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A new class of bi and trifunctional sugar oximes as antidotes against organophosphorus poisoning.

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Abstract

Recent events demonstrated that organophosphorus nerve agents are a serious threat for civilian and military populations. The current therapy includes a pyridinium aldoxime reactivator to restore the enzymatic activity of acetylcholinesterase located in the central nervous system and neuro-muscular junctions. One major drawback of these charged acetylcholinesterase reactivators is their poor ability to cross the blood-brain barrier to reach the centrally inhibited enzymes. Many strategies have been evaluated over the years to overcome this weakness. In this study, we propose to evaluate glucoconjugated oximes devoid of permanent charge as potential central nervous system reactivators. We determined their *in vitro* reactivation efficacy on organophosphorus inhibited human acetylcholinesterase and the crystal structure of two compounds in complex with the enzyme. We also determined their protective index on intoxicated mice as well as their pharmacokinetics. In order to gain a fine understanding of our compounds trafficking through the blood-brain barrier, we evaluated the endothelial

permeability coefficients of our molecules with a human *in vitro* model. This study shed light on the structural restraints of new sugar oximes designed to reach the central nervous system through the active glucose transporter located at the blood-brain barrier.

1. Introduction

The recent use of organophosphorus nerve agents (OPNAs) such as VX against Kim Jong-Nam in Malaysia in 2017 ¹ or Novichoks in march 2018 against a former Russian spy, Sergei Skripal and his daughter Yulia ² demonstrates that these compounds are still a real threat for the civilian and military populations. Commonly, nerve agents affect the cholinergic neurotransmission by phosphorylation of the catalytic serine residue of acetylcholinesterase (AChE, EC 3.1.1.7), a key enzyme for nerve impulse termination, located in the central nervous system (CNS) and at the neuromuscular junctions. This covalent binding leads to the irreversible inhibition of the enzyme, accumulation of the neurotransmitter acetylcholine, and subsequently to a cholinergic crisis characterized by nausea, dyspnea, seizure and death if not treated rapidly ³. The current therapy for OPNAs poisoning associates a muscarinic antagonist drug (e.g. atropine), an anticonvulsant drug (e.g. diazepam) and a pyridinium aldoxime reactivator (pralidoxime, trimedoxime, obidoxime, HI-6)⁴ able to remove the phosphyl group attached to the catalytic serine residue. These permanently charged quaternary oximes present an adequate pKa for the nucleophilic oxime residue increasing its nucleophilic character, and an electron deficient heteroaromatic moiety responsible for their binding affinity, and their positioning close to the phosphorylated serine residue in the enzyme catalytic site. However, these charged oximes are known cross the blood-brain barrier (BBB) poorly. Thus, if this medical countermeasure can limit the deadly peripheral cholinergic crisis, they do not reactivate cholinesterases of the CNS, causing long-lasting neurological disorders and side effects ⁵. Only 4 to 10 % of the oxime present in the plasma cross the BBB and are subsequently available for brain protection against OP poisoning ⁶. Moreover, despite the synthesis and evaluation of numerous new oximes over

the past years, no broad-spectrum oxime able to afford protection against all the main OPNAs has been identified ⁷.

In order to facilitate reactivators crossing of the BBB and improve the reactivation efficacy of new oximes in the CNS, many strategies and new compound designs have been explored. Amongst the different strategies developed recently, two have drawn our attention ^{8, 9}. Reactivators devoid of a permanent charge have been proposed to penetrate more efficiently the BBB and therefore to be more centrally active. *In vitro* results show an increased efficacy of these non-quaternary reactivators ¹⁰⁻¹². BBB crossing efficacy of some of these non-quaternary reactivators has been established ¹³, yet *in vivo* experiments did not show so far, a better protection in the mouse model compared to HI-6 ^{14, 15}. Another strategy to improve BBB penetration has implied the synthesis of sugar-oxime conjugates ¹⁶. Indeed, the glucose transporter GLUT-1 located at both sides of the BBB has been shown to potentially facilitate crossing of this physiological barrier of sugar conjugates ¹⁷. When applied to AChE reactivators, reports have demonstrated that glucose conjugated-2-PAM attenuates paraoxon-induced hypothermia in rats, suggesting a central effect ^{16, 18}.

Based on these previous results, herein we report the evaluation of the combination of these two strategies through the development and evaluation of a new class of uncharged oximes designed to reach inhibited AChE of the CNS through the glucose transporter GLUT-1 located at the BBB. Amongst the uncharged oximes evaluated so far, 6- substituted 3-hydroxypyridinaldoximes have shown the best *in vitro* AChE reactivation efficacy so far, we synthesized thus multi-functional molecules bearing this 3-hydroxypyridinaldoxime moiety as a reactivator function attached in position 6 or the pyridine to a sugar (glucose or ribose) aiming at GLUT-1 facilitated BBB crossing of the glycoconjugate. One of the prerequisites for efficient AChE reactivation is the ability of the reactivator to bind phosphorylated AChE near the phosphorylated serine residue. The 3-hydroxypyridinaldoxime moiety displaying a limited

affinity for AChE active site, structural studies prompted us to increase the affinity of the sugar-oximes for the phosphorylated AChE through the introduction of a triazole heterocycle between the sugar and the 3-hydroxypyridinaldoxime. Accordingly, docking experiments and previously obtained structures of different ligands bound to AChE have shown that such triazole moiety could improve binding to the gorge of acetylcholinesterase by stacking with aromatic amino-acids of the gorge^{19,20}. Binding of the triazole should thus 1) prevent the sugar moiety to interfere with the reactivation process and 2) increase the reactivator efficacy towards inhibited enzymes thanks to an increased binding affinity. In this study, we performed an exhaustive evaluation of these newly designed sugar-oximes. In order to guide the synthetic efforts, we first investigated the binding properties of the newly designed molecules by molecular docking. Next, the most promising candidates were synthesized, and we evaluated their reactivation efficacy on recombinant *h*AChE inhibited by various OPNAs and determined the crystallographic structures of the complexes formed between two of the synthesized sugar-oxime conjugates and *h*AChE. Then, we determined the protective index of selected sugar-oximes on mice exposed to OPNAs and finally, we checked the BBB crossing abilities of these new sugar-oximes molecules with an *in vitro* model mimicking the human BBB. Analysis of these findings allowed us to shed new light on the different issues associated with BBB crossing by such chemical counter-measures against OPNAs poisoning and opens the way to the development of a new family of reactivators.

2. Materials and Methods

2.1 Chemicals

2-PAM and HI-6 were obtained from Pharmacie Centrale des Armées (Orléans, France), NIMP (4-nitrophenyl isopropyl methylphosphonate), NEMP (4-nitrophenyl ethyl methylphosphonate) and NEDPA (4-nitrophenyl ethyl dimethylphosphoramidate) from UMR CNRS 7515 ICPEES (Strasbourg, France). Obidoxime, heparine, DTNB, acetylthiocholine

(ATC) and paraoxon were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). HI-6, 2-PAM, obidoxime (chlorhydrate salts), oxime 4 and 4' were dissolved into physiological serum (0.9 % NaCl) to obtain the concentration of 10 mM for *in vivo* experiments.

