

Molecular imaging of hypoxia with radiolabelled agents

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Abstract Tissue hypoxia results from an inadequate supply of oxygen (O₂) that compromises biological functions. Structural and functional abnormalities of the tumour vasculature together with altered diffusion conditions inside the tumour seem to be the main causes of tumour hypoxia. Evidence from experimental and clinical studies points to a role for tumour hypoxia in tumour propagation, resistance to therapy and malignant progression. This has led to the development of assays for the detection of hypoxia in patients in order to predict outcome and identify patients with a worse prognosis and/or patients that would benefit from appropriate treatments. A variety of invasive and non-invasive approaches have been developed to measure tumour oxygenation including oxygen-sensitive electrodes and hypoxia marker techniques using various labels that can be detected by different methods such as positron emission tomography (PET), single photon emission com-

puted tomography (SPECT), magnetic resonance imaging (MRI), autoradiography and immunohistochemistry. This review aims to give a detailed overview of non-invasive molecular imaging modalities with radiolabelled PET and SPECT tracers that are available to measure tumour hypoxia.

Keywords Hypoxia · PET · SPECT · Nitroimidazole

Hypoxia in tumour biology

The prevalence of hypoxic areas is a characteristic feature of locally advanced solid tumours and has been described in a wide range of human malignancies, including cancer of the breast, uterine cervix, vulva, head and neck, prostate, rectum, pancreas as well as in brain tumours, soft tissue sarcomas and malignant melanomas. Up to 50–60% of locally advanced solid tumours may exhibit hypoxic and/or anoxic tissue areas that are heterogeneously distributed within the tumour mass. These hypoxic areas result from an imbalance between oxygen supply and consumption which is caused by abnormal structure and function of the microvessels supplying the tumour (causing acute hypoxia), increased diffusion distances between the nutritive blood vessels and the tumour cells (causing chronic hypoxia), and reduced O₂ transport capacity of the blood due to the presence of disease- or treatment-related anaemia [1–3]. Recent studies have demonstrated a clear relevance of this hypoxic microenvironment to tumour-associated metabolic alterations, which are tightly linked to the biology of the tumour. In this respect, tumour hypoxia has been associated with an aggressive tumour phenotype, poor response to radiotherapy and chemotherapy, increased risk of invasion and metastasis, and worse prognosis in advanced squamous

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cell carcinoma of the cervix [4, 5], head and neck [6–8], and soft-tissue sarcomas [9].

Many of the adaptations to tumour hypoxia seem to be orchestrated by the transcription factor hypoxia-inducible factor (HIF)-1, which has been verified as a master regulator of oxygen homeostasis under hypoxic conditions. Its oxygen-dependent activity is regulated in a delicate interplay between several factors in which a family of prolyl hydroxylase domain-containing proteins or PHDs 1–3, the von Hippel-Lindau (VHL) tumour suppressor protein (pVHL) and an E3 ubiquitin ligase complex, amongst many others, play an important role. The HIF-1 pathway mediates critical hypoxic adaptations by the induction of target genes involved in glucose metabolism, angiogenesis, erythropoiesis and apoptosis. These target genes include vascular endothelial growth factor (VEGF), facilitative glucose transporters (GLUTs), hexokinases (HKs), erythropoietin (EPO), carbonic anhydrase IX (CAIX), ... The resulting adaptive changes in the proteome and genome of the tumour cells are believed to lead to more aggressive clones which are better adapted to survive in their compromised situation. Subsequent selection and clonal expansion of these clones lead to a more adapted and aggressive tumour cell population [1–3]. As noted above, the presence of tumour hypoxia appears to impair the effectiveness of common anticancer therapies like radiotherapy (RT) and chemotherapy. Hypoxia-induced radioresistance is multifactorial. Besides the above-mentioned proteomic and genomic changes that most likely contribute to resistance by increasing the number of mutated cells that are more resistant to apoptosis and by causing upregulation of several stress proteins, the main reason for radioresistance is the intrinsic dependence of RT on oxygen to cause damage to the tumour cell. For damage to be inflicted on tumour cells by ionizing radiation, the presence of oxygen is necessary because it mediates DNA damage through formation of free radicals by interaction of ionizing radiation with intracellular water. Hypoxia has also been shown to reduce chemotherapeutic efficacy by causing cells within hypoxic regions to cycle more slowly and by providing a selection mechanism for cells with reduced susceptibility for apoptosis. Additionally, due to limited drug penetration within solid tumours, hypoxic regions are often protected from the cytotoxic effects of chemotherapeutic agents further reducing drug efficacy [1–3].

All these mechanisms together ensure that tumour hypoxia is a negative prognostic factor. In order to predict outcome and identify patients with a worse prognosis and/or patients that would benefit from appropriate treatments, *in vivo* measurement of tumour hypoxia is required. At present, the gold standard for direct *in vivo* determination of tumour oxygenation is a commercially available oxygen electrode, commonly referred to as the Eppendorf electrode.

As mentioned above, tumour oxygenation measurements obtained with this technique correlated well with clinical outcome in several clinical trials [4–9]. However, this technically demanding procedure has a number of drawbacks and limitations like its sensitivity for sampling errors, its invasive nature and the fact that only easily accessible tumours can be studied. Therefore, the search for a non-invasive assay for tumour hypoxia continues. Non-invasive measurement of tumour hypoxia with PET and SPECT will be discussed below.

Non-invasive measurement of tumour hypoxia with PET

1. Nitroimidazole compounds

These compounds are reduced into reactive intermediary metabolites by intracellular reductases in a process which is directly related to the level of oxygenation/hypoxia. This causes a gradient which is favourable for detection of hypoxic cells. Subsequently, these metabolites covalently bind to thiol groups of intracellular proteins and thereby accumulate within viable hypoxic cells. When labelled with a PET tracer, these chemicals can be detected using PET imaging methods. Several nitroimidazole compounds with different properties and labelled with different PET radionuclides have been described [10, 11].

