CAIX

Citation for published version (APA):

van Kuijk, S. J. A. (2016). *CAIX: a potential target for cancer therapy*. [Doctoral Thesis, Maastricht University]. Datawyse / Universitaire Pers Maastricht. <https://doi.org/10.26481/dis.20161222sk>

Document status and date:

Published: 01/01/2016

DOI:

[10.26481/dis.20161222sk](https://doi.org/10.26481/dis.20161222sk)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
- The final author version and the galley proof are versions of the publication after peer review.
- The final published version features the final layout of the paper including the volume, issue and page numbers.

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CAIX:

A Potential Target for Cancer Therapy

Simon J.A. van Kuijk

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Cover: A golden ratio in cancer? Simon van Kuijk

ISBN: 978 94 6159 639 0

Print: Datawyse | Universitaire Pers Maastricht

CAIX:

A Potential Target for Cancer Therapy

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof dr. Rianne M. Letschert
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op donderdag 22 december 2016 om 14.00 uur

door

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Chapter 1

Introduction and outline thesis

CANCER AND CANCER TREATMENT

Cancer is a disease that has existed for over thousands of years, but is currently one of the leading causes of death in the world [1]. The rise in cancer incidence can be explained by an increased exposure to carcinogens, e.g. by smoking or alcohol consumption, causing DNA damage in normal cells, which may lead to mutations and disturb normal cellular functions. A single DNA mutation is however never sufficient to initiate the development of cancer. Instead, multiple mutations are required that need to deactivate tumor suppressor genes or activate oncogenes for a normal cell to eventually transform to a cancer cell. In cancer the accumulation of these acquired mutations, combined with genetic predisposed risk factors, e.g. BRCA1/2 mutations in breast cancer [2-4], and in some cases viral infections, e.g. HPV positivity in cervical cancer [5], results in an increased proliferative capacity and lifespan of the cell. In addition, several other cellular processes that aid in promoting carcinogenesis, i.e. the Hallmarks of Cancer, are also affected [6, 7]. Furthermore, cancer cells will continue to acquire mutations thereby giving rise to more aggressive phenotypes, of which a selective few will acquire the ability to invade surrounding tissue, migrate to distant organ sites and form metastases, which are the leading cause of death in the vast majority of cancer types [8-10].

The exact mechanisms responsible for cancer development however remain to be elucidated. Most cancer treatment options, including surgery, are therefore relatively blunt instruments. Chemotherapeutics for instance induce DNA damage in every fast proliferating cell in the body, which often results in systemic side-effects and often leads to acquired resistance to treatment [11]. Alternatively, the use of ionizing radiation allows for a more localized treatment delivery where the highest dose is administered to the tumor, whereas the surrounding normal tissue is spared as much as possible. Technical advances in radiotherapy continue to improve, but the successful treatment of micrometastases or undetectable tumors will remain problematic [12-15]. New treatment options that target tumor-specific molecular pathways, which might increase standard treatment efficacy and limit normal tissue toxicity therefore need to be investigated.

TUMOR MICROENVIRONMENT, HYPOXIA, AND THE HIF-PATHWAY

Identifying new targets for cancer treatment is however challenging since cancer is not a clump of one single cell population, but instead is a collection of many different cellular phenotypes, which all may have a varying response to treatment. In clinical practice however cancer is most of the time still only classified based on the organ site or cell type affected. Besides the so-called intrinsic sensitivity of cancer cells to treatment, e.g. via their DNA repair capacity, the response to treatment may also vary due to a heterogeneous tumor microenvironment [16] (Figure 1). Tumor hypoxia is such a microenvi-

ronmental characteristic in which certain areas in the tumor have a limited availability of oxygen. Hypoxia is associated with a worse prognosis in cancer patients [17] and promotes treatment resistance to surgery [18], chemotherapy [19], and radiotherapy [20]. This resistance to treatment is partly caused by a limited drug perfusion in the tumor in combination with a decrease in drug uptake [19], and limiting DNA damage fixation after ionizing radiation in the absence of oxygen [15, 20].

Figure 1: Schematic representation of the tumor microenvironment. The inadequate diffusion and supply of oxygen from blood vessels to tumor cells causes a decrease in oxygen availability with increasing distance from blood vessels. The areas in the tumor with extremely low levels of oxygen become hypoxic (blue), which is closely associated with a lower (acidic) extracellular pH and a decrease in treatment efficacy (TE).

Hypoxia arises when a tumor outgrows its supporting vasculature, thereby minimizing the levels of nutrients and oxygen that are available to the cell (Figure 1). These hypoxic areas require tumor cells to adapt to these hostile stresses for their continued survival and growth. Several mechanisms are involved of which the stabilization of Hypoxia-Inducible Factor 1 alpha (HIF1- α) is an important one [21, 22] (Figure 2). HIF1- α is expressed in every tissue in the human body, but is hydroxylated by prolyl-hydroxylase 2 (PHD2) during normoxic conditions [23]. This hydroxylation of HIF1- α allows for its recognition by the E3 ubiquitin ligase complex containing the von Hippel-Lindau tumor suppressor protein (VHL) and facilitates its subsequent proteasomal degradation. Hypoxia exposure inhibits PHD2 function, thereby stabilizing the HIF1- α protein, allowing for its dimerization with the abundantly expressed HIF1-beta and nuclear translocation.

The HIF protein complex together with the co-factor P300 then binds to hypoxia responsive element (HRE) regions in the promotor region of multiple genes, resulting in the transcription of these genes to promote cellular survival during hypoxia [21, 22].

Figure 2: Schematic representation of the HIF-pathway. The HIF pathway is activated during hypoxic conditions and modulates several cellular functions via the transcription of multiple genes. Abbreviations: Carbonic Anhydrase IX (CAIX); Hydroxyl group (OH); Hypoxia-inducible factor (HIF); Hypoxia responsive element (HRE); Prolyl hydroxylase 2 (PHD2); Transcriptional coactivator P300 (P300); Von Hippel-Lindau protein (VHL).

Tumors have long been known to use a more glycolytic energy production, i.e. the Warburg effect [24], which is less effective, but promotes carcinogenesis because certain glycolytic substrates can be utilized in macromolecule synthesis and thereby effectively stimulate cell growth [21]. This notion is supported by the observation that most cancer cells continue to use their glycolytic energy production when (re)exposed to normoxic conditions [6, 7, 24]. In addition, during hypoxic conditions the glycolytic energy production does not rely on the availability of oxygen, which thereby allow for the survival of the cancer cell in the hostile hypoxic environment. This survival is further promoted by HIF stabilization, which causes upregulation of glucose transporters (GLUT-1) expression to increase the availability of glucose as a substrate for the cells glycolytic energy metabolism. Nevertheless, glycolysis produces lactate as a waste product, which is excreted by several HIF-regulated ion transporters, e.g. monocarboxylate transporters (MCT), to prevent intracellular acidification [25, 26]. In addition, glycolysis also results in the production of the weak acid carbon dioxide, which can diffuse passively across the plasma membrane and requires a different protein for its inactivation, namely carbonic anhydrase IX (CAIX) [27].

CARBONIC ANHYDRASE IX

CAIX is a zinc-containing glycoprotein and a member of the carbonic anhydrase family of enzymes [28]. Carbonic anhydrases have an evolutionary conserved function to facilitate the hydration of carbon dioxide to bicarbonate and a proton. The family consists of 15 members of which two, i.e. CAIX and CAXII, have been associated with cancer and are expressed on the extracellular cell membrane [29, 30]. Expression of CAIX is transcriptionally regulated via HIF stabilization due to a HRE sequence in the promoter region in its DNA [31]. Furthermore, CAIX expression was found to be highly tumor specific with only marginal expression in epithelia of the gallbladder and stomach [32]. The predominant tumor specific expression pattern combined with its hypoxia-regulated transcription promoted the use of CAIX as a potential prognostic biomarker in cancer patients, e.g. [33-37].

The protons that are formed after hydration of carbon dioxide by CAIX accumulate in the extracellular space, thereby causing extracellular acidification and promoting tumor invasion by stimulating extracellular matrix degradation [38]. The bicarbonate that is formed however is transported back intracellular by sodium bicarbonate transporters (NBC) and anion exchangers (AE) (Figure 3). The intracellularly transported bicarbonate can be used as a buffer via CAII, which forms a metabolon with CAIX, and converts the bicarbonate and intracellular produced protons back to water and carbon dioxide. In addition, the intracellular bicarbonate concentrations cause a slightly alkaline intracellular pH, which promotes tumor cell proliferation and survival in the hostile acidic microenvironment [39-41]. CAIX is therefore involved in maintaining the balance between the alkaline intracellular and the acidic extracellular pH of tumor cells. In addition CAIX expression was found to be involved in the selection of a more aggressive phenotype of cancer cells. Firstly, CAIX expression was associated with several stem cell markers and may therefore be involved in the maintenance of cancer stem cells, which are more resistant to therapy [42]. Secondly, CAIX may promote metastases formation due to its ability to maintain a proton gradient, which resulted in CAIX re-localization to the invading lamellipodia of migrating tumor cells [43]. In addition, the glycosylated part of CAIX aids in cellular attachment and may thereby also aid in facilitating tumor cell migration [44, 45]. Together, these data suggest that CAIX is involved in selecting and maintaining a more aggressive cancer cell phenotype and promote the formation of metastases.

Figure 3: CAIX and other enzymes involved in maintaining the cellular pH balance. Many enzymes are involved in maintaining the cellular pH balance of tumor cells to compensate for the increased production of acid during glycolysis. Abbreviations: Anion exchanger (AE); Carbonic Anhydrase II (CAII); Carbonic Anhydrase IX (CAIX); Glucose transporter (GLUT); Monocarboxylate transporter (MCT); Sodium-bicarbonate cotransporter (NBC); Sodium-hydrogen antiporter 1 (NHE-1).

CAIX INHIBITORS

The tumor-specific expression of CAIX, its important role in maintaining the pH balance, and its association with a more aggressive tumor cell phenotype make it a promising target for therapy [25, 46]. Many transmembrane-specific carbonic anhydrase inhibitors are therefore being designed [47], which are hypothesized to lead to intracellular acid accumulation and tumor-specific cell death [48-50]. In addition, CAIX inhibitors have been shown to reduce tumor cell migration *in vitro* and decrease metastases formation *in vivo* [42, 49, 51]. Furthermore, since CAIX inhibitors only bind the zinc containing active site of CAIX when exposed to hypoxic conditions [52, 53], they allow for the specific targeting of the hypoxic areas of tumors and therefore increase the radiosensitivity of tumor xenografts [54]. Combination therapies of CAIXi with conventional treatment options, such as chemo- and radiotherapy, might therefore prove to be especially effective, as CAIXi have the potential to increase the efficacy of those conventional treatment modalities by affecting the hypoxic tumor microenvironment.

Firstly, the efficacy of weak basic chemotherapeutics, such as doxorubicin, might increase when combined with CAIX inhibitors, since the acidic extracellular environment of tumors hampers the cellular uptake of these drugs as explained by the ion trapping

model, i.e. formation of charged molecules that tend to remain cell impermeable [19, 55, 56]. Since CAIX inhibitors decrease extracellular acidification during hypoxia exposure [52, 54, 57], the combination treatment might allow for an increase in doxorubicin uptake and efficacy in the CAIX expressing tumor cell. Indeed, exposing cancer cells to the non-selective CA inhibitor acetazolamide resulted in an increased uptake and efficacy of doxorubicin *in vitro* [58]. Furthermore, specific CAIX inhibition was also reported to increase doxorubicin efficacy *in vivo* [59]. Secondly, the ability of CAIXi to specifically target and induce cell death in CAIX expressing tumor cells, which are closely associated with hypoxic areas in the tumor, may decrease the hypoxic fraction of those tumors, thereby increasing their radiosensitivity [60-62]. In general, CAIX inhibitor treatment might therefore be a valuable tool to specifically target the CAIX expressing hypoxic compartment of tumors and thereby increase the efficacy of standard treatment modalities, such as radio- and chemotherapy. In addition, the tumor specific expression of CAIX can be targeted for specific delivery of cytotoxic drugs to those CAIX expressing cells, e.g. via dual-targeting compounds existing of a conjugation of CAIX targeting CAIXi with cytotoxic drugs, to minimize normal tissue toxicity.

SOLUBLE ADENYLYL CYCLASE

Single treatment with a CAIX inhibitor may however not be sufficient enough to shift the tumor cells pH balance and cause intracellular acidification in hypoxic tumor cells. Besides CAIX many other HIF-regulated proteins aid in maintaining the pH balance of tumor cells [21, 25], e.g. MCT, NHE, NBC, and potentially compensate for a loss of CAIX function. A promising alternative therapeutic target might therefore be a protein that is involved in regulating the function of multiple proteins involved in pH regulation.

Soluble adenylyl cyclase (sAC) was identified in 1975 as a distinct member of the adenylyl cyclase family of proteins [63]. Adenylyl cyclases produce cyclic adenosine monophosphate (cAMP), one of the most common second messengers, which are intracellular signaling molecules released by the cell after external or internal stimuli to activate intracellular signal transduction cascades. Of the ten adenylyl cyclase family members only sAC is expressed in cytosolic microdomains instead of on the plasma membrane [64-66]. The function of sAC is not modulated by G-protein coupled receptors, but directly responds to bicarbonate as a surrogate of intracellular pH and therefore acts as an intracellular pH sensor [67, 68] (Figure 4). Upon sAC activation, vacuolar-type H⁺-ATPase (V-ATPase) and bicarbonate transporters translocate to the luminal cell membrane to aid in proton extrusion and thereby modulate the pH balance of the cells, as has been shown for clear cells of the epididymis and renal A-type cells [69-72]. In addition, non-selective CA inhibition with acetazolamide was reported to increase cAMP levels in a sAC-dependent manner in the non-pigmented ciliary epithelial cells of the eye, suggesting a link between carbonic anhydrase function and sAC [73].

Figure 4: Activation and function of soluble adenylyl cyclase. The bicarbonate that can activate sAC either enters the cell via bicarbonate transporters (NBC and AE), or is generated from mitochondrial metabolism, or originates from hydration of carbon dioxide by intracellular carbonic anhydrases. Activation of sAC produces cAMP, which can modulate several cellular functions, including the translocation of V-ATPase from intracellular vesicles to the cellular membrane, thereby modulating the cellular pH balance. Abbreviations: Adenosine triphosphate (ATP); Anion exchanger (AE); Carbonic anhydrase (CA); Cyclic adenosine monophosphate (cAMP); Soluble adenylyl cyclase (sAC); Vacuolar-type H⁺-ATPase (V-ATPase).

In cancer, specific expression patterns of sAC have been reported to be of diagnostic relevance in melanoma patients, e.g. pan-nuclear staining in lentigo maligna [74, 75]. Furthermore, sAC is overexpressed in prostate carcinoma and its inhibition decreased proliferation and survival of prostate cancer cells *in vitro* [76, 77]. In addition, sAC inhibition also increased the radiosensitivity of prostate cancer cells [76]. In contrast however, sAC has

also recently been described to be a tumor suppressor protein that reduces cellular transformation *in vitro* and malignant progression *in vivo* [78]. Together, these data show the incomplete understanding of the function of sAC in cancer and its function during hypoxia. Furthermore, a possible association between sAC and CAIX remains to be elucidated.

OUTLINE OF THE THESIS

The hypoxic microenvironment of solid tumors is a well-known adverse prognostic factor since hypoxia causes resistance to standard treatment modalities. CAIX expression is closely associated with the hypoxic areas of a tumor and is important for the cells survival in hostile acidic conditions. Together with the tumor-specific expression, CAIX might be a promising target for anti-cancer treatment. More specifically, because CAIX inhibition can modulate efficacy of basic chemotherapeutic agents as well as radiation, it is very interesting to study the effect of CAIXi in combination therapies. The general goal of this thesis is to study the potential of CAIX for anti-cancer therapy, either by increasing the efficacy of conventional treatment options by combining them with CAIXi, or by using CAIX expression as a target for anti-cancer drug delivery (Figure 5). In **Chapter 2** the clinical importance of CAIX expression is first evaluated, since conflicting data exists in literature about its prognostic value. Therefore, a meta-analysis has been performed, which included all available literature, to make a general conclusion about the prognostic value of CAIX.

Figure 5: Hypotheses investigated in this thesis. Several different CAIX inhibitors were studied in this thesis to modulate doxorubicin uptake, increase the radiosensitivity of tumors, or target cytotoxic compounds to CAIX expressing cells. Furthermore, the association between CAIX and prognosis was studied, as well as the involvement of sAC in the regulation of CAIX function. Abbreviations: Carbonic anhydrase IX (CAIX); Ionizing radiation (IR); Soluble adenylyl cyclase (sAC).

Since the uptake of certain chemotherapeutic agents is hampered upon hypoxia due to the enhanced extracellular acidification, combination with CAIX inhibitors might increase the therapeutic efficacy of these agents. In **Chapter 3** the ability of the cytotoxic ureido-substituted sulfamate CAIXi S4 to increase the efficacy of doxorubicin treatment is investigated. In **Chapter 4** a new class of CAIXi is described, which consists of a nitroimidazole moiety, i.e. a known radiosensitizer [61], conjugated to a CAIXi to allow for the specific targeting of CAIX expressing cells in tumors. This dual-target compound DH348 was previously found to increase the efficacy of doxorubicin, but its effect on the radiosensitivity of tumors is described in the present work. In **Chapter 5** the efficacy of the dual-target compound DH348 is further investigated after oral application and the attempts to validate the initial results using an identical experimental setup are described.

The design and development of several new classes of dual-target compounds is described in **Chapter 6** of this thesis. These compounds consist of different classes of anti-cancer drugs conjugated to a CAIXi moiety. The aim of these compounds is to exert their effect specifically in CAIX expressing cells, which should increase the tumor-specificity of the anti-cancer drugs and have the potential to decrease normal tissue toxicity. A potential novel therapeutic target for cancer therapy is discussed in **Chapter 7**, since sAC functions as an intracellular pH sensor and may act as a regulating mechanism for maintaining the pH balance, thereby possibly also affecting CAIX expression and function.

Finally, **Chapter 8** of this thesis will provide a general discussion on the use of CAIXi as an anti-cancer therapy, i.e. as single agent therapy, in combination strategies with conventional treatment options, or as a target for drug delivery. Furthermore, important future directions in the development of CAIX as a therapeutic target are addressed.

REFERENCES

1. Stewart, B.W., et al., *World cancer report 2014*. xiv, 630 pages.
2. Fu, R., et al., *Estimating risk of breast cancer in carriers of BRCA1 and BRCA2 mutations: a meta-analytic approach*. Statistics in medicine, 2007. **26**(8): p. 1775-87.
3. Molina-Montes, E., et al., *Cumulative risk of second primary contralateral breast cancer in BRCA1/BRCA2 mutation carriers with a first breast cancer: A systematic review and meta-analysis*. Breast, 2014. **23**(6): p. 721-42.
4. Hilgart, J.S., B. Coles, and R. Iredale, *Cancer genetic risk assessment for individuals at risk of familial breast cancer*. The Cochrane database of systematic reviews, 2012(2): p. CD003721.
5. Gonzalez Martin, A., *Molecular biology of cervical cancer*. Clinical & translational oncology : official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico, 2007. **9**(6): p. 347-54.
6. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer*. Cell, 2000. **100**(1): p. 57-70.
7. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation*. Cell, 2011. **144**(5): p. 646-74.
8. Mehlen, P. and A. Puisieux, *Metastasis: a question of life or death*. Nature reviews. Cancer, 2006. **6**(6): p. 449-58.
9. Monteiro, J. and R. Fodde, *Cancer stemness and metastasis: therapeutic consequences and perspectives*. European journal of cancer, 2010. **46**(7): p. 1198-203.
10. Nguyen, D.X., P.D. Bos, and J. Massague, *Metastasis: from dissemination to organ-specific colonization*. Nature reviews. Cancer, 2009. **9**(4): p. 274-84.
11. Dilruba, S. and G.V. Kalayda, *Platinum-based drugs: past, present and future*. Cancer chemotherapy and pharmacology, 2016. **77**(6): p. 1103-24.
12. Dinesh Mayani, D., *Proton therapy for cancer treatment*. Journal of oncology pharmacy practice : official publication of the International Society of Oncology Pharmacy Practitioners, 2011. **17**(3): p. 186-90.
13. Gomez, D. and R. Komaki, *Technical advances of radiation therapy for thymic malignancies*. Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer, 2010. **5**(10 Suppl 4): p. S336-43.
14. Nakano, T., et al., *Current advancement in radiation therapy for uterine cervical cancer*. Journal of radiation research, 2010. **51**(1): p. 1-8.
15. Joiner, M. and A.v.d. Kogel, *Basic clinical radiobiology*. 4th ed2009, London: Hodder Arnold ;. vi, 375 p.
16. Horsman, M.R. and P. Vaupel, *Pathophysiological Basis for the Formation of the Tumor Microenvironment*. Frontiers in oncology, 2016. **6**: p. 66.
17. Nordsmark, M., et al., *Prognostic value of tumor oxygenation in 397 head and neck tumors after primary radiation therapy. An international multi-center study*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2005. **77**(1): p. 18-24.
18. Hockel, M., et al., *Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix*. Cancer research, 1996. **56**(19): p. 4509-15.
19. Wojtkowiak, J.W., et al., *Drug resistance and cellular adaptation to tumor acidic pH microenvironment*. Molecular pharmaceutics, 2011. **8**(6): p. 2032-8.
20. Good, J.S. and K.J. Harrington, *The hallmarks of cancer and the radiation oncologist: updating the 5Rs of radiobiology*. Clinical oncology, 2013. **25**(10): p. 569-77.
21. Denko, N.C., *Hypoxia, HIF1 and glucose metabolism in the solid tumour*. Nature reviews. Cancer, 2008. **8**(9): p. 705-13.
22. Wouters, B.G. and M. Koritzinsky, *Hypoxia signalling through mTOR and the unfolded protein response in cancer*. Nature reviews. Cancer, 2008. **8**(11): p. 851-64.
23. Berra, E., et al., *HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia*. The EMBO journal, 2003. **22**(16): p. 4082-90.
24. Warburg, O., *On the origin of cancer cells*. Science, 1956. **123**(3191): p. 309-14.

25. Neri, D. and C.T. Supuran, *Interfering with pH regulation in tumours as a therapeutic strategy*. Nature reviews. Drug discovery, 2011. **10**(10): p. 767-77.
26. Parks, S.K., J. Chiche, and J. Pouyssegur, *pH control mechanisms of tumor survival and growth*. Journal of cellular physiology, 2011. **226**(2): p. 299-308.
27. Sedlakova, O., et al., *Carbonic anhydrase IX, a hypoxia-induced catalytic component of the pH regulating machinery in tumors*. Frontiers in physiology, 2014. **4**: p. 400.
28. Pastorek, J., et al., *Cloning and characterization of MN, a human tumor-associated protein with a domain homologous to carbonic anhydrase and a putative helix-loop-helix DNA binding segment*. Oncogene, 1994. **9**(10): p. 2877-88.
29. Supuran, C.T., *Carbonic anhydrases: novel therapeutic applications for inhibitors and activators*. Nature reviews. Drug discovery, 2008. **7**(2): p. 168-81.
30. Alterio, V., et al., *Multiple binding modes of inhibitors to carbonic anhydrases: how to design specific drugs targeting 15 different isoforms?* Chemical reviews, 2012. **112**(8): p. 4421-68.
31. Wykoff, C.C., et al., *Hypoxia-inducible expression of tumor-associated carbonic anhydrases*. Cancer research, 2000. **60**(24): p. 7075-83.
32. Pastorekova, S., et al., *Carbonic anhydrase IX, MN/CA IX: analysis of stomach complementary DNA sequence and expression in human and rat alimentary tracts*. Gastroenterology, 1997. **112**(2): p. 398-408.
33. Wykoff, C.C., et al., *Expression of the hypoxia-inducible and tumor-associated carbonic anhydrases in ductal carcinoma in situ of the breast*. The American journal of pathology, 2001. **158**(3): p. 1011-9.
34. Smeland, E., et al., *Prognostic impacts of hypoxic markers in soft tissue sarcoma*. Sarcoma, 2012. **2012**: p. 541650.
35. Stewart, D.J., et al., *Membrane carbonic anhydrase IX expression and relapse risk in resected stage I-II non-small-cell lung cancer*. J Thorac Oncol, 2014. **9**(5): p. 675-84.
36. Kim, S.J., et al., *Prognostic value of carbonic anhydrase IX and Ki-67 expression in squamous cell carcinoma of the tongue*. Japanese journal of clinical oncology, 2007. **37**(11): p. 812-9.
37. Douglas, C.M., et al., *Lack of prognostic effect of carbonic anhydrase-9, hypoxia inducible factor-1alpha and bcl-2 in 286 patients with early squamous cell carcinoma of the glottic larynx treated with radiotherapy*. Clinical oncology, 2013. **25**(1): p. 59-65.
38. Stock, C. and A. Schwab, *Protons make tumor cells move like clockwork*. Pflugers Archiv : European journal of physiology, 2009. **458**(5): p. 981-92.
39. Swietach, P., et al., *Tumor-associated carbonic anhydrase 9 spatially coordinates intracellular pH in three-dimensional multicellular growths*. The Journal of biological chemistry, 2008. **283**(29): p. 20473-83.
40. Swietach, P., et al., *The role of carbonic anhydrase 9 in regulating extracellular and intracellular pH in three-dimensional tumor cell growths*. The Journal of biological chemistry, 2009. **284**(30): p. 20299-310.
41. McIntyre, A., et al., *Disrupting hypoxia-induced bicarbonate transport acidifies tumor cells and suppresses tumor growth*. Cancer research, 2016.
42. Lock, F.E., et al., *Targeting carbonic anhydrase IX depletes breast cancer stem cells within the hypoxic niche*. Oncogene, 2013. **32**(44): p. 5210-9.
43. Svastova, E., et al., *Carbonic anhydrase IX interacts with bicarbonate transporters in lamellipodia and increases cell migration via its catalytic domain*. The Journal of biological chemistry, 2012. **287**(5): p. 3392-402.
44. Zavada, J., et al., *Human tumour-associated cell adhesion protein MN/CA IX: identification of M75 epitope and of the region mediating cell adhesion*. British journal of cancer, 2000. **82**(11): p. 1808-13.
45. Csaderova, L., et al., *The effect of carbonic anhydrase IX on focal contacts during cell spreading and migration*. Frontiers in physiology, 2013. **4**: p. 271.
46. Pastorek, J. and S. Pastorekova, *Hypoxia-induced carbonic anhydrase IX as a target for cancer therapy: from biology to clinical use*. Seminars in cancer biology, 2015. **31**: p. 52-64.
47. Monti, S.M., C.T. Supuran, and G. De Simone, *Anticancer carbonic anhydrase inhibitors: a patent review (2008 - 2013)*. Expert opinion on therapeutic patents, 2013. **23**(6): p. 737-49.

48. Chiche, J., et al., *Hypoxia-inducible carbonic anhydrase IX and XII promote tumor cell growth by counteracting acidosis through the regulation of the intracellular pH*. Cancer research, 2009. **69**(1): p. 358-68.
49. Gieling, R.G., et al., *Antimetastatic effect of sulfamate carbonic anhydrase IX inhibitors in breast carcinoma xenografts*. Journal of medicinal chemistry, 2012. **55**(11): p. 5591-600.
50. Winum, J.Y., et al., *Ureido-substituted sulfamates show potent carbonic anhydrase IX inhibitory and antiproliferative activities against breast cancer cell lines*. Bioorganic & medicinal chemistry letters, 2012. **22**(14): p. 4681-5.
51. Ward, C., et al., *Evaluation of carbonic anhydrase IX as a therapeutic target for inhibition of breast cancer invasion and metastasis using a series of in vitro breast cancer models*. Oncotarget, 2015. **6**(28): p. 24856-70.
52. Dubois, L., et al., *Imaging the hypoxia surrogate marker CA IX requires expression and catalytic activity for binding fluorescent sulfonamide inhibitors*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2007. **83**(3): p. 367-73.
53. Dubois, L., et al., *Imaging of CA IX with fluorescent labelled sulfonamides distinguishes hypoxic and (re)-oxygenated cells in a xenograft tumour model*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2009. **92**(3): p. 423-8.
54. Dubois, L., et al., *Specific inhibition of carbonic anhydrase IX activity enhances the in vivo therapeutic effect of tumor irradiation*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2011. **99**(3): p. 424-31.
55. Mahoney, B.P., et al., *Tumor acidity, ion trapping and chemotherapeutics. I. Acid pH affects the distribution of chemotherapeutic agents in vitro*. Biochemical pharmacology, 2003. **66**(7): p. 1207-18.
56. Raghunand, N., B.P. Mahoney, and R.J. Gillies, *Tumor acidity, ion trapping and chemotherapeutics. II. pH-dependent partition coefficients predict importance of ion trapping on pharmacokinetics of weakly basic chemotherapeutic agents*. Biochemical pharmacology, 2003. **66**(7): p. 1219-29.
57. Svastova, E., et al., *Hypoxia activates the capacity of tumor-associated carbonic anhydrase IX to acidify extracellular pH*. FEBS letters, 2004. **577**(3): p. 439-45.
58. Gieling, R.G., et al., *Inhibition of carbonic anhydrase activity modifies the toxicity of doxorubicin and melphalan in tumour cells in vitro*. Journal of enzyme inhibition and medicinal chemistry, 2013. **28**(2): p. 360-9.
59. Rami, M., et al., *Hypoxia-targeting carbonic anhydrase IX inhibitors by a new series of nitroimidazole-sulfonamides/sulfamides/sulfamates*. Journal of medicinal chemistry, 2013. **56**(21): p. 8512-20.
60. Dubois, L.J., et al., *New ways to image and target tumour hypoxia and its molecular responses*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2015. **116**(3): p. 352-7.
61. Overgaard, J., *Clinical evaluation of nitroimidazoles as modifiers of hypoxia in solid tumors*. Oncology research, 1994. **6**(10-11): p. 509-18.
62. Pettersen, E.O., et al., *Targeting tumour hypoxia to prevent cancer metastasis. From biology, biosensing and technology to drug development: the METOXIA consortium*. Journal of enzyme inhibition and medicinal chemistry, 2015. **30**(5): p. 689-721.
63. Braun, T. and R.F. Dods, *Development of a Mn-2+-sensitive, "soluble" adenylate cyclase in rat testis*. Proceedings of the National Academy of Sciences of the United States of America, 1975. **72**(3): p. 1097-101.
64. Zippin, J.H., et al., *Compartmentalization of bicarbonate-sensitive adenyllyl cyclase in distinct signaling microdomains*. FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2003. **17**(1): p. 82-4.
65. Zippin, J.H., et al., *Soluble adenyllyl cyclase defines a nuclear cAMP microdomain in keratinocyte hyperproliferative skin diseases*. The Journal of investigative dermatology, 2010. **130**(5): p. 1279-87.
66. Desman, G., C. Waintraub, and J.H. Zippin, *Investigation of cAMP microdomains as a path to novel cancer diagnostics*. Biochimica et biophysica acta, 2014. **1842**(12 Pt B): p. 2636-45.
67. Rahman, N., J. Buck, and L.R. Levin, *pH sensing via bicarbonate-regulated "soluble" adenyllyl cyclase (sAC)*. Frontiers in physiology, 2013. **4**: p. 343.

68. Wuttke, M.S., J. Buck, and L.R. Levin, *Bicarbonate-regulated soluble adenylyl cyclase*. JOP : Journal of the pancreas, 2001. **2**(4 Suppl): p. 154-8.
69. Tresguerras, M., J. Buck, and L.R. Levin, *Physiological carbon dioxide, bicarbonate, and pH sensing*. Pflugers Archiv : European journal of physiology, 2010. **460**(6): p. 953-64.
70. Pastor-Soler, N., et al., *Bicarbonate-regulated adenylyl cyclase (sAC) is a sensor that regulates pH-dependent V-ATPase recycling*. The Journal of biological chemistry, 2003. **278**(49): p. 49523-9.
71. Gong, F., et al., *Vacuolar H⁺-ATPase apical accumulation in kidney intercalated cells is regulated by PKA and AMP-activated protein kinase*. American journal of physiology. Renal physiology, 2010. **298**(5): p. F1162-9.
72. Paunescu, T.G., et al., *cAMP stimulates apical V-ATPase accumulation, microvillar elongation, and proton extrusion in kidney collecting duct A-intercalated cells*. American journal of physiology. Renal physiology, 2010. **298**(3): p. F643-54.
73. Shahidullah, M., et al., *Nonpigmented ciliary epithelial cells respond to acetazolamide by a soluble adenylyl cyclase mechanism*. Investigative ophthalmology & visual science, 2014. **55**(1): p. 187-97.
74. Magro, C.M., et al., *Expression of soluble adenylyl cyclase in lentigo maligna: use of immunohistochemistry with anti-soluble adenylyl cyclase antibody (R21) in diagnosis of lentigo maligna and assessment of margins*. Archives of pathology & laboratory medicine, 2012. **136**(12): p. 1558-64.
75. Magro, C.M., et al., *Soluble adenylyl cyclase antibody profile as a diagnostic adjunct in the assessment of pigmented lesions*. Archives of dermatology, 2012. **148**(3): p. 335-44.
76. Appukuttan, A., et al., *Inhibition of soluble adenylyl cyclase increases the radiosensitivity of prostate cancer cells*. Biochimica et biophysica acta, 2014. **1842**(12 Pt B): p. 2656-63.
77. Flacke, J.P., et al., *Type 10 soluble adenylyl cyclase is overexpressed in prostate carcinoma and controls proliferation of prostate cancer cells*. The Journal of biological chemistry, 2013. **288**(5): p. 3126-35.
78. Ramos-Espiritu, L., et al., *The metabolic/pH sensor soluble adenylyl cyclase is a tumor suppressor protein*. Oncotarget, 2016.

Chapter 2

Prognostic Significance of Carbonic Anhydrase IX expression in Cancer Patients: A Meta-Analysis

Published in: Front Oncol. 2016 Mar 29;6:69.

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ABSTRACT

Hypoxia is a characteristic of many solid tumors and an adverse prognostic factor for treatment outcome. Hypoxia increases the expression of carbonic anhydrase IX (CAIX), an enzyme that is predominantly found on tumor cells and is involved in maintaining the cellular pH balance. Many clinical studies investigated the prognostic value of CAIX expression, but most have been inconclusive, partly due to small numbers of patients included. The present meta-analysis was therefore performed utilizing the results of all clinical studies to determine the prognostic value of CAIX expression in solid tumors. Renal cell carcinoma was excluded from this meta-analysis due to an alternative mechanism of upregulation. 958 papers were identified from a literature search performed in PubMed and Embase. These papers were independently evaluated by two reviewers and 147 studies were included in the analysis. The meta-analysis revealed strong significant associations between CAIX expression and all endpoints: overall survival [hazard ratio (HR) = 1.76, 95% confidence interval (95%CI) 1.58–1.98], disease-free survival (HR = 1.87, 95%CI 1.62–2.16), locoregional control (HR = 1.54, 95%CI 1.22–1.93), disease-specific survival (HR = 1.78, 95%CI 1.41–2.25), metastasis-free survival (HR = 1.82, 95%CI 1.33–2.50), and progression-free survival (HR = 1.58, 95%CI 1.27–1.96). Sub-group analyses revealed similar associations in the majority of tumor sites and types. In conclusion, these results show that patients having tumors with high CAIX expression have higher risk of locoregional failure, disease progression, and higher risk to develop metastases, independent of tumor type or site. The results of this meta-analysis further support the development of a clinical test to determine patient prognosis based on CAIX expression and may have important implications for the development of new treatment strategies.

INTRODUCTION

Hypoxia is a characteristic of many different types of solid tumors and is caused by an inadequate vascular supply. Hypoxic areas are characterized by low oxygen concentrations, limited nutrient supply, and an acidic extracellular environment. Hypoxia is an independent prognostic factor of poor outcome in patients [1] and decreases the efficacy of standard treatment modalities, such as surgery, chemotherapy, and radiotherapy [2-4]. Many strategies are therefore being investigated to measure tumor hypoxia to predict treatment outcome and to overcome or target tumor hypoxia with newly designed treatments [5-8].

Tumor cells have adopted several mechanisms to survive the hostile conditions during hypoxia, of which one is the hypoxia-inducible factor (HIF) pathway [9, 10]. Upon hypoxic conditions, the expression of the dimeric zinc-containing glycoprotein carbonic anhydrase IX (CAIX) is enhanced as a consequence of HIF stabilization [11, 12]. CAIX is important in maintaining the cellular pH regulation and is located on the cell membrane where it hydrolyzes carbon dioxide, produced as a waste product during glycolysis, to bicarbonate and a proton. The bicarbonate is transported intracellularly by different proteins (e.g., anion exchangers), thereby slightly increasing the intracellular pH to promote tumor cell proliferation. The protons in turn add to an acidic extracellular environment causing extracellular matrix degradation favoring invasion, migration, and subsequent metastasis formation [12]. Hypoxia-induced CAIX expression, tumor-specific expression of CAIX, and its important role in maintaining the pH balance make CAIX a promising endogenous marker of tumor hypoxia and an attractive target for anti-cancer therapies with newly designed inhibitors [5, 11, 12].

Many clinical studies investigated the prognostic value of CAIX, and a recent meta-analysis of renal cell carcinoma (RCC) concluded that high CAIX expression was associated with a better overall survival (OS) [13]. By contrast, a meta-analysis in head and neck cancer patients showed high CAIX expression was associated with a decrease in both OS and disease-free survival (DFS) [14]. This discrepancy can be explained by the fact that RCCs are often characterized by an inactive mutant version of the Von Hippel–Lindau (VHL) protein preventing proteasomal degradation of CAIX upon normoxia and making its expression therefore independent of hypoxia [15, 16]. To the best of our knowledge, a comprehensive meta-analysis of the association between CAIX expression and treatment outcome in other tumor types has not been performed. The aim of this meta-analysis of published clinical studies is therefore to elucidate the prognostic value of CAIX expression in all solid tumor types besides RCC. In addition, current analysis has included sensitivity and subgroup analysis to be able to determine if the prognostic value of CAIX expression varies in patients with different tumor types.

METHODS

Literature Search

The research question of this meta-analysis was defined as follows: “what is the prognostic value of tumoral CAIX expression in patients with solid tumors?” From this research question, three distinctive keywords were identified, i.e., prognosis, CAIX, and tumor. Different formulations and truncations of the keywords were tested as free text searches to see if appropriate papers could be identified. The search algorithm was applied as a free text search and consisted of the combined mention of all three keywords, in any of the formulations or truncations (Data sheet 1 in Supplementary Material). The search for literature was performed on the 31st of August 2015 in both the PubMed and Embase databases. A total of 958 papers were identified from both databases (Figure 1).

Exclusion Criteria

From the total number of papers, 134 reviews, conference abstracts, commentaries, meta-analyses, editorials, or book chapters were excluded, as were 26 duplicates or non-English papers. From the remaining articles, 132 papers about RCC were excluded, since upregulation of CAIX in RCC is biologically different from other solid tumor types [15, 16]. Furthermore, from our experience, we know that papers that describe solely the development and synthesis of CAIX inhibitors do not include patient data, and 53 papers were therefore also excluded. The total number of papers for further screening was thereby reduced to 613 (Figure 1).

Screening of Papers

Two researchers (SK and AY) screened the remaining papers independently. The first round of screening was based on the title and abstract, whereas the second round consisted of a detailed evaluation of the full-text. Papers were evaluated based on the pre-determined inclusion criteria. First, only solid primary tumors of various types were included, thereby automatically excluding hematological cancer. Second, only immunohistochemical detection of CAIX was included, because mRNA upregulation of CA9 does not fully correlate with an increase in functional protein expression, possibly due to posttranscriptional processing and/or differences in stability [17-19]. Third, all end-points were included (see below) with a minimal median follow-up of 1 year. Fourth, all treatment modalities along with experimental treatments were included. Finally, we included every human patient population without making distinction based on tumor grades or stages. Discrepancies between the included papers by both reviewers were discussed and consensus was reached on all. An additional 14 papers were excluded

because their patient populations were similar or overlapping with other papers. Among these repetitive studies, the paper that was included contained the most detailed information about the patient population. A total of 144 papers were included in the meta-analysis (Figure 1).

Figure 1: Flowchart of selecting articles describing the association between tumoral CAIX expression and prognosis.

Data Extraction

Several different parameters, if reported, were extracted from each paper, i.e., the number and origin of patients, number of events, treatment modalities, tumor site, tumor stage, tumor type, group dichotomization, antibody supplier, expression pattern, cellular localization, and endpoints. The univariate hazard ratio (HR) was extracted to assess prognostic value of CAIX expression. When the univariate HR with corresponding 95% confidence interval (95%CI) was not reported, the method from Tierney et al. was used to estimate the HR [20]. Multivariate HR was only included in the meta-analysis when the univariate HR was not reported or could not be estimated. When insufficient data were reported for estimating HR, the authors were contacted to obtain additional data.

Quality Assessment

The methodological quality of the included papers was evaluated with an adjusted version of the Newcastle–Ottawa scale (NOS) to better suit the study design of the included papers (Data sheet 2 in Supplementary Material). The method of scoring based on awarding stars in different categories remained, however, identical. The NOS was commended in the 2011 version of the Cochrane Collaboration handbook and is an easy method to evaluate the methodological quality of cohort studies (available at http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp) [21].

Statistical Analysis and Sensitivity Analysis

Distribution and frequencies of the extracted data parameters were analyzed using SPSS (version 22). Meta-analysis was performed using R statistical software with the Metafor Library (version 1.9-8) [22]. Fixed-effect modeling was performed when no statistical significant heterogeneity between studies was observed. When the heterogeneity between studies was statistically significant, random-effects modeling was applied based on the DerSimonian and Laird method [23]. The assigned weight of each study in the analysis was based on its inverse variance. The following endpoints have been addressed: OS, DFS, locoregional control (LC), disease-specific survival (DSS), metastasis-free survival (MFS), and progression-free survival (PFS). Sensitivity analysis was performed by analyzing subgroups of studies separately, e.g., per tumor organ site. Funnel plots were created to visualize possible publication bias or heterogeneity between studies. Asymmetric funnel plots and studies outside the funnel plot suggest heterogeneity between them and/or publication bias [21]. p-Values <0.05 were considered as statistically significant.

RESULTS

This meta-analysis encompassed a total number of 24,523 patients across 147 independent studies. Many studies included only a small number of patients (median per study 93, range 15–3630) with a median follow-up time between 12.6 months and 13.9 years and are often inconclusive, which underlines the need for a meta-analysis. All papers were published between 2001 and 2015 of which approximately 50% were published after 2010. The majority of the included studies treated patients with surgery alone (36.7%) or in combination with either chemotherapy (8.8%) or standard radiotherapy (8.8%), or the combination of all three modalities (23.1%). Single radiotherapy treatment or combined with chemotherapy was reported in 5.4 and 6.1% of the papers, respectively. In 4.8% of the studies, a form of experimental treatment was administered, including experimental radiotherapy [24–28], hormonal treatment [29], and VEGF-targeted therapy [30]. Most of the studies reported on head and neck cancer patients (21.8%) followed by breast (16.3%) and brain cancer patients (10.2%). By contrast, cancers of the adrenal gland, the cartilage, and the penis were only described once.

Immunohistochemical staining of CAIX was predominantly performed using the M75 antibody (46.3%) targeting the proteoglycan domain of CAIX [31, 32]. Other studies used anti-CAIX antibodies obtained from different suppliers. A membranous expression of CAIX was described in 46.3% of the studies, although cytoplasmatic staining or a combination of the two was also reported (4.8 and 17.7%, respectively). Nuclear staining was only reported in one paper, whereas the rest did not state the staining localization. Different quantification methods and thresholds have been applied to stratify patients into groups with low and high tumoral CAIX expression. Taken together, 33.9% of the total tumors were classified as expressing high levels of CAIX.

Overall, patients suffering from tumors with high CAIX expression had a worse treatment outcome (Figure 2). This association was strong and significant for all endpoints. The negative association of CAIX expression with outcome was dominant for DFS and weaker for LC. Systematic heterogeneity in the present meta-analysis as demonstrated by an asymmetric funnel plot [21] for most of the endpoints (Image 1 in Supplementary Material) can at least in part be attributed to the considerable variation in tumor types and sites across the studies. Therefore, in addition, subgroup analysis based on organ site of the tumor was performed. The results of the subgroup analysis demonstrate a significant prognostic value of CAIX in most of the cancer types investigated (see below).

Figure 2: Summary plot of the overall HRs from each endpoint analyzed. Symbols represent the HR with 95%CI, and dashed line indicates no association between CAIX expression and prognosis.

Overall Survival

Effect of pretreatment expression of CAIX on OS could be evaluated in 104 studies. The complete data to estimate the HR could not be retrieved from 11 papers and were therefore not included in the analysis (Table 1 in Supplementary Material [33-43]). Overall, high CAIX expression was associated with a worse OS (HR = 1.76, 95%CI 1.58–1.98, $p < 0.0001$, Figure 3). Subgroup analysis of the different organ sites revealed a similar significant association between tumoral CAIX expression and OS in 11 organ sites: bladder (HR = 1.64, 95%CI = 1.21–2.22), brain (HR = 2.18, 95%CI 1.60–2.96), breast (HR = 1.90, 95%CI = 1.45–2.50), esophagus (HR = 1.97, 95%CI 1.50–2.60), gall bladder (HR = 2.35, 95%CI 1.33–4.15), gastroenteropancreatic tract (HR = 2.57, 95%CI 1.45–4.56), head and neck (HR = 1.66, 95%CI 1.29–2.13), lung (HR = 1.57, 95%CI 1.06–2.33), pancreas (HR = 2.37, 95%CI 1.04–5.43), soft tissue (HR = 2.97, 95%CI 1.65–5.34), and the stomach (HR = 1.92, 95%CI 1.39–2.67). The other six organ sites show a similar trend with worse OS, albeit not statistically significant (Table 1). Similar results were often, but not always, observed for different tumor types per organ site (Table 2 in Supplementary Material). A hypoxia-associated perinecrotic staining pattern was reported in 16 of these studies, whereas a diffuse staining pattern was reported in 3 papers. Interestingly, both patterns of CAIX expression significantly associated with OS (perinecrotic: HR = 1.99, 95%CI 1.60–2.48; diffuse: HR = 1.77, 95%CI 1.22–2.56). These results suggest that the expression pattern of CAIX does not affect its prognostic value.

Figure 3. Forest plot of the papers describing the association between CAIX expression and OS. Horizontal bars represent HR with corresponding 95%CI. Symbol size represents the assigned weight of the study. The overall HR with 95%CI is visualized with the diamond shape. Dashed line indicates no association between CAIX and prognosis. Esop, Stom, esophagus and stomach; GEP, gastroenteropancreatic tract [25-27, 29, 30, 44-129].

Table 1: Results of subgroup meta-analyses of different organ sites reported.

