Carbonic anhydrase IX is a pH-stat that sets an acidic tumour extracellular pH in vivo

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BACKGROUND: Tumour carbonic anhydrase IX (CAIX), a hypoxia-inducible tumour-associated cell surface enzyme, is thought to acidify the tumour microenvironment by hydrating CO2 to form protons and bicarbonate, but there is no definitive evidence for this in solid tumours in vivo.

METHODS: We used 1H magnetic resonance spectroscopic imaging (MRSI) of the extracellular pH probe imidazolyl succinic acid (ISUCA) to measure and spatially map extracellular pH in HCT116 tumours transfected to express CAIX and empty vector controls in SCID mice. We also measured intracellular pH in situ with 31P MRS and measured lactate in freeze-clamped tumours.

RESULTS: CAIX-expressing tumours had 0.15 pH-unit lower median extracellular pH than control tumours (pH 6.71 tumour vs pH 6.86 control, P = 0.01). Importantly, CAIX expression imposed an upper limit for tumour extracellular pH at 6.93. Despite the increased lactate concentration in CAIX-expressing tumours, 31P MRS showed no difference in intracellular pH, suggesting that CAIX acidifies only the tumour extracellular space.

CONCLUSIONS: CAIX acidifies the tumour microenvironment, and also provides an extracellular pH control mechanism. We propose that CAIX thus acts as an extracellular pH-stat, maintaining an acidic tumour extracellular pH that is tolerated by cancer cells and favours invasion and metastasis.
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non-tumour tissues), whereas 3-APP studies usually obtain only a single reading from a voxel encompassing the whole tumour.

Using $^1$H MRS imaging to detect ISUCA, we were able to measure pH$_e$ from multiple voxels in HCT116 colorectal tumour xenografts in mice bearing a tumour model in which all cells expressed CAIX (CA9 tumours) and also size-matched empty vector (EV) tumours that expressed lower levels of CAIX only in hypoxic areas (note that we use CAIX for the carbonic anhydrase IX enzyme, CA9 for the corresponding gene and CA9 for the HCT116 tumour line that constitutively expresses the enzyme). Hence, the entire extracellular viable volume of CA9 tumours was influenced by expression of CAIX, compared with the relatively small volume in EV tumours where CAIX was induced only at the hypoxic margin.

We observed that CAIX expression in the CA9 tumours imposed an upper limit on tumour pH$_e$, thus lowering the steady-state pH in their extracellular space. Furthermore, we demonstrated that CAIX acidifies only the extracellular space, since $^{31}$P MRS measurements showed no difference in tumour pH$_i$ despite increased lactate production. Our findings thus constitute definitive evidence for the long-postulated roles of CAIX in both acidifying the tumour microenvironment and acting as a pH-stat for it.

MATERIALS AND METHODS

Cell culture

HCT116 cancer cells transfected with the CA9 gene coding sequence (HCT116-CA9) or an empty vector (HCT116-EV) were grown in culture as described previously. The cell lines were grown in culture as described previously. The cell lines were obtained from Professor A.L. Harris (November 2008) and their identities were verified by short tandem repeat analysis using the PowerPlex 18 Primer Kit (Promega) and their ampiclons separated by capillary electrophoresis on ABI Prism genetic analysers. Cells were routinely tested for mycoplasma contamination.

pH$_e$ gradient measurements in 3D cell spheroids

HCT116 cell spheroids (radius 120–130 μm) were cultured using the hanging drop method described previously. Radial pH$_e$ measurements were performed on these spheroids by confocal microscopy (pixel volume of 2 × 2 μm), using a published method. Measurements were performed in the presence or absence of ISUCA (13.3 mM). The dye was membrane-tethered WGA (wheat germ agglutinin) conjugated fluorescein, excited at 488 nm and emission collected above 520 nm (fluorescence is quenched at low pH).

The average radial pH$_e$ gradients were measured in control spheroids (treated with the CA inhibitor ATZ only) and in spheroids incubated with ISUCA (13.3 mM) and ATZ for 2 h (n = 29). The diffusion coefficient of ISUCA (D$_{ISUCA}$) was estimated using a published modelling framework. To investigate the magnitude of pH measurement error introduced by ISUCA, pH$_e$ at the core of the spheroid mass was simulated for a model spheroid under different experimental conditions, using a previously published quantitative diffusion-reaction model.