(a) [^{18}F]FMISO ([^{18}F]fluoromisonidazole)

Pre-clinical data: Kubota et al. evaluated the tumour imaging potential of [^{18}F]FMISO in an AH109A tumour rat xenograft and examined the correlation between intratumoural distributions of [^{18}F]FMISO, ^{14}C -2-deoxyglucose and ^{14}C -methionine. Hypoxic and radioresistant tumours could be identified by increased [^{18}F]FMISO uptake. A large overlap in the distribution of [^{18}F]FMISO and ^{14}C -2-deoxyglucose and a small overlap in the distribution of [^{18}F]FMISO and ^{14}C -methionine were observed [12]. In a study by Rasey et al., an attempt was made to define the relationship between [^{18}F]FMISO uptake and radiobiologically hypoxic fraction in a 36B10 glioma rat xenograft. Although the relationship between classically defined radiobiologically hypoxic fraction and [^{18}F]FMISO time-activity data remained to be clarified, [^{18}F]FMISO retention provided useful correlations with the degree of hypoxia [13]. Bentzen et al. compared [^{18}F]FMISO uptake with invasive Eppendorf electrode pO_2 measurements in a C3H mammary carcinoma mouse xenograft and found no direct correlation between both methods [14].

A number of studies compared [^{18}F]FMISO uptake with immunohistochemical staining techniques. In a study by Dubois et al., [^{18}F]FMISO uptake was compared with the

exogenous hypoxia marker pimonidazole and the endogenous hypoxia marker carbonic anhydrase IX (CA IX) in a rhabdomyosarcoma rat xenograft. A statistically significant correlation was obtained between the hypoxic volumes defined with [^{18}F]FMISO PET and the volumes derived from the pimonidazole- and CA IX-stained tumour sections, indicating the value of [^{18}F]FMISO PET to measure hypoxia [15]. Troost et al. tried to validate [^{18}F]FMISO PET by comparing [^{18}F]FMISO uptake with pimonidazole staining in several xenograft models in two different studies. Both studies found a correlation between [^{18}F]FMISO uptake and pimonidazole immunohistochemistry [16, 17].

In a number of studies, [^{18}F]FMISO uptake was compared with [^{18}F]FDG uptake. Most of these studies demonstrated the feasibility and utility of [^{18}F]FMISO PET imaging to identify tumour hypoxia, whereas [^{18}F]FDG PET imaging seemed less suitable for this purpose [18–22].

Clinical data: Valk et al. were the first to demonstrate the feasibility of [^{18}F]FMISO PET to detect tumour hypoxia in three patients with malignant glioma [23]. Rasey et al. assessed pre-treatment hypoxia in a variety of human tumours using [^{18}F]FMISO PET and concluded that human tumour hypoxia is widely prevalent and highly variable between different tumours with the same histology and also between regions within the same tumour [24]. In a study by Bruehlmeier et al. where hypoxia was measured in 11 patients with various brain tumours, it was concluded that late [^{18}F]FMISO PET images provide a spatial description of tumour hypoxia which may develop irrespective of the magnitude of perfusion as measured with ^{15}O - H_2O PET [25].

A number of studies compared [^{18}F]FMISO uptake with invasive Eppendorf electrode pO_2 measurements. In some of these studies, [^{18}F]FMISO uptake in renal cell carcinoma and head and neck cancer correlated well with pO_2 measurements from polarographic needle oxygen electrodes, confirming the use of [^{18}F]FMISO PET to measure tumour hypoxia [26–29]. Bentzen et al., however, found no correlation between [^{18}F]FMISO uptake and pO_2 measurements in human soft tissue tumours [30]. Some of the above-mentioned studies also compared uptake of [^{18}F]FDG and [^{18}F]FMISO in patients with head and neck cancer. They found no correlation between [^{18}F]FDG uptake and pO_2 measurements, whereas an association between [^{18}F]FMISO uptake and pO_2 measurements existed [27–29]. Further comparison of [^{18}F]FDG and [^{18}F]FMISO indicated that no correlation exists between both tracers as both represent different tumour characteristics [31–35].

Several clinical studies have used [^{18}F]FMISO PET as a prognostic indicator in oncology. In a study by Rajendran et al., the prognostic effect of pre-therapy [^{18}F]FMISO PET

on survival was investigated in 73 patients with head and neck cancer, and pre-treatment [^{18}F]FMISO uptake proved to be an independent prognostic factor [36]. Another study of 12 patients with head and neck carcinoma who received a pre-radiotherapy [^{18}F]FMISO PET scan concluded that [^{18}F]FMISO uptake was predictive of treatment response to radiotherapy [35]. Similarly, in 40 patients with advanced head and neck cancer and non-small cell lung cancer, outcome after radiotherapy could be predicted on the basis of kinetic behaviour of [^{18}F]FMISO in tumour tissue [37]. Two studies investigating the prognostic significance of [^{18}F]FMISO PET in patients with head and neck cancer receiving chemoradiation in combination with the hypoxia sensitizer tirapazamine concluded that [^{18}F]FMISO uptake can predict prognosis and is associated with a high risk of locoregional failure [38, 39]. Cher et al. showed that in patients with malignant glioma [^{18}F]FMISO uptake is prognostic for treatment outcome in the majority of patients [34]. In a study with eight patients with non-small cell lung cancer receiving chemotherapy and/or radiotherapy, changes in [^{18}F]FMISO uptake measured early response to therapy and may predict freedom from disease as well as overall survival [40]. A recent study that was conducted to evaluate the reproducibility of [^{18}F]FMISO intratumour distribution in 20 patients with head and neck cancer showed considerable variability in the intratumour uptake that can occur between repeated [^{18}F]FMISO PET scans performed 3 days apart [41].

