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ABSTRACT

Tumour hypoxia and its molecular responses have been shown to be associated with poor prognosis. Detection of hypoxia, preferably in a non-invasive manner, could therefore predict treatment outcome and serve as a tool to individualize treatment. This review gives an overview of recent literature on hypoxia imaging markers currently used in clinical trials. Furthermore, recent progress made in targeting hypoxia (hypoxia-activated prodrugs) or hypoxia response (carbonic anhydrase IX inhibitors) is summarized. Last, window-of-opportunity trials implementing non-invasive imaging are proposed as an important tool to prove anti-tumour efficacy of experimental drugs early during drug development.

It is well established that tumours are not a collection of relatively homogeneous cancer cells, but act as organs with a complexity that might even exceed that of healthy tissues. Therefore to understand the biology of a tumour both the different individual cell types within a tumour as well as its microenvironment need to be studied [1]. Within this review, we will focus on hypoxia, a common characteristic of solid tumours, which has been associated with poor prognosis [2]. Detection of hypoxia, preferably in a non-invasive manner, could predict treatment outcome and serve as a tool to support treatment decisions. Such non-invasive imaging approaches that are routinely available in clinical practice including positron emission tomography (PET), magnetic resonance imaging (MRI) and perfusion computed tomography (CT) are able to accurately and reliably image hypoxia in tumours. Over the last decade, these diagnostic techniques are developing into versatile tools integrated in treatment monitoring, outcome prediction and treatment targeting. A meta-analysis evaluating the relationship between hypoxia imaging and outcome after radiation treatment demonstrated a uniform tendency for poor response when tumours were hypoxic. This was not only observed for widely used hypoxic PET tracers, but also when hypoxia was indirectly evaluated using perfusion-CT or DCE-MRI [3].

While the prognostic significance of tumoural hypoxia on outcome has been established more than two decades ago only recently compounds are being tested in clinical trials that enable monitoring and selective elimination of hypoxic tumours cells. Here we will provide an update on the current status of hypoxia imaging agents and strategies to combat tumour hypoxia.

Hypoxia PET imaging tracers

Multiple PET tracers suitable for the detection of hypoxia have been developed, validated and shown to exhibit different characteristics. The ideal hypoxia tracer has complete clearance of unbound tracer at time of imaging, thus only bound in oxygen deprived tissues resulting in high signal to noise ratios [3]. We recently reviewed the PET hypoxia tracers that were validated in preclinical and clinical studies and reported accurate quantification methods and clinical applications [4]. The most investigated PET hypoxia tracer is fluoromisonidazole (FMISO). However, due to concerns regarding FMISO stability, metabolite formation and slow clearance properties [5,6], alternative hypoxia PET tracers with different clearance and hydrophilicity characteristics have been developed trying to overcome these limitations:

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fluoroazomycin arabinoside (FAZA), fluoroerythronitroimidazole (FETNIM) and fluorinated etanidazole derivatives (EF1, EF3, EF5), which all have been extensively reviewed previously [3,4,7].

More recently, the hydrophilic flortanidazole (HX4), with preferred pharmacokinetics and clearance properties, has been synthesized through click-chemistry [8] showing 82% intact and 84% unmetabolized tracer at 2 h post injection (h p.i.) in plasma and urine respectively [9]. HX4 has been evaluated in a preclinical rhabdomyosarcoma rat tumour model, where binding was causally dependent on tumoural oxygenation status. Furthermore, a significant spatial relationship at tumour-microregional level between HX4 distribution and the exogenous hypoxia marker pimonidazole staining was observed [10,11]. Studies in primates and healthy volunteers [9] and in patients with histologically proven solid cancer [12] provided evidence for a good safety profile. Recently, in non-small cell lung cancer patients, image contrast was found to be superior 4 h p.i. compared with earlier time points and uptake patterns were strongly correlated between two scans [13]. Overlap studies between HX4 and the metabolism tracer FDG indicated that on average 24% of the hypoxic volume is outside the FDG volume [14]. Similar results have been obtained for head and neck cancer patients [15], suggesting that hypoxia PET imaging provides complementary information to FDG imaging.

