Interfering with pH regulation in tumours as a therapeutic strategy

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Abstract | The high metabolic rate of tumours often leads to acidosis and hypoxia in poorly perfused regions. Tumour cells have thus evolved the ability to function in a more acidic environment than normal cells. Key pH regulators in tumour cells include: isoforms 2, 9 and 12 of carbonic anhydrase, isoforms of anion exchangers, Na^+/HCO_3^- co-transporters, Na^+/H^+ exchangers, monocarboxylate transporters and the vacuolar ATPase. Both small molecules and antibodies targeting these pH regulators are currently at various stages of clinical development. These antitumour mechanisms are not exploited by the classical cancer drugs and therefore represent a new anticancer drug discovery strategy.

Warburg effect
The ability of cancer cells to predominantly produce energy by a high rate of glycolysis followed by lactic acid fermentation in the cytosol, rather than by glycolysis followed by oxidation of pyruvate in the mitochondria, as most normal cells do.

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The growth of solid tumours is characterized not only by the uncontrolled proliferation of cancer cells but also by changes in the tumour environment that support the growth of the neoplastic mass and the metastatic spread of cancer cells to distant sites[1]. The formation of new blood vessels from pre-existing ones (angiogenesis) provides oxygen and nutrients to the tumour, which are essential for tumour growth[2]. A complex dynamic interplay exists between the expanding neoplastic mass and the tumour environment, which is affected by the metabolic requirements of cancer cells and by their products, such as secreted proteins (for example, extracellular matrix components and proteases) and metabolites.

Upregulated glucose metabolism is a hallmark of invasive cancers[3]. In normal cells glucose is converted to glucose-6-phosphate and subsequently to pyruvate, which is then oxidized in the mitochondria to carbon dioxide and water; this releases 38 moles of ATP per mole of glucose[3]. However, inadequate oxygen delivery to some regions of tumours leads to hypoxia, which restricts oxidative phosphorylation. As a consequence, hypoxic tumour cells shift their metabolism towards glycolysis so that the pyruvate generated in the first step of the process is reduced to lactate, generating only 2 moles of ATP per mole of glucose. This is a less energy-efficient process but it does not depend on oxygen. Furthermore, glycolysis often persists even after reoxygenation because the obtained metabolic intermediates (that is, lactate and pyruvate) can be used for the biosynthesis of amino acids, nucleotides and lipids, thus providing a selective advantage to proliferating tumour cells. This explains Warburg's observation of high glucose consumption and high lactate production in tumour tissues[3–5] (known as the Warburg effect).

Oncogenic metabolism also generates an excess of protons and carbon dioxide, which are kept in equilibrium with carbonic acid by the enzyme carbonic anhydrase[3–9]. Thus, increased glucose metabolism in tumour cells leads to enhanced acidification of the extracellular milieu, which is frequently accompanied by various levels of hypoxia. This phenotype confers a substantial Darwinian growth advantage to tumour cells over normal cells, which undergo apoptosis in response to such an acidic extracellular environment[3].

Tumour cells have evolved various mechanisms to cope with the acidic and hypoxic stress mentioned above. They eliminate acidic catabolites by ion transporters and pumps to preserve a slightly alkaline intracellular pH (pH_i), which is optimal for cell proliferation and tumour survival[3–10]. Acid export leads to a reduction in the extracellular pH (pH_e) to values as low as 6.0 (the usual pH_e in tumours is in the range of 6.5–7.0)[3], which is a salient feature of the tumour microenvironment[3–9]. As well as triggering the overexpression of many proteins involved in glucose metabolism — such as the glucose transporter GLUT1 (also known as SLC2A1) and pH-regulating proteins such as carbonic anhydrase 9 (CA9)[3,4,10] — hypoxia also constitutes a detrimental feature for radiotherapy, as oxygen is needed to oxidize the radiation-induced DNA free radicals that subsequently lead to tumour cell death[4].

pH homeostasis in any cell type is a complicated process that involves many proteins and buffer systems[4]. In tumour cells, these processes are even more complex owing to the internal compartment being slightly more alkaline (pH 7.4 or more) and the external compartment being more acidic than in normal cells[4–9]. A variation

in the pH_i/pH_e ratio as low as 0.1 pH units may disrupt important biochemical and/or biological processes such as ATP synthesis, enzyme function and the proliferation, migration, invasion and metastasis of tumour cells[5]; consequently, a tight regulation of these processes has evolved[5–9]. Changes in the pH_i as low as 0.1–0.2 pH units also trigger mechanisms of alternative splicing of extracellular matrix components that generate different isoforms of tenascin and fibronectin, which are typical of tumour cells and not normal cells[11,12]. These alternatively spliced proteins are not involved in pH regulation but they may constitute a novel antitumour mechanism (BOX 1).

Several sophisticated molecular mechanisms are responsible for maintaining the alkaline pH_i and the acidic pH_e in tumour cells[5–10]. These include proteins that import weak bases (such as the HCO_3^- ion) into the cells and proteins that export the weak acids generated during metabolism — such as carbon dioxide, carbonic acid or lactic acid — out of the cells[4]. In addition, H^+ ions are directly extruded from the cells, either in exchange for other cations (such as Na^+) or by means of the vacuolar ATPase (V-ATPase)[5–9,13,14].

The main players involved in the regulation of tumour pH are shown in FIG. 1 and include: CA2, CA9 and CA12 (REFS 15–17); V-ATPase[18]; the anion exchangers AE1 (also known as SLC4A1), AE2 (also known as SLC4A2) and AE3 (also known as SLC4A3)[19,20]; Na^+/HCO_3^- co-transporters (NBCs)[14]; electroneutral Na^+-driven Cl^-/HCO_3^- exchanger (NDCBE; also known as SLC4A8)[14]; Na^+/H^+ exchanger 1 (NHE1; also known as SLC9A1)[14]; and the monocarboxylate transporters MCT1, MCT2, MCT3 and MCT4 (REFS 21,22). Many of these proteins exist as multiple isoforms, which represents a complication from the medicinal chemistry viewpoint, as a good drug should target only the desired isoform. Carbonic anhydrases[15,16], anion exchangers and MCTs all have several isoforms and not all of these are associated with tumours[14–23].

There are various approaches for targeting one or more of these proteins to restore both pH_i and pH_e in cancer cells towards normal values and halt tumour growth. This constitutes an antitumour mechanism that has not yet been exploited by any of the classical anticancer drugs. Proof-of-concept studies that were performed during the past few years have led to the validation of some of these proteins as new anticancer drug targets. This Review discusses recent advances in the development of pharmaceutical agents that interfere with pH regulation in tumours, with a special focus on carbonic anhydrases.

