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Evaluation of Enzyme Substrate Radiotracers as PET/MRS Hybrid Imaging Agents

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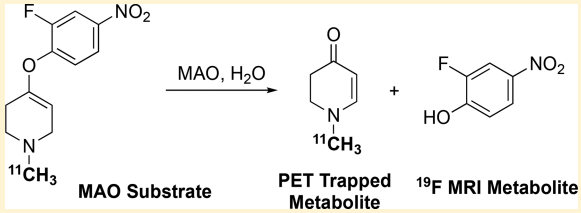
Supporting Information

ABSTRACT: The development of a positron emission tomography (PET)/magnetic resonance spectroscopy (MRS) hybrid imaging agent allows for functional imaging by both methods with a single imaging agent. Enzyme substrates that are cleaved to form two metabolites present an interesting opportunity, as the unique metabolites generated might each be detected by a different modality. To be successful, such enzyme substrates would require administration of doses that (a) reach the *in vivo* target tissue at concentrations necessary for MRS imaging, (b) do not show substrate inhibition of tissue uptake or enzymatic activity, and (c) provide PET images that still reflect the action of the enzyme. We report *in vitro* and *in vivo* proof-of-concept studies of a carbon-11 small molecule substrate for brain monoamine oxidases that, upon enzyme-mediated cleavage, produces two metabolites, one detectable by PET and the other by MRS.



KEYWORDS: *Hybrid imaging, carbon-11, positron emission tomography, magnetic resonance spectroscopy, neuroimaging*

Positron emission tomography (PET) imaging is a noninvasive molecular imaging method that has been utilized for decades in studies of physiology and pharmacology in living animals and human subjects.[1,2] Most applications of PET target high affinity but low capacity sites on proteins (e.g., receptors, enzymes, and transporters) and typically require high molar activities (radioactivity per mole), with clear decreases of specific binding as the mass of the injected radiotracer is increased.[3] The molar activities commonly acceptable for PET imaging (>37 GBq/μmol; > 1000 Ci/mmol) are thus usually considered incompatible with the higher concentrations (mmol) of imaging agents required for alternate functional imaging methods like magnetic resonance spectroscopy (MRS).[4]

The recent development of PET/MR imaging instrumentation has made it possible to simultaneously acquire imaging data for both modalities.[5−7] However, combining PET imaging with functional magnetic resonance spectroscopy (MRS) imaging has proven challenging. This would require a PET imaging agent that can function successfully at the high mass concentrations required for MRS imaging, despite the resulting low molar activity known to be problematic for PET radioligands. We hypothesized that the technique of metabolic trapping, whereby the product of an enzymatic reaction is trapped and retained in tissues at the site of the enzyme, might provide a suitable approach to combining PET and MRS: if the enzyme does not exhibit substrate inhibition *in vivo*, then a high mass/low molar activity PET radiotracer should still function well. To test this hypothesis, we have examined potential metabolically trapped substrates for monoamine oxidases (MAO-A and -B), enzymes of considerable interest in studies of brain biochemistry using PET, and report an example of a small molecule hybrid PET/MRS imaging agent.

Our prior radiotracer development efforts yielded several carbon-11 labeled *N*-methyl-4-aryloxy-1,2,3,6-tetrahydro-pyridines,[8] based on the work of Castagnoli and co-workers,[9,10] which are *in vitro* and *in vivo* substrates for MAO-A or MAO-B. These function *in vivo* as metabolic trapping PET agents, as the enzymatic reaction releases a carbon-11 labeled *N*-methylpyridinone that is retained in brain tissues. Inclusion of a coumarin group as the phenoxy substituent produced a radiotracer that released both radiolabeled and fluorescent metabolic products (Figure 1a), similar to a previous report of a MAO-B selective fluorogenic probe (which was not radiolabeled).[11]

To produce a combined PET/MRS radiotracer, we desired a 4-aryloxy substituent that would provide a metabolite with a unique signal visible by MRS following MAO oxidation. For this, we turned to the work of Yamaguchi et al., who reported an MAO substrate described as a chemical shift-switching agent (Figure 1b), as the ^{19}F NMR signal of the substrate (a 2-

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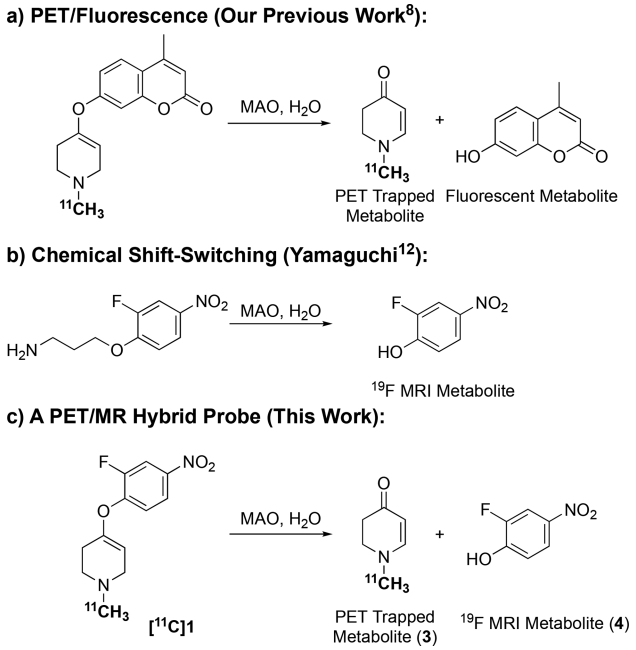


