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Dihydroquinoline Carbamate Derivatives as "Bio-oxidizable" Prodrugs for Brain Delivery of Acetylcholinesterase Inhibitors: [^{11}C] Radiosynthesis and Biological Evaluation

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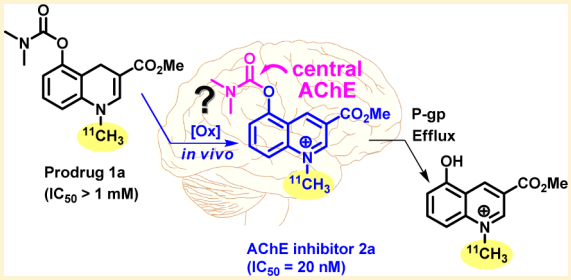
Supporting Information

ABSTRACT: With the aim of improving the efficiency of marketed acetylcholinesterase (AChE) inhibitors in the symptomatic treatment of Alzheimer's disease, plagued by adverse effects arising from peripheral cholinergic activation, this work reports a biological evaluation of new central AChE inhibitors based on an original "bio-oxidizable" prodrug strategy. After peripheral injection of the prodrug **1a** [IC_{50} > 1 mM (*h*AChE)] in mice, monitoring markers of central and peripheral cholinergic activation provided *in vivo* proof-of-concept for brain delivery of the drug **2a** [IC_{50} = 20 nM (*h*AChE)] through central redox activation of **1a**. Interestingly, peripheral cholinergic activation has been shown to be limited in time, likely due to the presence of a permanent positive charge in **2a** promoting rapid elimination of the AChE inhibitor from the circulation of mice. To support these assumptions, the radiosynthesis with carbon-11 of prodrug **1a** was developed for additional *ex vivo* studies in rats. Whole-body biodistribution of radioactivity revealed high accumulation in excretory organs along with moderate but rapid brain uptake. Radio-HPLC analyses of brain samples confirm rapid CNS penetration of [^{11}C]**1a**, while identification of [^{11}C]**2a** and [^{11}C]**3a** both accounts for central redox activation of **1a** and pseudoirreversible inhibition of AChE, respectively. Finally, Caco-2 permeability assays predicted metabolite **3a** as a substrate for efflux transporters (P-gp inter alia), suggesting that metabolite **3a** might possibly be actively transported out of the brain. Overall, a large body of evidence from *in vivo* and *ex vivo* studies on small animals has been collected to validate this "bio-oxidizable" prodrug approach, emerging as a very promising strategy in the rational design of selective central AChE inhibitors.



KEYWORDS: *Alzheimer's disease, central acetylcholinesterase inhibitors, prodrug, cyclic rivastigmine analogues, ^{11}C-radiolabeling*

Alzheimer's disease (AD) is one of the most prevalent neurodegenerative diseases within the elderly population affecting more than 20 million people worldwide. Symptoms of AD include gradual memory loss, difficulty in learning, diminished ability to execute routine tasks, and further cognitive impairments. Although the exact cause and progression of AD are still poorly understood, the two main pathological hallmarks are intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein and extracellular senile plaques of aggregated amyloid β-peptide, both contributing to neuronal cell death.[1,2] There is currently much effort being devoted to the design of new drugs capable of inhibiting the formation of amyloid plaques[3] and neurofibrillary tangles[4,5] to interrupt or prevent neuronal degeneration. The loss of basal forebrain cholinergic neurons, resulting in the reduction of synaptic availability of acetylcholine, is also a constant feature of AD, which is held responsible for the cognitive deficits observed in Alzheimer's disease. Despite huge research efforts made in developing curative pharmacological treatments, only symptomatic treatments mainly based on the cholinergic strategy are currently available.[6] This strategy relies on the inhibition of acetylcholinesterase (AChE), resulting in elevated levels of acetylcholine in brain areas involved in memory and cognitive functions (i.e., the hippocampus, nucleus basalis of Meynert, and cortex). Thus, rivastigmine, donepezil, and galantamine, which can be used in combination with the NMDA receptor antagonist memantine, are the only three marketed AChE inhibitors prescribed to patients with mild-to-moderate AD.[7,8] At early stages of AD, these AChE inhibitors

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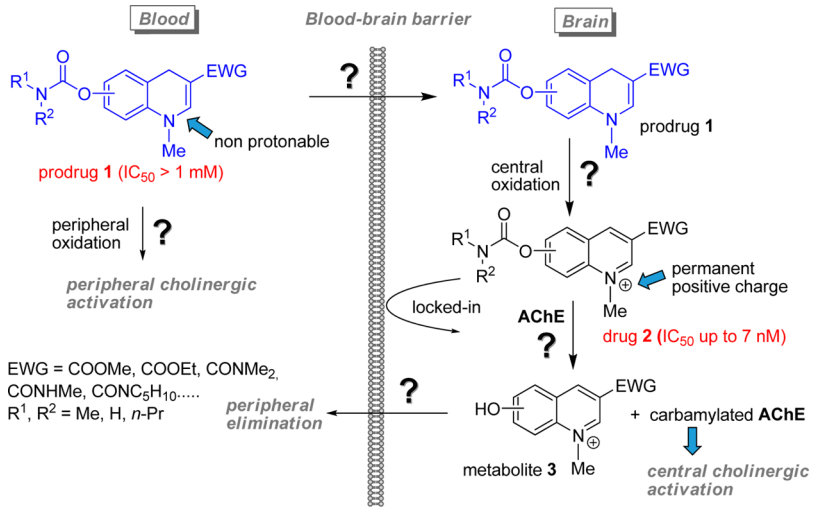

Figure 1. Rational design of central selective AChE inhibitors by means of a "bio-oxidizable" prodrug approach.