Tumour-associated carbonic anhydrases

α-carbonic anhydrases (EC 4.2.1.1) are metalloenzymes that are widespread in higher vertebrates, including humans[15,16]. So far, 16 isoforms of carbonic anhydrase have been characterized in mammals (15 in humans; CA15 is not expressed in primates), which differ in their subcellular localization, catalytic activity and susceptibility to different classes of inhibitors. There are cytosolic isozymes (CA1, CA2, CA3, CA7 and CA13), membrane-bound isozymes (CA4, CA9, CA12, CA14 and CA15), mitochondrial isozymes (CA5A and CA5B) and secreted isozymes (CA6). Three acatalytic isoforms — CA8, CA10 and CA11 — called carbonic anhydrase-related proteins have also been identified[24,25].

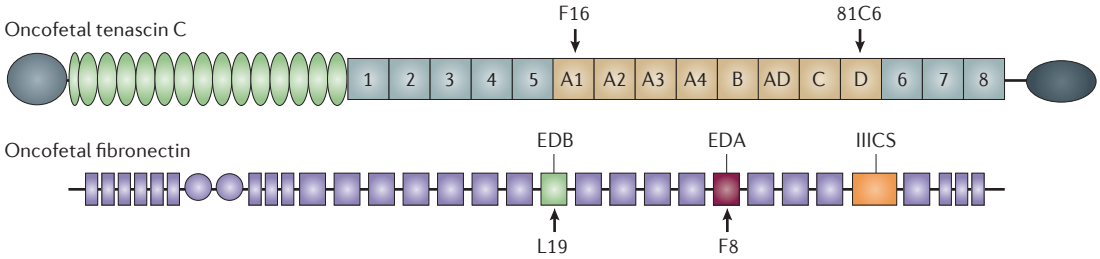
Most carbonic anhydrases are very efficient catalysts for the reversible hydration of carbon dioxide to bicarbonate and protons ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$), which is a vital reaction in most organisms[15,16]. The HCO_3^-–carbonic acid system is the main buffer of pH in all living cells[15,16]. Carbon dioxide is generated in high amounts as the final product of oxidative phosphorylation, and is immediately converted to HCO_3^- by carbonic anhydrases. The uncatalysed hydration reaction of this gas is too slow to meet the physiological needs of the cell, which is why so many carbonic anhydrase isoforms have evolved[15,16]. In mammals carbonic anhydrases are involved in processes such as respiration and acid–base regulation, electrolyte secretion, bone resorption and calcification, and in biosynthetic reactions that require HCO_3^- as a substrate (such as lipogenesis, gluconeogenesis and ureagenesis), whereas in plants and bacteria these enzymes are also involved in photosynthesis and carbon dioxide-fixation mechanisms[15,16,26]. Two carbonic anhydrase isozymes (CA9 and CA12) are overexpressed in many tumours, and are associated with cancer progression and response to therapy[15–17,27,28].

CA9 is expressed in the stomach and peritoneal lining, but it is ectopically induced and overexpressed in several types of solid tumours, predominantly owing to strong transcriptional activation of hypoxia-inducible transcription factor 1 (HIF1)[12,15–17,28,29]. Interestingly, *CA9* is the most strongly overexpressed gene in response to hypoxia in human cancer cells[28,30], and it is also the most active isoform of carbonic anhydrase for the carbon dioxide hydration reaction[29,31]. The X-ray crystal structure of CA9 reveals that it is a dimeric enzyme[32], unlike all other carbonic anhydrase isoforms known so far, which are monomeric. Like CA9, CA12 is a transmembrane isoform with an extracellular active site[27] but it has lower catalytic activity than CA9 (REFS 15,33). CA12 is also expressed in many tumours but it is more diffuse in some healthy tissues[27]. Given that carbonic anhydrases are considerably simple enzymes that are biochemically well characterized and have a multitude of known inhibitors[15,16] (FIG. 2), their potential as anticancer drug targets is very attractive.

Sulphonamides as carbonic anhydrase inhibitors. Primary sulphonamides, sulphamates and sulphamides act as carbonic anhydrase inhibitors (CAIs) by binding to the catalytic Zn^{2+} ion in the active site of the enzyme and blocking its function[15,16]. Some of these compounds inhibit both CA9 and CA12 (REFS 15,16,33–37). Many such compounds were specifically designed for targeting these tumour-associated isoforms of carbonic anhydrase[38]. The main approaches for developing such compounds are listed below.

One approach is the addition of fluorescent tags to generate fluorescent sulphonamides (compound 1; FIG. 2), which are used for imaging purposes and for determining the role of CA9 in tumour acidification[28,39]. Another

Box 1 | **Tumour-associated splice isoforms regulated by intracellular pH**



Elevation of the intracellular pH (pH_i) in tumour cells leads to the generation of alternatively spliced extracellular matrix components, which may serve as targets for antibody-based cancer therapy[11,12]. The insertion or omission of extra domains in tenascin C illustrates this process.

Tenascins are a family of four extracellular matrix glycoproteins that are found in vertebrates. They are typically present in many different connective tissues. Tenascins contribute to matrix structure and influence the behaviour of cells that are in contact with the extracellular matrix. Several isoforms of tenascin C can be generated as a result of different patterns of alternative splicing in the region between domains A1 and D (see figure). In normal tissues and at pH_i values of around 7.0, cells typically produce the 'small' tenascin C splice isoform, which does not include domains A1 to D. When cells are artificially exposed to basic pH values — for example, in cell culture experiments, fetal tissues (which typically have basic pH values of around 7.4–7.5 units) or in aggressive forms of cancer — changes of 0.2–0.3 pH units in the pH_i value lead to a complete switch towards the formation of the 'large' tenascin C splice isoform, which includes domains A1 to D[11,12]. Interestingly, tenascin C splice isoforms are commonly found in the tumour stroma and/or around tumour blood vessels[92–98] and serve as markers of angiogenesis[99]. The regulation of alternative splicing of tenascin C is complicated by the observation that the extra domain C displays a more restricted pattern of expression than the other domains between A1 and D[100,101]. However, whenever this domain is inserted into the tenascin C molecule (for example, in high-grade astrocytomas), it tends to accumulate around neovascular tumour structures[100–102]. The A1 domain of tenascin C is the target of the F16 antibody, whereas the 81C6 antibody recognizes domain D.

Similarly to tenascin C, other components of the extracellular matrix undergo alternative splicing, and the corresponding extra domains represent some of the best characterized markers of tumour angiogenesis and the tumour stroma. The alternatively spliced domains extra domain A (EDA) and extra domain B (EDB) of fibronectin, which are targeted by the F8 and L19 antibodies, respectively, exhibit a high level of conservation among species and are virtually undetectable in normal tissues; however, they are abundantly expressed in the stroma and neovasculature of most aggressive types of human cancers[96,102–110]. Similarly, systematic chemical proteomics studies[107,111–113] have recently shown that another component of the extracellular matrix, periostin, exists in several splice isoforms that are abundantly found in most types of cancers[111–114].

