potential utility of a dual drug approach as a new strategy for tumor-specific targeting.

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Elevated carbonic anhydrase (CA) IX expression has been associated with poor prognosis, tumor progression and aggressiveness [1]. The plasma membrane associated dimeric glycoprotein catalyzes the reversible hydration of carbon dioxide to bicarbonate and a proton. CAIX is therefore an important component of the pH regulation systems, which are activated upon anaerobic glycolysis [2]. Its main function during hypoxia is to preserve a neutral intracellular pH favorable for tumor growth and survival, while contributing to an acidic extracellular tumor microenvironment

favorable for invasiveness [3,4]. 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 [2,5]. Previously, we have demonstrated that inhibitors of CAIX activity require both protein expression and activation and this is dependent on the tumor oxygenation status [6–8]. Tumor-specific accumulation was found after administration of fluorescent sulfonamides at low dosage, while inhibition of tumor growth was observed at higher dose levels [7,9–11]. Treatment of mammary tumor-bearing mice with novel CAIX-specific (ureido)-sulfonamide and glycosylcoumarin inhibitors resulted in a significant inhibition of primary tumor growth and lung metastasis formation [11]. Recently, an underexplored class of sulfamate inhibitors proved to be excellent candidates for low dosage anti-metastatic drugs in breast cancer therapy [12]. These reports emphasize that specific inhibitors of CAIX activity are promising

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to pursue for their tumor-specific therapeutic properties in combination with conventional therapies. Recently, the CAIX dependent sensitizing effect of indanesulfonamides [8] and acetazolamide [13] on respectively radio- and chemotherapy has been demonstrated.

A different approach to sensitize tumors to irradiation is the use of the 2-nitroimidazole based hypoxic radiosensitizer misonidazole. This has shown to improve the radiation response both when administered in a single or fractionated radiation scheme, however with high clinical toxicity [14]. The less toxic 5-nitroimidazole derivative, nimorazole, has shown to reach similar enhancement ratios as misonidazole in fractionated schedules [14] and resulted in a highly significant benefit in terms of loco-regional tumor control and disease-free survival [15]. More specific targeting towards the hypoxic tumor regions using lower doses is therefore an important requisite for new compounds.

The aim of this study was to evaluate the antitumor activity of a newly designed class of dual targeting nitroimidazole based sulfamide drugs with high affinity for CAIX. We hypothesize that these compounds specifically block CAIX activity, reduce tumor growth and sensitize tumors to irradiation 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. HT-29 colorectal carcinoma cells harboring a shCAIX (KD) or control shRNA (EV) construct (kindly provided by Adrian Harris, Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe hospital, Oxford, UK) were engineered as previously described [8]. Low oxygen conditions were acquired in a hypoxic workstation (Ruskin INVIVO₂ 1000). The atmosphere in the chamber consisted of 0.2% O₂ (hypoxia) or <0.02% O₂ (anoxia, 0%), 5% CO₂ and residual N₂. In parallel, normoxic (20% O₂) dishes were incubated in air with 5% CO₂. pH of the culture medium was immediately measured at the end of each experiment as previously described [6].

Inhibition CAIX activity

The structure and Ki value of the different compounds are depicted in Fig. 1A. Compounds were dissolved in culture medium containing 0.5% DMSO at the indicated final concentrations just before addition to the cells. For the animal experiments, DH348 was dissolved in NaCl 0.9% containing 1% DMSO to a final concentration of 10 mg/kg and injected intravenously via a lateral tail vein.

Immunoblotting

Experiments were performed as previously described [6]. 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.

Clonogenic survival

Cells were exposed to normoxia or anoxia for 24 h, subsequently irradiated (225 kV Philips X-ray tube), trypsinized and plated in triplicate for clonogenic survival. Cells were allowed to form colonies during 10 days, which were fixed and stained with 0.4% methylene blue in 70% ethanol. Afterwards, colonies were counted in an automated way (Oxford Optronix).