2.2 Chemistry

General. Solvents were purified by a dry solvent station MB-SPS-800 (MBraun, Garching, Germany) immediately prior to use. Triethylamine was distilled from KOH. All reagents were obtained from commercial suppliers (Sigma-Aldrich, St Quentin Fallavier, France; Acros Illkirch, France; TCI Europe, Paris, France) unless otherwise stated. The melting points were recorded on a Stuart SMP30 apparatus (Stuart, Staffordshire, UK). Column chromatography purifications were performed with prepacked SI-HP (30 μ m) or SI-HC (15 μ m) columns from Interchim (Montluçon, France). Preparative normal phase chromatography was carried out on an automated flash purification apparatus, either Biotage Isolera One (Biotage, Uppsala, Sweden) or Interchim 420 PuriFlash. Thin-layer chromatography (TLC) was carried out on Merck DC Kieselgel 60F-254 aluminum sheets (Merck, Darmstadt, Germany). Compounds were visualized by UV irradiation and/or spraying with a solution of vanillin, followed by smooth heating. ^1H and ^{13}C NMR spectra were recorded with a Bruker DPX 300 spectrometer (Bruker, Wissembourg, France) and are presented in the Supporting Information (S9, NMR spectra). Chemical shifts are expressed in parts per million (ppm) from CDCl_3 ($\delta\text{H} = 7.26$ ppm, $\delta\text{C} = 77.16$ ppm). *J* values are expressed in hertz. Mass spectra were obtained with a Finnigan LCQAdvantage MAX (ion trap) apparatus equipped with an electrospray source (Thermo Electron Corporation, Waltham, MA). High-resolution mass spectra were obtained with a Varian MAT 311 spectrometer (Varian MAT, Bremen, Germany) using electrospray analysis. HPLC quality grade acetonitrile and Milli-Q purified water were used for analytical and preparative HPLC. Preparative HPLC run was carried out with an Interchim 4250 apparatus (Interchim, Montluçon, France) with an Interchim puriflash C18 column (Interchim,

Montluçon, France), 30 × 250 mm, C18AQ-5 μm. Analytical HPLC was performed on a ThermoFisher UHPLC Ultimate 3000 instrument (ThermoFisher, Waltham, MA) equipped with a PDA detector under the following conditions: Synchronis C18 column (3 μm, 3 × 100 mm) with MeCN and 20 mM NH₄OAc as eluents [using a gradient from 100% 20 mM NH₄OAc to 100% MeCN over 30 min] at a flow rate of 0.5 mL/min with UV detection at 254 nm. The synthesis of oxime 1, 2, 3, 4, 4', 5 and 15 are described below. The general chemistry, experimental information, and syntheses of all other compounds are supplied in the Supporting Information. The purity of all final compounds as determined by HPLC analysis is ≥ 95 %. The canonical SMILES of all compounds tested are provided in Table S1.

(*E*)-3-hydroxy-6-(4-(((2*R*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)butyl)picolinaldehyde oxime 1

General procedure of oxime formation/deacetylation applied on aldehyde **47** (496 mg, 0.94 mmol). The residue was purified by preparative HPLC (0 % for 5 min then 0 % to 30% MeCN in H₂O over 25 min then 30% to 100% over 5 min, 40 mL/min, Interchim puriflash prep C18AQ, 30x250 mm, 5 micro, PF5C18AQ-250/300) to afford the title compound as a solid (137 mg, 39%). mp = 47-53 °C. $[\alpha]_D^{20} = -22.8$ (c 0.50 MeOH). ¹H NMR (300 MHz, MeOD): δ 8.31 (s, 1H), 7.29 (d, *J* = 8.5 Hz, 1H), 7.19 (d, *J* = 8.5 Hz, 1H), 4.27 (d, *J* = 7.8 Hz, 1H), 3.96 (dt, *J* = 9.6, 6.4 Hz, 1H), 3.88 (dd, *J* = 11.9, 2.0 Hz, 1H), 3.72 – 3.64 (m, 1H), 3.59 (dt, *J* = 9.6, 6.3 Hz, 1H), 3.41 – 3.25 (m, 3H), 3.19 (dd, *J* = 8.9, 7.7 Hz, 1H), 2.81 – 2.72 (m, 2H), 1.87 – 1.73 (m, 2H), 1.73 – 1.61 (m, 2H). ¹³C NMR (75 MHz, MeOD): δ 153.3, 152.4, 151.4, 134.8, 124.7, 124.0, 103.0, 76.7, 76.5, 73.7, 70.3, 69.0, 61.4, 36.1, 28.8, 26.3. HRMS (ESI⁺): *m/z* calculated for [C₁₆H₂₅N₂O₈]⁺ 373.1611, found 373.1608.

(*E*)-3-hydroxy-6-(3-(((2*R*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)propyl)picolinaldehyde oxime 2

General procedure of oxime formation/deacetylation applied on aldehyde **46** (336 mg, 0.98 mmol). The residue was purified by preparative HPLC (0% for 5 min then 0 % to 40% MeCN in H₂O over 25 min then 30% to 100% over 5 min, 40 mL/min, Interchim puriflash prep C18AQ, 30x250 mm, 5 micro, PF5C18AQ-250/300) to afford the title compound as an oil (143 mg, 41%). $[\alpha]_D^{20} = -18.0$ (*c* 0,50 MeOH). ¹H NMR (300 MHz, MeOD): δ 8.32 (s, 1H), 7.29 (d, *J* = 8.5 Hz, 1H), 7.22 (d, *J* = 8.5 Hz, 1H), 4.27 (d, *J* = 7.7 Hz, 1H), 3.94 (dt, *J* = 9.6, 6.1 Hz, 1H), 3.91 – 3.84 (m, 1H), 3.69 (dd, *J* = 11.9, 5.1 Hz, 1H), 3.58 (dt, *J* = 9.8, 6.4 Hz, 1H), 3.43 – 3.25 (m, 4H), 3.22 (dd, *J* = 8.9, 7.7 Hz, 1H), 2.85 (dd, *J* = 8.6, 6.7 Hz, 2H), 2.07 – 1.91 (m, 2H). ¹³C NMR (75 MHz, MeOD): δ 152.9, 152.4, 151.5, 134.9, 124.7, 124.2, 103.1, 76.7, 76.5, 73.8, 70.3, 68.4, 61.4, 32.8, 29.9. HRMS (ESI⁺): *m/z* calculated for [C₁₅H₂₃N₂O₈]⁺ 359.1454, found 359.1458.

(*E*)-3-hydroxy-6-(3-(4-(4-(((2*R*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)butyl)-1*H*-1,2,3-triazol-1-yl)propyl)picolinaldehyde oxime 3

General procedure of oxime formation/deacetylation applied on aldehyde **48** (677 mg, 1.07 mmol). The residue was purified by normal phase flash chromatography (10% to 30% MeOH in DCM over 30 min, 12G SIHC) then reversed-phase flash chromatography (5 to 60% MeCN in H₂O over 30 min, 80 G, C18-15 micro, Interchim) to afford an off-white solid (384 mg, 75%). *R_f* = 0.12 (DCM/MeOH 90/10, v/v). $[\alpha]_D^{20} = -15.3$ (*c* 0,53 MeOH). ¹H NMR (300 MHz, MeOD): δ 8.28 (s, 1H), 7.73 (s, 1H), 7.25 (d, *J* = 8.4 Hz, 1H), 7.12 (d, *J* = 8.5 Hz, 1H), 4.87 (s, 7H), 4.41 (t, *J* = 6.9 Hz, 2H), 4.25 (d, *J* = 7.8 Hz, 1H), 3.94 (dt, *J* = 9.6, 6.3 Hz, 1H), 3.86 (dd, *J* = 11.9, 1.9 Hz, 1H), 3.67 (dd, *J* = 11.9, 5.3 Hz, 1H), 3.58 (dt, *J* = 9.6, 6.2 Hz, 1H), 3.39 – 3.32 (m, 1H), 3.32 – 3.24 (m, 1H), 3.28 – 3.25 (m, 1H), 3.18 (dd, *J* = 8.9, 7.7 Hz, 1H), 2.76 – 2.71 (m, 2H), 2.71 (t, *J* = 8.0 Hz, 2H), 2.29 (p, *J* = 6.9 Hz, 2H), 1.84 – 1.69 (m, 2H), 1.69 – 1.56 (m, 2H). ¹³C NMR (75 MHz, MeOD): δ 152.5, 151.6, 151.4, 147.7, 135.2, 124.6, 124.0, 121.9,

103.0, 76.7, 76.5, 73.7, 70.3, 68.9, 61.4, 49.3, 33.2, 29.8, 28.7, 25.6, 24.5. HRMS (ESI⁺): m/z
calculated for [C₂₁H₃₁N₅O₈Na]⁺ 504.2070, found 504.2072.