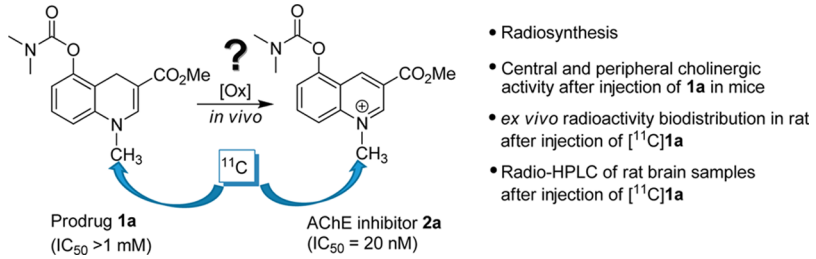

Figure 2. Prodrug **1a** candidate for *in vivo* assays in mice.

display a clear-cut preference for inhibition of AChE activity in the central nervous system (CNS) and demonstrate moderate but significant positive effects on cognitive functions. However, more pronounced cholinergic dysfunction in advanced stages of AD requires higher doses of AChE inhibitors, which trigger adverse peripheral cholinergic effects.[9] As a consequence, the design of new approaches aimed at developing highly CNS-selective AChE inhibitors free from adverse peripheral effects is therefore highly desirable. In addition, there has been renewed interest in the search for new AChE inhibitors since the peripheral anionic site of AChE has been identified as playing a key role in the pathological process of β-amyloid peptide aggregation.[10−12] It is worth noting that most common AChE inhibitors such as rivastigmine, donepezil, or galantamine are protonated at physiological pH, resulting in a positive charge, which binds to aromatic residues in the catalytic site or in the middle of the gorge via π−cation interaction. We recently reported on the rational design of CNS-selective AChE inhibitors by implementing a "bio-oxidizable prodrug" strategy based on the temporary masking of this positive charge, which is deemed to play a critical role in the binding with AChE (Figure 1).[13] Cyclic analogues of rivastigmine, namely, 1,4-dihydroquinoline carbamate derivatives, **1**, have appeared as relevant "bio-oxidizable prodrug" candidates. Indeed, the low basicity of the nitrogen enamine prevents protonation of the prodrug **1** at physiological pH, while ensuring high lipophilicity to cross the blood−brain barrier (BBB) by simple passive diffusion. Once in the CNS, the prodrug **1** is expected to be converted into the parent drug **2** thanks to a redox-activation step putatively mediated by the NAD(P)H/NAD(P)$^+$ coenzyme system. As rivastigmine, the resulting quinolinium salt **2** is expected to act as a pseudoirreversible AChE inhibitor by carbamylation of the serine hydroxyl group at the AChE catalytic site to produce the decarbamylated metabolite **3**. Interestingly, the presence of a permanent positive charge in **2** is expected not only to trap the AChE inhibitor in the CNS ("locked-in" effect) but also to promote rapid systemic elimination of the quinolinium salt **2** that could have been formed in the peripheral system. This redox approach has already been investigated by Bodor and Prokai by means of 1,4-dihydropyridines as carriers for targeting a variety of drugs to the brain[14,15] but remains almost unexplored to develop new prodrug strategies.[16]

In our previous work,[13] a large set of dihydroquinoline **1**/quinolinium salt **2** couples were synthesized by varying the position and the nature of both carbamate and electron withdrawing group (EWG) at C-3. This functional group at C-3 was found to be essential in the design of the prodrug **1** to stabilize the enamine character of the double bond. A preliminary *in vitro* evaluation on human AChE revealed that while several of these quinolinium carbamate derivatives **2** could display high AChE inhibitory activities (IC_{50} up to 7 nM), all the corresponding prodrugs **1** proved to be inactive (IC_{50} > 1 mM).[13] These promising *in vitro* assays give strong support to the assumption that the crucial positive charge in quinolinium salts **2** is entirely masked in the prodrug form **1**. At this stage, key to the success of this "bio-oxidizable" prodrug strategy is intimately tied to the preferential redox activation of prodrug **1** in the CNS versus periphery.

We report herein *in vivo* experiments to validate this critical activation step by investigating the central and peripheral cholinergic activity profile in mice after administration of the prodrug **1** (Figure 2). Most prodrugs **1** previously reported[13] have revealed too low solubility in physiological solution to be

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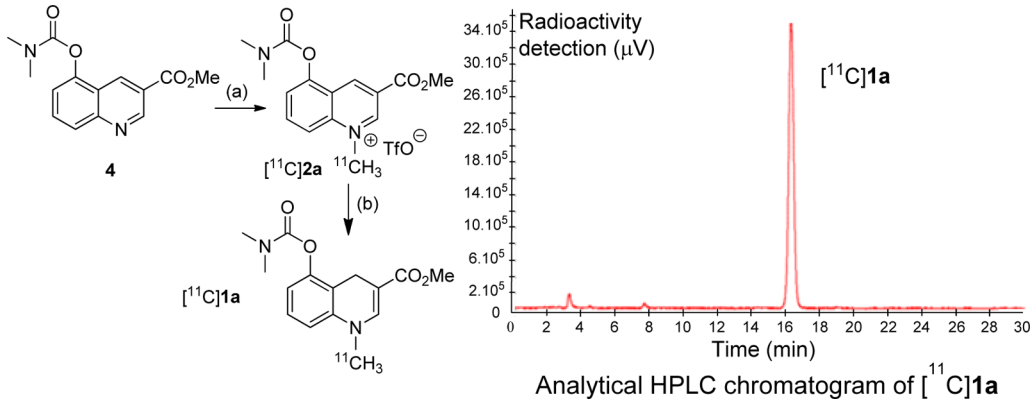
Scheme 1. Radiosynthesis of [^{11}C]-Prodrug 1a[a]



