Targeting ion transport in cancer

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The metabolism of cancer cells differs substantially from normal cells, including ion transport. Although this phenomenon has been long recognized, ion transporters have not been viewed as suitable therapeutic targets. However, the acidic pH values present in tumours which are well outside of normal limits are now becoming recognized as an important therapeutic target. Carbonic anhydrase IX (CAIX) is fundamental to tumour pH regulation. CAIX is commonly expressed in cancer, but lowly expressed in normal tissues and that presents an attractive target. Here, we discuss the possibilities of exploiting the acidic, hypoxic tumour environment as possible target for therapy. Additionally, clinical experience with CAIX targeting in cancer patients is discussed.

1. Introduction

Altered membrane transport of ions has been a subject of inquiry and speculation in cancer research since early in the twentieth century. Despite this long history, investigating these mechanisms, especially as therapeutic targets, has substantially lagged behind molecular genetic investigations of signal transduction. Searching Medline for ‘ion transport and cancer’ yields 3482 references, whereas there are 51 096 references for ‘p53 and cancer’; 16 708 references for ‘EGFR and cancer’ and 6664 references for ‘raf and cancer’. EGFR and b-raf are notable as they have been therapeutically targeted in the clinic. Yet, despite the fact that they can be inhibited with highly specific agents and they are well-validated targets, response to these therapies is fleeting and commonly results in the emergence of highly resistant cancers [1]. Hence, alternative therapeutic targets are needed, including ion transport mechanisms. One explanation for the lack of attention paid to ion transport is that cancer is canonically viewed as a disease of proliferation, while it is much more than that. For cancer cells to succeed, they have to establish complex communication between themselves and the surrounding stroma, and there is strong evidence that such communication requires ion gradients and currents. The second possible explanation is that there are few mutations in ion transporters, and thus ion transport can be viewed as too fundamental a process to be a therapeutic target. Indeed, the fundamental requirement for ion transport to the existence of living systems was first described by Claude Bernard over 150 years ago. However, as we will point out, complete abrogation of activity is not required for therapeutic effect. Even subtle modulation of ion transport activity leads to significant amplified effects. Recognizing the potential renewed interest in ion gradients in cancer has stimulated a series of recent reviews [2,3].

In considering the effects of inhibitors of ion transport, it is important to recognize that the endpoints of the intervention may be systemic and subtle. These effects may perhaps have no measurable impact on tumour growth, but may still have an impact on important components of tumour behaviour, for example invasion. For example, regulation of ion gradients entails much more than just transmembrane potentials of single cells. Ions are highly mobile and thus mediate cell–cell communication across long distances [4]. Through gap junction conductance, epithelial layers are electrical syncytia and through this activity, functional gap junctional proteins, connexions, are considered to be tumour suppressors [5]. Coordinated behaviour of electrically coupled cells can be mediated through activities of transient receptor potential K-channels [6] or voltage-gated calcium channels that can propagate as waves...
in single cells or across synthia [7]. Indeed, these bioelectric fields can be modulated to trigger patterning and stem cell differentiation in developing systems [8,9]. Such considerations must also account for the fact that the effects of extracellular ions are not simply transduced by similar effects on intracellular ion activities. Although intracellular ion activities are highly regulated, all cells contain cell surface ion sensors for, e.g. extracellular Ca\(^{2+}\) [10] and H\(^{+}\) through acid-stimulated ion channels [11,12] or G-protein-coupled receptors [13]. Notably, the Ca\(^{2+}\)-sensing receptor is epigenetically silenced during colorectal carcinogenesis, fuelling speculation that it plays an important tumour suppressor function [14].

As described above, an important aspect of modulating and investigating ion transport is that the most relevant endpoint effects relate to cell behaviour and not to cell proliferation or survival. For example, inhibition of ion transport will profoundly affect cell migratory behaviour, but will not be cytotoxic. Inhibition of migration can disrupt the metastasis paradigm. Ubiquitous Cl\(^{-}\) and Na\(^{+}\)K\(^{+}\)Cl channels are required for cell volume regulation [15], and regulatory volume changes are required for cell locomotion on three-dimensional collagen frameworks in vitro [16,17] and in vivo [18]. Furthermore, stimulating or inhibiting activity of cells to decrease their volumes can stimulate or inhibit metastasis, respectively [19,20]. Moreover, ion transport and pH regulation play a major role in cell migration and ion channels, and transporters are essential components in the regulation of cellular migration (reviewed in [21]). Another example wherein disruption of ion gradients affects cell behaviour and not survival is in pH buffer therapy. Ingestion of alkaline pH buffers has been shown to neutralize tumour acidity and prevent local invasion and metastases, with no effect on the growth of primary tumours in mice [22]. Furthermore, through an RNAi screen, ion transporters have been identified as resistance mechanisms to small molecule receptor tyrosine kinase inhibitors and that this activity therefore would only be evident under therapy [23].