(E)-3-hydroxy-6-(4-(4-(4-(((2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)butyl)-1H-1,2,3-triazol-1-yl)butyl)picolinaldehyde oxime 4

General procedure of oxime formation/deacetylation applied on aldehyde **49** (146 mg, 0.22 mmol). The residue was purified by normal phase flash chromatography (10% to 30% MeOH in DCM over 30 min, 12G SIHC) then reversed-phase flash chromatography (5 to 100 % MeCN in H₂O over 30 min, 80 G, C18-15 micro, Interchim) to afford an oil (112 mg, 100%). R_f = 0.14 (DCM/MeOH 9/1, v/v). $[\alpha]_D^{23} = -14.5$ (c 0.43 MeOH). ¹H NMR (300 MHz, MeOD): δ 8.26 (s, 1H), 7.74 (s, 1H), 7.26 (d, *J* = 8.4 Hz, 1H), 7.13 (d, *J* = 8.5 Hz, 1H), 4.39 (t, *J* = 6.8 Hz, 2H), 4.26 (d, *J* = 7.7 Hz, 1H), 3.93 (dt, *J* = 9.5, 6.2 Hz, 1H), 3.86 (dd, *J* = 12.1, 1.8 Hz, 1H), 3.67 (dd, *J* = 11.9, 4.8 Hz, 1H), 3.57 (dt, *J* = 9.6, 6.2 Hz, 1H), 3.44 – 3.11 (m, 3H), 3.18 (t, *J* = 8.3 Hz, 1H), 2.74 (t, *J* = 7.7 Hz, 2H), 2.71 (t, *J* = 7.7 Hz, 2H), 2.00 – 1.82 (m, 2H), 1.82 – 1.57 (m, 6H). ¹³C NMR (75 MHz, MeOD): δ 153.9, 153.8, 152.7, 149.1, 136.3, 126.1, 125.4, 123.2, 104.3, 78.0, 77.8, 75.1, 71.6, 70.3, 62.7, 51.0, 36.9, 30.7, 30.0, 27.8, 27.0, 25.9. HRMS (ESI⁺): m/z calculated for [C₂₂H₃₄N₅O₈]⁺ 496.2407 found 496.2413.

(E)-3-hydroxy-6-(4-(4-(4-(((2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)butyl)-1H-1,2,3-triazol-1-yl)butyl)picolinaldehyde oxime 4'

General procedure of oxime formation/deacetylation applied on aldehyde **50** (369 mg, 0.57 mmol). The residue was purified by normal phase flash chromatography (10 to 30% MeOH in DCM over 30 min, 12G SIHC) then reversed-phase flash chromatography (5 to 100 % MeCN in H₂O over 30 min, 80 G, C18-15 micro, Interchim) to afford an oil (225 mg, 80%). $[\alpha]_D^{26} = +65.1$ (c 0.42 MeOH). ¹H NMR (300 MHz, MeOD): δ 8.28 (s, 1H), 7.75 (s, 1H), 7.28 (d, *J* =

206 8.5 Hz, 1H), 7.14 (d, $J = 8.5$ Hz, 1H), 4.78 (d, $J = 3.7$ Hz, 1H), 4.40 (t, $J = 6.9$ Hz, 2H), 3.81
207 (dd, $J = 11.7, 2.4$ Hz, 2H), 3.86 – 3.71 (m, 1H), 3.67 (dd, $J = 11.7, 5.7$ Hz, 1H), 3.66 (dd, $J =$
208 9.7, 8.8 Hz, 1H), 3.58 (ddd, $J = 9.9, 5.6, 2.4$ Hz, 1H), 3.48 (dt, $J = 9.7, 5.9$ Hz, 1H), 3.40 (dd, J
209 = 9.7, 3.7 Hz, 1H), 3.29 (dd, $J = 9.8, 8.8$ Hz, 1H), 2.76 (t, $J = 7.7$ Hz, 2H), 2.74 (t, $J = 7.7$ Hz,
210 2H), 1.99 – 1.87 (m, 2H), 1.84 – 1.61 (m, 6H). ^{13}C NMR (75 MHz, MeOD): δ 153.9, 153.8,
211 152.9, 149.0, 136.4, 126.0, 125.3, 123.2, 100.1, 75.2, 73.7, 73.6, 71.9, 68.6, 62.8, 51.0, 37.0,
212 30.7, 29.9, 27.9, 27.2, 26.0. HRMS (ESI⁺): m/z calculated for $[\text{C}_{22}\text{H}_{34}\text{N}_5\text{O}_8]^+$ 496.2407, found
213 496.2399.

214 **(*E*)-6-(4-(4-(4-(((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-
215 *yl*)oxy)butyl)-1*H*-1,2,3-triazol-1-yl)butyl)-3-hydroxypicolinaldehyde oxime 5**

216 General procedure of oxime formation/deacetylation applied on aldehyde **51** (398 mg, 0.69
217 mmol). The residue was purified by normal phase flash chromatography (5 to 30% MeOH in
218 DCM over 30 min, 25G SIHC), then purified by preparative HPLC (0% for 5 min, then 0 to
219 40% MeCN in H₂O over 30 min, puriflash C18, 30x250 mm, C18AQ-5 micro) to afford the
220 title compound as an oil (233 mg, 73%). $R_f = 0.13$ (DCM/MeOH 9/1, v/v). $[\alpha]_D^{24} = -21.1$ (c
221 0.53 MeOH). ^1H NMR (300 MHz, MeOD): δ 8.14 (s, 1H), 7.59 (s, 1H), 7.13 (d, $J = 8.4$ Hz,
222 1H), 6.99 (d, $J = 8.4$ Hz, 1H), 4.73 (d, $J = 4.0$ Hz, 1H), 4.26 (t, $J = 6.9$ Hz, 2H), 3.92 (dd, $J =$
223 6.9, 4.7 Hz, 1H), 3.82 (td, $J = 6.7, 3.5$ Hz, 1H), 3.76 (dd, $J = 4.7, 1.0$ Hz, 1H), 3.71 – 3.57 (m,
224 1H), 3.60 (dd, $J = 11.8, 3.6$ Hz, 1H), 3.42 (dd, $J = 11.7, 6.5$ Hz, 1H), 3.28 (dt, $J = 9.5, 6.2$ Hz,
225 1H), 2.62 (t, $J = 6.5$ Hz, 2H), 2.59 – 2.51 (m, 2H), 1.78 (dd, $J = 15.0, 7.5$ Hz, 2H), 1.66 – 1.39
226 (m, 6H). ^{13}C NMR (75 MHz, MeOD): δ 152.5, 152.4, 151.5, 147.6, 135.0, 124.6, 123.9, 121.8,
227 107.4, 83.4, 74.9, 71.4, 67.0, 63.7, 49.6, 35.6, 29.3, 28.7, 26.5, 25.8, 24.6. HRMS (ESI⁺): m/z
228 calcd for $[\text{C}_{21}\text{H}_{32}\text{N}_5\text{O}_7]^+$ 466.2302, found 466.2303.