Organ Site	OS	DFS	LC	DSS	MFS	PFS
Bladder	1.64 (1.21 - 2.22)	2.63 (1.56 - 4.40)	0.88 (0.40 - 1.90)	0.82 (0.47 - 1.4)		0.68 (0.21 - 2.20)
Brain	2.18 (1.60 - 2.96)					1.44 (0.91 - 2.27)
Breast	1.90 (1.45 - 2.50)	1.74 (1.34 - 2.27)	1.37 (0.95 - 1.96)	1.75 (1.28 - 2.38)	1.76 (1.13 - 2.74)	1.88 (1.13 - 3.10)
Cartilage					6.46 (2.05 - 20.0)	
Cervix	1.11 (0.91 - 1.35)	1.12 (0.75 - 1.68)	1.17 (0.74 - 1.87)	2.19 (1.29 - 3.70)	2.37 (1.35 - 4.10)	1.76 (0.99 - 3.10)
Colorectal	1.41 (0.67 - 2.98)	3.31 (1.23 - 8.89)	3.33 (1.76 - 6.30)	1.31 (0.18 - 9.41)	5.17 (2.07 - 13.0)	2.38 (1.06 - 5.56)
Esophagus	1.97 (1.50 - 2.60)	2.70 (2.08 - 3.50)		2.78 (1.56 - 5.00)		
Esop, Stom	1.53 (1.00 - 2.30)					
Gall Bladder	2.35 (1.33 - 4.15)					
GEP	2.57 (1.45 - 4.56)					
H&N	1.66 (1.29 - 2.13)	1.98 (1.51 - 2.61)	1.54 (1.12 - 2.12)	2.21 (1.12 - 4.36)	0.77 (0.27 - 2.26)	1.62 (1.01 - 2.59)
Liver	1.41 (0.98 - 2.03)	1.51 (1.26 - 1.81)	1.39 (1.09 - 4.10)			
Lung	1.57 (1.06 - 2.33)	1.87 (1.27 - 2.74)		1.75 (0.59 - 5.15)		
Ovary	1.42 (0.82 - 2.45)					1.24 (0.67 - 2.30)
Pancreas	2.37 (1.04 - 5.43)	2.98 (0.56 - 15.9)		1.49 (1.07 - 2.10)		
Penis		1.35 (0.55 - 3.30)				
Small Intestine	2.58 (0.21 - 31.8)					
Soft tissue	2.97 (1.65 - 5.34)	3.41 (1.58 - 7.30)		1.65 (1.11 - 2.45)	1.65 (0.72 - 3.80)	
Stomach	1.92 (1.39 - 2.67)	1.27 (0.77 - 2.10)				
Vulva		1.52 (0.79 - 2.90)		1.34 (0.67 - 2.70)	2.25 (1.42 - 3.60)	

Overall HR (with 95%CI) are shown. When HR was available from only one paper, the values were adopted from that single paper. Bold numbers indicate statistical significant associations between CAIX expression and prognosis ($p < 0.01$). Esop, stom - oesophagus and stomach. GEP - gastro-entero-pancreatic tract.

Disease-Free Survival

A total of 40 from the selected 147 studies investigated the association between CAIX expression and DFS. Five studies could not be included in this analysis due to incomplete reporting (Table 1 in Supplementary Material [39, 40, 43, 124, 130]). Based on 35 studies, high CAIX expression was statistically significantly associated with a decreased DFS (HR = 1.87, 95%CI 1.62–2.16, $p < 0.001$) (Figure 4). Subgroup analysis based on organ site of the tumor showed that high CAIX expression was significantly associated with shorter DFS in bladder (HR = 2.63, 95%CI 1.56–4.40), breast (HR = 1.74, 95%CI 1.34–2.27), colorectal (HR = 3.31, 95%CI 1.23–8.89), esophagus (HR = 2.70, 95%CI 2.08–3.50), head and neck (HR = 1.98, 95%CI 1.51–2.61), liver (HR = 1.51, 95%CI 1.26–1.81), lung (HR = 1.87, 95%CI 1.27–2.74), and soft tissue tumors (HR = 3.41, 95%CI 1.58–7.30). By contrast, no significant association with DFS was observed for tumors in the cervix (HR = 1.12, 95%CI 0.75–1.68), pancreas (HR = 2.98, 95%CI 0.56–15.9), penis (HR = 1.35, 95%CI 0.55–3.30), stomach (HR = 1.27, 95%CI 0.77–2.10), and vulva (HR = 1.52, 95%CI 0.79–2.90) (Table 1). A similar trend was observed for all different tumor types per organ sites (Table 2 in Supplementary Material).

Figure 4: Forest plot of the papers describing the association between CAIX expression and DFS. Horizontal bars represent HR with corresponding 95%CI. Symbol size represents the assigned weight of the study. The overall HR with 95%CI is visualized with the diamond shape. Dashed line indicates no association between CAIX and prognosis [29, 33, 34, 48, 57, 59, 68, 73, 74, 82, 84, 85, 87, 89, 90, 97, 98, 112, 117, 119, 121, 125, 131-143].

Locoregional Control

The risk of locoregional relapse associated with CAIX expression was evaluated in 25 studies in 6 different organ sites. Figure 5 shows the overall LC outcome, which indicates that patients with high tumoral CAIX expression have a higher risk of locoregional recurrences than patients with low expression of CAIX in tumors (HR = 1.54, 95%CI 1.22–1.93, $p = 0.0002$). The negative association between high CAIX expression in tu-

mors and worse LC remained significant in head and neck (HR = 1.54, 95%CI 1.12–2.12) and liver tumors (HR = 1.39, 95%CI 1.09–4.10) (Table 1). A similar association was observed in most of the tumor types per organ sites (Table 2 in Supplementary Material).

Figure 5: Forest plot of the papers describing the association between CAIX expression and LC. Horizontal bars represent HR with corresponding 95%CI. Symbol size represents the assigned weight of the study. The overall HR with 95%CI is visualized with the diamond shape. Dashed line indicates no association between CAIX and prognosis [24-28, 52, 59, 94, 95, 112, 116, 122, 133, 144-155].

Disease-Specific Survival

Disease-specific survival was reported in 23 studies, of which 1 study provided incomplete data to estimate the HR (Table 1 in Supplementary Material [43]). In the remaining 22 studies, patients suffering from tumors with high CAIX expression had a significantly shorter DSS (HR = 1.78, 95%CI 1.41–2.25, $p < 0.0001$) (Figure 6). Subgroup analyses by organ site revealed significant associations between high CAIX expression and worse DSS in tumors of the breast (HR = 1.75, 95%CI 1.28–2.38), cervix (HR = 2.19, 95%CI 1.29–3.70), esophagus (HR = 2.78, 95%CI 1.56–5.00), head and neck (HR = 2.21, 95%CI 1.12–4.36), pancreas (HR = 1.49, 95%CI 1.07–2.10), and soft tissue (HR = 1.65, 95%CI 1.11–2.45) (Table 1). Subgroup analyses of the tumor types per organ sites revealed a

worse DSS to be associated with high CAIX expression in the majority of tumor types (Table 2 in Supplementary Material).

Figure 6: Forest plot of the papers describing the association between CAIX expression and DSS. Horizontal bars represent HR with corresponding 95%CI. Symbol size represents the assigned weight of the study. The overall HR with 95%CI is visualized with the diamond shape. Dashed line indicates no association between CAIX and prognosis [29, 78, 95, 102, 136, 138, 140, 144, 150, 152, 154, 156-165].

Metastasis-Free Survival

Metastasis-free survival was reported in 12 of the 147 included studies. Based on 11 of these studies, high CAIX expression was significantly associated with a shorter MFS (HR = 1.82, 95%CI 1.33–2.50, $p = 0.0002$) (Figure 7; Table 1 in Supplementary Material [166]). Subgroup analyses of the different organ sites of the tumors, independent of tumor types, revealed high CAIX expression to be significantly associated with a worse MFS in most of the organ sites reported, i.e., breast (HR = 1.76, 95%CI 1.13–2.74), cartilage (HR = 6.46, 95%CI 2.05–20.0), cervix (HR = 2.37, 95%CI 1.35–4.10), colorectal (HR = 5.17, 95%CI 2.07–13.0), and vulva (HR = 2.25, 95%CI 1.42–3.60), but not in head and neck (HR = 0.77, 95%CI 0.27–2.26) and soft tissue cancers (HR = 1.65, 95%CI 0.72–3.80) (Table 1). Interestingly, one study reported a significant positive association between high CAIX expression and better MFS in squamous cell carcinoma of the head and neck

(HR = 0.27, 95%CI 0.09–0.80) (Table 2 in Supplementary Material), which may be attributed to the hypoxia-modifying component of the treatment [24].

Figure 7: Forest plot of the papers describing the association between CAIX expression and MFS. Horizontal bars represent HR with corresponding 95%CI. Symbol size represents the assigned weight of the study. The overall HR with 95%CI is visualized with the diamond shape. Dashed line indicates no association between CAIX and prognosis [24, 27, 34, 52, 95, 102, 140, 150, 165, 167, 168].

Progression-Free Survival

Eleven out of 12 studies could be included to estimate the risk of disease progression after treatment based on CAIX expression in tumors (Table 1 in Supplementary Material [169]). Similar to the other endpoints, PFS was significantly shorter in patients with tumors expressing high levels of CAIX (HR = 1.58, 95%CI 1.27–1.96, $p < 0.0001$) (Figure 8). Subgroup analyses per organ site revealed that the association with PFS only remained statistically significant in breast (HR = 1.88, 95%CI 1.13–3.10), colorectal (HR = 2.38, 95%CI 1.06–5.56), and head and neck tumors (HR = 1.62, 95%CI 1.01–2.59) (Table 1). The subgroup analyses of tumor types per organ site showed similar associations between high CAIX expression and a worse PFS (Table 2 in Supplementary Material).

Figure 8: Forest plot of the papers describing the association between CAIX expression and PFS. Horizontal bars represent the HR with corresponding 95%CI. Symbol size represents the assigned weight of the study. The overall HR with 95%CI is visualized with the diamond shape. Dashed line indicates no association between CAIX and prognosis [30, 44, 47, 52, 62, 75, 80, 100, 123, 155].

High-Quality Papers

This meta-analysis used an adjusted version of the NOS to evaluate the quality of a study. The scores of this quality assessment ranged between 1 and 7 stars, i.e., the maximum, awarded per study. Approximately half of the studies (52.4%) were considered as high-quality studies, i.e., with a number of stars greater or equal to the median (5 stars). Meta-analysis of only the high-quality studies revealed significant prognostic values of CAIX expression for OS (HR = 1.81, 95%CI 1.57–2.09, $n = 50$), DFS (HR = 1.81, 95%CI 1.47–2.23, $n = 18$), DSS (HR = 1.71, 95%CI 1.16–2.51, $n = 10$), and PFS (HR = 1.59, 95%CI 1.21–2.07, $n = 7$). For both LC (HR = 1.90, 95%CI 1.58–2.30, $n = 14$) and MFS (HR = 2.47, 95%CI 1.92–3.19, $n = 7$), the association with CAIX expression became even stronger when only high-quality studies were included.

DISCUSSION

Many clinical studies investigated the prognostic association of CAIX expression with treatment outcome. Most of these studies, however, include only limited numbers of patients and remain inconclusive. This current meta-analysis is the first complete overview of all reported clinical studies investigating the impact of pretreatment CAIX expression in solid tumors on prognosis. Overall, these results clearly show that high CAIX expression is an adverse prognostic marker in solid tumors, irrespectively of the end-point evaluated, as summarized in Figure 2. A strong association between high CAIX expression and poor prognosis was also found in the majority of different tumor sites,

supporting an important role of CAIX in disease progression and treatment resistance in many cancer types.

The papers included in the current meta-analysis were all published between 2001 and 2015, which is likely attributed to the identification of the hypoxic responsive element in the promotor region of *ca9* in the end of 2000 [170]. This study identified a direct link between CAIX expression and its hypoxic upregulation through HIF stabilization. This crucial finding encouraged research to evaluate CAIX as an endogenous marker of tumor hypoxia, a known biological factor of therapy resistance [2-4]. Nevertheless, because alternative mechanisms can also regulate CAIX expression, e.g., via PI3K [171] or the unfolded protein response [10, 172], tumoral CAIX expression may not accurately identify hypoxic tumors. Apart from the hypoxia-associated mechanisms underlying resistance of tumor cells to several treatment modalities, CAIX can directly affect cancer prognosis as its main function is to maintain the balance between intracellular and extracellular pH, thereby generating an acidic extracellular microenvironment [11, 12]. This is supported by data demonstrating that CAIX is involved in promoting tumorigenesis and leads to a more aggressive phenotype of tumor cells [173]. This can partly be explained by the association between CAIX expression and the induction of tumor cell migration and invasion, which could be caused by the reduction in extracellular pH [174-176]. In addition, cancer stem cell markers also appear to be enriched in the CAIX expressing population of tumor cells [57, 177]. The important role of CAIX, either directly or indirectly, in cancer prognosis is also supported by the results of the current meta-analysis, which shows that tumors with high CAIX expression have higher risk of locoregional failure, disease progression, and higher risk to develop metastasis. Other proton exchangers and transporters have been shown preclinically and clinically to play an important role in the regulation of cellular pH homeostasis promoting survival and invasion as well as causing treatment resistance [178-180]. Therefore, assessment of several major pH regulators in tumors prior and/or during therapy may represent a more powerful prognostic and predictive biomarker as well as important targets for new anti-cancer treatments, which warrants further investigations.

A meta-analysis usually overestimates its results because of selective reporting and publication bias [21]. This meta-analysis identified a total of 147 studies reported in 144 papers of which 15 could not be included in final analysis because the HR could not be estimated due to incomplete reporting [33-43, 124, 130, 166, 169]. Non-significant association between CAIX and outcome was found in these studies (Table 1 in Supplementary Material). Including these 15 papers in the analysis might therefore decrease the magnitude of the prognostic values of CAIX expression reported here. This overestimation can be further increased by publication bias, i.e., when negative associations are not published at all and can therefore not be identified and included in this meta-analysis. Nevertheless, since the prognostic value of CAIX expression was highly statistically significant, we believe that the possible effect of publication bias on this association is minimal.

The different staining and scoring methods used in the included papers to quantify CAIX expression might be an additional source of bias. Visual quantification was used in the majority of the reports and could either be based on staining intensity, the number of stained cells, or a combination of both. In addition, different thresholds have been used to dichotomize patients based on their CAIX expression. This discrepancy in methods is one of the reasons of significant heterogeneity between studies, which therefore requires the use of a random-effect model in the meta-analysis [181, 182]. Additionally, tissue microarrays (TMAs) are used in the majority of included papers to visualize and quantify CAIX expression, even though TMAs may underestimate the actual expression levels of the protein [183]. The use of TMAs might therefore bias the prognostic value of CAIX when CAIX expression levels are dichotomized erroneously. Furthermore, this meta-analysis is limited by difficulties in obtaining homogenous endpoints and by non-uniform observation times, although most of the data are based on reports with a median follow-up of more than 1 year.

To identify possible bias in a selected study, an adjusted version of the NOS was used, which is a quick and easy method to assess the quality of studies that has been commended in the Cochrane handbook [21]. However, the validity and reproducibility of the NOS have been questioned because of the subjective interpretation of certain criteria, which require detailed guidelines to obtain a better inter-rater agreement [184–186]. The test–retest reliability of the NOS is, however, better, which allows for a single reviewer to continuously use uniform criteria while rating papers [185]. When only high-quality papers, i.e., those with minimal bias, were included in our analyses, there was no significant difference in the results as compared with all studies included.

It remains impossible to eliminate every source of bias in a meta-analysis. Nevertheless, the high statistical significance of the results presented here clearly show that CAIX expression is associated with worse prognosis in a global patient population and in the majority of tumor sites. These findings are similar to the results of the meta-analysis in head and neck cancer [14], but different from RCC [13] due to the alternative mechanism of CAIX upregulation in RCC [15, 16]. New treatment options are currently being developed to specifically inhibit CAIX function [5, 187], of which one is currently in a Phase I clinical trial (NCT02215850). These types of compounds might prove to be beneficial for the specific treatment of tumors with high CAIX expression. The results of this meta-analysis further support the development of a clinical test to determine patient prognosis based on CAIX expression, although a standardized protocol remains to be developed and validated.

SUPPLEMENTARY MATERIAL

Supplementary File 1: Literature search strategy to identify papers for the meta-analysis that describe an association between tumoral CAIX expression and prognosis.

Research question: What is the prognostic value of tumoral CAIX expression in patients with solid tumors?

Keywords identified as search terms:

CAIX, Tumor, and Prognosis

Search algorithm Pubmed:

Prognosis:

(Prognos*) OR (surviv*) OR (hazard) OR (disease-free) OR ("disease free") OR (progression-free) OR ("progression free") OR (Kaplan-meier) OR ("Kaplan meier") OR (predict*) OR (outcome) OR (efficacy) OR (effective*)

Hits: 4,589,956

CAIX:

(CAIX) OR (ca9) OR ("carbonic anhydrase IX") OR ("carbonic anhydrase 9") OR ("carbonic anhydrase-IX") OR ("carbonic anhydrase-9") OR (CA-IX) OR (ca-9) OR (G250)

Hits: 2,419

Tumor:

(tumor) OR (tumors*) OR (tumor's) OR (tumoral*) OR ("tumor associated") OR (tumor-associated) OR ("tumor related") OR (tumor-related) OR (tumorigen*) OR (tumorous*) OR (tumour*) OR (cancer) OR (cancers) OR (cancer's) OR (cancerogen*) OR (cancera*) OR (cancer-associate*) OR (cancerigen*) OR (cancerno*) OR (cancero*) OR (cancerp*) OR ("cancer related") OR (cancer-related) OR (*carcinoma) OR (*sarcoma) OR (neoplas*) OR (malignanc*) OR (melanoma)

Hits: 3,618,127

Prognosis AND CAIX AND Tumor:

((Prognos*) OR (surviv*) OR (hazard) OR (disease-free) OR ("disease free") OR (progression-free) OR ("progression free") OR (Kaplan-meier) OR ("Kaplan meier") OR (predict*) OR (outcome) OR (efficacy) OR (effective*)) AND ((CAIX) OR (ca9) OR ("carbonic anhydrase IX") OR ("carbonic anhydrase 9") OR ("carbonic anhydrase-IX") OR ("carbonic anhydrase-9") OR (CA-IX) OR (ca-9) OR (G250)) AND ((tumor) OR (tumors*) OR (tumor's) OR (tumoral*) OR ("tumor associated") OR (tumor-associated) OR ("tumor related") OR (tumor-related) OR (tumorigen*) OR (tumorous*) OR (tumour*) OR (cancer) OR (cancers) OR (cancer's) OR (cancerogen*) OR (cancera*) OR (cancer-associate*) OR (cancerigen*) OR (cancerno*) OR (cancero*) OR (cancerp*) OR ("cancer related") OR (cancer-related) OR (*carcinoma) OR (*sarcoma) OR (neoplas*) OR (malignanc*) OR (melanoma))

Hits: 940

Search algorithm Embase:

Prognosis:

(Prognos\$) OR (surviv\$) OR (hazard) OR (disease-free) OR ("disease free") OR (progression-free) OR ("progression free") OR (Kaplan-meier) OR ("Kaplan meier") OR (predict\$) OR (outcome) OR (efficacy) OR (effective\$)

Hits: 5,100

Prognostic Significance of CAIX in cancer patients

CAIX:

(CAIX) OR (ca9) OR ("carbonic anhydrase IX") OR ("carbonic anhydrase 9") OR ("carbonic anhydrase-IX") OR ("carbonic anhydrase-9") OR (CA-IX) OR (ca-9) OR (G250)

Hits: 512

Tumor:

(tumor) OR (tumors) OR (tumor's) OR (tumoral) OR ("tumor associated") OR (tumor-associated) OR ("tumor related") OR (tumor-related) OR (tumorigen) OR (tumorous) OR (tumour) OR (cancer) OR (cancers) OR (cancer's) OR (cancerogen) OR (cancera) OR (cancer-associate) OR (cancerigen) OR (cancerno) OR (cancero) OR (cancerp) OR ("cancer related") OR (cancer-related) OR (\$carcinoma) OR (\$sarcoma) OR (neoplas) OR (malignanc) OR (melanoma)

Hits: 3291

Prognosis AND CAIX AND Tumor:

((Prognos) OR (surviv) OR (hazard) OR (disease-free) OR ("disease free") OR (progression-free) OR ("progression free") OR (Kaplan-meier) OR ("Kaplan meier") OR (predict) OR (outcome) OR (efficacy) OR (effective)) AND ((CAIX) OR (ca9) OR ("carbonic anhydrase IX") OR ("carbonic anhydrase 9") OR ("carbonic anhydrase-IX") OR ("carbonic anhydrase-9") OR (CA-IX) OR (ca-9) OR (G250)) AND ((tumor) OR (tumors) OR (tumor's) OR (tumoral) OR ("tumor associated") OR (tumor-associated) OR ("tumor related") OR (tumor-related) OR (tumorigen) OR (tumorous) OR (tumour) OR (cancer) OR (cancers) OR (cancer's) OR (cancerogen) OR (cancera) OR (cancer-associate) OR (cancerigen) OR (cancerno) OR (cancero) OR (cancerp) OR ("cancer related") OR (cancer-related) OR (\$carcinoma) OR (\$sarcoma) OR (neoplas) OR (malignanc) OR (melanoma))

Hits: 18

Literature search was performed in Pubmed and Embase on the 31th of August 2015.

Chapter 2

Supplementary File 2: Adjusted version of the Newcastle-Ottawa Scale to assess the quality of the included papers.

In brief, for each criteria a single option can be registered. The amount of answers with stars behind them are counted and the total number of stars is a measurement of the study quality.

NEWCASTLE – OTTAWA QUALITY ASSESSMENT SCALE

Adjusted version

Note: A study can be awarded a maximum of one star for each numbered. When criteria are not reported no star can be awarded for that category.

First author:Year of publication:

Selection

- 1) Representativeness of the cohort
 - a. Truly representative of the average patient population ★
 - b. Somewhat representative of the average patient population ★
 - c. Selected group of patients based on certain criteria
 - d. No description of the derivation of the cohort

Grouping variable

- 1) Was the measurement performed blindly from the outcome
 - a. Yes ★
 - b. No
 - c. Not stated
- 2) How many persons performed the scoring
 - a. More than one ★
 - b. One
 - c. Not stated
- 3) Was scoring between investigators performed independently
 - a. Yes ★
 - b. No
 - c. Not stated

Outcome

- 1) Assessment of outcome
 - a. Independent blind assessment ★
 - b. Record linkage ★
 - c. Self-report
 - d. No description
- 2) Was follow-up long enough for outcomes to occur
 - a. Yes ★
 - b. No
- 3) Adequacy of follow-up of cohorts
 - a. Complete follow-up – all subjects accounted for ★
 - b. Subjects lost to follow-up unlikely to introduce bias ★
(Equally distributed/small numbers)
 - c. Follow-up rate unevenly distributed or large numbers lost
 - d. No statement

Supplementary File 3: Funnel plots of papers reporting on OS (A), DFS (B), LC (C), DSS (D), MFS (E), and PFS (F). The x-axis represents the HR for each paper with the corresponding standard error on the y axis. Heterogeneity between studies is present when papers are plotted outside the displayed funnel. Heterogeneity is significant for OS (A), DFS (B), LC (C), DSS (D), and MFS (E). For PFS (F) the lack of papers in the bottom right part of the funnel is suggestive of publication bias.

Chapter 2

Supplementary File 4: Table of papers that reported inadequate data to estimate the HR.

A brief description of the available data is described in the comments.

Study	Organ	Patients	Comments
OS papers			
Blank 2010	Adrenal Gland	111	"The survival depending on CA-9 offered no significant trend."
Cleven 2007	Colorectal	133	"CA9 epithelial expression had no impact on patient survival."
Huber 2015	Oesophagus	112	Median survival 20 vs 20 months for CAIX high and low, respectively
Jung 2013	Stomach	193	Mean survival time 39.1 vs 39.0 months for CAIX high and low (p=0.705)
Lancashire 2010	Breast	158	Chi-squared (2.976) with p-value (0.085) reported for OS.
Lee-Kong 2012	Colorectal	85	Univariate HR reported (1.27, p=0.01), no 95% CI could be estimated.
Noh 2014	Breast	334	P-value reported (0.392), without clear description of outcome.
Preusser 2005	Brain	84	"CA9 no significant influence on patient survival" (p=0.1065).
Seeber 2010	Cervix	93	"CAIX expression was not correlated with survival".
Trastour 2007	Breast	132	DFS data available, for OS only multivariate p-value shown (p=0.2).
Winter 2006	Head and Neck	149	"CA9 expression was not associated with different OS (p=0.3)."
DFS papers			
Lee-Kong 2012	Colorectal	85	Univariate HR reported (1.13, p=0.10), no 95% CI could be estimated.
Noh 2014	Breast	334	P-value reported (0.985), without clear description of outcome.
Rajaganeshan 2009	Colorectal	55	"A significant trend to worse DFS was not observed."
Winter 2006	Head and Neck	149	"CA9 was not associated with a survival difference for DFS (p=0.2)."
Woelber 2011	Cervix	175	"Association CAIX and survival failed statistical significance (p=0.220)."
DSS papers			
Winter 2006	Head and Neck	149	"CA9 was not associated with a survival difference for DSS (p=0.1)."
MFS papers			
Doyen 2014	Breast	770	No association reported between CAIX expression and MFS.
PFS papers			
Grigsby 2007	Cervix	15	Only p-value reported for univariate survival analysis (p=0.86).

Supplementary File 5: Table with the results from the subgroup meta-analysis of the tumor types per organ sites.

The corresponding number of papers included in each analysis is shown. When HR was available from only one paper, the values were adopted from that single paper. Bold numbers indicate statistical significant associations between CAIX expression and prognosis (p<0.01). SCC - squamous cell carcinoma. GBM - glioblastoma multiform.

Organ and Tumor Type	OS	DFS	LC	DSS	MFS	PFS
<u>Bladder</u>	1.64 (1.21 - 2.22) n=3	2.63 (1.56 - 4.40) n=1	0.88 (0.40 - 1.90) n=1	0.82 (0.47 - 1.40) n=1		0.68 (0.21 - 2.20) n=1
Carcinoma	1.83 (1.21 - 2.80) n=1	2.63 (1.56 - 4.40) n=1				
Transitional & SCC				0.82 (0.47 - 1.40) n=1		
Transitional Cell Carcinoma	1.44 (0.92 - 2.24) n=2		0.88 (0.40 - 1.90) n=1			0.68 (0.21 - 2.20) n=1
<u>Brain</u>	2.18 (1.60 - 2.96) n=14					1.44 (0.91 - 2.27) n=4
Anaplastic Oligodendroglioma	0.19 (0.02 - 1.60) n=1					0.47 (0.06 - 3.7) n=1
Astrocytoma	2.28 (1.19 - 4.37) n=3					
Astrocytoma & GBM	2.34 (1.47 - 3.70) n=1					
Glial Tumors	3.35 (1.55 - 7.20) n=1					
GBM	2.08 (0.69 - 6.26) n=2					
Pediatric Brain Tumors	3.96 (1.20 - 13.0) n=1					

Organ and Tumor Type	OS	DFS	LC	DSS	MFS	PFS
Meningioma	1.10 (0.62 - 2.00) n=1					1.31 (0.64 - 2.70) n=1
Neuroblastoma	4.20 (0.50 - 35.5) n=2					1.73 (0.83 - 3.60) n=1
Oligodendroglioma	2.75 (1.61 - 4.71) n=2					1.65 (0.50 - 5.50) n=1
<u>Breast</u>	1.90 (1.45 - 2.50) n=14	1.74 (1.34 - 2.27) n=10	1.37 (0.95 - 1.96) n=2	1.75 (1.28 - 2.38) n=5	1.76 (1.13 - 2.74) n=3	1.88 (1.13 - 3.10) n=1
Adenocarcinoma		2.57 (1.39 - 4.80) n=1			2.70 (1.20 - 6.10) n=1	
Cancer	2.02 (1.48 - 2.76) n=9	1.67 (1.26 - 2.23) n=9	1.37 (0.95 - 1.96) n=2	1.83 (1.29 - 2.58) n=4	1.60 (0.97 - 2.65) n=2	1.88 (1.13 - 3.10) n=1
Ductal Carcinoma	1.76 (0.68 - 4.58) n=3					
Male Carcinoma	1.37 (0.64 - 2.90) n=2					
Triple Negative Breast Cancer				1.36 (0.75 - 2.50) n=1		
<u>Cartilage</u>					6.46 (2.05 - 20.0) n=1	
Chrondrosarcoma					6.46 (2.05 - 20.0) n=1	
<u>Cervix</u>	1.11 (0.91 - 1.35) n=4	1.12 (0.75 - 1.68) n=3	1.17 (0.74 - 1.87) n=2	2.19 (1.29 - 3.70) n=1	2.37 (1.35 - 4.10) n=1	1.76 (0.99 - 3.10) n=1

Organ and Tumor Type	OS	DFS	LC	DSS	MFS	PFS
Cancer	1.61 (1.11 - 2.33) n=3	1.12 (0.75 - 1.68) n=3	1.18 (0.64 - 2.20) n=1			1.76 (0.99 - 3.10) n=1
Endometrial Cancer	0.96 (0.82 - 1.20) n=1					
SCC			1.16 (0.57 - 2.40) n=1	2.19 (1.29 - 3.70) n=1	2.37 (1.35 - 4.10) n=1	
<u>Colorectal</u>	1.41 (0.67 - 2.98) n=5	3.31 (1.23 - 8.89) n=3	3.33 (1.76 - 6.30) n=2	1.31 (0.18 - 9.41) n=2	5.17 (2.07 - 13.0) n=1	2.38 (1.06 - 5.56) n=1
Adenocarcinoma	1.20 (0.52 - 2.76) n=4	1.80 (0.98 - 3.31) n=2	3.33 (1.76 - 6.30) n=2	0.47 (0.21 - 1.00) n=1		
Cancer	2.86 (1.09 - 7.70) n=1	4.68 (2.59 - 8.50) n=1		3.52 (1.96 - 6.30) n=1	5.17 (2.07 - 13.0) n=1	2.38 (1.06 - 5.56) n=1
<u>Esophagus</u>	1.97 (1.50 - 2.60) n=1	2.70 (2.08 - 3.50) n=1		2.78 (1.56 - 5.00) n=1		
Cancer	1.97 (1.50 - 2.60) n=1	2.70 (2.08 - 3.50) n=1				
SCC				2.78 (1.56 - 5.00) n=1		
<u>Esophagus, Stomach</u>	1.53 (1.00 - 2.30) n=1					
Adenocarcinoma	1.53 (1.00 - 2.30) n=1					
<u>Gall Bladder</u>	2.35 (1.33 - 4.15) n=3					

Organ and Tumor Type	OS	DFS	LC	DSS	MFS	PFS
Adenocarcinoma	4.02 (1.27 - 13.0) n=1					
Cancer	1.42 (0.65 - 3.10) n=1					
(Adeno)SCC	4.02 (1.27 - 13.0) n=1					
<u>Gastro-Entero-Pancreatic</u>	2.57 (1.45 - 4.56) n=2					
Neuroendocrine Tumor	2.57 (1.45 - 4.56) n=2					
<u>Head and Neck</u>	1.66 (1.29 - 2.13) n=18	1.98 (1.51 - 2.61) n=6	1.54 (1.12 - 2.12) n=17	2.21 (1.12 - 4.36) n=5	0.77 (0.27 - 2.26) n=3	1.62 (1.01 - 2.59) n=2
Larynx SCC	0.72 (0.48 - 1.09) n=2		1.13 (0.38 - 3.39) n=4		0.70 (0.40 - 1.50) n=1	
Larynx & Pharynx SCC			1.20 (0.84 - 1.71) n=2	2.31 (1.04 - 5.10) n=1		
Oral Cavity SCC	2.24 (1.70 - 2.95) n=7	2.23 (1.22 - 4.09) n=3	1.80 (0.56 - 5.81) n=3	2.02 (0.79 - 5.16) n=4		
Pharynx Carcinoma	1.70 (1.03 - 2.81) n=2		1.96 (1.01 - 3.80) n=1		1.96 (1.02 - 3.80) n=1	1.62 (1.01 - 2.59) n=2
Salivary Gland Carcinoma	0.71 (0.23 - 2.20) n=1	1.77 (0.56 - 5.60) n=1	1.20 (0.34 - 4.20) n=1			
SCC	1.80 (1.36 - 2.36) n=5	1.88 (1.21 - 2.93) n=2	1.72 (1.04 - 2.86) n=6		0.27 (0.09 - 0.80) n=1	

Organ and Tumor Type	OS	DFS	LC	DSS	MFS	PFS
<u>Liver</u>	1.41 (0.98 - 2.03)	1.51 (1.26 - 1.81)	1.39 (1.09 - 4.10)			
Cancer	n=4	n=2	n=1			
	1.62 (1.08 - 2.44)	1.51 (1.26 - 1.81)	1.39 (1.09 - 4.10)			
Cholangiocarcinoma	n=3	n=2	n=1			
	0.84 (0.51 - 1.40)					
<u>Lung</u>	n=1					
	1.57 (1.06 - 2.33)	1.87 (1.27 - 2.74)		1.75 (0.59 - 5.15)		
Adenocarcinoma	n=8	n=3		n=3		
	1.83 (1.10 - 3.10)					
Mesothelioma	n=1					
				0.77 (0.31 - 1.90)		
Non-Small Cell Lung Cancer	1.84 (1.02 - 3.33)	1.87 (1.27 - 2.74)		n=1		
	n=4	n=3		2.46 (0.71 - 8.56)		
Small Cell Lung Cancer	1.04 (0.58 - 1.90)			n=2		
	n=1					
SCC	1.28 (0.41 - 3.98)					
	n=2					
<u>Ovary</u>	1.42 (0.82 - 2.45)					1.24 (0.67 - 2.30)
Cancer	n=4					n=1
	1.16 (0.45 - 2.99)					
	n=2					
Serous Carcinoma	1.82 (0.99 - 3.30)					1.24 (0.67 - 2.30)
	n=1					n=1

Organ and Tumor Type	OS	DFS	LC	DSS	MFS	PFS
Serous Cystadenocarcinoma	1.86 (0.85 - 4.00) n=1					
<u>Pancreas</u>	2.37 (1.04 - 5.43) n=5	2.98 (0.56 - 15.9) n=2		1.49 (1.07 - 2.10) n=1		
Adenocarcinoma	1.60 (1.12 - 2.29) n=3					
Cancer	1.07 (0.63 - 1.80) n=1					
Ductal Carcinoma		1.33 (0.97 - 1.80) n=1		1.49 (1.07 - 2.10) n=1		
Neuroendocrine Tumor	35.3 (10.3 - 121) n=1	7.36 (3.11 - 17.0) n=1				
<u>Penis</u>		1.35 (0.55 - 3.30) n=1				
SCC		1.35 (0.55 - 3.30) n=1				
<u>Small Intestine</u>	2.58 (0.21 - 31.8) n=2					
Cancer	0.73 (0.51 - 1.10) n=1					
Neuroendocrine Tumor	9.47 (4.6 - 19.5) n=1					
<u>Soft tissue</u>	2.97 (1.65 - 5.34) n=2	3.41 (1.58 - 7.30) n=1		1.65 (1.11 - 2.45) n=2	1.65 (0.72 - 3.80) n=1	

Organ and Tumor Type	OS	DFS	LC	DSS	MFS	PFS
Sarcoma	2.97 (1.65 - 5.34) n=2	3.41 (1.58 - 7.30) n=1		1.65 (1.11 - 2.45) n=2	1.65 (0.72 - 3.80) n=1	
<u>Stomach</u>	1.92 (1.39 - 2.67) n=3	1.27 (0.77 - 2.10) n=1				
Adenocarcinoma	5.59 (1.22 - 26.0) n=1					
Cancer	1.83 (1.30 - 2.56) n=2	1.27 (0.77 - 2.10) n=1				
<u>Vulva</u>		1.52 (0.79 - 2.90) n=1		1.34 (0.67 - 2.70) n=1	2.25 (1.42 - 3.60) n=1	
SCC		1.52 (0.79 - 2.90) n=1		1.34 (0.67 - 2.70) n=1	2.25 (1.42 - 3.60) n=1	

FUNDING

This study was performed with financial support from the EU 7th framework program METOXIA (ref. 2008-222741), NGI Pre-Seed grant (no. 93612005), and the Dutch Cancer Society (KWF UM 2011-5020 and KWF MAC 2013-6089).

REFERENCES

1. Nordmark, M., et al., Prognostic value of tumor oxygenation in 397 head and neck tumors after primary radiation therapy. An international multi-center study. *Radiother Oncol*, 2005. **77**(1): p. 18-24.
2. Good, J.S. and K.J. Harrington, The hallmarks of cancer and the radiation oncologist: updating the 5Rs of radiobiology. *Clinical oncology*, 2013. **25**(10): p. 569-77.
3. Hockel, M., et al., Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. *Cancer Res*, 1996. **56**(19): p. 4509-15.
4. Wojtkowiak, J.W., et al., Drug resistance and cellular adaptation to tumor acidic pH microenvironment. *Molecular pharmaceutics*, 2011. **8**(6): p. 2032-8.
5. Dubois, L.J., et al., New ways to image and target tumour hypoxia and its molecular responses. *Radiother Oncol*, 2015. **116**(3): p. 352-7.
6. Helbig, L., et al., BAY 87-2243, a novel inhibitor of hypoxia-induced gene activation, improves local tumor control after fractionated irradiation in a schedule-dependent manner in head and neck human xenografts. *Radiation oncology*, 2014. **9**: p. 207.
7. Peeters, S.G., et al., TH-302 in Combination with Radiotherapy Enhances the Therapeutic Outcome and Is Associated with Pretreatment [18F]HX4 Hypoxia PET Imaging. *Clin Cancer Res*, 2015. **21**(13): p. 2984-92.
8. Pettersen, E.O., et al., Targeting tumour hypoxia to prevent cancer metastasis. From biology, biosensing and technology to drug development: the METOXIA consortium. *J Enzyme Inhib Med Chem*, 2015. **30**(5): p. 689-721.
9. Denko, N.C., Hypoxia, HIF1 and glucose metabolism in the solid tumour. *Nature reviews. Cancer*, 2008. **8**(9): p. 705-13.
10. Wouters, B.G. and M. Koritzinsky, Hypoxia signalling through mTOR and the unfolded protein response in cancer. *Nature reviews. Cancer*, 2008. **8**(11): p. 851-64.
11. Neri, D. and C.T. Supuran, Interfering with pH regulation in tumours as a therapeutic strategy. *Nature reviews. Drug discovery*, 2011. **10**(10): p. 767-77.
12. Pastorek, J. and S. Pastorekova, Hypoxia-induced carbonic anhydrase IX as a target for cancer therapy: from biology to clinical use. *Seminars in cancer biology*, 2015. **31**: p. 52-64.
13. Zhao, Z., et al., Prognostic value of carbonic anhydrase IX immunohistochemical expression in renal cell carcinoma: a meta-analysis of the literature. *PLoS One*, 2014. **9**(11): p. e114096.
14. Peridis, S., et al., Carbonic anhydrase-9 expression in head and neck cancer: a meta-analysis. *European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies*, 2011. **268**(5): p. 661-70.
15. Gnarr, J.R., et al., Mutations of the VHL tumour suppressor gene in renal carcinoma. *Nature genetics*, 1994. **7**(1): p. 85-90.
16. Oosterwijk-Wakka, J.C., et al., Application of monoclonal antibody G250 recognizing carbonic anhydrase IX in renal cell carcinoma. *International journal of molecular sciences*, 2013. **14**(6): p. 11402-23.
17. Rafajova, M., et al., Induction by hypoxia combined with low glucose or low bicarbonate and high posttranslational stability upon reoxygenation contribute to carbonic anhydrase IX expression in cancer cells. *International journal of oncology*, 2004. **24**(4): p. 995-1004.
18. Said, H.M., et al., Distinct patterns of hypoxic expression of carbonic anhydrase IX (CA IX) in human malignant glioma cell lines. *J Neurooncol*, 2007. **81**(1): p. 27-38.

19. Tafreshi, N.K., et al., Evaluation of CAIX and CAXII Expression in Breast Cancer at Varied O Levels: CAIX is the Superior Surrogate Imaging Biomarker of Tumor Hypoxia. *Mol Imaging Biol*, 2015.
20. Tierney, J.F., et al., Practical methods for incorporating summary time-to-event data into meta-analysis. *Trials*, 2007. **8**: p. 16.
21. Higgins, J.P.T. and S. Green, eds. *Cochrane Handbook for Systematic Reviews of Interventions Vol. Version 5.1.0 [updated March 2011]*. 2011, The Cochrane Collaboration.
22. Viechtbauer, W., Conducting Meta-Analyses in R with the metafor Package. *Journal of Statistical Software*, 2010. **36**(3): p. 1-48.
23. DerSimonian, R. and N. Laird, Meta-analysis in clinical trials. *Controlled clinical trials*, 1986. **7**(3): p. 177-88.
24. Jonathan, R.A., et al., The prognostic value of endogenous hypoxia-related markers for head and neck squamous cell carcinomas treated with ARCON. *Radiother Oncol*, 2006. **79**(3): p. 288-97.
25. Koukourakis, M.I., et al., Endogenous markers of two separate hypoxia response pathways (hypoxia inducible factor 2 alpha and carbonic anhydrase 9) are associated with radiotherapy failure in head and neck cancer patients recruited in the CHART randomized trial. *J Clin Oncol*, 2006. **24**(5): p. 727-35.
26. Koukourakis, M.I., et al., Hypoxia inducible factor (HIF1alpha and HIF2alpha) and carbonic anhydrase 9 (CA9) expression and response of head-neck cancer to hypofractionated and accelerated radiotherapy. *Int J Radiat Biol*, 2008. **84**(1): p. 47-52.
27. Rademakers, S.E., et al., Pattern of CAIX expression is prognostic for outcome and predicts response to ARCON in patients with laryngeal cancer treated in a phase III randomized trial. *Radiother Oncol*, 2013. **108**(3): p. 517-22.
28. Bane, A.L., et al., Tumor factors predictive of response to hypofractionated radiotherapy in a randomized trial following breast conserving therapy. *Ann Oncol*, 2014. **25**(5): p. 992-8.
29. Brennan, D.J., et al., CA IX is an independent prognostic marker in premenopausal breast cancer patients with one to three positive lymph nodes and a putative marker of radiation resistance. *Clin Cancer Res*, 2006. **12**(21): p. 6421-31.
30. Hong, Y.S., et al., Carbonic anhydrase 9 is a predictive marker of survival benefit from lower dose of bevacizumab in patients with previously treated metastatic colorectal cancer. *BMC Cancer*, 2009. **9**: p. 246.
31. Pastorekova, S., et al., A novel quasi-viral agent, MaTu, is a two-component system. *Virology*, 1992. **187**(2): p. 620-6.
32. Zavada, J., et al., Human tumour-associated cell adhesion protein MN/CA IX: identification of M75 epitope and of the region mediating cell adhesion. *Br J Cancer*, 2000. **82**(11): p. 1808-13.
33. Lancashire, L.J., et al., A validated gene expression profile for detecting clinical outcome in breast cancer using artificial neural networks. *Breast Cancer Res Treat*, 2010. **120**(1): p. 83-93.
34. Trastour, C., et al., HIF-1alpha and CA IX staining in invasive breast carcinomas: prognosis and treatment outcome. *Int J Cancer*, 2007. **120**(7): p. 1451-8.
35. Blank, A., et al., SDHB loss predicts malignancy in pheochromocytomas/sympathetic paragangliomas, but not through hypoxia signalling. *Endocr Relat Cancer*, 2010. **17**(4): p. 919-28.
36. Clevon, A.H., et al., Stromal expression of hypoxia regulated proteins is an adverse prognostic factor in colorectal carcinomas. *Cell Oncol*, 2007. **29**(3): p. 229-40.
37. Huber, A.R., et al., High expression of carbonic anhydrase IX is significantly associated with glandular lesions in gastroesophageal junction and with tumorigenesis markers BMI1, MCM4 and MCM7. *BMC Gastroenterol*, 2015. **15**: p. 80.
38. Jung, J.H., et al., Clinicopathological implications of the expression of hypoxia-related proteins in gastric cancer. *Int J Med Sci*, 2013. **10**(9): p. 1217-23.
39. Lee-Kong, S.A., et al., Hypoxia-related proteins in patients with rectal cancer undergoing neoadjuvant combined modality therapy. *Dis Colon Rectum*, 2012. **55**(9): p. 990-5.
40. Noh, S., J.Y. Kim, and J.S. Koo, Metabolic differences in estrogen receptor-negative breast cancer based on androgen receptor status. *Tumour Biol*, 2014. **35**(8): p. 8179-92.

41. Preusser, M., et al., Vascularization and expression of hypoxia-related tissue factors in intracranial ependymoma and their impact on patient survival. *Acta Neuropathol*, 2005. **109**(2): p. 211-6.
42. Seeber, L.M., et al., Necrosis related HIF-1alpha expression predicts prognosis in patients with endometrioid endometrial carcinoma. *BMC Cancer*, 2010. **10**: p. 307.
43. Winter, S.C., et al., The relation between hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha expression with anemia and outcome in surgically treated head and neck cancer. *Cancer*, 2006. **107**(4): p. 757-66.
44. Abraham, S., N. Hu, and R. Jensen, Hypoxia-inducible factor-1-regulated protein expression and oligodendroglioma patient outcome: comparison with established biomarkers and preoperative UCSF low-grade scoring system. *J Neurooncol*, 2012. **108**(3): p. 459-68.
45. Ameis, H.M., et al., Carbonic anhydrase IX correlates with survival and is a potential therapeutic target for neuroblastoma. *J Enzyme Inhib Med Chem*, 2015: p. 1-6.
46. Beketic-Oreskovic, L., et al., Prognostic significance of carbonic anhydrase IX (CA-IX), endoglin (CD105) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) in breast cancer patients. *Pathol Oncol Res*, 2011. **17**(3): p. 593-603.
47. Betof, A.S., et al., Carbonic anhydrase IX is a predictive marker of doxorubicin resistance in early-stage breast cancer independent of HER2 and TOP2A amplification. *Br J Cancer*, 2012. **106**(5): p. 916-22.
48. Birner, P., et al., Carbonic anhydrase IX overexpression is associated with diminished prognosis in esophageal cancer and correlates with Her-2 expression. *Ann Surg Oncol*, 2011. **18**(12): p. 3330-7.
49. Brockton, N., et al., High stromal carbonic anhydrase IX expression is associated with decreased survival in P16-negative head-and-neck tumors. *Int J Radiat Oncol Biol Phys*, 2011. **80**(1): p. 249-57.
50. Chang, D.T., et al., Expression of p16(INK4A) but not hypoxia markers or poly adenosine diphosphate-ribose polymerase is associated with improved survival in patients with pancreatic adenocarcinoma. *Cancer*, 2010. **116**(22): p. 5179-87.
51. Chen, J., et al., Expression of carbonic anhydrase 9 at the invasion front of gastric cancers. *Gut*, 2005. **54**(7): p. 920-7.
52. Chen, Y., et al., Expression of HIF-1alpha and CAIX in nasopharyngeal carcinoma and their correlation with patients' prognosis. *Med Oncol*, 2014. **31**(12): p. 304.
53. Choi, S.W., et al., Expression of carbonic anhydrase IX is associated with postoperative recurrence and poor prognosis in surgically treated oral squamous cell carcinoma. *Hum Pathol*, 2008. **39**(9): p. 1317-22.
54. Choschzick, M., et al., Overexpression of carbonic anhydrase IX (CAIX) is an independent unfavorable prognostic marker in endometrioid ovarian cancer. *Virchows Arch*, 2011. **459**(2): p. 193-200.
55. Couvelard, A., et al., Expression of hypoxia-inducible factors is correlated with the presence of a fibrotic focus and angiogenesis in pancreatic ductal adenocarcinomas. *Histopathology*, 2005. **46**(6): p. 668-76.
56. Couvelard, A., et al., Microvascular density and hypoxia-inducible factor pathway in pancreatic endocrine tumours: negative correlation of microvascular density and VEGF expression with tumour progression. *Br J Cancer*, 2005. **92**(1): p. 94-101.
57. Currie, M.J., et al., Immunohistochemical analysis of cancer stem cell markers in invasive breast carcinoma and associated ductal carcinoma in situ: relationships with markers of tumor hypoxia and microvasculature. *Hum Pathol*, 2013. **44**(3): p. 402-11.
58. Deb, S., et al., Nuclear HIF1A expression is strongly prognostic in sporadic but not familial male breast cancer. *Mod Pathol*, 2014. **27**(9): p. 1223-30.
59. Debucquoy, A., et al., Molecular and clinico-pathological markers in rectal cancer: a tissue micro-array study. *Int J Colorectal Dis*, 2009. **24**(2): p. 129-38.
60. Deschamps, L., et al., The expression of the hypoxia markers CA9 and CXCR4 is correlated with survival in patients with neuroendocrine tumours of the ileum. *Neuroendocrinology*, 2012. **95**(3): p. 214-22.
61. Driessen, A., et al., Expression of carbonic anhydrase IX (CA IX), a hypoxia-related protein, rather than vascular-endothelial growth factor (VEGF), a pro-angiogenic factor, correlates with an extremely poor prognosis in esophageal and gastric adenocarcinomas. *Ann Surg*, 2006. **243**(3): p. 334-40.
62. Dungwa, J.V., L.P. Hunt, and P. Ramani, Carbonic anhydrase IX up-regulation is associated with adverse clinicopathologic and biologic factors in neuroblastomas. *Hum Pathol*, 2012. **43**(10): p. 1651-60.