Compound	Generic/ trade name	Product category	Indications	Highest phase	Organization
Splice isoforms of tenascin C					
^{131}I-81C6	Neuradiab	Radiolabelled antibody	Brain cancer	Phase III	Bradmer Pharmaceuticals
F16–IL-2	Teleukin	Antibody–cytokine fusion protein	Breast cancer, lung cancer	Phase II	Philogen
^{131}I-F16	Tenarad	Radiolabelled antibody	Hodgkin's lymphoma	Phase II	Philogen
^{124}I-F16	Tenapet	Radiolabelled antibody	Various tumours	Phase II	Philogen
Splice isoforms of fibronectin					
L19–IL-2	Darleukin	Antibody–cytokine fusion protein	Melanoma	Phase II	Philogen
^{131}I-L19	Radretumab	Radiolabelled antibody	Brain metastases	Phase II	Philogen
L19–TNF	Fibromun	Antibody–cytokine fusion protein	Melanoma (ILP)	Phase II	Philogen
huBC1–IL-12	AS1409	Antibody–cytokine fusion protein	Melanoma	Phase I/II	Antisoma
F8–IL-10	Dekavil	Antibody–cytokine fusion protein	Arthritis	Phase I	Philogen

IL-2, interleukin-2; ILP, isolated limb perfusion; TNF, tumour necrosis factor.

approach is the addition of a positive or negative charge to sulphonamide and sulphamate CAIs (compound 2; FIG. 2) so that they are unable to cross plasma membranes and thus selectively inhibit extracellular carbonic anhydrases, which include CA9 and CA12 (REF. 40). Such compounds are completely different from the classical sulphonamide inhibitor acetazolamide, (compound 3; FIG. 2) which is a promiscuous, potent inhibitor of most carbonic anhydrase isoforms. Acetazolamide has been an important lead for generating other inhibitors with a better selectivity profile (compounds 4 and 5; FIG. 2)[41], and has led to the development of compounds that are activated by hypoxia and exploit the reducing conditions of hypoxic tumours to convert an inactive prodrug

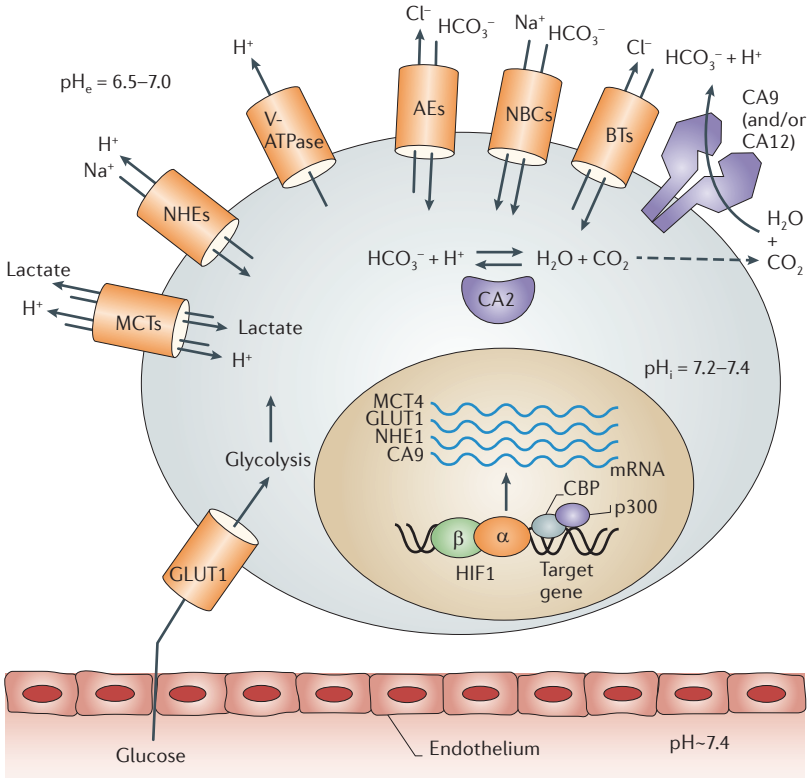


Figure 1 | **Proteins involved in pH regulation within a tumour cell.** The figure shows various proteins that are involved in regulating pH within tumours, including: monocarboxylate transporters (MCTs), which transport lactic acid and other monocarboxylates formed by the glycolytic degradation of glucose; Na^+/H^+ exchangers (NHEs); the plasma membrane proton pump vacuolar ATPase (V-ATPase); anion exchangers (AEs); Na^+/HCO_3^- co-transporters (NBCs); and carbonic anhydrases (CA2, CA9 and/or CA12). The glucose transporter GLUT1 (which is upregulated in most tumours) transports glucose into tumour cells. The intracellular tumour pH (pH_i) is slightly alkaline (pH 7.2–7.4), whereas the extracellular pH (pH_e) is slightly acidic (pH 6.5–7.0). BT, HCO_3^- transporter; CBP, cyclic AMP-responsive element-binding (CREB) protein; HIF1, hypoxia-inducible transcription factor 1; p300, histone acetyltransferase p300.

into an active CAI (compound 6; FIG. 2)[40]. The addition of sugar moieties to CAIs — leading to the formation of sugar-containing sulphonamides, sulphamates and sulphamides that are highly hydrophilic and unable to cross membranes easily — is another approach; these sugar-containing sulphonamides possess an enhanced selectivity for extracellular carbonic anhydrases such as CA9 and CA12 (compounds 7 and 8; FIG. 2)[15,16,40]. Other approaches for targeting these enzymes involve investigating different chemotypes of sulphonamides[42,43] or using nanoparticles to design CAIs[44].

The first study linking the inhibition of CA9 with tumour shrinkage used the fluorescent sulphonamide (compound 1, n=1) and the cationic compound 2 (REF 28). This study showed that these derivatives — which have a high affinity for CA9 and CA12 (inhibition constants of 5–24 nM)[36] — are useful probes for hypoxic tumours[28,45,46]. Compound 1 (n=1) inhibits CA9 only under hypoxic conditions *in vivo*, in cell cultures or in animals with transplanted tumours. Although the biochemical rationale for this phenomenon is not yet understood, these properties may be exploited in the design of diagnostic tools for imaging hypoxic tumours.

Orthotopic tumours
Tumours that occur at the normal place in the body in an animal model of cancer (for example, mouse orthotopic mammary tumours develop in the mammary gland of the animal).

It is interesting to note that in tumour cell cultures that were treated with compound 1 (n=1 or n=2) or compound 2, the acidic pH_e was reversed towards more normal pH values, which was undoubtedly due to the inhibition of the tumour-associated carbonic anhydrases[28,45,46]. Compound 1 (n=1), with a high affinity for CA9, was shown via non-invasive fluorescent imaging, to bind to cells only when the CA9 protein was expressed and when cells were hypoxic, in an *in vivo* model of cancer[46]. Fluorescently labelled sulphonamides may therefore provide a powerful tool for visualizing hypoxia responses in solid tumours and could be used to select patients for CA9-directed therapies[46].