229 **(*E*)-6-(6-(((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-
230 *yl*)oxy)hexyl)-3-hydroxypicolinaldehyde oxime 15**

General procedure of oxime formation/deacetylation applied on aldehyde **52** (318 mg, 0.66 mmol). The residue was purified by normal phase flash chromatography (5 to 30% MeOH in DCM over 30 min, 25G SIHC), then purified by preparative HPLC (5 to 50% MeCN in H₂O over 30 min, puriflash C18, 30x250 mm, C18AQ-5 micro) to afford the title compound as an oil (128 mg, 52%). $R_f = 0.22$ (90/10 DCM/MeOH, v/v). $[\alpha]_D^{20} = -26.3$ (c 0.51 MeOH). ¹H NMR (300 MHz, CDCl₃): δ 8.29 (s, 1H), 7.27 (d, $J = 8.5$ Hz, 1H), 7.15 (d, $J = 8.5$ Hz, 1H), 4.83 (s, 1H), 4.03 (dd, $J = 6.9, 4.7$ Hz, 1H), 3.93 (td, $J = 6.7, 3.5$ Hz, 1H), 3.87 (d, $J = 4.7$ Hz, 1H), 3.79 – 3.65 (m, 2H), 3.54 (dd, $J = 11.7, 6.6$ Hz, 1H), 3.36 (dt, $J = 9.3, 6.3$ Hz, 1H), 2.71 (dd, $J = 8.7, 6.7$ Hz, 2H), 1.67 (h, $J = 7.2$ Hz, 2H), 1.54 (q, $J = 6.6$ Hz, 2H), 1.37 (h, $J = 5.5, 4.5$ Hz, 4H). ¹³C NMR (75 MHz, CDCl₃): δ 154.8, 153.8, 152.8, 136.2, 126.1, 125.3, 108.7, 84.7, 76.3, 72.8, 68.8, 65.2, 37.8, 31.3, 30.6, 30.1, 27.1. HRMS (ESI⁺): m/z calculated for [C₁₇H₂₇N₂O₇]⁺ 371.1818, found 371.1824.

2.3 Molecular docking

Flexible dockings with the newly designed sugar-oxime conjugates have been performed using AutoDock Vina ²¹ as described previously ²² with the receptor files for GB-*hAChE* and VX-*hAChE*. For each phosphorylated *hAChE*, 10 poses per oxime were generated and the best identified poses were determined based on the minimal distance between the oxygen atom of the oxime and the phosphorus atom of the OP serine conjugated and by the higher binding affinity achieved.

2.4 Recombinant human acetylcholinesterase

Recombinant *hAChE* was produced and purified as previously described ²³.

2.5 Phosphorylation of recombinant human acetylcholinesterase

Stock solutions of OPNAs surrogates (NIMP as sarin surrogate, NEMP as VX surrogate and NEDPA as Tabun surrogate) or VX at 5 mM in isopropanol were used to inhibit the purified *hAChE* as previously described ²⁴. It is worth noting that using the OPNA surrogates gives the

same phosphyl residue than the real OP, and thus the same phosphylated AChE. Briefly, a ten-fold excess of OPNA surrogates or VX was used to perform the inhibition of *hAChE* in a sodium phosphate buffer (100 mM, pH 7.4, 0.1% BSA) at 25°C. Complete inhibition of *hAChE* was monitored by measuring the residual activity with a modified Ellman assay as previously described²⁵ and excess of OPNAs surrogates or VX were removed using a desalting PD-10 column (GE Healthcare).

2.6 IC₅₀ measurements

Oximes were dissolved in methanol to prepare a 40 mM stock solution and subsequently diluted in water to reach the desired concentrations. Recombinant *hAChE* activity was measured in a buffer containing 0,1 M phosphate buffer pH 7.4 / 0.1 % BSA / 0.1 mg/ml DTNB / 1 mM ATC and in presence of various oximes concentrations using a modified Ellman assay²⁵ measuring the released thiophenol concentration through the UV/Vis absorbance at 412 nm and 25°C. Measurements were performed at least in duplicate for each tested concentration and final methanol concentrations were kept below 5%. The compound concentration producing 50 % inhibition was determined by nonlinear fitting with ProFit (Quantumsoft) using the standard IC₅₀ equation:

$$\% \text{ activity} = \frac{100 \times IC_{50}}{(IC_{50} + [Cp])}$$

2.7 *In vitro* reactivation of phosphylated human acetylcholinesterase

The reactivation of OPNAs-inhibited *hAChE* was performed as previously described¹⁵. Briefly, the phosphylated enzymes were incubated at 37°C with various concentrations of oximes in 0,1 % BSA / 0,1 M phosphate buffer pH 7.4. Final concentration of methanol was kept below 2 % and had no effect on enzyme activity. Aliquots of the reactivation mixture were transferred at different time interval to cuvettes containing 1 mM ATC in 1 mL of Ellman's buffer (0.5 mM DTNB in 0.1 M sodium phosphate buffer, pH 7.4) to measure the *hAChE* activity through the

evaluation of the amount of thiophenol obtained by thiocholine cleavage of DTNB through measurement of its UV-Vis absorption at 412 nm and 25°C. The reactivation constants k_{obs} , K_D , k_r and k_{r2} have been calculated by non-linear fitting of the standard oxime-concentration-dependent reactivation equation with ProFit (Quantumsoft) derived from the following scheme.



$$k_{obs} = \frac{k_r[Ox]}{K_D + [Ox]} \quad \text{and} \quad k_{r2} = \frac{k_r}{K_D}$$

2.8 Human Acetylcholinesterase crystallization

Recombinant human acetylcholinesterase crystals were grown using the hanging drop vapor diffusion method as described previously²³. Crystals were soaked 60 min in the mother liquor containing 2 mM of each compound. Crystals were then washed with a cryoprotectant solution (1.6 M lithium sulfate, 100 mM HEPES pH 7.0, 60 mM magnesium sulfate and 18 % glycerol) and flash-cooled in liquid nitrogen.

2.9 Data collection, reduction and refinement

Diffraction data were collected at the European Synchrotron Radiation Facility (ESRF, Grenoble, France) at the ID23-2 beam line ($\lambda = 0.873 \text{ \AA}$) and processed with XDS²⁶ and scaled with XSCALE. The structure was solved by molecular replacement with PHASER²⁷ using pdb 4EY4 as starting model, iterative cycles of model building using Coot²⁸ and refinement using Phenix²⁹. Data collection and refinement statistics are presented in supporting information (table S2).

2.10 Animals

We used 9-week-old male Swiss mice (Janvier Labs, Le Genest-Saint-Isle, France), weighing 35-45 g at the experimentation time. The animals (3-4/cage) were housed for 14-18 days before the experiments in an environment maintained at $22 \pm 1^\circ\text{C}$ with controlled humidity on a 12 h

dark/light cycle with light provided between 7 a.m. and 7 p.m. They were given food and tap water *ad libitum*. All experiments were carried out in compliance with the European Directive on the protection of animals used for scientific purposes (2010/63/UE) and were approved by our Institutional Animal Care and Research Advisory Committee (approval n°239 of 10-09-2018).

2.11 Plasma test protocols: monitoring of the reactivability of blood samples

This protocol has been previously fully described ¹⁴.

Standard reactivation curves for pharmacokinetics

Naive mice plasma from Janvier Labs (Le Genest-Saint-Isle, France) was heated at 56°C for 30 min to inactivate endogenous cholinesterases. In a 96-well Greiner plate, VX-phosphylated *hAChE* solution in sodium phosphate buffer was incubated for 30 min at 37°C in presence of different concentrations of oximes diluted in heat-inactivated mice plasma (0, 1, 5, 10, 25, 50, 75, 100, 150, 200 and 500 µM). A mix of 2 mM acetylthiocholine and Ellman's buffer (0.5 mM DTNB in 0.1 M sodium phosphate buffer, pH 7.4, 25°C) was then simultaneously added in all wells for measurement of resulting *hAChE* activity at 412 nm every 5 s for 30 min with a SAFAS spectrophotometer (Monaco).

The percentage of reactivated enzyme (% E_{react}) was calculated as the ratio of the recovered VX-phosphylated *hAChE* activity and HI-6 reactivated *hAChE* activity, considering that maximal reactivation (*i.e.*, % E_{react} = 100 %) was achieved by incubating VX-phosphylated *hAChE* with 200 µM HI-6 diluted in heat-inactivated plasma. Standard curves were fitted with GraphPad Prism software using a simple linear regression.

