Evaluation of Deuterated $^{18}$F- and $^{11}$C-Labeled Choline Analogs for Cancer Detection by Positron Emission Tomography

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Abstract

**Purpose:** $^{11}$C-Choline–positron emission tomography (PET) has been exploited to detect the aberrant choline metabolism in tumors. Radiolabeled choline uptake within the imaging time is primarily a function of transport, phosphorylation, and oxidation. Rapid choline oxidation, however, complicates interpretation of PET data. In this study, we investigated the biologic basis of the oxidation of deuterated choline analogs and assessed their specificity in human tumor xenografts.

**Experimental Design:** $^{11}$C-Choline, $^{11}$C-methyl-[1,2-2H$_4$]-choline ($^{11}$C-D$_4$-choline), and $^{18}$F-D$_4$-choline were synthesized to permit comparison. Biodistribution, metabolism, small-animal PET studies, and kinetic analysis of tracer uptake were carried out in human colon HCT116 xenograft-bearing mice.

**Results:** Oxidation of choline analogs to betaine was highest with $^{11}$C-choline, with reduced oxidation observed with $^{11}$C-D$_4$-choline and substantially reduced with $^{18}$F-D$_4$-choline, suggesting that both fluorination and deuteration were important for tracer metabolism. Although all tracers were converted intracellularly to labeled phosphocholine (specific signal), the higher rate constants for intracellular retention ($K_i$ and $k_3$) of $^{11}$C-choline and $^{11}$C-D$_4$-choline, compared with $^{18}$F-D$_4$-choline, were explained by the rapid conversion of the nonfluorinated tracers to betaine within HCT116 tumors. Imaging studies showed that the uptake of $^{18}$F-D$_4$-choline in three tumors with similar radiotracer delivery ($K_1$) and choline kinase $\alpha$ expression—HCT116, A375, and PC3-M—were the same, suggesting that $^{18}$F-D$_4$-choline has utility for cancer detection irrespective of histologic type.

**Conclusion:** We have shown here that both deuteration and fluorination combine to provide protection against choline oxidation in vivo. $^{18}$F-D$_4$-choline showed the highest selectivity for phosphorylation and warrants clinical evaluation.

**Introduction**

Choline is required for the biosynthesis of phosphatidylcholine, a key component of the plasma membrane. Following transport into the cell, choline is phosphorylated by choline kinase to phosphocholine and then is further metabolized to phosphatidylcholine via CDP-choline, known as the Kennedy pathway. Once phosphorylated, phosphocholine is trapped within the cell. Diacylglycerol, a product of phosphatidylcholine degradation, is mitogenic, playing a role in the regulation of cell-cycle progression from G1 to S via increased cyclin D1 and cyclin D3 expression (1). Furthermore, aberrant activation and expression of several oncogenes results in elevated choline kinase activity and intracellular levels of phosphocholine (2–4). Choline kinase overexpression is a common feature of several human cancers (5) and in early stage non–small cell lung cancer, choline kinase has been shown to have prognostic significance (6). The expression of choline transporters, including CTL1 and OCT3, is also increased following malignant transformation and may contribute to radiotracer uptake (7, 8), with choline transport closely associated with cell growth (9). $^{11}$C-choline has become a viable alternative to $^{18}$F-fluoro-2-deoxyglucose for positron emission tomography (PET) imaging of the prostate (10–12), in which the increased choline kinase activity in tumors provides the basis for tumor-specific contrast in comparison with surrounding nonneoplastic tissues. A fluorinated analog, $^{18}$F-fluoromethylcholine, has also been developed for PET imaging of choline metabolism (13), with the longer half-life of fluorine-18 (109.8 vs. 20.4 minutes for...
Tranlsational Relevance

11C-Choline–positron emission tomography (PET) is a marker of choline kinase expression and activity, which is upregulated during carcinogenesis. To date, 11C-choline–PET has been used for the detection of a range of human cancers and has emerged as a viable alternative to 18F-2-fluoro-2-deoxyglucose for the imaging of prostate adenocarcinoma. 11C-choline, however, is rapidly oxidized to betaine in an unwanted side reaction, complicating data interpretation. Here, we designed novel choline analogs and tested their metabolic profiles and sensitivity for cancer detection. The doubly fluorinated and deuterated analog 18F-D4-choline showed lowest betaine oxidation. This radiotracer could be used for cancer detection, irrespective of histologic type. Therefore, the development of new choline radiotracers with an improved metabolic profile should provide a means to simplify interpretation of clinical PET data, while increasing selectivity for phosphorylation.

