Evaluation of deuterated $^{18}$F- and $^{11}$C-labeled choline analogs for cancer detection by positron emission tomography

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Translational Relevance

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

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 biological basis of the oxidation of deuterated choline analogues and assessed their specificity in human tumor xenografts. **Experimental Design:** $^{11}$C-Choline, $^{11}$C-methyl-$[1,2\cdot ^2\text{H}_4]$-choline ($^{11}$C-D4-choline) and $^{18}$F-D4-choline were synthesized to permit comparison. Biodistribution, metabolism, small-animal PET studies, and kinetic analysis of tracer uptake were performed.
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-D4-choline and substantially reduced with $^{18}$F-D4-choline; suggesting that both fluorination and deuteration were important for tracer metabolism. While 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-D4-choline, compared to $^{18}$F-D4-choline were explained by the rapid conversion of the non-fluorinated tracers to betaine within HCT116 tumors. Imaging studies showed that the uptake of $^{18}$F-D4-choline in three tumors with similar radiotracer delivery ($K_i$) and choline kinase $\alpha$ expression - HCT116, A375 and PC3-M - were the same, suggesting that $^{18}$F-D4-choline has utility for cancer detection irrespective of histological type. **Conclusion:** We have demonstrated here that both deuteration and fluorination combine to provide protection against choline oxidation *in vivo.* $^{18}$F-D4-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-2-fluoro-2-deoxyglucose (FDG) for PET imaging of the prostate (10-12), where the increased choline kinase activity in tumors provides the basis for tumor-specific contrast in comparison to surrounding non-neoplastic tissues. A fluorinated analogue, $^{18}$F-fluoromethylcholine, has also been developed for PET imaging of choline metabolism (13), with the longer half-life of fluorine-18 (109.8 min versus 20.4 min for carbon-11) potentially enabling more widespread adoption of choline imaging in the clinic and the ability to image at later time points post tracer injection.

Within the imaging time window (60 min), tumor radiolabeled choline uptake is a function of perfusion, transport of the radiotracer from the extracellular space into cells where it is either
converted into phosphocholine by the action of choline kinase or oxidized by choline oxidase to betaine. Further incorporation of phosphocholine to membrane phosphatidylcholine is negligible within this time window (7, 8, 14, 15); chromatographic analysis also indicates that further betaine metabolism or conversion to acetylcholine is negligible (16, 17). Hence radiotracer uptake broadly represents transport and phosphorylation on the one hand, and transport and oxidation on the other. One key limitation of choline-PET is the rapid oxidation to radiolabeled-betaine, making it difficult to assess choline kinase-specific trapping of activity (as phosphocholine) within tumors without plasma metabolite evaluation using complex kinetic analysis. We have recently developed a novel tracer, $^{18}$F-fluoromethyl-[1,2-$^2$H$_4$]-choline ($^{18}$F-D4-choline) with reduced in vivo oxidation to betaine and improved sensitivity for the detection of choline metabolism in comparison to the non-deuterated $^{18}$F-fluoromethylcholine (16). This improved metabolic profile was shown (16, 17) to be based on the deuterium isotope effect (18-21). Here, we sought to further evaluate the structural determinants of deuteration on substrate metabolism, as well as the effect of deuteration on tumor-specific uptake. To this end, we developed a novel choline tracer, $^{11}$C-[1,2-$^2$H$_4$]-choline ($^{11}$C-D4-choline), and compared its in vivo tumor uptake, kinetics and metabolic profile to $^{11}$C-choline and $^{18}$F-D4-choline PET tracers.
Materials and Methods

**Cell lines**

HCT116 colorectal carcinoma (LGC Standards, Teddington, Middlesex, UK) and PC3-M prostate adenocarcinoma cells (kind donation from Dr Matthew Caley, Prostate Cancer Metastasis Team, Imperial College London, UK) were grown in RPMI 1640 media, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U.mL\(^{-1}\) penicillin and 100 \(\mu\)g.mL\(^{-1}\) streptomycin (Invitrogen, Paisley, Refrewshire, UK). A375 malignant melanoma cells were a kind donation from Professor Eyal Gottlieb, Beatson Institute for Cancer Research, Glasgow, UK and were grown in high glucose (4.5 g/L) DMEM media, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U.mL\(^{-1}\) penicillin and 100 \(\mu\)g.mL\(^{-1}\) streptomycin (Invitrogen, Paisley, Refrewshire, UK). All cells were maintained at 37°C in a humidified atmosphere containing 5% CO\(_2\).

