Preclinical evaluation and validation of [^{18}F]HX4, a promising hypoxia marker for PET imaging

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Hypoxia has been shown to be an important microenvironmental parameter influencing tumor progression and treatment efficacy. Patient guidance for hypoxia-targeted therapy requires evaluation of tumor oxygenation, preferably in a noninvasive manner. The aim of this study was to evaluate and validate the uptake of [^{18}F]HX4, a novel developed hypoxia marker for PET imaging. A heterogeneous accumulation of [^{18}F]HX4 was found within rat rhabdomyosarcoma tumors that was significantly ($P < 0.0001$) higher compared with the surrounding tissues, with temporal increasing tumor-to-blood ratios reaching a plateau of 7.638 ± 0.926 and optimal imaging properties 4 h after injection. [^{18}F]HX4 retention in normal tissues was found to be short-lived, homogeneous and characterized by a fast progressive temporal clearance. Heterogeneity in [^{18}F]HX4 tumor uptake was analyzed based on 16 regions within the tumor according to the different orthogonal planes at the largest diameter. Validation of heterogeneous [^{18}F]HX4 tumor uptake was shown by a strong and significant relationship ($r = 0.722$; $P < 0.0001$) with the hypoxic fraction as calculated by the percentage pimonidazole-positive pixels. Furthermore, a causal relationship with tumor oxygenation was established, because combination treatment of nicotinamide and carbogen resulted in a 40% reduction ($P < 0.001$) in [^{18}F]HX4 tumor accumulation whereas treatment with 7% oxygen breathing resulted in a 30% increased uptake ($P < 0.05$). [^{18}F]HX4 is therefore a promising candidate for noninvasive detection and evaluation of tumor hypoxia at a macroscopic level.

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The presence of hypoxic regions due to abnormalities in tumor vasculature, heterogeneously spread within solid tumors influences clinical outcome; as it is an independent predictor of poor prognosis-free survival in several types of cancer (1). In contrast, this unique tumor characteristic makes it an attractive target for novel drugs to increase the therapeutic effect of conventional cancer treatment modalities. Another approach is the use of intensity-modulated radiotherapy to give a higher dose to hypoxic areas while sparing the surrounding normal tissue (2, 3). Although treatments to counteract the negative effect of intratumoral hypoxia are under investigation, not all patients will benefit from such selective treatments. Therefore, to guide hypoxia-directed therapies in individual patients, it is important to evaluate tumor oxygenation using a reliable noninvasive method.

To date, a variety of methods are available for assessment of tumor oxygenation in solid tumors, of which polarographic oxygen electrodes and immunohistological assays remain the gold standard (4). These standard invasive modalities have not yielded reliable 3D images of the whole tumor for clinical use, and therefore research has been focused on noninvasive imaging techniques, such as positron-emission tomography (PET) using nitroimidazoles. The 2-nitroimidazole derivative fluoromisonidazole (FMISO) has been evaluated extensively for different cancer types (5–7). [^{18}F]FMISO PET imaging has evolved as the gold standard;

however, clinical utility is limited, as there are concerns about the stability of the fluorine-18 linkage and the formation of metabolites in blood and urine. Furthermore, only modest signal-to-noise ratios are obtained due to its slow clearance, high lipophilicity, and liver and gut uptake (8). Second-generation, more water-soluble 2-nitroimidazoles, such as [^{18}F]fluoroazomycin arabinoside ([^{18}F]FAZA) and [^{18}F]fluoroerythronitroimidazole ([^{18}F]FETNIM), have been validated as hypoxia markers and have shown lower degradation resulting in higher tumor-to-background contrast (9, 10). On the other hand, more lipophilic fluorinated compounds, such as EF3, could be good alternatives for the detection of tumor hypoxia. However, in a comparative PET imaging study, we demonstrated no superiority of nitroimidazol-1H-yl-N-[^{18}F]-trifluoropropyl acetamide ([^{18}F]EF3) to [^{18}F]FMISO for the evaluation of hypoxia (11). Furthermore, it has been shown that the nonnitroimidazole hypoxia marker Copper(II)diacetyl-di(N^4 -methylthiosemicarbazone) (Cu-ATSM) is selective for hypoxia in several tumor types. Clinical data obtained from non-small-cell lung cancer (NSCLC) patients demonstrated the feasibility of ^{60}Cu -ATSM imaging (12). However, uptake of ^{64}Cu -ATSM in fibrosarcoma-bearing mice correlated with vascular perfusion as opposed to hypoxia, and the accumulation did not decrease upon oxygenation using carbogen breathing (13). Tumor hypoxia response can also be evaluated by imaging of molecular markers of hypoxia, such as carbonic anhydrase IX (CAIX) using specific sulfonamides (14).