Blood sampling

Twenty-four hours before the experiment, mice were anesthetized with isoflurane gas (Vetflurane®, Virbac, France) allowing the shaving of their hind limbs after a 3-min-long application of a commercial depilatory cream. Then, mice were returned to their cages to allow

recovery and complete anesthesia washout. The day of experimentation, mice received intraperitoneal (i.p.) injection of oxime at 100 $\mu\text{mol/kg}$ by analogy with the previous studies conducted in our department. At various times (0, 2, 5, 10, 15, 30, 60 and 180 min after oxime injection), the saphenous vein was drilled with a needle, approximately 20 μL of blood were collected with a heparinized capillary tube and put in a collection tube containing 2 μL of sodium heparin (Choay[®], Sanofi, France). Plasma was next isolated from erythrocytes by centrifugation at 4°C, 3 000 g for 10 min. Plasma samples were then heated 30 min at 56°C and treated as previously described to obtain the percentage of reactivated enzyme (% E_{react}) with a one-compartment model. T_{max} corresponds to the time when the curve reaches the peak of % E_{react} . The areas under the percentage of reactivation curve (AUC) and the first moment curve (AUMC) were calculated using the trapezoidal rule. Mean residence time (MRT) was calculated as the ratio of AUMC to AUC³⁰.

2.12 LD₅₀ estimation and protective index using the up-and-down method

LD₅₀ was estimated using the improved method of Dixon's up-and-down procedure described by Rispin et al.³¹. This method uses an iterative dose-selection algorithm. It consists of a single ordered dose progression in which mice are dosed, one at a time, at 24 h intervals. The first animal received a dose a step below the level of the best estimate of the LD₅₀. If the mouse survives, the dose for the next animal is increased by 1.1-fold the original dose; if it dies, the dose for the next animal is decreased by the same factor. In our particular conditions the testing stops when one of the following criteria is met: (1) three consecutive animals survive at the highest dose (which is normally 2000 mg/kg); (2) five reversals occur in any six consecutive animals tested; (3) at least four animals have followed the first reversal and the specified likelihood-ratios which compare the maximum likelihood estimate for LD₅₀ with LD₅₀ values above and below exceed the critical value of 2.5. Profile likelihood methods are used to estimate confidence intervals. In practice the stopping criteria, the resulting LD₅₀ and the corresponding

confidence interval were determined using the AOT 425 Pgm software as recommended by OECD (7). Antidotal efficacy of the oximes is expressed as a protective index (PI) with 95 % confidence interval. The PI corresponds to the ratio of LD₅₀ of the studied OP agent (either NIMP, NEMP or paraoxon) combined with oxime treatment on LD₅₀ of OP alone.

2.13 Blood-brain barrier permeability tests

Human Blood-brain barrier model setting up

The blood-brain barrier (BBB) permeability studies were performed using the *in vitro* human BBB model previously described and detailed ³². After infant's parents signed informed consents, endothelial cells were isolated and differentiated from cord blood CD34⁺-hematopoietic stem cells according to the protocol described by Pedroso et al. ³³ and then freezed. The preservation and preparation protocol of these cells issued from the human cord blood were approved by the French Ministry of Higher Education and Research (CODECOH number DC2011-1321). After thawing within 100 mm petri dishes (Corning, VWR, Switzerland), the endothelial cells derived from human stem cells reached the confluency and were then subcultured onto matrigel (BD Biosciences, Franklin Lakes, NJ, USA, 354230) coated Transwell inserts in the presence of bovine pericytes seeded at bottom of the wells, on other side of Transwell inserts, to induce the properties of the BBB. Renewal of the medium [ECM basal medium (Sciencell, Carlsbad, CA, USA) supplemented with 5% (v / v) fetal calf serum, 1% (v / v) EC growth supplement (Sciencell) and 50 µg / mL gentamycin (Biochrom AG, Berlin, Germany)] of the co-culture thus set up was carried out every other day. After six days under these culture conditions, the endothelial cells differentiated in human brain-like endothelial cells (hBLECs) reproduced characteristics of the *in vivo* BBB ³² and are widely used to predict molecule toxicity and passage to the CNS ³⁴⁻³⁷.

Endothelial permeability coefficient evaluation

The permeability of the BBB to the different oximes and ^{14}C D-glucose was evaluated by measuring the endothelial permeability coefficient (Pe) which represents the speed of diffusion through the BLECs monolayer^{38, 39}. Human BLECs monolayers developed after 6 days of coculture were transferred into new plates containing 1.5 mL per well (abluminal compartment) of HEPES buffered-Ringer's solution (RH; 150mM NaCl, 5.2mM KCl, 2.2mM NaCl₂, 0.2mM MgCl₂, 6mM NaHCO₃, 2.8mM glucose, 5mM HEPES). Medium in apical chambers (luminal compartment) was replaced by 0.5 mL of RH containing either one glycoconjugate oximes or 2-PAM or HI-6 or obidoxime used as oximes control. All compounds were tested at 50 μM (dose checked as non-toxic for the human BLECs) for a diffusion duration of an hour at 37°C. Then the amount of each oxime in the luminal and abluminal compartments was measured by mass spectrometry with a TripleTOF 5600+ System (AB SCIEX, Concord, ON, Canada). Percentages of recovery were checked between 86 and 106%. The quantification of radiolabeled ^{14}C D-glucose (PerkinElmer, Boston, MA, USA) was performed using a scintillation counter TriCarb 2100TR (PerkinElmer, USA).

The clearance principle was used to calculate a concentration-independent permeability coefficient. The mean compound cleared volume was plotted against time, and the slope was estimated by linear regression. The permeability values of the inserts (PSf for inserts with a Matrigel™ coating only) and the inserts with hBLECs (PSt, Matrigel™-coated inserts + endothelial cells) were taken into consideration by applying the following equation: $1/\text{PSe} = 1/\text{PSt} - 1/\text{PSf}$. To obtain the endothelial permeability coefficient (Pe expressed in cm/min), the permeability value (PSe) was divided by the insert's membrane surface area (1.13 cm²).

3 Results

3.1 Chemistry

In order to evaluate the requirement of an additional triazole moiety to increase reactivator affinity with phosphylated AChE, a first series of 3-

hydroxypyridinealdehyde - glucose conjugate was synthesized without the triazole ring. The sugar moiety was attached from its anomeric position (β isomer was chosen according to the literature describing efficient GLUT-1 mediated BBB crossing using such glycoconjugates) to the 6 position of the pyridine ring by an alkyl chain of respectively four, three or six carbons, yielding compounds **1**, **2** and **15** in 48%, 28%, and 23% yield, respectively, over four steps (