**Western blots**

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

**In vivo tumor models**

All animal experiments were performed 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, Wilmington, MA, USA) were used. Tumor cells (2 \(\times\) 10\(^6\)) were injected subcutaneously on the back of mice and animals were used when the xenografts reached ~ 100 mm\(^3\).
dimensions were measured continuously using a caliper and tumor volumes were calculated by the equation: volume = \( \frac{\pi}{6} \times a \times b \times c \), where \( a \), \( b \), and \( c \) represent three orthogonal axes of the tumor.

**In vivo tracer metabolism**

Radiolabeled metabolites from plasma and tissues were quantified using a method adapted from (17). Briefly, tumor-bearing mice under general anesthesia (2.5% isofluorane; non-recovery anesthesia) were administered a bolus i.v. injection of one of the following radiotracers: \(^{11}\text{C}\)-choline, \(^{11}\text{C}\)-D4-choline (~18.5 MBq) or \(^{18}\text{F}\)-D4-choline (~ 3.7 MBq), and sacrificed by exsanguination via cardiac puncture at 2, 15, 30 or 60 min post radiotracer 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 (14000 g, 5 min, 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 (14000 g; 4°C; 3 min). The supernatant was then decanted and evaporated to dryness on a rotary evaporator (bath temperature, 40°C), then resuspended in HPLC mobile phase (Solvent A: acetonitrile/water/ethanol/acetic acid/1.0 M ammonium acetate/0.1 M 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, MA., USA) 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, Staufen, Germany) and subsequently treated as per plasma samples.

Samples were analyzed on an Agilent 1100 series HPLC system (Agilent Technologies, Santa Clara, CA, USA), configured as described above, using the method of (16). A µBondapak C\textsubscript{18} HPLC column (Waters, Milford, MA, USA; 7.8×3000 mm), stationary phase and a mobile phase comprising of Solvent A (vide supra) and Solvent B (acetonitrile/water/ethanol/acetic acid/1.0 M ammonium acetate/0.1 M 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 min; 0% to 100% B in 10 min; 100% B for 0.5 min; 100% to 0% B in 2 min; 0% B for 2.5 min.

**PET imaging studies**

Dynamic \textsuperscript{11}C-choline, \textsuperscript{11}C-D4-choline and \textsuperscript{18}F-D4-choline imaging scans were carried out on a dedicated small animal PET scanner (Siemens Inveon PET module, Siemens Medical Solutions USA, Inc., Malvern, PA, USA) following a bolus \textit{i.v.} injection in tumor-bearing mice of either \textasciitilde3.7 MBq for \textsuperscript{18}F studies, or \textasciitilde18.5 MBq for \textsuperscript{11}C, accommodating for \textsuperscript{11}C’s substantially shorter half-life (20.38 min for \textsuperscript{11}C versus 109.77 min for \textsuperscript{18}F). Dynamic scans were acquired in list mode format over 60 min. The acquired data were then sorted into 0.5 mm sinogram bins and 19 time frames for image reconstruction (4 \times 15 s, 4 \times 60 s, and 11 \times 300 s), which was done by filtered back projection. For input function analysis, data were sorted into 25 time frames for image reconstruction (8 \times 5 s, 1 \times 20 s, 4 \times 40 s, 1 \times 80 s, and 11 \times 300 s). The Siemens Inveon Research Workplace software was used for visualization of radiotracer uptake in the tumor; 30 to 60 min cumulative images of the dynamic data were employed to define 3-dimensional (3D) regions of interest (ROIs). 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 min 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, Joure, The Netherlands), and expressed as percentage injected dose per mL tissue. The area under the TAC, calculated as the integral of %ID/mL from 0 – 60 min, and the normalized uptake of radiotracer at 60 min (%ID/mL\textsubscript{60}) 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 \textsuperscript{11}C-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. $K_1$ (radiotracer delivery; mL/mL/min) and $k_2$ (1/min) are the rate constants of transfer from plasma to tissue and from tissue to plasma, respectively. $k_3$ (1/min) represents the rate at which the parent tracer is phosphorylated. In this model the irreversible uptake rate constant $K_i$ (mL/mL/min) can be expressed as a function of the microparameters as $K_1 k_3 / (k_2 + k_3)$. $K_i'$ (mL/mL/min) and $k_2'$ (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 Supplemental Fig. 1.
Statistics