63. Eckert, A.W., et al., Co-expression of Hif1alpha and CAIX is associated with poor prognosis in oral squamous cell carcinoma patients. *J Oral Pathol Med*, 2010. **39**(4): p. 313-7.
64. Erpolat, O.P., et al., Hypoxia-related molecules HIF-1alpha, CA9, and osteopontin : predictors of survival in patients with high-grade glioma. *Strahlenther Onkol*, 2013. **189**(2): p. 147-54.
65. Eustace, A., et al., Necrosis predicts benefit from hypoxia-modifying therapy in patients with high risk bladder cancer enrolled in a phase III randomised trial. *Radiother Oncol*, 2013. **108**(1): p. 40-7.
66. Flynn, J.R., et al., Hypoxia-regulated protein expression, patient characteristics, and preoperative imaging as predictors of survival in adults with glioblastoma multiforme. *Cancer*, 2008. **113**(5): p. 1032-42.
67. Garcia, S., et al., Poor prognosis in breast carcinomas correlates with increased expression of targetable CD146 and c-Met and with proteomic basal-like phenotype. *Hum Pathol*, 2007. **38**(6): p. 830-41.
68. Generali, D., et al., Role of carbonic anhydrase IX expression in prediction of the efficacy and outcome of primary epirubicin/tamoxifen therapy for breast cancer. *Endocr Relat Cancer*, 2006. **13**(3): p. 921-30.
69. Giatromanolaki, A., et al., Expression of hypoxia-inducible carbonic anhydrase-9 relates to angiogenic pathways and independently to poor outcome in non-small cell lung cancer. *Cancer Res*, 2001. **61**(21): p. 7992-8.
70. Gu, M., CA9 overexpression is an independent favorable prognostic marker in intrahepatic cholangiocarcinoma. *Int J Clin Exp Pathol*, 2015. **8**(1): p. 862-6.
71. Gu, M.J. and K.W. Kwon, Carbonic anhydrase IX expression is associated with favorable prognostic factors in small intestinal carcinoma. *J Histochem Cytochem*, 2014. **62**(3): p. 205-10.
72. Haapasalo, J.A., et al., Expression of carbonic anhydrase IX in astrocytic tumors predicts poor prognosis. *Clin Cancer Res*, 2006. **12**(2): p. 473-7.
73. Han, M.W., et al., Role of FDG-PET as a biological marker for predicting the hypoxic status of tongue cancer. *Head Neck*, 2012. **34**(10): p. 1395-402.
74. Huang, W.J., et al., Expression of hypoxic marker carbonic anhydrase IX predicts poor prognosis in resectable hepatocellular carcinoma. *PLoS One*, 2015. **10**(3): p. e0119181.
75. Hui, E.P., et al., Coexpression of hypoxia-inducible factors 1alpha and 2alpha, carbonic anhydrase IX, and vascular endothelial growth factor in nasopharyngeal carcinoma and relationship to survival. *Clin Cancer Res*, 2002. **8**(8): p. 2595-604.
76. Hussain, S.A., et al., Hypoxia-regulated carbonic anhydrase IX expression is associated with poor survival in patients with invasive breast cancer. *Br J Cancer*, 2007. **96**(1): p. 104-9.
77. Hynninen, P., et al., Expression of transmembrane carbonic anhydrases IX and XII in ovarian tumours. *Histopathology*, 2006. **49**(6): p. 594-602.
78. Ilie, M., et al., High levels of carbonic anhydrase IX in tumour tissue and plasma are biomarkers of poor prognostic in patients with non-small cell lung cancer. *Br J Cancer*, 2010. **102**(11): p. 1627-35.
79. Jarvela, S., et al., Carbonic anhydrase IX in oligodendroglial brain tumors. *BMC Cancer*, 2008. **8**: p. 1.
80. Jensen, R. and J. Lee, Predicting outcomes of patients with intracranial meningiomas using molecular markers of hypoxia, vascularity, and proliferation. *Neurosurgery*, 2012. **71**(1): p. 146-56.
81. Jubb, A.M., et al., Expression of delta-like ligand 4 (Dll4) and markers of hypoxia in colon cancer. *Br J Cancer*, 2009. **101**(10): p. 1749-57.
82. Kang, H.J., et al., Expression of carbonic anhydrase 9 is a novel prognostic marker in resectable hepatocellular carcinoma. *Virchows Arch*, 2015. **466**(4): p. 403-13.
83. Kato, Y., et al., Expression of a hypoxia-associated protein, carbonic anhydrase-9, correlates with malignant phenotypes of gastric carcinoma. *Digestion*, 2010. **82**(4): p. 246-51.
84. Kaya, A.O., et al., Hypoxia inducible factor-1 alpha and carbonic anhydrase IX overexpression are associated with poor survival in breast cancer patients. *J BUON*, 2012. **17**(4): p. 663-8.
85. Kim, B.W., et al., Prognostic assessment of hypoxia and metabolic markers in cervical cancer using automated digital image analysis of immunohistochemistry. *J Transl Med*, 2013. **11**: p. 185.
86. Kim, H.M., W.H. Jung, and J.S. Koo, Site-specific metabolic phenotypes in metastatic breast cancer. *J Transl Med*, 2014. **12**: p. 354.
87. Kim, J.I., et al., Expression of hypoxic markers and their prognostic significance in soft tissue sarcoma. *Oncol Lett*, 2015. **9**(4): p. 1699-1706.

88. Kim, K., et al., Prognostic Relevance of the Expression of CA IX, GLUT-1, and VEGF in Ovarian Epithelial Cancers. *Korean J Pathol*, 2012. **46**(6): p. 532-40.
89. Kim, S.J., et al., Prognostic value of carbonic anhydrase IX and Ki-67 expression in squamous cell carcinoma of the tongue. *Jpn J Clin Oncol*, 2007. **37**(11): p. 812-9.
90. Klatte, T., et al., Carbonic anhydrase IX in bladder cancer: a diagnostic, prognostic, and therapeutic molecular marker. *Cancer*, 2009. **115**(7): p. 1448-58.
91. Kondo, Y., et al., Clinicopathological significance of carbonic anhydrase 9, glucose transporter-1, Ki-67 and p53 expression in oral squamous cell carcinoma. *Oncol Rep*, 2011. **25**(5): p. 1227-33.
92. Korkolopoulou, P., et al., Expression of hypoxia-related tissue factors in astrocytic gliomas. A multivariate survival study with emphasis upon carbonic anhydrase IX. *Hum Pathol*, 2007. **38**(4): p. 629-38.
93. Kornegoor, R., et al., Fibrotic focus and hypoxia in male breast cancer. *Mod Pathol*, 2012. **25**(10): p. 1397-404.
94. Koukourakis, M.I., et al., Hypoxia-regulated carbonic anhydrase-9 (CA9) relates to poor vascularization and resistance of squamous cell head and neck cancer to chemoradiotherapy. *Clin Cancer Res*, 2001. **7**(11): p. 3399-403.
95. Kyndi, M., et al., Carbonic anhydrase IX and response to postmastectomy radiotherapy in high-risk breast cancer: a subgroup analysis of the DBCG82 b and c trials. *Breast Cancer Res*, 2008. **10**(2): p. R24.
96. Lastraioli, E., et al., hERG1 Channels and Glut-1 as Independent Prognostic Indicators of Worse Outcome in Stage I and II Colorectal Cancer: A Pilot Study. *Transl Oncol*, 2012. **5**(2): p. 105-12.
97. Le, Q.T., et al., An evaluation of tumor oxygenation and gene expression in patients with early stage non-small cell lung cancers. *Clin Cancer Res*, 2006. **12**(5): p. 1507-14.
98. Le, Q.T., et al., Galectin-1: a link between tumor hypoxia and tumor immune privilege. *J Clin Oncol*, 2005. **23**(35): p. 8932-41.
99. Li, Y., et al., Roles of Carbonic Anhydrase IX in Development of Pancreatic Cancer. *Pathol Oncol Res*, 2015.
100. Liao, S.Y., et al., Prognostic relevance of carbonic anhydrase-IX in high-risk, early-stage cervical cancer: a Gynecologic Oncology Group study. *Gynecol Oncol*, 2010. **116**(3): p. 452-8.
101. Liu, Z., et al., Paxillin and carbonic anhydrase IX are prognostic markers in gallbladder squamous cell/adenosquamous carcinomas and adenocarcinomas. *Histopathology*, 2014. **64**(7): p. 921-34.
102. Maseide, K., et al., Carbonic anhydrase IX as a marker for poor prognosis in soft tissue sarcoma. *Clin Cancer Res*, 2004. **10**(13): p. 4464-71.
103. Nakao, M., et al., Prognostic significance of carbonic anhydrase IX expression by cancer-associated fibroblasts in lung adenocarcinoma. *Cancer*, 2009. **115**(12): p. 2732-43.
104. Nordfors, K., et al., The tumour-associated carbonic anhydrases CA II, CA IX and CA XII in a group of medulloblastomas and supratentorial primitive neuroectodermal tumours: an association of CA IX with poor prognosis. *BMC Cancer*, 2010. **10**: p. 148.
105. Ono, S., et al., Podooplanin-positive cancer-associated fibroblasts could have prognostic value independent of cancer cell phenotype in stage I lung squamous cell carcinoma: usefulness of combining analysis of both cancer cell phenotype and cancer-associated fibroblast phenotype. *Chest*, 2013. **143**(4): p. 963-70.
106. Ord, J.J., et al., An investigation into the prognostic significance of necrosis and hypoxia in high grade and invasive bladder cancer. *J Urol*, 2007. **178**(2): p. 677-82.
107. O'Toole, D., et al., Molecular markers associated with response to chemotherapy in gastro-entero-pancreatic neuroendocrine tumors. *Endocr Relat Cancer*, 2010. **17**(4): p. 847-56.
108. Perez-Sayans, M., et al., Expression of CA-IX is associated with advanced stage tumors and poor survival in oral squamous cell carcinoma patients. *J Oral Pathol Med*, 2012. **41**(9): p. 667-74.
109. Pinato, D.J., et al., An expression signature of the angiogenic response in gastrointestinal neuroendocrine tumours: correlation with tumour phenotype and survival outcomes. *Br J Cancer*, 2014. **110**(1): p. 115-22.
110. Polanski, R., et al., Activity of the monocarboxylate transporter 1 inhibitor AZD3965 in small cell lung cancer. *Clin Cancer Res*, 2014. **20**(4): p. 926-37.

111. Proescholdt, M.A., et al., Function of carbonic anhydrase IX in glioblastoma multiforme. *Neuro Oncol*, 2012. **14**(11): p. 1357-66.
112. Roh, J.L., et al., Prognostic values of pathologic findings and hypoxia markers in 21 patients with salivary duct carcinoma. *J Surg Oncol*, 2008. **97**(7): p. 596-600.
113. Sadlecki, P., et al., The role of Hypoxia-inducible factor-1 alpha , glucose transporter-1, (GLUT-1) and carbon anhydrase IX in endometrial cancer patients. *Biomed Res Int*, 2014. **2014**: p. 616850.
114. Sergeant, G., et al., The prognostic relevance of tumor hypoxia markers in resected carcinoma of the gallbladder. *Eur J Surg Oncol*, 2011. **37**(1): p. 80-6.
115. Sooman, L., et al., FGF2 as a potential prognostic biomarker for proneural glioma patients. *Acta Oncol*, 2015. **54**(3): p. 385-94.
116. Srivastava, S., et al., Expression of proteins associated with hypoxia and Wnt pathway activation is of prognostic significance in hepatocellular carcinoma. *Virchows Arch*, 2015. **466**(5): p. 541-8.
117. Stewart, D.J., et al., Membrane carbonic anhydrase IX expression and relapse risk in resected stage I-II non-small-cell lung cancer. *J Thorac Oncol*, 2014. **9**(5): p. 675-84.
118. Swinson, D.E., G. Cox, and K.J. O'Byrne, Coexpression of epidermal growth factor receptor with related factors is associated with a poor prognosis in non-small-cell lung cancer. *Br J Cancer*, 2004. **91**(7): p. 1301-7.
119. Tan, E.Y., et al., The key hypoxia regulated gene CAIX is upregulated in basal-like breast tumours and is associated with resistance to chemotherapy. *Br J Cancer*, 2009. **100**(2): p. 405-11.
120. Tomes, L., et al., Necrosis and hypoxia in invasive breast carcinoma. *Breast Cancer Res Treat*, 2003. **81**(1): p. 61-9.
121. Verstraete, M., et al., Investigation of possible endogenous hypoxia markers in colorectal cancer. *Int J Radiat Biol*, 2013. **89**(1): p. 9-15.
122. Wachters, J.E., et al., Prognostic significance of HIF-1a, CA-IX, and OPN in T1-T2 laryngeal carcinoma treated with radiotherapy. *Laryngoscope*, 2013. **123**(9): p. 2154-60.
123. Williams, E., et al., Co-expression of VEGF and CA9 in ovarian high-grade serous carcinoma and relationship to survival. *Virchows Arch*, 2012. **461**(1): p. 33-9.
124. Woelber, L., et al., Carbonic anhydrase IX in tumor tissue and sera of patients with primary cervical cancer. *BMC Cancer*, 2011. **11**: p. 12.
125. Yan, P., et al., High monocarboxylate transporter 4 protein expression in stromal cells predicts adverse survival in gastric cancer. *Asian Pac J Cancer Prev*, 2014. **15**(20): p. 8923-9.
126. Yang, J.S., et al., Carbonic anhydrase IX overexpression regulates the migration and progression in oral squamous cell carcinoma. *Tumour Biol*, 2015.
127. Yoo, H., et al., The expressions of carbonic anhydrase 9 and vascular endothelial growth factor in astrocytic tumors predict a poor prognosis. *Int J Mol Med*, 2010. **26**(1): p. 3-9.
128. Yu, M., et al., Metabolic phenotypes in pancreatic cancer. *PLoS One*, 2015. **10**(2): p. e0115153.
129. Zheng, G., et al., ZEB1 transcriptionally regulated carbonic anhydrase 9 mediates the chemoresistance of tongue cancer via maintaining intracellular pH. *Mol Cancer*, 2015. **14**: p. 84.
130. Rajaganesan, R., et al., Expression patterns of hypoxic markers at the invasive margin of colorectal cancers and liver metastases. *Eur J Surg Oncol*, 2009. **35**(12): p. 1286-94.
131. Aomatsu, N., et al., Carbonic anhydrase 9 is associated with chemosensitivity and prognosis in breast cancer patients treated with taxane and anthracycline. *BMC Cancer*, 2014. **14**: p. 400.
132. Choschick, M., et al., Overexpression of carbonic anhydrase IX (CAIX) in vulvar cancer is associated with tumor progression and development of locoregional lymph node metastases. *Virchows Arch*, 2010. **456**(5): p. 483-90.
133. De Schutter, H., et al., The prognostic value of the hypoxia markers CA IX and GLUT 1 and the cytokines VEGF and IL 6 in head and neck squamous cell carcinoma treated by radiotherapy +/- chemotherapy. *BMC Cancer*, 2005. **5**: p. 42.
134. Hedley, D., et al., Carbonic anhydrase IX expression, hypoxia, and prognosis in patients with uterine cervical carcinomas. *Clin Cancer Res*, 2003. **9**(15): p. 5666-74.

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135. Heo, K., et al., Hypoxia-induced up-regulation of apelin is associated with a poor prognosis in oral squamous cell carcinoma patients. *Oral Oncol*, 2012. **48**(6): p. 500-6.
136. Hiraoka, N., et al., Tumour necrosis is a postoperative prognostic marker for pancreatic cancer patients with a high interobserver reproducibility in histological evaluation. *Br J Cancer*, 2010. **103**(7): p. 1057-65.
137. Kim, S.J., et al., Expression of HIF-1alpha, CA IX, VEGF, and MMP-9 in surgically resected non-small cell lung cancer. *Lung Cancer*, 2005. **49**(3): p. 325-35.
138. Korkeila, E., et al., Expression of carbonic anhydrase IX suggests poor outcome in rectal cancer. *Br J Cancer*, 2009. **100**(6): p. 874-80.
139. Lee, S., et al., Tumor carbonic anhydrase 9 expression is associated with the presence of lymph node metastases in uterine cervical cancer. *Cancer Sci*, 2007. **98**(3): p. 329-33.
140. Lou, Y., et al., Targeting tumor hypoxia: suppression of breast tumor growth and metastasis by novel carbonic anhydrase IX inhibitors. *Cancer Res*, 2011. **71**(9): p. 3364-76.
141. Pinheiro, C., et al., GLUT1 and CAIX expression profiles in breast cancer correlate with adverse prognostic factors and MCT1 overexpression. *Histol Histopathol*, 2011. **26**(10): p. 1279-86.
142. Schmitt, A.M., et al., VHL inactivation is an important pathway for the development of malignant sporadic pancreatic endocrine tumors. *Endocr Relat Cancer*, 2009. **16**(4): p. 1219-27.
143. Zhu, Y., et al., Prognostic value of carbonic anhydrase IX expression in penile squamous cell carcinoma: a pilot study. *Urol Oncol*, 2013. **31**(5): p. 706-11.
144. Bernstein, J.M., et al., Prognostic value of hypoxia-associated markers in advanced larynx and hypopharynx squamous cell carcinoma. *Laryngoscope*, 2015. **125**(1): p. E8-15.
145. Douglas, C.M., et al., Lack of prognostic effect of carbonic anhydrase-9, hypoxia inducible factor-1alpha and bcl-2 in 286 patients with early squamous cell carcinoma of the glottic larynx treated with radiotherapy. *Clin Oncol (R Coll Radiol)*, 2013. **25**(1): p. 59-65.
146. Eriksen, J.G. and J. Overgaard, Lack of prognostic and predictive value of CA IX in radiotherapy of squamous cell carcinoma of the head and neck with known modifiable hypoxia: an evaluation of the DAHANCA 5 study. *Radiother Oncol*, 2007. **83**(3): p. 383-8.
147. Guedj, N., et al., Predictors of tumor response after preoperative chemoradiotherapy for rectal adenocarcinomas. *Hum Pathol*, 2011. **42**(11): p. 1702-9.
148. Kirkpatrick, J.P., et al., Elevated CAIX Expression is Associated with an Increased Risk of Distant Failure in Early-Stage Cervical Cancer. *Biomark Insights*, 2008. **3**: p. 45-55.
149. Kwon, O.J., et al., HIF-1alpha and CA-IX as predictors of locoregional control for determining the optimal treatment modality for early-stage laryngeal carcinoma. *Head Neck*, 2015. **37**(4): p. 505-10.
150. Lancaster, J.A., et al., Carbonic anhydrase (CA IX) expression, a potential new intrinsic marker of hypoxia: correlations with tumor oxygen measurements and prognosis in locally advanced carcinoma of the cervix. *Cancer Res*, 2001. **61**(17): p. 6394-9.
151. Nordsmark, M., et al., Differential risk assessments from five hypoxia specific assays: The basis for biologically adapted individualized radiotherapy in advanced head and neck cancer patients. *Radiother Oncol*, 2007. **83**(3): p. 389-97.
152. Roh, J.L., et al., The prognostic value of hypoxia markers in T2-staged oral tongue cancer. *Oral Oncol*, 2009. **45**(1): p. 63-8.
153. Sakata, K., et al., Brachytherapy for oral tongue cancer: an analysis of treatment results with various biological markers. *Jpn J Clin Oncol*, 2008. **38**(6): p. 402-7.
154. Silva, P., et al., Use of multiple biological markers in radiotherapy-treated head and neck cancer. *J Laryngol Otol*, 2010. **124**(6): p. 650-8.
155. Turner, K.J., et al., The hypoxia-inducible genes VEGF and CA9 are differentially regulated in superficial vs invasive bladder cancer. *Br J Cancer*, 2002. **86**(8): p. 1276-82.
156. Andersen, S., et al., Diverging prognostic impacts of hypoxic markers according to NSCLC histology. *Lung Cancer*, 2011. **72**(3): p. 294-302.
157. Brockton, N.T., et al., High stromal carbonic anhydrase IX expression is associated with nodal metastasis and decreased survival in patients with surgically-treated oral cavity squamous cell carcinoma. *Oral Oncol*, 2012. **48**(7): p. 615-22.

158. Hwa, J.S., et al., The prognostic value of immunohistochemical markers for oral tongue squamous cell carcinoma. *Eur Arch Otorhinolaryngol*, 2015. **272**(10): p. 2953-9.
159. Kivela, A.J., et al., Carbonic anhydrase IX in malignant pleural mesotheliomas: a potential target for anti-cancer therapy. *Bioorg Med Chem*, 2013. **21**(6): p. 1483-8.
160. Neumeister, V.M., et al., Hypoxia-induced protein CAIX is associated with somatic loss of BRCA1 protein and pathway activity in triple negative breast cancer. *Breast Cancer Res Treat*, 2012. **136**(1): p. 67-75.
161. Rasheed, S., et al., Assessment of microvessel density and carbonic anhydrase-9 (CA-9) expression in rectal cancer. *Pathol Res Pract*, 2009. **205**(1): p. 1-9.
162. Sherwood, B.T., et al., Carbonic anhydrase IX expression and outcome after radiotherapy for muscle-invasive bladder cancer. *Clin Oncol (R Coll Radiol)*, 2007. **19**(10): p. 777-83.
163. Smeland, E., et al., Prognostic impacts of hypoxic markers in soft tissue sarcoma. *Sarcoma*, 2012. **2012**: p. 541650.
164. Tanaka, N., et al., Expression of carbonic anhydrase 9, a potential intrinsic marker of hypoxia, is associated with poor prognosis in oesophageal squamous cell carcinoma. *Br J Cancer*, 2008. **99**(9): p. 1468-75.
165. van de Nieuwenhof, H.P., et al., Hemoglobin level predicts outcome for vulvar cancer patients independent of GLUT-1 and CA-IX expression in tumor tissue. *Virchows Arch*, 2010. **457**(6): p. 693-703.
166. Doyen, J., et al., Expression of the hypoxia-inducible monocarboxylate transporter MCT4 is increased in triple negative breast cancer and correlates independently with clinical outcome. *Biochem Biophys Res Commun*, 2014. **451**(1): p. 54-61.
167. Boeuf, S., et al., Correlation of hypoxic signalling to histological grade and outcome in cartilage tumours. *Histopathology*, 2010. **56**(5): p. 641-51.
168. Schweiger, T., et al., Carbonic anhydrase IX is associated with early pulmonary spreading of primary colorectal carcinoma and tobacco smoking. *Eur J Cardiothorac Surg*, 2014. **46**(1): p. 92-9.
169. Grigsby, P.W., et al., Comparison of molecular markers of hypoxia and imaging with (60)Cu-ATSM in cancer of the uterine cervix. *Mol Imaging Biol*, 2007. **9**(5): p. 278-83.
170. Wykoff, C.C., et al., Hypoxia-inducible expression of tumor-associated carbonic anhydrases. *Cancer Res*, 2000. **60**(24): p. 7075-83.
171. Kaluz, S., et al., Lowered oxygen tension induces expression of the hypoxia marker MN/carbonic anhydrase IX in the absence of hypoxia-inducible factor 1 alpha stabilization: a role for phosphatidylinositol 3'-kinase. *Cancer Res*, 2002. **62**(15): p. 4469-77.
172. van den Beucken, T., et al., Deficient carbonic anhydrase 9 expression in UPR-impaired cells is associated with reduced survival in an acidic microenvironment. *Radiother Oncol*, 2009. **92**(3): p. 437-42.
173. Lock, F.E., et al., Targeting carbonic anhydrase IX depletes breast cancer stem cells within the hypoxic niche. *Oncogene*, 2013. **32**(44): p. 5210-9.
174. Svastova, E., et al., Carbonic anhydrase IX interacts with bicarbonate transporters in lamellipodia and increases cell migration via its catalytic domain. *The Journal of biological chemistry*, 2012. **287**(5): p. 3392-402.
175. Csaderova, L., et al., The effect of carbonic anhydrase IX on focal contacts during cell spreading and migration. *Frontiers in physiology*, 2013. **4**: p. 271.
176. Ward, C., et al., Evaluation of carbonic anhydrase IX as a therapeutic target for inhibition of breast cancer invasion and metastasis using a series of in vitro breast cancer models. *Oncotarget*, 2015. **6**(28): p. 24856-70.
177. Ledaki, I., et al., Carbonic anhydrase IX induction defines a heterogeneous cancer cell response to hypoxia and mediates stem cell-like properties and sensitivity to HDAC inhibition. *Oncotarget*, 2015. **6**(23): p. 19413-27.
178. Papagerakis, S., et al., Proton pump inhibitors and histamine 2 blockers are associated with improved overall survival in patients with head and neck squamous carcinoma. *Cancer prevention research*, 2014. **7**(12): p. 1258-69.
179. Fais, S., Evidence-based support for the use of proton pump inhibitors in cancer therapy. *J Transl Med*, 2015. **13**(1): p. 368.

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180. Spugnini, E.P., et al., Proton channels and exchangers in cancer. *Biochimica et biophysica acta*, 2015. **1848**(10 Pt B): p. 2715-26.
181. Borenstein, M., et al., A basic introduction to fixed-effect and random-effects models for meta-analysis. *Research synthesis methods*, 2010. **1**(2): p. 97-111.
182. Liu, Z., et al., A step-by-step guide to the systematic review and meta-analysis of diagnostic and prognostic test accuracy evaluations. *Br J Cancer*, 2013. **108**(11): p. 2299-303.
183. Adams, A., et al., The potential of hypoxia markers as target for breast molecular imaging--a systematic review and meta-analysis of human marker expression. *BMC Cancer*, 2013. **13**: p. 538.
184. Hartling, L., et al., Testing the Newcastle Ottawa Scale showed low reliability between individual reviewers. *Journal of clinical epidemiology*, 2013. **66**(9): p. 982-93.
185. Oremus, M., et al., Inter-rater and test-retest reliability of quality assessments by novice student raters using the Jadad and Newcastle-Ottawa Scales. *BMJ open*, 2012. **2**(4).
186. Stang, A., Critical evaluation of the Newcastle-Ottawa scale for the assessment of the quality of nonrandomized studies in meta-analyses. *European journal of epidemiology*, 2010. **25**(9): p. 603-5.
187. Monti, S.M., C.T. Supuran, and G. De Simone, Anticancer carbonic anhydrase inhibitors: a patent review (2008 - 2013). *Expert opinion on therapeutic patents*, 2013. **23**(6): p. 737-49.

Chapter 3

The sulfamate small molecule CAIX inhibitor S4 modulates doxorubicin efficacy

Published in: PLoS One. 2016 Aug 11;11(8):e0161040.

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ABSTRACT

Carbonic anhydrase IX (CAIX) is a tumor-specific protein that is upregulated during hypoxic conditions where it is involved in maintaining the pH balance. CAIX causes extracellular acidification, thereby limiting the uptake of weak basic chemotherapeutic agents, such as doxorubicin, and decreasing its efficacy. The aim of this study was to determine if doxorubicin efficacy can be increased when combined with the selective sulfamate CAIX inhibitor S4.

The effect of S4 on doxorubicin efficacy was tested *in vitro* using cell viability assays with MDA-MB-231, FaDu, HT29 – CAIX high and HT29 – CAIX low cell lines. In addition, the efficacy of this combination therapy was investigated in tumor xenografts of the same cell lines.

The addition of S4 *in vitro* increased the efficacy of doxorubicin in the MDA-MB-231 during hypoxic exposure (IC₅₀ is 0.25 versus 0.14 μ M, $p=0.0003$). Similar results were observed for HT29 - CAIX high with S4 during normoxia (IC₅₀ is 0.20 versus 0.08 μ M, $p<0.0001$) and in the HT29 – CAIX low cells (IC₅₀ is 0.09 μ M, $p<0.0001$). *In vivo* doxorubicin treatment was only effective in the MDA-MB-231 xenografts, but the efficacy of doxorubicin was decreased when combined with S4.

In conclusion, the efficacy of doxorubicin treatment can be increased when combined with the selective sulfamate CAIX inhibitor S4 *in vitro* in certain cell lines. Nevertheless, in xenografts S4 did not enhance doxorubicin efficacy in the FaDu and HT29 tumor models and decreased doxorubicin efficacy in the MDA-MB-231 tumor model. These results stress the importance of better understanding the role of CAIX inhibitors in intratumoral pH regulation before combining them with standard treatment modalities, such as doxorubicin.

INTRODUCTION

The immature and inadequate vasculature of solid tumors prevents oxygen and nutrients supply to certain areas in those tumors. This hypoxic microenvironment selects for a more aggressive tumor phenotype and promotes invasion, migration, and thereby metastases formation [1, 2]. These hypoxic tumor cells are known to be more resistant to standard treatment modalities (e.g. radio- and chemotherapy), highlighting the importance of developing new, or increasing the efficacy of already available, therapies to specifically target hypoxic tumor cells [2-5].

To meet with the high energy demand, tumor cells switch their energy metabolism to glycolysis in hypoxic but also in well-oxygenated areas in the tumors, i.e. the Warburg effect. The increase in glycolytic energy production leads to the production of high concentrations of acids (e.g. lactate and carbon dioxide) [6]. To aid in maintaining cellular pH homeostasis, carbonic anhydrase IX (CAIX) expression becomes transcriptionally upregulated through stabilization of hypoxia-inducible factor 1 (HIF1- α), although alternative hypoxia responses also play a role [7]. CAIX catalyzes the conversion of water and the cellular produced carbon dioxide to bicarbonate and protons. The protons contribute to the hostile acidic extracellular environment, whereas the bicarbonate is transported back intracellularly to aid in maintaining a slightly alkaline pH [8, 9]. The important role that CAIX plays in maintaining the cellular pH balance combined with its predominant expression on hypoxic tumor cells make it an attractive target for treatment [2-4, 10]. The importance of CAIX on tumors is strengthened further by the significant association between high tumoral CAIX expression and a worse prognosis in patients with many different cancer types [11]. CAIX expression might therefore also be a valuable imaging tool for future clinical practice [12-14]. Previously, several CAIX inhibitors have been shown to be effective in reducing primary tumor growth *in vivo*, either as a single treatment or in combination with radiotherapy [15-17].

CAIX inhibition is also able to increase the efficacy of certain chemotherapeutics, such as the anthracycline antitumor antibiotic doxorubicin [18, 19]. This chemotherapeutic agent is commonly used in combination treatment regimens in different cancer types, of which breast cancer is a prime example [20]. Doxorubicin diffuses passively across the cell membrane, intercalates the DNA and induces cell death. However, since doxorubicin is a weak basic compound the reduced extracellular pH in hypoxic areas of tumors prevents passive drug uptake, referred to as the ion trapping model. This model predicts pH modulating therapies are capable of increasing the uptake and efficacy of doxorubicin [21-23]. Inhibiting CAIX function reduces the degree of extracellular acidification [8, 15, 16, 19, 24], which may thereby increase the uptake and efficacy of doxorubicin. In line with the ion trapping model non-specific carbonic anhydrase inhibition with acetazolamide has been shown previously to increase doxorubicin uptake *in vitro* [18], although the carbonic anhydrase isoform responsible for this effect remained

elusive, but was suggested to be CAIX. Furthermore, evidence exist that CAIX inhibition is able to increase doxorubicin efficacy *in vivo* [19].

The ureido-substituted sulfamate S4 is a member of an alternative class of CAIX inhibitory molecules that were synthesized with high selectivity for CAIX [25] and exhibited significant anti-proliferative efficacy *in vitro* in different breast cancer tumor models [26, 27]. Although S4 was ineffective in reducing primary tumor growth *in vivo*, the compound decreased spontaneous lung metastases formation in an orthotopic MDA-MB-231 breast cancer model [25]. This study aims to determine for the first time whether the specific inhibition of CAIX with the sulfamate S4 is able to increase doxorubicin efficacy, both *in vitro* and *in vivo*, and determine if specific CAIX targeting or CAIX knockdown will be able to increase doxorubicin efficacy.

RESULTS

For both MDA-MB-231 and FaDu cell lines an increased CAIX expression upon hypoxic exposure was observed (Figs 1A and 1B). Cell viability of MDA-MB-231 cells decreased with increasing concentrations of doxorubicin under both normoxic and hypoxic conditions but this effect was slightly more pronounced during normoxia: IC₅₀ 0.13 vs. 0.25 μ M, $p=0.0025$ (Fig 1C). While S4 did not increase the sensitivity of MDA-MB-231 cells during normoxia, IC₅₀ reduced to 0.14 μ M during hypoxia ($p=0.0003$), suggesting that S4 mediated CAIX inhibition increases doxorubicin efficacy during hypoxia exposure in MDA-MB-231 cells. In contrast, viability of FaDu cells decreased with increasing doxorubicin concentration independent of oxygen concentrations and this effect could not be enhanced by S4 under hypoxia (Fig 1D).

Figure 1: Effect of S4 on doxorubicin sensitivity in MDA-MB-231 and FaDu cells. CAIX protein expression is higher during hypoxia in MDA-MB-231 and FaDu cells (A). Quantification of three independent biological repeats shows an almost twofold increase in CAIX expression (normalized to actin expression levels) in both cell lines (B). Cell viability assays of MDA-MB-231 (C) and FaDu cells (D) with increasing concentrations of doxorubicin. Cells were exposed to vehicle (black) or S4 (green) during normoxia (N), or to vehicle (red) or S4 (blue) during hypoxia (H). Results of three independent biological repeats are shown (mean $\pm$ SEM).

To test if the efficacy of S4 is dependent on CAIX expression, HT29 cells with a doxycycline-inducible knockdown (KD) of *CA9* were generated. HT29 cells were chosen since these cells are shown to be sensitive to CAIX inhibition [15, 16]. Exposing these HT29 cells to doxycycline (1 μ g/ml) for one week significantly reduced the mRNA levels of CAIX, but not of a different HIF1- α target, i.e. VEGF, thereby indicating the change in CAIX mRNA levels to be independent on changes in HIF1- α (Fig 2A). Similar results were obtained for protein expression of CAIX which was decreased by doxycycline exposure of HT29 cells both in normoxia and hypoxia (Figs 2B and 2C). Without doxycycline CAIX expression of HT29 cells was however similar to the parental HT29 cells (Figs 2B and 2C). These HT29 cells are therefore defined as HT29 – CAIX high, when not exposed to doxycycline, and HT29 – CAIX low, when a doxycycline-induced CAIX knockdown is present.

Figure 2: Effect of S4 on doxorubicin efficacy in HT29 – CAIX high and HT29 – CAIX low cells. Exposing HT29 cells to doxycycline (doxy) reduced CA9 mRNA levels during normoxia (N) and hypoxia (H), whereas VEGF levels were unaffected (**A**). CAIX protein levels minimized in HT29 – CAIX low cells when exposed to doxycycline (**B**). Quantification of three independent biological repeats showed minimal residual CAIX protein expression in HT29 – CAIX low cells as compared to parental cells or HT29 – CAIX high cells without a KD (**C**). Cell viability assays of HT29 – CAIX high (**D**) or HT29 – CAIX low cells (**E**) with increasing concentrations of doxorubicin. Cells were exposed to vehicle (black) or S4 (green) during normoxia (N), or to vehicle (red) or S4 (blue) during hypoxia (H). Results of three independent biological repeats are shown (mean $\pm$ SEM).

Contrary to what was expected, HT29 – CAIX high cells were more sensitive to doxorubicin under hypoxic as compared to normoxic conditions (IC₅₀ 0.10 vs 0.20 μ M, $p=0.0013$) (Fig 2D). Doxorubicin efficacy however did increase when cells were exposed to S4 during normoxic conditions (IC₅₀ 0.20 vs 0.08 μ M, $p<0.0001$, Fig 2D), but decreased slightly during hypoxia (IC₅₀ is 0.10 vs 0.18 μ M, $p=0.0039$, Fig 2D). Sensitivity to doxorubicin under normoxic conditions was increased for HT29 – CAIX low as compared to HT29 – CAIX high cells (IC₅₀ 0.09 vs 0.20 μ M, $p<0.0001$, Fig 2D and 2E), suggestive of a CAIX dependent mechanism of increased doxorubicin efficacy. The decreased CAIX expression in HT29 – CAIX low cells might also explain the lack of effect of S4 on doxorubicin sensitivity in these cells during normoxic conditions (IC₅₀ 0.09 vs 0.10 μ M, $p=0.0972$, Fig 2E), or the similar sensitivity during hypoxia (IC₅₀ 0.09 vs 0.08 μ M, $p=0.6465$, Fig 2E). In contrast however, doxorubicin sensitivity decreased when HT29 – CAIX low cells were exposed to S4 during hypoxic conditions (IC₅₀ 0.08 vs 0.17 μ M, $p=0.0002$, Fig 2E). Higher serum concentrations abrogated the effect of S4 on doxorubi-

cin efficacy (S1 Fig), which may be because of the high binding affinity of S4 to bovine serum albumin (data not shown).

To further study and validate the *in vitro* findings, the combination treatment of S4 with doxorubicin was tested in tumor-bearing mice. No acute toxicity in these animals was observed up to three treatment cycles, as no significant changes in total body weight were observed (S2 Fig). Doxorubicin treatment significantly inhibited MDA-MB-231 tumor growth as compared to vehicle (Fig 3A): time to reach 5 times starting volume (T5xSV) were 14.3 and 8.2 days, respectively ($p < 0.01$, Fig 3B). Contradictory to the *in vitro* data and our hypothesis, S4 treatment abrogated the effect of doxorubicin ($p < 0.01$) in MDA-MB-231. In FaDu xenografts doxorubicin treatment either alone or in combination with S4 was ineffective in reducing tumor growth as compared to vehicle treated mice (T7xSV 10.8, 12.6 and 11.8 days, respectively, Fig 3C and Fig 3D).

Figure 3: Effect of S4, doxorubicin, or the combination of both, on MDA-MB-231 and FaDu tumor xenograft growth. Relative tumor volume (mean $\pm$ SEM) of MDA-MB-231 (A) and FaDu (C) xenografts treated with vehicle (black), S4 (green), vehicle with doxorubicin (red), or S4 with doxorubicin (blue). Linear fits from relative tumor growth were used to estimate the mean time to reach 5 times starting volume (T5xSV) of MDA-MB-231 (B) or 7 times start volume (T7xSV) of FaDu (D) xenografts. ** indicates statistical significance with $p < 0.01$.

To test whether the efficacy of combined treatment of doxorubicin with S4 *in vivo* depends on CAIX expression we employed the HT29 – CAIX high and HT29 – CAIX low tumor models. The HT29 – CAIX low tumor model exhibited a markedly reduced expression of CAIX by doxycycline exposure in the drinking water, although residual CAIX expression could still be observed. In both the HT29 – CAIX high and HT29 – CAIX low tumor models pimonidazole-positive labeling demonstrated presence of hypoxia (S3 Fig). Single S4 or doxorubicin treatment did not inhibit the growth of HT29 – CAIX high (Fig 4A) or HT29 – CAIX low (Fig 4B) tumors. Tumor response to doxorubicin treatment was not improved by knockdown of CAIX as time to reach 2 times start volume (T2XSV) was not statistically different (19.4 vs 25.7 days, $p=0.5144$, Fig 4C and 4D). In addition, combination of S4 with doxorubicin was unable to increase doxorubicin efficacy in HT29 – CAIX high (T2XSV 19.4 vs 22.4, $p=0.5801$, Fig 4C), or in HT29 – CAIX low tumors (T2XSV 25.7 vs 15.8, $p=0.3274$, Fig 4D). Additional treatment cycles were also ineffective in exerting an effect on tumor growth and caused severe toxicity in animals.

Figure 4: Effect of S4, doxorubicin, or the combination of both, on HT29 – CAIX high and HT29 – CAIX low tumor xenograft growth. Relative tumor volume (mean $\pm$ SEM) of HT29 – CAIX high (A) or HT29 – CAIX low xenografts (B) treated with vehicle (black), S4 (green), vehicle with doxorubicin (red), or S4 with doxorubicin (blue). Linear fits from relative tumor growth were used to estimate the mean time to reach 2 times start volume (T2XSV) of HT29 – CAIX high (C) or HT29 – CAIX low (D) xenografts.

DISCUSSION

In the present study we investigated whether doxorubicin treatment efficacy can be enhanced when combined with the CAIX specific sulfamate inhibitor S4 [25-28]. Previously, CAIX inhibition has been shown to potentiate the efficacy of standard treatment modalities, such as radio- and chemotherapy [15, 16, 19]. Furthermore, non-isoform specific CA inhibition with acetazolamide can increase doxorubicin uptake, thereby making it more effective [18]. The ion trapping model explains the higher efficacy of the weak basic anthracycline antitumor antibiotic doxorubicin due to an increased uptake when the balance between intracellular and extracellular pH is shifted towards a reduced extracellular acidification upon hypoxia when treated with selective CAIX inhibitors [21-23]. The results of this study demonstrate that the selective CAIX inhibitor S4 increased doxorubicin efficacy *in vitro* in MDA-MB-231 cells, specifically in hypoxic conditions. These results could however not be found in FaDu, HT29 – CAIX low and HT29 – CAIX high cells; although for the latter an increased efficacy of doxorubicin was observed during normoxia when combined with S4. This increase in doxorubicin efficacy therefore appeared to depend on the levels of CAIX expression in normoxic HT29 cells. Doxorubicin efficacy however was not modulated by S4 under hypoxic conditions, which are essential for full CAIX activation [24, 29]. Nevertheless, this could be due to an incomplete CAIX inhibition that would still reduce the extracellular pH and thereby reduce doxorubicin uptake and efficacy.

To validate the efficacy of S4 treatment *in vivo* experiments were conducted using the same cell lines, since tumor hypoxic and acidic microenvironment might be an important factor influencing the potential of S4 to enhance doxorubicin cytotoxicity. Similar to the previous report [25] S4 monotherapy did not inhibit the growth of any of the tumor models included in this study. These results are in line with histological evaluation of tumor microenvironmental characteristics in SCCNij202 xenografts demonstrating that S4 treatment did not have an effect on proliferation, apoptosis, necrosis, and hypoxia [30]. Taken together the data indicate that S4 lacks anti-cancer efficacy *in vivo* in primary tumors. Previously S4 has been described to reduce migration of MDA-MB-231 tumor cells, and prevent invasion of *ex vivo* breast cancer spheroids [25, 26]. Furthermore, treatment of orthotopic MDA-MB-231 xenografts with S4 reduced the number of spontaneous lung metastases [25]. In addition, it has been shown that S4 can increase ectodomain shedding of CAIX, both *in vitro* and *in vivo*, although the exact relevance and mechanism of this finding requires additional studies [28, 30].

In this study doxorubicin treatment was only effective in reducing tumor growth in the MDA-MB-231 xenografts, whereas no effect was observed in the FaDu, HT29 – CAIX high and HT29 – CAIX low tumor models. This difference between models is unlikely caused by any intrinsic variation in doxorubicin sensitivity, as no differences were observed *in vitro* or in previous reports [31]. The tumor microenvironment might therefore be responsible for this difference in doxorubicin response. Quantification of the

exogenous hypoxia marker pimonidazole in FaDu xenografts revealed the average hypoxic fraction to range between 10 – 20% [32-34]. The hypoxic fraction of the subcutaneous HT29 – CAIX high tumors was also approximately 10% and increased to 20% in the HT29 – CAIX low tumors in previous experiments (data not shown). In contrast however, orthotopic MDA-MB-231 xenografts implanted in the mammary fat pad have a hypoxic fraction of only 5% [35], whereas the hypoxic fraction is estimated to be approximately 7% for subcutaneous tumors (data not shown). Hypoxic areas in tumors are characterized by extracellular acidification, which would reduce doxorubicin uptake and thereby decrease its efficacy. The relatively lower amount of hypoxia in MDA-MB-231 tumors as compared to FaDu and HT29 tumors could therefore explain the reduced efficacy of doxorubicin in the latter tumor model. Furthermore, the difference in doxorubicin efficacy between HT29 and MDA-MB-231 tumors can likewise be explained by a relatively higher activity of CAIX in HT29 cells as compared to the MDA-MB-231 cells [18], leading to a higher degree of extracellular acidification and as a result reduced doxorubicin efficacy. This is further supported by the results obtained in spheroid models showing that MDA-MB-231 cells are more sensitive to doxorubicin as compared to HT29 cells [36].

Treating HT29 tumor-bearing mice with doxorubicin was reported previously to be effective [19]. The lack of effect observed in this study might partly be explained by retention of doxorubicin in the abdominal cavity after intraperitoneal injections [37], as opposed to intravenous injections used previously [19]. This alternative administration route might therefore prevent adequate distribution of doxorubicin to xenograft tumors with poorer vasculature and higher hypoxic fraction, i.e. FaDu and HT29. We were unable to increase the doses of doxorubicin in this study due to severe cardiac toxicity caused by doxorubicin after multiple treatment cycles [38].

In conclusion, treatment with the sulfamate CAIX inhibitor S4 has no monotherapeutic effect and is unable to increase doxorubicin efficacy. In contrast, S4 abrogated the effect of doxorubicin in MDA-MB-231 tumors. The mechanism underlying this reduction in doxorubicin efficacy remains unclear. Nevertheless, these results, in combination with the pharmacokinetic characteristics of S4 after oral and intravenous administration (S4 Fig) suggest sufficient concentrations of S4 might have reached the tumor. Previously a different class of CAIX inhibitor has been shown to increase doxorubicin efficacy and also exert an effect as single agent therapy [19]. These contradictory findings stress the importance of further investigations to better understand the mechanism of action of different classes of CAIX inhibitors before being implemented as anti-cancer agents in combination with standard treatment modalities such as doxorubicin treatment.

METHODS

Cell culture

MDA-MB-231 triple negative breast adenocarcinoma (HTB-26), FaDu pharynx squamous cell carcinoma (HTB-43), and HT29 colorectal adenocarcinoma (HTB-38) tumor cells, all obtained from ATCC, were cultured in Dulbecco's Modified Eagle Medium (DMEM, Lonza) supplemented with 10% Fetal Bovine Serum (FBS, Lonza). Cells were maintained in humidified incubators with 5% carbon dioxide in which they were allowed to attach overnight before the start of experiments.

Transfection

Inducible genetic CA9 knockdown (KD) cells were constructed using the pTRIPZ doxycycline inducible system (Open Biosystems). Specific shRNA targeting CA9, based on Sigma TRCN0000180210, was inserted in the EcoR1 – Xho1 site of the vector. Lentivirus was made in HEK293FT cells (Thermo Fisher Scientific, # R700-07) and used to infect HT29 cells. These HT29 cells were exposed to low concentrations of doxycycline (1 µg/ml, Sigma-Aldrich) one week prior to the start of experiments to induce a sufficient CAIX KD, as CAIX half-life is approximately 38 hours [39]. HT29 cells exposed to doxycycline, i.e. with a CAIX KD, are defined as HT29 – CAIX low cells, whereas HT29 cells not exposed to doxycycline, i.e. with normal CAIX levels, are defined as HT29 – CAIX high cells.