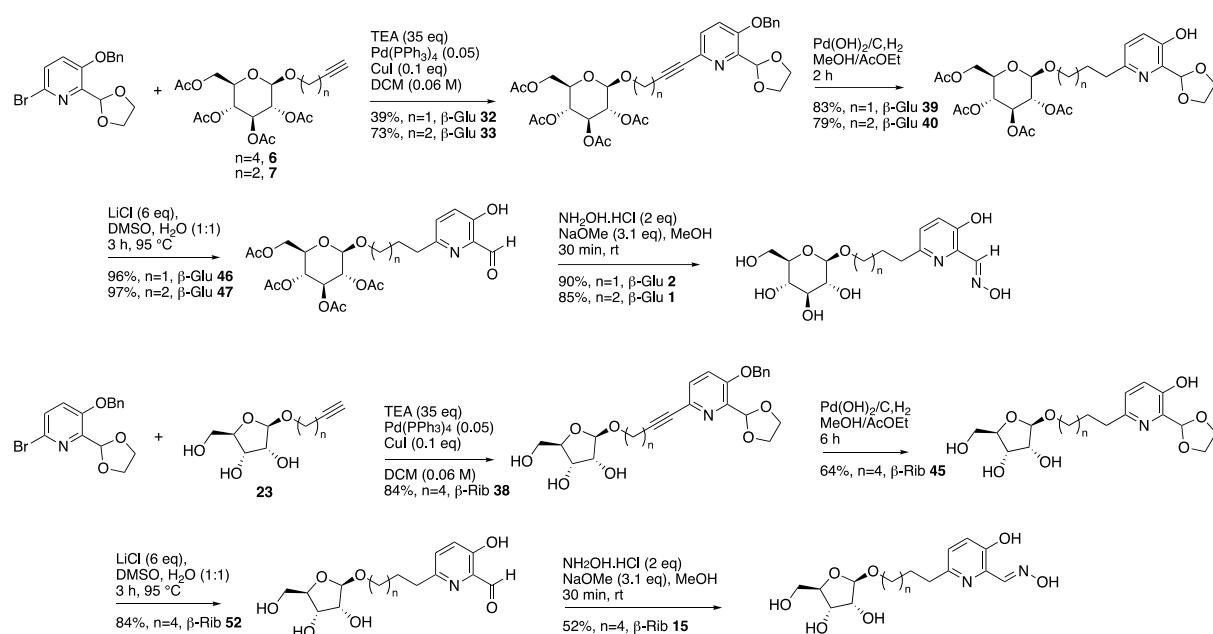
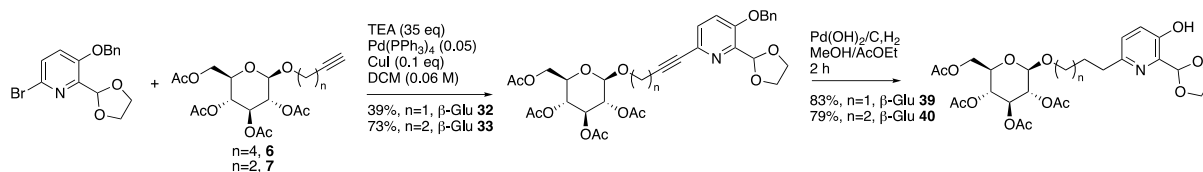
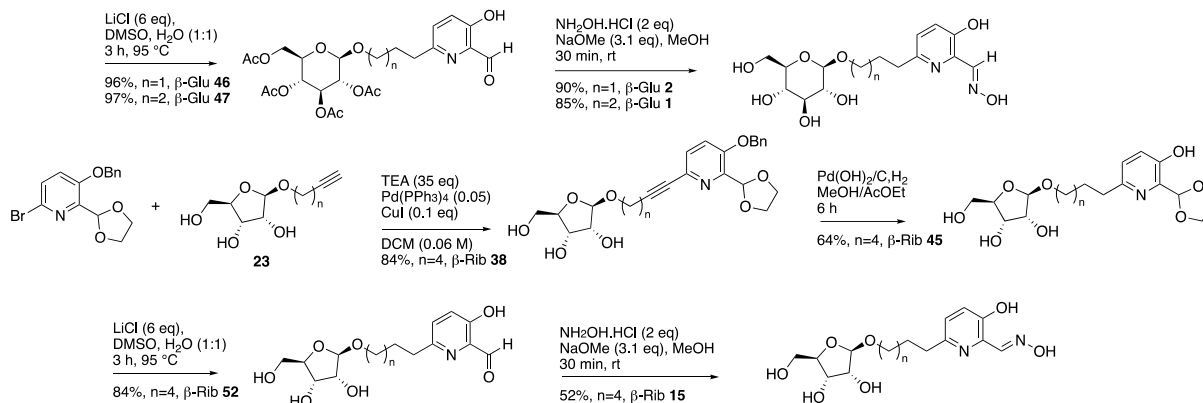


Figure 1). Peracylated glucose bearing a terminal alkyne moiety at its anomeric position of different lengths was obtained as previously described⁴⁰. A Sonogashira cross-coupling reaction was used to link the glycosyl moiety and a pre-functionalized pyridine ring using standard procedure (CuI, Pd(PPh₃)₄) followed by hydrogenation in the presence of Pearlman palladium catalyst⁴¹. Finally, the acetals were removed by a mild and efficient procedure (LiCl in DMSO/H₂O)⁴² forming the corresponding 3-hydroxypiconaldehyde which was converted to the aldoxime through reaction with hydroxylamine in methanol. Preparative RP-HPLC purified final compounds, and their purity (see 2.1) was determined by RP-HPLC.

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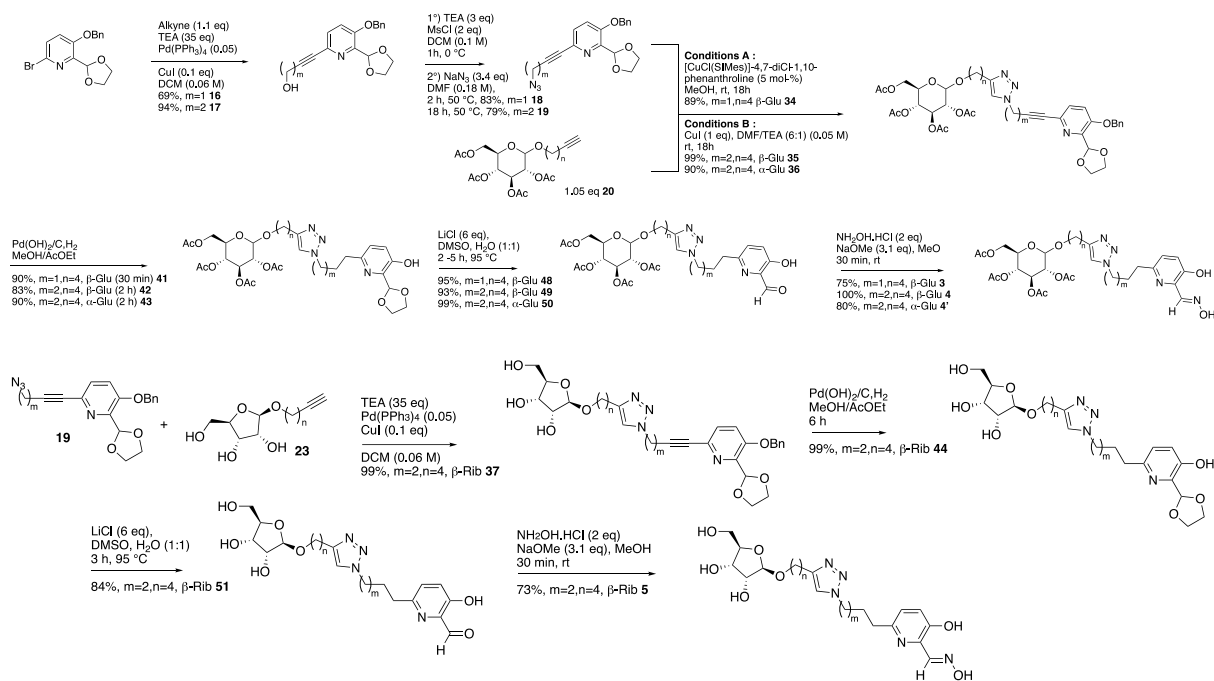


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Figure 1. Alkylchain-containing aldoxime synthetic pathway

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Aldoximes **3**, **4**, **4'** and **5** (

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Figure 2) bearing a triazole heterocycle between the sugar moiety and the position 6 of the pyridinaldoxime moiety were obtained using Huisgen cycloaddition between an alkyne chain linked to the glycosyl and an azidoalkynylpyridine. Backed by the docking experiments, a 4 carbons atom linker was found sufficient between the triazole moiety and the sugar to keep the sugar moiety away from AChE peripheral site. Docking experiments (vide infra) showed that

the best binding affinities were obtained with a 3 or 4 carbon atoms distance between the oxime and the triazole ring, thus the β -glucoconjugate **3** and **4** were first targeted. In addition, α -glucoconjugate **4'** and β -riboconjugate **5** were also synthesized to evaluate the role of the sugar moiety (glucose vs. ribose) and of the stereochemistry at the anomeric position (α -glucose vs β -glucose) in the hexose series. Particular attention was paid to the Huisgen cycloaddition with the azidopropynylpyridine which required the use of 5-mol-% of [CuCl(SIMes)]-4,7-dichloro-1,10-phenanthroline⁴³ to avoid by-product formation. The same sequence was used to form the oxime moiety (overall yields over the four final steps: 57%, 76%, 64% and 60% respectively for **3**, **4**, **4'** and **5**).