Due to the large heterogeneity in uptake, differences in tumour and animal models, different time points of imaging and anaesthesia observed in the literature, it is difficult to compare different hypoxia markers. Although characterization of new hypoxia markers should be preferably performed in multiple cancer models, highly additive data can be expected from comparisons of different tracers within the same tumour models [3,11]. Recently, we performed a comparative study characterizing the clinically approved hypoxia markers FMISO, FAZA and HX4 on tumour to blood ratio (TBR), reproducibility and reversibility within a rat rhabdomyosarcoma model [16]. Blood clearance for FAZA and HX4 became similar 3 h p.i., while for FMISO as expected clearance from normal tissues was significantly lower. Differences in tumour uptake resulted in significantly higher TBR for HX4 compared to the other tracers. Reproducibility and spatial overlap between two PET acquisitions over a 48 h time period was high for both FMISO and HX4. Furthermore, decreasing the hypoxic fraction using carbogen resulted in loss of FMISO uptake, while increased hypoxia achieved by breathing 7% oxygen, further enhanced FAZA and HX4 uptake. Another study performed a similar comparison in a SQ20b head and neck xenograft mouse model and found similar tumour to muscle ratios for FMISO, FAZA and HX4 [10]. However, these results were obtained at 80–90 min p.i., a time point which is probably too early for evaluation since normal tissue clearance is still ongoing. A comparative study in head and neck cancer patients found similar tumour to muscle ratios for HX4 imaging at 1.5 h p.i. and FMISO imaging at 2 h p.i. [17]. For HX4 higher uptake and increasing ratios would be expected at later time points based on our clinical results [13]. Recently a simulation study, comparing FMISO, FAZA and HX4 based on their respective physical and chemical properties, revealed that tracer clearance and diffusion are the major parameters influencing image contrast. Highest clearance and image contrast was observed for HX4, but also the largest patient-to-patient variation, which might be a concern for clinical imaging to define tumour hypoxia based on a reliable threshold value [18].

Current available tracers have proven to be reliable for evaluation of tumour hypoxia, although with inherent problems resulting in clinical limitations. Alternative tracers, such as HX4, are promising with respect to deliver higher contrast images, whereas FMISO remains a robust reproducible hypoxia marker. It is not inconceivable that more tracers will be developed; but currently existing PET tracers should rather be used in clinic with standardized protocols enabling comparisons between different institutes. Furthermore, applicability and clinical validation should be proven in multiple cancer types and tracers need be tested with respect to their prognostic and predictive value.

Hypoxia targeting

The compelling evidence for hypoxia in tumour tissue and its therapeutic importance makes hypoxia a high priority target for cancer therapy. Bioreductive prodrugs selectively activated under hypoxia and drugs that inhibit molecular targets in hypoxic cells (vide infra) are currently extensively investigated. A recent overview described the challenges and opportunities of these strategies [19]. The clinically most advanced hypoxia-activated prodrug is tirapazamine (TPZ). Although promising results have been reported in a number of Phase 2 trials, TPZ failed in several Phase 3 clinical trials since no survival benefit was observed when incorporated into standard therapy regimens. Possible explanations are its poor tumour penetration, low *in vivo* potency at tolerable doses and unacceptable toxicity levels and lack of patient selection with high levels of tumour hypoxia [20]. A more potent hypoxia-activated prodrug currently undergoing early clinical testing is TH-302. It is a 2-nitroimidazole conjugated to bromo-isophosphoramide mustard, which is released and activated upon very low levels of oxygen [21] and diffuses to surrounding cells creating a cytotoxic bystander effect [19]. TH-302 displayed clinical activity when used as single agent, which makes it unique compared to earlier generation hypoxia-activated cytotoxins which demonstrate anti-tumour activity only when used in combination with radiation or chemotherapy [22]. Furthermore, TH-302 efficacy was correlated with the hypoxic fraction across different tumour models [23–26]. Phase 1 trials have proved TH-302 safety with nausea, vomiting and fatigue as the most frequently occurring toxicities. Other trials successfully combined TH-302 with doxorubicin in patients with advanced soft tissue sarcoma [27] or with gemcitabine in patients with advanced pancreatic cancer [28]. A phase 3 double-blind, placebo-controlled trial has been initiated in which patients with advanced pancreatic cancer were randomized to gemcitabine combined with TH-302 or placebo [29]. Recently our group has evaluated the efficacy of TH-302 in a rat rhabdomyosarcoma and a human H460 xenograft model, using growth delay as endpoint. TH-302 in both models significantly inhibited tumour growth and markedly sensitized tumours to radiation. Furthermore, the therapeutic effect of TH-302 was dependent on the tumour oxygenation status prior to local radiotherapy that was modified by either carbogen (to improve oxygenation) or low oxygen containing gas (to increase hypoxia) breathing [30].