Immunoblotting

Adherent cells were exposed to hypoxic conditions for 24 hours in a hypoxic chamber (MACS VA500 microaerophilic workstation, Don Whitley Scientific, UK). The atmosphere in the chamber consisted of 0.2% O₂, 5% CO₂ and residual N₂. Normoxic dishes were incubated in parallel in ambient air with 5% CO₂. Protein isolates were prepared by scraping cells in RIPA, sonicating the samples, and centrifugation them to remove cellular debris. Protein concentrations were measured using Bradford protein quantification reagent (Bio-rad). Western Blot was performed using primary antibodies against CAIX (M75, kindly provided by Professor Silvia Pastorekova, Institute of Virology, Slovak Academy of Science, Slovak Republic), and beta-actin (MP Biomedicals, #691001) as a reference protein. Primary antibodies were incubated overnight at 4°C, whereas HRP (horseradish peroxidase)-linked secondary antibody (Cell Signalling, #7076) was incubated for 1 hr at room temperature (RT).

qPCR

Cells were exposed to 0.2% O₂ for 24 hours after which RNA was isolated using the NucleoSpin® RNA kit (Macherey Nagel). Thereafter cDNA was synthesized using iScript mix (Bio-Rad) and gene expression of CAIX (F-CATCCTAGCCCTGGTTTTTGG, R-GCTCACACCCCTTTGGTT) and vascular endothelial growth factor (VEGF) (F-GACTCCGGCGGAAGCAT, R- TCCGGGCTCGGTGATTTA) was determined using power SYBR® Green I (Applied Biosystems). Expression of 18S RNA (F- AGTCCCTGCCCTTTGTACACA, R- GATCCGAGGGCCTCACTAAAC) levels was used as a reference.

Cell viability assays

Tumor cells were seeded with 6000, 1000, or 4000 cells per well for MDA-MB-231, FaDu, or HT29, respectively, in flat bottom 96-well plates. The next day cells were exposed to hypoxia (0.2% O₂) and medium was replaced by low serum medium (0.2% FBS) containing vehicle (DMSO, final concentration 0.75%), or the sulfamate CAIX inhibitor S4 (33 µM, kindly provided by Professor Claudiu Supuran, University of Florence, Italy). It has previously been demonstrated that 33 µM of S4 was effective in reducing cell viability [25-27]. Simultaneously, cells were exposed to different concentrations of doxorubicin (ranging from 0.01 to 5 µM, Sigma-Aldrich). After 24 hours medium with compounds was replaced with normal DMEM with 10% FBS. Cells were allowed to grow for an additional 72 hrs under normoxic conditions and cell viability was measured thereafter using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) for FaDu, or Alamar Blue (Life Technology) in MDA-MB-231 and HT29 cells. Half the HT29 were exposed to doxycycline (1 µg/ml) one week prior to the experiment and for the entire duration of the experiment to test both HT29 – CAIX high and HT29 – CAIX low cells. Treatment response was quantified in terms of IC₅₀, i.e. the concentration of a drug that gives half-maximal response

In vivo experiments

All animal experiments were ethically approved by the ethical committee on animal experimentation of the university of Manchester (PPL 70/7760) and the university of Maastricht (DEC 2008-025) and performed in accordance to local legislation and guidelines. Eight weeks old NMRI nu/nu mice (Charles River) were subcutaneously injected with MDA-MB-231, FaDu, or HT29 tumor cells resuspended in Matrigel (Corning). Half of the mice implanted with the HT29 cells were provided with water containing doxycycline (2 g/l) and sucrose (5%) ad libitum during the entire experiment to obtain HT29 – CAIX high and HT29 – CAIX low tumor xenografts. For each treatment group 6 – 8 animals were used. Tumors were measured three times a week in three orthogonal dimensions to calculate tumor volume based on the formula of an ellipsoid. Treatment started

when tumors reached a volume of 100 - 150 mm³. A treatment cycle consisted of a 'five days on, two days off' schedule with either vehicle (4% DMSO in saline) or S4 (10 mg/kg ip for MDA-MB-231 and FaDu, and 25 mg/kg ip for HT29). Doxorubicin or vehicle control (PBS) was administered ip once a week (5 mg/kg on day 4 of treatment cycle). Total body weight was measured repeatedly for the duration of the study to monitor possible treatment-induced toxicity. In the experiment with the HT29 – CAIX high and HT29 – CAIX low models a parallel group of mice was sacrificed directly after the first treatment cycle, i.e. after the last S4 injection, to investigate levels of CAIX expression in the tumors. Treatment response was quantified as time to reach 2, 5 or 7 times start volume (T2xSV, T5xSV, and T7xSV, respectively) depending on the growth of the tumor model.

Immunohistochemical staining

Frozen 7 µm tumor sections were fixed in acetone (4°C, 10 min), air-dried and rehydrated in PBS. The sections were incubated with a mixture of polyclonal rabbit anti-CAIX (Novus Biologicals NB100-417) and FITC-conjugated IgG1 mouse monoclonal anti-pimonidazole (clone 4.3.11.3, Hypoxyprobe™-1 Plus Kit) antibodies overnight at 4°C, followed by incubation with secondary goat anti-rabbit AlexaFluor 594 (Invitrogen) antibody for 1 hr at RT. Images were acquired as described previously in detail [40].

Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 5.03). Curve fits of cell viability assays were compared with the extra sum-of-squares F test. Average tumor growth curves were fitted with linear regression. Differences in means between groups were compared with unpaired t-tests. $P < 0.05$ indicates statistical significant difference.

SUPPORTING INFORMATION

Supplementary Figure 1: Cell viability assay of the HT29 – CAIX high and HT29 – CAIX low cells with 10% FBS. Cell viability assays of HT29 – CAIX high (A) or HT29 – CAIX low cells (B) with increasing concentrations of doxorubicin. Cells were exposed to vehicle (black) or S4 (green) during normoxia (N), or to vehicle (red) or S4 (blue) during hypoxia (H). Results of three independent biological repeats are shown (mean ± SEM).

Supplementary Figure 2: Relative total body weight of mice with different tumor xenografts. Mice were implanted with MDA-MB-231 (A), FaDu (B), or HT29 – CAIX high (C) or HT29 – CAIX low (D) xenografts. Relative total body weight (mean $\pm$ SEM) of mice treated with vehicle (black), S4 (green), vehicle with doxorubicin (red), or S4 with doxorubicin (blue) showed no signs of acute toxicity in any of the treatment groups for any of the included tumor models.

Supplementary Figure 3: Validation CAIX expression in HT29 – CAIX high and HT29 – CAIX low xenografts. CAIX expression (red) and pimonidazole-labelled hypoxia (green) in the HT29 – CAIX high tumors, and the HT29 – CAIX low xenografts by addition of doxycycline in the drinking water of the mice.

Supplementary Figure 4: Pharmacokinetic studies with S4. A single dose of S4 was administered either intravenously (5 mg/kg) (A) or orally (50 mg/kg) (B) in male CD1 mice. S4 was dissolved in 12.5% ethanol, 37.5% triethylene glycol, and 50% saline. Blood samples were taken at 7 different time points (n=3 mice per time point) after injection and plasma was isolated. Plasma was mixed with methanol, centrifuged and the supernatant was transferred to mass spectrometry plate for LC-MS/MS analysis. From the single intravenous administration several parameters could be estimated (C). From the oral administration these parameters could not be estimated because the concentration curve over time was too inaccurate. The curve after oral injection however does suggest S4 to be slowly resorbed out of the intestine. Intraperitoneal injection of S4 might therefore form a depot at the injection site, thereby only releasing limited concentrations in the blood and eventually reaching the tumor. S4 might thereby only exert an effect in certain sensitive tumor models. These studies were performed by Cyprotex Ltd. (Macclesfield, UK) as a part of the EU 7th framework program METOXIA (ref. 2008-222741) funded initiative.

ACKNOWLEDGEMENTS

Authors would like to thank Professor Silvia Pastorekova (Institute of Virology, Slovak Academy of Science, Slovak Republic) for providing us with the M75 antibody targeting CAIX. Authors would also like to thank Professor Claudiu Supuran (University of Florence, Italy) for providing us with S4. Pharmacokinetic studies were performed by Cyprotex Ltd. (Macclesfield, UK) as a part of the EU 7th framework program METOXIA (ref. 2008-222741) funded initiative.

REFERENCES

1. Hockel, M. and P. Vaupel, *Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects*. Journal of the National Cancer Institute, 2001. **93**(4): p. 266-76.
2. Pettersen, E.O., et al., *Targeting tumour hypoxia to prevent cancer metastasis. From biology, biosensing and technology to drug development: the METOXIA consortium*. Journal of enzyme inhibition and medicinal chemistry, 2015. **30**(5): p. 689-721.
3. Dubois, L.J., et al., *New ways to image and target tumour hypoxia and its molecular responses*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2015. **116**(3): p. 352-7.
4. Ebbesen, P., et al., *Taking advantage of tumor cell adaptations to hypoxia for developing new tumor markers and treatment strategies*. Journal of enzyme inhibition and medicinal chemistry, 2009. **24 Suppl 1**: p. 1-39.
5. Walsh, J.C., et al., *The clinical importance of assessing tumor hypoxia: relationship of tumor hypoxia to prognosis and therapeutic opportunities*. Antioxidants & redox signaling, 2014. **21**(10): p. 1516-54.
6. Gatenby, R.A. and R.J. Gillies, *Why do cancers have high aerobic glycolysis?* Nature reviews. Cancer, 2004. **4**(11): p. 891-9.
7. van den Beucken, T., et al., *Hypoxia-induced expression of carbonic anhydrase 9 is dependent on the unfolded protein response*. The Journal of biological chemistry, 2009. **284**(36): p. 24204-12.
8. Svastova, E., et al., *Hypoxia activates the capacity of tumor-associated carbonic anhydrase IX to acidify extracellular pH*. FEBS letters, 2004. **577**(3): p. 439-45.
9. Swietach, P., R.D. Vaughan-Jones, and A.L. Harris, *Regulation of tumor pH and the role of carbonic anhydrase 9*. Cancer metastasis reviews, 2007. **26**(2): p. 299-310.
10. Neri, D. and C.T. Supuran, *Interfering with pH regulation in tumours as a therapeutic strategy*. Nature reviews. Drug discovery, 2011. **10**(10): p. 767-77.
11. van Kuijk, S.J., et al., *Prognostic Significance of Carbonic Anhydrase IX Expression in Cancer Patients: A Meta-Analysis*. Frontiers in oncology, 2016. **6**: p. 69.
12. Akurathi, V., et al., *Development and biological evaluation of (9)(9)mTc-sulfonamide derivatives for in vivo visualization of CA IX as surrogate tumor hypoxia markers*. European journal of medicinal chemistry, 2014. **71**: p. 374-84.
13. Akurathi, V., et al., *Synthesis and biological evaluation of a 99mTc-labelled sulfonamide conjugate for in vivo visualization of carbonic anhydrase IX expression in tumor hypoxia*. Nuclear medicine and biology, 2010. **37**(5): p. 557-64.
14. Peeters, S.G., et al., *[(18)F]VM4-037 MicroPET Imaging and Biodistribution of Two In Vivo CAIX-Expressing Tumor Models*. Molecular imaging and biology : MIB : the official publication of the Academy of Molecular Imaging, 2015. **17**(5): p. 615-9.
15. Dubois, L., et al., *Specific inhibition of carbonic anhydrase IX activity enhances the in vivo therapeutic effect of tumor irradiation*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2011. **99**(3): p. 424-31.
16. Dubois, L., et al., *Targeting carbonic anhydrase IX by nitroimidazole based sulfamides enhances the therapeutic effect of tumor irradiation: a new concept of dual targeting drugs*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2013. **108**(3): p. 523-8.
17. Monti, S.M., C.T. Supuran, and G. De Simone, *Anticancer carbonic anhydrase inhibitors: a patent review (2008 - 2013)*. Expert opinion on therapeutic patents, 2013. **23**(6): p. 737-49.
18. Gieling, R.G., et al., *Inhibition of carbonic anhydrase activity modifies the toxicity of doxorubicin and melphalan in tumour cells in vitro*. Journal of enzyme inhibition and medicinal chemistry, 2013. **28**(2): p. 360-9.
19. Rami, M., et al., *Hypoxia-targeting carbonic anhydrase IX inhibitors by a new series of nitroimidazole-sulfonamides/sulfamides/sulfamates*. Journal of medicinal chemistry, 2013. **56**(21): p. 8512-20.

20. Crown, J., et al., *Chemotherapy for metastatic breast cancer-report of a European expert panel*. The Lancet. Oncology, 2002. **3**(12): p. 719-27.
21. Mahoney, B.P., et al., *Tumor acidity, ion trapping and chemotherapeutics. I. Acid pH affects the distribution of chemotherapeutic agents in vitro*. Biochemical pharmacology, 2003. **66**(7): p. 1207-18.
22. Raghunand, N., B.P. Mahoney, and R.J. Gillies, *Tumor acidity, ion trapping and chemotherapeutics. II. pH-dependent partition coefficients predict importance of ion trapping on pharmacokinetics of weakly basic chemotherapeutic agents*. Biochemical pharmacology, 2003. **66**(7): p. 1219-29.
23. Wojtkowiak, J.W., et al., *Drug resistance and cellular adaptation to tumor acidic pH microenvironment*. Molecular pharmaceutics, 2011. **8**(6): p. 2032-8.
24. Dubois, L., et al., *Imaging the hypoxia surrogate marker CA IX requires expression and catalytic activity for binding fluorescent sulfonamide inhibitors*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2007. **83**(3): p. 367-73.
25. Gieling, R.G., et al., *Antimetastatic effect of sulfamate carbonic anhydrase IX inhibitors in breast carcinoma xenografts*. Journal of medicinal chemistry, 2012. **55**(11): p. 5591-600.
26. Ward, C., et al., *Evaluation of carbonic anhydrase IX as a therapeutic target for inhibition of breast cancer invasion and metastasis using a series of in vitro breast cancer models*. Oncotarget, 2015.
27. Winum, J.Y., et al., *Ureido-substituted sulfamates show potent carbonic anhydrase IX inhibitory and antiproliferative activities against breast cancer cell lines*. Bioorganic & medicinal chemistry letters, 2012. **22**(14): p. 4681-5.
28. Hektoen, H.H., et al., *Sulfamate inhibitor S4 influences carbonic anhydrase IX ectodomain shedding in colorectal carcinoma cells*. Journal of enzyme inhibition and medicinal chemistry, 2015: p. 1-8.
29. Dubois, L., et al., *Imaging of CA IX with fluorescent labelled sulfonamides distinguishes hypoxic and (re)-oxygenated cells in a xenograft tumour model*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2009. **92**(3): p. 423-8.
30. Meijer, T.W., et al., *Tumor microenvironmental changes induced by the sulfamate carbonic anhydrase IX inhibitor S4 in a laryngeal tumor model*. PloS one, 2014. **9**(9): p. e108068.
31. Hartlieb, K.J., et al., *Anticancer activity expressed by a library of 2,9-diazaperopyrenium dications*. ACS nano, 2015. **9**(2): p. 1461-70.
32. Santiago, A., et al., *Effect of cetuximab and fractionated irradiation on tumour micro-environment*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2010. **97**(2): p. 322-9.
33. Yaromina, A., et al., *Co-localisation of hypoxia and perfusion markers with parameters of glucose metabolism in human squamous cell carcinoma (hSCC) xenografts*. International journal of radiation biology, 2009. **85**(11): p. 972-80.
34. Yaromina, A., et al., *Pimonidazole labelling and response to fractionated irradiation of five human squamous cell carcinoma (hSCC) lines in nude mice: the need for a multivariate approach in biomarker studies*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2006. **81**(2): p. 122-9.
35. Franco, M., et al., *Targeted anti-vascular endothelial growth factor receptor-2 therapy leads to short-term and long-term impairment of vascular function and increase in tumor hypoxia*. Cancer research, 2006. **66**(7): p. 3639-48.
36. Doillon, C.J., et al., *Three-dimensional culture system as a model for studying cancer cell invasion capacity and anticancer drug sensitivity*. Anticancer research, 2004. **24**(4): p. 2169-77.
37. Nagai, K., et al., *Pharmacokinetic evaluation of intraperitoneal doxorubicin in rats*. Die Pharmazie, 2014. **69**(2): p. 125-7.
38. Eckman, D.M., et al., *Weekly doxorubicin increases coronary arteriolar wall and adventitial thickness*. PloS one, 2013. **8**(2): p. e57554.
39. Rafajova, M., et al., *Induction by hypoxia combined with low glucose or low bicarbonate and high posttranslational stability upon reoxygenation contribute to carbonic anhydrase IX expression in cancer cells*. International journal of oncology, 2004. **24**(4): p. 995-1004.

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40. Peeters, S.G., et al., *TH-302 in Combination with Radiotherapy Enhances the Therapeutic Outcome and Is Associated with Pretreatment [18F]HX4 Hypoxia PET Imaging*. Clinical cancer research : an official journal of the American Association for Cancer Research, 2015. **21**(13): p. 2984-92.

Chapter 4

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

Published in: Radiother Oncol. 2013 Sep;108(3):523-8.

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[#]*Validation experiments are described in Chapter 5*

Corrigendum attached:

STR analyses revealed transfected HT29 cells were HCT116 cells

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.

INTRODUCTION

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 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.

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× 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 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 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.

Figure 1: (A) Chemical structure and affinity (K_i) 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 $\pm$ 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 4x the volume at irradiation time (T_{4xSV}). Data represent the mean $\pm$ SD of six to nine independent animals. Asterisks indicate statistical significance (* $P < 0.05$; ** $P < 0.01$).

Figure 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).

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).

Figure 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 4x the volume at irradiation time (T4xSV). Data represent the mean $\pm$ 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 ($T4 \times SV$) 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.

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 K_i 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. 1.5×10^6 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× the volume at irradiation time (T4×SV) and treatment toxicity was scored by body weight measurements. Radiation sensitization enhancement ratios (SER) are calculated as the ratios of the mean T4×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 4X) 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.

SUPPORTING INFORMATION

Supplementary Figure 1: DH348 (10 mg/kg, 5 days) was administered to 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. Treatment toxicity was evaluated by monitoring body weight 3x/week. Data represent the mean $\pm$ SD of all animals within a treatment group (independent of the type of tumor).

ACKNOWLEDGEMENTS

This work has been funded with the support of the Mastro Cancer Foundation, KWF UM2012-5394 and the EU 7th framework (Metoxia project ref. 2008-222741).

REFERENCES

- Potter, C.P. and A.L. Harris, *Diagnostic, prognostic and therapeutic implications of carbonic anhydrases in cancer*. British journal of cancer, 2003. **89**(1): p. 2-7.
- McDonald, P.C., et al., *Recent developments in targeting carbonic anhydrase IX for cancer therapeutics*. Oncotarget, 2012. **3**(1): p. 84-97.
- Svastova, E., et al., *Hypoxia activates the capacity of tumor-associated carbonic anhydrase IX to acidify extracellular pH*. FEBS letters, 2004. **577**(3): p. 439-45.
- Swietach, P., et al., *The role of carbonic anhydrase 9 in regulating extracellular and intracellular pH in three-dimensional tumor cell growths*. The Journal of biological chemistry, 2009. **284**(30): p. 20299-310.
- Neri, D. and C.T. Supuran, *Interfering with pH regulation in tumours as a therapeutic strategy*. Nature reviews. Drug discovery, 2011. **10**(10): p. 767-77.
- Dubois, L., et al., *Imaging the hypoxia surrogate marker CA IX requires expression and catalytic activity for binding fluorescent sulfonamide inhibitors*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2007. **83**(3): p. 367-73.
- Dubois, L., et al., *Imaging of CA IX with fluorescent labelled sulfonamides distinguishes hypoxic and (re)-oxygenated cells in a xenograft tumour model*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2009. **92**(3): p. 423-8.
- Dubois, L., et al., *Specific inhibition of carbonic anhydrase IX activity enhances the in vivo therapeutic effect of tumor irradiation*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2011. **99**(3): p. 424-31.
- Ahlskog, J.K., et al., *In vivo targeting of tumor-associated carbonic anhydrases using acetazolamide derivatives*. Bioorganic & medicinal chemistry letters, 2009. **19**(16): p. 4851-6.
- Buller, F., et al., *Selection of Carbonic Anhydrase IX Inhibitors from One Million DNA-Encoded Compounds*. ACS chemical biology, 2011. **6**(4): p. 336-44.
- Lou, Y., et al., *Targeting tumor hypoxia: suppression of breast tumor growth and metastasis by novel carbonic anhydrase IX inhibitors*. Cancer research, 2011. **71**(9): p. 3364-76.
- Gieling, R.G., et al., *Antimetastatic effect of sulfamate carbonic anhydrase IX inhibitors in breast carcinoma xenografts*. Journal of medicinal chemistry, 2012. **55**(11): p. 5591-600.
- Gieling, R.G., et al., *Inhibition of carbonic anhydrase activity modifies the toxicity of doxorubicin and melphalan in tumour cells in vitro*. Journal of enzyme inhibition and medicinal chemistry, 2013. **28**(2): p. 360-9.
- Overgaard, J., et al., *A comparative investigation of nimorazole and misonidazole as hypoxic radiosensitizers in a C3H mammary carcinoma in vivo*. British journal of cancer, 1982. **46**(6): p. 904-11.
- Overgaard, J., et al., *A randomized double-blind phase III study of nimorazole as a hypoxic radiosensitizer of primary radiotherapy in supraglottic larynx and pharynx carcinoma. Results of the Danish Head and Neck Cancer Study (DAHANCA) Protocol 5-85*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 1998. **46**(2): p. 135-46.
- van den Beucken, T., et al., *Deficient carbonic anhydrase 9 expression in UPR-impaired cells is associated with reduced survival in an acidic microenvironment*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2009. **92**(3): p. 437-42.
- Nordsmark, M., et al., *Prognostic value of tumor oxygenation in 397 head and neck tumors after primary radiation therapy. An international multi-center study*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2005. **77**(1): p. 18-24.
- Yaromina, A., et al., *Radiobiological hypoxia, histological parameters of tumour microenvironment and local tumour control after fractionated irradiation*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2010. **96**(1): p. 116-22.
- Sattler, U.G., et al., *Glycolytic metabolism and tumour response to fractionated irradiation*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2010. **94**(1): p. 102-9.

Chapter 4

20. McIntyre, A., et al., *Carbonic anhydrase IX promotes tumor growth and necrosis in vivo and inhibition enhances anti-VEGF therapy*. Clinical cancer research : an official journal of the American Association for Cancer Research, 2012. **18**(11): p. 3100-11.
21. Sugie, C., et al., *Reevaluation of the radiosensitizing effects of sanazole and nimorazole in vitro and in vivo*. Journal of radiation research, 2005. **46**(4): p. 453-9.

Corrigendum attached:

STR analyses revealed transfected HT29 cells were HCT116 cells

Dear Prof. Dr. J. Overgaard, Prof. Dr. AJ van der Kogel,

Recently we have discovered a discrepancy in the origin of cell lines used in two publications in Radiotherapy and Oncology (PMID 21676479 and PMID 23849171) (Dubois et al 2011 and Dubois et al 2013). The two cell lines in question were referred to as HT-29-EV and shCA9 KD. These lines were constructed by Dr. Adrian Harris at Oxford University and provided to us for these studies, and have been also used by in 2 additional publications (McIntyre et al 2012, Gondi et al 2013). We have determined that these two cell lines, containing an empty vector control plasmid, and a plasmid expressing a shRNA against CA9, are both derived from the colorectal HCT116 cell line *and not* the colorectal HT-29 cell line as previously indicated in these papers.

Using short tandem repeat (STR) analysis with the Powerplex 18 primer kit (Promega) we found that the STR profile of the two cell lines in question were highly similar to HCT116 and not to HT-29, 89% compared to 39% respectively. STR analysis was performed by Wolfgang Eicheler (OncoRay, Dresden) for 14 STR loci, including the 9 ATCC described STR loci. Based on these results, we conclude it is therefore highly unlikely that these cells are of HT29 origin.

The purpose of using these cells was to investigate if a causal relationship existed between CAIX expression and the therapeutic efficacy of inhibitors of CAIX activity. Although the used cells were assigned HT-29 instead of HCT116, the proper empty vector controls were used in the paired cell lines for both publications. Therefore, the conclusion that the therapeutic efficacy of blocking CAIX activity is causally dependent on CAIX expression is still valid. We are disappointed that this error in assigning cells happened and propose the corrigendum below. This corrigendum has also been emailed through the Radiotherapy Oncology submission website.

Yours sincerely,

Ludwig Dubois and Philippe Lambin

Sarah Peeters, Natasja Lieuwes, Nele Geusens, Anne Thiry, Simon Wigfield, Fabrizio Carta, Alan McIntyre, Andrea Scozzafava, Jean-Michel Dogné, Claudiu Supuran, Adrian Harris, Bernard Masereel, Simon van Kuijk, Ala Yaromina, Ruchi Saraya, Rianne Biemans, Marouan Rami, Nanda Kumar Parvathaneni, Daniela Vullo, Marc Vooijs, Jean-Yves Winum.

Corrigendum for:

Dubois L, Peeters S, Lieuwes NG, Geusens N, Thiry A, Wigfield S, Carta F, McIntyre A, Scozzafava A, Dogné JM, Supuran CT, Harris AL, Masereel B, Lambin P. **Specific inhibition of carbonic anhydrase IX activity enhances the *in vivo* therapeutic effect of tumour irradiation.** Radiother Oncol. 2011 Jun;99(3):424-31. doi: 10.1016/j.radonc.2011.05.045. Epub 2011 Jun 14. PMID 21676479

Dubois L, Peeters SG, van Kuijk SJ, Yaromina A, Lieuwes NG, Saraya R, Biemans R, Rami M, Parvathaneni NK, Vullo D, Vooijs M, Supuran CT, Winum JY, Lambin P. **Targeting carbonic anhydrase IX by nitro-imidazole based sulfamides enhances the therapeutic effect of tumour irradiation: A new concept of dual targeting drugs.** Radiother Oncol. 2013 Sep;108(3):523-8. doi: 10.1016/j.radonc.2013.06.018. Epub 2013 Jul 9. PMID 23849171

In both articles (Volume 99, Issue 3, Pages 424–431 and Volume 108, Issue 3, Pages 523–528) published in the June 2011 and September 2013 issues of Radiotherapy and Oncology, we reported results using cell lines designated as HT-29 EV (containing an empty vector as a control) and shCA9 (expressing a shRNA against Carbonic anhydrase IX/CA9). Using Short tandem repeat (STR) analysis we recently discovered that these cell lines are both derived from the colorectal HCT116 cell line rather than the HT-29 colorectal cell line. Since the proper empty vector control CAIX expressing cell line was included in the paired cell lines for both publications, our conclusion stating that the therapeutic efficacy of blocking CAIX activity is causally dependent on CAIX expression is still valid. The authors regret this error.

Chapter 5

Oral Applicability of the Dual-Target Nimorazole-Based Carbonic Anhydrase Inhibitor DH348 and Validation of Its Efficacy

*Simon J.A. van Kuijk, Ala Yaromina, Raymon Niemans, Nanda Kumar Parvathaneni,
Ludwig J. Dubois, Jean-Yves Winum, Philippe Lambin*

ABSTRACT

Carbonic anhydrase IX may be a promising therapeutic target to increase the efficacy of standard treatment modalities in the resistant hypoxic tumor microenvironment. Previously, the dual-target CAIXi DH348, a radiosensitizing nimorazole conjugated to a CAIXi moiety, increased the efficacy of both radio- and chemotherapy in xenograft tumors. Here we aim to investigate the feasibility of oral administration of DH348 and validate its efficacy in multiple cell lines.

Pharmacokinetic characteristics of DH348 were assessed with Caco-2 absorption assays *in vitro* and by quantifying plasma concentrations after intravenous and oral administration *in vivo*. The efficacy of DH348 after oral administration with or without radiotherapy was tested in HT29 and H460 tumor-bearing mice. Validation of DH348 efficacy was performed with intravenous administration in HT29, H460 and Fadu tumors with the exact same experimental conditions as previously described.

Pharmacokinetic characterization of DH348 supported its oral applicability and the human equivalent dose of DH348 caused no signs of acute toxicity in tumor-bearing mice. Oral administration of DH348 however was ineffective as monotherapy and appeared to abrogate the effect of radiotherapy in both HT29 and H460 tumors. In addition, validation experiments using the previously described experimental setup also showed no increased radiosensitivity of tumors after intravenous DH348 treatment.

In conclusion, here DH348 does not sensitize human tumor xenografts to radiation, both after oral and intravenous administration, which contradicts our previously published findings. The most likely explanation is a difference in batch of DH348 compound that was used, which unfortunately cannot be investigated further.

INTRODUCTION

Elevated carbonic anhydrase (CA) IX expression has been associated with poor prognosis [1] and is closely associated with hypoxic areas in the tumor that are known to be more resistant to treatment [2-5]. CAIX visualization using diagnostic imaging techniques (e.g. PET) is therefore a promising approach to identify therapy resistant tumors and is currently under investigation with the testing of several CAIX-specific probes [6-11]. The main function of CAIX is to catalyze the conversion of carbon dioxide to a free proton and bicarbonate, where the bicarbonate preserves a neutral intracellular pH after anion exchanger transport, which favors tumor growth and survival. The produced protons contribute to an acidic extracellular tumor microenvironment favorable for invasiveness and metastases formation [4, 5, 12]. Since CAIX is implicated in the regulation of extra- and intracellular pH and its expression is highly tumor specific, it has been proposed as a potential therapeutic target.

A possible approach to target CAIX is via inhibiting its enzymatic activity with specific pharmacological inhibitors, of which many different classes are being developed and tested [4, 13, 14]. These CAIX inhibitors are hypothesized to cause cell death via intracellular acidification [15-17], but also reduce metastases formation [18-21] and increase radio- and chemotherapy efficacy [22-25]. Previously we have reported a novel class of dual targeting 5-nitroimidazole based sulfamide drugs with high binding affinity for the transmembrane CA isoforms CAIX and CAXII [23, 24]. The goal of these compounds is to increase the radiosensitivity of tumors via the nitroimidazole moiety, i.e. a fixator of radiation induced DNA damage in the absence of oxygen [26]. Conjugating these nitroimidazoles with sulfamides is hypothesized to specifically target the CAIX expressing cells of a tumor, thereby reducing normal tissue toxicity, but also inhibit CAIX activity, resulting in a reduced tumor growth and an increase in the tumors radiosensitivity [22]. These compounds are based on the less toxic radiosensitizing 5-nitroimidazole derivative, nimorazole, which showed similar enhancement ratios as misonidazole in fractionated radiotherapy schedules with a highly significant benefit in terms of locoregional tumor control and disease-free survival [26]. As expected, the hypoxic extracellular acidification of tumor cells was decreased when exposed to these compounds, which was dependent on the cells CAIX expression levels [23, 24]. In addition, treatment of tumor-bearing mice with one of these dual-target compounds (i.e. DH348) was found to delay tumor growth and to increase the efficacy of radiotherapy, i.e. in a CAIX dependent manner, and chemotherapy [23, 24].

Previously, DH348 was administered intravenously, but to increase patient compliance, the aim of this study was to determine the oral applicability of DH348 treatment and evaluate its efficacy in multiple tumor models with varying CAIX expression. However, the lack of DH348 efficacy in the present study was in contrast to the previously published data [23, 24] and prompted us to perform a series of validation experiments using an identical experimental setup and conditions as before [23]. Here we summa-

size all experiments conducted in order to elucidate potential factors contributing to this discrepancy.

RESULTS

DH348 shows a good oral pharmacokinetic profile

Based on the previous encouraging *in vivo* radio- and chemo-sensitization properties of DH348, screening of oral liquid formulations was performed. Using the organic solvent PEG400, a solubility level of 64.6 mg/ml was reached (Table 1), which was the highest observed amongst all tested solvents. Addition of acid could not further improve the solubility. The highest soluble concentration of DH348 is higher than the calculated human equivalent dose (HED) of nimorazole (400 mg/kg, based on a 1.2 g/m² daily oral delivery of nimorazole) [27]. Since PEG400 is a commonly used solvent for oral drug delivery these data support the use of oral administration of DH348 at doses comparable with the HED of nimorazole.

Table 1: Solubility of DH348 in organic solvents and organic solvent mixtures

Medium	Solubility (mg/ml)
PEG400	64.6
PEG400 + 0.1 N HCl	66.2
PEG400 + 0.1 N citric acid	66.9
PEG400 / 50 mM citrate buffer (pH 2, 50/50 v/v)	15.1
Propylene glycol	13.3
Ethanol	2.7

In addition, *in vitro* and mouse pharmacokinetic studies were initiated. Based on the absorption assay using Caco-2 cells (Table 2), DH348 mean permeability coefficient (P_{app}) is $1.81 \cdot 10^{-6}$ cm/s, indicative for human intestinal absorption between 50 and 60%. For comparison, a $P_{app} > 3.0 \cdot 10^{-6}$ cm/s corresponds to an uptake of >80% in humans [28, 29]. The coefficient indicating high absorption (P_{active}) value of $-0.325 \cdot 10^{-6}$ cm/s furthermore shows a lack of significant blood brain barrier (BBB) permeability, since a value ranging from -2.5 to $-1.5 \cdot 10^{-5}$ cm/s corresponds with significant BBB permeability [30–32]. DH348 showed an efflux ratio of 0.638 indicative of low active transport out of the cell by P-glycoproteins, which is a measurement of multidrug resistance. An efflux value >2 corresponds with active transport out of the cell [33]. A high P_{app} combined with a low efflux ratio suggests reasonable bioavailability following oral administration. Furthermore, in male CD1 mice the half-life after intravenous administration of DH348

was calculated to be 1.9 hours and the oral bioavailability was approximately 100%. Together these results strengthen the oral applicability of higher doses of DH348.

Table 2: Results of Caco-2 absorption assay

Compound	Mean P_{app}^a (10^{-6} cm/s)	Mean % Recovery ^b	Efflux Ratio ^c	P_{active}^d (10^{-6} cm/s)
DH348	1.81	103	0.638	-0.325
Atenolol ^e	0.615	99.9	1.13	0.04
Propranolol ^f	43.2	85.3	0.544	-9.85
Talinolol ^g	0.675	88.7	15.9	5.01

^a P_{app} is the permeability coefficient = $(dQ/dt)/(C_0 \cdot A)$, where dQ/dt is the rate of permeation of the drug across the cells, C_0 is the concentration in the donor compartment at time zero, and A is the area of the cell monolayer.

^bMean % Recovery = total compound in donor and receiver at end of experiment (nmol) / initial compound present (nmol) * 100. If the mean recovery is low problems may have occurred including solubility, binding of compound to the plate, metabolism by the cells, or accumulation of the compound in the monolayer.

^cEfflux ratio = Mean P_{app} B2A / Mean P_{app} A2B, with P_{app} B2A is permeability coefficient from basolateral to apical membrane, and P_{app} A2B is the permeability coefficient in the apical to basolateral direction.

^d P_{active} is a coefficient for high absorption = $(P_{app} B2A - P_{app} A2B) / 2$, where P_{app} B2A is permeability coefficient from basolateral to apical membrane, and P_{app} A2B is the permeability coefficient in the apical to basolateral direction.

^eAtenolol is a control with human absorption of 50%

^fPropranolol is a control with human absorption of 90%

^gTalinolol is a control for a P-glycoprotein substrate

High oral DH348 dose is tolerable

Next the acute toxicity of DH348 with the HED (400 mg/kg), i.e. based on its nimorazole moiety [27], was assessed in HT29 tumor-bearing mice, which previously was shown to be a DH348 sensitive model [23, 24]. Throughout the entire duration of the experiment, i.e. until the tumors reached 1000 mm³, none of the mice showed any signs of toxicity caused by DH348 treatment with the HED, or with lower dosing levels (i.e. 100, 200 or 300 mg/kg) or vehicle (PEG). During the treatment period (5 consecutive days), and up to two weeks after start of treatment, total body weight was measured daily and did not decrease more than 10% from the start of treatment (Figure 1).

Figure 1: Relative total body weight of HT29 tumor-bearing mice treated with varying doses of oral injections of DH348 for five consecutive days. Average relative body weights of three mice treated with vehicle, and five mice treated with different DH348 concentrations are shown $\pm$ SEM.

As a secondary endpoint the average time to reach 4 times the tumor volume at start of treatment (T4xSV) was determined for each treatment group (Figure 2). These results suggest that oral treatment with DH348 for five consecutive days did not have an effect on tumor growth with lower dosing levels, since even the effect on T4xSV of 100 mg/kg was not statistically different as compared to vehicle: 28.1 days vs 18.4 days, $p=0.4147$. In contrast, the highest concentration of DH348 tended to reduce T4xSV as compared with vehicle: 14.8 days vs 18.4 days, $p=0.0516$).

Figure 2: Relative tumor volume (mean $\pm$ SEM), normalized to start of treatment, of HT29 tumor-bearing mice after oral administration of varying doses of DH348 for five consecutive days (A). From the exponential fits the times to reach 4x start volume (T4xSV) were calculated and summarized (B). Symbols represent T4xSV for each animal and a bar represents mean value per group.

Oral administration of DH348 has no radiosensitizing effect

To elucidate the efficacy of orally administered DH348, the HED (i.e. 400 mg/kg) was tested in combination with irradiation in two tumor models, i.e. the colorectal HT29 and

non-small cell lung cancer H460. In both tumor models a single dose of ionizing radiation (i.e. 10 Gy) caused a significant growth delay (HT29: T4xSV 18.6 vs 36.0 days, $p=0.030$; and H460: 8.4 days vs 13.3 days, $p=0.013$,) as compared to sham treatment. Monotherapy with DH348 alone however had no effect ($p=0.191$, and $p=0.189$ for HT29 and H460 respectively) on tumor growth in both models as compared to vehicle control. In addition, the results obtained in both tumor models suggest that the combination of DH348 with radiotherapy counteracted the effect of radiotherapy, i.e. tumor growth delay induced by RT was abrogated although this trend was not statistically significant ($p=0.072$, and $p=0.131$ for HT29 and H460 respectively).

Figure 3: Relative tumor volume (mean $\pm$ SEM) of HT29 (A) and H460 (C) tumor-bearing mice orally treated with vehicle (black), DH348 (blue), 10 Gy radiation (green), or the combination of DH348 with 10 Gy radiation (red). From the exponential fits the times to reach 4x start volume (T4xSV) were calculated and summarized for HT29 (B) and H460 (D) tumors. Symbols represent T4xSV for each animal and a bar represents mean value per group. * indicates statistical significance with $p < 0.05$.

Previously, high dose of nitroimidazoles have been reported to cause a vascular collapse, thereby increasing the hypoxic fraction resulting in a more radioresistant tumor [34]. Therefore, we evaluated several histological parameters, i.e. hypoxic fraction, relative vascular area, fraction of perfused vessels, and necrotic fraction, to test the impact of DH348 on the tumor microenvironment (Figure 4). In contrast to the reported data, the DH348 nitroimidazole derivative did not enhance tumor hypoxia in H460 tumors, since hypoxic fraction was 14.4% vs 13.2% for vehicle and DH348 treated mice,

respectively ($p=0.734$). All other investigated histological parameters also remained unchanged (Figure 4). These results thus do not support a vascular collapse induced by high dose of DH348 as an explanation for an increased radioresistance after DH348 treatment.

Figure 4: Representative images of H460 tumors after oral treatment for five consecutive days with vehicle (A) or DH348 (B) showing distribution of the hypoxia marker pimonidazole (green), vessels (CD31, red), and the perfusion marker Hoechst 33342 (blue). Quantification of the hypoxic fraction (C), necrotic fraction (D), relative vascular area (E), and the fraction of perfused vessels (F) is shown for each included tumor with the corresponding mean of the groups.

Validation of DH348 radiosensitization after intravenous administration

The contradictory results on the efficacy of DH348 as a radiosensitizer required a validation experiment using the identical experimental setup as before, i.e. intravenous administration of DH348 (10 mg/kg dissolved in 1% DMSO) combined with radiotherapy on day 3 [23]. This validation experiment was performed in the same colorectal HT29 model, which was reported to be responsive to DH348 treatment [23, 24]. In addition we also evaluated DH348 efficacy in the head and neck squamous cell carcinoma FaDu and non-small cell lung cancer H460 model. In contrast to the previous findings, in all three tumor models intravenous DH348 treatment did not enhance treatment response of radiation (Figure 5). Furthermore, the results in the HT29 tumor model were unaffected by the day of treatment with ionizing radiation (i.e. on day 3 or day 5 of DH348 treatment: 41.6 vs 42.6 days, $p=0.910$). In Kaplan-Meier analyses eight HT29 tumors did not reach endpoint (1000 mm^3) and were censored, i.e. three in the vehicle and DH348 treated group with irradiation on day 3 and two in the DH348 treated group with irradiation on day 5. In H460 tumors two tumors were censored in the DH348 treated group of mice. Excluding these censored cases when comparing T4xSV between groups however did not affect the results. Therefore, both with Kaplan-Meier survival analyses and comparing the T4xSV between groups DH348 treatment did not show any significant enhancement of radiosensitivity in all three tumor models and the previous study results could therefore not be confirmed [23].

Figure 5: Kaplan-Meier curves of HT29 (A), H460 (C), and FaDu (E) xenograft tumors after radiation (10 Gy on Day 3) in combination with intravenous administration of vehicle (green) or DH348 (red) for five consecutive days. A separate group of HT29 tumors received radiation on Day 5 of treatment instead of Day 3 (orange). An event is defined as a tumor reaching 4x start volume. From the exponential fits of the tumor growth the times to reach 4x start volume (T4xSV) were calculated and summarized for the HT29 (B), H460 (D), and FaDu (F) tumors. Symbols represent T4xSV for each animal and the bar represents mean value per group. Open symbols indicate tumors that have not reached endpoint (i.e. 1000 mm³) at the end of the study and were censored in the Kaplan-Meier curves. Overall results are not affected when these tumors are excluded from analyses.

DISCUSSION

The CAIX dual targeting 5-nitroimidazole based sulfamide DH348 neither inhibited tumor growth when applied as a single agent, nor sensitized tumors to radiation in several

human tumor models using various schedules and routes of administration, despite of its favorable pharmacokinetic profile. These results therefore do not confirm the previously reported radiosensitizing effect of DH348 [23]. In contrast, oral administration of high dose of DH348 even abrogated the effect of radiotherapy, which could not be explained by DH348 induced elevated hypoxia levels prior to irradiation. To explain the discrepancy between the results obtained in this study and previous findings we repeated the same experiments using the same tumor model, treatment schedule and experimental conditions. The results of this validation experiment are in discord with previous experiments showing significant tumor growth inhibition by DH348 in HT29, HCT116, and MDA-MB-231 xenograft tumors [21, 23, 24]. The reduction in MDA-MB-231 tumor growth was associated with decreased proliferation and a reduced percentage of viable cells upon DH348 treatment [21]. Moreover, treating tumor-bearing animals with DH348 combined with radiation using the exact same protocol as reported previously [23] did not confirm the radiosensitizing potential of DH348.

Several potential explanations for the discrepancy in results between studies need to be addressed. Firstly, an important difference between the initial [23] and the present experiments was the batch of DH348 used. The initial study has been performed with the first generation batch DH348 (i.e. a paste-like formulation [23]), while the rest of the studies were done with a large-scale second generation batch of DH348 (i.e. a white powder formulation). Both batches of compound however resulted in a similar reduction in hypoxia-induced extracellular acidification, i.e. in line with the effect of sulfamide CAIX inhibitors, thereby assuming both behaved similarly (data not shown). No compound was available anymore from the initial batch, although other derivatives from the same series of compounds were still available [24]. All available compounds were tested for their stability and the paste-like compounds appeared to be degraded, while the white powder batches remained stable (data not shown). The presence of stable DH348 or degraded metabolites in tumor tissue however cannot be examined and compared between the studies, because of the loss of tumor samples from the initial intravenous study [23]. The question what degradation product was tested in the initial study therefore remains unanswered. We can speculate that degradation of DH348 may have occurred by hydrolysis of the NO_2 group of the nitroimidazole moiety resulting in a functional sulfamide and non-functional nitroimidazole moiety. The remaining functional CAIXi moiety may have been sufficient to enable therapeutic efficacy. In line with this speculation, previously, treatment with an indanesulfonamide was also effective as single agent treatment and furthermore acted as a radiosensitizer in tumor xenografts [22].

Secondly, the analysis of the data may have affected the results as previous studies normalized the tumor volume to the time of irradiation [35-37], whereas all data of subsequent experiments were normalized to start of treatment [38]. The latter method of normalization allows better estimation of the combined efficacy of the compound and radiation treatment. Nevertheless, re-analysis of the data obtained in the present

experiments in the same way as previously, i.e. normalization of tumor volumes to the start of irradiation, did not change the conclusion of the present study.

Thirdly, different growth rates were observed in the HT29 tumors in the validation study with intravenous administration of DH348 as compared to the previous studies [23, 24]. The relatively slower growth of HT29 tumors in the present validation study can be explained by the formation of the external necrosis during tumor regrowth after irradiation, which may affect the accuracy of the tumor volume estimations. Because this phenomenon was observed in both vehicle and drug treated tumors in all tumor models, it is unlikely that formation of the external necrosis could confound the results.

In general, from these results it can be concluded that DH348 does not sensitize human tumor xenografts to radiation, both after oral and intravenous administration, which contradicts our previously published findings [23]. Unfortunately, no definitive conclusion about the responsible factor for this discrepancy in results can be provided. A lesson to be learned however is that a better and more rigorous administration is required to allow for validation of compound stability and proper control of experiments. In addition, differences in experimental setup between experiments need to be kept to a minimum during drug design and development, which will allow for an earlier detection of results that are contradictory to the original hypothesis. Dual-target drugs could however still be promising to pursue as it might allow for the targeting of effector molecules, e.g. cytotoxic drugs, towards CAIX expressing regions in the tumors that are normally more resistant to standard therapies. This hypothesis on the efficacy of dual-target drugs remains to be studied further.

METHODS

Solution in organic solvents and organic solvent mixtures

The solubility of DH348 in different solvents was determined by amatsiSEPS (Ghent, Belgium). DH348 was dissolved in 2 ml PEG400, propylene glycol or ethanol, as well as in acidified conditions of these solvents (i.e. 0.1M citric acid or 0.1M HCl) until saturation. After 24h stirring at room temperature, the mixtures were filtered using a 0.2 μ M PVDF syringe filter (BD Biosciences). The concentration of DH348 in solution was determined with an Acquity UPLC H-Class (Waters Corp., Milford, Massachusetts, USA) in combination with a (PDA) UV detector. A single point calibration was made to a concentration of 0.20 mg/ml DH348 and samples were diluted in purified water.

Caco-2 absorption assay

Cyprotex Ltd. (Macclesfield, United Kingdom) assessed the permeability of DH348 using a Caco-2 absorption assay. Cells were seeded on Millipore Millicell plates 20 days prior to

the experiment to allow them to form a confluent monolayer. DH348 (10 μ M) was added to the apical side of the membrane and the transport across the monolayer was monitored over a 2 h time period. To study drug efflux the transport of the compound from the basolateral compartment to the apical compartment was also investigated. The permeability coefficient (P_{app}) is calculated as: $P_{app} = (dQ/dt)/(C_0 \cdot A)$, where dQ/dt is the rate of permeation of the drug across the cells, C_0 is the donor compartment concentration at time zero, and A is the area of the cell monolayer. C_0 was obtained from analysis of the dosing solution at the start of the experiment. In addition, atenolol and propranolol were included as these compounds have a known human absorption of 50% and 90%, respectively [28]. Finally, talinolol was included as a positive control of a P-glycoprotein substrate [33].

Animal experimentation

All animal experiments were performed according to the local ethical guidelines. Study design and experimental protocols were evaluated and approved prior to the start by the local ethical committee on animal welfare.

Mouse pharmacokinetic analysis

In vivo pharmacokinetic characteristics of DH348 were determined by Cyprotex Ltd. (Macclesfield, United Kingdom) as described before [25]. In short, male CD1 mice received DH348 either injected intravenously (5 mg/kg, iv) or orally (50 mg/kg) and DH348 concentrations were measured in plasma up to 8 h after administration ($n=3$ per time point). The half-life ($t_{1/2}$) was estimated from the curve after intravenous administration. Bioavailability was calculated with the area under the curve (AUC) of both administration routes with: $B_{app} = 100\% \cdot (AUC_{PO} \cdot Dose_{IV}) / (AUC_{IV} \cdot Dose_{PO})$, where AUC_{PO} is AUC after oral administration, $Dose_{IV}$ is the IV dose, AUC_{IV} is AUC after iv administration, and $Dose_{PO}$ is the oral dose of DH348.