Data were expressed as mean ± standard error of the mean (SEM), unless otherwise shown. The significance of comparison between two data sets was determined using Student's \( t \) test. ANOVA was used for multi-parametric analysis (Prism v5.0 software for windows, GraphPad Software, San Diego, CA, USA). Differences between groups were considered significant if \( P \leq 0.05 \).

Results

Deuteration leads to enhanced renal radiotracer uptake

Time course biodistribution was performed in non-tumor-bearing male nude mice with \( ^{11}\text{C}-\text{choline} \), \( ^{11}\text{C}-\text{D4-choline} \) and \( ^{18}\text{F}-\text{D4-choline} \) tracers. Supplemental Fig. 2 shows tissue distribution at 2, 15, 30 and 60 min. There were minimal differences in tissue uptake between the three tracers over 60 min, with uptake values in broad agreement with data previously published for \( ^{18}\text{F}-\text{choline} \) and \( ^{18}\text{F}-\text{D4-choline} \) (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 min post injection. Deuteration of \( ^{11}\text{C}-\text{choline} \) led to a significant 1.8-fold increase in kidney retention over 60 min (\( P < 0.05 \); Supplemental Fig. 2A and B), with a 3.3-fold increase in kidney retention observed for \( ^{18}\text{F}-\text{D4-choline} \) when compared to \( ^{11}\text{C}-\text{choline} \) at this time point (\( P < 0.01 \); Supplemental Fig. 2A and C respectively). There was a trend towards increased urinary excretion for \( ^{11}\text{C}-\text{D4-choline} \) and \( ^{18}\text{F}-\text{D4-choline} \), in comparison to the nature identical tracer, \( ^{11}\text{C}-\text{choline} \), 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 performed 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 (Supplemental Fig. 3 and 4, respectively) (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 min. Deuteration of $^{11}$C-choline provided significant protection against oxidation in the liver at 2 min post injection, with 24.5 ± 2.1 % radioactivity as betaine (51.2 % decrease in betaine levels; $P = 0.037$), although this protection was lost by 15 min. Notably, a high proportion of liver radioactivity (~80 %) was present as $^{18}$F-D4-phosphocholine by 15 min with $^{18}$F-D4-choline (Fig. 1C). This corresponded to a much reduced liver-specific oxidation when compared to the two carbon-11 tracers (15.0 ± 3.6 % of radioactivity as betaine at 60 min; $P = 0.002$).

In contrast to the liver, deuteration of $^{11}$C-choline resulted in protection against oxidation in the kidney over the entirety of the 60 min time course (Fig. 1D and E). With $^{11}$C-D4-choline there was a 20 – 40 % decrease in betaine levels over 60 min when compared to $^{11}$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 three tracers were compared ($R^2 = 0.504$; Supplemental Fig. 5). 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 min, 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 79.5 ± 2.9 % at 15 min. Betaine levels were significantly reduced with $^{18}$F-D4-choline, with 43.7 ± 12.4 % of activity present as betaine at 15 min. A further increase in plasma betaine was not observed with $^{18}$F-D4-choline over the remainder of the time course.