[a]Reagents and conditions: (a) [^{11}C]MeOTf/CH_3CN, 20 °C, 8 min; (b) *N*-benzyl-1,4-dihydronicotinamide/CH_3CN, 20 °C, 5 min.

used as such during *in vivo* experiments on mice. To circumvent this issue, we decided to create inclusion complexes between 1,4-dihydroquinolines **1** and the readily available hydroxypropyl-β-cyclodextrin (HPBCD). Among the various prodrugs **1** tested, only prodrug **1a** (EWG = CO_2Me, $R^1 = R^2 =$ Me at C-5) provided an inclusion complex stable in physiological solution for several days (up to 3 days; the solubility of the prodrug **1a** in such a complex was found to reach 20 g/L). Consequently, on the basis of *in vitro* AChE inhibitory activity of the quinolinium salts **2** and the ability of the corresponding 1,4-dihydroquinolines **1** to form stable complexes with HPBCD in physiological solution, prodrug **1a** was selected for *in vivo* activity studies (Figure 2). In parallel, we undertook the radiosynthesis with carbon-11 ($t_{1/2}$ = 20.4 min, β^+ emitter) of **1a** in order to perform *ex vivo* experiments in rats. These studies will enable us (a) to evaluate the whole-body biodistribution of the radioactivity after injection of the prodrug [^{11}C]**1a** and (b) to study the oxidation rate of the 1,4-dihydroquinoline [^{11}C]**1a** and to follow the behavior of the parent drug [^{11}C]**2a** in the brain.

RESULTS AND DISCUSSION

Radiosynthesis of the Prodrug [^{11}C]1a. In our previous studies, 1,4-dihydroquinoline-based derivatives were labeled with carbon-11 according to a two-step procedure involving the quaternization of the corresponding quinolines with [^{11}C]-methyl triflate followed by regioselective 1,4-reduction of the resulting quinolinium salts.[17,18] This approach could be successfully applied to the radiosynthesis of prodrug [^{11}C]**1a** with a good radiochemical yield (Scheme 1). Thereby, quaternization reaction of quinoline **4** with [^{11}C]methyl triflate afforded quinolinium salt [^{11}C]**2a** with a 95% incorporation rate of [^{11}C]CH_3OTf. The subsequent reduction step was carried out by means of *N*-benzyl-1,4-dihydronicotinamide (BNAH) to give the desired 1,4-dihydroquinoline [^{11}C]**1a** with an excellent radiochemical yield of 95%. It is worth noting that the radiosynthesis was conducted in an efficient one-pot strategy without any intermediate purification of the quinolinium salt [^{11}C]**2a**. To ensure a reproducible production for *in vivo* preclinical studies, these conditions were used to perform the automation on a GE TRACERlab FX-MeI and FX-M. Prodrug [^{11}C]**1a** was obtained with a radiochemical purity greater than 95% in only 50 min from quinoline **4**, including HPLC purification and formulation.

Central and Peripheral AChE Activity Profile of Prodrug 1a in Mice. The permeability of prodrug **1a** was first evaluated using the cell-based Caco-2 (pH 7.4/7.4) system as a surrogate BBB model. Bidirectional analysis was conducted to assess the propensity of prodrug **1a** to act as a substrate for efflux mechanisms expressed in Caco-2 as well as a measure of passive diffusion. By this assay, the so-obtained data [P_{app}(A–B) = 53 × 10^{-6} cm·s^{-1} and P_{app}(B–A) = 32 × 10^{-6} cm·s^{-1}, **1a** at 10^{-5} mol L^{-1}] suggest that **1a** both displayed no active efflux and should be highly permeable to the blood–brain barrier. The same permeability assay was performed for **2a** [P_{app}(A–B) = 0.2 × 10^{-6} cm·s^{-1} and P_{app}(B–A) = 1.3 × 10^{-6} cm·s^{-1}, **2a** at 10^{-5} mol L^{-1}], predicting both poor brain penetration and no efflux at the BBB of the parent drug **2a** expected to be formed centrally by redox activation of the prodrug **1a**. After having determined the median lethal dose (LD_{50}) of prodrug **1a** (25 mg/kg), we then considered various markers of central and peripheral cholinergic activation for prodrug **1a** using tacrine as reference compound. Hypothermia is a useful index of central cholinergic activation in experimental animals.[19] After intraperitoneal injection of **1a** (10 mg/kg), body temperature was measured at regular time intervals, and the resulting data were compared with those obtained with tacrine. Whereas prodrug **1a** induced a significant hypothermic response with a maximum effect at 60 min, tacrine displayed maximum hypothermia at 30 min and a similar response profile when administered at the same dose (10 mg/kg). In a parallel *ex vivo* experiment, residual AChE activity was determined in mice cortex after injection of prodrug **1a**. Data revealed a tight correlation between inhibition of AChE activity in mice cortex and hypothermic response elicited by prodrug **1a**, thus confirming that hypothermia is a reliable marker of central cholinergic activation for prodrug **1a**. More importantly, the central cholinergic activation observed also gave strong evidence that redox-activation of prodrug **1a** occurred within the CNS (Figure 3).