Tumor cell implantation and measurement

NMRI-nu mice (Charles River) were subcutaneously injected with 1.5×10^6 tumor cells suspended in Matrigel (Corning). Tumors were measured three times per week in three orthogonal directions. Tumor volume was calculated according to $A \cdot B \cdot C \cdot \pi / 6$, where A , B and C represent length, width and height in mm, respectively, each corrected for thickness of the skin (i.e. 0.5 mm).

Acute toxicity after oral administration of DH348 at human equivalent dose

The human equivalent dose (HED) of DH348 was based on nimorazole administration in patients of 1.2 g/m^2 with daily oral delivery [27], and amounted to 400 mg/kg when correcting for body surface area as calculated with the method of Reagan-Shaw *et al* [39]. Colorectal HT29 tumor-bearing mice were randomized to receive 0, 100, 200, 300, or 400 mg/kg DH348, dissolved in polyethylene glycol (PEG), by oral gavage in a dose volume of 100 μl /10 g body weight for five consecutive days when tumor volume was greater than 150 mm^3 (median 155 mm^3 , range 118 – 240 mm^3). During the treatment period and two weeks after the start of oral administration the weight of the mice was measured daily. Acute toxicity was defined as a sudden loss in body weight, i.e. more than 20% per day or more than 30% during the entire study. The study lasted until mice were sacrificed, i.e. when maximum tumor volume was reached (i.e. 1000 mm^3), or when signs of acute toxicity caused by treatment were observed.

Oral administration of DH348 in combination with ionizing radiation

Colorectal HT29 or non-small cell lung cancer H460 tumor-bearing mice were randomized to receive oral administration of vehicle (triethyleneglycol, [TEG]) or DH348 (400 mg/kg), in combination with sham irradiation or a 10 Gy single radiation dose. Tumor irradiation was performed on day 5 of treatment up to 6 hours after the last oral application. During irradiation animals were anaesthetized with ketamine/xylazine (100 mg/kg + 10 mg/kg, intraperitoneal [ip]). Mice were sacrificed when tumor volumes reached 1000 mm^3 . In parallel, in histology groups, mice were sacrificed at an average tumor volume of 150 mm^3 or directly after the last oral injection. In the histology groups, 1 h prior to sacrifice mice were injected with the hypoxia marker pimonidazole (60 mg/kg, [ip]) and the proliferation marker BrdU (30 mg/kg, ip.). The perfusion marker Hoechst 33342 (15 mg/kg, iv) was injected 1 min prior to sacrifice. Tumors were isolated, frozen in liquid nitrogen, and stored in -80°C until histological evaluation.

Validation of radiosensitization effect of DH348

To validate previous findings, i.e. radiosensitizing effect of DH348 in HT29 colorectal adenocarcinoma xenografts, a validation experiment was performed under the same experimental conditions as described previously [23]. In addition to HT29, this experiment also included non-small cell lung cancer H460, and head and neck squamous cell carcinoma FaDu xenografts. When tumor volumes reached $150 - 200 \text{ mm}^3$ mice were randomized to receive either intravenous administration of DH348 (10 mg/kg) or vehicle (1% DMSO in saline) for five consecutive days. On day 3 of treatment all mice were irradiated with a single dose of 10 Gy. A separate group of HT29 xenografts was included that received irradiation on day 5 of treatment. On the day of irradiation DH348 was

administered 30 min prior to irradiation of the animals. Mice were sacrificed when tumor volumes reached 1000 mm³.

Immunohistochemical staining and analyses

Frozen H460 xenograft sections were stained for immunohistochemistry and analyzed as described previously [38]. In short, sections were stained with rabbit-anti-pimonidazole (HP3-1000, Bio-Connect) and rat-anti-mouse CD31 (BD Biosciences) to visualize hypoxic regions and vessels in the tumor, respectively. Perfusion in tumor sections was estimated via the fluorescent intensity of Hoechst 33342. After scanning the images, the hypoxic fraction, necrotic fraction, microvessel density, and perfused fraction were quantified.

Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 5.03). Average tumor growth curves were fitted to the exponential growth equation, which was used to estimate the tumor response as the time to reach 4 times start volume (T4xSV). Differences in mean, i.e. between the average T4xSV between treatment groups or between immunohistochemical parameters, were compared with unpaired t-test. Differences between Kaplan-Meier curves were tested with log-rank test. A p-value <0.05 indicates a statistically significant difference.

REFERENCES

1. van Kuijk, S.J., et al., *Prognostic Significance of Carbonic Anhydrase IX Expression in Cancer Patients: A Meta-Analysis*. *Frontiers in oncology*, 2016. **6**: p. 69.
2. Good, J.S. and K.J. Harrington, *The hallmarks of cancer and the radiation oncologist: updating the 5Rs of radiobiology*. *Clinical oncology*, 2013. **25**(10): p. 569-77.
3. Horsman, M.R. and P. Vaupel, *Pathophysiological Basis for the Formation of the Tumor Microenvironment*. *Frontiers in oncology*, 2016. **6**: p. 66.
4. Neri, D. and C.T. Supuran, *Interfering with pH regulation in tumours as a therapeutic strategy*. *Nature reviews. Drug discovery*, 2011. **10**(10): p. 767-77.
5. Pastorek, J. and S. Pastorekova, *Hypoxia-induced carbonic anhydrase IX as a target for cancer therapy: from biology to clinical use*. *Seminars in cancer biology*, 2015. **31**: p. 52-64.
6. Akurathi, V., et al., *Development and biological evaluation of (9)(9)mTc-sulfonamide derivatives for in vivo visualization of CA IX as surrogate tumor hypoxia markers*. *European journal of medicinal chemistry*, 2014. **71**: p. 374-84.
7. Akurathi, V., et al., *Synthesis and biological evaluation of a 99mTc-labelled sulfonamide conjugate for in vivo visualization of carbonic anhydrase IX expression in tumor hypoxia*. *Nuclear medicine and biology*, 2010. **37**(5): p. 557-64.
8. Dubois, L., et al., *Imaging the hypoxia surrogate marker CA IX requires expression and catalytic activity for binding fluorescent sulfonamide inhibitors*. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology*, 2007. **83**(3): p. 367-73.
9. Dubois, L., et al., *Imaging of CA IX with fluorescent labelled sulfonamides distinguishes hypoxic and (re)-oxygenated cells in a xenograft tumour model*. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology*, 2009. **92**(3): p. 423-8.
10. Peeters, S.G., et al., *[(18)F]VM4-037 MicroPET Imaging and Biodistribution of Two In Vivo CAIX-Expressing Tumor Models*. *Molecular imaging and biology : MIB : the official publication of the Academy of Molecular Imaging*, 2015. **17**(5): p. 615-9.
11. Sneddon, D., et al., *Synthesis and in Vivo Biological Evaluation of (68)Ga-Labeled Carbonic Anhydrase IX Targeting Small Molecules for Positron Emission Tomography*. *Journal of medicinal chemistry*, 2016. **59**(13): p. 6431-43.
12. Sedlakova, O., et al., *Carbonic anhydrase IX, a hypoxia-induced catalytic component of the pH regulating machinery in tumors*. *Frontiers in physiology*, 2014. **4**: p. 400.
13. Lomelino, C. and R. McKenna, *Carbonic anhydrase inhibitors: a review on the progress of patent literature (2011-2016)*. *Expert opinion on therapeutic patents*, 2016: p. 1-10.
14. Monti, S.M., C.T. Supuran, and G. De Simone, *Anticancer carbonic anhydrase inhibitors: a patent review (2008 - 2013)*. *Expert opinion on therapeutic patents*, 2013. **23**(6): p. 737-49.
15. Chiche, J., et al., *Hypoxia-inducible carbonic anhydrase IX and XII promote tumor cell growth by counteracting acidosis through the regulation of the intracellular pH*. *Cancer research*, 2009. **69**(1): p. 358-68.
16. Swietach, P., et al., *The role of carbonic anhydrase 9 in regulating extracellular and intracellular pH in three-dimensional tumor cell growths*. *The Journal of biological chemistry*, 2009. **284**(30): p. 20299-310.
17. Swietach, P., et al., *Tumor-associated carbonic anhydrase 9 spatially coordinates intracellular pH in three-dimensional multicellular growths*. *The Journal of biological chemistry*, 2008. **283**(29): p. 20473-83.
18. Csaderova, L., et al., *The effect of carbonic anhydrase IX on focal contacts during cell spreading and migration*. *Frontiers in physiology*, 2013. **4**: p. 271.
19. Gieling, R.G., et al., *Antimetastatic effect of sulfamate carbonic anhydrase IX inhibitors in breast carcinoma xenografts*. *Journal of medicinal chemistry*, 2012. **55**(11): p. 5591-600.
20. Svastova, E., et al., *Carbonic anhydrase IX interacts with bicarbonate transporters in lamellipodia and increases cell migration via its catalytic domain*. *The Journal of biological chemistry*, 2012. **287**(5): p. 3392-402.

21. Ward, C., et al., *Evaluation of carbonic anhydrase IX as a therapeutic target for inhibition of breast cancer invasion and metastasis using a series of in vitro breast cancer models*. *Oncotarget*, 2015. **6**(28): p. 24856-70.
22. Dubois, L., et al., *Specific inhibition of carbonic anhydrase IX activity enhances the in vivo therapeutic effect of tumor irradiation*. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology*, 2011. **99**(3): p. 424-31.
23. Dubois, L., et al., *Targeting carbonic anhydrase IX by nitroimidazole based sulfamides enhances the therapeutic effect of tumor irradiation: a new concept of dual targeting drugs*. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology*, 2013. **108**(3): p. 523-8.
24. Rami, M., et al., *Hypoxia-targeting carbonic anhydrase IX inhibitors by a new series of nitroimidazole-sulfonamides/sulfamides/sulfamates*. *Journal of medicinal chemistry*, 2013. **56**(21): p. 8512-20.
25. van Kuijk, S.J., et al., *The Sulfamate Small Molecule CAIX Inhibitor S4 Modulates Doxorubicin Efficacy*. *PloS one*, 2016. **11**(8): p. e0161040.
26. Overgaard, J., *Clinical evaluation of nitroimidazoles as modifiers of hypoxia in solid tumors*. *Oncology research*, 1994. **6**(10-11): p. 509-18.
27. Bentzen, J., et al., *Locally advanced head and neck cancer treated with accelerated radiotherapy, the hypoxic modifier nimorazole and weekly cisplatin. Results from the DAHANCA 18 phase II study*. *Acta oncologica*, 2015. **54**(7): p. 1001-7.
28. Zhao, Y.H., et al., *Evaluation of human intestinal absorption data and subsequent derivation of a quantitative structure-activity relationship (QSAR) with the Abraham descriptors*. *Journal of pharmaceutical sciences*, 2001. **90**(6): p. 749-84.
29. Testa, B., et al., eds. *Pharmacokinetic Optimization in Drug Research: Biological, Physicochemical, and Computational Strategies*. 2001.
30. Garberg, P., et al., *In vitro models for the blood-brain barrier*. *Toxicology in vitro : an international journal published in association with BIBRA*, 2005. **19**(3): p. 299-334.
31. Lundquist, S. and M. Renftel, *The use of in vitro cell culture models for mechanistic studies and as permeability screens for the blood-brain barrier in the pharmaceutical industry--background and current status in the drug discovery process*. *Vascular pharmacology*, 2002. **38**(6): p. 355-64.
32. Lundquist, S., et al., *Prediction of drug transport through the blood-brain barrier in vivo: a comparison between two in vitro cell models*. *Pharmaceutical research*, 2002. **19**(7): p. 976-81.
33. Anderle, P., et al., *P-Glycoprotein (P-gp) mediated efflux in Caco-2 cell monolayers: the influence of culturing conditions and drug exposure on P-gp expression levels*. *Journal of pharmaceutical sciences*, 1998. **87**(6): p. 757-62.
34. Dische, S., et al., *A trial of Ro 03-8799 (pimonidazole) in carcinoma of the uterine cervix: an interim report from the Medical Research Council Working Party on advanced carcinoma of the cervix*. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology*, 1993. **26**(2): p. 93-103.
35. Dubois, L., et al., *Inhibition of 4E-BP1 sensitizes U87 glioblastoma xenograft tumors to irradiation by decreasing hypoxia tolerance*. *International journal of radiation oncology, biology, physics*, 2009. **73**(4): p. 1219-27.
36. Rouschop, K.M., et al., *The unfolded protein response protects human tumor cells during hypoxia through regulation of the autophagy genes MAP1LC3B and ATG5*. *The Journal of clinical investigation*, 2010. **120**(1): p. 127-41.
37. Rouschop, K.M., et al., *PERK/eIF2alpha signaling protects therapy resistant hypoxic cells through induction of glutathione synthesis and protection against ROS*. *Proceedings of the National Academy of Sciences of the United States of America*, 2013. **110**(12): p. 4622-7.
38. Peeters, S.G., et al., *TH-302 in Combination with Radiotherapy Enhances the Therapeutic Outcome and Is Associated with Pretreatment [18F]HX4 Hypoxia PET Imaging*. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 2015. **21**(13): p. 2984-92.
39. Reagan-Shaw, S., M. Nihal, and N. Ahmad, *Dose translation from animal to human studies revisited*. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 2008. **22**(3): p. 659-61.

Chapter 6

New approach of delivering cytotoxic drugs towards CAIX expressing cells: A concept of dual-target drugs

In Press Eur J Med Chem

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Highlights

- New dual-target drugs combining cytotoxic agents with CAIX inhibitors were designed.
- Dual-targeting drugs may allow for specific drug delivery to hypoxic tumor areas.
- Increased binding affinity to CAIX was found for most of the dual-targeting drugs.
- Higher efficacy of dual-target ATR inhibitor was found in CAIX overexpressing cells.
- Design of alternative CAIX-targeting drugs may increase their therapeutic window.

ABSTRACT

Carbonic anhydrase IX (CAIX) is a hypoxia-regulated and tumor-specific protein that maintains the pH balance of cells. Targeting CAIX might be a valuable approach for specific delivery of cytotoxic drugs, thereby reducing normal tissue side-effects. A series of dual-target compounds were designed and synthesized incorporating a sulfonamide, sulfamide, or sulfamate moiety combined with several different anti-cancer drugs, including the chemotherapeutic agents chlorambucil, tirapazamine, and temozolomide, two Ataxia Telangiectasia and Rad3-related protein inhibitors (ATRi), and the anti-diabetic biguanide agent phenformin. An ATRi derivative (**12**) was the only compound to show a preferred efficacy in CAIX overexpressing cells versus cells without CAIX expression when combined with radiation. Its efficacy might however not solely depend on binding to CAIX, since all described compounds generally display low activity as carbonic anhydrase inhibitors. The hypothesis that dual-target compounds specifically target CAIX expressing tumor cells was therefore not confirmed. Even though dual-target compounds remain an interesting approach, alternative options should also be investigated as novel treatment strategies.

INTRODUCTION

Solid tumors are characterized by a hypoxic microenvironment caused by their immature and inadequate vascular supply of oxygen and nutrients. These hostile hypoxic conditions result in a phenotype that is associated with a worse prognosis [1] and resistance to standard treatment options such as radiotherapy, chemotherapy, and surgery [2-4]. Several different approaches are currently being investigated to target these hypoxic areas to make tumors more sensitive to standard treatment modalities [5-7].

Carbonic anhydrase IX (CAIX) can be a valuable therapeutic target since it plays an important role in maintaining the intracellular pH homeostasis [8, 9]. Furthermore its expression is predominantly tumor specific [5, 8, 10] and directly regulated via the hypoxia-inducible factor (HIF) pathway [11]. Even though alternative pathways are also able to modulate CAIX expression [12-14], its significant prognostic value in many different tumor types [15] has promoted investigations in its use as an imaging agent for diagnostic and prognostic purposes [5, 16-19]. Together these characteristics of CAIX support investigations into the therapeutic targeting of CAIX to improve efficacy of standard treatments. The function of CAIX is evolutionary conserved and catalyzes the hydration of carbon dioxide to bicarbonate at the cell membrane. The bicarbonate is transported back intracellular from the extracellular space, whereas the free proton is extruded in the extracellular space. CAIX thereby maintains the balance between an acidic extracellular and alkaline intracellular pH of tumor cells, the latter of which would otherwise acidify due to the increased acid production resulting from their glycolytic metabolism [8, 9]. Many different inhibitors are currently being developed to specifically target the tumor-associated CAIX isoform and have shown promise in reducing tumor cell survival, migration, invasion, and reduce tumor xenograft growth and metastases formation [20-23]. Furthermore, the combination therapy of CAIX inhibitors (CAIXi) with standard treatment options was previously found to increase the efficacy of radiotherapy [24] and of weakly basic chemotherapeutic agents such as doxorubicin [25].

The predominant expression of CAIX on hypoxic tumor cells can also be exploited to direct cytotoxic agents specifically to those CAIX expressing cancer cells thereby possibly minimizing normal tissue toxicity. This can be achieved by conjugating anti-cancer drugs with CAIX inhibiting molecules that bind to the Zn^{2+} active site of CAIX and hence inhibit its enzymatic function [8, 26, 27], *i.e.* a so-called dual-targeting approach. Our group showed previously that such a dual-target approach with a sulfamide CAIXi moiety coupled to the radiosensitizing compound nitroimidazole to be a more effective radiosensitizer than an indanesulfonamide CAIXi [28]. Alternative novel dual-target compounds have been developed to investigate this strategy of dual-targeting further in the context of anti-cancer agents to target CAIX. Here we have designed five different classes of dual-target compounds conjugated to CAIXi (sulfonamide, sulfamide, or sulfamate), which included the chemotherapeutic anti-cancer agents chlorambucil, tirapazamine, and temozolomide, two ataxia telangiectasia and Rad3-related protein inhibitors (ATRI), and

the biguanide agent phenformin, previously used in diabetes treatment. We hypothesize that these new dual-target compounds will have the ability to specifically target CAIX expressing cells and modulate their efficacy in a CAIX-dependent manner.

RESULTS AND DISCUSSION

Chemistry

Chlorambucil was converted to its acid chloride [29] **1** by using oxalyl chloride. This chlorambucil acid chloride reacted with different benzene sulfonamides under basic condition to obtain good yields of chlorambucil derivatives **2a**, **2b** and **2c**. Chlorambucil carbamate derivatives were obtained by converting compound **1** into a methyl ester [30] using methanol. This ester was reduced to alcohol [31], *i.e.* compound **3**, after treating with lithium aluminium hydride. Compound **3** was treated with triphosgene to obtain its respective chloroformate [32], *i.e.* compound **4** (Scheme 2). The reaction of chlorambucil chloroformate (**4**) with different benzene sulfonamides resulted in compounds **5a**, **5b** and **5c** (Scheme 3).

Scheme 1: Reagents and conditions: (i) $(\text{COCl})_2$, DMF, DCM, 0 °C–rt; (ii) DIPEA, THF, 0 °C–rt.

Scheme 2: Reagents and conditions: (i) MeOH, DCM; (ii) LAH, THF; (iii) Triphosgene, Na₂CO₃, Toluene, DMF.

Scheme 3: Reagents and conditions: (i) DIPEA, THF, 0 °C–rt.

Tirapazamine derivatives **8** and **11** were synthesized from **6** and **9** with the previously described procedure [33]. In short, **6** and **9** reacted with 4-(2-aminoethyl) benzene sulfonamide under reflux conditions and was followed by oxidation of the mono-oxides (Scheme 4).

The ATRi derivatives **12** and **13** were synthesized from commercially available VE-821 and VE-822 (MedChemTronica) using a classical synthetic strategy described previously [25] (Scheme 5).

Commercially purchased temozolomide (SelleckChem) was converted into its respective acid by treating with concentrated sulfuric acid and sodium nitrate at 0 °C to 15 °C (Scheme 6) [34]. Reacting the temozolomic acid with different benzenesulfonamides, aminoxysulfonamide [35] and 5-amino-1, 3,4-thiadiazole-2-sulfonamide hydrochloride under known amide bond formation conditions [34] resulted in compounds **15a**, **15b**, **15c** and **15d** (Scheme 7).

Scheme 4: Reagents and conditions: (i) RNH_2 (3 equiv.), DME, reflux; (ii) TFAA, H_2O_2 , DCM, rt.

Scheme 5: Reagents and conditions: (i) ClSO_2NCO , tBuOH, NEt_3 , DCM, 0°C to rt; (ii) 20% TFA-DCM.

Scheme 6: Reagents and conditions: (i) Con. H_2SO_4 , NaNO_2 , 0 °C to 15 °C

Scheme 7: Reagents and conditions: (i) BOP, NEt_3 , DCM, rt.

The compound **18** was obtained by a slight modification based on the method reported by Kelarev et al. [36]. The commercially available compounds 4-(2-aminoethyl) benzenesulfonamide **16** and cyanoguanidine **17** were coupled in *n*-butanol using a stoichiometric amount of hydrochloric acid (Scheme 8).

Scheme 8: Reagents and conditions: (i) 6.0 M HCl aq, *n*BuOH, reflux.

Binding affinity human CAs

Increased binding affinity to human carbonic anhydrases (CAs) as compared with their respective parental compound are observed (Table 1) for most of the compounds, ex-

cept for the CAIXi conjugated ATRi (**12** and **13**), which do not bind to any of the four tested human CA isoforms included ($K_i > 50000$ nM). The K_i values of the other dual-targeting compounds are higher than of the previously reported CAIXi [22] with **15a** showing relatively good K_i for the CAII and CAIX isoforms, but not for CAXII. Only the phenformin derivative **18** was found to be selective for the transmembrane CAIX and CAXII isoforms. To investigate whether the biological efficacy of the functionalized compounds is dependent on CAIX expression, canine kidney epithelial (MDCK) cells without CAIX (CAIX⁻), *i.e.* both human and canine [37], or MDCK cells transfected with human CAIX [37], *i.e.* overexpressing CAIX (CAIX⁺), were used. Western blotting confirmed differential expression of CAIX in these cells both under normoxic and hypoxic conditions (Supplementary Figure S1).

Table 1: Binding affinity (K_i) to human CAI, CAII, CAIX, and CAXII of the parental compounds (bold) and their CAIXi conjugated derivatives.

Compound	K_i (nM) ^a				Selectivity Ratios ^b	
	hCA I	hCA II	hCA IX	hCA XII	K_i hCA II / K_i hCA IX	K_i hCA II / K_i hCA XII
Chlorambucil	>50000	>50000	>50000	>50000		
2a	73.0	9.0	172	689	0.05	0.01
2b	5950	747	8970	7340	0.08	0.10
2c	8400	450	4610	10160	0.10	0.04
5a	5580	553	2740	9380	0.20	0.06
5c	6140	265	4130	9570	0.06	0.03
5c	5670	504	3850	13600	0.13	0.04
Tirapazamine	>50000	>50000	>50000	>50000		
8	567	7.1	383	14600	0.02	<0.01
11	428	8.1	307	624	0.03	0.01
Temozolomide	>50000	>50000	>50000	>50000		
15a	91.3	9.2	37.1	9300	0.25	<0.01
15b	>50000	>50000	>50000	>50000		
15c	539	90.5	271	12400	0.33	0.01
15d	743	15.7	176	92.7	0.09	0.17
ATRi VE-821	>50000	>50000	>50000	>50000		
12	>50000	>50000	>50000	>50000		
ATRi VE-822	>50000	>50000	>50000	>50000		
13	>50000	>50000	>50000	>50000		
Phenformin	>50000	>50000	>50000	>50000		
18	4435	501	20.2	1.7	24.8	295
Acetazolamide ^c	250	12.1	25.3	5.7	0.48	2.12

^aValues reported (in nM) are the average of three different estimations with errors between 5–10% of the reported values. Reported values >50000 indicates no binding of the compound towards the CA isoforms.

^bSelectivity ratios of the cytosolic hCAII over the tumor-associated hCAIX and hCAXII isoforms.

^cNon-specific CAi acetazolamide is included as a reference.

Chlorambucil derivatives

Chlorambucil (4-[p-[bis(2-chloroethyl)amino]phenyl]butyric acid) is a nitrogen mustard that acts as a bifunctional alkylating agent used for decades to treat cancers originating in the blood and lymphatic system, *e.g.* chronic lymphocytic leukemia and lymphomas [38]. Even though reported data suggest chlorambucil efficacy to increase in an acidic microenvironment [39], the CAIXi moiety (benzenesulfonamides) with different linkers (*i.e.* amide, carbamate) might allow for specific targeting of the compounds to these areas in the tumor. The six CAIXi conjugated chlorambucil derivatives (**2a**, **2b**, **2c**, **5a**, **5b**, and **5c**, Scheme 1 and 2) lead to reduced cell viability as compared to the parental compound, which was only marginally effective (Table 2, Supplementary Figure S1). The therapeutic efficacy of these compounds however was not increased in the CAIX⁺ MDCK cells as compared with the CAIX⁻ MDCK cells. Furthermore, none of the six compounds showed an increased efficacy upon hypoxia exposure (0.2% O₂). In contrast, some of the chlorambucil dual-target derivatives were less cytotoxic (*i.e.* higher IC₅₀, Table 2) in CAIX expressing cells independent of oxygen levels, which contradicts the studies demonstrating an increased efficacy of chlorambucil in an acidic micromilieu [39]. All together from these results it can be concluded that the CAIXi conjugated chlorambucil derivatives do not show an increased efficacy in a CAIX or hypoxia dependent manner.

Table 2: Estimated IC₅₀ of the cytotoxic parental compounds (bold) and their CAIXi conjugated derivatives obtained with cell viability assays for MDCK CAIX⁻ and MDCK CAIX⁺ cells exposed to normoxic and hypoxic conditions.

Compound	Normoxia (μM) ^a		Hypoxia (μM) ^a	
	CAIX ⁻	CAIX ⁺	CAIX ⁻	CAIX ⁺
Chlorambucil ^b	93	>100	~100	>100
2a	~100	87	~100	95
2b	18	98	14	92
2c	18	~100	18	~100
5a	8	56	8	62
5b	86	98	52	100
5c	89	99	81	~100
Tirapazamine ^c	>300	>300	<50	95
8	>300	>300	~300	>300
11	>300	>300	>300	>300
Temozolomide ^d	775	>1000	~1000	>1000
15a	>1000	>1000	>1000	>1000
15b	719	>1000	~1000	>1000
15c	>1000	>1000	>1000	>1000
15d	>1000	>1000	>1000	>1000

^aNo IC₅₀ reached is indicated with >. Estimated IC₅₀ value higher than the maximum concentration included is indicated with ~.

^bIncluded concentrations for chlorambucil were 1, 10, and 100 μM.

^cIncluded concentrations for tirapazamine were 50, 100, 200, and 300 μM.

^dIncluded concentrations for temozolomide were 100, 400, 700, and 1000 μM.

Tirapazamine derivatives

The hypoxia-activated prodrug tirapazamine (3-amino-1,2,4-benzotriazine-1,4-dioxide) has been tested in several clinical trials in combination with chemo- and/or radiotherapy [40]. The cytotoxicity of tirapazamine results from activation by reductive enzymes that add an electron to the parent drug to produce a radical species that causes DNA damage. Nevertheless, no definitive conclusions regarding its clinical efficacy can be drawn since addition of tirapazamine to standard treatment (*i.e.* radio-chemotherapy) did not result in an increased benefit in phase 3 clinical trials. In addition, tirapazamine treatment was often characterized by toxic side-effects, such as nausea, vomiting, and diarrhea, which limited its therapeutic gain [40]. Targeting tirapazamine towards the CAIX expressing (hypoxic) areas in tumors by conjugating tirapazamine with the benzenesulfonamide CAIXi might thereby prove a valuable approach to reduce normal tissue toxicity and increase the efficacy of the compounds (**8** and **11**) in CAIX expressing (hypoxic) cells. Cell viability assays (Table 2, Supplementary Figure S2) confirmed that the parental compound was specifically effective in hypoxic cells, and more effective in CAIX⁺ cells (IC₅₀ <50 versus 95 μ M, $p=0.037$). The CAIXi conjugated tirapazamine derivatives however abrogated the effect observed for the parental compound, both during hypoxia and normoxia, which was independent of CAIX levels (Table 2).

Temozolomide derivatives

The current treatment of glioblastoma is based on radiotherapy combined with temozolomide, which has been shown to increase survival in phase III clinical trials [41]. Temozolomide is a methylating agent that spontaneously hydrolyzes to its active metabolite 3-methyl-(triazene-1-yl)imidazole-4-carboxamide (MTIC) at physiological pH [42]. The acidic extracellular pH in tumors might therefore reduce spontaneous temozolomide conversion and thereby decrease its efficacy. Inhibiting CAIX function is known to decrease extracellular acidification *in vitro* [24, 25, 28, 43] and we hypothesized that conjugating temozolomide with a sulfonamide or sulfamate moiety (**15a**, **15b**, **15c**, and **15d**) will specifically target hypoxic tumors and increase temozolomide conversion and thereby its efficacy. Nevertheless, while temozolomide resulted in lower cell viability in CAIX⁺ cells, consistent with the pH-dependent mechanism of activation, the CAIXi conjugated temozolomide derivatives **15a**, **15c**, and **15d** were ineffective in reducing cell viability in both MDCK cell lines during normoxic and hypoxic conditions within the concentration range tested in the present study (Table 2, Supplementary Figure S3). In contrast, **15b** was similarly effective as the parental temozolomide compound (Table 2).

This dual-target compound was therefore investigated further in clonogenic survival assays in which the medium of the cells was acidified because of CAIX function during hypoxic conditions (Supplementary Figure S1) [44]. Temozolomide was again more effective in reducing clonogenic cell survival in the CAIX⁺ MDCK cells as compared with

the CAIX⁺ MDCK cells (Figure 1), similarly to its efficacy on cell viability. During hypoxia however temozolomide caused no difference in clonogenic survival as compared to normoxia, even though hypoxia is required to activate CAIX and cause extracellular acidification [43, 45] and is therefore hypothesized to reduce temozolomide conversion and efficacy. In contrast, the CAIXi conjugated derivative **15b** significantly reduced clonogenic cell survival in hypoxic versus normoxic conditions in the CAIX⁺ cells (surviving fraction is 46.1 ± 3.1 versus 26.1% ± 7.9 during normoxia and hypoxia respectively, $p < 0.05$). Nevertheless, the effect of **15b** on survival was not significantly different from the parental temozolomide compound. In addition, the low binding affinity of the compound (Table 1) combined with its relatively low efficacy in the CAIX⁺ as compared to the CAIX⁻ cells minimizes its potential for further development. These results furthermore suggest that temozolomide efficacy is not affected by CAIX dependent changes in extracellular pH during hypoxia. A reduction of temozolomide conversion and efficacy might require lower pH levels, *i.e.* pH < 6.6, which may have not been achieved in the present experiments [8, 9, 39].

Figure 1: Clonogenic cell survival of confluent MDCK CAIX⁻ and CAIX⁺ cells during normoxia (21% O₂) and hypoxia (0.02% O₂) when exposed to temozolomide (TMZ) and the CAIXi conjugated derivative **15b**. Surviving fraction (%) was normalized to vehicle control. Average ± SEM of three independent biological repeats is shown. Asterisks indicate statistical significance (* $p < 0.05$; *** $p < 0.001$).

ATR inhibitor derivatives

Preclinical experiments have shown that Ataxia Telangiectasia and Rad3-related protein inhibitors (ATRi) reduce the DNA repair capacity resulting in enhanced cell death and decreased tumor growth when combined with either chemo- or radiotherapy [46-48]. However ATRi are not highly tumor specific, thus targeting these compounds towards the CAIX expressing areas of a tumor might increase their therapeutic benefit. The effect on cell viability of the parental ATRi (VE-821 and VE-822) and their CAIXi conjugated derivatives (**12** and **13**) was tested in combination with radiotherapy to induce DNA damage where a higher radiation dose was applied to anoxic cells, since those are more radioresistant [49, 50]. The parental ATRi and the CAIXi conjugated derivatives in combination with radiation decreased cell viability as compared to radiation only in the

CAIX⁺ cells ($p < 0.05$) under both normoxic and anoxic conditions, but not in the CAIX⁻ cells (Figure 2). The only exception is the derivative **13**, which had no significant effect on cell viability during anoxic conditions in both cell lines as compared to radiation alone ($p = 0.09$ and $p = 0.08$ for CAIX⁻ and CAIX⁺ cells, respectively). More importantly, the CAIXi conjugated derivative **12** was more effective than its respective parental ATRi (VE-821) in the CAIX⁺ ($p < 0.01$ during normoxia and anoxia), but not the CAIX⁻ cells ($p = 0.52$ and $p = 0.72$ for normoxia and anoxia, respectively), suggesting a CAIX specific effect. In contrast, the CAIXi conjugated derivative **13** in combination with radiation was less effective in reducing cell viability than the parental compound VE-822 in CAIX⁺ cells ($p < 0.001$ and $p < 0.01$ during normoxic and anoxic conditions, respectively). Although radiation induced similar effects on cell viability during normoxic and anoxic conditions, the efficacy of derivative **12** did not increase further during anoxic conditions as compared to normoxic conditions, even though CAIX expression is upregulated under hypoxic conditions (Supplementary Figure S1) and these conditions are essential for CAIXi binding [43, 45]. Although derivative **12** indeed proved to be more effective in CAIX⁺ than in CAIX⁻ cells in combination with radiation, its efficacy might however not be solely dependent on binding to CAIX, which is consistent with unfavorable K_i values of the compound (Table 1). Exposing both cell lines to ATRi without irradiation decreased cell viability of both cell lines during normoxic and anoxic conditions, although this effect appeared to be slightly more pronounced in the CAIX⁻ cells (Supplementary Figure S5). Differences in ATRi response between the cell lines when combined with radiation might be explained by a lower number of cells in the resistant S-phase of the cell cycle [51], or by a decreased DNA repair capacity in cells with lower intracellular pH [52-54], *i.e.* those that do not express CAIX. This may also explain the difference in sensitivity to cytotoxic drugs between both cell lines, although further investigations are required to prove this causal relationship.

Figure 2: Relative cell viability (%) in MDCK CAIX⁻ and CAIX⁺ cells exposed to ATR inhibitors (VE-821 and VE-822) or the CAIXi conjugated derivatives (**12** and **13**) in combination with radiation during normoxia (21% O₂) and anoxia (<0.02% O₂). Normoxic cells were irradiated with 2 Gy and anoxic cells with 4 Gy to induce similar effects on cell viability. Cells were exposed to 500 nM VE-821 and **12**, and to 50 nM VE-822 and **13**. Average $\pm$ SEM of three independent biological repeats is shown. Asterisks indicate statistical significance (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Phenformin derivatives

Phenformin (1-(diaminomethylidene)-2-(2-phenylethyl)guanidine) is a drug used to treat diabetes, but was withdrawn from the North-American market in the 1970s by the Food and Drug Administration (FDA) due to a high risk of developing lactic acidosis [55]. Treating patients with a similar but less potent drug metformin was found to be associated with a decrease in cancer incidence and an increased life span of cancer patients [56]. The repurposing of these compounds as anti-cancer agents is therefore being investigated where phenformin is found to be more lipophilic, thereby requiring less active transport than metformin [57]. The proposed mechanism of action of phenformin is its ability to inhibit mitochondrial respiration, which will consequently result in a decreased ATP production, thereby reducing tumor cell growth and improving tumor oxygenation as a result of decreased oxygen consumption [58, 59]. Conjugating phenformin with CAIXi might make the drug more tumor-specific leading to reduced normal tissue toxicity. Since tumor cells are more sensitive to phenformin treatment due to their altered energy metabolism, human colorectal HCT116 cells, with or without CAIX knockdown [24, 28] were used to study the effect of phenformin and its CAIXi conjugated derivative **18** on mitochondrial respiration. Western blotting confirms low expression of CAIX in CAIX KD cells under hypoxic conditions as compared with control cells (Supplementary Figure S1B). As expected, CAIX levels were low in both cell lines under normoxic conditions. Phenformin significantly reduced oxygen consumption rate (OCR) in both cell lines ($p < 0.05$), independent of CAIX expression levels (Figure 3). In contrast, the CAIXi conjugated derivative **18** was ineffective in reducing OCR, even when a four-fold higher concentration was used.

Figure 3: Oxygen consumption rate (OCR) of HCT116 cells with CAIX (shSCR) or without CAIX expression (shCAIX) exposed to phenformin or the CAIXi conjugated derivative **18**. OCR was normalized to baseline OCR levels before compound injection. Average $\pm$ SEM of four independent biological repeats is shown. Asterisks indicate statistical significance ($*p < 0.05$).

Conclusion

Overall our hypothesis that newly designed dual-target drugs are more selective for CAIX expressing cells and are able to modulate their own efficacy by inhibiting CAIX function was not confirmed. Of all derivatives included, only one (*i.e.* the ATRi derivative **12**) proved more effective than its parental compound when combined with irradiation in CAIX⁺ cells versus CAIX⁻ cells. Nevertheless, the effect of this compound may not only be related to binding of the compound to CAIX due to limited binding affinity and the lack of further increase in its efficacy under hypoxic conditions. The rest of the derivatives included in this study did not show an increased efficacy in CAIX⁺ versus CAIX⁻ cells, or an efficacy that depended on oxygen levels, *i.e.* hypoxia versus normoxia. Nevertheless, since the parental compounds proved effective in these experiments the conjugation of the CAIXi moiety with the cytotoxic compounds may have caused conformational changes, thereby altering the compounds efficacy. In addition, these conformational changes may have also limited the binding of the CAIXi moiety (sulfonamide, sulfamide or aminoxysulfonamide) into the Zn²⁺ containing active pocket of CAIX, which explains the lack of CAIX specificity and binding affinity for human CAs (Table 1) of the compounds. Alternative strategies to target the CAIX expressing cells in a tumor, *e.g.* antibody targeting or an increased number of CAIXi conjugated molecules [60], might therefore be more promising options to pursue in the future.

EXPERIMENTAL SECTION CHEMISTRY.

General

Unless otherwise specified, reagents and solvents were of commercial quality and were used without further purification. All reactions were carried out under an inert atmosphere of nitrogen. TLC analyses were performed on silica gel 60 F₂₅₄ plates (Merck Art.1.05554). Spots were visualized under 254nm UV illumination, or by ninhydrin solution spraying. Melting points (mp) were determined on a Büchi Melting Point 510 and are uncorrected. ¹H and ¹³C NMR spectra were recorded on Bruker DRX-400 spectrometer using DMSO-*d*₆ as a solvent and tetramethylsilane as an internal standard. For ¹H NMR spectra, chemical shifts are expressed in δ (ppm) downfield from tetramethylsilane, and coupling constants (*J*) are expressed in Hertz. Electron Ionization mass spectra were recorded in positive or negative mode on a Waters MicroMass ZQ. All compounds that were tested in the biological assays were analyzed by High-resolution ESI mass spectra (HRMS) using on a Q-ToF I mass spectrometer fitted with an electrospray ion source in order to confirm the purity of >95%.

4-(4-(Bis(2-chloroethyl)amino)phenyl)butanoyl chloride (1)

Oxalyl chloride (32.8 mmol, 2.0 equiv.) was added slowly over a period of 1.0 hr at 5–10 °C to a stirred solution of chlorambucil (16.4 mmol, 1.0 equiv.) in DCM (25.0 mL, 5.0 vol.) and a catalytic amount of *N,N*-dimethylformamide. The reaction mixture was stirred at ambient temperature for 2–3 hr, after which excess oxalyl chloride and DCM were removed under reduced pressure. The chlorambucil acid chloride that was obtained was a pale green solid in quantitative yield, which was used as such for the synthesis of Compounds **2a–c**.

4-(4-(Bis(2-chloroethyl)amino)phenyl)butan-1-ol (3)

Compound **1** (15.5 mmol, 1.0 equiv.) was dissolved in DCM (125 mL) and methanol (75 mL, 3 vol.) was slowly added over a period of 1 hr at 15–20 °C. The reaction mixture was stirred at ambient temperature for 2 hr. The reaction mixture was concentrated and the residue was dissolved in ethyl acetate (125 mL, 5 vol.) and washed successively with a 5% aq. NaHCO₃. Evaporation of the solvent under reduced pressure resulted in the chlorambucil methyl ester (16.4 mmol, 1.0 equiv.) in 95% yield as a light brown oil, which was added to a suspension of lithium aluminium hydride (32.8 mmol, 2 equiv.) in anhydrous THF (100 mL, 4 vol.) at 0–5 °C for a period of 1 hr. The reaction mixture was thereafter allowed to stir at ambient temperature for 2–3 hr. Next, the reaction mixture was cooled to 0–5 °C and quenched slowly with ethyl acetate (250 mL, 10 vol.) followed by water (100 mL, 4 vol.). The reaction mixture was filtered through celite and ethyl acetate (50 mL, 2 vol.) was used to wash the celite bed. The organic layer was washed with water (100 mL, 4 vol.), dried over anhydrous Na₂SO₄, and filtered. Evaporation of the solvent under reduced pressure resulted in 99% yield of the crude alcohol as a pale yellow oil, which was used in the next step.

4-(4-(Bis(2-chloroethyl)amino)phenyl)butyl carbonochloridate (4)

DMF (1.4 g) and sodium carbonate (75.79 mmol, 1.1 equiv.) were added to a solution of triphosgene (37.89 mmol, 0.55 equiv.) in toluene (300 mL, 15 vol.) at ambient temperature. The reaction mixture was cooled to 0–5 °C and maintained at the same temperature for 30 minutes. Next, a solution of **3** (68.9 mmol, 1.0 equiv.) in toluene (100 mL, 5 vol.) was added to the stirred reaction mixture at 0–5 °C during 30 min. This reaction mixture was stirred for 4–5 hr at room temperature. The reaction mixture was filtered thereafter and the solid was washed with toluene (100 mL, 5 vol.). Evaporation of the solvent under reduced pressure resulted in the chloroformate **4** with a 64% yield as a yellow viscous liquid, which was used for the synthesis of carbamates (compounds **5a–c**).

General procedure for the preparation of compounds (2a–c and 5a–c)

To a solution of aminoalkylbenzene sulfonamide (1.0 equiv.) in acetonitrile (225 mL, 15 vol.) and N,N-diisopropylethylamine (2.5 equiv.) a solution of compound **1** (**2a–c**) or compound **4** (**5a–c**) (1.0 equiv.) in acetonitrile (75 mL, 5 vol.) was added over a period of 1 hr and stirred overnight at ambient temperature. After completion, the reaction mixture was concentrated and the residue obtained was dissolved in ethyl acetate (150 mL, 10 vol.). The organic layer was successively washed with 2N HCl solution (100 mL × 2) in water, dried over anhydrous Na₂SO₄, and filtered. After evaporation of the solvent under reduced pressure a pale yellow solid was obtained as a crude product. This crude product was purified with column chromatography using a silica gel (40% ethyl acetate in hexanes) to obtain compound **2a–c** and **5a–c** in a 51–87% yield.

General procedure for Amination of 3-Chlorobenzotriazine-1,4-Di-N-Oxides (7 and 10)

4-(2-Aminoethyl) benzene sulfonamide (8.25 mmol, 3.0 equiv.) was added to a stirring solution of 3 chlorobenzotriazine-1,4-di-N-oxide (2.75 mmol, 1.0 equiv.) in dimethoxyethane (30 mL) and the mixture was stirred overnight at reflux temperature. The next day mixture was cooled to room temperature and concentrated under vacuum, after which the residue was dissolved in ammonium hydroxide solution and extracted with ethyl acetate. The organic layer was dried over Na₂SO₄, filtered and concentrated under vacuum. The residue was purified by chromatography using a silica gel with methylene chloride- methanol 98:2 v-v as an eluent to obtain the expected compound as a yellow powder with an 85–94% yield.

General procedure for Oxidation (8 and 11)

Hydrogen peroxide (12.9 mmol, 10 equiv.) was added dropwise to a stirred solution of trifluoroacetic anhydride (12.9 mmol, 10 equiv.) in DCM at 0 °C. This reaction mixture was stirred at 0 °C for 5 min, warmed to room temperature for 10 min, and cooled to 5 °C. Next, the mixture was added to a stirred solution of mono oxide (1.29 mmol, 1.0 equiv.) in DCM at 0 °C and stirred at room temperature for 2 to 3 days. The reaction mixture was carefully diluted with water and basified with aqueous NH₄OH and extracted with CHCl₃. The organic fraction was dried over Na₂SO₄, filtered and evaporated to obtain the residue. This residue was purified by chromatography using a silica gel with methylene chloride- methanol 98:2 v-v as an eluent to obtain the expected compound as an orange red powder with a 46–96% yield.

General procedure for synthesis of ATRi derivatives (12 and 13)

A solution of VE-821 or VE-822 (0.54 mmol, 1.0 equiv.) and triethylamine (1.62 mmol, 3.0 equiv.) in 10 mL of methylene chloride was added to a mixture of chlorosulfonyl isocyanate (0.68 mmol, 1.2 equiv.) and *tert*-butanol (0.648 mmol, 1.2 equiv.) in 2 mL of methylene chloride. The mixture was stirred at room temperature for 1.0 hr, diluted with ethyl acetate, and washed with water. The organic layer was then dried over anhydrous Na_2SO_4 , filtered and concentrated under vacuum. The residue was purified by chromatography with a silica gel and methylene chloride-methanol 98:2 as an eluent. This intermediate was thereafter diluted in a solution of trifluoro acetic acid in methylene chloride (20% vol.) and stirred at room temperature for 6 hr. Next, the mixture was concentrated under vacuum and co-evaporated with diethyl ether multiple times to obtain the expected compound with a 58–65% yield.

General procedure for synthesis of Temozolomide derivatives

To a slurry of 3-methyl-4-oxo-3,4-dihydroimidazo [5,1-d][1,2,3,5] tetrazine-8-carboxylic acid (1.0 mmol, 1.0 equiv.) in DCM Bop (1.0 mmol, 1.0 equiv.), amine (1.1 mmol, 1.1 equiv.) and tri ethylamine (2.5 mmol, 2.5 equiv.) were added. This reaction mixture was stirred overnight at room temperature and filtered to obtain the expected compounds with a 41–95% yield.

4-(4-(Bis(2-chloroethyl)amino)phenyl)-N-(4-sulfamoylphenyl)butanamide (2a).

mp: 155–157 °C; ^1H NMR (400 MHz, $\text{DMSO}-d_6$), δ 10.22 (s, 1H), 7.73 (d, J = 4.4, 4H), 7.23 (s, 2H), 7.05 (d, J = 8.7, 2H), 6.67 (d, J = 8.7, 2H), 3.70 (d, J = 8.6, 8H), 2.54–2.50 (m, 2H), 2.34 (t, J = 8.6, 2H), 1.90–1.78 (m, 2H); ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$), δ 171.68, 144.46, 142.23, 138.03, 129.53, 126.65, 118.51, 111.90, 52.22, 41.17, 35.84, 33.54, 26.87; MS (ESI^+) m/z 458.11 $[\text{M}+\text{H}]^+$, 460.10 $[\text{M}+2]^+$. HRMS (ESI) $[\text{M}+\text{H}]^+$ calculated for $[\text{C}_{20}\text{H}_{26}\text{N}_3\text{O}_3\text{SCl}_2]^+$: 458.1072, found: 458.1075.