Animal experiments

All experiments were in accordance with local institutional guidelines for animal welfare and were approved by the Animal Ethics Committee of the University. 1.5e6 cells were resuspended in Basement Membrane Matrix (Matrigel™ BD Biosciences) and injected subcutaneously into the lateral flank of adult NMRI-nu mice (28–32 g). Intravenous DH348 treatment started at a tumor volume of $193 \pm 66 \text{ mm}^3$ for 5 days (10 mg/kg daily) and irradiation (10 Gy single dose [8]) was performed at day 3. Tumor growth was monitored until reaching $4 \times$ the volume at irradiation time (T4 $\times$ SV) and treatment toxicity was scored by body weight measurements. Radiation sensitization enhancement ratios (SER) are calculated as the ratios of the mean T4 $\times$ SV of the DH348 compared to the vehicle treated irradiation groups.

Immunohistochemistry

Frozen sections were fixed in 4% formalin and incubated with normal goat serum. Contiguous sections were stained using rabbit anti-pimonidazole (1:250, NPI Inc.) or rabbit anti-CAIX (1:1000, Novus Biologicals). Secondary incubation was done with biotinylated goat anti-rabbit antibody (1:200 and 1:400 for CAIX and pimonidazole, respectively, DAKO). Detection was done with Vectastain ABC kit (Vector Laboratories) followed by 0.033% hydrogen peroxide in 10% diaminobenzidine (DAB; Sigma–Aldrich). Staining without primary antibody served as negative control. The sections were viewed (magnification 4 $\times$) by means of a Nikon Eclipse E800 microscope (Nikon Instruments Inc.).

Statistics

All statistical analyses were performed with GraphPad Prism version 5.03 for Windows (GraphPad Software, 2009, California, USA). A non-parametric Mann–Whitney *U* test for small groups was used to determine the statistical significance 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 CAIX protein levels in response to anoxia (Fig. 1B) and its expression is heterogeneous across tumors, overlapping the pimonidazole-positive hypoxic areas (Fig. 1C). To examine the necessity for CAIX activity for extracellular acidification under hypoxic conditions, the effect of the dual targeting compound DH348 on hypoxia-induced changes in extracellular pH was tested. A significant dose-dependent (*P* < 0.05) reduction in hypoxia-induced extracellular acidosis was observed, while the effect on cells exposed to ambient air was negligible (Fig. 1D). To explore the effect of CAIX activity inhibition on tumor growth and radiotherapy response, HT-29 tumor bearing mice were treated with DH348 and subsequently irradiated. Tumor growth was significantly (*P* < 0.01) inhibited in DH348-treated mice versus mice receiving vehicle only. The time to reach $4 \times$ irradiation starting volume (T4 $\times$ SV) was increased (*P* < 0.001) when treated with single dose irradiation, which was further enhanced (*P* < 0.05) upon combination with DH348 (Fig. 1E) resulting in a radiation sensitization enhancement ratio (SER) of 1.50.

To establish a causal relationship between CAIX expression and its therapeutic effect in more detail, CA9 was genetically silenced in a constitutive manner. A clear reduction in CAIX protein levels was observed for the CAIX knock-down (KD) cell line as compared with a control shRNA (EV) cell line (Fig. 2A). HT-29 EV xenografts demonstrated a robust and heterogeneous CAIX expression, which co-localized with the hypoxia marker pimonidazole. CAIX levels