(b) [^{18}F]FAZA ([^{18}F]fluoroazomycin-arabinofuranoside)

Pre-clinical data: Sorger et al. compared the selective uptake of [^{18}F]FMISO and [^{18}F]FAZA in hypoxic cells in vitro and in a Walker 256 rat sarcoma model. The in vitro study showed that [^{18}F]FAZA is able to indicate reduced oxygen supply in the same order of magnitude of [^{18}F]FMISO. The in vivo study, however, indicated that [^{18}F]FMISO displayed a slightly higher standardized uptake value and tumour to muscle ratio compared to [^{18}F]FAZA though the elimination of the latter was much faster [42]. Two other studies also compared [^{18}F]FMISO and [^{18}F]FAZA in various tumour mice xenografts and reported superior biokinetics for [^{18}F]FAZA compared with [^{18}F]FMISO. In both studies [^{18}F]FAZA displayed higher tumour to background, tumour to muscle and tumour to blood ratios due to its more rapid clearance from blood and non-target tissues [43, 44]. Beck et al. evaluated the predictive value of [^{18}F]FAZA PET for success of radiotherapy in combination with tirapazamine in EMT6 tumour mice xenografts. High [^{18}F]FAZA uptake was identified as an independent adverse prognostic factor for tumour progression, and hypoxia imaging with [^{18}F]FAZA PET was able to predict the success of radiochemotherapy [45]. In a study by Busk et al., [^{18}F]FAZA uptake was

compared with Eppendorf electrode measurements and the hypoxia marker pimonidazole. The distribution of [^{18}F]FAZA proved to be consistent with tumour hypoxia, as identified with the Eppendorf electrode measurements and the hypoxia marker pimonidazole [46]. The same group compared the *in vitro* hypoxia specificity of cellular [^{18}F]FDG and [^{18}F]FAZA retention and tested tracer distribution between hypoxic and non-hypoxic areas in different mice xenografts. The *in vitro* as well as the *in vivo* experiments indicated that [^{18}F]FAZA is an excellent marker for tumour hypoxia, whereas [^{18}F]FDG is not [47].

Clinical data: Souvatzoglou et al. evaluated the feasibility of [^{18}F]FAZA PET for the imaging of tumour hypoxia in 11 patients with head and neck cancer and concluded that PET imaging with [^{18}F]FAZA is feasible and that adequate image quality is achieved [48]. Another study, which included 18 patients with advanced squamous cell head and neck cancer, evaluated the role of [^{18}F]FAZA PET imaging to identify hypoxia in order to plan radiation treatment. It was concluded that radiation treatment planning and intensity-modulated radiotherapy based on [^{18}F]FAZA uptake measurements are feasible [49].

(c) [^{18}F]FETA ([^{18}F]fluoroetanidazole)

In a study by Rasey et al., four cultured rodent cell lines were incubated with [^{18}F]FETA for various times under graded O_2 concentrations. The biodistributions of [^{18}F]FETA and [^{18}F]fluoromisonidazole (FMISO) at 2 and 4 h post-injection in C3H mice bearing KHTn tumours were also compared. [^{18}F]FMISO and [^{18}F]FETA demonstrated similar oxygen dependency of binding in cultured cells. However, differences in biodistribution suggested advantages of [^{18}F]FETA over [^{18}F]FMISO because [^{18}F]FETA appeared to be less metabolized *in vivo* than [^{18}F]FMISO [50]. In another study, the cellular transport and retention of [^{18}F]FETA were determined *in vitro* under air and nitrogen and the biodistribution and metabolism were determined in mice bearing several different xenografts. It was concluded that [^{18}F]FETA has suitable physicochemical properties and is stable to non-hypoxic degradation *in vivo*. It was also demonstrated that the tumour retention of the radiotracer is related to radiobiological hypoxia and pO_2 status as determined with polarographic needle oxygen electrodes [51].

(d) [^{18}F]FETNIM ([^{18}F]fluoroerythronitroimidazole)

Pre-clinical data: Yang et al. reported on the synthesis and evaluation of [^{18}F]FETNIM. Their results indicated that at 4 h after injection, tumour to blood and tumour to muscle ratios in mammary tumour-bearing rats were significantly higher with [^{18}F]FETNIM than with [^{18}F]FMISO [52]. In a later study by Grönroos et al. where the pharmacokinetic properties and metabolite formation of [^{18}F]FETNIM were

studied, [^{18}F]FETNIM showed low peripheral metabolism, little defluorination and possible metabolic trapping in hypoxic tumour tissue [53]. In a pre-clinical study by the same group, the hypoxia imaging ability of [^{18}F]FETNIM was compared with that of [^{18}F]FMISO in a C3H mammary carcinoma mice xenograft under different oxygenation conditions. Additionally, the biodistribution of both markers in normal tissues was assessed under similar conditions. Uptake of both tracers correlated with the oxygenation status in the tumours, but [^{18}F]FETNIM showed a low and favourable background signal in normal tissues as compared with [^{18}F]FMISO [54].

Clinical data: Most of the clinical studies with [^{18}F]FETNIM were performed in patients with head and neck cancer. A study investigating the accurate radiation dosimetry in 27 patients with head and neck cancer concluded that the effective dose of [^{18}F]FETNIM PET is well within the range of several related nuclear medicine procedures [55]. A lot of clinical studies were performed by researchers at the University of Turku in Finland. They found that [^{18}F]FETNIM uptake in the early phase of tissue accumulation, as measured using [^{15}O]H $_2$ O and PET, was highly variable and depended for the most part on perfusion [56]. Tumour to plasma ratio provided the best estimate for tumour hypoxia [57]. In another study, the radiotherapy response was assessed by hypoxia imaging with [^{18}F]FETNIM PET in 21 patients with head and neck cancer and high uptake of [^{18}F]FETNIM prior to radiation therapy was associated with a trend towards poor overall survival [58].