Further evidence of a redox activation of prodrug **1a** in the CNS was provided by investigating the effect of prodrug **1a** on shivering, which is also a reliable index of central cholinergic activation in experimental animals (Figure 4).[20] Analysis of the shivering score after peripheral administration of prodrug **1a** (3 mg/kg, ip) revealed a significant response, which confirms a central cholinergic stimulation ascribable to the presence of quinolinium salt **2a** in the CNS. Interestingly, a 10-fold higher dose of tacrine (30 mg/kg, ip) was necessary to attain identical response profile with the same amplitude level. Thus, these

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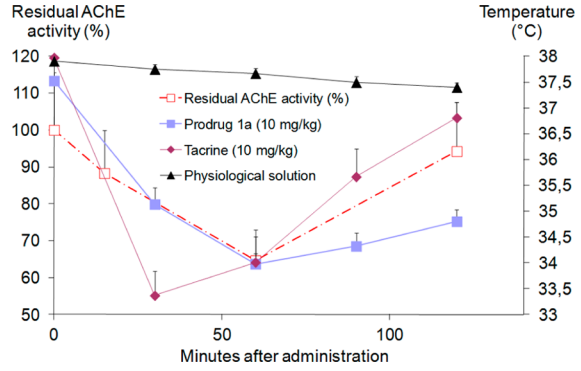

Figure 3. Central cholinergic activation. Hypothermic effect of prodrug **1a** (■), tacrine (◆), and physiological solution (▲) in mice. Residual cortex AChE activity (□) after administration of prodrug **1a**.

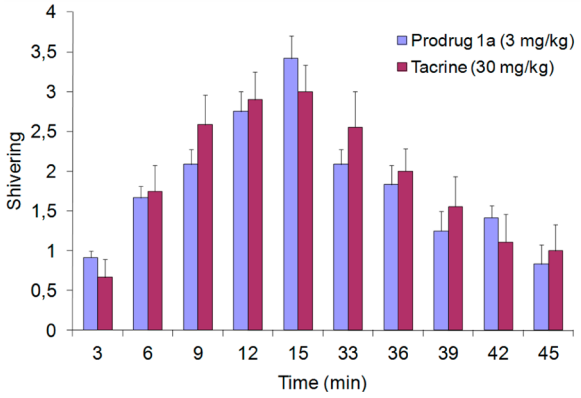

Figure 4. Central cholinergic activation. Induction of shivering after injection of prodrug **1a** (3 mg/kg) and tacrine (30 mg/kg).

preliminary *in vivo* observations of central cholinergic stimulation (hypothermia and shivering) validate the fundamental step of our strategy, demonstrating that after having reached the brain through the BBB by passive diffusion, prodrug **1a** is subsequently converted to the parent drug **2a** to exhibit a central cholinergic activity.

We then investigated the peripheral activity profile of prodrug **1a** by monitoring salivation score, a good marker of peripheral cholinergic activation.[20] In a comparative study, prodrug **1a** (3 mg/kg) and tacrine (30 mg/kg) were administered intraperitoneally. These doses were chosen so that a comparison of the peripheral cholinergic activation is made while, as determined in Figure 4, prodrug **1a** and tacrine are equipotent in inhibiting central acetylcholinesterase. As shown in Figure 5, salivation was observed after 15 min with both prodrug **1a** and tacrine. However, while tacrine still induced salivation after 30 and 45 min, no peripheral cholinergic sign could be detected with prodrug **1a** after 15 min. Interestingly, although this study revealed a partial peripheral oxidation of prodrug **1a**, the resultant permanently charged quinolinium salt **2a** is rapidly eliminated from the peripheral circulation resulting in only limited peripheral activity. Further optimization of prodrug **1a** should lead to a good compromise between stability of the 1,4-dihydroquinoline ring in the peripheral system and its redox activation in the CNS to optimize brain delivery of these AChE inhibitors.

Thereafter, we sought to correlate central and peripheral AChE activity profile of prodrug **1a** observed in mice with

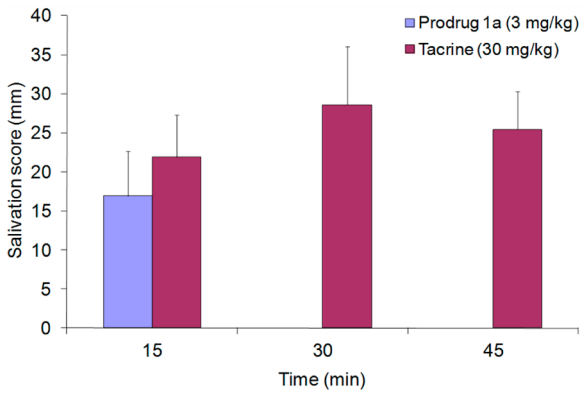

Figure 5. Peripheral cholinergic activation. Induction of salivation after injection of prodrug **1a** (3 mg/kg) and tacrine (30 mg/kg).

further *ex vivo* experiments in rats by means of the radiolabeled prodrug [^{11}C]**1a**.[21] Although the amount of [^{11}C]**1a** administered in rats is well below pharmacological doses of **1a** administered in mice, whole-body biodistribution of the radioactivity in rats and radio-HPLC analyses of rat brain may be useful to predict the pharmacokinetics of **1a** and to highlight the main crucial steps of this "bio-oxidizable" prodrug approach; namely, (1) brain penetration and (2) central redox activation of prodrug **1a** prior to (3) carbamylation of AChE by drug **2a**.