4-(4-(Bis(2-chloroethyl)amino)phenyl)-N-(4-sulfamoylbenzyl)butanamide (2b).

mp: 130–132 °C; ^1H NMR (400 MHz, $\text{DMSO}-d_6$), δ 8.41 (t, J = 5.9, 1H), 7.76 (d, J = 8.3, 2H), 7.41 (d, J = 8.3, 2H), 7.31 (s, 2H), 7.02 (d, J = 8.6, 2H), 6.66 (d, J = 8.6, 2H), 4.31 (d, J = 5.9, 2H), 3.69 (s, 8H), 2.45 (t, J = 7.5, 2H), 2.15 (t, J = 7.5, 2H), 1.82–.72 (m, 2H); ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$), δ 172.15, 144.42, 143.92, 142.54, 129.86, 129.33, 127.46, 125.68, 111.89, 52.22, 41.67, 41.17, 34.84, 33.66, 27.39; MS (ESI^+) m/z 472.12 $[\text{M}+\text{H}]^+$, 474.12 $[\text{M}+2]^+$. HRMS (ESI) $[\text{M}+\text{H}]^+$ calculated for $[\text{C}_{21}\text{H}_{28}\text{N}_3\text{O}_3\text{SCl}_2]^+$: 472.1228, found: 472.1236.

4-(4-(Bis(2-chloroethyl)amino)phenyl)-N-(4-sulfamoylphenethyl)butanamide (2c).

mp: 108–110 °C; ^1H NMR (400 MHz, DMSO- d_6), δ 7.89 (t, J = 5.6, 1H), 7.74 (d, J = 8.3, 2H), 7.38 (d, J = 8.3, 2H), 7.30 (s, 2H), 7.00 (d, J = 8.6, 2H), 6.66 (d, J = 8.6, 2H), 3.70 (d, J = 8.9, 8H), 3.29 (dd, J = 13.0, 5.6, 2H), 2.78 (t, J = 7.5, 2H), 2.41 (t, J = 7.5, 2H), 2.08–1.98 (m, 2H), 1.76–1.65 (m, 2H); ^{13}C NMR (101 MHz, DMSO- d_6), δ 171.95, 144.39, 143.80, 142.01, 129.89, 129.21, 125.67, 111.87, 52.23, 41.17, 34.87, 33.61, 27.32; MS (ESI $^+$) m/z 486.14 [M+H] $^+$, 488.14 [M+2] $^+$. HRMS (ESI) [M+H] $^+$ calculated for [C₂₂H₃₀N₃O₃SCl₂] $^+$: 486.1385, found 486.1387.

4-(4-(Bis(2-chloroethyl)amino)phenyl)butyl (4-sulfamoylphenyl)carbamate (5a).

mp: 156–158 °C; ^1H NMR (400 MHz, DMSO- d_6), δ 10.01 (s, 1H), 7.76–7.69 (m, 2H), 7.64–7.56 (m, 2H), 7.22 (s, 2H), 7.04 (d, J = 8.7, 2H), 6.66 (d, J = 8.7, 2H), 4.12 (t, J = 6.0, 2H), 3.77–3.63 (m, 8H), 1.70–1.54 (m, 4H); ^{13}C NMR (101 MHz, DMSO- d_6), δ 153.50, 144.40, 142.34, 137.48, 130.06, 129.30, 126.77, 117.50, 111.89, 64.43, 52.22, 41.17, 33.68, 28.09, 27.62; MS (ESI $^+$) m/z 488.12 [M+H] $^+$, 490.12 [M+2] $^+$. HRMS (ESI) [M+H] $^+$ calculated for [C₂₁H₂₈N₃O₄SCl₂] $^+$: 488.1178, found: 488.1184.

4-(4-(Bis(2-chloroethyl)amino)phenyl)butyl (4-sulfamoylbenzyl)carbamate (5b).

mp: 98–100 °C; ^1H NMR (400 MHz, DMSO- d_6), δ 7.76 (t, J = 8.5, 3H), 7.41 (d, J = 8.5, 2H), 7.31 (s, 2H), 7.02 (d, J = 8.6, 2H), 6.66 (d, J = 8.6, 2H), 4.23 (d, J = 6.1, 2H), 3.98 (s, 2H), 3.75–3.63 (m, 8H), 2.47 (s, 2H), 1.55 (m, 4H); ^{13}C NMR (101 MHz, DMSO- d_6), δ 156.63, 144.37, 143.97, 142.61, 130.13, 129.26, 127.27, 125.70, 111.88, 63.84, 52.23, 43.37, 41.18, 33.68, 28.31, 27.64; MS (ESI $^+$) m/z 502.13 [M+H] $^+$, 504.13 [M+2] $^+$. HRMS (ESI) [M+H] $^+$ calculated for [C₂₂H₃₀N₃O₄SCl₂] $^+$: 502.1334, found: 502.1338.

4-(4-(Bis(2-chloroethyl)amino)phenyl)butyl (4-sulfamoylphenethyl)carbamate (5c).

mp: 100–102 °C; ^1H NMR (400 MHz, DMSO- d_6), δ 7.77–7.70 (m, 2H), 7.37 (d, J = 8.2, 2H), 7.30 (s, 2H), 7.19 (t, J = 5.5, 1H), 7.02 (d, J = 8.6, 2H), 6.66 (d, J = 8.6, 2H), 3.94 (d, J = 5.5, 2H), 3.69 (s, 8H), 3.21 (dd, J = 13.3, 6.6, 2H), 2.76 (dd, J = 16.9, 9.8, 2H), 2.46 (s, 2H), 1.52 (s, 4H); ^{13}C NMR (101 MHz, DMSO- d_6), δ 156.30, 144.37, 143.58, 142.03, 130.15, 129.19, 125.67, 111.88, 63.51, 52.23, 41.28, 35.09, 33.67, 28.32, 27.63; MS (ESI $^+$) m/z 516.15 [M+H] $^+$, 518.15 [M+2] $^+$. HRMS (ESI) [M+H] $^+$ calculated for [C₂₃H₃₂N₃O₄SCl₂] $^+$: 516.1491, found: 516.1490.

3-(4-Sulfamoylphenethylamino) benzo [e][1,2,4] triazine 1-oxide (7).

Compound **7** was synthesized from **6** by a general amination method and resulted in a yellow solid with a yield of 94%. mp: 250–252 °C; ^1H NMR (400 MHz, DMSO- d_6), δ 8.15 (s, 1H), 8.13 (s, 1H), 8.03 (s, 1H), 7.82–7.71 (m, 3H), 7.60 (d, J = 8.0, 1H), 7.47 (d, J = 8.0, 2H), 7.38–7.30 (m, 1H), 7.28 (s, 2H), 3.60 (d, J = 6.2, 2H), 3.01 (dd, J = 6.2, 2H); ^{13}C NMR (101 MHz, DMSO) δ 158.80, 143.70, 142.07, 135.76, 129.25, 125.92, 124.66, 119.93,

41.92, 34.09; MS (ESI⁺) m/z 346.10 [M+H]⁺. HRMS (ESI) [M+H]⁺ calculated for [C₁₅H₁₆N₅O₃S]⁺: 346.0974, found: 346.0973.

3-((4-Sulfamoylphenethyl)amino)benzo[e][1,2,4]triazine 1,4-dioxide (8).

Compound **8** was synthesized from **7** by a general oxidation method, resulting in an orange red solid with a yield of 96%. mp: 210–212 °C; ¹H NMR (400 MHz, DMSO-*d*₆), δ 8.31 (t, *J* = 6.1, 1H), 8.22 (d, *J* = 8.1, 1H), 8.13 (d, *J* = 8.1, 1H), 7.97–7.89 (m, 1H), 7.75 (d, *J* = 8.3, 2H), 7.61–7.53 (m, 1H), 7.47 (d, *J* = 8.3, 2H), 7.29 (s, 2H), 3.67 (dd, *J* = 7.2, 6.1, 2H), 3.03 (t, *J* = 7.2, 2H); ¹³C NMR (101 MHz, DMSO-*d*₆), δ 149.67, 143.19, 142.16, 138.19, 135.48, 130.07, 129.26, 127.04, 125.71, 121.13, 116.89, 41.76, 34.18; MS (ESI⁺) m/z 362.09 [M+H]⁺. HRMS (ESI) [M+H]⁺ calculated for [C₁₅H₁₆N₅O₄S]⁺: 362.0923, found: 362.0928.

*3-(4-Sulfamoylphenethylamino)-7,8-dihydro-6H-indeno [5,6-*e*][1,2,4] triazine 1-oxide (10).*

Compound **10** was synthesized from **9** by using a general amination method, which resulted in a yellow solid with a 85% yield. mp: 238–240 °C; ¹H NMR (400 MHz, DMSO-*d*₆), δ 7.95 (s, 1H), 7.82 (s, 1H), 7.74 (t, *J* = 10.0, 2H), 7.49–7.39 (m, 3H), 7.28 (s, 2H), 3.57 (dd, *J* = 13.0, 6.8, 2H), 3.02–2.90 (m, 6H), 2.11–1.99 (m, 2H); ¹³C NMR (101 MHz, DMSO-*d*₆), δ 157.56, 153.64, 142.79, 141.97, 130.10–127.99, 125.35, 112.78, 41.77, 32.35, 31.60, 25.25; MS (ESI⁺) m/z 386.13 [M+H]⁺. HRMS (ESI) [M+H]⁺ calculated for [C₁₈H₂₀N₅O₃S]⁺: 386.1287, found: 386.1291.

*3-((4-Sulfamoylphenethyl) amino)-7,8-dihydro-6H-indeno [5,6-*e*][1,2,4] triazine 1,4-dioxide (11).*

Compound **11** was synthesized from **10** by using a general oxidation method resulting in an orange red solid with a yield of 46%. mp: 218–220 °C; ¹H NMR (400 MHz, DMSO-*d*₆), δ 8.19 (s, 1H), 7.98 (d, *J* = 24.2, 2H), 7.75 (d, *J* = 7.8, 2H), 7.46 (d, *J* = 7.7, 2H), 7.29 (s, 2H), 3.65 (d, *J* = 6.2, 2H), 3.12–2.92 (m, 6H), 2.17–1.99 (m, 2H); ¹³C NMR (101 MHz, DMSO-*d*₆), δ 154.56, 149.25, 145.07, 143.22, 142.15, 129.23, 125.71, 41.76, 32.74, 31.80, 25.24; MS (ESI⁺) m/z 402.12 [M+H]⁺. HRMS (ESI) [M+H]⁺ calculated for [C₁₈H₂₀N₅O₄S]⁺: 402.1236, found: 402.1234.

6-(4-(Methylsulfonyl)phenyl)-N-phenyl-3-(sulfamoylamino)pyrazine-2-carboxamide (12).

Compound **12** was synthesized from commercially purchased VE-821 by using the general procedure described above, which resulted in a yellow solid with an overall yield of 58%. mp: 233–235 °C; ¹H NMR (400 MHz, DMSO-*d*₆), δ 11.27 (s, 1H), 10.82 (s, 1H), 9.28 (s, 1H), 8.64 (d, *J* = 8.6, 2H), 8.08 (d, *J* = 8.6, 2H), 7.83–7.76 (m, 2H), 7.68 (s, 2H), 7.48–7.39 (m, 2H), 7.23 (dd, *J* = 14.0, 6.6, 1H), 3.30 (s, 3H); ¹³C NMR (101 MHz, DMSO-*d*₆), δ 164.59–163.27, 149.32–147.73, 144.46–143.38, 141.16, 139.57, 137.17–136.45,

128.69, 127.46, 125.15, 122.08, 43.46; MS (ESI⁺) m/z 448.07 [M+H]⁺. HRMS (ESI) [M+H]⁺ calculated for [C₁₈H₁₈N₅O₂S₂]⁺: 448.0749, found: 448.0748.

5-(4-(Isopropylsulfonyl) phenyl)-3-(3-(4-((methylamino) methyl) phenyl) isoxazol-5-yl) pyrazin-2-carboxamide (13).

Compound **13** was synthesized from commercially purchased VE-822 by using the general procedure described above, which resulted in a yellow solid with an overall yield of 65%. mp: 242–244 °C; ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.94 (s, 1H), 8.38 (d, *J* = 8.5, 2H), 8.02 (d, *J* = 8.2, 2H), 7.93 (d, *J* = 8.5, 2H), 7.79 (s, 1H), 7.54 (d, *J* = 8.2, 2H), 7.20 (s, 2H), 6.96 (s, 2H), 4.17 (s, 2H), 3.54–3.38 (m, 1H), 2.58 (s, 3H), 1.17 (t, *J* = 14.1, 6H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 167.67, 162.00, 151.75, 142.47, 141.04, 139.53, 137.62, 135.78, 129.00, 127.17, 125.69, 124.47, 102.16, 54.22, 53.49, 34.94, 15.19; MS (ESI⁺) m/z 543.15 [M+H]⁺. HRMS (ESI) [M+H]⁺ calculated for [C₂₄H₂₇N₆O₅S₂]⁺: 543.1484, found: 543.1484.

*3-Methyl-4-oxo-N-(4-sulfamoylphenethyl)-3,4-dihydroimidazo[5,1-*d*][1,2,3,5]tetrazine-8-carboxamide (15a).*

Compound **15a** was synthesized from **14** by reacting it with 4-(2-aminoethyl) benzene sulfonamide using the general procedure for synthesizing temozolomide derivatives described above. This reaction resulted in a white solid with a yield of 50%. mp: 195–197 °C; ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.83 (s, 1H), 8.58 (t, *J* = 5.9, 1H), 7.74 (d, *J* = 8.3, 2H), 7.44 (d, *J* = 8.3, 2H), 7.30 (s, 2H), 3.86 (s, 3H), 3.58 (dd, *J* = 13.4, 6.8, 2H), 2.96 (t, *J* = 7.1, 2H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 159.67, 143.64, 142.05, 139.20, 134.45, 130.30, 129.14, 128.46, 125.73, 36.16, 34.78; MS (ESI⁺) m/z 378.10 [M+H]⁺. HRMS (ESI) [M+H]⁺ calculated for [C₁₄H₁₆N₇O₄S]⁺: 378.0984, found: 378.0986.

*3-Methyl-4-oxo-N-(sulfamoyloxy)-3,4-dihydroimidazo[5,1-*d*][1,2,3,5]tetrazine-8-carboxamide (15b).*

Compound **15b** was synthesized from **14** by reacting it with aminoxysulfonamide using the general procedure for synthesizing temozolomide derivatives described above. This reaction resulted in a white solid with a yield of 41%. mp: 195–197 °C; ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.81 (s, 1H), 7.80 (s, 1H), 7.67 (s, 1H), 7.30 (s, 2H), 3.86 (s, 3H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 161.51, 139.16, 134.57, 130.51, 128.37, 45.72;

*3-Methyl-4-oxo-N-(4-sulfamoylbenzyl)-3,4-dihydroimidazo[5,1-*d*][1,2,3,5]tetrazine-8-carboxamide (15c).*

Compound **15c** was synthesized from **14** by reacting it with 4-(aminomethyl) benzene sulfonamide hydrochloride using the general procedure for synthesizing temozolomide derivatives described above. This reaction resulted in a white solid with a yield of 88%. mp: 185–187 °C; ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.20 (t, *J* = 6.2, 1H), 8.87 (s, 1H), 7.77 (d, *J* = 8.3, 2H), 7.50 (d, *J* = 8.3, 2H), 7.31 (s, 2H), 4.55 (d, *J* = 6.3, 2H), 3.87 (s, 3H); ¹³C

NMR (101 MHz, DMSO- d_6), δ 159.96, 143.85, 142.63, 142.63, 138.96, 134.69, 130.11, 129.28, 128.62, 127.68, 125.77, 41.94, 36.23; MS (ESI⁺) m/z 364.08 [M+H]⁺. HRMS (ESI) [M+H]⁺ calculated for [C₁₃H₁₄N₇O₄S]⁺: 364.0828, found: 364.0826.

3-Methyl-4-oxo-N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxamide (15d).

Compound **15d** was synthesized from **14** by reacting it with 5-amino-1, 3,4-thiadiazole-2-sulfonamide hydrochloride using the general procedure for synthesizing temozolomide derivatives described above. This reaction resulted in a light yellow solid with a yield of 95%. mp: 128–130 °C; ¹H NMR (400 MHz, DMSO- d_6), δ 8.63 (s, 1H), 8.06 (s, 1H), 7.81 (s, 1H), 7.35 (s, 2H), 3.83 (s, 3H); ¹³C NMR (101 MHz, DMSO- d_6), δ 171.73, 170.89, 165.39, 161.04, 157.91, 139.53, 134.24, 127.88; MS (ESI⁺) m/z 358.01 [M+H]⁺. HRMS (ESI) [M+H]⁺ calculated for [C₈H₈N₉O₄S₂]⁺: 358.0141, found: 358.0140.

Synthesis of 4-(2-(3-carbamimidoylguanidino)ethyl)benzenesulfonamide hydrochloride salt (18)

4-(2-Aminoethyl)benzenesulfonamide **16** (0.5g, 1.0 equiv.) and cyanoguanidine **17** (0.21g, 1.0 equiv.) were suspended in *n*-butanol (5.0 mL) and treated with a 6.0 M aqueous hydrochloric acid solution (1.0 equiv., 0.4 mL). The mixture was treated at 100 °C overnight and the solvents were removed under vacuum. The residue was thereafter crystallized from isopropyl alcohol (IPA) to obtain compound **18** as a white solid with a 75% yield. mp: 154–159 °C; ¹H NMR (400 MHz, DMSO- d_6), δ 7.87 (d, 2H, J = 8.4, Ar-H), 7.82 (brs, 2H, exchangeable with D₂O), 7.50 (d, 2H, J = 8.4, Ar-H), 7.38 (brs, 1H, exchangeable with D₂O), 6.62 (brs, 2H, exchangeable with D₂O), 3.15 (t, 2H, J = 6.7), 2.98 (t, 2H, J = 6.7); ¹³C NMR (101 MHz, DMSO- d_6), δ 164.0, 143.7, 142.0, 130.3, 127.0, 119.3, 61.4, 34.1; MS (ESI⁺) m/z 285.11 [M+H]⁺.

CA INHIBITION ASSAYS

To measure the CA-catalyzed CO₂ hydration activity an Applied Photophysics stopped-flow instrument was used [61]. To maintain ionic strength Na₂SO₄ (20 mM) was used with HEPES (20 mM, pH 7.5) as a buffer and Phenol red (0.2 mM) as an indicator working at the maximum absorbance of 557 nm, which was used to follow the initial rates of the CA-catalyzed CO₂ hydration for a duration of 10–100 seconds. To determine the kinetic parameters and inhibition constants varying CO₂ concentrations were included (1.7–17 mM). Initial velocity was assayed with at least six traces of the initial 5–10% of the reaction for each compound. Compounds were dissolved in distilled-deionized water (0.01 nM). The combined enzyme solutions and compounds were incubated for 15 minutes at room temperature to allow for the E-I complex formation prior to measurements. The nonlinear least-squares method of PRISM 3 was used to estimate the inhibi-

tion constants and the mean of three independent estimations is reported. The CA isoforms included are recombinant proteins obtained in house.

BIOLOGICAL ASSAYS

Cells

All cell lines used were cultured in DMEM supplemented with 10% fetal bovine serum. Canine kidney epithelial MDCK cells overexpressing human CAIX (CAIX⁺) or a scrambled control vector (CAIX⁻) have been described before [37, 44]. The HCT116 constitutive CAIX knockdown cell line and its scrambled control have also been described before and were kindly provided by Professor Adrien Harris (Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe hospital, Oxford, UK) [24, 28, 62]. Cells were exposed to hypoxic or anoxic conditions in a hypoxic chamber (MACS VA500 microaerophilic workstation, Don Whitley Scientific, UK) with 0.2 or <0.02% O₂, respectively, and 5% CO₂ and residual N₂ to upregulate and activate CAIX. Normoxic cells were grown in normal incubators with 21% O₂, 5% CO₂ at 37 °C.

Cell viability assays

The efficacy of the cytotoxic derivatives was compared to their respective parental compounds in cell viability assays using alamarBlue® (Invitrogen). In short, MDCK cells were seeded in 96-well plates and allowed to attach overnight. The next day plates were exposed to hypoxia and DMEM was replaced with pre-incubated hypoxic DMEM. In contrast, testing the ATRi was performed in anoxic conditions to decrease the radiosensitivity of the cells. In parallel normoxic 96-well plates were incubated in normal incubators with 21% O₂ and 5% CO₂. Compounds were dissolved in DMSO (0.5%, Sigma-Aldrich) and final concentrations were made with pre-incubated hypoxic or normoxic DMEM and added to the wells after 24 hr of exposure. To test the ATR inhibitors cells were exposed to the compounds 1 hr prior to irradiation and the 96-well plates were irradiated (225 kV Philips X-ray tube) with 2 Gy (normoxia) or 4 Gy (anoxia). Cells were exposed to compounds for a total of 2 hr for chlorambucil and tirapazamine, or 72 hr for temozolomide and the ATR inhibitors, after which medium was washed off and replaced with fresh medium. For chlorambucil, tirapazamine, and ATR inhibitor derivatives cells were allowed to grow for an additional 72 hr under normoxic conditions prior to measurement, whereas cells exposed to temozolomide derivatives remained in hypoxic conditions prior to measurement. Cells were allowed to convert alamarBlue® for two hr during normoxic conditions, which corresponds with their metabolic function and is a measure for cell viability.

Clonogenic assays

Clonogenic survival of MDCK cells was determined with high cell numbers to allow for CAIX-dependent extracellular acidification [44]. These cells were exposed to temozolomide or **15b** for 24 hr during normoxic or hypoxic conditions after which cells were trypsinized and reseeded in triplicate with known cell numbers. Cells were allowed to grow for 7 days to form colonies that were quantified after staining and fixation with 0.4% methylene blue in 70% ethanol. Surviving fraction was normalized to vehicle (0.5% DMSO).

Basal respiration measurements

Oxygen consumption rates (OCR) were determined using the Seahorse XF96 extracellular Flux analyzer (Agilent Technologies). Cells were seeded in a XF96 cell plate with normal growth medium at an optimized cell density of 1.5×10^4 cells/well. Plates were placed in a 5% CO₂ incubator at 37 °C in order to let the cells attach. Subsequently cells were incubated for 18 hr under hypoxic conditions (0.2% O₂). Culture medium was exchanged with DMEM containing 25 mM D-glucose, 4 mM L-glutamine and 1 mM sodiumpyruvate (GIBCO, Thermo Fisher) 60 minutes prior to the assay and plates were placed in a CO₂-free incubator at 37 °C. Prior to the first injection, baseline OCR was determined using a mixing period of 5 minutes and a measurement period of 3 minutes followed by 3 loops of mixing and measuring for 3 minutes each. Medium containing vehicle (PBS, Lonza), Phenformin Hydrochloride (Sigma-Aldrich), or the CAIXi conjugated phenformin derivative **18** were injected followed by several mixing and measurements cycles. Subsequently cells were washed with PBS and lysed in a 0.05% SDS (Sigma-Aldrich) solution. Protein quantification for normalization purposes was performed using Pierce™ BCA Protein Assay Kit (Thermo Fisher).

Western Blot

To validate CAIX expression in the genetically modified cell lines protein immunoblotting was performed after 24 hr of hypoxia exposure (0.2% O₂) as described previously [43]. Primary antibodies used included the anti-CAIX M75 antibody (kindly provided by Professor Silvia Pastorekova, Institute of Virology, Slovak Academy of Science, Slovak Republic), and anti-β-actin (MP Biomedicals, #691001) as a reference protein.

Statistical analyses

GraphPad Prism (version 5.03) was used for all statistical analyses. For the cytotoxic compounds IC₅₀ values were estimated with the curve of the log(inhibitor) vs. normalized response (Variable slope). Means between groups were compared using unpaired t-tests, where p<0.05 indicated statistical significance.

SUPPLEMENTARY MATERIAL

Supplementary Figure 1: CAIX protein expression during normoxia and hypoxia of the MDCK (A) and HCT116 (B) cells and pH measurements of MDCK cells (C). Expression of β -actin was included as a reference protein. CAIX⁺ MDCK cells are overexpressing CAIX, and CAIX⁻ cells are control cells lacking both human and canine CAIX expression. HCT116 scrambled control vector cells (SCR) show hypoxia-dependent CAIX expression, whereas CAIX knockdown (KD) cells do not. During normoxic conditions HCT116 SCR cells have no detectable levels of CAIX, since the dot (B) is an artefact on film. The pH of the culture medium was measured of the MDCK cells after 24 hours of normoxic or hypoxic exposure (C). Mean $\pm$ SEM of three independent biological repeats are shown. Asterisks indicate statistical significance (*p < 0.05; **p < 0.01).

Supplementary Figure 2: Relative cell viability of CAIX⁺ and CAIX⁻ MDCK cells exposed to increasing concentrations of chlorambucil or the CAIXi conjugated derivatives during normoxic (N) and hypoxic (H) conditions. Relative cell viability was normalized to vehicle control (0.5% DMSO). Average $\pm$ SEM of three independent biological repeats is shown.

Supplementary Figure 3: Relative cell viability of CAIX⁺ and CAIX⁻ MDCK cells exposed to increasing concentrations of tirapazamine or the CAIXi conjugated derivatives during normoxic (N) and hypoxic (H) conditions. Relative cell viability was normalized to vehicle control (0.5% DMSO). Average $\pm$ SEM of three independent biological repeats is shown.

Supplementary Figure 4: Relative cell viability of CAIX⁺ and CAIX⁻ MDCK cells exposed to increasing concentrations of temozolomide or the CAIXi conjugated derivatives during normoxic (N) and hypoxic (H) conditions. Relative cell viability was normalized to vehicle control (0.5% DMSO). Average $\pm$ SEM of three independent biological repeats is shown.

Supplementary Figure 5: Relative cell viability (%) in MDCK CAIX⁻ and CAIX⁺ cells exposed to ATR inhibitors (VE-821 and VE-822) or the CAIXi conjugated derivatives (**12** and **13**) without radiation during normoxia (21% O₂) and anoxia (<0.02% O₂). Cells were exposed to 500 nM VE-821 and **12**, and to 50 nM VE-822 and **13**. Average $\pm$ SEM of three independent biological repeats is shown. Asterisks indicate statistical significance (* p < 0.05; ** p < 0.01; *** p < 0.001).

NMR spectra 1: 4-(4-(bis(2-chloroethyl)amino)phenyl)-N-(4-sulfamoylphenyl)butanamide (2a).

NMR spectra 2: 4-(4-(bis(2-chloroethyl)amino)phenyl)-N-(4-sulfamoylbenzyl)butanamide (2b).

NMR spectra 3: 4-(4-(bis(2-chloroethyl)amino)phenyl)-N-(4-sulfamoylphenethyl)butanamide(2c).

NMR spectra 4: 4-(4-(bis(2-chloroethyl)amino)phenyl)butyl (4-sulfamoylphenyl)carbamate (**5a**).

NMR spectra 5: 4-(4-(bis(2-chloroethyl)amino)phenyl)butyl (4-sulfamoylbenzyl)carbamate(**5b**).

NMR spectra 6: 4-(4-(bis(2-chloroethyl)amino)phenyl)butyl (4-sulfamoylphenethyl)carbamate(**5c**).

NMR spectra 7: 3-(4-sulfamoylphenethylamino) benzo [e][1,2,4] triazine 1-oxide (7).

NMR spectra 8: 3-((4-sulfamoylphenethyl)amino)benzo[e][1,2,4]triazine 1,4-dioxide (**8**).

NMR spectra 9: 3-(4-sulfamoylphenethylamino)-7,8-dihydro-6H-indeno [5,6-e][1,2,4] triazine 1-oxide (10).

NMR spectra 10: 3-((4-sulfamoylphenethyl) amino)-7,8-dihydro-6H-indeno [5,6-e][1,2,4] triazine 1,4-dioxide (11).

NMR spectra 11: 6-(4-(methylsulfonyl)phenyl)-N-phenyl-3-(sulfamoylamino)pyrazine-2-carboxamide (**12**).

NMR spectra 12: 5-(4-(isopropylsulfonyl) phenyl)-3-(3-(4-((methylamino) methyl) phenyl) isoxazol-5-yl) pyrazin-2-carboxamide (**13**).

NMR spectra 13: 3-methyl-4-oxo-N-(4-sulfamoylphenethyl)-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxamide (**15a**).

NMR spectra 14: 3-methyl-4-oxo-N-(sulfamoyloxy)-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxamide (15b).

NMR spectra 15: 3-methyl-4-oxo-N-(4-sulfamoylbenzyl)-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxamide (**15c**).

Chapter 6

NMR spectra 16: 3-methyl-4-oxo-N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxamide (**16d**).

NMR spectra 17: 4-(2-(3-carbamimidoylguanidino)ethyl)benzenesulfonamide hydrochloride salt (**18**)

ACKNOWLEDGEMENTS:

Authors acknowledge the aid of Advinus Therapeutics Ltd. for synthesis of compounds **1**, **2a**, **2b**, **2c**, **3**, **4**, **5a**, **5b** and **5c**. This work was supported by METOXIA (Metastatic Tumors Facilitated by Hypoxic Micro-Environment; EU 7th Research Framework Programme - Theme HEALTH; Grant no.: 222741) NGI Pre-Seed grant (n° 93612005), Kankeronderzoekfonds Limburg from the Health Foundation Limburg and the Dutch Cancer Society (KWF UM 2011-5020, KWF UM 2009-4454, KWF MAC 2013-6425, KWF MAC 2013-6089, KWF 2015-7635).

REFERENCES

1. Nordsmark, M., et al., *Prognostic value of tumor oxygenation in 397 head and neck tumors after primary radiation therapy. An international multi-center study*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2005. **77**(1): p. 18-24.
2. Good, J.S. and K.J. Harrington, *The hallmarks of cancer and the radiation oncologist: updating the 5Rs of radiobiology*. Clinical oncology, 2013. **25**(10): p. 569-77.
3. Hockel, M., et al., *Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix*. Cancer research, 1996. **56**(19): p. 4509-15.
4. Wojtkowiak, J.W., et al., *Drug resistance and cellular adaptation to tumor acidic pH microenvironment*. Molecular pharmaceutics, 2011. **8**(6): p. 2032-8.
5. Dubois, L.J., et al., *New ways to image and target tumour hypoxia and its molecular responses*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2015. **116**(3): p. 352-7.
6. Ebbesen, P., et al., *Taking advantage of tumor cell adaptations to hypoxia for developing new tumor markers and treatment strategies*. Journal of enzyme inhibition and medicinal chemistry, 2009. **24 Suppl 1**: p. 1-39.
7. Pettersen, E.O., et al., *Targeting tumour hypoxia to prevent cancer metastasis. From biology, biosensing and technology to drug development: the METOXIA consortium*. Journal of enzyme inhibition and medicinal chemistry, 2015. **30**(5): p. 689-721.
8. Neri, D. and C.T. Supuran, *Interfering with pH regulation in tumours as a therapeutic strategy*. Nature reviews. Drug discovery, 2011. **10**(10): p. 767-77.
9. Pastorek, J. and S. Pastorekova, *Hypoxia-induced carbonic anhydrase IX as a target for cancer therapy: from biology to clinical use*. Seminars in cancer biology, 2015. **31**: p. 52-64.
10. Pastorek, J., et al., *Cloning and characterization of MN, a human tumor-associated protein with a domain homologous to carbonic anhydrase and a putative helix-loop-helix DNA binding segment*. Oncogene, 1994. **9**(10): p. 2877-88.
11. Wykoff, C.C., et al., *Hypoxia-inducible expression of tumor-associated carbonic anhydrases*. Cancer research, 2000. **60**(24): p. 7075-83.
12. Kaluz, S., et al., *Lowered oxygen tension induces expression of the hypoxia marker MN/carbonic anhydrase IX in the absence of hypoxia-inducible factor 1 alpha stabilization: a role for phosphatidylinositol 3'-kinase*. Cancer research, 2002. **62**(15): p. 4469-77.
13. Kopacek, J., et al., *MAPK pathway contributes to density- and hypoxia-induced expression of the tumor-associated carbonic anhydrase IX*. Biochimica et biophysica acta, 2005. **1729**(1): p. 41-9.
14. van den Beucken, T., et al., *Hypoxia-induced expression of carbonic anhydrase 9 is dependent on the unfolded protein response*. The Journal of biological chemistry, 2009. **284**(36): p. 24204-12.
15. van Kuijk, S.J., et al., *Prognostic Significance of Carbonic Anhydrase IX Expression in Cancer Patients: A Meta-Analysis*. Frontiers in oncology, 2016. **6**: p. 69.
16. Akurathi, V., et al., *Development and biological evaluation of (9)(9)mTc-sulfonamide derivatives for in vivo visualization of CA IX as surrogate tumor hypoxia markers*. European journal of medicinal chemistry, 2014. **71**: p. 374-84.
17. Akurathi, V., et al., *Synthesis and biological evaluation of a 99mTc-labelled sulfonamide conjugate for in vivo visualization of carbonic anhydrase IX expression in tumor hypoxia*. Nuclear medicine and biology, 2010. **37**(5): p. 557-64.
18. Peeters, S.G., et al., *[(18)F]VM4-037 MicroPET Imaging and Biodistribution of Two In Vivo CAIX-Expressing Tumor Models*. Molecular imaging and biology : MIB : the official publication of the Academy of Molecular Imaging, 2015. **17**(5): p. 615-9.
19. Sneddon, D., et al., *Synthesis and in Vivo Biological Evaluation of 68Ga-Labeled Carbonic Anhydrase IX Targeting Small Molecules for Positron Emission Tomography*. Journal of medicinal chemistry, 2016.

20. Gieling, R.G., et al., *Antimetastatic effect of sulfamate carbonic anhydrase IX inhibitors in breast carcinoma xenografts*. Journal of medicinal chemistry, 2012. **55**(11): p. 5591-600.
21. Lock, F.E., et al., *Targeting carbonic anhydrase IX depletes breast cancer stem cells within the hypoxic niche*. Oncogene, 2013. **32**(44): p. 5210-9.
22. Monti, S.M., C.T. Supuran, and G. De Simone, *Anticancer carbonic anhydrase inhibitors: a patent review (2008 - 2013)*. Expert opinion on therapeutic patents, 2013. **23**(6): p. 737-49.
23. Ward, C., et al., *Evaluation of carbonic anhydrase IX as a therapeutic target for inhibition of breast cancer invasion and metastasis using a series of in vitro breast cancer models*. Oncotarget, 2015. **6**(28): p. 24856-70.
24. Dubois, L., et al., *Specific inhibition of carbonic anhydrase IX activity enhances the in vivo therapeutic effect of tumor irradiation*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2011. **99**(3): p. 424-31.
25. Rami, M., et al., *Hypoxia-targeting carbonic anhydrase IX inhibitors by a new series of nitroimidazole-sulfonamides/sulfamides/sulfamates*. Journal of medicinal chemistry, 2013. **56**(21): p. 8512-20.
26. McDonald, P.C., et al., *Recent developments in targeting carbonic anhydrase IX for cancer therapeutics*. Oncotarget, 2012. **3**(1): p. 84-97.
27. Supuran, C.T., *Carbonic anhydrases: novel therapeutic applications for inhibitors and activators*. Nature reviews. Drug discovery, 2008. **7**(2): p. 168-81.
28. Dubois, L., et al., *Targeting carbonic anhydrase IX by nitroimidazole based sulfamides enhances the therapeutic effect of tumor irradiation: a new concept of dual targeting drugs*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2013. **108**(3): p. 523-8.
29. Barman, S., et al., *Coumarin-benzothiazole-chlorambucil (Cou-Benz-Cbl) conjugate: an ESIPT based pH sensitive photoresponsive drug delivery system*. Journal of Materials Chemistry B, 2015. **3**(17): p. 3490-97.
30. Bekele, T., et al., *Catalytic, enantioselective [4 + 2]-cycloadditions of ketene enolates and o-quinones: efficient entry to chiral, alpha-oxygenated carboxylic acid derivatives*. Journal of the American Chemical Society, 2006. **128**(6): p. 1810-1.
31. Gill, M. and A.F. Smrdel, *Pigments of fungi, part 16. Synthesis of methyl (R)-(+)-tetrahydro-2-methyl-5-oxo-2-furanacetate and its (S)-(-)-antipode, chiroptical references for determination of the absolute stereochemistry of fungal pre-antraquinones*. Tetrahedron Asymmetry, 1990. **1**(7): p. 453-64.
32. Deshmukh, A.R. and V. Gumaste, *Process for preparing alkyl/aryl chloroformates*. 2005, Google Patents.
33. Hay, M.P., et al., *Tricyclic [1,2,4]triazine 1,4-dioxides as hypoxia selective cytotoxins*. Journal of medicinal chemistry, 2008. **51**(21): p. 6853-65.
34. Arrowsmith, J., et al., *Antitumor imidazotetrazines. 41. Conjugation of the antitumor agents mitozolomide and temozolomide to peptides and lexitropsins bearing DNA major and minor groove-binding structural motifs*. Journal of medicinal chemistry, 2002. **45**(25): p. 5458-70.
35. Ombouma, J., et al., *Carbonic Anhydrase Glycoinhibitors belonging to the Aminoxysulfonamide Series*. ACS medicinal chemistry letters, 2015. **6**(7): p. 819-21.
36. Kelarev, V.I., et al., *Synthesis of N-substituted 6-alkyl-2,4-diamino-1,3,5-triazines containing long alkyl radicals*. Zhurnal Organicheskoi Khimii, 1988. **24**: p. 1100-1105.
37. Svastova, E., et al., *Carbonic anhydrase IX reduces E-cadherin-mediated adhesion of MDCK cells via interaction with beta-catenin*. Experimental cell research, 2003. **290**(2): p. 332-45.
38. Goede, V., et al., *Past, present and future role of chlorambucil in the treatment of chronic lymphocytic leukemia*. Leukemia & lymphoma, 2015. **56**(6): p. 1585-92.
39. Horsman, M.R. and P. Vaupel, *Pathophysiological Basis for the Formation of the Tumor Microenvironment*. Frontiers in oncology, 2016. **6**: p. 66.
40. Reddy, S.B. and S.K. Williamson, *Tirapazamine: a novel agent targeting hypoxic tumor cells*. Expert opinion on investigational drugs, 2009. **18**(1): p. 77-87.
41. Omuro, A. and L.M. DeAngelis, *Glioblastoma and other malignant gliomas: a clinical review*. JAMA, 2013. **310**(17): p. 1842-50.

42. Fukushima, T., H. Takeshima, and H. Kataoka, *Anti-glioma therapy with temozolomide and status of the DNA-repair gene MGMT*. Anticancer research, 2009. **29**(11): p. 4845-54.
43. Dubois, L., et al., *Imaging the hypoxia surrogate marker CA IX requires expression and catalytic activity for binding fluorescent sulfonamide inhibitors*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2007. **83**(3): p. 367-73.
44. Ditte, P., et al., *Phosphorylation of carbonic anhydrase IX controls its ability to mediate extracellular acidification in hypoxic tumors*. Cancer research, 2011. **71**(24): p. 7558-67.
45. Dubois, L., et al., *Imaging of CA IX with fluorescent labelled sulfonamides distinguishes hypoxic and (re)-oxygenated cells in a xenograft tumour model*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2009. **92**(3): p. 423-8.
46. Fokas, E., et al., *Targeting ATR in vivo using the novel inhibitor VE-822 results in selective sensitization of pancreatic tumors to radiation*. Cell death & disease, 2012. **3**: p. e441.
47. Hall, A.B., et al., *Potentiation of tumor responses to DNA damaging therapy by the selective ATR inhibitor VX-970*. Oncotarget, 2014. **5**(14): p. 5674-85.
48. Josse, R., et al., *ATR inhibitors VE-821 and VX-970 sensitize cancer cells to topoisomerase α inhibitors by disabling DNA replication initiation and fork elongation responses*. Cancer research, 2014. **74**(23): p. 6968-79.
49. Gray, L.H., et al., *The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy*. The British journal of radiology, 1953. **26**(312): p. 638-48.
50. Wright, E.A. and P. Howard-Flanders, *The influence of oxygen on the radiosensitivity of mammalian tissues*. Acta radiologica, 1957. **48**(1): p. 26-32.
51. Doyen, J., et al., *Knock-down of hypoxia-induced carbonic anhydrases IX and XII radiosensitizes tumor cells by increasing intracellular acidosis*. Frontiers in oncology, 2012. **2**: p. 199.
52. De Milito, A. and S. Fais, *Tumor acidity, chemoresistance and proton pump inhibitors*. Future oncology, 2005. **1**(6): p. 779-86.
53. Kulshrestha, A., et al., *Selective inhibition of tumor cell associated Vacuolar-ATPase ' α 2' isoform overcomes cisplatin resistance in ovarian cancer cells*. Molecular oncology, 2016.
54. Liao, C., et al., *Genomic screening in vivo reveals the role played by vacuolar H⁺ ATPase and cytosolic acidification in sensitivity to DNA-damaging agents such as cisplatin*. Molecular pharmacology, 2007. **71**(2): p. 416-25.
55. van Gisbergen, M.W., et al., *How do changes in the mtDNA and mitochondrial dysfunction influence cancer and cancer therapy? Challenges, opportunities and models*. Mutation research. Reviews in mutation research, 2015. **764**: p. 16-30.
56. Pollak, M., *Potential applications for biguanides in oncology*. The Journal of clinical investigation, 2013. **123**(9): p. 3693-700.
57. Pernicova, I. and M. Korbonits, *Metformin--mode of action and clinical implications for diabetes and cancer*. Nature reviews. Endocrinology, 2014. **10**(3): p. 143-56.
58. Liu, Z., et al., *Phenformin Induces Cell Cycle Change, Apoptosis, and Mesenchymal-Epithelial Transition and Regulates the AMPK/mTOR/p70s6k and MAPK/ERK Pathways in Breast Cancer Cells*. PloS one, 2015. **10**(6): p. e0131207.
59. Shackelford, D.B., et al., *LKB1 inactivation dictates therapeutic response of non-small cell lung cancer to the metabolism drug phenformin*. Cancer cell, 2013. **23**(2): p. 143-58.
60. Krall, N., et al., *A small-molecule drug conjugate for the treatment of carbonic anhydrase IX expressing tumors*. Angewandte Chemie, 2014. **53**(16): p. 4231-5.
61. Khalifah, R.G., *The carbon dioxide hydration activity of carbonic anhydrase. I. Stop-flow kinetic studies on the native human isoenzymes B and C*. The Journal of biological chemistry, 1971. **246**(8): p. 2561-73.
62. McIntyre, A., et al., *Carbonic anhydrase IX promotes tumor growth and necrosis in vivo and inhibition enhances anti-VEGF therapy*. Clinical cancer research : an official journal of the American Association for Cancer Research, 2012. **18**(11): p. 3100-11.

Chapter 7

Soluble Adenylyl Cyclase is Involved in Regulating the Activity and Expression of Carbonic Anhydrase IX

In preparation

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ABSTRACT

The increased glycolytic energy production in tumor cells requires compensatory mechanisms to maintain the cellular pH balance. Carbonic anhydrase IX (CAIX) is a tumor-specific and hypoxia-associated enzyme involved in this process. The intracellular pathways involved in regulating the function of CAIX, and other enzymes involved in maintaining the pH balance, are however largely unknown. Here we propose the intracellular pH sensor soluble adenylyl cyclase (sAC) might be responsible, although its function in cancer cells remains to be elucidated.

Total intracellular cAMP levels of several cell lines were measured during normoxic and hypoxic conditions in combination with the specific sAC inhibitor KH7. The effects of KH7 on extracellular acidification, CAIX expression, and cell viability were also investigated.

Hypoxic conditions decreased cAMP levels in Hela and HT29 cells, whereas the sAC inhibitor KH7 only significantly reduced cAMP levels in H460 cells. Total cAMP levels were unaffected by CAIX expression levels in MDCK cells. Furthermore, KH7 decreased extracellular acidification in a CAIX-dependent manner and significantly increased normoxic CAIX expression levels. Finally, KH7 was able to decrease cell viability, although its efficacy was cell-type specific.

In conclusion, these results suggest several cell types may depend differently on sAC signaling. More importantly, the results of this study suggest that sAC regulates CAIX activity and expression. Further investigations are required to elucidate the exact pathways responsible.

INTRODUCTION

Several methods are being investigated to target the hypoxic tumor cells [1, 2], which are known to be more resistant to conventional treatment options [3-5]. The hypoxic microenvironment develops due to immature and inadequate vascular supply of oxygen and is thought to give rise to a more aggressive phenotype of cancer cells. Hypoxic tumor cells are known to require compensatory mechanisms to maintain their cellular pH balance due to the increased production of acid waste products during glycolysis [6-8]. One of the enzymes involved in this process is carbonic anhydrase IX (CAIX), which hydrolyzes carbon dioxide to form protons and bicarbonate. The combined tumor-specific and hypoxia-associated expression of CAIX makes it an interesting target for therapy [9-11]. Many CAIX inhibitors are therefore being developed aimed to induce intracellular acidification causing tumor-specific cell death [12, 13].

The intracellular pathways involved in regulating the function of CAIX, and other enzymes involved in maintaining the pH balance, are however largely unknown. Soluble adenylyl cyclase (sAC) functions as an intracellular pH sensor in response to intracellular bicarbonate levels and is ubiquitously expressed in the cytosol [14-16]. In contrast, the 9 remaining members of the adenylyl cyclase family of proteins are located on the cell membrane (tmAC) and are activated by external stimuli via G-protein coupled receptors [17]. Adenylyl cyclases catalyze the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP), one of the most common cellular second messengers. It has been proposed that sAC locally stimulates the cAMP responsive pathways of the cell, since sAC is expressed in specific cellular microdomains [18, 19]. Activation of sAC furthermore can modulate the cellular pH balance via translocation of vacuolar-type H^+ -ATPase (V-ATPase) to the luminal membrane, as was reported for clear cells of the epididymis and renal A-type cells [16, 20-22]. The intracellular bicarbonate responsible for sAC activation can originate from different sources, e.g. exogenous or via metabolic production in mitochondria, where carbonic anhydrase (CA) enzymes play an important role [16]. Hence, non-specific CA inhibition with acetazolamide was found to increase cAMP levels in non-pigmented ciliary epithelial cells of the eye [23].

The specific role of sAC in cancer however remains unclear, since contradictory evidence suggests that sAC functions either as a tumor suppressor protein [24], or is involved in stimulating the survival and proliferation of cancer cells [25]. Nevertheless, specific sAC expression patterns in melanoma patients were identified as possibly be useful for diagnostic purposes [26, 27]. Furthermore, sAC inhibition may also have therapeutic potential since the radiosensitivity of prostate cancer cells was increased after sAC inhibition [28]. In the present study we therefore hypothesize that sAC activation is also important in regulating and maintaining the cellular pH balance of hypoxic tumor cells. The aim of this study is therefore to investigate the function of sAC during hypoxic conditions and determine if cancer cells depend on sAC function for their survival. Furthermore we aim to elucidate if sAC activity is associated with CAIX function.

RESULTS

Activity of sAC during hypoxic conditions

The effect of hypoxia (0.2% O₂) on total intracellular cAMP levels was assessed in different tumor cell lines (Figure 1). After hypoxia exposure the total intracellular cAMP levels either remain constant, i.e. in MDA-MB-231 ($p=0.821$) and H460 ($p=0.977$) cells, or significantly decreased, i.e. in Hela ($p=0.041$) and HT29 ($p=0.018$) cells, which suggests an association with hypoxia-induced CAIX expression levels (Figure 3). The influence of sAC activity on total cAMP content in these tumor cells was evaluated by exposing them to the selective sAC inhibitor KH7 [29]. KH7 was only able to significantly decrease total cAMP levels in the H460 cells, i.e. both during normoxic ($p<0.001$) and hypoxic conditions ($p=0.008$) (Figure 1A-D). A small decrease in cAMP levels after KH7 exposure was also observed during normoxic conditions in Hela and HT29 cells, albeit not statistically significant ($p=0.092$ and $p=0.066$, respectively), whereas both these cell lines showed no effect of KH7 on cAMP levels during hypoxic conditions. Total intracellular cAMP levels of MDA-MB-231 cells remained unaltered after sAC inhibition with KH7, i.e. both during normoxic and hypoxic conditions. These results suggest that the difference in response to the sAC inhibitor KH7 may be due to a different degree of sAC signalling in these cell lines.