(e) [^{18}F]EF5

In a study by Ziemer et al., the biodistribution of [^{18}F]EF5 was assessed using hepatoma and glioma rodent tumour models. [^{18}F]EF5 was rapidly and uniformly distributed to all tissues. This together with its high drug stability *in vivo* suggests that [^{18}F]EF5 is a promising agent for the non-invasive assessment of tumour hypoxia [59]. Another study investigated hypoxia in androgen-dependent, androgen-independent and regressing Shionogi tumours using [^{18}F]EF5. Differences in hypoxia between the different types of tumours could be detected with [^{18}F]EF5 [60]. Recently, the first human study with [^{18}F]EF5 was performed in 15 patients with squamous cell carcinoma of the head and neck (HNSCC) in which the time course of [^{18}F]EF5 uptake after intravenous injection was evaluated to determine the most suitable PET protocol [61].

(f) [^{18}F]EF3

Pre-clinical data: In the framework of the pre-clinical evaluation, Mahy et al. studied the pharmacokinetics, biodistribution, metabolism and specificity for hypoxia of [^{18}F]EF3 in different tumour-bearing C3H mice breathing carbogen (5% CO_2 , 95% O_2), 21% oxygen and 10%

oxygen. They also compared [^{18}F]EF3 uptake and EF5 adducts detected by immunofluorescence in the same model. [^{18}F]EF3 uptake was inversely correlated with oxygen concentration, and a significant correlation was found between the [^{18}F]EF3 tumour to muscle ratio and the fluorescence intensity of EF5 [62, 63]. Pharmacokinetics, biodistribution and metabolism of [^{18}F]EF3 were assessed and compared with [^{18}F]FMISO uptake in rodent tumour models. It was concluded that both exhibited similar pharmacokinetics, biodistribution and metabolism and that [^{18}F]FMISO was able to detect tumour hypoxia to a similar extent as [^{18}F]EF3, although it seemed less specific than the latter tracer [64]. The same group tried to increase the tumour to noise ratio in C3H mice by increasing [^{18}F]EF3 elimination. Several chemicals increasing renal filtration rate, decreasing tubular reabsorption or stimulating gastrointestinal elimination were tested. Only phenobarbital induced a trend toward an increase in tumour to noise ratio [65]. In another study, [^{18}F]EF3 was quantitatively compared with [^{18}F]FMISO in rats bearing syngeneic rhabdomyosarcoma tumours. It was shown that [^{18}F]EF3 is cleared faster from the blood compared to [^{18}F]FMISO. Both had a similar tumour uptake at 4 h post-injection, a similarly fast and uniform distribution in normal tissues and a comparable intratumoural distribution, indicating that [^{18}F]EF3 is not superior to [^{18}F]FMISO [66].

Clinical data: In a recent phase I study by Mahy et al., pharmacokinetics, biodistribution and metabolism of [^{18}F]EF3 were assessed in ten patients with head and neck squamous cell carcinoma. Administration of [^{18}F]EF3 seemed feasible and safe in head and neck cancer patients. Uptake and retention of the tracer was observed in the tumour, indicating the presence of hypoxia [67].

(g) [^{18}F]EF1

Imaging with this marker was studied in two rat tumour types whereby the drug's biodistribution was assessed and optimized. [^{18}F]EF1 proved an excellent radiotracer for non-invasive imaging of tumour hypoxia [68].

(h) [^{124}I]IAZA ([^{124}I]iodoazomycin arabinoside)

Although IAZA has been frequently labelled with gamma rays-emitting isotopes of iodine ([^{123}I] and [^{125}I]), several studies report on the use of IAZA labelled with [^{124}I]. In a recent study by Reischl et al., the hypoxia imaging capacities of [^{124}I]IAZA, [^{18}F]FAZA and [^{18}F]FMISO were compared in female Balb/c nude mice bearing A431 tumours with a small animal PET scanner. [^{18}F]FAZA displayed significantly higher tumour to background ratio compared to [^{18}F]FMISO and [^{124}I]IAZA. Although the tumour to background ratio for [^{124}I]IAZA increased with time, ratios were still lower than those for [^{18}F]FAZA at shorter time periods. The study demonstrated the superior

biokinetics of [^{18}F]FAZA compared to [^{18}F]FMISO and [^{124}I]IAZA [44].

Newer agents based on the azomycin-nucleoside structure such as iodoazomycin galactoside (IAZG) [69, 70] and iodoazomycin galactopyranoside (IAZGP) [71] have been developed and evaluated. Two studies compared [^{124}I]IAZG uptake with [^{18}F]FMISO uptake. Zanzonico et al. studied the use of [^{124}I]IAZG as a hypoxia imaging agent in MCa and FsaII tumour-bearing mice using microPET imaging by comparing it with [^{18}F]FMISO imaging and provided data showing the potential of this tracer for hypoxia imaging [69]. A similar study by Riedl et al. in Morris hepatoma (RH7777)-bearing nude rats, however, found that although [^{18}F]FMISO localized in the same intratumoural regions as [^{124}I]IAZG, a superior diagnostic image quality was obtained with [^{18}F]FMISO [70]. A recent study evaluated hypoxia imaging using [^{124}I]IAZGP in a Morris hepatoma RH7777 tumour rat model by comparing it with fluorescence fiberoptic oxygen probe measurements, pimonidazole and EF5 distribution and tried to determine the optimal time after injection to depict hypoxia. [^{124}I]IAZG distribution correlated positively with pimonidazole and EF5 distributions, and the optimal ratio between signal intensity and tumour to liver contrast occurred 6 h after tracer administration [71].