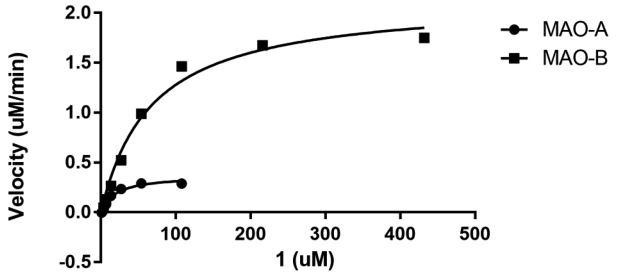
Figure 1. Design of a PET/MRS hybrid probe for monoamine oxidases, building on published examples of PET/fluorescence and chemical shift-switching *in vitro* MAO substrates.

fluoro-4-nitrophenyl ether) could be distinguished from that of the metabolite (2-fluoro-4-nitrophenol).[12] We thus hypothesized that combining 2-fluoro-4-nitrophenol with the *N*-[^{11}C]methyl-1,2,3,6-tetrahydro-pyridine moiety should provide a molecule suitable for testing our hypothesis that a combined PET/MRS radiotracer is feasible using the metabolic trapping concept (Figure 1c).

The authentic standard 4-(2-fluoro-4-nitrophenoxy)-1-methyl-1,2,3,6-tetrahydropyridine (**1**) and corresponding *N*-desmethyl compound (**2**) were prepared using methods previously reported for syntheses of 4-aryloxy substituted tetrahydropyridines.[8,13] To first demonstrate that the proposed radiotracer was a substrate for monoamine oxidases, kinetic studies were done with solutions of human MAO (hMAO)-A or -B and using the change in absorbance from substrate to metabolite in a spectrophotometric assay. As shown in Figure 2, **1** is a substrate for both hMAO-A and -B, with similar catalytic efficiencies (k_{cat}/K_M) for both isozymes.

To next demonstrate that the compound would be useful as a chemical shift-switching agent for MRS, enzymatic reactions with hMAO-A and **1** were run for 90 min and quenched by addition of an excess of the irreversible inhibitor clorgyline. ^{19}F NMR analyses showed that over the time period the signal for substrate **1** at −130 ppm was replaced by that of the phenol metabolite **4** at −136 ppm, as well as a secondary metabolite at −129 ppm (Figure 3).

With **1** confirmed *in vitro* as a MAO chemical shift-switching agent, the last step was to radiolabel with carbon-11 and evaluate whether [^{11}C]**1** could be utilized as a PET imaging agent at low molar activity. The procedure to radiolabel [^{11}C]**1** was similar to the method previously described.[8,13] Briefly, [^{11}C]carbon dioxide was converted by standard procedure into [^{11}C]CH_3OTf and sparged through a solution of the pyridine precursor (1 mg) in ethanol (0.2 mL). The [^{11}C]-methylpyridinium intermediate was transferred to a conical vial containing $NaBH_4$ (2 mg) in ethanol (0.3 mL) and stirred



	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M ($min^{-1}\mu M^{-1}$)
MAO-A	0.0416 ± 0.0016	19 ± 2.3	0.0022
MAO-B	0.240 ± 0.048	67 ± 4.5	0.0036

Figure 2. Michaelis−Menten kinetic analysis data for MAO-A and -B by absorbance assay.

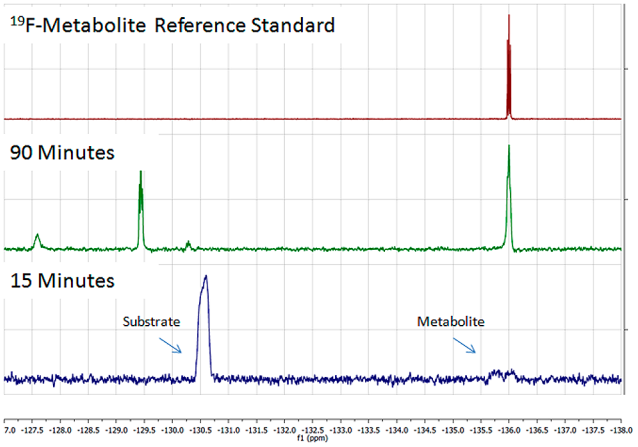


Figure 3. ^{19}F NMR experiments (376 MHz) demonstrating time-dependent formation of 2-fluoro-4-nitrophenol metabolite (−136 ppm) and secondary metabolite (−129 ppm) via oxidation of substrate **1** (−130 ppm) by MAO-A.

for 5 min. [^{11}C]**1** was then purified by HPLC and reformulated into a 10% ethanol in saline dose (392 ± 207 MBq; nondecay corrected 1.2% radiochemical yield based on starting [^{11}C]-CH_3OTf; 30 min from end of bombardment) for use in animal imaging studies. Final product was obtained in high molar activities (>259 GBq/μmol), and to prepare doses of low molar activity [^{11}C]**1**, unlabeled standard was added to the saline used for reformulation. Final molar activities were confirmed using HPLC analysis of the UV-254 nm absorbances.