To determine if CAIX expression influences sAC activity levels, the intracellular cAMP levels were measured in MDCK – CAIX⁻ and MDCK – CAIX⁺ cells (Figure 1E-F). Similar as found for the MDA-MB-231 and H460 cells, no change in cAMP levels was observed upon hypoxia exposure. Furthermore, no significant effect of KH7 on cAMP levels was observed during either normoxic or hypoxic conditions in both cell lines. In addition, no differences in cAMP levels were present between these two cell lines, thereby indicating that cAMP levels and sAC activity are not associated with cellular CAIX expression in these cells. As a positive control all cell lines were also exposed to forskolin, which should stimulate cAMP production by activating tmAC [30, 31]. Indeed total intracellular cAMP levels in all cell lines increased after exposure to forskolin, i.e. both during normoxic and hypoxic conditions.

Figure 1: Effect of normoxic (black) or hypoxic (white) conditions on total intracellular cAMP levels, with or without the selective sAC inhibitor KH7 (20 μ M) of Hela (A), MDA-MB-231 (B), HT29 (C), H460 (D), MDCK – CAIX⁻ (E), and MDCK – CAIX⁺ (F) cells. As a positive control cells were exposed to the tmAC activator forskolin (10 μ M). Total intracellular cAMP concentrations were normalized to protein concentrations of each sample. Asterisks indicate statistical significance (*p<0.05; **p<0.01; ***p<0.001).

Inhibition of sAC decreases extracellular acidification

Similarly what was previously described for V-ATPase [16, 20-22], sAC activity may also regulate CAIX activity to maintain the cellular pH balance. MDCK – CAIX⁻ and MDCK – CAIX⁺ cells were therefore allowed to acidify their extracellular environment, i.e. their medium, when exposed to either KH7 or vehicle (Figure 2). In line with previous reports [32, 33], the pH of the medium was significantly lower during hypoxic as compared to normoxic conditions (p=0.006 and p=0.002 for MDCK – CAIX⁻ and MDCK – CAIX⁺ cells, respectively). In addition, the degree of extracellular acidification was more pronounced in the MDCK – CAIX⁺ as compared to the MDCK – CAIX⁻ cells (p=0.031 and p=0.002 dur-

ing normoxic and hypoxic conditions, respectively). Inhibiting the function of sAC by exposing MDCK – CAIX⁺ cells to KH7 significantly decreased the extracellular acidification during hypoxic ($p=0.038$), but not during normoxic conditions ($p=0.561$). Even though a similar trend was observed in the MDCK – CAIX⁻ cells during hypoxic conditions, the effect of KH7 on pH in this cell line was relatively smaller and not statistically significant ($p=0.058$). Together these results suggest that sAC inhibition might be able to modulate the activity of CAIX during hypoxic, but not normoxic conditions.

Figure 2: Measurements of extracellular pH of the MDCK – CAIX⁻ (A) and MDCK – CAIX⁺ (B) cells exposed to normoxic (black) or hypoxic (white) conditions in combination with or without KH7 (20 μ M) for 24 hours. Asterisks indicate statistical significance (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Increased expression of CAIX after sAC inhibition

CAIX expression is increased during hypoxic conditions in four different tumor cell lines (Figure 3). Strikingly, CAIX protein expression was also increased during normoxic conditions in all cell lines when exposed to the selective sAC inhibitor KH7. Exposure to KH7 even resulted in similar CAIX expression levels during normoxic and hypoxic conditions in the Hela, MDA-MB-231, and H460 cells. In contrast, KH7 exposure doubled CAIX expression levels in HT29 cells during normoxic conditions, but those expression levels were not as high as observed during hypoxia. Furthermore, CAIX expression levels were unaltered after KH7 exposure during hypoxic conditions in all included tumor cell lines. Together these results suggest that sAC function might be involved in regulating CAIX expression, which seems to occur preferably during normoxic conditions.

Figure 3: CAIX expression of cells exposed to normoxic or hypoxic conditions in combination with (black) or without (white) the sAC inhibitor KH7 (20 μ M). Representative immunoblots with quantification of three independent experiments are shown of HeLa (A), MDA-MB-231 (B), HT29 (C), and H460 (D). Graphs depict mean $\pm$ SEM, where CAIX expression is corrected for protein content via normalization to actin expression.

Inhibition of sAC with KH7 decreases cell viability

Previously, sAC has been reported to be a tumor suppressor protein [24], but also a regulator of tumor cell proliferation [25]. We therefore evaluated the effect of the selective sAC inhibitor KH7 on cell viability of the four tumor and two MDCK cell lines (Figure 4). All cell lines showed decreased cell viability with increasing concentration of KH7 under both normoxic and hypoxic conditions. H460 cells were most sensitive to KH7 as 20 μ M, i.e. the concentration used in the other experiments, significantly decreased cell viability to 49.4% ($p=0.005$) and 50.2% ($p=0.002$) during normoxic and hypoxic conditions, respectively. This result is in line with the pronounced decrease in cAMP after KH7 treatment under normoxic and hypoxic conditions (Figure 1D). In addition, HeLa cells showed a more intermediate sensitivity to KH7 treatment, since 20 μ M KH7 decreased cell viability to 70% ($p=0.013$) and 73% ($p=0.007$) during normoxic and hypoxic conditions, respectively. In contrast, a minimal or no effect of 20 μ M KH7 on cell viability was observed in either MDA-MB-231 (91.6%, $p=0.115$ and 85.2%, $p=0.044$) or HT29 cells (89.1%, $p=0.226$ and 90.8%, $p=0.124$) during normoxic or hypoxic conditions, respectively. MDCK cells showed a similar sensitivity to KH7 as did the HeLa cells, but no difference existed between MDCK – CAIX⁻ and MDCK – CAIX⁺ cells, either during normoxic or hypoxic conditions ($p=0.742$ and $p=0.185$, respectively), thereby indicating that the effect of KH7 on cell viability is independent of CAIX expression levels. Furthermore, the sensitivity to KH7 of all included cell lines was not different between normoxic and hypoxic conditions. All together these results suggest that sAC is involved in maintaining (tumor) cell viability, although the dependency of cells on sAC function for survival may be cell type specific.

Figure 4: Relative cell viability of cells exposed to increasing concentrations of the sAC inhibitor KH7 during normoxic (A) or hypoxic (B) conditions for 24 hours. Cell lines included are the Hela (black, circle), MDA-MB-231 (yellow, square), HT29 (purple, triangle), H460 (blue, reversed triangle), MDCK – CAIX⁻ (red, reversed open triangle), and MDCK – CAIX⁺ cells (green, open triangle). Relative cell viability is normalized to vehicle.

DISCUSSION

The second messenger cAMP is involved in regulating a wide variety of intracellular pathways and can originate from different sources, i.e. the transmembrane adenylyl cyclases (tmAC) or the intracellular soluble adenylyl cyclase (sAC) [17]. The intracellular pH sensor sAC directly responds to bicarbonate [15, 16] and could be a valuable intracellular signaling pathway in glycolytic cancer cells as these require bicarbonate for buffering their intracellular pH in an acidic extracellular environment [6, 7]. Activation of sAC in hypoxic tumor cells may be closely related to the CAIX produced bicarbonate, although the function of sAC during hypoxic conditions has not been reported previously. In this study intracellular cAMP levels decreased in Hela and HT29 cells during hypoxic conditions, but remained unchanged in MDA-MB-231 and H460 cells. The efficacy of the sAC inhibitor KH7 on intracellular cAMP levels was however unaltered during hypoxic as compared to normoxic conditions. Nevertheless, sAC inhibition decreased total cAMP levels significantly in H460 during both normoxic and hypoxic conditions, and a similar trend was observed during normoxic conditions in Hela and HT29 cells. The activity of sAC may therefore vary between tumor cells, but the biological relevance of these results remains to be investigated further.

Hypoxic conditions are essential for CAIX activation and bicarbonate production [33–35], which may stimulate sAC to produce cAMP. An increased sAC activity could therefore explain the previously reported increased cAMP levels during hypoxic conditions in MDCK – CAIX⁺ cells [32]. In contrast, in the present study cAMP levels in the MDCK – CAIX⁺ cells were unaffected by hypoxic conditions. The discrepancy between the previously reported and the current results may at least in part be explained by different oxygen concentrations used, i.e. 0.2% O₂ versus 1% O₂ [32]. Furthermore, no differences in cAMP levels were observed between MDCK – CAIX⁻ and MDCK – CAIX⁺ cells,

thereby suggesting that sAC activation is not solely dependent on CAIX expression in these cell lines under experimental conditions used here. Further investigations are therefore required to study the activity of sAC during exposure to various oxygen concentrations and elucidate an association with CAIX expression levels, or extracellular acidification.

More importantly however, the results of this study suggest that sAC may be able to regulate CAIX, i.e. both on a functional and expression level (Figure 5). Here we show that sAC decreased the extracellular acidification of MDCK cells during hypoxic conditions, which was most pronounced in the CAIX overexpressing cells. Previously, full activation of CAIX, i.e. the highest extracellular acidification, was reported to require phosphorylation via protein kinase A (PKA) [32]. Since PKA is activated via cAMP, exogenous stimulation of MDCK – CAIX⁺ cells with cAMP also increased phosphorylated CAIX levels [32]. The origin of the cAMP that modulates phosphorylated CAIX levels however remains to be identified. The results of this study suggest that sAC, as opposed to tmAC, is herein involved, since sAC inhibition with KH7 decreased extracellular acidification in a CAIX-dependent manner. Nevertheless, sAC activity was not sufficient enough to translate to efficacy of KH7 on total intracellular cAMP levels, suggesting tmAC are the predominant supplier of cAMP in the cell. The effect of sAC inhibition on phosphorylated CAIX levels therefore remains to be elucidated.

Besides affecting CAIX function, sAC inhibition was also able to modulate CAIX expression (Figure 5), since exposure to KH7 increased the CAIX expression levels during normoxic conditions of all included tumor cell lines. In Hela, MDA-MB-231, and H460, this increased expression even appeared to be similar to hypoxic expression levels, i.e. a known regulator of CAIX expression via HIF stabilization [11]. Previously, the cAMP response element-binding protein (CREB) was identified as a negative regulator of CAIX expression that can bind upstream of HIF on the promoter region of CAIX [36]. Since CREB is also activated via PKA [37], the cAMP required to downregulate CAIX expression may originate from sAC. Nevertheless, during hypoxic conditions no effect on CAIX expression levels were observed as the HIF signal may have been too strong to be affected by a reduced CREB signaling after sAC inhibition. Further investigation is however required to exclude any possible effects of KH7 on HIF stabilization, and prove that a reduced activation of CREB is responsible for the change in CAIX expression.

Figure 5: Schematic representation of hypothesized modulation of CAIX activity and expression via sAC function. Activation of sAC via different sources of HCO_3^- produces cAMP, which might activate PKA and result in CAIX phosphorylation, thereby modulating CAIX activity. PKA activation by sAC may also phosphorylate CREB, which translocates to the nucleus and downregulates the transcription of CA9 mRNA, thereby decreases CAIX expression. Question marks indicate validation experiments are required to prove sAC is responsible for activation of these pathways. Abbreviations used: adenosine triphosphate (ATP); anion exchanger (AE); carbonic anhydrase 9 mRNA (CA9); carbonic anhydrase IX (CAIX); cyclic adenosine monophosphate (cAMP); cAMP response element-binding protein (CREB); mitochondria (Mito); sodium-bicarbonate cotransporter (NBC); phosphate group (P); protein kinase A (PKA); soluble adenylyl cyclase (sAC).

Even though sAC activity did not appear to depend on either hypoxia or CAIX expression levels in this study, it may still be an important signaling pathway in cancer as it can modulate both CAIX activity and expression, as has been shown in the present study using a panel of cell lines. Additional research however is required to identify alternative enzymes involved in maintaining the pH balance, e.g. V-ATPase or bicarbonate transporters [16, 20-22], that can also be modulated via sAC activity in cancer cells. Furthermore, sAC inhibition may prove a feasible anti-cancer therapy as it affects cell viability, albeit to a different extent between cell lines. Importantly, the effect of KH7 on cAMP levels may be closely associated with its effect on cell viability, since the H460 and MDA-

MB-231 cells were the most and least responsive, respectively, in both experiments. These results therefore suggest that certain cancer cells depend on sAC signaling to a greater extent than other cells for their proliferation and survival, which may explain the existing contradictory data about the role of sAC in cancer, i.e. as a tumor suppressor [24] or stimulator of proliferation [25]. Nevertheless, before definitive conclusions can be drawn regarding the feasibility of sAC inhibition as anti-cancer therapy, future research should elucidate the exact roles of sAC in cancer. In addition, the ubiquitous expression of sAC in all tissues requires targeted drug delivery of sAC inhibitors to cancer cells, which requires further investigation.

METHODS

Cells

Cervical Hela (CCL-2), breast MDA-MB-231 (HTB-26) and colorectal HT29 (HTB-38) adenocarcinoma, and lung H460 (HTB-177) carcinoma cell lines were all purchased from the ATCC. Canine kidney MDCK cells without CAIX expression (MDCK – CAIX⁻) and MDCK cells transfected to overexpress human CAIX (MDCK – CAIX⁺) have been described previously [32, 33, 38] and were a kind gift from Prof. Silvia Pastorekova (Bratislava, Slovak Republic). Cell lines were cultured in DMEM, except for H460 cells, which were cultured in RPMI 1640. Medium was supplemented with 10% fetal calf serum (FCS). Cells were exposed to hypoxic conditions in a hypoxic chamber (MACS VA500 microaerophilic workstation, Don Whitley Scientific, UK) with 0.2% O₂, 5% CO₂ and residual N₂ at 37°C. Normoxic cells were grown in regular incubators with 21% O₂, 5% CO₂ at 37°C.

cAMP ELISA

Total intracellular cAMP levels were measured with the Direct cAMP ELISA kit (Enzo Life Sciences, #ADI-900-066) according to the manufacturers protocol. In short, 500,000 cells were seeded in 6 cm dishes and allowed to attach overnight. The next day cells were exposed to vehicle, the sAC specific inhibitor KH7 (20 µM, Sigma-Aldrich, #K3394) [29], or the tmAC activator Forskolin (10 µM, Sigma-Aldrich, #F6886) as a positive control [30, 31]. Immediately after compound addition the cells were exposed to normoxic (21% O₂) or hypoxic (0.2% O₂) conditions for 24 hours. Afterwards, intracellular cAMP levels were measured immediately and corrected for protein concentrations of the samples, as determined with Bradford protein quantification reagent (Bio-rad, #500-0006).

pH assay

Measurements of pH were performed as described before [34, 39, 40] where both MDCK – CAIX⁻ and MDCK – CAIX⁺ cells were seeded in 6 cm dishes to obtain 100% confluence at time of measurement. Cells were exposed to vehicle (0.1% DMSO) or the sAC specific inhibitor KH7 (20 μ M) [29] for 24 hours during normoxic (21% O₂) or hypoxic conditions (0.2% O₂) and pH was measured thereafter.

Western Blot

CAIX expression was determined by immunoblotting as described previously [34]. Protein samples were isolated after exposing cells to normoxic (21% O₂) or hypoxic conditions (0.2% O₂) for 24 hours, in combination with KH7 (20 μ M,) or vehicle (0.1% DMSO). CAIX was visualized with the primary M75 antibody (kindly provided by Prof. Silvia Pastorekova, Bratislava, Slovak Republic), whereas β -actin (MP Biomedicals, #691001) was used as a reference protein.

Cell viability assays

Cell viability was measured with alamarBlue® cell viability reagent (Invitrogen, #DAL1100) immediately after exposing cells to increasing concentrations of the sAC inhibitor KH7 for 24 hours. Parallel 96-well plates were included to allow for testing of KH7 efficacy during both normoxic (21% O₂) and hypoxic conditions (0.2% O₂).

Statistical analyses

Statistical testing of the difference between groups was performed with independent t-tests. In the cell viability assays the effect of KH7 was compared to vehicle using one-sample t-test. To test differences in responses between cell lines to KH7 one-way ANOVA with a post-hoc Tukey's multiple comparison test was used.

REFERENCES

1. Dubois, L.J., et al., *New ways to image and target tumour hypoxia and its molecular responses*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2015. **116**(3): p. 352-7.
2. Pettersen, E.O., et al., *Targeting tumour hypoxia to prevent cancer metastasis. From biology, biosensing and technology to drug development: the METOXIA consortium*. Journal of enzyme inhibition and medicinal chemistry, 2015. **30**(5): p. 689-721.
3. Good, J.S. and K.J. Harrington, *The hallmarks of cancer and the radiation oncologist: updating the 5Rs of radiobiology*. Clinical oncology, 2013. **25**(10): p. 569-77.
4. Hockel, M., et al., *Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix*. Cancer research, 1996. **56**(19): p. 4509-15.
5. Wojtkowiak, J.W., et al., *Drug resistance and cellular adaptation to tumor acidic pH microenvironment*. Molecular pharmaceutics, 2011. **8**(6): p. 2032-8.
6. Neri, D. and C.T. Supuran, *Interfering with pH regulation in tumours as a therapeutic strategy*. Nature reviews. Drug discovery, 2011. **10**(10): p. 767-77.
7. Pouyssegur, J., F. Dayan, and N.M. Mazure, *Hypoxia signalling in cancer and approaches to enforce tumour regression*. Nature, 2006. **441**(7092): p. 437-43.
8. Parks, S.K., J. Chiche, and J. Pouyssegur, *pH control mechanisms of tumor survival and growth*. Journal of cellular physiology, 2011. **226**(2): p. 299-308.
9. Pastorek, J., et al., *Cloning and characterization of MN, a human tumor-associated protein with a domain homologous to carbonic anhydrase and a putative helix-loop-helix DNA binding segment*. Oncogene, 1994. **9**(10): p. 2877-88.
10. Pastorekova, S., et al., *Carbonic anhydrase IX, MN/CA IX: analysis of stomach complementary DNA sequence and expression in human and rat alimentary tracts*. Gastroenterology, 1997. **112**(2): p. 398-408.
11. Wykoff, C.C., et al., *Hypoxia-inducible expression of tumor-associated carbonic anhydrases*. Cancer research, 2000. **60**(24): p. 7075-83.
12. Lomelino, C. and R. McKenna, *Carbonic anhydrase inhibitors: a review on the progress of patent literature (2011-2016)*. Expert opinion on therapeutic patents, 2016: p. 1-10.
13. Monti, S.M., C.T. Supuran, and G. De Simone, *Anticancer carbonic anhydrase inhibitors: a patent review (2008 - 2013)*. Expert opinion on therapeutic patents, 2013. **23**(6): p. 737-49.
14. Wuttke, M.S., J. Buck, and L.R. Levin, *Bicarbonate-regulated soluble adenylyl cyclase*. JOP : Journal of the pancreas, 2001. **2**(4 Suppl): p. 154-8.
15. Rahman, N., J. Buck, and L.R. Levin, *pH sensing via bicarbonate-regulated "soluble" adenylyl cyclase (sAC)*. Frontiers in physiology, 2013. **4**: p. 343.
16. Tresguerras, M., J. Buck, and L.R. Levin, *Physiological carbon dioxide, bicarbonate, and pH sensing*. Pflugers Archiv : European journal of physiology, 2010. **460**(6): p. 953-64.
17. Steegborn, C., *Structure, mechanism, and regulation of soluble adenylyl cyclases - similarities and differences to transmembrane adenylyl cyclases*. Biochimica et biophysica acta, 2014. **1842**(12 Pt B): p. 2535-47.
18. Zippin, J.H., et al., *Bicarbonate-responsive "soluble" adenylyl cyclase defines a nuclear cAMP microdomain*. The Journal of cell biology, 2004. **164**(4): p. 527-34.
19. Desman, G., C. Waintraub, and J.H. Zippin, *Investigation of cAMP microdomains as a path to novel cancer diagnostics*. Biochimica et biophysica acta, 2014. **1842**(12 Pt B): p. 2636-45.
20. Gong, F., et al., *Vacuolar H⁺-ATPase apical accumulation in kidney intercalated cells is regulated by PKA and AMP-activated protein kinase*. American journal of physiology. Renal physiology, 2010. **298**(5): p. F1162-9.
21. Pastor-Soler, N., et al., *Bicarbonate-regulated adenylyl cyclase (sAC) is a sensor that regulates pH-dependent V-ATPase recycling*. The Journal of biological chemistry, 2003. **278**(49): p. 49523-9.

22. Paunescu, T.G., et al., *cAMP stimulates apical V-ATPase accumulation, microvillar elongation, and proton extrusion in kidney collecting duct A-intercalated cells*. American journal of physiology. Renal physiology, 2010. **298**(3): p. F643-54.
23. Shahidullah, M., et al., *Nonpigmented ciliary epithelial cells respond to acetazolamide by a soluble adenylyl cyclase mechanism*. Investigative ophthalmology & visual science, 2014. **55**(1): p. 187-97.
24. Ramos-Espiritu, L., et al., *The metabolic/pH sensor soluble adenylyl cyclase is a tumor suppressor protein*. Oncotarget, 2016.
25. Flacke, J.P., et al., *Type 10 soluble adenylyl cyclase is overexpressed in prostate carcinoma and controls proliferation of prostate cancer cells*. The Journal of biological chemistry, 2013. **288**(5): p. 3126-35.
26. Magro, C.M., et al., *Soluble adenylyl cyclase antibody profile as a diagnostic adjunct in the assessment of pigmented lesions*. Archives of dermatology, 2012. **148**(3): p. 335-44.
27. Magro, C.M., et al., *Expression of soluble adenylyl cyclase in lentigo maligna: use of immunohistochemistry with anti-soluble adenylyl cyclase antibody (R21) in diagnosis of lentigo maligna and assessment of margins*. Archives of pathology & laboratory medicine, 2012. **136**(12): p. 1558-64.
28. Appukuttan, A., et al., *Inhibition of soluble adenylyl cyclase increases the radiosensitivity of prostate cancer cells*. Biochimica et biophysica acta, 2014. **1842**(12 Pt B): p. 2656-63.
29. Hess, K.C., et al., *The "soluble" adenylyl cyclase in sperm mediates multiple signaling events required for fertilization*. Developmental cell, 2005. **9**(2): p. 249-59.
30. Florio, C., et al., *Amplification of the cyclic AMP response to forskolin in pheochromocytoma PC12 cells through adenosine A(2A) purinoceptors*. The Journal of pharmacology and experimental therapeutics, 1999. **290**(2): p. 817-24.
31. Seamon, K.B., W. Padgett, and J.W. Daly, *Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells*. Proceedings of the National Academy of Sciences of the United States of America, 1981. **78**(6): p. 3363-7.
32. Ditte, P., et al., *Phosphorylation of carbonic anhydrase IX controls its ability to mediate extracellular acidification in hypoxic tumors*. Cancer research, 2011. **71**(24): p. 7558-67.
33. Svastova, E., et al., *Hypoxia activates the capacity of tumor-associated carbonic anhydrase IX to acidify extracellular pH*. FEBS letters, 2004. **577**(3): p. 439-45.
34. Dubois, L., et al., *Imaging the hypoxia surrogate marker CA IX requires expression and catalytic activity for binding fluorescent sulfonamide inhibitors*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2007. **83**(3): p. 367-73.
35. Dubois, L., et al., *Imaging of CA IX with fluorescent labelled sulfonamides distinguishes hypoxic and (re)-oxygenated cells in a xenograft tumour model*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2009. **92**(3): p. 423-8.
36. Wang, G., et al., *CREB is a key negative regulator of carbonic anhydrase IX (CA9) in gastric cancer*. Cellular signalling, 2015. **27**(7): p. 1369-79.
37. Kobrinsky, E., et al., *Microdomain organization and frequency-dependence of CREB-dependent transcriptional signaling in heart cells*. FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2011. **25**(5): p. 1544-55.
38. Svastova, E., et al., *Carbonic anhydrase IX reduces E-cadherin-mediated adhesion of MDCK cells via interaction with beta-catenin*. Experimental cell research, 2003. **290**(2): p. 332-45.
39. Dubois, L., et al., *Targeting carbonic anhydrase IX by nitroimidazole based sulfamides enhances the therapeutic effect of tumor irradiation: a new concept of dual targeting drugs*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2013. **108**(3): p. 523-8.
40. Rami, M., et al., *Hypoxia-targeting carbonic anhydrase IX inhibitors by a new series of nitroimidazole-sulfonamides/sulfamides/sulfamates*. Journal of medicinal chemistry, 2013. **56**(21): p. 8512-20.

Chapter 8

General Discussion and Future Perspectives

RATIONALE OF USING CAIX AS A THERAPEUTIC TARGET

The Warburg effect, i.e. an increased glycolytic energy metabolism in cancer cells [1], is one of the hallmarks of cancer and has the potential to be exploited for therapy [2]. This enhanced glycolytic energy metabolism in cancer cells increases the production of acid waste products, which requires compensatory mechanisms to maintain the balance between the intracellular and extracellular pH [3-5]. Several proteins are involved in this process, of which CAIX is an interesting and promising potential therapeutic target [3, 4, 6, 7]. The identification of a hypoxia-responsive element (HRE) in its promoter region [8] and its predominant tumor-specific expression [9] are the main reasons why CAIX might be suited for therapy. The direct link between CAIX expression and hypoxia furthermore stimulated many studies to investigate the prognostic significance of CAIX expression in tumors, e.g. [10-14]. A meta-analysis of these data (Chapter 2, [15]) clearly shows CAIX expression to be associated with worse prognosis in a wide variety of cancer types, thereby strengthening its clinical importance.

SPECIFIC TARGETING AND INHIBITION OF CAIX ON CANCER CELLS

Recent years have brought forward the development of a large variety of different CAIX inhibitors (CAIXi) [16, 17], of which several have been described in this thesis (Figure 1). The proposed general mechanism of these inhibitors is to bind within the Zn^{2+} containing active pocket of CAIX, thereby inhibiting the conversion of carbon dioxide and water to bicarbonate and a free proton [7, 16, 17]. Subsequently, CAIXi treatment decreases extracellular acidification, but more importantly, causes intracellular acidification to induce cell death [18, 19]. Furthermore, binding of CAIXi was described to be highest during hypoxic conditions [20-22]. The reduction in extracellular acidification by the dual-targeting nimorazole based sulfamide DH348 was therefore far more pronounced during hypoxic conditions [23] and, more importantly, depended on the cellular expression levels of CAIX (Chapter 4, [24]). Together these results therefore suggest CAIXi treatment to be effective specifically in the hypoxic regions of tumors. The effect on extracellular acidification of the other CAIXi described in this thesis, i.e. the ureido-substituted sulfamate S4 (Chapter 3) and the dual-targeting cytotoxic compounds (Chapter 6), could however not be tested due to their relatively higher cytotoxicity. Therefore, biological efficacy of these compounds was proven by assessing their activity in cell viability experiments. In line with previous results, S4 reduced cell viability of tumor cells, although this effect might not be depended on either CAIX expression or hypoxic conditions [25-27].

Figure 1: Summary of the results described in this thesis. High tumoral CAIX expression was associated with a worse prognosis in patients. Next different CAIX inhibitors were tested, but were unable to increase doxorubicin efficacy, or target CAIX overexpressing cells. Furthermore, DH348 was effective as single agent and in combination with radiotherapy, but validation experiments proved unsuccessful. Finally, sAC may be involved in regulating both CAIX function and expression. Abbreviations: Carbonic anhydrase IX (CAIX); Carbonic anhydrase IX overexpressing (CAIX⁺); Ionizing radiation (IR); Soluble adenylyl cyclase (sAC).

Cytotoxic compounds are frequently used as anti-cancer agents, but have limited therapeutic success due to unwanted adverse effects. Several cytotoxic dual-target compounds were therefore designed to specifically exert their effect on CAIX expressing cells via binding of a CAIXi moiety to CAIX (Chapter 6). Of all tested dual-target compounds, only an ATRi derivative proved to be more effective when combined with radiation in CAIX overexpressing cells as compared to cells that do not express CAIX. The poor binding affinity of this compound for recombinant CAIX and the absence of an increased efficacy during hypoxic conditions however suggested that the effect of the compound not solely depends on binding to CAIX. Alternatively, the difference in response to ATRi when combined with radiation between cells overexpressing CAIX and those that lack CAIX expression might be the result of a lower number of cells in the resistant S-phase of the cell cycle [28], or by a decrease in DNA repair capacity in cells with lower intracellular pH [29-31], i.e. those that do not express CAIX. These possible explanations may also explain the different response between cells overexpressing CAIX and those that lack CAIX expression when exposed to cytotoxic drugs, or the dual-target derivatives of these drugs (Chapter 6), although further investigations are required to prove this causal relationship.

CAIX is further implicated in the invasion and migration of tumor cells [25, 27, 32-35], which may partly be due via its ability to cause extracellular acidification thereby stimulating extracellular matrix degradation, e.g. via MMP activation [36]. In addition, the ability of CAIX to maintain a localized proton gradient was suggested to facilitate

migration by relocalization of CAIX to lamellipodia of migrating tumor cells [35]. Suppression of CAIX was furthermore described to decrease focal adhesion of cancer cells and decrease cell spreading [33]. The only CAIX inhibitor included in this thesis that has been described to decrease cellular migration and invasion of tumor cells is S4 [25, 27], although this effect may partly be affected by a decrease in cell viability after S4 exposure [25-27]. The ability of the other CAIXi in this thesis to modulate CAIX facilitated migration and invasion of cancer cells however has not been tested, due to their lack of specificity for targeting CAIX expressing cells, i.e. the cytotoxic dual-target compounds (Chapter 6), or because results of validation experiments were contradictory, i.e. DH348 (Chapter 4 and 5, and described below).

CAIXI TREATMENT IN TUMORS

The tumor microenvironment is markedly different from *in vitro* experimental conditions due to the existence of gradients in pH, oxygen, and nutrients, but also the presence of different cell types [37]. It therefore remains essential to also test the efficacy of CAIXi in tumor-bearing mice, until reliable alternatives become available [38, 39]. Treating xenograft tumors with CAIXi is hypothesized to specifically affect the CAIX expressing subpopulation of cancer cells, which are often present in the treatment resistant hypoxic areas of a tumor. Single therapy with S4 however proved ineffective in decreasing primary tumor growth (Chapter 3) [25], and was previously described to have no effect on microenvironmental characteristics of xenograft tumors, i.e. hypoxic fraction, proliferation and apoptosis [40]. Treating orthotopic MDA-MB-231 tumor-bearing mice with S4 however did decrease the amount of spontaneous lung metastases [25]. S4 treatment may therefore still be able to affect the circulating tumor cells to prevent metastases formation, since adequate levels of the compound are present in the blood (Chapter 3). Nevertheless, this proposed effect of S4, and possibly also of alternative CAIXi, e.g. DH348, on circulating tumor cells remains to be elucidated. Treatment of tumor-bearing mice with DH348 however did decrease tumor growth after intraperitoneal administration [27] and decreased Ki67 expression and the number of viable cells. In contrast, oral administration of DH348 showed no effect on primary tumor growth (Chapter 5), whereas results after intravenous administration were contradictory (Chapter 4 and 5).

COMBINING CAIXI WITH STANDARD TREATMENT MODALITIES

Treatment with CAIXi is furthermore hypothesized to increase the uptake and efficacy of certain chemotherapeutics, such as doxorubicin. This phenomenon can be explained with the ion-trapping model where a decrease in extracellular acidification, i.e. via inhi-

bition of CAIX function, leads to an increased passive diffusion of weak basic compounds, e.g. doxorubicin, across the cell membrane [41, 42]. Non-specific CA inhibition with acetazolamide indeed increased doxorubicin uptake *in vitro* [43], whereas CAIX specific inhibition with DH348 has been described to significantly increase doxorubicin efficacy *in vivo* [23]. Nevertheless, combination treatment of doxorubicin with S4 failed to increase doxorubicin efficacy in all of the included tumor models (Chapter 3). Strikingly the effect of doxorubicin was even abrogated when combined with S4 in the MDA-MB-231 model, of which the mechanism responsible is unknown. These results therefore stress the incomplete understanding of the mechanism of action of certain CAIX inhibitors in combination with chemotherapeutics. Care therefore needs to be taken before implementing such combination strategies in clinical practice without rigorous prior investigation.

Besides being able to increase doxorubicin efficacy in xenograft tumors, DH348 treatment effectively decreased primary tumor growth and sensitized tumors to radiation (Chapter 4, [24, 27]). Oral administration with high dose of DH348, i.e. based on the HED of clinical nimorazole treatment (i.e. daily oral delivery of 1.2 g/m^2 , [44]), was therefore investigated next and proved safe to administer as no signs of acute toxicity were observed in tumor-bearing mice (Chapter 5). Strikingly however, this high dose of DH348 with oral administration was no longer effective as single agent in both the H460 and HT29 tumor model, whereas the latter proved responsive previously. In addition, similar to what was observed with the combination of S4 and doxorubicin in the MDA-MB-231 model (Chapter 3), the effect of radiotherapy was counter-acted when combined with DH348 in both included tumor models (Chapter 5). Treatment with high dose of nitroimidazoles was previously reported to result in a vascular collapse, thereby increasing the hypoxic fraction and hence radioresistance of tumors [45]. Treatment with DH348 however showed no effect on the tumor microenvironment, including the hypoxic fraction, in H460 xenograft tumors. CAIXi treatment is however still hypothesized to induce cell death in the CAIX expressing subpopulation of tumor cells [27], which are closely associated with the hypoxic regions of a tumor. Effective CAIXi treatment should therefore decrease the hypoxic fraction of tumors, although this has not been reported yet and remains to be investigated further.

To validate the radiosensitizing effect of DH348 (Chapter 4, [24]) the exact same protocol was implemented (Chapter 5), which again also included the previously responsive colorectal HT29 tumor model [24]. The radiosensitivity of the three different tumor models however was unaltered after intravenous DH348 administration (Chapter 5). Several possible reasons can explain these contradictory results, of which the most plausible one was a difference between the batches of DH348 that were used. The initial DH348 study (Chapter 4, [24]) may have been performed with unstable compound. The stability of DH348 from the initial batch of compound however cannot be confirmed, because no compound is remaining from this study. In addition, the loss of tumor material from this initial study also prevents testing for the presence of stable

compound or degraded intermediates in the tumors. Combination therapy of CAIXi with radiation may however still prove valuable for future clinical practice, since an indane-sulfonamide previously showed xenograft tumors to be sensitized to radiation [46]. Similar observations were reported with a CAIX knockdown, which lead to an lower number of cells in the S-phase of the cell cycle, thereby increasing their radiosensitivity [28]. The exact mechanism responsible for increasing the radiosensitivity of tumors after CAIXi treatment, e.g. by affecting DNA repair after a decrease in intracellular pH [29-31], or by modulating the hypoxic fraction [40], however remains to be elucidated.

ALTERNATIVE THERAPEUTIC TARGETS

Targeting alternative proteins than CAIXi to disturb the cellular pH balance of tumor cells are currently also being investigated [4, 47]. Decreasing the function of several of these proteins, either by genetic silencing of the protein or by pharmacological inhibition, also effectively decreases cancer cell proliferation and survival, e.g. [48-51]. However, the loss of function of a single protein to disturb the cellular pH balance, including CAIX, may allow the tumor cell to adapt and compensate through activation or upregulation of several other proteins involved in the same process. Combination therapy with different inhibitors may therefore be more effective than single agent therapy. Combination of S4 with the proton pump inhibitor lansoprazole indeed showed synergism in their efficacy [52], but these combination strategies do require further investigation and validation.

A therapeutic target that is involved in the regulation of the activity of multiple proteins involved in the maintenance of the tumoral pH balance might also be promising. The intracellular pH sensor protein soluble adenylyl cyclase (sAC) might be such a target as it produces cAMP, i.e. one of the most versatile second messengers in the cell, after direct stimulation by intracellular bicarbonate levels [53-55]. The function of sAC in cells of the collecting duct of the kidney and of the epididymis has furthermore been described to regulate V-ATPase translocation to the luminal membrane, thereby directly regulating the pH balance of these cells [56-58]. It is unknown if sAC function is also involved in regulating any other proteins involved in maintaining the cellular pH balance, or how it functions during hypoxic conditions. Previously the full activation of CAIX was reported to require phosphorylation mediated via PKA, which is stimulated by cAMP [59]. The source of cAMP in this process is however unknown, but inhibition of sAC was able to decrease the extracellular acidification of CAIX overexpressing cells, which was more pronounced during hypoxic conditions (Chapter 7). These results thereby suggest that the degree of CAIX activation may be dependent on sAC activity, but it remains to be investigated if phosphorylated CAIX levels are indeed altered when sAC is inhibited.

The function of sAC in cancer however remains poorly understood. Previously, expression of sAC in melanoma has been reported to be a possible valuable diagnostic marker [60, 61]. Nevertheless, conflicting data exists about the function of sAC in cancer cells where it either acts as a tumor suppressor protein [62] or is overexpressed and stimulates cellular proliferation [63]. In addition, sAC inhibition was also reported to increase the radiosensitivity of prostate cancer cells [64]. In this thesis the sAC inhibitor KH7 was shown to decrease cell viability of some, but not all, tumor cell lines (Chapter 7), which was not dependent on either CAIX expression or hypoxia. The mechanism responsible for the difference in response between cell lines therefore remains to be elucidated. Furthermore, inhibition of sAC resulted in an increased CAIX expression during normoxic conditions in all of the four tumor cell lines included (Chapter 6). This effect could have been mediated via CREB, which previously has been described as a downregulating mechanism of CAIX expression [65], although the role of sAC herein still requires further investigation. These results however do suggest sAC function might be able to regulate CAIX, both on an expression and functional level, and may be important for the survival of certain tumor cells. The application of sAC inhibition as a new anti-cancer therapy therefore should require future investigation.

FUTURE DIRECTIONS

Recently a Phase I clinical trial with the CAIXi SLC-0111 (NCT02215850) has been completed and the results are eagerly awaited. Nevertheless, before implementing this compound in clinical practice its efficacy in combination with chemo- and radiotherapy should be studied, since the results described in this thesis indicate that the mechanisms of action of CAIXi might not yet be completely understood when combined with conventional anti-cancer therapies (Chapter 3, 4 and 5). In addition, with the wide variety of CAIX inhibitors currently available and still being developed, more focus should be with identifying the most promising compounds. This decision should be based on the combined results of the compounds binding affinity, pharmacokinetic characteristics, *in vitro*, and *in vivo* results. Furthermore, these *in vitro* and *in vivo* studies should require characterization of the compounds efficacy in several oncogenic processes (e.g. proliferation, survival, migration, invasion), either as single agent therapy or in combination with standard treatment modalities. Unfortunately, the perfect models to test these processes and determine the influence of CAIX therein are still unavailable. Currently the CRISPR/Cas9 technology is therefore investigated to generate CAIX and/or CAXII knockout tumor cells.

Overall, the idea of targeting the hypoxic tumor cells remains an interesting approach to increase the efficacy of standard treatment modalities. One such an approach, i.e. the use of dual-target compounds to target CAIX expressing cells, has been described in Chapter 6. The efficacy of these compounds however might not solely

depend on CAIX binding. Alternative methods, e.g. antibody targeting [66-68], might therefore prove to be more valuable in the future and require further investigation. Such a targeting strategy might also be beneficial for the delivery of a sAC inhibitor towards CAIX expressing tumor cells, thereby minimizing normal tissue toxicity as sAC is ubiquitously expressed in all tissues [54, 55, 69]. In addition, the tumor specificity and the prognostic value of CAIX expression in a wide variety of cancer types (Chapter 2, [15]) also make it a promising marker for diagnostic, prognostic, or predictive purposes in clinical practice. Several methods are therefore currently being investigated to visualize CAIX expression, e.g. via PET imaging [70-75]. The focus now however should be with developing a standardized method that is suitable for implementation in clinical practice in the near future. The tumor-specific expression of CAIX also promotes its use as an antigen for immunotherapy. The results of therapeutic vaccines against CAIX in renal cell carcinoma are promising [76], but further research is required in the use of CAIX targeted immunotherapy in alternative cancer types.

REFERENCES

1. Warburg, O., *On the origin of cancer cells*. Science, 1956. **123**(3191): p. 309-14.
2. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation*. Cell, 2011. **144**(5): p. 646-74.
3. Dubois, L.J., et al., *New ways to image and target tumour hypoxia and its molecular responses*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2015. **116**(3): p. 352-7.
4. Neri, D. and C.T. Supuran, *Interfering with pH regulation in tumours as a therapeutic strategy*. Nature reviews. Drug discovery, 2011. **10**(10): p. 767-77.
5. Parks, S.K., J. Chiche, and J. Pouyssegur, *pH control mechanisms of tumor survival and growth*. Journal of cellular physiology, 2011. **226**(2): p. 299-308.
6. Pastorek, J. and S. Pastorekova, *Hypoxia-induced carbonic anhydrase IX as a target for cancer therapy: from biology to clinical use*. Seminars in cancer biology, 2015. **31**: p. 52-64.
7. Supuran, C.T., *Carbonic anhydrases: novel therapeutic applications for inhibitors and activators*. Nature reviews. Drug discovery, 2008. **7**(2): p. 168-81.
8. Wykoff, C.C., et al., *Hypoxia-inducible expression of tumor-associated carbonic anhydrases*. Cancer research, 2000. **60**(24): p. 7075-83.
9. Pastorekova, S., et al., *Carbonic anhydrase IX, MN/CA IX: analysis of stomach complementary DNA sequence and expression in human and rat alimentary tracts*. Gastroenterology, 1997. **112**(2): p. 398-408.
10. Douglas, C.M., et al., *Lack of prognostic effect of carbonic anhydrase-9, hypoxia inducible factor-1alpha and bcl-2 in 286 patients with early squamous cell carcinoma of the glottic larynx treated with radiotherapy*. Clinical oncology, 2013. **25**(1): p. 59-65.
11. Kim, S.J., et al., *Prognostic value of carbonic anhydrase IX and Ki-67 expression in squamous cell carcinoma of the tongue*. Japanese journal of clinical oncology, 2007. **37**(11): p. 812-9.
12. Smeland, E., et al., *Prognostic impacts of hypoxic markers in soft tissue sarcoma*. Sarcoma, 2012. **2012**: p. 541650.
13. Wykoff, C.C., et al., *Expression of the hypoxia-inducible and tumor-associated carbonic anhydrases in ductal carcinoma in situ of the breast*. The American journal of pathology, 2001. **158**(3): p. 1011-9.
14. Stewart, D.J., et al., *Membrane carbonic anhydrase IX expression and relapse risk in resected stage I-II non-small-cell lung cancer*. Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer, 2014. **9**(5): p. 675-84.
15. van Kuijk, S.J., et al., *Prognostic Significance of Carbonic Anhydrase IX Expression in Cancer Patients: A Meta-Analysis*. Frontiers in oncology, 2016. **6**: p. 69.
16. Lomelino, C. and R. McKenna, *Carbonic anhydrase inhibitors: a review on the progress of patent literature (2011-2016)*. Expert opinion on therapeutic patents, 2016: p. 1-10.
17. Monti, S.M., C.T. Supuran, and G. De Simone, *Anticancer carbonic anhydrase inhibitors: a patent review (2008 - 2013)*. Expert opinion on therapeutic patents, 2013. **23**(6): p. 737-49.
18. Swietach, P., et al., *The role of carbonic anhydrase 9 in regulating extracellular and intracellular pH in three-dimensional tumor cell growths*. The Journal of biological chemistry, 2009. **284**(30): p. 20299-310.
19. Swietach, P., et al., *Tumor-associated carbonic anhydrase 9 spatially coordinates intracellular pH in three-dimensional multicellular growths*. The Journal of biological chemistry, 2008. **283**(29): p. 20473-83.
20. Dubois, L., et al., *Imaging the hypoxia surrogate marker CA IX requires expression and catalytic activity for binding fluorescent sulfonamide inhibitors*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2007. **83**(3): p. 367-73.
21. Dubois, L., et al., *Imaging of CA IX with fluorescent labelled sulfonamides distinguishes hypoxic and (re)-oxygenated cells in a xenograft tumour model*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2009. **92**(3): p. 423-8.
22. Svastova, E., et al., *Hypoxia activates the capacity of tumor-associated carbonic anhydrase IX to acidify extracellular pH*. FEBS letters, 2004. **577**(3): p. 439-45.

23. Rami, M., et al., *Hypoxia-targeting carbonic anhydrase IX inhibitors by a new series of nitroimidazole-sulfonamides/sulfamides/sulfamates*. Journal of medicinal chemistry, 2013. **56**(21): p. 8512-20.
24. Dubois, L., et al., *Targeting carbonic anhydrase IX by nitroimidazole based sulfamides enhances the therapeutic effect of tumor irradiation: a new concept of dual targeting drugs*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2013. **108**(3): p. 523-8.
25. Gieling, R.G., et al., *Antimetastatic effect of sulfamate carbonic anhydrase IX inhibitors in breast carcinoma xenografts*. Journal of medicinal chemistry, 2012. **55**(11): p. 5591-600.
26. Winum, J.Y., et al., *Ureido-substituted sulfamates show potent carbonic anhydrase IX inhibitory and antiproliferative activities against breast cancer cell lines*. Bioorganic & medicinal chemistry letters, 2012. **22**(14): p. 4681-5.
27. Ward, C., et al., *Evaluation of carbonic anhydrase IX as a therapeutic target for inhibition of breast cancer invasion and metastasis using a series of in vitro breast cancer models*. Oncotarget, 2015. **6**(28): p. 24856-70.
28. Doyen, J., et al., *Knock-down of hypoxia-induced carbonic anhydrases IX and XII radiosensitizes tumor cells by increasing intracellular acidosis*. Frontiers in oncology, 2012. **2**: p. 199.
29. Kulshrestha, A., et al., *Selective inhibition of tumor cell associated Vacuolar-ATPase 'a2' isoform overcomes cisplatin resistance in ovarian cancer cells*. Molecular oncology, 2016. **10**(6): p. 789-805.
30. De Milito, A. and S. Fais, *Tumor acidity, chemoresistance and proton pump inhibitors*. Future oncology, 2005. **1**(6): p. 779-86.
31. Liao, C., et al., *Genomic screening in vivo reveals the role played by vacuolar H⁺ ATPase and cytosolic acidification in sensitivity to DNA-damaging agents such as cisplatin*. Molecular pharmacology, 2007. **71**(2): p. 416-25.
32. Chiche, J., et al., *Membrane-bound carbonic anhydrases are key pH regulators controlling tumor growth and cell migration*. Advances in enzyme regulation, 2010. **50**(1): p. 20-33.
33. Csaderova, L., et al., *The effect of carbonic anhydrase IX on focal contacts during cell spreading and migration*. Frontiers in physiology, 2013. **4**: p. 271.
34. Lock, F.E., et al., *Targeting carbonic anhydrase IX depletes breast cancer stem cells within the hypoxic niche*. Oncogene, 2013. **32**(44): p. 5210-9.
35. Svastova, E., et al., *Carbonic anhydrase IX interacts with bicarbonate transporters in lamellipodia and increases cell migration via its catalytic domain*. The Journal of biological chemistry, 2012. **287**(5): p. 3392-402.
36. Stock, C. and A. Schwab, *Protons make tumor cells move like clockwork*. Pflugers Archiv : European journal of physiology, 2009. **458**(5): p. 981-92.
37. Horsman, M.R. and P. Vaupel, *Pathophysiological Basis for the Formation of the Tumor Microenvironment*. Frontiers in oncology, 2016. **6**: p. 66.
38. Barriuso, J., R. Nagaraju, and A. Hurlstone, *Zebrafish: a new companion for translational research in oncology*. Clinical cancer research : an official journal of the American Association for Cancer Research, 2015. **21**(5): p. 969-75.
39. Doke, S.K. and S.C. Dhawale, *Alternatives to animal testing: A review*. Saudi pharmaceutical journal : SPJ : the official publication of the Saudi Pharmaceutical Society, 2015. **23**(3): p. 223-9.
40. Meijer, T.W., et al., *Tumor microenvironmental changes induced by the sulfamate carbonic anhydrase IX inhibitor S4 in a laryngeal tumor model*. PloS one, 2014. **9**(9): p. e108068.
41. Mahoney, B.P., et al., *Tumor acidity, ion trapping and chemotherapeutics. I. Acid pH affects the distribution of chemotherapeutic agents in vitro*. Biochemical pharmacology, 2003. **66**(7): p. 1207-18.
42. Raghunand, N., B.P. Mahoney, and R.J. Gillies, *Tumor acidity, ion trapping and chemotherapeutics. II. pH-dependent partition coefficients predict importance of ion trapping on pharmacokinetics of weakly basic chemotherapeutic agents*. Biochemical pharmacology, 2003. **66**(7): p. 1219-29.
43. Gieling, R.G., et al., *Inhibition of carbonic anhydrase activity modifies the toxicity of doxorubicin and melphalan in tumour cells in vitro*. Journal of enzyme inhibition and medicinal chemistry, 2013. **28**(2): p. 360-9.