Biodistribution Studies in Rat after Injection of [^{11}C] 1a. We first evaluated the *in vitro* stability of [^{11}C]**1a** in rat blood by radio-HPLC showing that neither traces of oxidation product [^{11}C]**2a** nor any other degradation product were detected after 1 h. We then investigated the profile of the radioactivity in various rat organs (salivary glands, lung, heart, liver, spleen, adrenal glands, kidney, bladder, intestine, blood, and brain) after [^{11}C]**1a** administration. The animals were euthanized followed by microdissection at 5, 10, 20, and 30 min postinjection, and an extra time of 60 min has been chosen for the brain. Uptakes were examined by measuring percentage of injected dose per gram of tissue (%ID/g) and are summarized in Figure 6. The results show a widespread distribution of the radioactivity among peripheral organs (Figure 6a). However, the highest uptake was seen in the adrenal glands (1.7 %ID/g) at 10 min after injection of [^{11}C]**1a** and decreased to about 0.7% at 30 min. This finding is consistent with a recent *in vivo* study highlighting the relationship between the high level of radioactivity observed in the adrenal glands after administration of [^{11}C]donepezil in the rat and the high activity of AChE measured by fluorometric assays in this organ.[22] In our case, the radioactivity uptake observed in adrenal glands would emanate from peripheral redox activation of prodrug [^{11}C]**1a** into [^{11}C]**2a**. Accumulation of the radioactivity was significant in the excretory organs, the kidneys and the liver (0.84−0.88 %ID/g), at 10 min after [^{11}C]**1a** injection and lower in the spleen, heart, and lung (0.26−0.39 %ID/g). At 30 min after [^{11}C]**1a** injection, a high accumulation of radioactivity (1.32 % ID/g) was found in the bladder indicating a fast elimination. The radioactivity kinetics of rat brain uptake were also investigated (Figure 6b). The radioactivity brain uptake was 0.27 %ID/g at 5 min, 0.28 % ID/g at 10 min, and declining from 0.11 to 0.06 %ID/g at 20 and 60 min, respectively. In line with the central and peripheral AChE activity profile of **1a** observed in mice, whole-body biodistribution of [^{11}C]**1a** in rat revealed high

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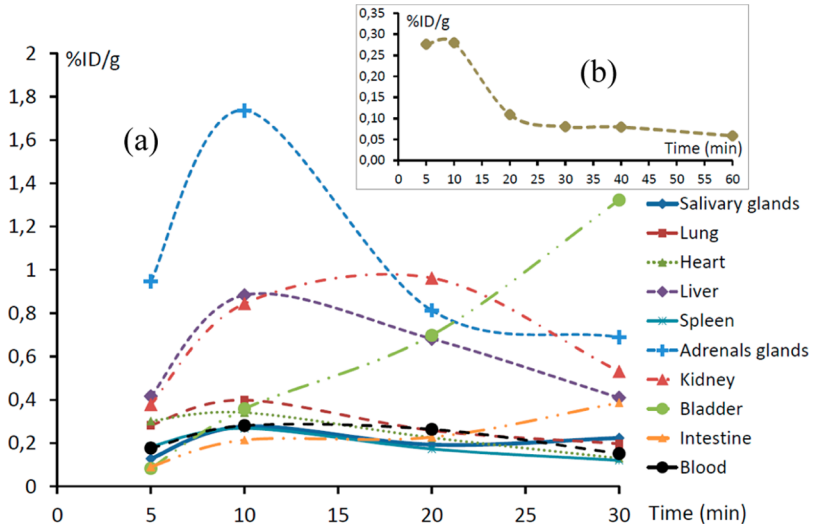

Figure 6. *Ex vivo* biodistribution of the radioactivity (a) in various tissues and (b) in brain at different time intervals following tail vein injection of [^{11}C]**1a** into the rat. Results are expressed in percent of injected dose per gram (%ID/g). Each point represents an average value of three samples. Uptake in adrenal glands is significantly different at 5 min vs tissue ($P < 0.05$) and at 10 min vs time and tissue ($P < 0.001$).

accumulation in excretory organs, while showing a moderate but rapid brain uptake with a progressive and slow clearance.

HPLC Analyses of Brain after Injection of [^{11}C]1a in Rats. Thereafter, we aimed to identify and monitor over time two radioactive species, namely, the quinolinium salt [^{11}C]**2a** and the decarbamylated product [^{11}C]**3a** putatively formed according to this "bio-oxidizable" prodrug strategy. Rats were then injected with [^{11}C]**1a** and subsequently euthanized at 5, 10, 20, 30, and 60 min postinjection. After treatment, brain samples were analyzed by radio-HPLC to determine the nature of the radioactivity in the brain (Figure 7). In addition to the

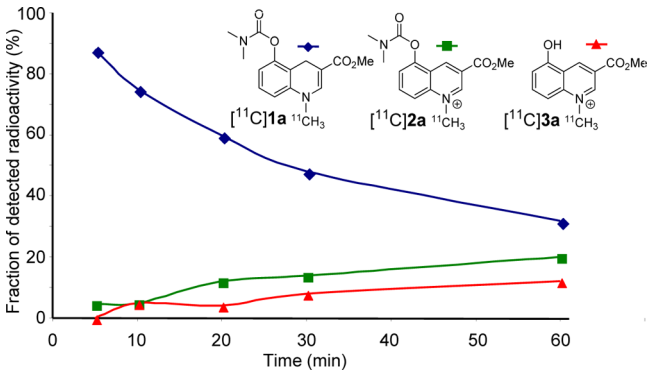

Figure 7. Percentage of [^{11}C]**1a**, [^{11}C]**2a**, and [^{11}C]**3a** in brain and at different time intervals after tail vein injection of [^{11}C]**1a** into rat.