MAO is known to be a highly conserved enzyme between species with, for example, high sequence homology between hMAO and rat MAO (rMAO).[14] Therefore, kinetic PET imaging studies were conducted in Sprague−Dawley rats. Experiments conducted with high (259 GBq/μmol) and low (0.0185 GBq/μmol) molar activity [^{11}C]**1** gave very similar kinetic curves for whole brain radioactivity (Figure 4). These data were also used in conjunction with specific activity to generate curves estimating mass of tracer in the brain (inset, Figure 4). For the low specific activity PET studies, 1.29 ± 0.86 μmol of **1** was administered, similar to the mass administered for prior rodent ^{19}F-MRI studies,[15] and peak

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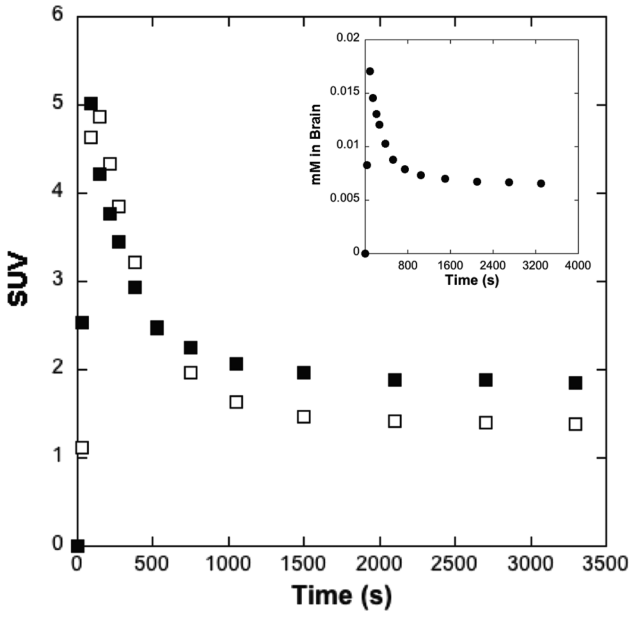

Figure 4. Main panel: averaged (n = 2) rodent whole brain time-radioactivity curves (0−60 min post-iv-injection of high (259 GBq/μmol, □) and low (0.0185 GBq/μmol, ■) molar activity doses of [^{11}C]**1**). Inset: whole brain concentration curve (0−60 min post-iv-injection of low (●) molar activity doses of [^{11}C]**1**).

uptake of **1** in the rodent brain was ∼0.02 mM. Given that the total concentration of MAO (A + B) in the rodent brain is ∼11 pmol/mg protein,[16] and that rat brain is ∼10% protein,[17] this corresponds to an estimated total rMAO concentration of ∼1.1 pmol/mg tissue (0.0011 mM). Thus, [**1**]/[rMAO] ≈ 18 and, given the high catalytic efficiency of MAO (Figure 2), we would not expect to overwhelm MAO at this concentration of **1** in the brain. Reflecting this, there was indeed no evidence that the MAO enzymes in the brain tissues were subject to *in vivo* substrate inhibition at this concentration.

In summary, enzyme substrates that form trapped metabolites appear as a promising way forward to design PET/MRS hybrid imaging agents. In the system evaluated here, the enzyme reaction produces two metabolites that are separately visible by PET or NMR. When considering potential *in vivo* applications of such a hybrid PET-MRS imaging agent in the future, if both metabolites are trapped in the tissue, they will yield a measure of the same parameter, MAO oxidation. Of more interest would be if the two methods gave different biochemical measures. How might that be achieved? Two possibilities come to mind. First, if the MRS-visible metabolite is freely diffusible across the BBB but is designed to have high affinity but reversible binding to a specific CNS site (e.g., receptor), then the retention and delayed clearance from the brain tissue might reflect the amount of specific binding (the pharmacokinetics of delivery are identical to the radiotracer trapping curve). As an alternative, if the metabolite is designed not to be BBB permeable but instead is a substrate for an efflux transporter (e.g., P-glycoprotein, Pgp), then the rate of clearance from the tissues will reflect the transporter function, as previously described for the glutathione-conjugated metabolites of radiolabeled purines.[18] Future work will address the interesting possibilities of designing bifunctional PET/MRS brain imaging tracers based on MAO, and the extension to other enzymes that can be studied using the metabolic trapping principle.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.8b00402.

Experimental procedures for chemistry and radiochemistry as well as spectra/chromatograms for all novel compounds synthesized; procedures for *in vitro* assays and *in vivo* rodent PET imaging (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BBB, blood−brain barrier; Bq, Becquerel; CNS, central nervous system; MAO, monoamine oxidase; PET, positron emission tomography; Pgp, P-glycoprotein

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