2. Tumour acidity

There are unequivocal and historical roles for ions in the aetiology of cancer. As described above, the ubiquitous Cl\(^{-}\) counteranion plays a role in cancer progression, especially in volume regulation related to metastasis. In addition, there are significant amounts of data supporting roles for divalent cations, such as Ca\(^{2+}\), Cu\(^{2+}\) and Zn\(^{2+}\) [24,25]. Sodium levels can be measured in vivo by MRI, have altered distributions in cancer and change with effective therapies [26,27]. Potassium channels have also been strongly implicated in carcinogenesis, for example breast cancer [28,29].

Although it has been studied for decades [30], there is renewed interest in the roles that H\(^{+}\) play in cancer aetiology and therapy. All metabolism, whether oxidative or fermentative, ends in the production of acids (CO\(_2\) or lactic) and, hence, cells, organs and systems have evolved complex and redundant pathways to maintain pH within strict limits, reviewed in [31,32]. Because they are highly metabolic and poorly perfused, tumours have acidic pH values that are well outside of normal limits, and this is becoming recognized as an important therapeutic target [33]. It is proposed that the acidity of tumours provides cancer cells with a selective advantage over the tissues into which they invade [34]. Targeting the pH of tumours for therapeutic benefit can take two divergent approaches: (i) the low pH itself can be used to enhance the distribution of targeted chemotherapeutics; or (ii) agents can be developed to inhibit tumour acidosis and thus reduce the selective advantage of cancer cells over their microenvironment.

(a) Exploiting tumour acidity

The most straightforward approach to exploiting tumour acidity is to use drugs that are preferentially distributed into cells that are within an acidic microenvironment. This ‘ion trapping’ phenomenon favours weakly acidic drugs, such as vincristine or topotecan [35]. Further, reversing the pH gradient with systemic buffers can enhance the efficacy of the more common weakly basic drugs, for example the anthracyclines [36–38]. A more direct approach is to develop agents that are selectively activated by the low pH of tumours. This has been an area of intense interest over the past decade. For example, Engleman has developed a low pH inserting peptide, pHLP, that has been used to develop molecular imaging agents as well as a targeting moiety to distribute chemotherapeutics to low pH regions [39–41]. Additionally, whole families of pH-sensitive nanoparticles, nanogels, liposomes and micelles have been developed to release their chemotherapeutic payloads only under acidic conditions [42–48]. This is a very dynamic area of research, which has shown dramatic anti-tumour effects.

(b) Targeting acidosis

By contrast, the acidosis of tumours itself can be targeted either directly or indirectly. Direct targeting can be achieved through the use of systemic buffers, which selectively increase the pH of tumours without affecting systemic pH values [49,50]. Systemic buffers include sodium bicarbonate, imidazole-free bases, alkaline lysine or TRIS free base and all of these have been shown to reduce spontaneous or experimental metastases [22,51,52] in mice, and are now in clinical trials. Figure 1 is an example of such an approach, wherein experimental metastases were induced in immune-deficient mice through intravenous injection of PC3M metastatic prostate cancer cells. In control mice, fulminant metastases are evident after seven weeks, whereas there was a significant retardation of metastasis formation in mice provided 200 mM TRIS base in their drinking water. Indirect methods of targeting acidity involve the use of selective agents to inhibit pH-regulatory pathways, including sodium–hydrogen exchangers, NHE-1 and carboxic anhydrases, specifically CAIX [53].

NHE-1 has long been a target for anti-cancer therapy, as inhibitors are widely available and clinically approved as diuretics [54]. In early studies, the use of these agents in the clinic was associated with unacceptable toxicities. However, more selective and less-toxic agents, for example cariporide, have more recently been developed [55,56], increasing the possibility of selectively inhibiting NHE-1 as an anti-cancer therapy by disrupting cancer cells’ ability to regulate their intracellular pH and migrational capacity in the face of an acidic microenvironment [21,57]. More recently, and more exciting, however, has been the development of selective inhibitors for CAIX. CAIX is commonly expressed in cancer, lowly expressed in normal tissues and is fundamental to tumour pH regulation [58,59]. Treatment with these selective
inhibitors reduced metastatic burden in preclinical models of cancer, suggesting that CAIX might also be involved in cell migration [60–62].

c) Carbonic anhydrase IX as an anti-cancer target
Initially, CAIX was reported as a human tumour-associated protein detected in HeLa cells [63]. Subsequent studies demonstrated that CAIX is one of the most important factors regulating tumour pH [59]. In view of the importance of CAIX in regulating tumour pH and its membrane localization, the protein has been suggested as potential target for cancer therapy.