An interesting study reported that in hypoxic LS174Tr tumour cells that overexpress both CA9 and CA12, it was possible to genetically silence one or both carbonic anhydrase isoforms[47]. Genetic silencing of CA9 alone caused a 40% reduction in xenograft tumour volume and led to the overexpression of CA12. However, these cells were kept at an artificially low HCO_3^- concentration, and this observation has not been reproduced[45,48]. Genetic silencing of both CA9 and CA12 led to an 85% reduction in tumour growth, which suggests that CA9 and CA12 are major tumour prosurvival pH-regulating enzymes[47].

Membrane-impermeable sulphonamide derivatives (compounds 4 and 5), which are based on the acetazolamide scaffold to which either fluorescein–carboxylic acid or albumin-binding moieties were attached, suppressed tumour growth in mice with xenografts of a clear-cell renal cell carcinoma cell line when they were administered alone or in combination with other anticancer drugs[41]. These data were the first to demonstrate *in vivo* the potential of sulphonamides (or related agents acting on CA9 or CA12) as alternative anticancer drugs[15,16,41]. Since then, potent bis-sulphonamide CA9 inhibitors that accumulate in colorectal adenocarcinoma xenografted tumour sections in mice have also been identified in a library of one million DNA-encoded chemical compounds[41].

Interestingly, in a mouse model of breast cancer the overexpression of CA9 in hypoxic conditions led to the formation of spontaneous lung metastasis[48]. Treatment of the mice harbouring such orthotopic tumours with compound 1 (n=2), at a dose of 75 mg per kg, led to the regression of orthotopic mammary tumours and inhibited the formation of lung metastases[48]. However, even stronger effects were observed with the ureidosulphonamide inhibitor (compound 9; FIG. 2), a CA9 inhibitor with low nanomolar potency, which substantially inhibited both the growth of primary tumours and metastases in the same animal model, at doses of 30–45 mg per kg[47,50]. Thus, sulphonamide CAIs that target CA9 seem to be effective at inhibiting the growth of both the primary tumour and metastases, at least in this animal model[47,49]. Some of these compounds are in advanced preclinical evaluation[47,50] (TABLE 1). It has also been observed that combining CA9 inhibition (via sulphonamide or sulphamate compounds) with tumour irradiation has a synergistic antitumour effect in HT29 carcinoma cells xenografted in mice[51].

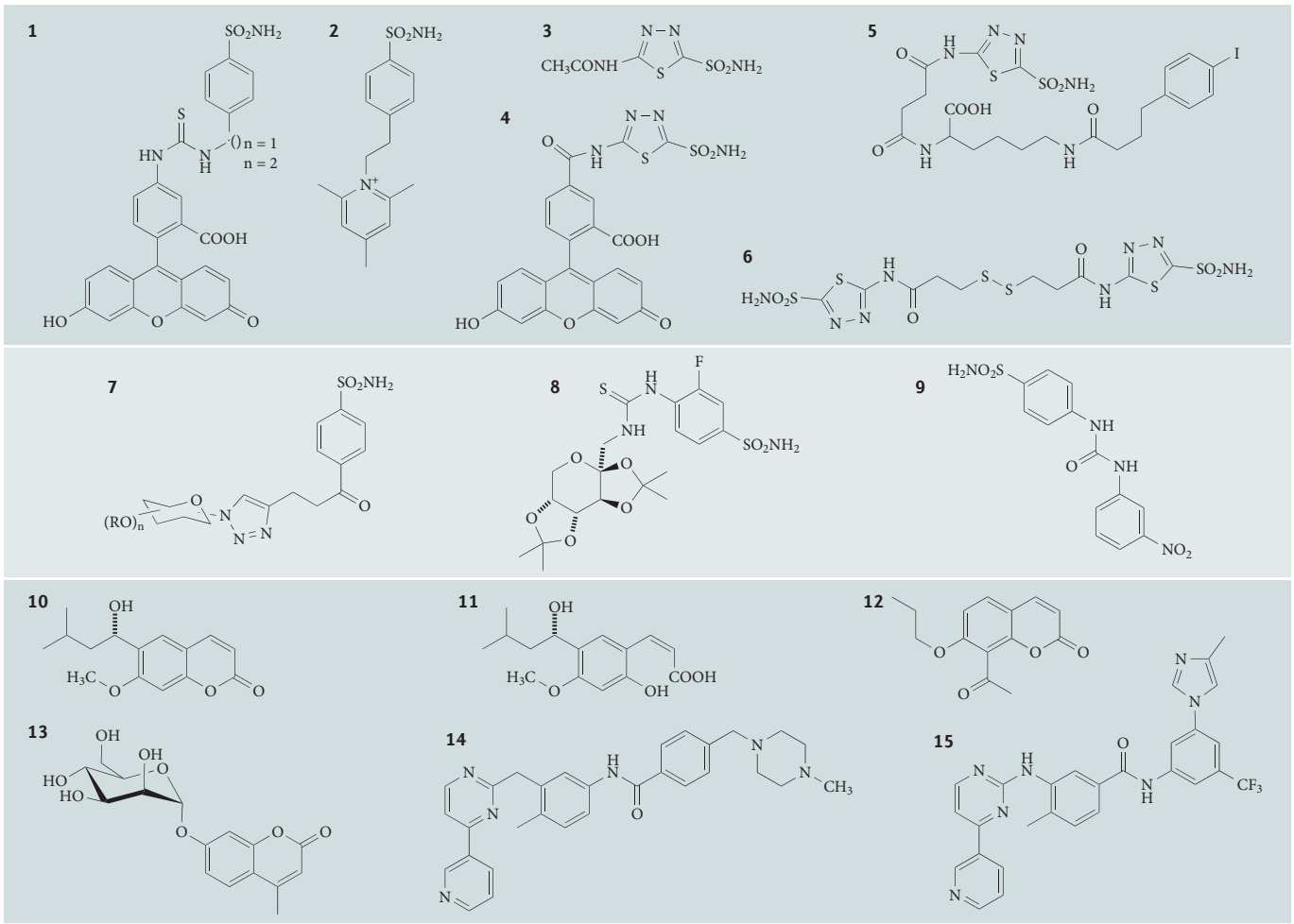


Figure 2 | **Carbonic anhydrase inhibitors of the sulphonamide type and alternative chemotypes.** Fluorescent sulphonamides (compound **1**, n=1, n=2) and compound **2** were used in the proof-of-concept study showing carbonic anhydrase 9 (CA9)-mediated tumour acidification[28]. Acetazolamide (compound **3**) is a classical carbonic anhydrase inhibitor, and was used as a basis for the development of several compounds with carbonic anhydrase-inhibiting properties (compounds **4**, **5**, **6**). Sugar sulphonamides (compound **7**, **8**), as well as the ureido derivative (compound **9**) showed potent *in vitro* and *in vivo* CA9 inhibition and antitumour effects[48]. Coumarins (compounds **10**, **11**, **12**, **13**) and the protein tyrosine kinase inhibitors imatinib (compound **14**) and nilotinib (compound **15**) belong to the alternative chemotypes that inhibit tumour-associated carbonic anhydrases.