2. Non-imidazole imaging agents

(a) [^{18}F]FDG (2-deoxy-2-[^{18}F]fluoro-D-glucose)

[^{18}F]FDG-PET is a non-invasive functional imaging method that is routinely used for cancer detection, staging and monitoring of response in several tumour types. Because the uptake of [^{18}F]FDG during FDG PET imaging relies largely on the expression of proteins that are under control of HIF-1, the degree of [^{18}F]FDG uptake by tumours might indirectly reflect the level of hypoxia. Reports trying to relate [^{18}F]FDG uptake with tumour hypoxia have, however, given inconsistent results. In vitro studies have suggested that FDG should be accumulated in hypoxic cancer cells compared to normoxic cancer cells because of changed metabolism [21, 47, 72–77]. However, in vivo experiments (pre-clinical and clinical) have given conflicting results when showing a correlation between the uptake of [^{18}F]FDG and the existence of hypoxia in tumours [18–20, 22, 27–29, 32–35, 40, 78–84]. A recent review by Dierckx et al. addresses this subject matter [85].

(b) Cu-ATSM

Another alternative PET agent for hypoxia imaging that holds great promise is based on a metal complex of radioactive copper with ATSM, diacetyl-bis(N^4 -methylthiosemicarbazone). Cu(II)-ATSM is a neutral lipophilic molecule, which is highly membrane permeable. It can undergo reduction by cellular reducing equivalents and can be converted to

[Cu(I)-ATSM]⁻, which becomes entrapped in cells because of its negative charge when cells are hypoxic. There are four different positron-emitting copper isotopes that each have their own decay scheme: ⁶⁰Cu ($t_{1/2}$ =0.40 h), ⁶¹Cu ($t_{1/2}$ =3.32 h), ⁶²Cu ($t_{1/2}$ =0.16 h), ⁶⁴Cu ($t_{1/2}$ =12.7 h) [86].

Pre-clinical data: After reports on the use of Cu⁶²-ATSM to detect hypoxia in hypoxic myocardial tissue [87], numerous pre-clinical studies have evaluated and validated its use for imaging of hypoxia in tumours. In an in vitro study by Dearling et al., several ⁶⁴Cu-labelled bis (thiosemicarbazone) complexes were prepared and tested for tumour hypoxia selectivity by incubation with CHO320 Chinese hamster ovary cells under normoxic and hypoxic conditions. A number of molecules, including ⁶⁴Cu-ATSM, showed significant hypoxia selectivity [88]. Later, attempts were made to improve the hypoxia selectivity of the copper complexes by identification of the physicochemical properties that control hypoxia selectivity [89]. Lewis et al. evaluated ⁶⁴Cu-ATSM in vitro in the EMT6 carcinoma cell line under varying pO₂ and compared it with [¹⁸F]FMISO and further evaluated ⁶⁴Cu-ATSM in vivo in a murine animal model. ⁶⁴Cu-ATSM was selectively trapped in vitro in EMT6 cells under hypoxic conditions and in vivo in solid EMT6 tumours, confirming its role as an agent to successfully detect tumour hypoxia [90]. A study by Burgman et al. indicated, after determining the in vitro uptake of ⁶⁴Cu-ATSM as a function of oxygenation conditions and incubation time in several tumour cell lines of rodent and human origin, that the uptake and retention of ⁶⁴Cu-ATSM and their relation to oxygenation conditions were cell line dependent [91]. The pO₂ dependence of Cu-ATSM was confirmed in a 9L gliosarcoma rat model by comparison of Cu-ATSM uptake with direct oxygen measurements using needle oxygen electrodes while tumour oxygen concentration was manipulated [92]. A study by Yuan et al., on the other hand, concluded after comparing the autoradiographic distributions of ⁶⁴Cu-ATSM with the hypoxia markers EF5, pimonidazole and CAIX in R3230 mammary adenocarcinomas, fibrosarcomas and 9L gliomas that ⁶⁴Cu-ATSM is a suitable PET hypoxia marker in most tumour types, but not for all [93].

A number of studies compared ⁶⁴Cu-ATSM uptake with [¹⁸F]FMISO uptake and [¹⁸F]FDG uptake in vivo. O'Donoghue et al. reported that the uptake of ⁶⁴Cu-ATSM 4 h after injection in an R3327-AT anaplastic rat prostate tumour model did not correlate with [¹⁸F]FMISO uptake and does not reflect the level of hypoxia, as assessed by pimonidazole immunostaining and invasive oxygen needle probes. ⁶⁴Cu-ATSM imaging at 16–20 h after injection, however, corresponded with [¹⁸F]FMISO uptake and showed a good correlation with the distribution of tumour hypoxia. In a FaDu tumour model, early and late ⁶⁴Cu-ATSM images were in concordance with [¹⁸F]FMISO

imaging, indicating a tumour-specific dependence of ⁶⁴Cu-ATSM uptake and retention under hypoxic conditions [94]. In another study, ⁶⁴Cu-ATSM tumour uptake was unable to predictably detect changes in varying amounts of tumour hypoxia when oxygenation levels in SCCVII tumours were modulated, whereas [¹⁸F]FMISO tumour uptake was more responsive to changing levels of hypoxia. Tumour hypoxia was also assessed independently using pimonidazole [95].

Two studies comparing ⁶⁴Cu-ATSM uptake with [¹⁸F]FDG uptake in different animal models concluded that both tracers have a different distribution pattern [81, 82]. ⁶⁴Cu-ATSM accumulated in hypoxic but viable tumour cells, whereas [¹⁸F]FDG uptake was highest in pre-necrotic regions where the cells were believed to lack the necessary reductive mechanisms to accumulate ⁶⁴Cu-ATSM [81]. It was also shown that regions with high ⁶⁴Cu-ATSM uptake were hypovascular and consisted of tumour cells arrested in the cell cycle, whereas regions with high [¹⁸F]FDG uptake were hypervascular and consisted of proliferating cells, as confirmed by histological analysis with Ki67, CD34 and TUNEL assay [82]. Finally Dence et al. compared the regional distribution of ⁶⁴Cu-ATSM, [¹⁸F]FDG and [¹⁸F]FMISO in 9L gliosarcoma tumours. It was shown that the regional distribution of [¹⁸F]FMISO at 2 h correlates highly with the distribution of ⁶⁴Cu-ATSM at 10 min or 24 h. A poor correlation existed however between ⁶⁴Cu-ATSM (10 min) and [¹⁸F]FDG [22].