Materials and Methods

Cell lines

HCT116 colorectal carcinoma (LGC Standards) and PC3-M prostate adenocarcinoma cells (kind donation from Dr Matthew Caley, Prostate Cancer Metastasis Team, Imperial College London, United Kingdom) were grown in RPMI-1640 media, supplemented with 10% fetal calf serum (FCS), 2 mmol/L l-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen). A375 malignant melanoma cells were a kind donation from Professor Eyal Gottlieb, Beatson Institute for Cancer Research, Glasgow, United Kingdom, and were grown in high glucose (4.5 g/L) Dulbecco’s modified Eagle’s medium media, supplemented with 10% FCS, 2 mmol/L l-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen). All cells were maintained at 37°C in a humidified atmosphere containing 5% CO2.

Western blots

Western blotting was done using standard techniques (22, 23). For detailed methodology, see Supplementary Materials.

In vivo tumor models

All animal experiments were conducted by licensed investigators in accordance with the United Kingdom Home Office Guidance on the Operation of the Animal (Scientific Procedures) Act 1986 and within the newly published guidelines for the welfare and use of animals in cancer research (24). Male BALB/c nude mice (aged 6–8 weeks; Charles River) were used. Tumor cells (2 × 10⁶) were injected subcutaneously on the back of mice and animals were used when the xenographs reached approximately 100 mm³. Tumor dimensions were measured continuously using a caliper and tumor volumes were calculated by the equation: volume = (π/6) × a × b × c, in which a, b, and c represent 3 orthogonal axes of the tumor.

In vivo tracer metabolism

Radiolabeled metabolites from plasma and tissues were quantified using a method adapted from Smith and colleagues (17). Briefly, tumor-bearing mice under general anesthesia (2.5% isofluorane; nonrecovery anesthesia) were administered a bolus intravenous injection of one of the following radiotracers: 11C-choline, 11C-D4-choline (~18.5 MBq), or 18F-D4-choline (~3.7 MBq), and sacrificed by exsanguination via cardiac puncture at 2, 15, 30, or 60 minutes postradiotracer injection. For automated radiosynthesis methodology see Supplementary Materials. Tumor, kidney, and liver samples were immediately snap-frozen in liquid nitrogen. Aliquots of heparinized blood were rapidly centrifuged (14,000 × g, 5 minutes, 4°C) to obtain plasma. Plasma samples were subsequently snap-frozen in liquid nitrogen and kept on dry ice prior to analysis.

For analysis, samples were thawed and kept at 4°C immediately before use. To ice-cold plasma (200 µL) was...
added ice-cold methanol (1.5 mL) and the resulting suspension centrifuged (14,000 × g; 4°C; 3 minutes). The supernatant was then decanted and evaporated to dryness on a rotary evaporator (bath temperature, 40°C), then resuspended in high-performance liquid chromatography (HPLC) mobile phase [solvent A: acetonitrile/water/ethanol/acetic acid/1.0 mol/L ammonium acetate/0.1 mol/L sodium phosphate (800/127/68/2/3/10); 1.1 mL]. Samples were filtered through a hydrophilic syringe filter (0.2-µm filter, Millex PTFE filter, Millipore) and the sample (~1 mL) then injected via a 1-mL sample loop onto the HPLC for analysis. Tissues were homogenized in ice-cold methanol (1.5 mL) using an Ultra-Turrax T-25 homogenizer (IKA Werke GmbH and Co. KG) and subsequently treated as per plasma samples.

Samples were analyzed on an Agilent 1100 series HPLC system (Agilent Technologies), configured as described above, using the method of Leyton and colleagues (16). A μBondapak C18 HPLC column (7.8 × 3,000 mm; Waters), stationary phase and a mobile phase comprising of solvent A (vide supra) and solvent B [acetonitrile/water/ethanol/acetic acid/1.0 mol/L ammonium acetate/0.1 mol/L sodium phosphate (400/400/68/44/88/10)], delivered at a flow rate of 3 mL/min were used for analyte separation. The gradient was set as follows: 0% B for 5 minutes; 0% to 100% B in 10 minutes; 100% to 0% B in 2 minutes; 0% B for 5 minutes; 0% to 0% B in 2 minutes; 0% B for 2.5 minutes.