Fluorination protects against choline oxidation in tumor

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

Choline tracers have similar sensitivity for imaging tumors by PET

Despite the low systemic oxidation of $^{18}$F-D4-choline, tumor radiotracer uptake in mice by PET was no higher than with $^{11}$C-choline or $^{11}$C-D4-choline (Fig. 3). Fig. 3A-C shows typical (0.5 mm) transverse PET image slices showing accumulation of all three tracers in HCT116 tumors. For all three tracers there was heterogeneous tumor uptake, but tumor
signal-to-background levels were identical; confirmed by normalized uptake values at 60 min 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 $^{11}$C-choline or $^{11}$C-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 three choline tracers, detected by PET (Fig. 3D). The kinetics for the three tracers were characterized by rapid tumor influx over the initial 5 min, followed by stabilization of tumor retention. Initial delivery of $^{18}$F-D4-choline over the first 14 min of imaging was higher than for both $^{11}$C-choline and $^{11}$C-D4-choline (expanded TAC for initial 14 min shown in Supplemental Fig. 6). Slow wash-out of activity was observed with both $^{18}$F-D4-choline and $^{11}$C-D4-choline between 30 and 60 min, in contrast to the gradual accumulation detected with $^{11}$C-choline. Parameters for the irreversible trapping of radioactivity in the tumor, $K_i$ and $k_3$, were calculated from a two-tissue irreversible model, using metabolite-corrected TAC from the heart cavity as input function (Fig. 4A and B). A double input (DI) model, accounting for the contribution of metabolites to the tissue TAC, was used for kinetic analysis, described in supplemental data. There was no significant difference in flux constant measurements between deuterated and undeuterated $^{11}$C-choline. Addition of methylfluoride, however, resulted in 49.2 % ($n = 3; P = 0.022$) and 75.2 % ($n = 3; P = 0.005$ decreases in $K_i$ and $k_3$, respectively; i.e., when $^{18}$F-D4-choline was compared to $^{11}$C-D4-choline. $K_1$ values were similar between all three tracers: $0.106 \pm 0.026; 0.114 \pm 0.019; 0.142 \pm 0.027$ for $^{11}$C-choline, $^{11}$C-D4-choline and $^{18}$F-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 388 and 230%
increase in the ratio of betaine:phosphocholine at 15 and 60 min respectively (P = 0.045 and 0.036) with $^{11}$C-choline in comparison to $^{18}$F-D4-choline (Fig. 4C).

$^{18}$F-D4-choline shows good sensitivity for the PET imaging of prostate adenocarcinoma and malignant melanoma

Having confirmed that $^{18}$F-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 $^{18}$F-D4-choline was similar in the three cell lines over 30 min (Supplemental Fig. 7), relating to near-identical levels of choline kinase expression (Supplemental Fig. 7 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 > 90 % decrease in intracellular tracer radioactivity in all three cell lines. Similar intracellular trapping of $^{18}$F-D4-choline in these cancer models were translated to their uptake in vivo (Fig. 5A)), showing similar values for flux constant measurements showing similar values for flux constant measurements, including rates of delivery ($K_i$; Supplemental Table 1), and other and PET imaging variables . There was a trend towards increased tumor retention of $^{18}$F-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 three cell cancer models at either 15 or 60 min post injection (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 \textit{ex vivo} and from non-invasive spectroscopic imaging measurements \textit{in vivo} (27). MRS, however, is hampered by inherently poor \textit{in vivo} sensitivity, making it difficult to resolve individual choline metabolite resonances, complicating data interpretation; whereas \textit{ex vivo} measurements requires 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 non-invasive tumor imaging. PET-labeled choline tracers provide vastly improved sensitivity when compared to MRS, and enable dynamic measurements of choline metabolism, but without the chemical resolution of MRS. To date, \textsuperscript{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, \textsuperscript{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 analogue, \textsuperscript{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 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 to partial deuteration of the fluorocholine (17). Urinary radioactivity however is higher with fluorinated choline analogues 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 min time course (Supplemental Fig. 2), with total kidney radioactivity shown to be proportional to the % radioactivity retained as labeled phosphocholine (Supplemental Fig. 5; $R^2 = 0.504$). 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 min post injection when compared to $^{11}C$-choline (Fig. 1). This effect is presumably due to the lower capacity of choline oxidase in rodent kidney compared to liver. $^{18}F$-D₄-choline provided substantially reduced betaine oxidation in the liver over 60 min post injection, again
suggesting that fluorination in part drives this reduced capacity to oxidize choline pseudo-substrates to betaine.