Increasing tumour oxygenation has shown potential for improving radiotherapy efficacy in several randomized clinical trials [31,32]. In spite of positive results, these strategies using hyperbaric oxygen or carbogen combined with vasodilating agents have not gained clinical traction due to practical limitations, toxicity and relatively modest clinical benefit [33]. An alternative strategy to achieve improved tumour oxygenation is to decrease cellular oxygen consumption using for example metformin, an inhibitor of the mitochondrial NADH dehydrogenase, also known as complex 1, activity in the mitochondrial electron transport respiration chain [34]. Recently, it has been demonstrated that metformin increases tumour response to radiotherapy, through a reduction in oxygen consumption and improved tumour oxygenation [35]. For future personalized cancer medicine, evaluation of hypoxia biomarkers and patient stratification will be essential to apply hypoxia targeting treatments to change radiotherapy response.

Hypoxia molecular response

As tumours progress from an early to later stage disease, supply of oxygen becomes limited. Cancer cells must therefore alter their metabolism to an anaerobic glycolytic phenotype, resulting in a less efficient energy production and intracellular acidosis. In order to survive cancer cells must adapt to this acidic microenvironment, which helps promote metastases [36]. One of the important molecular responses to hypoxia is the stabilization of the hypoxia inducible factor (HIF)-1α, enabling interaction with HIF-1β. The complex translocates to the nucleus where it binds to hypoxia responsive elements (HRE) in the promoter region of target genes, such as vascular endothelial growth factor (VEGF), glucose transporters 1 (GLUT-1) and carbonic anhydrase IX (CAIX) [37]. CAIX as well as other membrane transporters, like the sodium-proton exchanger 1 (NHE-1) and the monocarboxylate transporters (MCT), are upregulated to counteract the hypoxia-induced intracellular acidosis. CAIX is a tumour specific dimeric membrane bound zinc metallo-enzyme, which catalyses the reversible hydration of carbon dioxide to bicarbonate and a proton to help maintain the cells pH homeostasis [38]. High tumoural CAIX expression has been associated with poor prognosis, tumour progression and aggressiveness [39]. Inhibition of its function would therefore be a promising anticancer approach to target the hypoxic compartment of tumours.

CAIX imaging tracers

A molecular imaging approach based on selective ligands to accessible proteins overexpressed at sites of hypoxia is desired. Such an agent could help physicians to decide which patients would benefit from adjuvant hypoxia-targeted therapy, e.g. anti-CAIX therapy. One strategy is using antibodies or antibody fragments targeted against transmembrane CAIX expression. The highly specific antibody M75 recognizes the extracellular proteoglycan-like domain of CAIX and is used for Western blotting and immunohistochemistry [40]. Specific accumulation of iodine-125 radiolabelled derivative has been observed in HT29 tumour-bearing mice [41]. Independently, the monoclonal G250 antibody was developed as a biomarker for renal cell carcinoma [42]. A chimeric version of G250 (cG250) has been radiolabelled with iodine-124, zirconium-89 or indium-111 for diagnostic purposes [43]. However, no apparent correlation has been observed between cG250 uptake and pimonidazole labelling or CAIX staining in head and neck tumour models attributed to the large interval between cG250 injection and immunohistochemical evaluation [44]. Pepsin degradation of intact cG250 antibodies resulted in $F(ab')_2$ fragments with a smaller molecular weight resulting in faster clearance from blood and healthy tissues. Zirconium-89 labelled cG250-$F(ab')_2$ fragments were found to spatially correlate with CAIX expression [45]. Furthermore, fully human CAIX single-chain variable fragment (scFv) minibodies have been generated using phage-display technology. They recognize the extracellular carbonic anhydrase domain, but do not inhibit CAIX activity and do not bind to the other transmembrane enzyme CAXII [46]. Recently, near-infrared (NIR) fluorescent monoclonal antibodies against CAIX and CAXII have been successfully tested for the non-invasive detection of breast cancer metastasis [47]. A dual labelled antibody combining indium-11 or iodine-125 nuclear with NIR imaging has proven feasible for preoperative and intraoperative detection of CAIX expressing renal cell carcinomas [48,49].

A second approach is the use of small molecules specifically targeting the active site of CAIX. Several classes of small molecules with low (nM) affinity have been extensively described, but due to the high degree of homology among CA isotypes, small molecules are generally not specific for one isoform [50]. To prevent interaction with the intracellular CA isoforms charged species or bulky groups such as FITC, albumin or sugar moieties are added to the small molecule. These strategies prevent transportation across membranes, but do not guarantee selectivity between CAIX and CAXII, both transmembrane enzymes with their catalytic domains oriented extracellularly. Attempts to design specific compounds targeting each isoform separately have been recently reviewed [51].