Alternative CAIs. Coumarins and thiocoumarins were only recently discovered to have carbonic anhydrase-inhibiting properties, and their mechanism of inhibition has subsequently been deciphered[42,43]. The natural product 6-(1*S*-hydroxy-3-methylbutyl)-7- methoxy-2*H*-chromen-2-one (compound 10; FIG. 2) is hydrolysed within the carbonic anhydrase active site, leading to the formation of the 2-hydroxycinnamic acids (compound 11; FIG. 2), which represent the *de facto* enzyme inhibitor. This new class of CAIs binds to carbonic anhydrase in its hydrolysed form at the entrance of the active site, and does not interact with the metal ion, thus constituting a class of CAIs with a new mechanism of action[42,43].

As well as the natural coumarin compound (compound 10), compounds 12 and 13 (GC-205) (FIG. 2) that were discovered thereafter have been shown to be potent and selective inhibitors against some human isoforms of carbonic anhydrase. Compounds such as 12 and GC-205 are selective CA9 and CA12 inhibitors with a low nanomolar potency that do not substantially inhibit the off-target isoforms CA1 and CA2 (REFS 51,52). Furthermore, like the sulphonamide CAIs, GC-205 was recently shown to inhibit the growth of primary tumours and metastases in an animal model of breast cancer[47] and is in advanced preclinical evaluation as an anticancer and antimetastasis agent[47] (TABLE 1).

It has been demonstrated that the protein tyrosine kinase (PTK) inhibitors that are in clinical use[53] — imatinib (Gleevec; Novartis (compound 14; FIG. 2)) and nilotinib (Tasigna; Novartis (compound 15; FIG. 2)) — also inhibit some physiologically relevant carbonic anhydrase isoforms, albeit less efficiently than sulphonamides and coumarins[54]. Indeed, imatinib and nilotinib can inhibit CA1, CA2, CA9 and CA12 at nanomolar concentrations; however, their mechanism of inhibition is not yet understood as X-ray crystal structures for the adducts of any carbonic anhydrase isozymes with these compounds have not been obtained so far[54]. Nonetheless, this is an important finding

Table 1 | **Carbonic anhydrase 9 inhibitors in clinical development or in use**

Compound	Generic/trade name	Product category	Indications	Highest phase	Organization
cG250	Girentuximab	Chimeric antibody	Kidney cancer	Phase III	Wilex
^{124}I-cG250	^{124}I-girentuximab	Radiolabelled antibody	Cancer imaging	Phase III	Wilex
3ee9–MMAE	BAY-79-4620	Antibody–drug conjugate	Solid tumours	Phase I	Bayer/MorphoSys
Imatinib	Gleevec	PTK inhibitor and/or CAI	GISTs, CML	In clinical use	Novartis
Nilotinib	Tasigna	PTK inhibitor and/or CAI	GISTs, CML	In clinical use	Novartis
U-104	Not available	Sulphonamide CAI	Solid tumours and/or metastases	Preclinical	MetaSignal Therapeutics
GC-205	Not available	Coumarin CAI	Solid tumours and/or metastases	Preclinical	MetaSignal Therapeutics

CAI, carbonic anhydrase inhibitor; CML, chronic myeloid leukaemia; GIST, gastrointestinal stromal tumours; MMAE, monomethyl auristatin E; PTK, protein tyrosine kinase.

that may explain their potent antitumour effects in malignancies such as gastrointestinal stromal tumours, which overexpress CA2 (REF. 55). A synergy between PTK inhibition and the inhibition of cancer-associated carbonic anhydrase isoforms could explain the potent therapeutic effect observed with imatinib and nilotinib in various types of metastatic cancer. Studies in experimental models of cancer combining a selective PTK inhibitor and a selective CAI would be required to investigate this hypothesis.

Monoclonal antibodies may represent the most convenient avenue for the selective inhibition of CA9 and CA12 (REFS 56,57). Compared to rodent antibodies, chimeric and humanized antibodies are less immunogenic in humans[58], can be generated easily and with excellent specificity[59], and do not spontaneously cross the cell membrane[60].

M75 is a monoclonal antibody with a high specificity for CA9 that targets the proteoglycan domain of the enzyme[61]. It has been used in immunohistochemical and western blot studies for identifying CA9 in various types of tumours, and a radiolabelled form of this antibody is used for imaging tumours via positron emission tomography (PET). The biological distribution of this radiolabelled monoclonal antibody in nude mice with HT-29 human colorectal carcinoma xenografts that overexpressed CA9 in hypoxic conditions demonstrated that the radioactivity accumulated only in the tumour[61]. Thus, monoclonal antibodies labelled with various PET tracers may be used successfully for imaging hypoxic tumours that overexpress CA9.

WX-G250 is another CA9-specific chimeric monoclonal antibody that is in Phase III clinical trials as an adjuvant therapy for the treatment of non-metastasized renal cell carcinoma in patients who are at a high risk of recurrence after resection of the primary tumour[62]. Phase III trials are also underway at Wilex for the treatment of metastatic renal cell carcinoma in combination with interleukin-2 or interferon-α[62]. Antibody binding to CA9 on tumour cells triggers antibody-dependent cellular cytotoxicity, an immune reaction in which effector cells such as natural killer cells are engaged in a binding interaction via their Fcγ receptors and are activated to release toxic granuli[60]. Recently, CA12-specific monoclonal antibodies have been generated and characterized. Such monoclonal antibodies inhibit the enzymatic activity of CA12 in the low nanomolar or subnanomolar range and also inhibit tumour growth[56].

Somewhat surprisingly, considerable differences in tumour-targeting performance have been observed among different CA9-specific antibodies, with the approaches developed by the Boerman group resulting in some of the highest tumour uptake values ever reported in the field of antibody-based tumour targeting[63,64]. The observation that pre-targeting strategies (that is, the sequential administration of a bi-specific antibody, which is specific to CA9 and a metal chelator, followed by the intravenous injection of the complex of the metal chelator with a suitable radionuclide) lead to an extremely high and rapid uptake of radioactivity in mouse models of renal cell carcinoma[64] suggests that small high-affinity ligands may be distributed more rapidly into tumour tissues than antibodies.