Tumours

All animal experiments were performed under UK Home Office Licences 80/2203 and 70/7676. Tumours were grown subcutaneously as described previously but in female severe combined immunodeficient (SCID) mice (CB17/ScSprKrdc$^{-}$Cd/crlCrI) obtained from Charles River Laboratories (Margate, UK) at 6 to 8 weeks of age. Tumours were initiated after a minimum of 7 days post-delivery acclimatisation and scanned at a volume of 300–500 mm$^3$. Mice were housed in individually ventilated cages, up to 5 per cage, with food and water ad libitum and kept under a light–dark cycle.
In vitro characterisation of ISUCA
The chemical synthesis of ISUCA and generation of a pH calibration curve using the chemical shift of the ISUCA H2 proton have been previously described.20 The spectrum contains three peaks of equal area for the H2, H4 and H5 protons (Fig. 1a); the chemical shift of the H2 peak is most pH sensitive (Fig. 1b), and this peak was used for pH measurement relative to the water resonance, which served as the internal standard. Calibration of pH was confirmed on a 600 MHz Bruker AVANCE NMR Spectrometer (Bruker Biospin, Coventry UK), using solutions of ISUCA in murine plasma at 37 °C with pH values over the range 2.06–9.44 (Fig. 1c).

Measurement of membrane CA catalytic activity
CA catalytic activity was determined on intact HCT116, EV and CA9 cells in the presence and absence of 100 µM ATZ by means of live-cell fluorescence imaging methods published previously.11 In previous studies on the HCT116 line we had shown that its CAIX activity was blocked by a CAIX-specific monoclonal antibody.22 The CA-catalysed CO2 hydration rate was determined in fractionated cell lysates, using a previously published activity assay.23

In vivo magnetic resonance spectroscopy of tumours
We devised an experimental protocol for the delivery and detection of ISUCA in tumour-bearing mice, followed by automated chemical shift assignment and peak quantitation of the ISUCA H2 peak by 1H MRS imaging (MRSI), which permitted direct in vivo measurement and spatial mapping of the voxel-wise chemical shift of the ISUCA H2 peak by 1H MRS imaging (MRSI), which permitted direct in vivo measurement and spatial mapping of the voxel-wise distribution of pHe. This method allowed us to obtain data from multiple voxels in each tumour and to discard any in which the ISUCA concentration was inadequate or in which skeletal muscle was present in the T2-weighted image.

Our method requires consistent delivery of high ISUCA concentrations into the tumour extracellular space, so timing of ISUCA delivery is crucial for obtaining spectra of adequate quality. For 1H MRS of ISUCA to measure pHe, anaesthesia was induced by inhalation of 2% isofluorane in oxygen and maintained with 1.0 to 1.5% isofluorane. With the mouse in an appropriate jig, the tumour was positioned centrally in a 20 mm-diameter receive-only surface coil (Rapid Biomedical, Rimpar, Germany). Respiratory rate was determined in fractionated cell lysates, using a previously published activity assay.23

Histopathology
Tumours were excised immediately after pH spectroscopy, fixed for 24 h in 10% formalin and embedded into paraffin blocks (Leica ASP300S automated processor). The 3 µm sections were cut and where possible adjacent sections were stained with haematoxylin and eosin and for CAIX and Ki67. Automated immunohistochemistry was performed on a Leica BOND-MAX system. The CAIX antibody (monoclonal mouse anti-human, M75 clone) was a gift from Dr. Pastorek, Bratislava, Slovakia, and was used in a 1:100 dilution with pH 6.0 citrate buffer antigen retrieval; the Ki67 antibody (monoclonal mouse anti-human, Dako M7240) was a gift from Dr. Pastorek, Bratislava, Slovakia, and was used in a 1:200 dilution with pH 9.0 Tris-EDTA antigen retrieval. DAB enhancer (Leica 9432) was used for both antibodies. Slides were scanned using the Aperio (UK) ScanScope system at ×20 magnification.