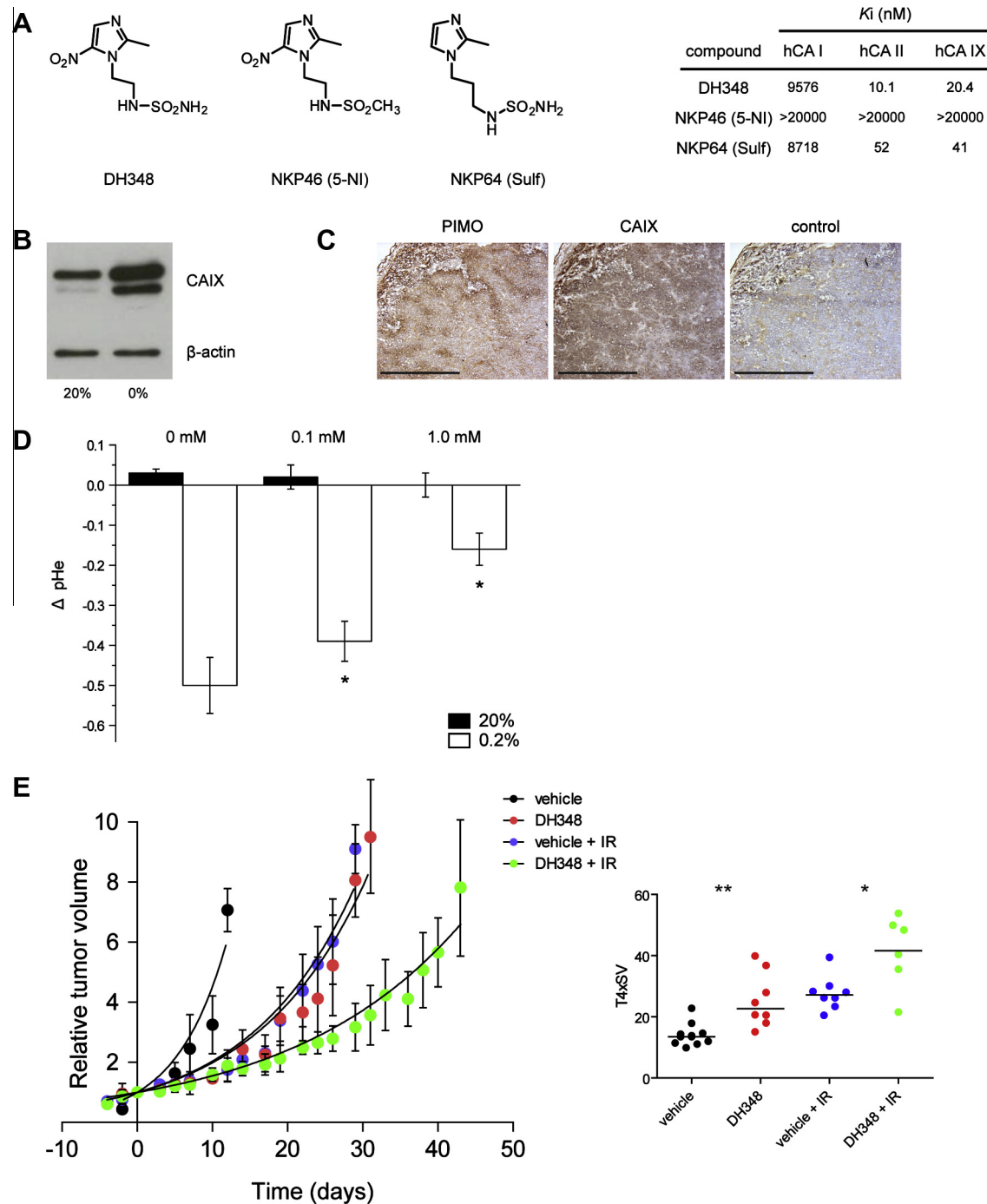


Fig. 1. (A) Chemical structure and affinity (Ki) for CAI, CAII and CAIX of the dual targeting compound DH348 and its respective single targeting controls. (B) Western blot analysis of CAIX expression in HT-29 cells under normoxic (20%) and anoxic (0%) conditions. β-Actin was used as loading control. (C) Representative pimonidazole (PIMO) and CAIX immunohistochemical staining of HT-29 tumors resected 5 days after inhibitor treatment. Negative control indicates staining without the primary antibody. Bar indicates 1 mm length. (D) Extracellular acidification of cells exposed to normoxia (20%) and hypoxia (0.2%) upon treatment with 0.1 or 1.0 mM DH348. Data show the mean ± SD of at least three independent experiments and are expressed as the difference between pHe values ($\Delta pHe = pH_{\text{after incubation}} - pH_{\text{before incubation}}$). (E) DH348 (10 mg/kg, 5 days) was administered intravenously when tumors reached an average size of 200 mm³ and tumors were irradiated (10 Gy) at day 3 (set to 0) of this treatment. Tumor growth was monitored until reaching 4× the volume at irradiation time (T4×SV). Data represent the mean ± SD of six to nine independent animals. Asterisks indicate statistical significance (**P* < 0.05; ***P* < 0.01).