We and others have demonstrated *in vitro* that small molecule binding requires not only CAIX expression but also its hypoxic activation [52,53]. This offers a big advantage compared with antibodies against CAIX, since these small molecules can distinguish cells that are currently hypoxic from those that were previously hypoxic, while antibodies do not since their long half-life after reoxygenation [54]. *In vivo*, we have reported significant accumulation of fluorescent sulfonamides in HT29 xenografts, which was causally related with tumour oxygenation. Furthermore, bound sulfonamide decreased rapidly upon tumour reoxygenation [55]. Similar results have been obtained using fluorescent acetazolamide derivatives showing preferential targeting of CAIX overexpressing SK-RC-52 renal cell xenografts [56]. Recently, a series of sulfonamide derivatives conjugated with NIR fluorescent dyes having up to 50-fold higher selectivity for CAIX compared to the intracellular and other transmembrane isoforms has been designed. High tumoural uptake with little accumulation in other organs, except for the kidneys, has been observed using fluorescence molecular tomography [57]. Several synthesis strategies have been proposed to enable nuclear imaging, however most attempts were not successful in showing specific enhanced tumour uptake. We have synthesized and evaluated a technecium-99 m labelled sulfonamide for visualization of CAIX expression by SPECT imaging. Despite favourable affinity values maximum tumour uptake was low (<0.5% ID/g) even after varying physicochemical properties of the molecules [58,59]. A range of sulfonamides conjugated metal complexes have recently been designed for metallic radionuclide imaging. Although high *in vitro* uptake was observed, cellular binding between CAIX positive and negative cell lines was not obviously different [60]. Besides metal chelation approaches, direct fluorine-18 radiolabelling of CAIX targeted molecules has been assessed. Several compounds have been synthesized, such as 7-(2-fluoroethoxy)coumarin (FEC) and U-104 [61], the tertiary sulfonamides 4a-c [62] and VM4-037A [63]. Although all derivatives showed good affinity for CAIX with excellent plasma stability, uptake in HT-29 xenografts was minimal which precludes their application as CAIX imaging agents.

CAIX targeting

Inhibiting CAIX can be done either by the use of monoclonal antibodies or with small molecule inhibitors. Antibody approaches are mostly based on the concept of antibody dependent cell cytotoxicity (ADCC). A leading example is cG250, marketed as RENCAREX®, which is extensively investigated as an anticancer immunotherapy [64]. Phase 1 and 2 trials have demonstrated safety and efficacy as monotherapy or in combination with interferon (IFN)-α for the treatment of renal cell carcinoma (RCC) [65]. This antibody was also tested in the double-blind, placebo-controlled phase 3 aRISER trial for adjuvant therapy of clear cell RCC, but as announced by WILEX AG the antibody failed to meet the primary endpoint, since no improvement in median disease-free survival was observed compared to placebo. Several new antibodies currently tested in preclinical settings show promising results regarding anti-tumour effects [66,67].

Specific inhibition of different carbonic anhydrase isoforms using small molecules is an active field of research and has been extensively reviewed [50,68,69]. Membrane-impermeable acetazolamide derivatives [56] and aromatic sulfonamides [70] were able to reduce tumour growth and proliferation. Treatment of mammary-tumour bearing mice with CAIX-specific sulfonamide and glycosylcoumarin inhibitors resulted in a significant reduction in tumour growth and lung metastasis formation [71]. One of the several potent CAIX inhibitors identified from a DNA-encoded chemical library screen has shown high and specific accumulation in tumour models [72]. A new class of sulfamate inhibitors proved to be excellent candidates for low dosage anti-metastatic drugs [73], but were ineffective in reducing primary tumour growth [73,74]. Combining small molecules targeting CAIX with conventional therapies might yield even better efficacy. Recently, a CAIX dependent sensitizing effect of indanesulfonamides [75] and acetazolamide [76] on respectively radio- and chemotherapy has been demonstrated. Similarly combination of paclitaxel with orally administered U-104 significantly affected primary tumour growth and metastasis formation by reducing the breast cancer stem cell population [77]. Nitroimidazole and sulfamide based dual targeting drugs reduced hypoxic extracellular acidification *in vitro*, inhibited tumour growth at low dosage and sensitized tumours to both radiation [78] and doxorubicin [79]. This dual-targeting strategy appeared to be more effective than single targeting molecules. Recently, a family of novel small-molecule drug conjugates comprising of a linker cleavable in the extracellular space and a potent cytotoxic payload targeting CAIX has been designed and characterized. The disulfide-linked conjugate with maytansinoid DM1 as cytotoxic payload and an acetazolamide derivative as the targeting ligand has shown potent anti-tumour effects in renal cell carcinoma models with only minimal toxicity [80]. These results indicate that targeted delivery of potent cytotoxic agents using CAIX directed ligands may provide therapeutic benefits over current standard of care. The first clinical trial (NCT02215850) testing a small molecule CAIX inhibitor, named SLC-0111, is currently ongoing and is focused on testing the safety in subjects with advanced solid tumours.