**PET imaging studies**

Dynamic 11C-choline, 11C-D4-choline, and 18F-D4-choline imaging scans were carried out on a dedicated small animal PET scanner (Siemens Inveon PET module, Siemens Medical Solutions USA, Inc.) following a bolus intravenous injection in tumor-bearing mice of either approximately 3.7 MBq for 18F studies or approximately 18.5 MBq for 11C, accommodating for substantially shorter half-life of 11C (20.38 minutes for 11C vs. 109.77 minutes for 18F). Dynamic scans were acquired in list mode format over 60 minutes. The acquired data were then sorted into 0.5-mm sinogram bins and 19 time frames for image reconstruction (4 × 15, 4 × 60, and 11 × 300 seconds), which was done by filtered back projection. For input function analysis, data were sorted into 25 time frames for image reconstruction (8 × 5, 1 × 20, 4 × 40, 1 × 80, and 11 × 300 seconds). The Siemens Inveon Research Workplace software was used for visualization of radiotracer uptake in the tumor; 30- to 60-minute cumulative images of the dynamic data were employed to define 3-dimensional (3D) regions of interest (ROI). Arterial input function was estimated as follows: a single voxel 3D ROI was manually drawn in the center of the heart cavity using 2 to 5 minutes of cumulative images. Care was taken to minimize ROI overlap with the myocardium. The count densities were averaged for all ROIs at each time point to obtain a time versus radioactivity curve (TAC). Tumor TACs were normalized to injected dose, measured by a VDC-304 dose calibrator (Veenstra Instruments) and expressed as percentage injected dose per mL tissue. The area under the TAC, calculated as the integral of %ID/mL from 0 to 60 minutes, and the normalized uptake of radiotracer at 60 minutes (%ID/mL60) were also used for comparisons.

**Kinetic analysis in HCT116 tumors**

A 2-tissue irreversible compartmental model was employed to fit the TACs, as has been previously established for 11C-choline (25, 26), described extensively in Supplementary Data. Here, both a Single Input 3k model (irreversible binding of the parent) and Double Input [3+2]k model (irreversible binding of the parent, reversible binding of the metabolite) were used to describe radiotracer kinetics. Kf (radiotracer delivery; mL/min/mL/min) and k0 (1/min) are the rate constants of transfer from plasma to tissue and from tissue to plasma, respectively. k5 (1/min) represents the rate at which the parent tracer is phosphorylated. In this model the irreversible uptake rate constant Kf (mL/mL/min) can be expressed as a function of the microparameters as Kf[kf/(k2 + k3)]. Ki (mL/mL/min) and k0 (1/min) are the rate constants of transfer from plasma to tissue and from tissue to plasma of labeled betaine. A schematic describing the kinetic models used here is described in Supplementary Fig. S1.

**Statistics**

Data were expressed as mean ± SEM, unless otherwise shown. The significance of comparison between 2 data sets was determined using Student t test. ANOVA was used for multiparametric analysis (Prism v5.0 software for windows, GraphPad Software). Differences between groups were considered significant if *P* ≤ 0.05.

**Results**

**Deuteration leads to enhanced renal radiotracer uptake**

Time course biodistribution was done in nontumor-bearing male nude mice with 11C-choline, 11C-D4-choline, and 18F-D4-choline tracers. Supplementary Fig. S2 shows tissue distribution at 2, 15, 30, and 60 minutes. There were minimal differences in tissue uptake between the 3 tracers over 60 minutes, with uptake values in the 3 tracers over 60 minutes, with uptake values in broad agreement with data previously published for 18F-choline and 18F-D4-choline (13, 17). In all tracers there was rapid extraction from blood, with the majority of radioactivity retained within the kidneys, evident as early as 2 minutes postinjection. Deuteration of 11C-choline led to a significant 1.8-fold increase in kidney retention over 60 minutes (*P* < 0.05; Supplementary Fig. S2A and B), with a 3.3-fold increase in kidney retention observed for 18F-D4-choline when compared with 11C-choline at this time point (*P* < 0.01; Supplementary Fig. S2A and C, respectively). There was a trend toward increased urinary excretion for 11C-D4-choline and 18F-D4-choline, in comparison with the nature identical tracer, 11C-choline, although this increase did not reach statistical significance.
Deuteration of $^{11}$C-choline results in modest resistance to oxidation in vivo

Tracer metabolism in tissues and plasma was done by radio-HPLC (Fig. 1). Peaks were assigned as choline, betaine, betaine aldehyde, and phosphocholine, using enzymatic (alkaline phosphatase and choline oxidase) methods to determine their identity (Supplementary Figs. S3 and S4, respectively; ref. 16).