were dramatically reduced in KD xenografts, however without affecting the presence of hypoxia as assessed with pimonidazole (Fig. 2B). Next we examined whether CAIX depletion in vitro resulted in a decreased extracellular acidification upon hypoxia. Extracellular acidosis upon hypoxia was significantly reduced (30%, *P* < 0.01) upon CAIX depletion (Fig. 2C). Furthermore, we investigated if pharmacological inhibition of CAIX activity would

mimic the effect of CAIX silencing. Besides the dual targeting drug DH348, the single targeting controls (NKP46 = 5-nitroimidazole: 5-NI and NKP64 = sulfamide: Sulf) were evaluated for their capacity to reduce the CAIX dependent extracellular acidification. Both DH348 and Sulf reduced the hypoxic extracellular acidosis (43% and 29% for 1.0 and 0.1 mM, respectively, *P* < 0.05) to similar levels as seen for the KD cells. No effect was seen when cells were

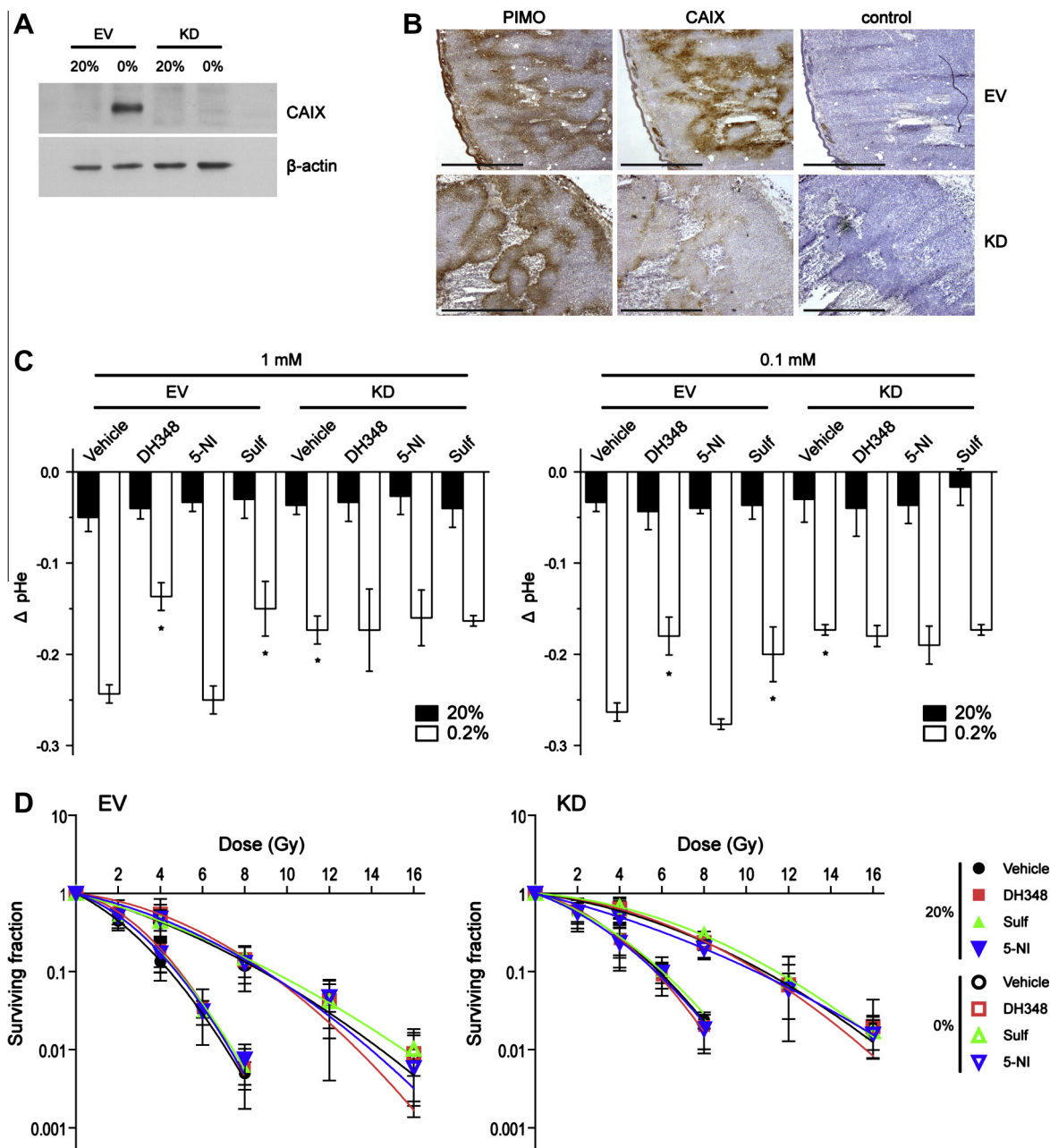


Fig. 2. (A) Western blot analysis of CAIX expression in HT-29 CAIX expressing (EV) and CAIX silenced (KD) cells under normoxic (20%) and anoxic (0%) conditions. (B) Representative pimonidazole (PIMO) and CAIX immunohistochemical staining of EV and KD tumors resected 5 days after inhibitor treatment. (C) Extracellular acidification of EV and KD cells exposed to normoxia (20%) and hypoxia (0.2%) upon treatment with 0.1 or 1.0 mM DH348 or controls. Data represent the mean $\pm$ SD of at least three independent experiments. (D) Clonogenic survival assay after exposure to different doses of irradiation upon normoxia (20%; 0, 2, 4, 6 and 8 Gy) and anoxia (0%; 0, 4, 8, 12 and 16 Gy) exposed EV and KD