As mentioned, several CAIX-selective small molecule inhibitors have been developed and tested [64]. Administration of a nitroimidazole and sulfamide dual-targeting drug reduced hypoxic extracellular acidification, decreased tumour growth and sensitized tumours to irradiation in a CAIX-dependent manner. These studies demonstrated that manipulation of tumour pH through CAIX-selective inhibitors could lead to significant anti-tumour effects [60–62,65]. Interestingly, these sulfonamide derivatives only accumulated on CAIX-expressing cells during hypoxia and not under normoxia, consistent with the suggestion that CAIX may undergo oxygen-dependent conformational changes [65]. This makes these small molecule drugs interesting as they may be able to specifically accumulate in hypoxic tumour areas, areas that are known to be inherently therapy resistant. Moreover, treatment with these selective inhibitors reduced metastatic burden in preclinical models of cancer, suggesting that CAIX targeting with these inhibitors might be useful for treating cancers not responsive to classic chemo- and radiotherapy [60–62].

Parallel to functional CAIX studies involving its relevance for tumour physiology, studies with monoclonal antibody G250 (mAbG250), were performed in patients with renal cell carcinoma (RCC). Initially, the target antigen of mAbG250 was not known, but the mAb showed tumour specificity, whereas cross-reactivity with other tissues was restricted [66]. Subsequently, more detailed fine-specificity analysis revealed slight reactivity with some normal tissues, albeit antigen expression was low [67]. Upon cloning of the molecule recognized by mAbG250, it became evident that mAbG250 recognized CAIX [68].

Promoter studies revealed that expression of CAIX is heavily dependent on the transcription factor HIF-1α [69]. Under normoxic conditions, prolyl hydroxylated HIF-1α is polyubiquitylated for subsequent degradation via the 26S proteasome [70,71]. This results in a HIF-1α half-life of minutes. When oxygen levels drop, a situation that occurs

Figure 1. SCID Beige mice were inoculated by intravenous injection with PC3M prostate cancer cells transfected to express firefly luciferase. Ad libitum drinking water for experimental animals contained 200 mM TRIS base, pH 8.4, whereas that for control animals was unadulterated. Tumour growth was monitored periodically using in vivo bioluminescence following injection of luciferin substrate. (Online version in colour.)
frequently in tumours, and hypoxia sets in, HIF-1α hydroxylation stops, HIF-1α concentrations increase, leading to HIF-1α nuclear translocation where it associates with the constitutively stable partner HIF-1β and formation of an active heterodimeric HIF-1 transcription factor. HIF-1 then binds to hypoxia-responsive elements located in the promoter/enhancer regions of hypoxia-inducible genes whereupon several hundred genes are upregulated, including CAIX. Thus, separate from its importance in tumour pH maintenance, CAIX can be viewed as a marker for hypoxia [72,73].

The exclusive dependence on HIF-1 explained the almost ubiquitous expression of CAIX in clear cell renal cell cancer (ccRCC), the most prominent type of RCC. Multiple studies have demonstrated the presence of aberrations in the Von Hippel Lindau (VHL) gene in sporadic ccRCC [74]. Aberrant VHL leads to upregulation of hypoxic inducible genes as VHL acts as an E3 ubiquitin-ligase catalyzing HIF-1α degradation [70,71]. If VHL is mutated as in ccRCC, the ubiquitous expression of CAIX is the functional consequence of a non-functional VHL gene product in ccRCC. CAIX expression in non-ccRCC malignancies also leads to CAIX expression, but this is generally a consequence of hypoxia [72]. This implies that CAIX is expressed in the vast majority of ccRCC and CAIX has emerged as an attractive target for ccRCC.

The almost ubiquitous expression of CAIX in ccRCC was immediately recognized as an important asset for a potential diagnostic imaging agent, despite CAIX expression in normal tissues. Already in the first phase I clinical trial, the clarity of mAbG250 imaging was noted, as well as its possibility to image primary as well as metastatic RCC (mRCC) [75]. The only organ accumulating mAbG250 was the liver. This liver uptake was dose-dependent and appeared to be saturable and did not hamper the image quality; with higher protein doses, liver uptake became invisible as only a small fraction of the labelled material ended up in the liver compartment. This liver uptake was attributed to the CAIX expression in the larger bile ducts. Other CAIX-expressing organs did not show mAbG250 uptake. This can be explained by the difference of the liver vasculature from the rest of the body: the majority of the liver’s blood supply is venous blood and blood flows through sinusoids, vessels with highly fenestrated endothelial cells. Thus, CAIX is much more accessible in the liver than in other organs where endothelial cells connect through tight junctions.