Alternatively, the 2-nitroimidazole nucleoside analog [^{18}F]HX4 was developed as a potential marker for hypoxic tumor cells. It represents a novel, click chemistry-based generation of derivatives in which structure–activity relationships, focused on the incorporation of a 1,2,3-triazole moiety, have been used to design an agent with preferred pharmacokinetic and clearance properties (15). Biodistribution and dosimetry studies in healthy monkeys and humans showed that the amount of unmetabolized [^{18}F]HX4 in blood and urine samples remained stable, liver and gastrointestinal tract uptake was relatively low, and the highest uptake of [^{18}F]HX4 was found in the urinary bladder, indicating an excretion primarily through the kidneys (15, 16). Because of the better water solubility and faster clearance, we hypothesized that

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[^{18}F]HX4 would generate a suitable tumor-to-background signal ratio earlier compared with other established hypoxia markers. The aim of this study was to evaluate the *in vivo* uptake of [^{18}F]HX4 over time in an experimental rat tumor model by noninvasive PET imaging and to define the optimal imaging time point. Secondary aim was to validate the heterogeneity of the [^{18}F]HX4 accumulation by comparison with the exogenous hypoxia marker pimonidazole, and third aim was to establish a causal relationship between [^{18}F]HX4 uptake and tumor oxygenation.

Results

Synthesis and Metabolite Analysis. To increase the hydrophilicity of HX4, a metabolically stable 1,2,3-triazole compound was incorporated using the Cu-catalyzed Huisgen click-chemistry reaction. Radiolabeling with fluorine-18 afforded [^{18}F]HX4 with a yield of 3–7 GBq [^{18}F]HX4 at the end of synthesis (14–35% decay-corrected yield) within 85 min total synthesis time. Radiochemical purity was higher than 95% and specific activity was typically in the area of 24 GBq/ μmol . Calculation of the partition coefficients confirmed the hydrophilic character of HX4 [$\log P$ (octanol/water) = -0.69 ± 0.02 and $\log P$ (octanol/PBS) = -0.68 ± 0.01]. Radio-HPLC analysis showed that >90%, >85%, 60%, and 10% of plasma, urine, kidney, and liver radioactivity, respectively, represented intact [^{18}F]HX4 60 min after injection.

In Vivo Accumulation of [^{18}F]HX4 in an Experimental Rat Tumor Model. To evaluate the potential of [^{18}F]HX4 as a noninvasive marker of hypoxia, a clinically relevant (heterogeneous hypoxic pimonidazole-positive areas and low-percentage necrosis even at larger volumes) experimental rat rhabdomyosarcoma R1 tumor model (5, 11) was used at an average tumor volume of $12.894 \pm 5.104 \text{ cm}^3$ (Fig. S1A). A clear heterogeneous accumulation of [^{18}F]HX4 was observed in the tumor (Fig. 1B), whereas equal [^{18}F]HX4 activity levels were found in surrounding normal tissues. Furthermore, a high accumulation of [^{18}F]HX4 was found in the bladder, confirming its renal clearance. Quantification of [^{18}F]HX4 accumulation was done by volume of interest (VOI) analysis of the heart, hind leg muscles, and tumor. The heart was selected as reference tissue to noninvasively analyze whole-blood [^{18}F] activity, as previously shown (11). Mean [^{18}F]HX4 activity levels in tumor and muscle tissue were comparable ($P = 0.795$) during the first hour after injection (Fig. 1C Left), whereas the blood pool mean activity level was found to be significantly higher ($P < 0.0001$). Maximum activity levels showed a similar pattern (Fig. 1C Right). No significant differences were observed between mean and maximum activities in the blood pool ($P = 0.566$) and muscle ($P = 0.227$). However, maximum activity levels in tumors were significantly higher ($P = 0.0004$) compared with the corresponding mean accumulation. From 2 h after injection onward, a significantly ($P < 0.0001$) faster clearance of [^{18}F]HX4 from the blood and muscle was observed compared with the tumor (Table S1). This resulted in a significant ($P < 0.001$) increase in mean tumor-to-blood (T/B) ratios until 4 h after injection, where a mean T/B of 2.512 ± 0.578 was reached (Fig. 1D Left). No further increase was observed at later time points, making 4 h after injection an optimal time point for [^{18}F]HX4 evaluation in tumors. Because tumors are known to be heterogeneous, we also analyzed the maximum T/B signal ratios and found a similar pattern of increase, with a maximum of 7.638 ± 0.926 at 4 h post injection (Fig. 1D Right). A progressive clearance from normal tissues was observed because muscle-to-blood (M/B) ratios gradually decreased with time, resulting in significantly ($P < 0.05$) lower M/B ratios from 4 h post injection on. Both mean and maximum [^{18}F]HX4 uptake (percentage injected radioactivity per mL; %ID/mL) was independent of the tumor volume ($r^2 < 0.050$; $P > 0.4$) for all investigated time points.