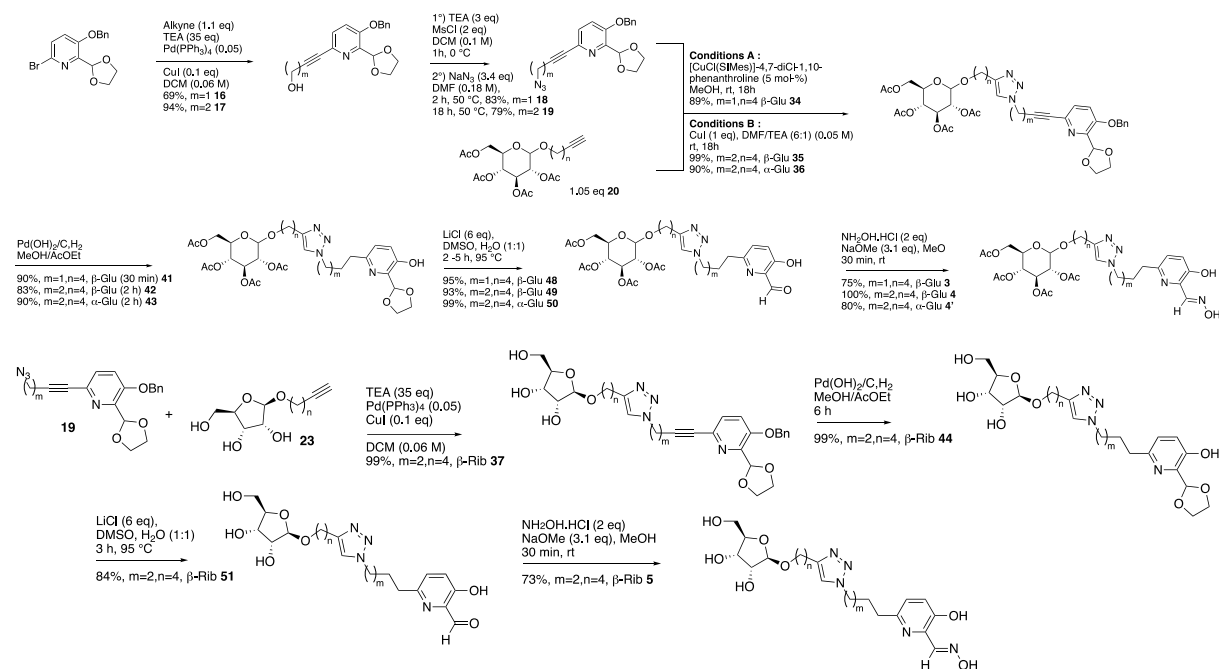
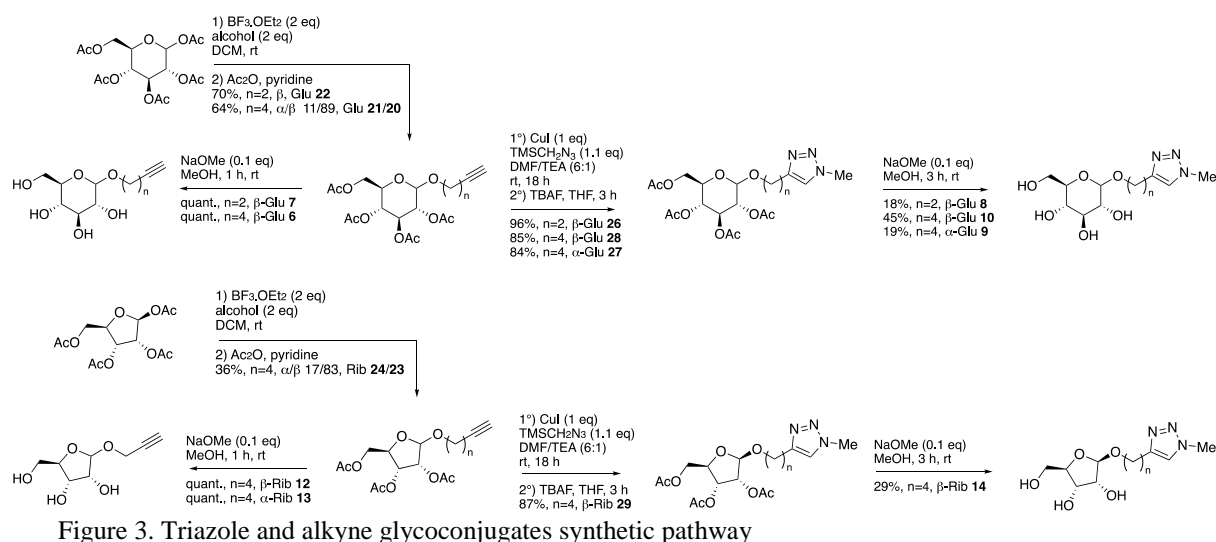
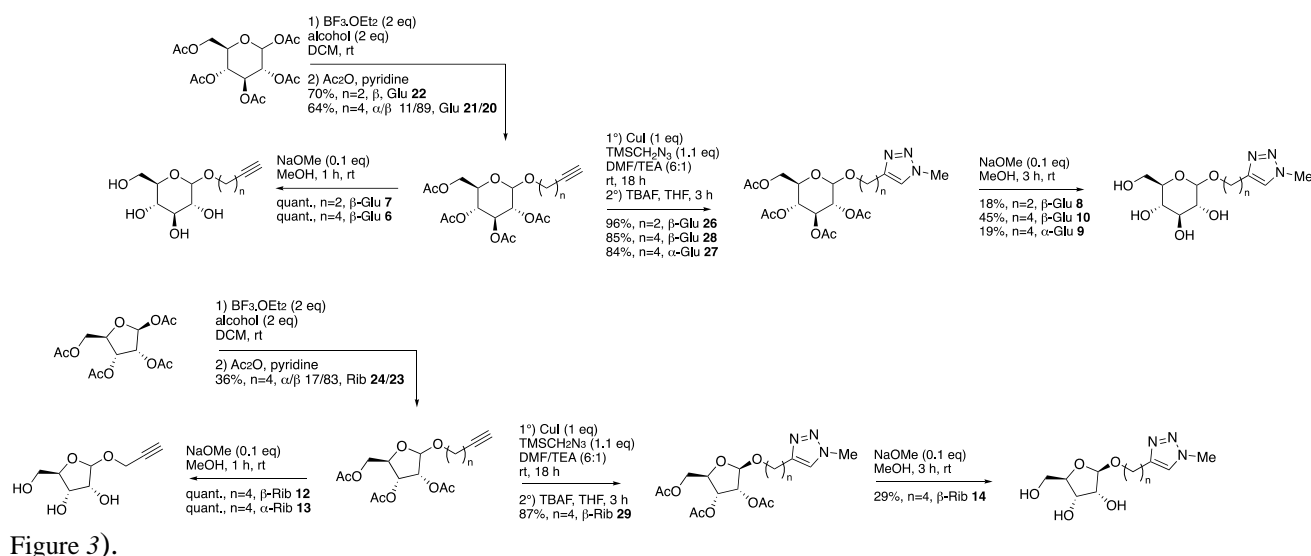