This situation of normal organs expressing the molecule of interest will also occur when other CA or ion channels are pursued as possible tumour targets. Thus, it is critical to identify possible normal organs that express the molecule of interest and, if possible, estimate the amount and accessibility of the target. This will prevent unwanted side effects and preferential accumulation in non-malignant tissue.

Moreover, the vasculature of solid tumours is irregular and poorly organized heavily influencing drug distribution. Theoretical analyses suggest that IgG-sized macromolecular constructs exhibit the most favourable balance between systemic clearance and vascular extravasation, resulting in maximal tumour uptake and contrast [76]. This may explain the success of CAIX targeting with mAbG250. Whether hypoxia in tumours can be imaged adequately with small drugs remains to be established. Therapy studies with a high CAIX-affinity indanesulfonyamide have demonstrated that these enhanced the effect of external radiation, suggesting that adequate drug levels can be reached to influence extracellular acidification and thus increase radiation efficacy [65]. Whether this will lead to new treatment strategies combining CAIX inhibition with irradiation treatment remains to be established.

The PET tracer 124I-mAbG250 has been investigated extensively to study whether the presence of ccRCC could be predicted pre-operatively. This is of particular importance because almost 50% of renal lesions are detected incidentally in the course of non-renal cancer-related imaging. Because approximately 30% of incidentilomas are benign, adequate presurgical stratification of patients might therefore reduce unnecessary surgery. After a successful proof-of-concept phase I trial in 26 patients scheduled for nephrectomy [77], a large multicentre study confirmed the high accuracy of 124I-mAbG250 in visualizing ccRCC [78]. An example of CAIX imaging in ccRCC is shown in figure 2. This patient presented with a renal cyst and a lesion suspect for RCC. Clear targeting of 111Indium-labelled mAbG250 was seen on SPECT/CT and minimal physiological uptake of mAb in the GI tract. Also, no uptake in the cyst was observed. Histopathological examination confirmed ccRCC in the imaged lesion. Thus, with CAIX imaging, malignant lesions can be distinguished from non-malignant lesions.

Preclinical experiments and clinical trials with CAIX therapy highlight some of the intrinsic difficulties of targeted therapy and this might be an even bigger challenge when ion channels are targets. Thus far, over 1000 patients have received CAIX-directed therapy with unmodified mAbG250, without any antibody-related toxicity. This demonstrates that molecules like CAIX can be therapeutic targets without induction of
serious side effects. The trials have suggested that the natural course of mRCC could be altered by CAIX-targeted treatment [79]. However, patients were not randomized and patient bias may have occurred. The natural disease course of mRCC is highly variable, and periods with stable disease and/or partial regression can occur, even in the absence of treatment. It is therefore difficult to judge the value of these observations.

In a large adjuvant phase 3 trial aimed to reduce the recurrence of disease in nephrectomised RCC patients who have a high risk of relapse, CAIX treatment did not improve the median disease free survival (approx. 72 months) compared with placebo. A biomarker analysis showed that, with increasing density of CAIX expression in tumour tissue, as quantified by a CAIX score, the more significant the treatment effect became [80]. This emphasizes that also when ion channels are considered as targets, target density should be taken into consideration.

Finally, the observation that the CAIX-selective inhibitors appear to bind under hypoxia conditions only, suggests that these drugs cannot be used in ccRCC, because CAIX expression occurs under normoxic conditions and conformational changes that are possibly needed for sulfonamide derivative binding does not occur. Hypoxia imaging with mAbG250 has been achieved with $\text{Zr}$-zirconium-labelled cG250-F(\text{ab'})$_2$ [81]. In view of the extensive clinical experience with mAbG250, further research along these lines seems warranted.

3. Conclusion
The acidic, hypoxic tumour environment provides an attractive target for therapy either through direct neutralization with buffers, agents that selectively release payloads under acidic conditions or though targeting of pH-regulatory molecules, for example CAIX. New, selective small drugs are becoming available and CAIX-specific mAbs are available. One of the main challenges is to deliver sufficient selective drugs to poorly diffused areas. The true challenge is to translate our knowledge on ion transporters in such a way that it leads to effective anti-tumour therapies.

References

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