Bayer, MorphoSys and Seattle Genetics have advanced a CA9-specific antibody, coupled to the potent cytotoxic drug auristatin, to Phase I clinical trials in patients with various types of solid cancer (TABLE 1). In the future, it may be possible to bypass antibodies with high-affinity CA9 ligands (for example, sulphonamide derivatives), which could serve as modular targeting agents for pharmacodelivery applications. These innovative biomedical strategies bear certain analogies to the successful use of folate derivatives for targeting tumours overexpressing folate receptors, as pioneered by the Low group[65]. Indeed, many studies have shown that antibodies are restricted in their ability to access the most hypoxic and poorly vascularized regions of tumours[63–65]. Such problems are not normally encountered with small molecules such as CA9 and CA12 inhibitors[15].

V-ATPase inhibitors and proton pump inhibitors

V-ATPase is an ATP-dependent proton pump that is involved in the acidification of intracellular compartments and the extrusion of protons through the cytoplasmic membrane of the cell[18,66,67]. This enzyme has a crucial role in regulating the pH of normal cells and tumour cells. It consists of multiple subunits (formed from several multisubunit proteins) with both cytosolic and transmembrane domains[18,66,67]. In addition to proton transfer processes, V-ATPase is involved in a host of physiological

and pathological processes, such as endocytosis, intracellular trafficking and the acidification of endosomes, as well as the creation of an appropriate microenvironment for protein transport[18]. It is therefore not improbable that V-ATPase also participates in the regulation of pH_e[66,67].

There are various classes of V-ATPase inhibitors, such as the macrocyclic antibiotics bafilomycin A and concanamycin A, a large series of benzolactone enamides incorporating a salicylic acid scaffold, the macrocyclic antibiotic archazolid and some free radical indolyls[18], but all of these inhibitors are highly toxic and difficult to use as tools for understanding the role of V-ATPase in tumorigenesis. However, one study has suggested that bafilomycin A retards the growth of pancreatic xenograft tumours[68].

The similarity between V-ATPase and H^+,K^+ ATPase, the enzyme involved in proton secretion in the stomach, prompted the investigation of proton pump inhibitors (PPIs) of the omeprazole type for inhibiting V-ATPase[66,69]. Such compounds — including omeprazole, pantoprazole, lansoprazole and rabeprazole (compounds 16, 17, 18 and 19; FIG. 3), among others — are widely used clinically as antacids and are prodrugs that require acidity to be activated[66,67,69,70]. In an acidic environment they undergo a transformation that leads to the formation of a sulphenamide, which reacts with cysteine residues of the proton pump and results in its inactivation[70]. PPIs may also target the acidic (hypoxic) tumour mass, where they are metabolized as in the stomach and block the formation and trafficking of protons[6,66]. Proton pump inhibition was shown to trigger rapid cell death as a result of intracellular acidification, caspase activation and early accumulation of reactive oxygen species in tumour cells[66]. Many human tumours — including melanomas, osteosarcomas, lymphomas and various adenocarcinomas — were shown to be responsive to PPIs[6,66]. Metastatic tumours seemed to be even more responsive to PPIs as they are generally more acidic than most primary tumours[66,69].

Two clinical trials are testing the effectiveness of PPIs in patients with chemosensitizing melanoma and osteosarcoma[66]. Indeed, tumour acidity represents a very potent mechanism of chemoresistance[71], as most cytotoxic agents are weak bases that are quickly protonated outside the cell and prevented from reaching specific cellular targets. Compounds that show enhanced uptake by hypoxic tumours (with a pH_e value in the range of 6.5–7.0) are those that possess a logarithmic acid dissociation constant (pKa) in the range of 5–6 — the carboxylic acids[71]. Further studies will determine whether PPIs represent a new class of antitumour agents with a low level of systemic toxicity compared with standard chemotherapeutic agents.

Metabolon
A temporary structural–functional complex formed between sequential enzymes or proteins of a metabolic pathway. It is held together by non-covalent interactions and structural elements of the cell, such as integral membrane proteins and proteins of the cytoskeleton.

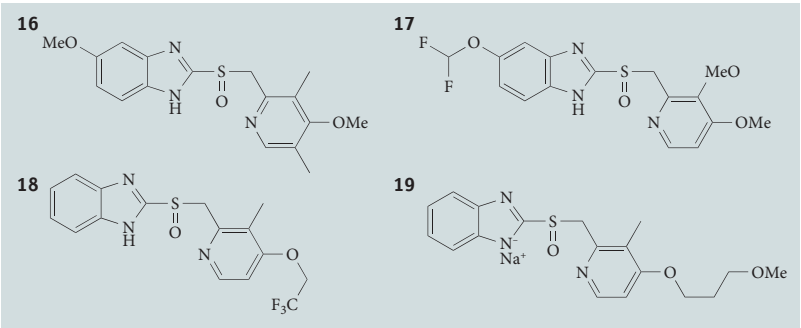


Figure 3 | **Proton pump inhibitors in clinical use.** Omeprazole (compound **16**), pantoprazole (compound **17**), lansoprazole (compound **18**) and rabeprazole (compound **19**) are used in the treatment of gastric ulcers but have also shown promising antitumour effects in animal models of cancer by a mechanism of action that is not yet completely understood[66].

Interference with HCO_3^- transport

HCO_3^- transporters facilitate the movement of the membrane-impermeant HCO_3^- ion across biological membranes[72]. They are phylogenetically clustered into three classes: electroneutral Cl^-/HCO_3^- exchangers of the solute carrier 4A (SLC4A) family, the anion exchangers; the NBC family of Na^+/HCO_3^- co-transporters; and anion transporters of the SLC26 family[19,20,72].

Inhibiting the anion exchangers. The anion exchanger family is composed of three isoforms: AE1, AE2 and AE3. Human AE1 is formed from a 43 kDa amino-terminal domain, a 55 kDa membrane-spanning domain (which is responsible for Cl^-/HCO_3^- exchange activity) and a 33 kDa cytoplasmic carboxy-terminal domain that contains the binding site for CA2 (REF. 72). AE2 is the most widely expressed isoform, whereas AE3 is predominantly expressed in excitable tissues and throughout the gastrointestinal tract[72]. The physical and functional interactions between these HCO_3^- transporters and the various carbonic anhydrase isoforms[72] suggest that they could form a complex. This has been demonstrated for the AE1–CA2 metabolon[73]. In this complex, carbonic anhydrases supply the HCO_3^- substrate for transport (by catalysing their physiological reaction: the hydration of CO_2 to HCO_3^- and H^+), whereas the HCO_3^- transporter translocates the membrane-impermeant HCO_3^- into or out of the cell[72,73]; however, carbonic anhydrases may also remove HCO_3^- by catalysing the reverse reaction.