cells after pre-treatment (24 h) with 33 μ M DH348 or controls. Data show mean $\pm$ SD of at least three independent experiments. Asterisks indicate statistical significance compared to EV treated with vehicle under hypoxia (* $P < 0.05$; ** $P < 0.01$).

incubated in ambient air or upon addition to KD cells. Similarly, 5-NI did not affect extracellular acidosis under any experimental conditions (Fig. 2C). To examine if inhibition of CAIX activity influences radiosensitivity, the in vitro response of EV and KD cells to varying radiation doses after pre-incubation with the different compounds was measured under normoxia and anoxia (Fig. 2D). As expected, both cell lines demonstrated a more radioresistant phenotype under anoxic conditions. However, none of the compounds when added before irradiation resulted in enhancement of the irradiation effect, suggesting the need for a naive tumor microenvironment.

To exclude non-specific activity of the dual targeting compound in vivo when investigating the effect of CAIX inhibition on radiation response, growth delay experiments were repeated for EV and KD xenografts. All animals were treated with DH348 at a similar volume for both tumor models. DH348 treatment resulted in a significant growth delay ($P < 0.01$) compared to vehicle controls and sensitized EV tumors to radiation ($P < 0.05$) with a SER of 1.45. These effects were only observed for the CAIX expressing EV tumor model, supporting the specificity of the CAIX targeted treatment (Fig. 3). Additionally, no body weight loss was observed for any of the treatment schedules suggesting no observable systemic toxicity (Fig. S1).