Pimonidazole Staining, Hypoxic Fraction, and the Relationship with [^{18}F]HX4 Accumulation. To investigate the relationship between [^{18}F]HX4 accumulation and pimonidazole hypoxic fraction, taking into account the heterogeneity in uptake of both chem-

Fig. 1. Heterogeneous [^{18}F]HX4 accumulation occurs at optimal imaging properties 4 h after injection. (A) Structure of [^{18}F]HX4. (B) Representative CT and PET/CT image of a rhabdomyosarcoma R1 tumor-bearing rat 4 h after injection of [^{18}F]HX4. (C) Quantification of mean and maximum [^{18}F]HX4 uptake up to 6 h after injection. Uptake data ($n = 21$) for tumor (squares), heart (triangles), and muscles from the hind legs (diamonds) are shown and data are expressed as mean $\pm$ SD %ID/mL. (D) Mean and maximum tissue-to-blood ratios of [^{18}F]HX4 uptake up to 6 h after injection. Ratios represent tumor (squares) and muscle (triangles) uptake divided by blood pool uptake. Data are expressed as mean $\pm$ SD. Asterisks indicate significant increase in tumor-to-blood ratios between individual time points (* $P < 0.01$, ** $P < 0.001$). NS, not significant.

icals, tumors were divided into 16 pieces based on the computed tomography (CT) image according to their orthogonal planes (Fig. 2A). HX4 percentage injected dose, based on the PET image, was heterogeneously distributed across the tumor (Fig. 2A and B) and ranged from 1 to 19%.

Pimonidazole-positive staining areas (range: $0.713\text{--}19.346 \text{ mm}^2$) were observed in all tumor pieces, with a heterogeneous distribution across the pieces (Fig. 2C). Staining was localized in most cases at a distance (several cell layers) from a perfused (Hoechst-positive) blood vessel, most often near areas of necrosis, indicative of diffusion-limited hypoxia (Fig. 2D Lower). However, there is also evidence for the presence of perfusion-limited hypoxia (Fig. 2D Upper), indicated by pimonidazole-positive staining in close proximity to perfusion marker-negative vessels. The hypoxic fraction ranged from 0.006 to 0.146 and was independent ($r = 0.152$; $P = 0.662$) of the tumor volume.

On whole-tumor level, a relationship between [^{18}F]HX4 accumulation and pimonidazole hypoxic fraction was observed ($r = 0.600$) but not significant ($P = 0.285$). On the other hand, on tumor-regional level, a strong and significant ($r = 0.722$; $P < 0.0001$) relationship (Fig. 2E) was observed between the hypoxic fractions as assessed using [^{18}F]HX4 noninvasive imaging and pimonidazole staining.

Fig. 2. [^{18}F]HX4 accumulation correlates with the hypoxic fraction of the tumors. (A) Division of the tumor into 16 pieces based on the CT image according to their coronal (XY), sagittal (XZ), and transaxial (YZ) planes at the largest diameter and quartiles. A representative heat map of the distribution of the percentage HX4 positivity is shown. (B) Representative axial PET/CT views of the mid and quartile axes of the heat map. (C) Pseudocolored gray-value images of the areas highlighted in the axial PET/CT views showing pimonidazole staining (green), vessels (red), and perfusion (blue). (D) Higher-magnification images of the areas highlighted in the original stainings. Areas of perfusion-limited (white) or diffusion-limited (gray) hypoxia are indicated with arrowheads. (E) Relationship at the regional level ($n = 76$) between [^{18}F]HX4 accumulation and pimonidazole hypoxic fraction.