44. Bentzen, J., et al., *Locally advanced head and neck cancer treated with accelerated radiotherapy, the hypoxic modifier nimorazole and weekly cisplatin. Results from the DAHANCA 18 phase II study*. Acta oncologica, 2015. **54**(7): p. 1001-7.
45. Dische, S., et al., *A trial of Ro 03-8799 (pimonidazole) in carcinoma of the uterine cervix: an interim report from the Medical Research Council Working Party on advanced carcinoma of the cervix*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 1993. **26**(2): p. 93-103.
46. Dubois, L., et al., *Specific inhibition of carbonic anhydrase IX activity enhances the in vivo therapeutic effect of tumor irradiation*. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 2011. **99**(3): p. 424-31.
47. Pouyssegur, J., F. Dayan, and N.M. Mazure, *Hypoxia signalling in cancer and approaches to enforce tumour regression*. Nature, 2006. **441**(7092): p. 437-43.
48. Lee, J.Y., et al., *MCT4 as a potential therapeutic target for metastatic gastric cancer with peritoneal carcinomatosis*. Oncotarget, 2016.
49. Lu, Y., et al., *ZT-25, a new vacuolar H(+)-ATPase inhibitor, induces apoptosis and protective autophagy through ROS generation in HepG2 cells*. European journal of pharmacology, 2016. **771**: p. 130-8.
50. Guan, B., A. Hoque, and X. Xu, *Amiloride and guggulsterone suppression of esophageal cancer cell growth in vitro and in nude mouse xenografts*. Frontiers in biology, 2014. **9**(1): p. 75-81.
51. Canitano, A., et al., *Proton pump inhibitors induce a caspase-independent antitumor effect against human multiple myeloma*. Cancer letters, 2016. **376**(2): p. 278-83.
52. Federici, C., et al., *Lansoprazole and carbonic anhydrase IX inhibitors synergize against human melanoma cells*. Journal of enzyme inhibition and medicinal chemistry, 2016: p. 1-7.
53. Wuttke, M.S., J. Buck, and L.R. Levin, *Bicarbonate-regulated soluble adenylyl cyclase*. JOP : Journal of the pancreas, 2001. **2**(4 Suppl): p. 154-8.
54. Rahman, N., J. Buck, and L.R. Levin, *pH sensing via bicarbonate-regulated "soluble" adenylyl cyclase (sAC)*. Frontiers in physiology, 2013. **4**: p. 343.
55. Tresguerres, M., J. Buck, and L.R. Levin, *Physiological carbon dioxide, bicarbonate, and pH sensing*. Pflugers Archiv : European journal of physiology, 2010. **460**(6): p. 953-64.
56. Pastor-Soler, N., et al., *Bicarbonate-regulated adenylyl cyclase (sAC) is a sensor that regulates pH-dependent V-ATPase recycling*. The Journal of biological chemistry, 2003. **278**(49): p. 49523-9.
57. Gong, F., et al., *Vacuolar H+-ATPase apical accumulation in kidney intercalated cells is regulated by PKA and AMP-activated protein kinase*. American journal of physiology. Renal physiology, 2010. **298**(5): p. F1162-9.
58. Paunescu, T.G., et al., *cAMP stimulates apical V-ATPase accumulation, microvillar elongation, and proton extrusion in kidney collecting duct A-intercalated cells*. American journal of physiology. Renal physiology, 2010. **298**(3): p. F643-54.
59. Ditte, P., et al., *Phosphorylation of carbonic anhydrase IX controls its ability to mediate extracellular acidification in hypoxic tumors*. Cancer research, 2011. **71**(24): p. 7558-67.
60. Magro, C.M., et al., *Soluble adenylyl cyclase antibody profile as a diagnostic adjunct in the assessment of pigmented lesions*. Archives of dermatology, 2012. **148**(3): p. 335-44.
61. Magro, C.M., et al., *Expression of soluble adenylyl cyclase in lentigo maligna: use of immunohistochemistry with anti-soluble adenylyl cyclase antibody (R21) in diagnosis of lentigo maligna and assessment of margins*. Archives of pathology & laboratory medicine, 2012. **136**(12): p. 1558-64.
62. Ramos-Espiritu, L., et al., *The metabolic/pH sensor soluble adenylyl cyclase is a tumor suppressor protein*. Oncotarget, 2016.
63. Flacke, J.P., et al., *Type 10 soluble adenylyl cyclase is overexpressed in prostate carcinoma and controls proliferation of prostate cancer cells*. The Journal of biological chemistry, 2013. **288**(5): p. 3126-35.
64. Appukuttan, A., et al., *Inhibition of soluble adenylyl cyclase increases the radiosensitivity of prostate cancer cells*. Biochimica et biophysica acta, 2014. **1842**(12 Pt B): p. 2656-63.
65. Wang, G., et al., *CREB is a key negative regulator of carbonic anhydrase IX (CA9) in gastric cancer*. Cellular signalling, 2015. **27**(7): p. 1369-79.

66. Zatovicova, M., et al., *Monoclonal antibody G250 targeting CA : Binding specificity, internalization and therapeutic effects in a non-renal cancer model*. International journal of oncology, 2014. **45**(6): p. 2455-67.
67. Bauer, S., et al., *Targeted therapy of renal cell carcinoma: synergistic activity of cG250-TNF and IFN γ* . International journal of cancer, 2009. **125**(1): p. 115-23.
68. Duan, Y., et al., *A tumor targeted gene vector modified with G250 monoclonal antibody for gene therapy*. Journal of controlled release : official journal of the Controlled Release Society, 2008. **127**(2): p. 173-9.
69. Steegborn, C., *Structure, mechanism, and regulation of soluble adenylyl cyclases - similarities and differences to transmembrane adenylyl cyclases*. Biochimica et biophysica acta, 2014. **1842**(12 Pt B): p. 2535-47.
70. Akurathi, V., et al., *Development and biological evaluation of (9)(9)mTc-sulfonamide derivatives for in vivo visualization of CA IX as surrogate tumor hypoxia markers*. European journal of medicinal chemistry, 2014. **71**: p. 374-84.
71. Akurathi, V., et al., *Synthesis and biological evaluation of a 99mTc-labelled sulfonamide conjugate for in vivo visualization of carbonic anhydrase IX expression in tumor hypoxia*. Nuclear medicine and biology, 2010. **37**(5): p. 557-64.
72. Peeters, S.G., et al., *[(18)F]VM4-037 MicroPET Imaging and Biodistribution of Two In Vivo CAIX-Expressing Tumor Models*. Molecular imaging and biology : MIB : the official publication of the Academy of Molecular Imaging, 2015. **17**(5): p. 615-9.
73. Minn, I., et al., *[64Cu]XYMSR-06: A dual-motif CAIX ligand for PET imaging of clear cell renal cell carcinoma*. Oncotarget, 2016.
74. Sneddon, D., et al., *Synthesis and in Vivo Biological Evaluation of (68)Ga-Labeled Carbonic Anhydrase IX Targeting Small Molecules for Positron Emission Tomography*. Journal of medicinal chemistry, 2016. **59**(13): p. 6431-43.
75. Cheal, S.M., et al., *Pairwise comparison of 89Zr- and 124I-labeled cG250 based on positron emission tomography imaging and nonlinear immunokinetic modeling: in vivo carbonic anhydrase IX receptor binding and internalization in mouse xenografts of clear-cell renal cell carcinoma*. European journal of nuclear medicine and molecular imaging, 2014. **41**(5): p. 985-94.
76. Combe, P., et al., *Trial Watch: Therapeutic vaccines in metastatic renal cell carcinoma*. Oncoimmunology, 2015. **4**(5): p. e1001236.

Summary

The hypoxic tumor microenvironment exists in many different solid tumor types and originates due to an inadequate and immature vascular supply resulting in a decreased delivery of oxygen and nutrients. These hypoxic areas are associated with resistance to standard treatment modalities such as radiotherapy, chemotherapy, and surgery, thereby causing a worse outcome for patients. These hostile microenvironmental stresses require tumor cells to adapt, which can partly be mediated via the stabilization of the hypoxia-inducible factor 1 alpha (HIF1- α) during hypoxic conditions. HIF1- α regulates the transcription of many genes, one of them being carbonic anhydrase IX (CAIX). During hypoxic conditions, stabilized HIF1- α binds to the hypoxic response element in the promoter regions of CAIX resulting in elevated expression levels. CAIX is an important protein that is involved in maintaining the balance between an acidic extracellular, and an alkaline intracellular environment by hydrolyzing carbon dioxide to bicarbonate and a free proton. In addition, the expression of CAIX was found to be highly tumor-specific, which makes CAIX an attractive target for anti-cancer therapy. The aim of this thesis was therefore to investigate whether inhibition of CAIX affects tumor growth and to evaluate whether the efficacy of standard treatment modalities such as chemo- and radiotherapy is affected by CAIX inhibitor (CAIXi) treatment. In this thesis several different CAIXi were therefore investigated.

First we evaluated the prognostic value of tumoral CAIX expression (chapter 2). Ever since CAIX expression was identified to be directly regulated via HIF stabilization many clinical studies have investigated the prognostic value of CAIX expression as a hypoxia surrogate marker. Nevertheless a definitive conclusion about its association with prognosis remained unavailable, since most of these clinical studies were inconclusive, partly due to a limited number of patients included. A meta-analysis was therefore performed, which combined all available published clinical data to determine the prognostic value of CAIX expression in solid tumors. This meta-analysis revealed that patients having tumors with high CAIX expression have a higher risk of locoregional failure, disease progression, and a higher risk to develop metastases, independent of the tumor type or site. The results of this meta-analysis therefore support the development of a clinically applicable test to assess CAIX expression prior to treatment to estimate patient prognosis.

The prognostic value of CAIX also supports its use as a target for anti-cancer therapy, thus the efficacy of several CAIXi in combination with standard treatment modalities such as chemo- and radiotherapy were evaluated in this thesis. Chapter 3 describes the effect of the selective sulfamate CAIXi S4 on doxorubicin efficacy. CAIX is involved in extracellular acidification, which limits the uptake of weak basic chemotherapeutic drugs, such as doxorubicin, and thereby decreases its efficacy. The efficacy of doxorubicin treatment might therefore increase when combined with a selective CAIXi like the sulfamate CAIXi S4. Treatment with S4 however did not enhance doxorubicin efficacy in two of the investigated tumor models and doxorubicin efficacy even decreased in one

tumor model. These results therefore stress the need for better understanding the mechanism of interaction between CAIXi and chemotherapeutic agents.

In chapter 4 the efficacy of a novel dual-target CAIXi, i.e. DH348 consisting of a radiosensitizing nimorazole conjugated to a sulfamide CAIXi moiety, was evaluated in combination with ionizing radiation. *In vitro*, DH348 reduced the hypoxia-induced and CAIX-dependent extracellular acidification, but had no influence on the intrinsic radiosensitivity of tumor cells. *In vivo* however, DH348 monotherapy decreased tumor growth and increased their radiosensitivity, which was dependent on CAIX expression levels. In chapter 5 the feasibility of a more attractive administration route for patients, i.e. oral application of DH348, was evaluated and we attempted to validate its efficacy in multiple tumor models. The pharmacokinetic characteristics of DH348 supported its oral applicability and the human equivalent dose of DH348 was found safe to be administered to tumor-bearing mice. Nevertheless, monotherapy of DH348 after oral administration was ineffective and even appeared to abrogate the effect of radiotherapy. Validation experiments using the identical experimental setup as described in chapter 4 were therefore performed and again showed no increase in radiosensitivity of tumors after DH348 treatment. Based on the results from chapter 5 it can therefore be concluded that DH348 does not sensitize human tumor xenografts to radiation, both after oral and intravenous administration, which contradicts the results described in chapter 4. The most likely explanation for these conflicting findings is that a different batch of DH348 was used in both studies.

A similar dual-target approach may however still be useful to target cytotoxic drugs towards CAIX expressing cells, which may reduce normal tissue side-effects. Chapter 6 therefore describes the design and synthesis of new series of dual-target compounds incorporating a CAIXi moiety combined with several anti-cancer drugs, including the chemotherapeutic agents chlorambucil, tirapazamine, and temozolomide, two ataxia telangiectasia and Rad3-related protein inhibitors (ATRI), and the anti-diabetic biguanide agent phenformin. Only one compound, i.e. an ATRI derivative, showed a higher efficacy in combination with radiation in CAIX overexpressing cells as compared to cells lacking CAIX expression. Nevertheless, the preferred efficacy of this compound for CAIX expressing cells might not solely depend on binding of the compound to CAIX, since all of the synthesized compounds exhibit a low binding affinity to CAIX and other human carbonic anhydrases. The hypothesis that these dual-target compounds specifically affect CAIX expressing tumor cells was therefore not confirmed, but targeting cytotoxic drugs towards CAIX expressing regions in tumors using alternative approaches continues to be an interesting area for future drug design.

In addition, other mechanisms, besides CAIX, are also involved in maintaining the pH balance of tumor cells. The intracellular pathways involved in regulating these processes however remain largely unknown. In chapter 7 we describe that the intracellular pH sensor soluble adenylyl cyclase (sAC) might be involved herein, since pharmacological inhibition of sAC decreased the CAIX-dependent extracellular acidification and signifi-

cantly increased CAIX expression levels during normoxic conditions. In addition, cell viability was also affected by sAC inhibition, although these effects appeared to be cell-type specific. These results therefore suggest that sAC is involved in regulating the activity and expression of CAIX, although the exact role of sAC in cancer and the intracellular pathways involved remain to be elucidated.

In conclusion, this thesis showed that tumor CAIX expression is strongly associated with a worse prognosis for cancer patients. Furthermore, even though CAIXi were hypothesized to increase the efficacy of standard treatment modalities like chemo- and radiotherapy, the CAIXi studied in this thesis were ineffective as such. Targeting alternative intracellular pathways, e.g. sAC, may therefore represent a better approach to treat cancer, but additional research is essential to identify and elucidate these pathways. Targeting CAIX with cytotoxic drugs to increase the therapeutic window of those drugs may therefore be more promising and requires further investigation.

Samenvatting

Een hypoxisch micromilieu is aanwezig in verschillende typen solide tumoren. Dit hypoxisch micromilieu ontstaat door een verminderde toevoer van zuurstof en voedingsstoffen door onvoldoende bloedvoorziening. Deze hypoxische gebieden in de tumor zijn minder sensitief voor standaard behandelopties zoals radio- en chemotherapie en chirurgie, wat de prognose voor patiënten verslechterd. Dit vijandige micromilieu in tumoren maakt het noodzakelijk voor de kankercel om zich aan te passen. Deze aanpassingen kunnen gedeeltelijk gefaciliteerd worden door de stabilisatie van hypoxie-induceerbare factor 1-alfa (HIF1- α) tijdens hypoxische condities. HIF1- α reguleert de transcriptie van verschillende genen, waaronder “carbonic anhydrase IX” (CAIX). Tijdens hypoxische omstandigheden kan het gestabiliseerde HIF1- α zich binden aan het hypoxie-reagerend element in de promotor regio van CAIX. Dit resulteert in verhoogde CAIX expressie levels. CAIX is een eiwit dat belangrijk is voor het onderhouden van de balans tussen het zure extracellulaire en het alkalische intracellulaire milieu door middel van de hydrolyse van koolstofdioxide naar bicarbonaat en een vrije proton. Daarnaast is gebleken dat de expressie van CAIX zeer tumor-specifiek is, wat CAIX daarmee een aantrekkelijk doelwit voor kankertherapie maakt. Daarom is het doel van deze thesis te onderzoeken of inhibitie van CAIX een effect heeft op tumorgroei en te evalueren of de effectiviteit van standaard behandelopties zoals chemo- en radiotherapie beïnvloed wordt door behandeling met CAIX inhibitoren (CAIXi). In deze thesis worden daarom verschillende typen CAIXi getest.

Ten eerste is de prognostische waarde van CAIX expressie in de tumor bepaald (hoofdstuk 2). Sinds de bevinding dat CAIX expressie direct gereguleerd wordt door HIF stabilisatie hebben vele klinische studies de prognostische waarde van CAIX expressie onderzocht als een surrogaat marker voor hypoxie. Echter, een definitieve conclusie aangaande de associatie tussen CAIX en prognose bleef onduidelijk, omdat de resultaten van de meeste van deze klinische studies niet overtuigend waren door een gelimiteerd aantal geïncludeerde patiënten. Een meta-analyse is daarom uitgevoerd waarin de resultaten van alle beschikbare en gepubliceerde klinische studies gecombineerd zijn om de algehele prognostische waarde van CAIX expressie in solide tumoren te bepalen. De resultaten van deze meta-analyse lieten zien dat patiënten met een hoge CAIX expressie in de tumor een hoger risico hebben op locoregionale recidieven, progressie van de ziekte en een hoger risico op het ontwikkelen van metastasen, wat onafhankelijk van het tumor type of orgaan is. De resultaten van deze meta-analyse ondersteunen daarom de ontwikkeling van een klinische bruikbare test om de CAIX expressie vast te stellen voorafgaand aan de start van behandeling om daarmee de prognose voor patiënten te bepalen.

De prognostische betekenis van CAIX ondersteund tevens het gebruik van CAIX als een doelwit voor therapeutische doeleinden. Vervolgens is de effectiviteit van verschillende CAIXi in combinatie met standaard behandelopties zoals chemo- en radiotherapie verder onderzocht in deze thesis. Hoofdstuk 3 beschrijft het effect van de selectieve sulfamaat CAIXi S4 op de effectiviteit van doxorubicine. CAIX is betrokken bij de extra-

cellulaire verzuring, wat de opname van basische chemotherapeutische medicijnen verminderd, zoals doxorubicine, en verlaagt daardoor de effectiviteit van deze medicijnen. De effectiviteit van doxorubicine zou daarom verhoogd kunnen worden wanneer deze gecombineerd wordt met een selectieve CAIXi, zoals de sulfamaat CAIXi S4. Desalniettemin had de behandeling met S4 geen effect op de effectiviteit van doxorubicine in twee van de drie onderzochte tumor modellen. S4 verminderde zelfs de doxorubicine effectiviteit in één van deze tumor modellen. Uit deze resultaten blijkt dat het noodzakelijk is om de mogelijke interacties tussen CAIXi en chemotherapieën beter te begrijpen.

Daarnaast is de effectiviteit van een nieuwe “dual-target” CAIXi genaamd DH348 in hoofdstuk 4 onderzocht in combinatie met bestraling. Deze CAIXi DH348 bestaat uit een radiosensibiliserende nimorazole geconjugeerd met een sulfamide CAIXi. DH348 verminderde de hypoxische CAIX afhankelijke extracellulaire verzuring *in vitro*, maar had geen invloed op de intrinsieke radiosensitiviteit van tumorcellen. *In vivo* zorgde DH348 echter voor een reductie in tumorgroei en verhoogde de radiosensitiviteit van tumoren. Deze effecten bleken afhankelijk te zijn van de hoeveelheid CAIX expressie in de tumoren. In hoofdstuk 5 werd daarom een aantrekkelijkere, orale toedieningsvorm voor patiënten onderzocht en werd geprobeerd om de eerder behaalde resultaten te valideren in meerdere tumor modellen. De farmacokinetische karakteristieken van DH348 ondersteunde de orale toediening, waarbij het ook veilig bleek om de humaan equivalente dosis van DH348 toe te dienen aan tumor-dragende muizen. Orale toediening van DH348 had echter geen effect op tumor groei en leek zelfs het effect van radiotherapie tegen te werken. Validatie experimenten met een identieke experimentele opzet zoals in hoofdstuk 4 werden daarom uitgevoerd en lieten wederom geen verschil in radiosensitiviteit zien in tumoren behandeld met DH348. Gebaseerd op de resultaten van hoofdstuk 5 blijkt de gevoeligheid voor bestraling van tumoren onveranderd na zowel oraal als intraveneuze toediening van DH348. De resultaten van hoofdstuk 5 spreken daarmee de resultaten van hoofdstuk 4 tegen, wat waarschijnlijk veroorzaakt wordt door een verschillende partij DH348 die gebruikt is tijdens de studies.

Een soortgelijke “dual-target” methode kan echter nog steeds potentieel gebruikt worden om cytotoxische medicijnen specifiek te leiden naar CAIX positieve cellen, wat daardoor mogelijk de toxische bijwerkingen in normale weefsels kan verminderen. Hoofdstuk 6 beschrijft de synthese van nieuwe “dual-target” stoffen bestaande uit een CAIXi gecombineerd met verschillende kanker medicijnen, zoals de chemotherapeutica chlorambucil, tirapazamine en temozolomide, twee ataxia telangiectasia en Rad3-gerelateerde eiwit inhibitoren (ATRI) en het diabetes guanide middel fenformine. Slechts één van deze stoffen, een ATRI derivaat, had een hogere effectiviteit in combinatie met bestraling in cellen met een overexpressie van CAIX in vergelijking tot cellen waarbij de CAIX expressie ontbreekt. Echter, de voorkeur van deze stof voor CAIX positieve cellen is naar alle waarschijnlijkheid niet uitsluitend afhankelijk van de binding van de stof aan CAIX, aangezien alle gesynthetiseerde stoffen slechts een lage affiniteit

hebben voor binding met CAIX en andere humane carbonic anhydrases. De hypothese dat deze “dual-target” stoffen specifiek hun invloed uitoefenen op CAIX positieve cellen kon daarom niet bevestigd worden, maar het specifiek leiden van cytotoxische medicijnen naar CAIX positieve tumor regio’s blijft interessant voor toekomstige medicijnontwikkeling.

Buiten CAIX zijn alternatieve mechanismen ook verantwoordelijk voor het onderhouden van een correcte pH balans in tumor cellen. De intracellulaire processen die deze mechanismen reguleren zijn echter nog steeds grotendeels onbekend. In hoofdstuk 7 wordt de mogelijke betrokkenheid van de intracellulaire pH sensor “soluble adenylyl cyclase” (sAC) beschreven, aangezien farmacologische inhibitie van sAC de CAIX geïnduceerde extracellulaire verzuring verminderde en de expressie van CAIX deed toenemen tijdens normoxische condities. Daarbij was de levensvatbaarheid van cellen ook aangedaan door inhibitie van sAC, maar deze effecten bleken specifiek te zijn voor bepaalde cel types. Deze resultaten geven daardoor aan dat sAC mogelijk betrokken is bij de regulatie van de activiteit en expressie van CAIX, hoewel de exacte rol van sAC in kanker en de intracellulaire processen die hierbij betrokken zijn nog steeds opgehelderd dienen te worden.

Deze thesis laat zien dat de expressie van CAIX in tumoren van groot prognostisch belang is voor kanker patiënten. Verder bleken de in deze thesis onderzochte CAIXi ineffectief in het verhogen van de effectiviteit van standaard behandelopties, zoals chemo- en radiotherapie, ook al was dat wel de originele hypothese. Het ontwikkelen van medicijnen gericht tegen alternatieve intracellulaire processen, zoals bijvoorbeeld sAC, zou daarom een betere optie kunnen zijn voor de behandeling van kanker. Allereerst dienen deze processen echter geïdentificeerd en opgehelderd te worden. Het specifiek leiden van cytotoxische medicijnen naar CAIX om de effectiviteit van die medicijnen te verhogen en bijwerkingen te verminderen is daarom mogelijk veelbelovender en behoeft verder onderzoek.

Valorization addendum

This thesis describes the potential of increasing the efficacy of standard treatment modalities, such as chemo- and radiotherapy, through inhibition or targeting of carbonic anhydrase IX (CAIX). The main aim of this thesis was the development and evaluation of compounds targeting the hypoxic tumor microenvironment to decrease normal tissue side-effects of currently clinically used anti-cancer chemotherapeutic agents. Developing new anti-cancer therapies however remains challenging, since cancer is an extremely heterogeneous disease consisting of distorted versions of a person's own cells. Nevertheless, the increase in cancer incidence has a severe socio-economic impact and the development of novel anti-cancer therapies therefore remains essential. In this valorization addendum the societal benefit of targeting CAIX expression in cancer is evaluated.

CLINICAL RELEVANCE

The heterogeneity of cancer requires personalized medicine, which consists of treatment options specifically tailored for each individual patient's needs. Many studies aim to identify biomarkers to predict treatment response and to stratify patients based on their sensitivity to a certain treatment. One such a biomarker might be CAIX as its expression is highly tumor-specific and is generally associated with a worse prognosis for cancer patients (Chapter 2). Clinical evaluation of CAIX expression in tumors may therefore prove beneficial for patient stratification; although the specific treatments best suited for this type of stratification remain to be identified. An interesting approach to evaluate tumoral CAIX expression levels is by using PET imaging with specific CAIX targeting PET tracers that are currently being developed. This non-invasive method of visualizing CAIX expression might also be promising for the detection and diagnosis of early malignant lesions in patients.

In addition, CAIX has been implicated in several carcinogenic processes (e.g. invasion and migration), but the clinical benefit of using CAIX inhibitors in patients remains to be investigated. The CAIX inhibitors described in this thesis (Chapter 3, 4, 5 and 6) will unlikely be implemented in clinical practice due to limited efficacy, but an alternative CAIX inhibitor (i.e. SLC-0111) is currently under investigation in a phase I clinical trial to evaluate its safety for use in patients. The results of this clinical trial however are not yet available and will provide data on safety and some insights into the efficacy of CAIX inhibitors in patients. Prior to implementing CAIX inhibitors in clinical practice, their interactions with standard treatment modalities need to be investigated in detail, since unexpected adverse effects may occur, as described in this thesis (Chapter 3) where the efficacy of doxorubicin decreased in combination with CAIX inhibitor treatment in one of the investigated tumor models.

The CAIX targeting molecules may also be utilized in clinical practice as a carrier to deliver cytotoxic compounds towards CAIX expressing tumor cells (e.g. the dual-targeting concept described in Chapter 4, 5 and 6). It is hypothesized that such an ap-

proach would decrease normal tissue side-effects, thereby allowing dose escalation in the tumor and enhancement of overall treatment efficacy. Even though dual-targeting compounds (Chapter 6) in general showed no preference for CAIX expressing cells, the tumor-specific and extracellular expression of CAIX continues to make it a promising target for drug delivery using alternative approaches.

GAIN FOR SOCIETY

Improving the efficacy of anti-cancer treatment through targeting CAIX will also be beneficial for society in general. Firstly, the use of tumoral CAIX expression as a biomarker (Chapter 2) may select treatment-sensitive patient populations. This type of personalized medicine will lead to a general increase in treatment efficacy as the non-responsive patient population may be treated with alternative more effective therapies, which eventually will decrease the overall treatment costs. Secondly, treatment costs may also decrease when normal tissue side-effects caused by anti-cancer therapy can be reduced, e.g. via targeting cytotoxic drugs towards CAIX expressing tumor cells. In general, any benefit in cancer treatment has the potential to be a gain for society as it can decrease treatment costs and prolong patient's survival.

IMPROVEMENT IN HEALTH CARE

Even though tumoral CAIX expression is hypothesized to be suited for theragnostic purposes, single CAIX inhibitor treatment may not be sufficiently potent enough to significantly improve health care (Chapter 3, 4, and 5). Nevertheless definitive conclusions regarding the efficacy of single CAIX inhibitor treatment can only be drawn after completing multiple clinical trials, for which the CAIX inhibitor SLC-0111 is a likely candidate that is currently being evaluated in a Phase I clinical trial. The extracellular and tumor-specific CAIX expression in tumors might also be alternatively exploited by designing a method for specific drug delivery to those regions (Chapter 6). Such an approach is expected to improve health care as patients will experience less toxic side-effects that otherwise may require additional interventions. Alternatively, CAIX inhibitors may modulate the efficacy of standard treatment options, although rigorous validation is required before clinical implementation (Chapter 3). Taken together both these methods can potentially increase the therapeutic window of anti-cancer therapy and thereby result in an improvement in the health care of cancer patients.

In addition, CAIX expression is tumor-specific and has prognostic value in a wide variety of cancer types (Chapter 2). The expression levels of CAIX could therefore be an excellent biomarker for patient selection, although the relevant therapies remain to be identified. Patient selection improves health care when it can prevent treating unre-

sponsive patients who would otherwise experience the side-effects of treatment without the therapeutic gain. Furthermore, CAIX may also be of diagnostic importance when it is proven to identify malignant lesions, e.g. via PET imaging. Early detection of these malignant lesions, e.g. by implementing CAIX visualization in screening programs, can be a great improvement in health care, since diagnosis and treatment can start earlier thereby increasing the patient's chance of survival.

NOVELTY OF THE CONCEPT

The application of CAIX as either a biomarker or a therapeutic target in cancer is not novel. Studies investigating the prognostic value of CAIX exponentially increased after the identification of a hypoxia-responsive element (HRE) in its promotor region in 2001. A wide variety in protocols and patient populations were however used in these studies and this thesis is therefore the first summarizing all available data regarding the tumoral CAIX expression and its association with prognosis (Chapter 2). The clinical application of this finding however remains to be elucidated and requires a standardized quantification method for evaluating the levels of CAIX expression.

Furthermore, CAIX has long been hypothesized to be a valuable therapeutic target due to its tumor-specific expression and its involvement in maintaining the pH balance of tumor cells. In addition, CAIX has been implicated in several other carcinogenic processes (e.g. proliferation, migration, and invasion). In general, however, the efficacy of CAIX inhibitors varies greatly (Chapter 3, 4, 5 and 6) and single CAIX inhibitor treatment may therefore not be potent enough to exert a significant effect on tumor cells. This thesis therefore focused on the potential of CAIX inhibitors to modulate the efficacy of standard treatment modalities, i.e. chemo- and radiotherapy (Chapter 3, 4, and 5). The counterintuitive results in this thesis (Chapter 3) where an adverse effect of CAIX inhibition on doxorubicin efficacy was observed in one tumor model indicates the incomplete understanding of the mechanism of CAIX inhibitors in combination with standard treatment options. An alternative novel therapeutic target involved in multiple intracellular pathways may therefore be sAC (Chapter 7). The use of sAC inhibition in cancer is however still relatively new, since only limited but conflicting data is currently available in literature. Therefore, before sAC can be used as an anti-cancer therapeutic target its exact mechanism of action remains to be elucidated.

The last approach described in this thesis where cytotoxic drugs are targeted towards CAIX expressing cells has been described previously, although alternative approaches were used and these studies predominantly focused on renal cell carcinoma where CAIX expression becomes upregulated due to a mutation in the VHL protein preventing HIF1- α degradation. This thesis described a novel method of targeting anti-cancer drugs towards CAIX expressing cells via dual-target compounds (Chapter 6), which consist of a CAIX inhibitor moiety conjugated to an anti-cancer drug. In general,

these dual-target compounds however showed little preference for CAIX expressing cells, which minimized their practical applicability. Alternative methods to target cytotoxic anti-cancer drugs towards CAIX expressing tumors may therefore be more potent and require further investigation.

ROAD TO MARKET

Even though several aspects of tumoral CAIX expression may be promising for future marketing, additional preclinical and clinical experiments remain essential. Firstly, the prognostic value of CAIX requires quantification of its expression levels via either immunohistochemistry or non-invasive imaging, e.g. PET. Immunohistochemical evaluation of CAIX expression requires the development of a specific antibody to detect CAIX, which can potentially be marketed once its specificity is sufficiently proven. As an example, the M75 antibody is currently marketed by Bayer. However, many different CAIX-specific antibodies are already available from many different suppliers, thereby increasing the competition. The development of a fast and easy to use kit to evaluate CAIX expression might therefore be more promising, which will provide a standardized method for CAIX detection and quantification, which is currently unavailable. Evaluation of CAIX expression levels with non-invasive PET imaging may therefore be more promising. Future research should therefore focus on developing a specific CAIX tracer that can be implemented in clinical practice. This tracer can potentially be marketed once CAIX expression is found to be of clinical importance such as when CAIX is identified as a diagnostic marker to detect early malignant and/or invasive tumor lesions. In addition, such a tracer can also be potentially marketed when CAIX expression levels are proven to be predictive for treatment outcome. These expression levels can then be used to stratify patients for specific therapies that have proven to be effective, e.g. specific drug delivery approaches to CAIX expressing tumor cells. Prior to marketing however the cost-effectiveness and added benefit of this CAIX specific imaging as compared to standard clinical care needs to be evaluated, since PET imaging is currently not cost-effective enough for implementation in clinical practice.

Secondly, CAIX inhibitors will be relatively easy to market once they prove to be effective in preclinical and clinical experiments. The CAIX inhibitors described in this thesis however will not be pursued further as they were either ineffective as single agents, or were unable to increase the efficacy of standard treatment modalities (Chapter 3, 4, 5, and 6). A wide variety of alternative CAIX inhibitors are however still being developed and one of these, i.e. SLC-0111, has recently completed a phase I clinical trial. When the results of this clinical trial show that SLC-0111 is safe to administer to patients, its efficacy will remain to be evaluated in phase II/III clinical trials. These future clinical trials should also investigate the influence of SLC-0111 on the efficacy of standard treatment modalities as CAIX inhibitors may also cause adverse effects on chemotherapy efficacy

(Chapter 3). Alternative options, such as targeting CAIX expressing tumors with cytotoxic drugs however continue to be potentially clinically relevant and should therefore also be pursued further. An alternative approach than dual-targeting drugs (Chapter 6) however remains to be developed, which requires both preclinical and clinical validation before eventual marketing.

In general, targeting or imaging CAIX in tumors may be clinically relevant, improve health care, and provide gain for society. Several approaches have been described in this thesis, but the most promising approaches to utilize CAIX are most likely via imaging for diagnostic and predictive purposes, or via targeted drug delivery of cytotoxic compounds. In addition, the identification of alternative therapeutic targets in cancer remains essential, but it requires further preclinical and clinical research in order to assess the influence on health care and to estimate a gain for society.

Dankwoord / Acknowledgements

En dan is het ineens tijd voor het laatste onderdeel van mijn thesis, het dankwoord. Dit is misschien wel het onderdeel waar ik het meest tegen op heb gezien, omdat je toch bang bent om iemand te vergeten. Daarom ben ik dit onderdeel ook uit blijven stellen tot het laatste moment, maar met de rest van de hoofdstukken al bij de drukker kan ik er nu echt niet meer onderuit. De afgelopen vier jaar zijn zwaar geweest waarbij weinig gegaan is zoals ik van te voren bedacht had. Gelukkig was het niet alleen maar negatief was en kreeg ik ook steun vanuit alle hoeken. Om iedereen daarvoor te bedanken zal ik me nu toch aan een dankwoord gaan wagen. Desalniettemin wil ik meteen van het begin af aan al aangeven dat het me spijt als ik vergeet om iemand te noemen. Ik ga er daarom gewoon van uit dat iedereen weet wat hun bijdrages geweest zijn en wat dat allemaal voor mij betekent heeft.

Allereerst wil ik Philippe bedanken voor het vertrouwen om mij een PhD te laten doen bij Maastricht. Tijdens de werkbesprekingen en brainstorm sessies vond ik je wetenschappelijke input en manier van denken zeer nuttig. Tijdens de afgelopen vier jaar heb ik veel van je kunnen leren.

Of course I would also like to thank my co-promoters, Ludwig and Ala. Ik herinner me de eerste keer nog goed dat ik Ludwig ontmoette op het NVRB congres in Noordwijkerhout. Daar vertelde je me dat je een vacature had in Maastricht, waar ik nog niet meteen enthousiast over was vanwege de gedachte aan het verre zuiden. With Ala I still remember that we started at Maastricht Lab on the same day, together with Nicolle. Both of you supervised my PhD and we experienced a lot of setbacks including experiments that didn't work, projects that failed, and many unexpected results. Fortunately we also had many laughs during work discussions and at conferences. I would like to thank you both for your motivation, optimism and continuous drive to get to the bottom of things. I like your way of thinking and your critical evaluation of results. I hope some of these qualities slightly rubbed off on me. I would like to thank you both for all the chances and support that you gave me and I am grateful for all the stuff I learned from you two.

Ik wil graag de leden van de leescommissie: Prof. dr. F.C.S. Ramaekers, Prof. dr. T.M. Hackeng, Prof. dr. J.H.A.M. Kaanders, Prof. dr. I. Kunkler, en Dr. J.W. Voncken bedanken voor het lezen en beoordelen van mijn thesis.

I would also like to thank all coauthors for their contributions and scientific output.

Raymon bedankt dat je mijn paranimf wil zijn en voor al je bijdrages in deze thesis. Toen je begon met jouw PhD hebben we samen aan CAIX gewerkt. Gelukkig bleken we een goed team en hadden we genoeg raakvlakken en onderwerpen om over te praten. De uitstapjes buiten het werk, waaronder spelletjesavonden en pretparken, moeten we er daarom maar in houden. Veel succes nog met je laatste jaar.

Ook Marike en Nicolle bedankt voor alle gezelligheid op de kantoren de afgelopen jaren. Het is altijd fijn geweest om onze frustraties samen te kunnen ventileren. Gelukkig hebben we samen ook veel kunnen lachen. Ik weet dat het waarschijnlijk als een cliché klinkt, maar het afronden van jullie PhD gaat wel goed komen. Probeer niet te veel te stressen, maar ook te genieten, dan loopt alles wel los. Succes alvast met jullie carrière en jullie leven na een PhD.

Rianne en Natasja bedankt voor alle ondersteuning met mijn experimenten de afgelopen jaren. Deze hulp begon al met mijn eerste dierexperiment waarbij we intensief samen moesten werken en dagenlang beneden zaten. Gelukkig zorgde jullie er wel voor dat het altijd gezellig bleef. Ik waardeer al jullie hulp, maar wil jullie ook bedanken voor alle leuke momenten in de afgelopen vier jaar.

Een goede, soms ietwat vroege, werkdag begint natuurlijk met koffie. Ik wil Barry, Maud en Sarah bedanken voor de daarbij behorende leuke gesprekken die gelukkig niet continue over de wetenschap gingen. Natuurlijk ook Hanneke bedankt hiervoor, want jij was het laatste jaar alleen met mij nog over van dit oude clubje. Succes met de vroege ochtenden nu ik er niet meer ben.

I now would like to thank all of my office roommates. There have been quite a few, since I changed offices multiple times. I therefore won't include all the names, since this will only increase the chance that I forget someone. Thank you all for the fun conversations and support during the past few years. In addition I would also like to thank the rest of Maastricht Lab. Thanks for all the scientific input, discussions, help, and important moments of relaxation. It has been fun, both inside and outside the lab. Thanks for making me feel welcome in the far south of the Netherlands.

Ook Anika en Paul bedankt, want door jullie heb ik kennis gemaakt met de wetenschappelijke wereld. Mede door jullie enthousiasme ben ik gemotiveerd geraakt om een PhD te gaan doen, wat uiteindelijk tot deze thesis geleid heeft.

Verder wil ik de mensen bedanken die ik al jaren ken en die iedere keer weer mijn geklaag aan hebben moeten horen. Bedankt Maarten, Koen, Tim, Stefan, Toby en Job. In de afgelopen jaren hebben we elkaar misschien toch wat minder gezien dan we gedacht hadden, maar het was toch altijd weer vanouds gezellig. Maarten ook natuurlijk bedankt dat je mijn paranimf wil zijn.

Drea en Berry wil ik graag bedanken voor alle belangstelling die jullie de afgelopen jaren getoond hebben. Jullie hebben constant het beste met zowel mij als Sulakshana voor gehad. Bedankt voor alle visites en leuke momenten, waaronder natuurlijk ook onze reis naar India.

Toon en Jo, ons opa en ons oma, maar ook de rest van de familie. Jullie wil ik ook allemaal bedanken voor jullie belangstelling de afgelopen jaren tijdens feestjes, visites, of gewoon tussendoor.

Marloes en Michael, bedankt voor de benodigde afleiding de afgelopen jaren. We hebben met zijn allen veel kunnen lachen als we samen waren. Lekker geinen ook tijdens spelletjes avonden en dierentuin bezoeken, zowel in de regen en de zon. Deze ontspanningsmomenten zorgde ervoor dat ik de rest van de week weer fris en opgeladen aan de slag kon.

Marion en Peter, pap en mam, bedankt voor jullie steun tijdens deze moeilijke jaren van mijn PhD. Natuurlijk ook bedankt voor alle steun en liefde in de jaren ervoor. Jullie hebben er altijd voor gezorgd dat ik de kans had om te doen wat ik wilde en me daardoor kon ontwikkelen zoals ik zelf wilde. Sommige dingen zijn lastig te verwoorden, maar dat is zoals jullie weten een trekje van beide kanten van de familie. Ik hoop dat jullie weten wat jullie voor me betekenen en hoe ik jullie steun, motivatie, gesprekken, etc. waardeer.

En dan, last but not least, Sulakshana. Ik heb je meerdere pagina's aan dankwoord beloofd, maar dat is misschien toch een beetje te ambitieus geweest. Ik wil echter wel graag zeggen dat ik je ontzettend dankbaar ben dat je de sprong in het diepe hebt durven wagen en samen met mij naar Maastricht verhuisd bent. Misschien hadden we het met zijn tweeën iets anders voorgesteld in het begin, maar uiteindelijk is toch alles goed gekomen. Bedankt voor je optimisme, je luisterend oor, je kritische blik, je begrip, je goede ideeën, en al het andere wat me nu nog ontschiet. Zonder jou had ik deze afgelopen vier jaar sowieso niet gered. Om het daarom maar gewoon kort samenvattend te zeggen: je bent geweldig. De toekomst blijft spannend omdat we niet kunnen voorspellen wat er allemaal zal gaan gebeuren, maar zolang we samen zijn weet ik zeker dat we alles aan kunnen : Yin en yang, Duo penotti, Sus(h)i, etc. Ik hou van je lieverd.

Het lijkt er op dat het me toch nog gelukt is om een dankwoord te schrijven. Ik blijf dit echter nog steeds het lastigste onderdeel van heel de thesis vinden. Je blijft namelijk twijfelen of je het wel goed gedaan hebt. Een willekeurig hoofdstuk schrijven is daarmee vele malen makkelijker. Toch is het gelukt en is het nu toch echt voorbij. Mijn thesis is klaar en daarmee is het tijd voor de volgende uitdaging. As I already stated in the beginning, I may have forgotten to mention some people. I am however convinced that everybody knows what they have meant for me during my PhD. For that I want to thank you all!

Curriculum Vitae

Simon Johannes Antonius van Kuijk was born on September 16th 1988 in Goirle, the Netherlands, where he also grew up. After finishing his pre-university education (VWO) in 2006 at the Mill-Hill College in Goirle, the Netherlands, he started the bachelor Bio-medical Sciences in Nijmegen, the Netherlands, at the Radboud University. The major of his bachelor was human pathobiology, which led to an internship at the department of Radiation Oncology of the Radboud University Medical Centre and his first scientific publication in 2011. After finishing his bachelor in 2010 he pursued the major human pathobiology further in his masters at the Radboud University Nijmegen. In addition, he included epidemiology as a minor to gain a broader scientific background. During his minor he therefore performed an internship in 2011 at the department of Primary and Community Care of the Radboud University Medical Centre and these results were made freely available on Researchgate. He however wanted to continue the topic of his bachelor internship and therefore performed his major internship again at the department of Radiation Oncology of the Radboud University Medical Centre. The results of this internship again led to a scientific publication, now in Biomarkers in Medicine. More importantly however, during his major internship he attended his first scientific conference, which proved to be a stepping-stone that gave him the opportunity to start his PhD directly after his graduation in 2012 at the department of Radiation Oncology (Maastrro Lab) of the Maastricht University. His PhD research investigated the therapeutic potential of carbonic anhydrase IX to target the hypoxic tumor microenvironment and the results are described in this thesis.

List of Publications

van Kuijk SJ, Niemans R, Lieuwes NG, Yaromina A, Dubois LJ, Lambin P
Soluble Adenylyl Cyclase is Involved in Regulating the Activity and Expression of Carbonic Anhydrase IX.
In preparation

van Kuijk SJ*, Parvathaneni NK*, Niemans R*, van Gisbergen MW*, Carta F, Vullo D, Pastorekova S, Yaromina A, Supuran CT, Dubois LJ**, Winum JY**, Lambin P**
New approach of delivering cytotoxic drugs towards CAIX expressing cells: a concept of dual-target drugs.
In Press Eur J Med Chem

van Kuijk SJ*, Gieling RG*, Niemans R, Lieuwes NG, Biemans R, Telfer BA, Haenen GR, Yaromina A, Lambin P, Dubois LJ**, Williams KJ**.
The Sulfamate Small Molecule CAIX Inhibitor S4 Modulates Doxorubicin Efficacy.
PLoS One. 2016 Aug 11;11(8):e0161040. doi: 10.1371/journal.pone.0161040.

Sneddon D*, Niemans R*, Bauwens M, Yaromina A, **van Kuijk SJ**, Lieuwes NG, Biemans R, Pooters I, Pellegrini PA, Lengkeek NA, Greguric I, Tonissen KF, Supuran CT, Lambin P, Dubois L, Poulsen SA.
Synthesis and in Vivo Biological Evaluation of (68)Ga-Labeled Carbonic Anhydrase IX Targeting Small Molecules for Positron Emission Tomography.
J Med Chem. 2016 Jul 14;59(13):6431-43.

van Kuijk SJ*, Yaromina A*, Houben R, Niemans R, Lambin P**, Dubois LJ**.
Prognostic Significance of Carbonic Anhydrase IX Expression in Cancer Patients: A Meta-Analysis.
Front Oncol. 2016 Mar 29;6:69.

Dubois LJ, Niemans R, **van Kuijk SJ**, Panth KM, Parvathaneni NK, Peeters SG, Zegers CM, Rekers NH, van Gisbergen MW, Biemans R, Lieuwes NG, Spiegelberg L, Yaromina A, Winum JY, Vooijs M, Lambin P.
New ways to image and target tumour hypoxia and its molecular responses.
Radiother Oncol. 2015 Sep;116(3):352-7.

Nagelkerke A, **van Kuijk SJ**, Martens JW, Sweep FC, Hoogerbrugge N, Bussink J, Span PN. Poor prognosis of constitutive γ -H2AX expressing triple-negative breast cancers is associated with telomere length. Biomark Med. 2015;9(4):383-90.

Rami M, Dubois L, Parvathaneni NK, Alterio V, **van Kuijk SJ**, Monti SM, Lambin P, De Simone G, Supuran CT, Winum JY. Hypoxia-targeting carbonic anhydrase IX inhibitors by a new series of nitroimidazole-sulfonamides/sulfamides/sulfamates. J Med Chem. 2013 Nov 14;56(21):8512-20.

Dubois L, Peeters SG^{*}, **van Kuijk SJ^{*}**, Yaromina A, Lieuwes NG, Saraya R, Biemans R, Rami M, Parvathaneni NK, Vullo D, Vooijs M, Supuran CT, Winum JY, Lambin P. Targeting carbonic anhydrase IX by nitroimidazole based sulfamides enhances the therapeutic effect of tumor irradiation: a new concept of dual targeting drugs. Radiother Oncol. 2013 Sep;108(3):523-8.

Nagelkerke A, **van Kuijk SJ**, Sweep FC, Nagtegaal ID, Hoogerbrugge N, Martens JW, Timmermans MA, van Laarhoven HW, Bussink J, Span PN. Constitutive expression of γ -H2AX has prognostic relevance in triple negative breast cancer. Radiother Oncol. 2011 Oct;101(1):39-45.

^{*} Indicates equal contribution

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