prodrug [^{11}C]**1a** (t_R = 16 min), the HPLC profile disclosed three radioactive species more hydrophilic than [^{11}C]**1a**, and two of them have retention times matching perfectly with those of the drug **2a** (t_R = 3.5 min) and the decarbamylated metabolite **3a** (t_R = 2.5 min). Various hypotheses have been put forward to elucidate the chemical structure of the third radioactive species. Unfortunately, we faced several difficulties during the preparation of these putative metabolites, which brought our efforts toward identification of this third radioactive species to a halt. While [^{11}C]**1a** represents 88% of the radioactivity at 5 min postinjection to drop after 60 min to 32%, the percentage of radioactivity arising from [^{11}C]**2a** and [^{11}C]**3a** slightly increases over time, not exceeding 20% and 12%, respectively. Both disappearance of [^{11}C]**1a** and low levels of [^{11}C]**2a** are fully consistent with a central redox activation of [^{11}C]**1a** followed by carbamylation of AChE by [^{11}C]**2a**. The poor accumulation of the resulting decarbamylated quinolinium salt [^{11}C]**3a** suggests that efflux transporters expressed at the BBB may be involved in the active transport of **3a** out of the brain. To shed light on this hypothesis, a bidirectional Caco-2 permeability assay was used to probe whether the positively charged quaternary ammonium metabolite **3a** is a substrate for efflux pumps. While no A−B permeability was observed, the permeability value in the B−A direction foresees **3a** as a substrate for efflux transporters [P_{app}(B−A) = 23 × 10^{-6} cm·s^{-1}, **3a** at 10^{-5} mol L^{-1}, pH 7.4/7.4]. An additional Caco-2 permeability assay in the presence of verapamil, a known P-gp inhibitor, revealed that efflux of **3a** is significantly reduced [P_{app}(B−A) = 7 × 10^{-6} cm·s^{-1}, **3a** at 10^{-5} mol L^{-1} (verapamil), pH 7.4/7.4]. Thus, efflux of [^{11}C]**3a** across the BBB, mediated by P-gp efflux transporter, accounts for the progressive elimination of the radioactivity from the brain observed in Figure 6b.

CONCLUSION

To conclude, *in vivo* and *ex vivo* studies in small animals for evaluation of new central selective AChE inhibitors based on an original "bio-oxidizable" prodrug strategy were performed. Investigation of the central and peripheral activity profile of prodrug **1a** in mice demonstrated a substantial preference for central activity as demonstrated by a pronounced induction of hypothermia and shivering (markers of central cholinergic activation), while eliciting relatively modest salivation effect (index of peripheral cholinergic activation). These findings provided *in vivo* evidence that after crossing the BBB, prodrug **1a** is subjected to central redox activation to furnish drug **2a**, which can subsequently exert a central cholinergic activation likely by carbamylation of AChE. This scenario was supported by *ex vivo* studies in rats carried out with the radiolabeled prodrug [^{11}C]**1a**. A two-step one-pot protocol was developed for the radiosynthesis with carbon-11 of prodrug **1a** affording [^{11}C]**1a** in 50 min with a good overall radiochemical yield and

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high radiochemical purity (>99%). While rapid brain penetration of [^{11}C]**1a** was confirmed by measurement of brain radioactivity uptake, radioactive species [^{11}C]**2a** and [^{11}C]**3a** could be also identified by HPLC. These results highlighted not only the central redox activation of the prodrug **1a** but also the pseudoirreversible nature of AChE inhibition by **2a**. The profile of the brain metabolites is a crucial aspect to pursue the development of this new prodrug strategy and needs to be further investigated through more detailed studies. Interestingly, *in vitro* bidirectional permeability and transporter assays on Caco-2 cell line disclosed that metabolite **3a** is a substrate of P-gp efflux transporter, suggesting that once central AChE is carbamylated by drug **2a**, metabolite **3a** would efflux back into the circulation. Although further investigations remain to be conducted, especially to adjust the balance between peripheral stability and central activation of the prodrug, this "bio-oxidizable" prodrug approach appears as a promising strategy to develop new centrally active AChE inhibitors devoid of adverse effects for improving current symptomatic treatment of AD. Further research works are ongoing in our laboratory to apply this prodrug strategy in the design of new central dual-binding-site AChE inhibitors[23,24] that, beyond restoring the cholinergic balance, might also prevent amyloid plaque formation.

METHODS

General Methods. In the radiochemistry procedure, [^{11}C]CO_2 was produced by the ^{14}N(p,α)^{11}C nuclear reaction from a cyclotron (IBA, Cyclone 18/9) using a gas target filled with nitrogen containing 0.5% oxygen and irradiated with a proton beam. All chromatography solvents were purchased from Sigma-Aldrich and were used without further purification. Analytical HPLC was realized with a system equipped with a Merck L-6200 pump and a Merck L-4250 detector in series with a Novelec β^+ flow detector. The identity of the labeled compounds was confirmed by its coelution with the unlabeled reference compound onto the HPLC system. For each described analysis, the characteristics of the column and solvent used will be described.