In the liver, both $^{11}$C-choline and $^{11}$C-D4-choline were rapidly oxidized to betaine (Fig. 1A and B), with 49.2 ± 7.7% of $^{11}$C-choline radioactivity already oxidized to betaine by 2 minutes. Deuteration of $^{11}$C-choline provided significant protection against oxidation in the liver at 2 minutes postinjection, with 24.5 ± 2.1% radioactivity as betaine (51.2% decrease in betaine levels; $P = 0.037$), although this protection was lost by 15 minutes. Notably, a high proportion of liver radioactivity (≈80%) was present as $^{18}$F-D4-phosphocholine by 15 minutes with $^{18}$F-D4-choline (Fig. 1C). This corresponded to a much reduced liver-specific oxidation when compared with the 2 carbon-11 tracers (15.0 ± 3.6% of radioactivity as betaine at 60 minutes; $P = 0.002$).

In contrast to the liver, deuteration of $^{11}$C-choline resulted in protection against oxidation in the kidney over the entirety of the 60-minute time course (Fig. 1D and E). With $^{11}$C-D4-choline there was a 20% to 40% decrease in betaine levels over 60 minutes when compared with $^{11}$C-choline ($P < 0.05$), corresponding to a proportional increase in labeled phosphocholine ($P < 0.05$). As shown in Fig. 1F, $^{18}$F-D4-choline was more resistant to oxidation in the kidney than both carbon-11–labeled choline tracers. There was a relationship between levels of radiolabeled phosphocholine and kidney retention when data from all 3 tracers were compared ($R^2 = 0.504$; Supplementary Fig. S5). In the plasma, the temporal levels of betaine for both $^{11}$C-choline and $^{11}$C-D4-choline were almost identical; it should be noted that total radioactivity levels were low for all radiotracers. At 2 minutes, 12.1 ± 2.6% and 8.8 ± 3.8% of radioactivity was in the form of betaine for $^{11}$C-choline and $^{11}$C-D4-choline, respectively, rising to 78.6 ± 4.4% and
radioactivity was phosphocholine for all 3 tracers, with significantly more radioactivity corresponding to labeled phosphocholine than 11C-choline; 43.8% of activity present as betaine at 15 minutes. A further increase in plasma betaine was not observed with 18F-D4-choline over the remainder of the time course.

**Fluorination protects against choline oxidation in tumor**

11C-choline, 11C-D4-choline, and 18F-D4-choline metabolism were measured in HCT116 tumors (Fig. 2). With all tracers, choline oxidation was greatly reduced in the tumor in comparison with levels in the kidney and liver. At 15 minutes, both 11C-D4-choline and 18F-D4-choline had significantly more radioactivity corresponding to labeled phosphocholine than 11C-choline; 43.8 ± 1.5% and 45.1 ± 3.2% for 11C-D4-choline and 18F-D4-choline, respectively, in comparison with 30.5 ± 4.0% for 11C-choline (P = 0.035 and P = 0.046, respectively). By 60 minutes, the majority of radioactivity was phosphocholine for all 3 tracers, with labeled phosphocholine levels increasing in the order of 11C-choline < 11C-D4-choline < 18F-D4-choline. There was no difference in the tumor metabolic profile for 11C-choline and 11C-D4-choline at 60 minutes, although reduced choline oxidation was observed for 18F-D4-choline; 14.0 ± 3.0% betaine radioactivity with 18F-D4-choline compared with 28.1 ± 2.9% for 11C-choline (P = 0.026).

**Choline tracers have similar sensitivity for imaging tumors by PET**

Despite the low systemic oxidation of 18F-D4-choline, tumor radiotracer uptake in mice by PET was no higher than with 11C-choline or 11C-D4-choline (Fig. 3). Figure 3A–C shows typical (0.5 mm) transverse PET image slices showing accumulation of all 3 tracers in HCT116 tumors. For all 3 tracers, there was heterogeneous tumor uptake, but tumor signal-to-background levels were identical, confirmed by normalized uptake values at 60 minutes and values for the tumor area under the time versus radioactivity curve (data not shown). It should be noted that the PET data represent total radioactivity. In the case of 11C-choline or 11C-D4-choline, a significant proportion of this radioactivity is betaine (Fig. 2).