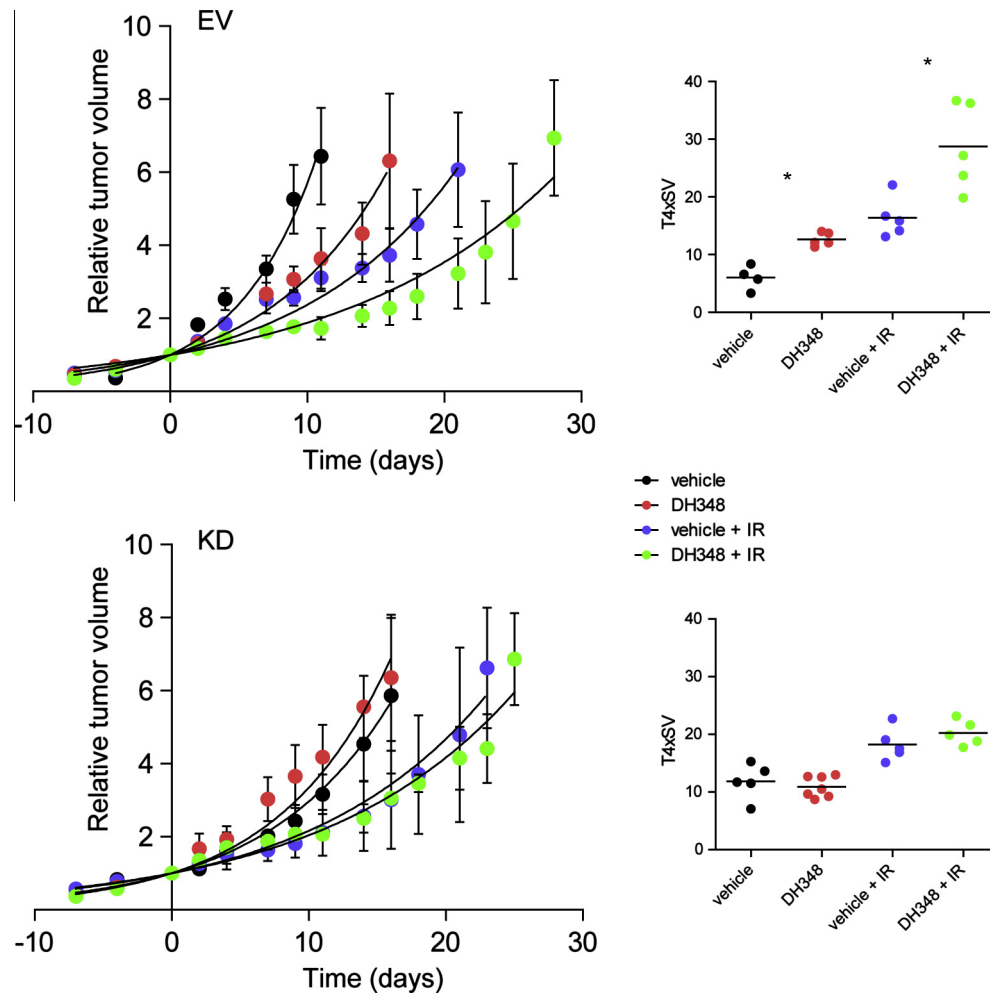


Fig. 3. DH348 (10 mg/kg, 5 days) was administered to EV or KD tumor-bearing animals when tumors reached an average size of 200 mm³ and tumors were irradiated (10 Gy) at day 3 (set to 0) of this treatment. Tumor growth was monitored until reaching 4× the volume at irradiation time (T4×SV). Data represent the mean ± SD of five to eight independent animals. Asterisks indicate statistical significance (**P* < 0.05; ***P* < 0.01).

Discussion

CAIX has been associated with cancer progression, metastasis and poor prognosis through its capacity to regulate intra- and extracellular acidity of hypoxic tumors [1,4] and is therefore a potential therapeutic target. Recently, CAIX inhibitors have been proposed as potential anti-tumor agents [2,5]. Previously, others and we have demonstrated that not only CAIX expression, but also hypoxia is a requirement to enable accumulation of different classes of CAIX inhibitors [3,6–8]. Hypoxia causes an acidification of the extracellular environment and studies have demonstrated that CAIX is the main contributor to this process [3,8,16]. Also in the current study, genetic silencing of CAIX reduced the hypoxia-induced extracellular acidosis. Additionally, blocking CAIX activity using sulfamide-based CAIX inhibitors was also able to reduce extracellular acidification up to 40% only upon hypoxia in a CAIX-dependent manner, in agreement with previously obtained results using sulfonamide-based inhibitors [6,8].

Hypoxia is a common feature of solid tumors and experimental and clinical studies have demonstrated that its presence is an important factor influencing tumor resistance to radiation therapy [17,18]. An extensively investigated approach to sensitize tumor to irradiation, is the use of nitroimidazole-based hypoxic radiosensitizers. The 2-nitroimidazole misonidazole has been shown to be a very efficient radiosensitizer in experimental and human tumors,

however associated with unacceptable systemic toxicity, which prevented the drug to be given in therapeutically relevant doses [14]. The less toxic 5-nitroimidazole derivative, nimorazole, has been shown to reach similar sensitizer enhancement ratios (SER 1.26) in a fractionated radiation schedule (300 mg/kg) as compared to misonidazole (SER 1.32) but with far less toxicity [14] and is therefore now used in standard clinical treatment for head and neck cancer patients in Denmark [15]. Recently, it has been shown that the tumor response to fractionated irradiation is also dependent on the glycolytic metabolism and therefore on extracellular acidosis [19]. We have previously shown that CAIX inhibition using sulfonamide 11c is able to counteract the hypoxic-induced extracellular acidification resulting in a sensitization to radiation [8]. Times to reach 4 times the starting volumes (T4×SV) were 32.5 (11c) vs 27.8 (vehicle) days for HT-29 parental and 22.2 (11c) vs 17.2 (vehicle) days for EV xenografts [8], resulting in a respective SER of 1.17 and 1.29.