Radiochemistry. In highly radioactive chemistry, radiosyntheses were performed in a hot cell using an automatic apparatus (GE Healthcare TRACERlab FX-MeI and FX-M). [^{11}C]Methyl triflate was trapped at 20 °C during 8 min in a solution containing the quinoline **4** (1.5 mg, 5.4 μmol) in acetonitrile (350 μL). Then, a solution of BNAH (2 mg, 9.3 μmol) in CH_3CN (200 μL) was added to the mixture. The resulting mixture was kept at 20 °C for 5 min. The purification was performed by reverse-phase semipreparative HPLC (Phenomenex Gemini column 10 mm × 250 mm, 4 mL/min, λ = 254 nm, CH_3CN/Na_2HPO_4 10 mM, 50:50). The fraction containing the labeled product [^{11}C]**1a** (t_R = 9 min) was collected into a flask containing water (50 mL) and passed through a solid phase extraction cartridge (C18ec, Macherey Nagel chromabond). [^{11}C]**1a** was eluted with ethanol (700 μL) and diluted with an isotonic saline solution (7 mL). The radiolabeled product was filtered through a sterile filter 0.22 μm. The radiochemical and chemical purities were determined by reverse-phase analytical HPLC (Phenomenex Gemini column 4.6 mm × 250 mm, 1 mL/min, λ = 254 nm, $CH_3CN/AcONH_4$ 10 mM, 40:60, t_R([^{11}C]**1a**) = 13 min). Radiochemical purity of [^{11}C]**1a** exceeded 99%, and batches of 2000−2200 MBq were ready for injection in about 50 min.

***Ex Vivo* AChE Inhibition Assay in Brain Cortex of Mice.** Groups of six Swiss albino mice, weighing 20−25 g upon arrival, purchased from Charles River Laboratories (Domaines des Oncins, Saint Germain sur l'Arbresle, France), were treated with prodrug **1a** (10 mg/kg, ip) and killed at various times thereafter (15 min, 1 h, and 2 h postinjection). The brains were immediately isolated and frozen at −80 °C until next step of the procedure. Then, the frontal cortex of each mouse was isolated from other cerebral structures as fast as possible at 0 °C and homogenized in a physiological solution (NaCl 9 g/L, 500 μL). From the resulting suspension, 20 μL were collected and subjected to Ellman's test[25] (brain suspensions replacing RBC membranes as source of acetylcholinesterase). The results were expressed as total cholinesterase activity in UI. Protein content was determined by the method of Lowry in order to deduct the cholinesterase activity per milligram of fresh tissue. From this experiment, we could deduce the percentage of central residual cholinesterase activity in treated mice compared with that of mouse cortex of a control group.

***In Vivo* Studies.** Swiss albino mice, weighing 20−25 g upon arrival, were purchased from Charles River Laboratories (Domaines des Oncins, Saint Germain sur l'Arbresle, France). The animals had free access to water and food and were kept in a well-ventilated room at a temperature of 21 ± 1 °C, under a 12-h light/dark cycle (lights on between 7:00 h and 19:00 h). The procedures used in this study are in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Inclusion of prodrug **1a** in hydroxypropyl-β-cyclodextrin (HPBC) was required to improve its poor solubility. A solution of prodrug **1a** (61 mg, 0.21 mmol) and hydroxypropyl-β-cyclodextrin (DS-0.58, MW = 1386 g/mol) in CH_2Cl_2 (15 mL) and MeOH (10 mL) was prepared. The clear solution was evaporated under reduced pressure at room temperature. The resulting solid was then dissolved in Milli-Q water (3 mL) and filtered through a 45 μm pore size filter. The filter was rinsed with Milli-Q water (9 mL). The aqueous solution was freeze-dried affording a pale yellow powder containing 4 wt % of prodrug **1a** (mol/mol ratio for the complex HPBCD/prodrug **1a**, 5/1). ^{1}H NMR of the complex HPBCD/prodrug **1a** (D_2O) δ 7.30 (s, 1H), 7.20 (dd, J=7.7 and 8.9 Hz, 1H), 6.74 (m, 2H), 4.60−5.30 (HPBCD), 3.30−4.10 (HPBCD), 3.18 (s, 3H), 2.95 (s, 3H), 1.11 (HPBC). The solubility of the included prodrug **1a** was found to reach up to 20 g/L in a 9 g/L NaCl aqueous solution. The resulting complex HPBCD/prodrug **1a** was used in all following *in vivo* studies.

Acute Toxicity (Determination of the LD_{50} of Prodrug 1a). The toxicity study was carried out using male Swiss albino mice (20−25 g). Animals were kept in a temperature-controlled environment (21 ± 1 °C) with a 12 h light/dark cycle, and food and water were freely available. The animals were divided into one control group and five treated groups, each group consisting of six animals. The control group received saline, and each treated group received compound **1a** in a dose of 1, 3, 10, 30, and 100 mg/kg by intraperitoneal injection. The animals were observed continuously for 3 h, and then they were observed every 8 h during 72 h after administering the compound to observe any changes in general behavior or other physiological activities. At the end of the experiment, animals were sacrificed by cervical dislocation. The lethal dose 50 (LD_{50}) is the injected dose that causes mortality of 50% of animals. For the compound **1a**, the LD_{50} was approximately equal to 25 mg/kg.

Central Cholinergic Activation. *Hypothermia.* Temperature measurements were performed in a temperature-controlled room (21 ± 1 °C). Colonic temperature was measured with a thermistor probe (Physitemp TH5, sonde RM6, Clifton, NJ, USA) gently inserted to a depth of 2.5 cm into the rectum and maintained about 20 s. After isolating mice during a period of at least 30 min, their initial colonic temperature (t = 0) was recorded. Then, different groups of mice (n = 6−12) received either prodrug **1a** at various concentrations (0.1 to 30 mg/kg, ip) or solvent (NaCl 9 g/L in water). The colonic temperature was measured for each animal at 30 min intervals, starting 30 min postinjection and ending after 120 min.