**Tumor tracer kinetics**

Despite there being no difference in overall tracer retention in the tumor, the kinetic profiles of tracer uptake varied between the 3 choline tracers, detected by PET (Fig. 3D). The kinetics for the 3 tracers were characterized by rapid tumor influx over the initial 5 minutes, followed by stabilization of tumor retention. Initial delivery of 18F-D4-choline over the first 14 minutes of imaging was higher than for both 11C-choline and 11C-D4-choline (expanded TAC for initial 14 minutes shown in Supplementary Fig. S6). Slow wash-out of activity was observed with both 18F-D4-choline and 11C-D4-choline between 30 and 60 minutes, in contrast to the gradual accumulation detected with 11C-choline. Parameters for the irreversible trapping of radioactivity in the tumor, Kᵢ and kᵢ, were calculated from a 2-tissue irreversible model, using metabolite-corrected TAC from the heart cavity as input function (Fig. 4A and B). A double input model, accounting for the contribution of metabolites to the tissue TAC, was used for kinetic analysis, described in Supplementary Data. There was no significant difference in flux constant measurements between deuterated and undeuterated 11C-choline. Addition of methylfluoride, however, resulted in 49.2% (n = 3; P = 0.022) and 75.2% (n = 3; P = 0.005) decreases in Kᵢ and kᵢ, respectively; that is, when 18F-D4-choline was compared with 11C-D4-choline. Kᵢ values were similar between all 3 tracers: 0.106 ± 0.026, 0.114 ± 0.019, and 0.142 ± 0.027 for 11C-choline, 11C-D4-choline, and 18F-D4-choline, respectively. It is possible that intracellular betaine formation (not just presence of betaine in the extracellular space) led to a higher than expected irreversible uptake; there was a significant 368% and 230% increase in the ratio of betaine:phosphocholine at 15 and 60 minutes, respectively (P = 0.045 and 0.036) with 11C-choline in comparison with 18F-D4-choline (Fig. 4C).
18F-D4-choline shows good sensitivity for the PET imaging of prostate adenocarcinoma and malignant melanoma

Having confirmed that 18F-D4-choline has the most desirable metabolic profile for in vivo studies, with good sensitivity for the imaging of colon adenocarcinoma, we wanted to evaluate its suitability for cancer detection in other models of human cancer, including malignant melanoma A375 and prostate adenocarcinoma PC3-M. In vitro uptake of 18F-D4-choline was similar in the 3 cell lines over 30 minutes (Supplementary Fig. S7), relating to near-identical levels of choline kinase expression (Supplementary Fig. S7 insert). Retention of radioactivity was shown to be dependent on both choline transport and choline kinase activity, as treatment of cells with the dual choline transport and choline kinase inhibitor, hemicholinium-3, resulted in more than 90% decrease in intracellular tracer radioactivity in all 3 cell lines. Similar intracellular trapping of 18F-D4-choline in these cancer models was translated to their uptake in vivo (Fig. 5A), showing similar values for flux constant measurements, including rates of delivery (Kd; Supplementary Table S1) and other PET imaging variables. There was a trend toward increased tumor retention of 18F-D4-choline in the order of A375 < HCT116 < PC3-M, reflected by the expression of choline kinase in these lines (Fig. 5C). There was no discernible difference in tumor metabolite profiles between the 3 cell cancer models at either 15 or 60 minutes of postinjection (Fig. 5B).