[¹⁸F]HX4 Accumulation Is Dependent on Available Oxygen. To test this hypothesis, we used two short-term treatments to modify tumor oxygenation. To increase or decrease tumor hypoxia, animals were exposed to an environment containing, respectively, 7% oxygen or a combination of nicotinamide and carbogen. No adverse effects were observed for either treatment regimen. First, a baseline [¹⁸F]HX4 acquisition was performed on tumor-bearing (13.029 ± 5.149 cm³) animals breathing normal air. Two days later (volume 15.882 ± 6.284 cm³), animals were randomized into the two treatment groups and [¹⁸F]HX4 accumulation was compared with the baseline uptake. Maximum [¹⁸F]HX4 uptake at the optimal imaging time point in the same animal was found to be significantly lower (~40%; *P* < 0.001) after treatment with a combination of nicotinamide and carbogen (Fig. 3*A* and *C*). This reduced accumulation persisted over time until 4 h after injection (Fig. S1*B Left*) and was lost at later time points. Also, the mean [¹⁸F]HX4 uptake was significantly lower (*P* < 0.01) after treatment, but to a smaller extent (25%).

Fig. 3. [^{18}F]HX4 accumulation depends on tumor oxygenation. (A and B) Representative set of CT and PET/CT images within the same animal 4 h after injection of [^{18}F]HX4 before and after treatment (interval of 2 d) with (A) a combination of nicotinamide and carbogen or (B) 7% oxygen breathing. Images are scaled to the maximum-intensity values of all images. (C) Quantification of maximum [^{18}F]HX4 uptake in animals treated (white bars) with nicotinamide and carbogen ($n = 7$) or 7% oxygen breathing ($n = 6$) compared with no treatment (black bars) at the optimal imaging time point 4 h after injection. Data are expressed as mean $\pm$ SD. Asterisks indicate significant difference ($^*P < 0.05$, $^{**}P < 0.001$) compared with no treatment.

Furthermore, no significant differences in [^{18}F]HX4 accumulation were observed after treating the animals with a combination of nicotinamide and carbogen for blood and muscle tissue (Table S2), indicating that normal tissues can indeed cope with changes in oxygenation. On the other hand, when animals were treated with 7% oxygen, a significant increase ($\sim 30\%$; $P < 0.05$) in maximum [^{18}F]HX4 uptake in the tumor was observed (Fig. 3 B and C) persisting over time (Fig. S1B Right). The effect of 7% oxygen breathing on normal tissues was also negligible (Table S2). Evaluation of the mean [^{18}F]HX4 activity in the tumor after 7% oxygen treatment resulted in increased accumulation, but statistical significance was lost. Furthermore, also the mean and maximum [^{18}F]HX4 uptake after oxygen modification were independent ($r = 0.356$; $P > 0.3$ and $r = 0.368$; $P > 0.3$ for 7%

positive voxels (above background) was assessed and divided by the number of voxels in the tumor VOI to calculate the percentage [^{18}F]HX4 positivity.

Immunohistochemical Staining and Analysis. The exogenous hypoxia marker pimonidazole hydrochloride (PIMO; 60 mg/kg, i.p.) and the perfusion marker Hoechst 33342 (15 mg/kg, i.v.) were injected 1 h and immediately before euthanizing the animals, respectively. From each region, 5- μ m sections were cut and mounted on poly-L-lysine-coated slides. After fixation in cold acetone, slides were scanned for fluorescent signal of Hoechst before staining for PIMO and vessels. Staining was done as previously described (22), using rabbit anti-pimonidazole (1:1,000; gift of J. A. Raleigh., University of North Carolina, NC) and goat anti-collagen IV (1:100; Southern Biotechnology Association). Grayscale images for each staining were obtained and converted into binary images. After thresholding above background staining for each individual marker, the hypoxic fraction represented as the ratio of the sum

of all PIMO-positive pixels and the sum of all tumor pixels, including areas of necrosis, was calculated.

Statistics. All statistical analyses were performed with GraphPad Prism version 5.01 for Windows. An unpaired Student's *t* test and nonparametric Mann-Whitney *U* test for small groups were used to determine the statistical significance of differences between two independent groups of variables. Linear regression analysis was used to assess correlations (Spearman's *r*) between the different parameters. For all tests, $P < 0.05$ was considered significant.

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