Clinical data: In numerous studies, ⁶⁰Cu-ATSM uptake proved to be predictive of tumour behaviour and response to therapy in patients with non-small cell lung cancer [83], cervical cancer [96, 97] and rectal carcinoma [84]. In a study by Dehdashti et al. in 14 patients with biopsy-proven cervical cancer, an arbitrarily selected tumour to muscle threshold of 3.5 was able to discriminate those patients that were likely to develop recurrence so that ⁶⁰Cu-ATSM uptake was inversely related to progression-free survival and overall survival. Additionally, no correlation was found between ⁶⁰Cu-ATSM uptake and [¹⁸F]FDG uptake [96]. To confirm these results, a study with a larger group of patients was performed by the same group. Tumour ⁶⁰Cu-ATSM uptake (T/M threshold of 3.5) in 38 patients with cervical cancer was inversely related to progression-free survival and cause-specific survival. Again, no correlation was found between ⁶⁰Cu-ATSM uptake and [¹⁸F]FDG uptake [97]. Similar results were obtained in a study where semiquantitative analysis of the ⁶⁰Cu-ATSM tumour to muscle ratio in 14 patients with non-small cell lung cancer was able to discriminate responders from non-responders. However, there was no significant difference in mean tumour SUV of non-responders and responders. Again, no correlation was found between ⁶⁰Cu-ATSM uptake and [¹⁸F]FDG uptake [83]. In a recent study, an effort was made

to predict the response of rectal cancers to neoadjuvant chemoradiotherapy and prognosis in 17 patients. The results of this small pilot study suggested that ^{60}Cu -ATSM tumour to muscle ratio may be predictive of survival and, possibly, tumour response. Again, no correlation was found between ^{60}Cu -ATSM uptake and ^{18}F FDG uptake [84]. To determine if hypoxia-related molecular markers were associated with ^{60}Cu -ATSM retention, the PET imaging data of 15 patients with cancer of the cervix were compared with the expression of tissue molecular markers, which included VEGF, cyclo-oxygenase-2 (COX-2), epidermal growth factor receptor (EGFR), carbonic anhydrase IX (CA-9) and apoptotic index. Hypoxia as identified with ^{60}Cu -ATSM imaging was correlated with overexpression of VEGF, EGFR, COX-2, CA-9, an increase in apoptosis and a poor outcome [98]. Chao et al. further demonstrated the feasibility of using ^{60}Cu -ATSM imaging to identify the hypoxic tumour subvolume through coregistration of CT and ^{60}Cu -ATSM PET images in order to plan a patient's course of radiotherapy and perform intensity-modulated radiation therapy (IMRT) [99]. As most clinical Cu-ATSM studies used the agent with the short-lived positron-emitting radionuclide of copper, ^{60}Cu , a recent study compared the image quality and tumour uptake of ^{60}Cu -ATSM and ^{64}Cu -ATSM in ten patients with cervical carcinoma to evaluate the use of Cu-ATSM with one of the longer-lived positron-emitting copper nuclides, ^{64}Cu . It was concluded that ^{64}Cu -ATSM was a safe radiopharmaceutical that can be used to obtain high quality images of tumour hypoxia in human cancers [100].

Non-invasive measurement of tumour hypoxia with SPECT

1. ^{123}I IAZA and ^{125}I IAZA (^{123}I / ^{125}I iodoazomycin arabinoside)

Pre-clinical data: In one of the first studies with IAZA, its synthesis and labelling with ^{125}I was described. Its elimination and biodistribution were also studied in vivo in EMT-6 tumours in BALB/c mice, and it was shown that IAZA undergoes hypoxia-dependent binding in EMT-6 cells in vitro [101]. Moore et al. investigated the oxygenation status and tumour perfusion of rats with Dunning R3327-AT tumours who were treated with photodynamic therapy (PDT) with ^{123}I IAZA and $^{99\text{m}}\text{Tc}$ HMPAO. Increased retention of ^{123}I IAZA was observed in tumours treated with PDT together with an inverse correlation between tumour hypoxia as measured with ^{123}I IAZA and tumour perfusion as measured with $^{99\text{m}}\text{Tc}$ HMPAO [102].

Clinical data: The first clinical study assessing hypoxia with IAZA investigated the uptake of ^{123}I IAZA in patients

with advanced malignancies. Radiotracer avidity was observed in three of ten tumours, and it was concluded that the use of gamma emitter-labelled 2-nitroimidazoles as diagnostic radiopharmaceuticals is feasible and safe and that metabolic binding of ^{123}I IAZA is observed in some, but not all tumours [103]. In 22 patients, ^{123}I IAZA uptake showed a significant inverse correlation with the perfusion marker $^{99\text{m}}\text{Tc}$ HMPAO, and severe perfusion deficits were usually associated with an increased uptake of the hypoxic marker [104]. After observing uptake of radioactivity in the brain after administration of ^{123}I IAZA, a study was undertaken to investigate the proposed metabolites of IAZA in normal and tumour-bearing murine models. Neither of the proposed metabolites' biodistribution did support its involvement in brain radioactivity uptake in patients [105]. A study investigating the use of ^{123}I IAZA in 51 human patients with newly diagnosed malignancies demonstrated hypoxia in small cell lung cancer and squamous cell carcinoma of head and neck but not in malignant gliomas. The study did, however, demonstrate the feasibility of ^{123}I IAZA imaging in a clinical setting [106]. Stypinski et al. reported the clinical pharmacokinetics of IAZA, the radio-pharmacokinetics of ^{123}I IAZA, total radioactivity kinetics and the radiation dosimetry estimates for six healthy volunteers and concluded that all supported its clinical use for imaging tissue hypoxia [107, 108].