We therefore designed a dual targeting CAIX-specific compound (DH348) composed of a 5-nitroimidazole radiosensitizing hypoxic cells and a sulfamide targeting active CAIX. We hypothesize that this compound shows an enhanced sensitization to irradiation in a CAIX-dependent manner. Reduced CAIX expression was observed in KD xenografts without affecting the presence of hypoxia as assessed with pimonidazole staining. A recent study reported an enhanced pimonidazole labeling upon CAIX silencing. However, this

association was only found in spheroids, but not in xenograft models, emphasizing the requirement of host tissue [20]. Single treatment with DH348 resulted in a slower tumor growth compared to vehicle treatment only in CAIX expressing tumors. Similar results were obtained in mammary or colorectal tumor-bearing mice for which the effect is dependent on the treatment schedule and the administered dose [8,9,11]. In contrary, a new class of CAIX-specific sulfamate inhibitors did not affect primary tumor growth at low doses, but had significant anti-metastatic properties in breast cancer therapy [12]. Recently, the general CA inhibitor acetazolamide has been found to enhance the toxicity of the chemotherapeutic agent doxorubicin, with the highest effect for CAIX expressing cell lines [13]. Therefore, it is promising to pursue specific inhibitors of CAIX activity for their capacity to enhance the effect of conventional therapies.

In the current study, upon treatment with the CAIX-specific dual targeting sulfamide DH348, the therapeutic effect of irradiation was enhanced in a CAIX dependent manner (SER 1.50 and 1.45 for HT-29 parental and EV tumors, respectively) without systemic toxicity as evaluated by body weight loss. Higher SER was found for parental HT-29, which might be explained by higher CAIX activity, since hypoxic extracellular acidification was larger compared to the EV tumor cells. Furthermore, no sensitization (SER 1.04) could be observed for the KD tumors. The enhancement ratios found in the present study are 30% higher compared with the previously investigated indanesulfonamide [8]. It can be argued that we did not include the single targeting agents in our animal study. Previously, we have demonstrated that a single CAIX targeting agent showed smaller sensitization effects compared with the currently investigated dual targeting drug (vide supra) [8]. Additionally, we observed that the SER of the dual targeting compound DH348 (10 mg/kg) was also higher than the well-established hypoxic radiosensitizers misonidazole and nimorazole for which a minimal dose of 100 mg/kg was needed to achieve an SER above unity [14]. In vitro, no hypoxic radiosensitization was observed using previously described effective dose regimens [12]. Since also no radiosensitization was found for the 5-NI single compound when using equimolar doses, in vitro drug dose levels might be too low. Nimorazole has been shown to radiosensitize tumor cells, but this at dose levels 30-fold higher than used in the current study [21]. We are currently investigating if higher drug doses can radiosensitize hypoxic cells in vitro. In the present proof-of-principle study only one tumor line was investigated and single dose was applied, which warrants confirmatory investigations using tumor models of other histology and fractionated radiation schemes. These experiments as well as mechanistic studies have been initiated in our laboratory. In order to prepare the first in human clinical trial, pharmacokinetic, pharmacodynamic and extensive toxicity studies are currently ongoing.

In conclusion, a newly designed nitroimidazole and sulfamide based dual targeting drug is able to reduce hypoxic extracellular acidification, to inhibit tumor growth at nontoxic doses and to sensitize tumors to irradiation in a CAIX dependent manner proving the absence of any "off-target" effect. The dual drug enhances radiation response more effectively as compared to single published CAIX targeting compounds. Our data therefore indicate the potential utility of a dual drug approach as a new strategy for tumor-specific targeting to eventually improve the response to radiation treatment.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.radonc.2013.06.018>.

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