Shivering. Experiments were performed in a temperature-controlled room (21 ± 1 °C). The intensity of shivering was classified into four grades as follows: the quotation was 4 if the whole body of the mouse trembled with convulsions, 3 if the whole body of the mouse trembled, 2 if the head and superior limb of the mouse trembled, 1 if only the head trembled, and 0 if no shivering was observed. This evaluation was performed on different groups of mice (n = 6−12) receiving either prodrug **1a** at various concentrations (0.1 to 30 mg/kg, ip) or solvent (NaCl 9g/L in water). This evaluation was made for each animal

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during 3 min at 3 min intervals, starting immediately after injection and ending after 90 min.

Peripheral Cholinergic Activation. *Salivation.* Salivation measurements were performed in a temperature-controlled room (21 ± 1 °C). A blotting paper was applied on the corner of the animal's mouth. The migration of saliva on this paper was measured in millimeters. In a physiological normal state, no migration was observed. This evaluation was performed on different groups of mice (n = 6−12) receiving either prodrug **1a** at various concentrations (0.1 to 30 mg/kg, ip) or solvent (NaCl 9 g/L in water). This evaluation was made for each animal at 15 min intervals, starting immediately after injection and ending after 45 min.

Biological Evaluation of the Prodrug [^{11}C]1a. *Animals.* The animal investigations were performed under the European directive (86/609/EU) and the French National Committee (decree 87/848) for the care and use of laboratory animals (GIP Cyceron; approval D14-118-001). Permission was sought and obtained for all experimental procedures from the regional committee on animal ethics (CENOMEXA 1112.17). Sprague−Dawley male rats weighing 260−410 g were used in all experiments. The animals were kept at constant temperature (22 °C) and humidity (50%) with 12 h light/dark cycles and were allowed free access to food and water until experiment time. Anesthesia was induced with 5% halothane in a gas mixture of nitrous oxide/oxygen (70/30%) and maintained with 2−2.5% halothane during the entire surgical procedure. They were placed on a padded heating pad (37 °C). A catheter was inserted into the tail vein.

Radioactivity Uptake in Rat Brain. Rats were injected via a tail vein with [^{11}C]**1a** (22−157 MBq) formulated in a solution containing NaCl 0.9%/ethanol (v/v 90/10) and were killed by decapitation at 5, 10, 20, 30, 40, and 60 min postinjection. Whole brain was quickly dissected. The brain sample was weighed, and the radioactivity was measured in a γ-counter (Cobra 2 gamma counter, Packard). Data were expressed as the percentage of injected dose (decay-corrected) per gram of tissue (% ID/g).

HPLC Analyses in Rat Blood and Brain Samples. Rats were injected via a tail vein with [^{11}C]**1a** (22−157 MBq) formulated in a solution containing NaCl 0.9%/ethanol (v/v 90/10) and were sacrificed at various time points after the administration (5, 10, 20, 30, 40, and 60 min). Whole brain was quickly dissected and rinsed with saline solution to minimize residual blood. Brain was crushed (UltraTurrax T25, Janke and Kunkel) in methanol (2.5 mL) and centrifuged (4000g, 10 min, 4 °C). The radioactivity of the precipitate was measured to quantify the efficiency of the methanol extraction. Supernatants were filtered and injected onto HPLC (Phenomenex Gemini column 7.8 mm × 250 mm, 5 mL/min, λ = 254 nm, CH_3CN/$AcONH_4$ 10 mM, 40:60, t_R([^{11}C]**1a**) = 16 min). An intracardiac blood sample was collected, and after centrifugation (4000g, 3 min, 4 °C), plasma was mixed with 3 volumes of methanol and centrifuged (4000g, 7 min, 4 °C). The radioactivity of the precipitate was measured to quantify the efficiency of the methanol extraction. Supernatants were filtered and injected onto HPLC (conditions described above). The detected peaks were integrated, and their areas were expressed as a percentage of the sum of areas of all radioactive compounds present (decay-corrected).

Biodistribution Studies. Rats were injected via a tail vein with [^{11}C]**1a** (22−157 MBq) formulated in a solution containing NaCl 0.9%/ethanol (v/v 90/10) and were killed by decapitation at 5, 10, 20, and 30 min postinjection. Organs of interest were removed, weighed, and gamma counted using a NaI crystal well-type gamma counter, and the activity in organs and tissues was subsequently calculated as percentage of the injected dose per gram per organ (%ID/g). Results were expressed as mean ± standard error of mean (SEM). Statistical analyses were performed by SigmaStat software (Jandel Scientific, Germany). Data were analyzed by a two-way analysis of variance (ANOVA) with tissue and time as factors. If a significant difference appeared (p values <0.05) a *post hoc* Tukey test was performed.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedure for the preparation of compound **3a** including ^{1}H NMR and ^{13}C NMR spectra, high-resolution MS data, *in vitro* AChE inhibition assay, and Caco-2 permeability assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

P.B., F.M., F.G., L.B., and V.L. conceived of the project; F.G. and M.I. developed the radiosynthesis of [^{11}C]**1a** and performed metabolites studies; V.G., F.A., and M.-L.T. synthesized compounds **4**, **1a**, **2a**, and **3a** and prepared inclusion complex of **1a** with hydroxypropyl-β-cyclodextrin; P.B. performed *in vivo* experiments in mice; M.D. performed *ex vivo* experiments in rats; C.P., F.G., and V.L. wrote the manuscript. All authors edited and approved the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AD, Alzheimer's disease; AChE, acetylcholinesterase; BBB, blood−brain barrier; CNS, central nervous system; HPBCD, hydroxypropyl-β-cyclodextrin; IC_{50}, drug concentration causing 50% inhibition of acetylcholinesterase; ip, intraperitoneally; LD_{50}, median lethal dose; P-gp, P-glycoprotein

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