Lactate analysis
Tumours (CA9 constitutive expressers and EV5 empty vectors) were excised from terminally anaesthetised mice and immediately freeze-clamped using tongs previously immersed in liquid nitrogen. Samples were powdered in liquid nitrogen and extracted with 4 volumes of 6% perchloric acid followed by centrifugation and neutralisation. Lactate was assayed in the extracts using the L-Lactate Assay Kit (Colorimetric) ab65331 (Abcam) according to the manufacturer’s instructions. Since lactate is a passively distributed solute, total tissue measurements cannot attribute its location relative to the cell membrane. The pH distribution was therefore used to infer the intracellular to extracellular lactate gradient.

Statistics
Statistical analyses were performed using GraphPad Prism software, Version 5.01 (GraphPad Software, Inc., La Jolla, CA, USA). For pH or rate constant data, differences between means were tested by a two-tailed t-test. For animal experiments, statistical differences were tested with the non-parametric Mann–Whitney test, while correlations were tested with the non-parametric Spearman’s rank correlation coefficient. For the in vitro studies, the data are presented as the means ± standard errors and were tested for statistical differences with a two-tailed t-test. Differences were considered statistically significant if P < 0.05.
RESULTS
Using fluorescent dye measurements of cell surface pH transients in response to a 30 s pulse of NH$_3$/NH$_4^+$-containing buffer, we verified the presence of exofacial carbonic anhydrase activity in CA9 cells (Fig. 2ai) and its absence in EV cells in vitro (Fig. 2aii). ATZ (100 µM) was added to block CA activity and interrogate pH transients in the absence of CA catalysis. NH$_3$/NH$_4^+$ (100 µM) was added to block CA activity and interrogate pH transients in the absence of CA catalysis. NH$_3$/NH$_4^+$-evoked pH$_{ex}$ transients were significantly smaller (i.e., better buffered) in CA9 cells (Fig. 2aiii). Note that ATZ is membrane permeable, but inhibition of intracellular CAs cannot meaningfully affect extracellular pH dynamics; therefore, its inhibitory effect is attributable only to exofacial catalytic sites. Using a functional assay for CA activity, CA9 cell membrane fragments were shown to significantly inhibit CO$_2$ hydration rate, whereas EV membranes showed no catalytic activity (Fig. 2b). The exofacial CA activity in CA9 cells was comparable to endogenous levels of membrane CA activity in wild-type MDA-MB-468 breast cancer cells, a cancer cell line known to constitutively express CAIX (Fig. 2b), which confirms that CAIX-transfected HCT116 cells have exofacial CA activity in the physiological range.

EV tumours expressed CAIX only in cells around hypoxic regions, whereas CAIX was uniformly expressed by all viable CA9 tumour cells (Fig. 3), as had been found by McIntyre et al. We mapped the pH$_{ex}$ of CA9 and EV tumours in vivo by $^1$H MRS of the $^1$H MRS probe ISUCA. Mapping 5 mm-thick spectroscopic images of in vivo pH$_{ex}$ onto MRI images of CA9 and EV tumours (Fig. 4a, Supplementary Figures 1 and 2) revealed a median pH$_{ex}$ of 6.71 in the CA9 tumours (n = 8), which was significantly more acidic (P = 0.001, Mann–Whitney test) than the median pH$_{ex}$ of 6.86 in the EV tumours (n = 9) (Fig. 4b, c). The range of pH$_{ex}$ values in CA9 tumours was 6.23 to 6.93, whereas the range of pH$_{ex}$ values in EV tumours was 6.20 to 7.53. Intriguingly, we observed a striking absence of any pH$_{ex}$ value above pH 6.93 in the CA9 tumours, whereas 27% of the EV tumour pH$_{ex}$ values were above that cut-off value (Fig. 4d). These results suggest that CAIX expression by HCT116 cells imposes an upper pH$_{ex}$ limit in solid tumours, akin to the action of a pH-stat.