CA9 has been demonstrated to form a metabolon with isoform AE2 (REF. 74). The concerted action of the enzyme and the anion exchanger may lead to the high pH_i that is characteristic of tumour cells and to the acidification of the pH_e, through the shuttling of HCO_3^- into or out of the cell. This mechanism may also explain why an intracellular isoform (CA2) and two extracellular isoforms (CA9 and CA12) are involved in pH regulation in cancer cells[15,72]. 4,4′-Diisothiocyano-2,2′-stilbenedisulphonic acid (DIDS) (compound 20; FIG. 4)and structurally related stilbenes act as inhibitors of anion exchangers, but they also indiscriminately inhibit other HCO_3^- transporters[75]. These compounds initially block the anion exchangers reversibly and later block them irreversibly[75]. DIDS has antitumour activity and it induces apoptosis in HA22T hepatocellular carcinoma cells overexpressing AE2 (REF. 76).

Another potent CA9 and CA12 inhibitor, celecoxib (Celebrex; Pfizer (compound 21; FIG. 4))[15], and several other potent sulphonamide CAIs such as compound 22 (FIG. 4) substantially inhibited the Cl^-/HCO_3^- exchange

activity of AE1, with EC_{50} (the effector concentration for half-maximum response) values in the range of 0.22–2.8 μM. It was evident that bulkier compounds such as compound 22 inhibited AE1 more potently than sulphonamides incorporating less bulky moieties. The maximum inhibition achieved, using 40 μM of each compound, was only 22–53% of AE1 transport activity, possibly because the assays were performed in the presence of competing substrates. In the Cl^-/HCO_3^- exchange assays, which depend on functional carbonic anhydrase to produce the transport substrate, 40 μM of celecoxib inhibited AE1 by 62%. Thus, some sulphonamide CAIs, including the clinically used analgesic celecoxib, can inhibit AE1-mediated HCO_3^- transport at clinically significant concentrations[20]. Celecoxib shows substantial antitumour activity, possibly owing to its cyclooxygenase 2-, CA9- and CA12-inhibiting properties, although other mechanisms of action may also be involved[77,78]. Compounds that specifically inhibit anion exchangers and do not interfere with other enzymes or transport proteins are not yet available.

Inhibiting other HCO_3^- exchangers. The NBC family of co-transporters is composed of: electrogenic Na^+/HCO_3^- co-transporter 1 (NBC1; also known as SLC4A4), electrogenic NBC4 (also known as SLC4A5), electroneutral NBC3 (also known as SLC4A7), NDCBE and the Na^+-driven Cl^-/HCO_3^- exchanger NCBE (also known as SLC4A10). NBCs have a widespread tissue distribution[72]. These co-transporters facilitate the transport of Na^+ and HCO_3^- across the plasma membrane with an electroneutral or electrogenic (two or three HCO_3^- per Na^+) mechanism[72]. NBC3 is an electroneutral co-transporter that is involved in the regulation of pH_i in the heart, skeletal muscle and kidney. Like other HCO_3^- transporters, a region in the C terminus of NBC3 (D1135–D1136) binds to CA2, and this interaction was shown to be essential for maximal HCO_3^- transport[72]. NBC1 is an electrogenic co-transporter, which operates with either a 3:1 or 2:1 HCO_3^-:Na^+ stoichiometry. NBC1 mediates HCO_3^- efflux from the cell to the blood, and normally works with a stoichiometry of three HCO_3^- per Na^+. CA4 and NBC1 have been shown to colocalize in the mammalian kidney, pancreas and heart[19].

DIDS is an inhibitor of these transporters, but owing to its toxicity it cannot be used as a therapeutic agent[79]. Another inhibitor of these HCO_3^- transporters — S3705 — has been reported and evaluated for the inhibition of proliferation and apoptosis of the human cholangiocarcinoma cell lines HUH-28 and Mz-ChA-1[80]; the structure of S3705, however, has not been disclosed. The pH_i of the treated cells was shown to decrease following inhibition with S3705. Incubation of HUH-28 cells with cariporide (compound 24; FIG. 4) and/or S3705 reduced cell proliferation and induced apoptosis[80]. The same two agents (cariporide and S3705) were investigated for their effects on the toxicity of the anticancer drug melphalan in two human breast cancer cell lines (MDA-MB231 and MCF7)[81], but the effects of the two drugs on pH_e were too small to warrant clinical studies. Like anion exchangers, there is a lack of selective ligands for these other HCO_3^- exchangers.

Inhibitors of Na^+/H^+ exchanger 1

NHE1 is expressed in cells all over the body and is one of the crucial proteins involved in pH regulation[5,6]. NHE1 is involved in tumorigenesis, as deletion of this protein drastically reduces tumour growth[82]. The same effects have been observed using pharmacological inhibitors[6,83] such as amiloride or cariporide[84] (compound 23 and 24; FIG. 4). There are numerous NHE1 inhibitors that are structurally related to amiloride and cariporide, all of them possessing the pyrazine–acylguanidine scaffold that is present in amiloride along with various other substituents to the pyrazine ring[84].

The inhibition of NHE1 is useful not only for the management of tumours but also for the treatment of acute renal failure. Cardioprotective and cerebroprotective effects of NHE1 have also been reported[84]. However, owing to the diffuse presence of NHE1 in many tissues and its fundamental role in crucial physiological processes, there is a potential risk of life-threatening side

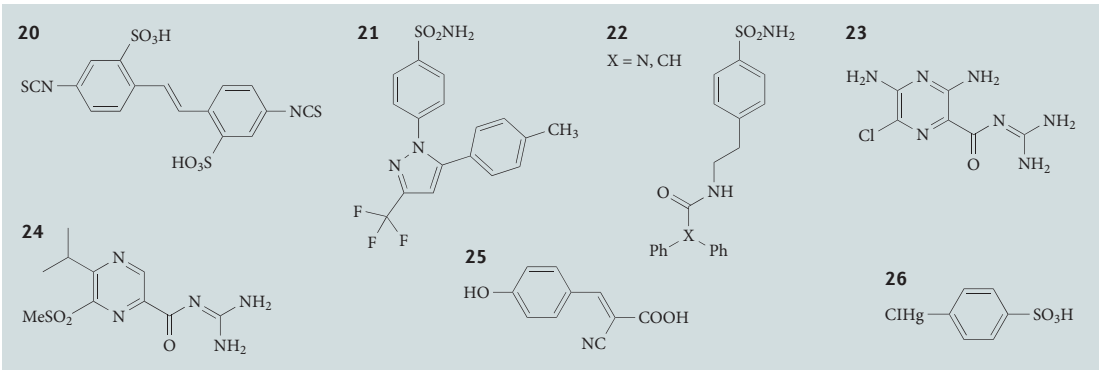


Figure 4 | **Compounds interfering with Na^+/HCO_3^- co-transporters, anion exchangers and Na^+/H^+ exchanger 1.** 4,4′-Diisothiocyano-2,2′-stilbenedisulphonic acid (DIDS) (compound **20**), celecoxib (compound **21**) and structurally related sulphonamides — such as compound **22**, amiloride (compound **23**) and cariporide (compound **24**), as well as the stilbene derivative α-cyano-4-hydroxycynnamic acid (compound **25**) or the organomercuric compound 4-chloromercuribenzene sulphonic acid (compound **26**) — interfere with several proteins that are involved in the transport of HCO_3^-, such as the anion exchangers, Na^+/HCO_3^- co-transporters and Na^+/H^+ exchanger 1.

effects associated with this class of agents. In fact, Sanofi has stopped the clinical development of cariporide owing to the unexpected incidence of stroke in a large Phase III clinical trial[85]. To exploit the benefits of NHE1 inhibition in the treatment of cancer, it would be crucial to develop agents that selectively target NHE1 in tumours and do not target the enzyme in healthy tissues.