Discussion

Aberrant phospholipid metabolism is a hallmark of many cancers (5), resulting in upregulated mitotic signaling and an increase in plasma membrane biosynthesis. One such mediator of phospholipid metabolism, choline kinase, has been shown to be a biomarker of malignant transformation (2). Proton and phosphorous magnetic resonance spectroscopic (MRS) techniques have provided a means to measure the product of choline kinase activity, phosphocholine, from tumor tissue biopsies ex vivo and from noninvasive spectroscopic imaging measurements in vivo (27). MRS, however, is hampered by inherently poor in vivo sensitivity, making it difficult to resolve individual choline metabolite resonances, complicating data interpretation, whereas ex vivo measurements require invasive sampling from a small, possibly unrepresentative, region of interest. Given the current drawbacks of choline metabolite analysis by MRS, a more viable alternative has been the use of radiolabeled choline for noninvasive tumor imaging. PET-labeled choline tracers provide vastly improved...
sensitivity, when compared with MRS, and enable dynamic measurements of choline metabolism, but without the chemical resolution of MRS. To date, $^{11}$C-choline has successfully been used for the clinical imaging of prostate, brain, breast, and esophageal carcinomas (10, 25, 28–30). Despite its relative success, $^{11}$C-choline-PET imaging has not been widely adopted in the clinic; the short half-life of carbon-11 requires an on-site cyclotron and rapid metabolism of the choline tracer presents complications for data interpretation and limits the imaging time frame to early time frames (25). We have recently developed a fluorinated choline analog, $^{18}$F-D4-choline, labeled with a longer lived isotope and with improved metabolic profile, required for late tumor imaging (16). The substitution of deuterium for carbon-11 requires an on-site cyclotron and rapid metabolism of the choline tracer presents complications for data interpretation and limits the imaging time frame to early time frames (25).
hydrogen on the ethyl alcohol portion of choline resulted in a large observed isotope effect in the oxidation of choline to betaine by choline oxidase. Further studies showed that the magnitude of the $^{1}H/^{2}D$ isotope effect was more profound when all protons were substituted for deuterium, in comparison with partial deuteration of the fluorocholine (17).

Urinary radioactivity, however, is higher with fluorinated choline analogs relative to $^{11}C$-choline (16, 31), potentially limiting their use for the detection of pelvic cancers, such as prostate adenocarcinoma. Here, we developed a novel choline tracer, $^{11}C$-D4-choline, which, based on previous work with fluorinated and deuterated choline tracers, was predicted to have reduced oxidation to betaine and a favorable urinary excretion profile.

The kidney has high choline kinase activity along the nephron (32), shown to exhibit the greatest tissue retention for choline-PET and, therefore, is the radiation-dose-critical organ (13, 17). Kidney retention increased in the order of $^{11}C$-choline $<^{11}C$-D4-choline $<^{18}F$-D4-choline over the 60-minute time course (Supplementary Fig. S2), with total kidney radioactivity shown to be proportional to the % radioactivity retained as labeled phosphocholine (Supplementary Fig. S5; $R^2 = 0.504$). The increased conversion of choline to phosphocholine with $^{11}C$-D4-choline relative to $^{11}C$-choline corresponded with a significant decrease in choline oxidation to betaine and could be ascribed to increased substrate availability for phosphorylation. Further attenuation of choline oxidation was observed with $^{18}F$-D4-choline, indicating that the magnitude of the $^{1}H/^{2}D$ isotope effect is influenced by fluorination. Protection against choline oxidation by deuteration of $^{11}C$-choline was shown to be tissue specific, with a decrease in betaine radioactivity measured in the liver at just 2 minutes post-injection when compared with $^{11}C$-choline (Fig. 1). This effect is presumably due to the lower capacity of choline oxidase in rodent kidney compared with liver. $^{18}F$-D4-choline provided substantially reduced betaine oxidation in the liver over 60 minutes postinjection, again suggesting that fluorination, in part, drives this reduced capacity to oxidize choline pseudosubstrates to betaine.

Despite systemic protection against choline oxidation with $^{18}F$-D4-choline, the reduction in the rate of choline oxidation was much more subtle in implanted HCT116 tumors (Fig. 2). At 15 minutes postinjection, there were 43.6% and 47.9% higher levels of radiolabeled phosphocholine when $^{11}C$-D4-choline and $^{18}F$-D4-choline, respectively, were injected relative to $^{11}C$-choline. By 60 minutes there was no difference in labeled phosphocholine levels between the 3 tracers, although there was a significant decrease in betaine-specific radioactivity with $^{18}F$-D4-choline. This equilibration of phosphocholine-specific activity can be explained by a saturation effect, with parent tracer levels reduced to a minimum by 60 minutes, severely limiting substrate levels available for choline kinase activity. Lower betaine levels were observed in the tumor with all 3 tracers over the entire time course when compared with liver and kidney, likely resulting from a lower capacity for choline oxidation or increased washout of betaine. It should be noted that the capacity of rodents to metabolize choline is substantially higher than that of humans (14, 33). The slower metabolic rate in humans may, therefore, provide a better differential between these choline tracers. Despite this, deuteration of $^{11}C$-choline per se provided less than expected protection against choline oxidation in the liver, tumor, and kidney, especially in the context of improved metabolic profile shown with deuterated fluorocholine versus nondeuterated fluorocholine (16, 17).