To confirm our conclusions, we investigated whether the presence of ISUCA in the extracellular fluid affects the accuracy of tumour pH$_{ex}$ measurements. ISUCA was capable of buffering absolute changes in pH in cell spheroids but only slightly diminished the size of the pH$_{ex}$ gradients (Supplementary Figure 3A, B). The average ISUCA concentration in tumours (13.3 mM, Supplementary Figure 4A) only accelerated CAIX activity in spheroids by 12% (Supplementary Figure 3C), possibly because this imidazole derivative may augment the transfer of H$^+$ ions between the bulk solution and the catalytic site. Despite these minor effects, the lack of positive correlation between ISUCA concentration and tumour pH$_{ex}$ suggests that ISUCA is unlikely to have a significant effect on the magnitude of facilitated CO$_2$ diffusion in vivo (Supplementary Figure 4B, C and D). In silico modelling of pH$_{ex}$ dynamics at the core of spheroids suggests that the presence of ISUCA may lead to a minor underestimation of extracellular acidification (Supplementary Figure 5). Collectively, these lines of evidence suggest that the increased acidification observed in CA9 tumours is unlikely to be due to artefacts induced by the chemical buffering properties of ISUCA. Furthermore, even if one of these potential artefacts were to have an unexpectedly large effect, it would only induce spurious results if higher concentrations of ISUCA were present in one of the tumour types.
whereas we found that there was no significant difference in these concentrations (Supplementary Figure 4A).

To investigate whether extracellular acidification was accompanied by intracellular alkalinisation in vivo, we obtained the pHi of CA9 and EV tumours from 31P MRS measurements of the endogenous inorganic phosphate signal, the chemical shift of which is pH sensitive in the physiological range and which predominantly arises from the intracellular compartment of tumours.3 The ranges of pHe and pHi values in the CA9 tumours (Fig. 5) show minimal overlap, demonstrating that markedly differing extracellular and intracellular pH environments were probed, and we found no detectable differences between the pHi values of the CA9 tumours and the EV tumours (7.00 ± 0.04 vs 6.99 ± 0.04; mean ± SEM; Fig. 6a).

To confirm the validity of our pH imaging results, we performed a biochemical assay for lactate to test whether there are differences in lactate retention between CA9 and EV tumours. Lactate ions are known to distribute passively across the cell membrane, both in tumour cells27 and solid tumours,28 transported by MCT proteins,29 with the intracellular to extracellular lactate concentration ratio depending on the transmembrane pH gradient. Cells bathed in a more acidic milieu, such as CA9 tumour cells, are therefore expected to retain more lactate. We measured total lactate concentrations in freeze-clamped CA9 and EV tumours (see Supplementary Methods). The CA9 tumours had significantly higher total lactate content than the EV tumours (10.01 ± 0.55 µmol/g, n = 10 vs 6.6 ± 0.89 µmol/g, n = 6; P = 0.01, see Fig. 6b). Since the mean pHi and pHė for each tumour type are known, we can therefore calculate the ratio of lactate on either side of the membrane. Using the mean values from Fig. 6b we calculate the intracellular and extracellular lactate values shown in Fig. 6c: the CA9 tumours had markedly higher intracellular lactate than the EV tumours, whereas the difference in extracellular lactate was much less. This calculation assumed an intracellular volume fraction of 0.6, but a sensitivity analysis (Fig. 6d) shows qualitatively similar results across the full reasonable range of intracellular volume fractions (0.4–0.8); for comparison, Panagiotaki et al.30 reported intracellular volume fractions of 0.84 ± 0.02 and 0.68 ± 0.02 in two other human colorectal xenograft models.

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Fig. 3 CAIX is expressed in all viable CA9 tumour tissue (a) but only at the hypoxic necrotic/viable interface in EV tumours (b). For both (a) and (b): upper parts i, ii and iii show close-cut whole tumour sections, scale bar: 3 mm; lower parts iv, v and vi show higher magnification of the area indicated by the black rectangle, scale bar: 800 µm. Parts i and iv: haematoxylin and eosin staining; viable areas are dark purple; necrotic areas are pale. Parts ii and v: CAIX staining in brown, showing staining in all viable areas in CA9 tumours but absence of staining in the viable areas of EV tumours. Parts iii and vi: Ki67 staining (for proliferating cells) in brown, which confirms the proliferative areas and further defines the hypoxic periphery of viable tissue, adjacent to necrosis. Necrotic areas are non-perfused and so do not give an ISUCA signal.
In conclusion, we demonstrated (i) that exofacial CAIX expression by HCT116 tumours resulted in reduced tumour pH_e; (ii) that the maximum pH_e of their extracellular space was held below 6.93; (iii) that their pH_i was unaffected; and (iv) that they would therefore retain intracellular lactate.