Monocarboxylate transporter inhibitors

There are at least nine different isoforms of proteins that are involved in the transport of monocarboxylates into and out of cells, but MCT1, MCT2, MCT3 and MCT4 seem to have crucial physiological roles[21]. Tumour cells metabolize glucose and glutamine to meet the high bioenergetic and biosynthetic demands of continuous proliferation[86]. As mentioned above, hypoxia and oncogenic mutations favour glycolysis and promote the conversion of pyruvate to lactate, which results in the production of high amounts of lactic acid[86]. The preferential use of lactate for oxidative metabolism involves the uptake of lactate by bystander, non-hypoxic tumour cells, thus sparing glucose for hypoxic tumour cells, which cannot carry out oxidative metabolism[5,6,86]. MCTs (mainly MCT1) regulate the entry and exit of lactate from tumour cells[86], and their inhibition favours the switch from lactate-fuelled respiration to glycolysis, which consecutively kills hypoxic tumour cells via glucose starvation[6].

Indeed, MCTs are overexpressed in many tumours, with MCT4 also being induced by hypoxia through HIF1 signalling, although this remains a controversial issue[87]. MCT inhibitors might therefore have utility as alternative antitumour agents, and various classes of such inhibitors — including DIDS, compound 25 and 26, (FIG. 4) — are under investigation[22]. The efficacy of these inhibitors on the different MCTs varies, as well as their mechanisms of action[22].

In a proof-of-concept study, the inhibition of MCT1 with compound 25 induced a switch from lactate-fuelled respiration to glycolysis, which was accompanied by a retardation of tumour growth in a mouse model of lung carcinoma as well as in xenotransplanted human colorectal carcinoma cells[6]. This study validated MCT1 as an antitumour target[6]. In another study the expression of MCT4 in RAS-transformed fibroblasts substantially increased the gradient between pH_i and pH_e from 0.14 to 0.43, when compared to CCL39 wild-type tumours that do not express MCT4 (REF. 88). The increased pH gradients were paralleled by increased tumour growth and diminished necrotic regions, thus proving the important role of proton–lactate co-transport in tumorigenesis[88]. Although the involvement of MCTs in tumours is well established, the lack of potent, non-toxic, isoform-selective MCT inhibitors has prevented further investigation of their potential antitumour properties. Such agents are urgently needed.

Current status and future prospects

TABLE 1 lists CA9 inhibitors that are in clinical development or in use. It is worth noting that antibodies are the predominant agents in clinical development compared to small-molecule inhibitors. Apart from the splice isoforms of tenascin C and fibronectin (BOX 1), other proteins that are involved in pH regulation either still need to be validated as antitumour targets or their inhibitors have not yet reached clinical trials.

As mentioned above, two PTK inhibitors in clinical use — imatinib and nilotinib — also target CA2 and CA9. Three monoclonal antibodies — girentuximab, its radiolabelled variant ^{124}I-girentuximab and a CA9-specific auristatin antibody (BAY79-4620) — are in Phase I–III trials for kidney cancer, other solid tumours or as imaging agents. Two small-molecule CA9 inhibitors, the sulphonamide U-104 and the glycosyl coumarin GC-205, are in advanced preclinical evaluation both for imaging and for the treatment of solid tumours and metastases in which CA9 is overexpressed[47].

Several antibodies or antibody–cytokine conjugates targeting splice isoforms of tenascin C are also in Phase II–III clinical development (BOX 1). Such agents might be useful for the management of various cancers, such as brain, breast and lung cancer, as well as Hodgkin's lymphomas. Several antibody–cytokine fusion products targeting splice isoforms of fibronectin are in Phase I–II clinical development for the treatment of melanoma, brain metastases or arthritis (BOX 1).

As outlined in this Review, the regulation of pH in tumours involves the interplay of many proteins, including: carbonic anhydrases (specifically, CA9 and CA12), anion exchangers (AE1, AE2 and AE3), NBCs, NDCBE, MCTs, NHE1 and V-ATPase. Some of these proteins are overexpressed in tumours but their particular contribution to cancer cell proliferation is poorly understood. The concerted action of these proteins ensures a slightly alkaline pH_i and an acidic pH_e within the tumours, which favours the proliferation of the primary tumour and the formation of metastases. The inhibition of one or more of these pH regulators causes both the pH_i and the pH_e values to return to normal, with the consequent impairment of tumour growth. This constitutes an antitumour mechanism that is not exploited by the classical anticancer drugs. Among these new possible antitumour targets, the inhibition of CA9 and CA12 with monoclonal antibodies or with sulphonamide- or coumarin-based small-molecule inhibitors has been proven to reverse the effects of tumour acidification, leading to the inhibition of cancer cell growth in both primary tumours and metastases. This approach may be useful for both imaging and treating tumours that overexpress these two enzymes.

There are few specific, non-toxic and effective compounds that interfere with the other pH-regulating proteins discussed in this article, but antibodies may also be useful for this purpose. Indeed, a polyclonal antibody that targets NBC3, an isoform that is mainly present in heart, has been reported to inhibit the cardiac electrogenic Na^+/HCO_3^- co-transporter[89]. Similarly, antibodies that target the cardiac isoforms of other NBCs and AE3 (REFS 90,91) have been investigated. However, studies to examine their potential role in blocking tumour acidification have not yet been performed. Nevertheless, monoclonal antibodies may circumvent some of the toxicity and selectivity issues encountered with the small-molecule compounds.

Some sulphonamides also seem to inhibit anion exchangers, whereas PPIs of the omeprazole type show antitumour effects — possibly by inhibiting V-ATPase. Potent, specific and non-toxic compounds that inhibit one or more of these proteins may represent valuable new antitumour drugs. Several antibodies, antibody–cytokine conjugates and small molecules are currently in various stages of clinical development, suggesting that agents that interfere with pH regulation in tumours might soon be available in the armamentarium of anti-tumour drugs. Combination therapies comprising such agents and other anticancer drugs may lead to even better antitumour responses, but this hypothesis warrants further studies.

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Competing interests statement

The authors declare competing financial interests: see web version for details.