Comparison of the 3 choline radiotracers by PET showed no significant differences in overall tumor radiotracer uptake and hence sensitivity (Fig. 3), despite large changes observed in other organs. Initial tumor kinetics (at time points when metabolism was lower), however, varied between tracers, with $^{18}F$-D4-choline characterized by rapid delivery over approximately 5 minutes, followed by slow wash-out of activity from the tumor. This compared with the slower uptake, but continuous tumor retention of $^{11}C$-choline. At 60 minutes, a 2.7-fold and 4.0-fold higher unmetabolized parent tracer was seen with $^{18}F$-D4-choline in tumor compared with $^{11}C$-choline and $^{11}C$-D4-choline, respectively, (Fig. 2). Deuteration did not, however, alter total tumor radioactivity levels and the modeling approach used did not distinguish between different intracellular species. Although all tracers were converted intracellularly to phosphocholine, the higher rate constants for intracellular retention ($K_i$ and $k_{11}$, Fig. 3A and B) of $^{11}C$-choline and $^{11}C$-D4-choline, compared with $^{18}F$-D4-choline, were explained by the rapid conversion of the nonfluorinated tracers to betaine within HCT116 tumors, indicating greater specificity with $^{18}F$-D4-choline. Compared with $^{18}F$-D4-choline, the tumor betaine-to-phosphocholine metabolite ratio increased by 388% ($P = 0.045$) and 259% ($P = 0.061$, nonsignificant) for $^{11}C$-choline and $^{11}C$-D4-choline, respectively (Fig. 4C). It is also important to note that the compartmental modeling is subject to some minor experimental limitations. In humans and larger animals, a more accurate input function can be obtained by continuous blood sampling following radiotracer injection. Individual plasma metabolite data can also be easily obtained and fitted instead of the averaged data taken from a separate cohort of animals used here.

It has been reported elsewhere that fluorination increases urinary excretion in comparison with $^{11}C$-choline (13, 16). However, in this study, we did not observe these undesirable urinary excretion properties. This may be due to use of anesthesia for immobilizing mice during imaging. There was a trend toward increased urinary excretion in the two deuterated tracers (suggesting a trade-off between reduced oxidation and renal excretion) when compared with $^{11}C$-choline, although these did not reach significance. Low radioactivity levels in the urine prevented accurate metabolite analysis (data not shown). The low radioactivity levels in the urine should enable accurate prostate imaging with $^{18}F$-D4-choline, especially if patients void to reduce bladder radioactivity prior to late time point imaging. Given the favorable urinary excretion properties and greatly superior systemic metabolic profile of $^{18}F$-D4-choline, PET imaging
was carried out in 2 further models of human cancer to assess generic utility in tumors of different origins: A375 malignant melanoma and PC3-M prostate adenocarcinoma. PC3-M cells were chosen as a clinically relevant model for choline imaging, whereas A375 have constitutively active mitogen-activated protein kinase (MAPK) due to a BRAFV600E mutation (34); the MAPK pathway is known to regulate choline kinase activity (35). 18F-D4-choline uptake in vivo (where betaine formation is negligible) was similar in all 3 cell lines, reflecting near-identical levels of choline kinase α expression. The delivery of 18F-D4-choline (K1) was similar between the different tumor types in vivo, suggesting that choline transporter expression was probably not deficient in any of the tumors. These in vivo findings translated well in vitro, with comparable tumor uptake, kinetics, choline kinase α expression, and metabolism for all the tumor types, suggesting that 18F-D4-choline may have utility for tumor detection, irrespective of histologic type. In conclusion, we have shown here that deuteration of 11C-choline provides a smaller than expected protection against choline oxidation. Despite a significant increase in labeled phosphocholine at early time points, this did not increase the overall sensitivity for the detection of choline metabolism in vivo. More promising is the substantial decrease in betaine oxidation illustrated here with 18F-D4-choline, which may permit the clinical imaging of choline without invasive blood sampling. Fluorine-18 labeling may also lead to wider clinical adoption and permit imaging at late time points. We have further validated 18F-D4-choline using 3 models of human cancer, including a clinically relevant model of human prostate adenocarcinoma.

Disclosure of Potential Conflicts of Interest
A patent on novel choline imaging agents has been filed.

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