Despite systemic protection against choline oxidation with $^{18}\text{F}$-D4-choline, the reduction in the rate of choline oxidation was much more subtle in implanted HCT116 tumors (Fig. 2). At 15 min post injection there were 43.6 % and 47.9 % higher levels of radiolabeled-phosphocholine when $^{11}\text{C}$-D4-choline and $^{18}\text{F}$-D4-choline, respectively, were injected relative to $^{11}\text{C}$-choline. By 60 min there was no difference in labeled phosphocholine levels between the three tracers, although there was a significant decrease in betaine-specific radioactivity with $^{18}\text{F}$-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 min, severely limiting substrate levels available for choline kinase activity. Lower betaine levels were observed in the tumor with all three tracers over the entire time course when compared to 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 $^{11}\text{C}$-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 non-deuterated fluorocholine (16, 17)

Comparison of the three 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 $^{18}\text{F}$-D4-choline characterized by rapid delivery over ~5 min,
followed by slow wash-out of activity from the tumor. This compared to the slower uptake, but continuous tumor retention of $^{11}$C-choline. At 60 min a 2.7-fold and 4.0-fold higher unmetabolized parent tracer was seen with $^{18}$F-D4-choline in tumor compared to $^{11}$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. While all tracers were converted intracellularly to phosphocholine, the higher rate constants for intracellular retention ($K_i$ and $k_3$; Fig. 3A and B) of $^{11}$C-choline and $^{11}$C-D4-choline, compared to $^{18}$F-D4-choline were explained by the rapid conversion of the non-fluorinated tracers to betaine within HCT116 tumors, indicating greater specificity with $^{18}$F-D4-choline. Compared to $^{18}$F-D4-choline, the tumor betaine-to-phosphocholine metabolite ratio increased by 388% ($P = 0.045$) and 259% ($P = 0.061$, non-significant) 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 to $^{11}$C-choline (13, 16). In this study however 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 towards increased urinary excretion in the two deuterated tracers (suggesting a trade-off between reduced oxidation and renal excretion) when compared to $^{11}$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 performed in two 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 MAPK due to a $BRAF^{V600E}$ mutation (34); the MAPK pathway is known to regulate choline kinase activity (35). $^{18}$F-D4-choline uptake $\textit{in vitro}$ (where betaine formation is negligible) was similar in all three cell lines, reflecting near-identical levels of choline kinase $\alpha$ expression. The delivery of $^{18}$F-D4-choline ($K_i$) was similar between the different tumor types $\textit{in vivo}$ suggesting that choline transporter expression was probably not deficient in any of the tumors. These $\textit{in vitro}$ findings translated well $\textit{in vivo}$, with comparable tumor uptake, kinetics, choline kinase $\alpha$ expression and metabolism for all the tumor types, suggesting that $^{18}$F-D4-choline may have utility for tumor detection irrespective of histological type.

In conclusion, we have shown here that deuteration of $^{11}$C-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 $\textit{in vivo}$. More promising is the substantial decrease in betaine oxidation illustrated here with $^{18}$F-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 $^{18}$F-D4-choline using three 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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References


Figure legends

**Figure 1.** Metabolic profile of $^{11}$C-choline (A + D), $^{11}$C-D4-choline (B + E) and $^{18}$F-D4-choline (C + F) in the liver (A – C) and kidney (D – F) of BALB/c nude mice. Radiolabelled metabolite profile was assessed at 2, 15, 30 and 60 min after *i.v.* injection of parent radiotracers using radio-HPLC. Mean values ($n = 3$) and SEM are shown. *a*, $P < 0.05$ when $^{11}$C-D4-choline is compared to $^{11}$C-choline; *b*, $P < 0.05$ when $^{18}$F-D4-choline is compared to $^{11}$C-choline; *c*, $P < 0.05$ when $^{18}$F-D4-choline is compared to $^{11}$C-D4-choline. Abbreviations: Bet-ald, betaine aldehyde; p-Choline, phosphocholine.