**DISCUSSION**

The HCT116 CA9 colorectal tumours uniformly expressing high levels of CAIX protein, along with their EV counterparts in which CAIX is induced only in hypoxic regions, offer a tractable experimental platform to study the effect of CAIX expression on tumour pH_e in vivo, independent of changes in tumour vascularity, oxygenation and metabolism. The CA9 tumours present an adequate volume of viable tissue in which pH_e is influenced by CAIX to allow accurate pH_e measurements that can be compared with similar volumes of EV tissue which have much less CAIX expression. Using the ISUCA method, we have obtained multiple pH_e values from each tumour and discarded voxels with inadequate ISUCA uptake or skeletal muscle contamination. Our results thus provide strong evidence that exofacial expression of CAIX on cancer cells results in a lower pH_e. We have also shown that exofacial CAIX catalysis acts in vivo as a pH-stat mechanism that tends to maintain a mildly acidic pH_e. We infer that this will occur not only within regions of solid tumours in which CAIX is expressed because of hypoxia, but also in any other area where CAIX is expressed independently of hypoxia. While CAIX is usually associated with tumour hypoxia and frequently used as a hypoxia marker, it is expressed in normoxia in the oestrogen receptor-positive MCF7 cell line and multiple studies have shown that it is often expressed in vivo in areas where hypoxia is not detected.

In previous studies on CAIX expression in HCT116 spheroids, the extracellular acidiﬁcation was accompanied by an intracellular alkalisation. We therefore hypothesised that if extracellular acidiﬁcation by CAIX activity was due to a transmembrane redistribution of acidity, the intracellular compartment of CA9 tumours would be more alkaline than that of the EV tumours. The absence of detectable differences between the pH_i values of the CA9 tumours and the EV tumours (7.00 ± 0.04 vs 6.99 ± 0.04; mean ± SEM) (Fig. 6a) suggests that exofacial CAIX expression has little net effect on tumour pH_i in vivo and that the effect of CAIX expression is primarily on tumour pH_e. This might be due to the weaker buffering capacity and smaller volume of the extracellular space compared with the intracellular space, both of which would tend to amplify acid-load induced pH_e changes. The calculated intracellular and extracellular lactate concentrations (Fig. 6c) predict that the more positive pH_i−pH_e gradient in CA9 tumours would retain lactate intracellularly at the steady state, and this was
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Fig. 6 CAIX-expressing HCT116 tumours do not show increased alkalisation in vivo and have a higher lactate retention. a ^{31}P MRS measurements of pH in 8 CA9 and 8 EV5 tumours. The pH of CA9 tumours was 7.00 ± 0.04 (mean ± SEM) and of EV tumours was 6.99 ± 0.04 (P = ns). This data group, with a pooled error variance of 0.15-unit alkalinisation using a one-sided t-test. b Total lactate levels measured in excised tumours. c Calculated intracellular and extracellular lactate concentrations, assuming an intracellular fraction of 0.6; note that there are no error bars as the calculation was based on the mean lactate levels. d Variation of calculated intracellular and extracellular lactate concentrations with intracellular fraction.

demonstrated by measuring lactate in excised tumours (Fig. 6b) and calculating intra- and extracellular lactate (Fig. 6c). Exofacial carbonic anhydrases (mostly CAIX) facilitate CO₂ diffusion across extracellular spaces, and by doing so acidify the extracellular space en route. The fall in pH₆ reduces the thermodynamic driving force for removing lactate from cells and therefore raises intracellular lactate. Notwithstanding the different methods for assay, the minimal overlap in the pH₆ and pH₇ values of the CA9 tumours (Fig. 5) also demonstrates that ISUCA reports pH₆ in vivo, substantiating the previous in vitro report in cultured C6 cells.