Newer agents based on the azomycin-nucleoside structure such as IAZG [109, 110], iodoazomycin pyranoside (IAZP) [111], IAZGP [112, 113] and iodoazomycin xylopyranoside (IAZXP) [109] have been developed and evaluated. Iyer et al. demonstrated that microelectrode measurements in R3327-AT tumour-bearing rats did not correlate with ^{125}I IAZGP uptake [112]. Furthermore, a study by Saitoh et al. showed high accumulation of IAZGP in FM3A mouse tumours 24 h after administration [113].

2. $^{99\text{m}}\text{Tc}$ -labelled agents
 - (a) BMS 181321

This was the first $^{99\text{m}}\text{Tc}$ -labelled 2-nitroimidazole to be widely studied for imaging [114]. A number of experimental studies have evaluated the use of BMS 181321 for the detection of ischaemic and hypoxic myocardium [115–118]. Ballinger et al. showed selective accumulation in hypoxic cells in vitro and in vivo but concluded that BMS 181321 was not optimal for tumour hypoxia imaging because of in vitro and in vivo instabilities and a high partition coefficient, resulting in slow clearance from the blood and high background levels in normal tissues [119].

- (b) BRU59-21

Pre-clinical data: BRU59-21, previously known as BMS 194796, is a second-generation analogue of BMS 181321 which shows greater stability in vitro and more rapid

clearance from the circulation *in vivo*, resulting in higher tumour to blood and tumour to muscle ratios. It showed selective localization in tumour cells incubated under hypoxic conditions and following intravenous injection in animal models representative of poorly perfused tumours [120]. In a study by Zhang et al., BRU59-21 and HL91 were compared directly in the same *in vitro* systems. Both tracers proved suitable for hypoxia imaging [121].

Clinical data: Hoebbers et al. assessed the safety and biodistribution of [^{99m}Tc]BRU59-21 in ten patients with head and neck cancer and correlated uptake *in vivo* with pimonidazole staining. *In vivo* evaluation of tumour hypoxia with [^{99m}Tc]BRU59-21 appeared to be safe and feasible, and uptake and retention of the marker seemed to be indicative of tumour hypoxia, as confirmed by pimonidazole staining [122].

(c) [^{99m}Tc]HL-91

Pre-clinical data: Zhang et al. evaluated the efficacy of [^{99m}Tc]HL91 as a non-invasive marker of tumour hypoxia *in vitro* (Chinese hamster ovary cells) and *in vivo* (C3H mice bearing KHT-C tumours) and observed selective accumulation of [^{99m}Tc]HL91 in hypoxic cells and hypoxic tumours [123]. A similar study assessed the retention of [^{99m}Tc]HL91 in mice bearing three different tumours under control and enhanced oxygenation conditions and correlated these data with the oxygenation status as assessed by Eppendorf pO_2 histogram measurements. A very good correlation between [^{99m}Tc]HL91 retention and hypoxia, as measured by the Eppendorf histogram, was observed [124]. Yutani et al. found that [^{99m}Tc]HL91 accumulated to significantly higher levels in hypoxic tumour areas and that [^{99m}Tc]HL91 uptake was strongly correlated with the expression of GLUT1 in the viable cancer cell area [125]. In a study by Tatsumi et al., a dual-tracer autoradiography was performed with HL91 and IAP (^{14}C -iodoantipyrine) in Walker 256 tumour-bearing rats to elucidate the relationship between hypoxia and blood flow. The study confirmed that high HL91 uptake is related to low blood flow [126]. Kinuya et al. reported on an increase in [^{99m}Tc]HL91 uptake after exposure to X-ray radiation [127]. Siim et al. examined whether [^{99m}Tc]HL91 uptake could be used as a marker for the inhibition of tumour blood flow by the antivasular agents DMXAA (5,6-dimethylxantenone-4-acetic acid) and CA4P (combretastatin A4 phosphate) and observed that tumour hypoxia as a result of the acute inhibition of blood flow by antivasular agents caused increased tumour uptake of [^{99m}Tc]HL91 [128]. Another study demonstrated that microelectrode measurements in R3327-AT tumour-bearing rats did not correlate with [^{99m}Tc]HL91 uptake [112]. After having determined the biodistribution of [^{99m}Tc]HL91 [129], Suzuki et al. investigated the relationship between [^{99m}Tc]HL91 uptake and tumour response to radiation in athymic

mice bearing different human tumours. They concluded that [^{99m}Tc]HL91 uptake did not always relate to their sensitivities to radiation therapy [130]. In a study by Kinuya et al., an attempt was made to determine whether oxygenation status affected [^{99m}Tc]MIBI (sestamibi) uptake. They observed enhanced [^{99m}Tc]HL91 accumulation in hypoxic tumour cells after treatment with N_2 gas (*in vitro*) and hydralazine (*in vivo*) [131]. A recent study by Lee et al. investigated the selectivity of [^{99m}Tc]HL91 for hypoxia *in vitro* in A549 human lung cancer cells and LL2 murine Lewis lung cancer cells under varying oxygen concentrations and *in vivo* in different xenograft mouse models after chemically altering the degree of tumour hypoxia with hydralazine. The *in vitro* studies identified hypoxia-selective uptake of [^{99m}Tc]HL91, with significantly increased uptake in the hypoxic state compared to the normoxic state. The *in vivo* studies showed that [^{99m}Tc]HL91 was markedly increased in mice treated with hydralazine compared with controls [132].