**Figure 2.** Metabolic profile of $^{11}$C-choline, $^{11}$C-D4-choline and $^{18}$F-D4-choline in HCT116 tumors. Radiolabelled metabolite profile in HCT116 tumor xenografts was assessed at 15 min (A) and 60 min (B) after *i.v.* injection of parent radiotracers using radio-HPLC. Mean values ($n = 3$) and SEM are shown. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Abbreviations: p-Choline, phosphocholine.

**Figure 3.** $^{11}$C-choline (○), $^{11}$C-D4-choline (▲) and $^{18}$F-D4-choline (■) PET image analysis. HCT116 tumor uptake profiles were examined following 60 min dynamic PET imaging. A – C, representative axial PET-CT images of HCT116 tumor-bearing mice (30 – 60 min summed activity) for $^{11}$C-choline (A), $^{11}$C-D4-choline (B) and $^{18}$F-D4-choline (C). Tumor margins, indicated from CT image, are outlined in red. D, The tumor time versus radioactivity curve (TAC). Mean ± SEM ($n = 4$ mice per group).
Figure 4. Pharmacokinetics of $^{11}$C-choline, $^{11}$C-D4-choline and $^{18}$F-D4-choline in HCT116 tumors. A, Modified compartmental modeling analysis, taking into account plasma metabolites and their flux into the exchangeable space in tumor, was used to derive $K_i$, a measure of irreversible retention within the tumor. B, The kinetic parameter, $k_3$, an indirect measure of choline kinase activity, was calculated using a two site compartmental model as previously described (36, 37). C, Ratio of betaine to phosphocholine in tumors. Metabolites were quantified by radio-HPLC at 15 and 60 min post injection of tracer. Mean values ($n = 4$) and SEM are shown. * $P < 0.05$; *** $P < 0.001$. Abbreviations: p-choline, phosphocholine.

Figure 5. Dynamic uptake and metabolite analysis with $^{18}$F-D4-choline in tumors of different histological origin. A, The tumor time versus radioactivity curve (TAC) obtained from 60 min dynamic PET imaging. Mean ± SEM ($n = 3-5$ mice per group). B, Metabolic profile of $^{18}$F-D4-choline in tumors. Radiolabelled metabolite profile in HCT116 tumor xenografts was assessed post PET imaging using radio-HPLC. Mean values ($n = 3$) and SEM are shown. C, Choline kinase expression in malignant melanoma, prostate adenocarcinoma and colon carcinoma tumors. Representative western blot from tumor lysates ($n = 3$ xenografts per tumor cell line). Actin was used as a loading control. Abbreviations: CK$\alpha$, choline kinase alpha; %ID, percentage injected dose.
A

\[ \text{% Radioactivity} \]

\[ \begin{array}{c}
\text{\textsuperscript{11}C-choline} \\
\text{\textsuperscript{11}C-D4-choline} \\
\text{\textsuperscript{18}F-D4-choline} \\
\end{array} \]

\[ \begin{array}{c}
\text{Betaine} \\
\text{Choline} \\
\text{p-Choline} \\
\end{array} \]

B

\[ \text{% Radioactivity} \]

\[ \begin{array}{c}
\text{\textsuperscript{11}C-choline} \\
\text{\textsuperscript{11}C-D4-choline} \\
\text{\textsuperscript{18}F-D4-choline} \\
\end{array} \]

\[ \begin{array}{c}
\text{Betaine} \\
\text{Choline} \\
\text{p-Choline} \\
\end{array} \]
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Evaluation of deuterated $^{18}$F- and $^{11}$C-labeled choline analogs for cancer detection by positron emission tomography


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