Window-of-opportunity trial

Although there is a high number of new promising anti-cancer agents under preclinical and clinical investigation, the success rate of approved drugs for clinical practice has not been significantly increased. Improved clinical trial designs, such as 'window-of-opportunity' trials will help to select effective drugs at an earlier stage and to identify patients which potentially will benefit of the drug. In this trial, the patient agrees to delay combined conventional anti-cancer therapy to first receive the experimental drug, with the aim to obtain knowledge about anti-tumour activity in a disease state that is not disturbed by previous or simultaneous treatments [81]. The question has been raised whether these trials should be more widely applied in early phases of drug development knowing the progress in imaging and monitoring tumour progression [82] to prevent expensive long-lasting classical clinical testing of inefficacious drugs. Using this trial approach, hypoxia imaging can be used as a biomarker of response, especially suitable in the context of testing hypoxia (response) targeting drugs. Upon patient inclusion, baseline hypoxia should be acquired followed by the experimental targeting drug. A post-treatment hypoxia PET scan will assess the effect of the single treatment by comparison of the hypoxic fractions between the two scans (Fig. 1). This window-of-opportunity trial can precede a phase 1 trial testing safety of the experimental drug in combination with conventional treatment, e.g. radiotherapy, or a randomized phase 2 clinical trial.

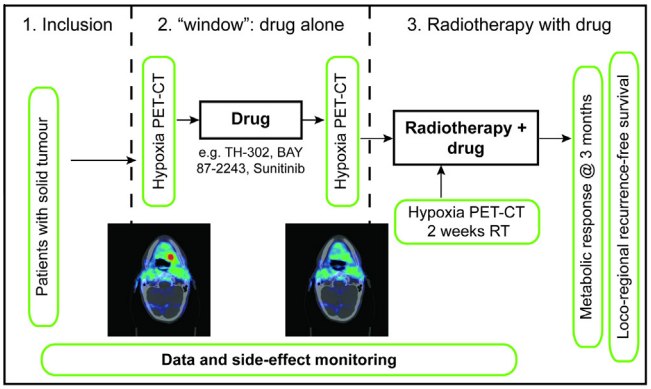

Fig. 1. Window-of-opportunity trial concept implementing non-invasive hypoxia imaging before and after drug administration followed by phase 1 or 2 trial combining radiotherapy and experimental drug. Additional hypoxia imaging can be included in step 3 for early response monitoring. HX4 hypoxia PET-CT images from a patient with head and neck cancer are shown as proof of concept.

We have used this concept preclinically to proof efficacy of the hypoxia-activated cytotoxic prodrug TH-302. The hypoxic fraction assessed with HX4 PET imaging in the rhabdomyosarcoma model was significantly reduced at day 4 upon TH-302 treatment, while vehicle treatment was ineffective. Additionally, TH-302 was not only effective as monotherapy, but also sensitized tumours to a single dose of radiation [30]. Similarly, BAY 87-2243, an inhibitor of mitochondrial complex 1, resulted in reduced HIF-1α activity and pimonidazole binding prior to radiotherapy improving local tumour control [83,84]. Its efficacy has also been shown by a dramatic reduction in FAZA PET signal before significant changes in tumour volume were observed [85]. Finally sunitinib treatment resulted in improved tumour oxygenation as FAZA uptake in Caki-1 renal cell xenografts [86] and in patients with soft-tissue sarcomas [87] was significantly reduced during therapy. Upon withdrawal of sunitinib therapy, FAZA uptake increased again, indicating a rebound in tumour hypoxia. These examples clearly highlight the importance of imaging the hypoxic fraction of tumours to monitor treatment response.

Conclusions

Current clinically available hypoxia PET tracers, although showing different characteristics, have proven to be reliable for evaluation of tumour hypoxia. Much progress has been made in the synthesis and evaluation of high affinity small molecules targeting CAIX. Nevertheless, proper clinically-suited diagnostic tools are still lacking. The window-of-opportunity trial concept implementing non-invasive imaging to monitor treatment response is an important tool to provide evidence of anti-tumour efficacy in earlier stages of drug development.

Conflict of interest statement

None of the authors have any conflict of interest to declare.

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