Clinical data: Clinical studies concerning the clinical evaluation of [^{99m}Tc]HL91 are limited. In a pilot study, Cook et al. compared [^{99m}Tc]HL91 uptake with [^{18}F]FDG PET imaging in ten patients with a variety of tumours and showed visible [^{99m}Tc]HL91 tumour uptake in all seven patients where the tumour could be clearly identified with [^{18}F]FDG PET [133]. Another phase I pilot study evaluated the usefulness of [^{99m}Tc]HL91 imaging for the visualization of local recurrence in nine men with squamous cell carcinoma of the head and neck (SCCHN) as compared to CT and biopsy and concluded that [^{99m}Tc]HL91 is a safe radioligand and that metabolic binding in a large fraction but not all of local SCCHN recurrences may be expected [134]. Finally, in a study with 32 patients with non-small cell lung cancer, Li et al. showed that hypoxia imaging with [^{99m}Tc]HL91 before radiotherapy may predict tumour response and patient survival [135].

Discussion

There are several prerequisites to which the ideal non-invasive hypoxic marker should comply: (1) It should be specific for hypoxia and thus distinguish normoxia, hypoxia and anoxia or necrosis. (2) It should image acute and chronic hypoxia and possibly distinguish between both. (3) It should be simple, non-toxic, fast, easy to perform and allow repeated measurements. (4) It should be lipophilic to have a homogeneous biodistribution in all tissues including tumours but at the same time hydrophilic to have a faster elimination, thus allowing larger tumour to normal tissue ratio of radioactivity. (5) It should have little hypoxia-independent degradation *in vivo* leading to non-specific tracer metabolites and/or little aspecific tissue binding, so

that only oxygen-specific retention mechanisms determine the amount of tracer that is temporarily or permanently trapped. (6) It should reflect intracellular pO_2 rather than blood flow or some consequence of subsequent biochemistry. (7) It should be sensitive at pO_2 levels relevant to tumour therapy. (8) It should offer the ability to quantify. Unfortunately, none of the present hypoxia tracers available completely fulfils these requirements.

Although SPECT is more commonly used than PET, and, in particular, ^{99m}Tc has a number of practical advantages that include ready availability at low cost, convenient half-life for hypoxia measurements and versatile chemistry as compared with ^{18}F , the superior spatial resolution and more accurate quantitation with PET makes the latter a better candidate for detection of tumour hypoxia. Of all the PET tracers that are being evaluated as possible markers of tumour hypoxia, only three have been thoroughly evaluated in a clinical situation: $[^{18}F]FMISO$, $[^{18}F]FDG$ and Cu-ATSM.

$[^{18}F]FMISO$ is the most widely used and investigated hypoxia marker and has been validated in multiple studies both in humans and animals. Studies using $[^{18}F]FMISO$ have demonstrated variable, but significant levels of hypoxia in several tumour types. In addition, $[^{18}F]FMISO$ PET imaging has been used as a prognostic indicator in several other studies. It has, however, failed to gain wider acceptance for routine clinical application because of a number of limitations such as: (1) slow accumulation in hypoxic tumours; (2) a low target to background ratio due to high non-specific binding resulting from its relatively high lipophilicity; and (3) significant non-oxygen dependent metabolism leading to a considerable amount of radioactive metabolite products.

Several studies have tried to validate $[^{18}F]FDG$ as an alternative marker for hypoxia imaging. The rationale behind this is that $[^{18}F]FDG$ uptake during FDG PET imaging relies largely on the expression of proteins that are under control of HIF-1. As a result, the degree of $[^{18}F]FDG$ uptake by tumours might indirectly reflect the level of hypoxia. This would obviate the need for more specific radiopharmaceuticals for hypoxia imaging. Reports trying to relate $[^{18}F]FDG$ uptake with tumour hypoxia have given inconsistent results. In vitro studies have suggested that FDG is preferably accumulated in hypoxic cancer cells compared to normoxic cancer cells because of changed metabolism. However, in vivo experiments (pre-clinical and clinical) have given conflicting results when showing a correlation between the uptake of $[^{18}F]FDG$ and the existence of hypoxia in tumours. It appears that in those tumours where HIF-1 activation is mainly hypoxia driven, the degree of $[^{18}F]FDG$ uptake may be a surrogate marker of hypoxia. Further evaluation of $[^{18}F]FDG$ uptake by various tumour types in relation with invasive and non-

invasive markers of tumour hypoxia is needed to fully elucidate the role of $[^{18}F]FDG$ as a marker of tumour hypoxia. Furthermore, most studies to date assessing the value of FDG as a measure of hypoxia calculated SUV. Possibly, dynamic PET studies and compartment analysis may offer an advantage over standard uptake measurements.

The molecule that holds the greatest promise for the future is Cu-ATSM. Although its mechanism of hypoxic retention is not yet fully elucidated, numerous in vitro and in vivo studies have shown its selectivity for hypoxic tissue. It has a small molecular weight and a high cell membrane permeability allowing it to diffuse easily from the bloodstream to surrounding cells. This combined with a rapid blood clearance and its rapid reduction and retention in hypoxic tissues ensures that Cu-ATSM shows a rapid delineation of tumour hypoxia and high tumour to background ratios. Clinical results clearly suggest further clinical evaluation is warranted and positive results are expected to follow.

Conclusion

As the importance of tumour hypoxia is being recognized, so is the importance of its detection. Because of the limitations of the current gold standard, a non-invasive technique to predict outcome and identify patients with a worse prognosis and/or patients that would benefit from appropriate treatments is needed. Several candidates are rapidly being developed and investigated. Of the several candidate techniques and molecules, each has its advantages and disadvantages, and it may be possible that a particular technique or molecule is best suited for a certain tumour type, grade or stage. Evaluation of the several candidate molecules is taking place in patients at this moment and it is hoped that this eventually will lead to further characterization and optimization.

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