The acidic pH₆ of solid tumours has been hypothesised to advantage cancer cell growth by killing adjacent normal cells, promoting extracellular matrix degradation and thus remodelling normal tissue architecture and modulating various steps along the invasion-metastasis cascade. Importantly, recent studies have uncovered evidence for increased mitochondrial oxidative metabolism (and thus CO₂ production) in migratory/invasive cancer cells and also in vivo in a variety of preclinical tumour models and human cancers. Estrella et al. using a window-chamber model, confirmed that tumours invaded the normal tissue that was most acidic, at pH₆ values below a threshold that ranged from 7.1 to 6.8 in individual mice (their Fig. 2b, d). In our study 44% of the measured CA9 tumour pH₆ values were below 6.7, whereas the EV tumours had only 15% (Fig. 3), showing that CA9 tumours produced sufficient CAIX-related acidification in vivo to favour invasion and/or metastatic cell escape. This notion is supported by reports of reduction in the incidence of metastases in breast cancer models by CAIX inhibition40-42 and in a prostate cancer model by CAIX silencing.43 Thus, the pH-stat function of CAIX is likely to extend to the growing margin of the solid tumour, distant from optimal oxygen supplies, allowing leading-edge cells to set tissue pH₆ at a level inhibitory to normal tissue and thus facilitating invasion of cancer cells. CAIX is also known to be expressed in the lamellipodia at the leading edge of motile normal cells and has been postulated to acidify the adjacent extracellular fluid, a mechanism that might facilitate the invasion of motile cancer cells into the surrounding normal tissue. Perhaps cancer cells induce extracellular acidification by co-opting a method used by normal cells to facilitate tissue remodelling.

The idea that there should be a homeostatic mechanism that maintains pH₆ within a physiologically appropriate range was hypothesised by Stubbs et al.16 Recent publications have suggested that CAIX could be acting as both the H⁺ ion generator and the pH sensor in such a mechanism. Studies in vitro have found CAIX to be active even at relatively acidic levels, with a pKₐ of 6.4913 or 6.81-6.8612 and others have suggested that CAIX has its highest catalytic activity at pH 6.817. Moreover, compared to other CA isoforms, CAIX appears to be more sensitive to pH changes near the pKₐ with a Hill cooperativity number of ~2; these properties suit it ideally to the role of an acidic pH-stat.12,17 In contrast, CAXII (another exofacial cancer-related isoform) is active across the pH range and would not act as a pH-stat, since the persistence of CAXII activity at low pH₆ means that the reaction will continue unopposed.

This study constitutes the first evidence from solid tumours in vivo for the presence of a pH-stat mechanism, which, when supplied with CO₂, would tend to stabilise an acidic pH₆ within CAIX-expressing regions of tumours. We propose, therefore, that
whereas other membrane H^+ transport mechanisms such as the Na^+/H^+ -exchanger and the vacuolar ATP-dependent H^+ pump are primarily concerned with maintaining a constant pH. Extracellular CA catalysis endows CAIX-expressing cancer cells with the ability to set the pH of their environment at an acidic level. We suggest that this pH-stat mechanism may be found to operate in other human cancers, and perhaps in other disease states involving carbonic anhydrases, tissue ischaemia and pH changes, such as ischaemic cardiomyopathy^66 and diabetic retinopathy.^67

Our findings support the involvement of CAIX in determining tissue pH independently of hypoxia status, and add to the therapeutic rationale for pharmacologically inhibiting CAIX, which is already an emerging drug target.^68 Abrogation of this pH-stat function might reduce the acidity of the tumour microenvironment and impinge on the downstream disease mechanisms governed by this pathophysiologic feature of cancer.

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AUTHOR CONTRIBUTIONS

S.-H.L., D.M., D.H., J.P.-T., P.S. and A.H. designed and conducted experiments and analysed data. D.M. designed the MR analyses. J.R.G., A.L.H., P.S. and S.C. designed the project. S.-H.L., J.R.G., D.M. and D.H. drafted the manuscript. All authors helped to revise the manuscript and approved the final version. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

ADDITIONAL INFORMATION

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