Figure 2. Triazole-containing aldoxime synthetic pathway

The glycosylation steps were carried out in the presence of either 3-butyn-1-ol, 5-hexyn-1-ol as a glycol acceptor and the corresponding glycosyl donor in the form of peracetylated glucopyranose or ribofuranose (



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458 In order to gain a better understanding of the glycoconjugate trafficking according to the

459 sugar derivative, and the glycosyl stereochemistry, several intermediates bearing simpler

460 loads (alkyne or methyltriazoles) were synthesized (

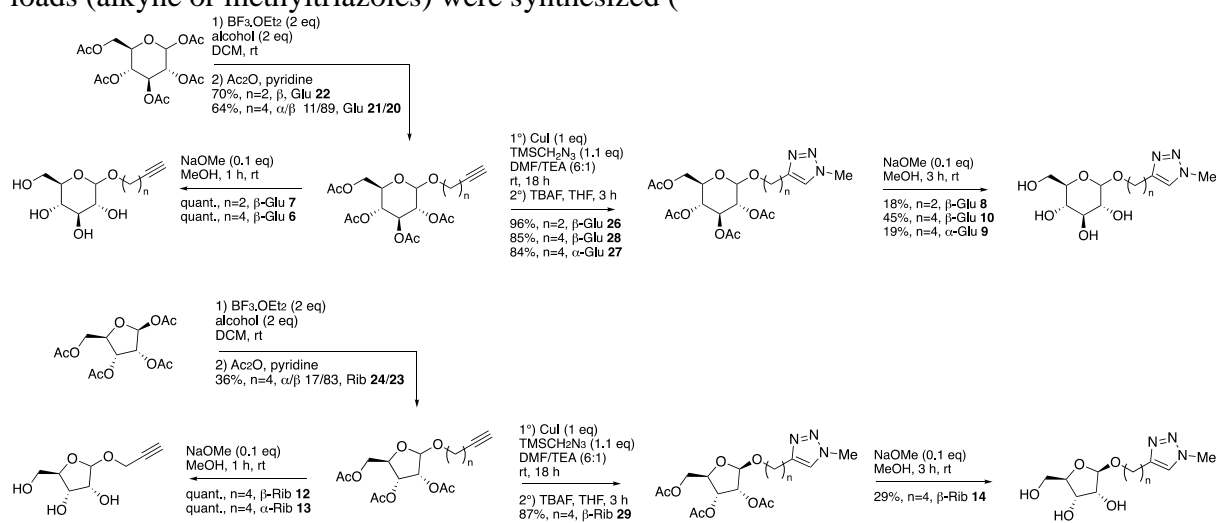


Figure 3). The alkyne chain glycoconjugates **6**, **7**, **12** and **13** were synthesized as a single diastereoisomer in two steps from the corresponding peracetylated sugar in respectively 57%, 70%, 36% and 6% yield by a glycosylation reaction followed by Zemplén procedure to study the influence of possible inhibiting interaction. The triazole glycoconjugates, **8**, **9**, **10** and **14** were synthesized, as pure diastereoisomer, in 3 steps and respectively in 12%, 1%, 22 % and 8% overall yields using Huisgen cycloaddition with 1.1 equivalent of trimethylsilylmethyl azide followed by TBAF treatment and Zemplén transesterification procedure to uncover the *N*-methyltriazole glycoconjugates as free hydroxyl groups.

Compound **11**, bearing the triazole alkyl chain on position 6 of the sugar, was also synthesized to investigate the impact of the position of the triazole-comprising chain on the BBB permeability. Compound **11** was obtained in 5 steps and with 24 % overall yield from glucose.

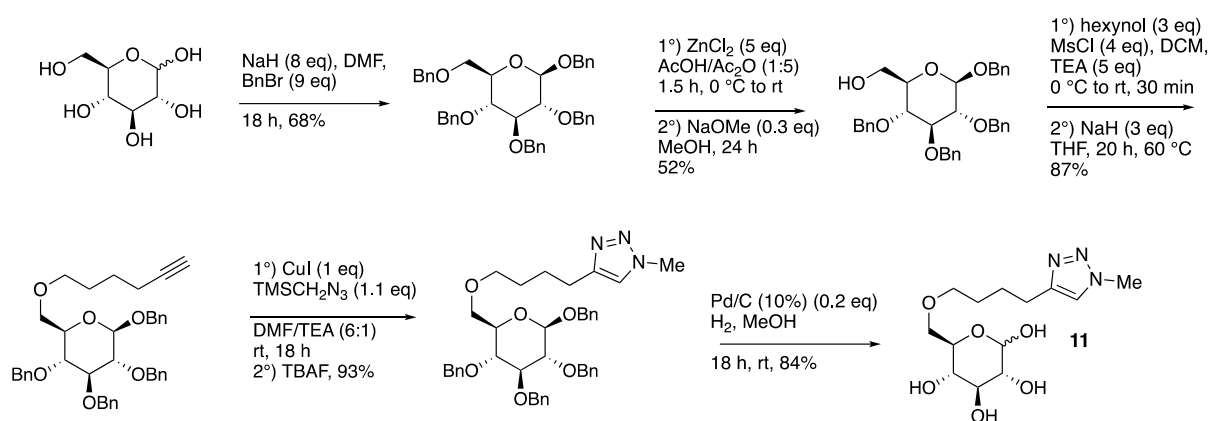


Figure 4. Position 6-substituted alkyl-triazole glycoconjugate

3.2 Molecular Docking

To determine if the candidates oximes **1,2,3** and **4** can bind in a productive way to the OPNAs phosphorylated *hAChE* close to the catalytic OP-serine adduct, we performed flexible molecular docking on *hAChE* phosphorylated by either VX or GB (Sarin) as previously described²². The side chain of aromatic residues (Trp 286, Tyr72, Tyr341, Tyr124, Tyr337) of the active site of *hAChE* as well as the alkoxy chain of the nerve agent were allowed to span freely from their native position. The binding energy determined by the scoring function of Autodock Vina and

the distances between the phosphorus atoms of nerve agents and the oxime oxygen atom are reported in table 1.

Table 1: Binding affinity and distances between the phosphorus atom of VX and GB-inhibited *hAChE* and the oxime oxygen atom of the best molecular docking pose of selected oximes. Binding affinity in kcal/mol and P-O distances in angstrom.

Oxime	VX		GB	
	ΔG (kcal/mol)	Distance P-O (\AA)	ΔG (kcal/mol)	Distance P-O (\AA)
1	-9	10.7	-8.2	9.2
2	-8.6	4.2	-7.7	6.3
3	-10.1	10.2	-8.7	9.6
4	-9.7	10.8	-8.1	11

Results for bi-functional sugar-oximes **1** and **2** showed a uniform low binding affinity for all OPNAs tested indicating a poor positioning of the oximes inside the gorge of the *hAChE*. The sugar moiety of compound **1** does not interact with the aromatic residues of the gorge to give a possible stabilizing interaction, as shown in figure 5. Interestingly, the sugar moiety of compound **2**, which has a 3 carbon atoms linker, is able to bind between Tyr341 and Tyr337 of the VX-inhibited *hAChE*. The resulting 4.2 \AA distance observed between the oxime oxygen atom and the phosphorus atom of the phosphorylated *hAChE* is sufficiently short to allow a possible reactivation of the enzyme by compound **2**. As expected, the binding affinity observed for tri-functional sugar oximes **3** and **4** appeared to be higher than those observed for **1** and **2** (Table 1). The triazole moiety of compounds **3** and **4** act as a ligand of the peripheral site by forming a π stacking interaction with Tyr341, stabilizing the molecule in a productive way. Overall, the sugar moiety of these new oximes appears to be localized most of the time at the entrance of the gorge without strong interaction with the peripheral site. In this position, the sugar moiety cannot interfere with the binding of the oxime moiety down into the gorge, allowing the oxime oxygen atom to reach a short and productive distance to the phosphorus atom.

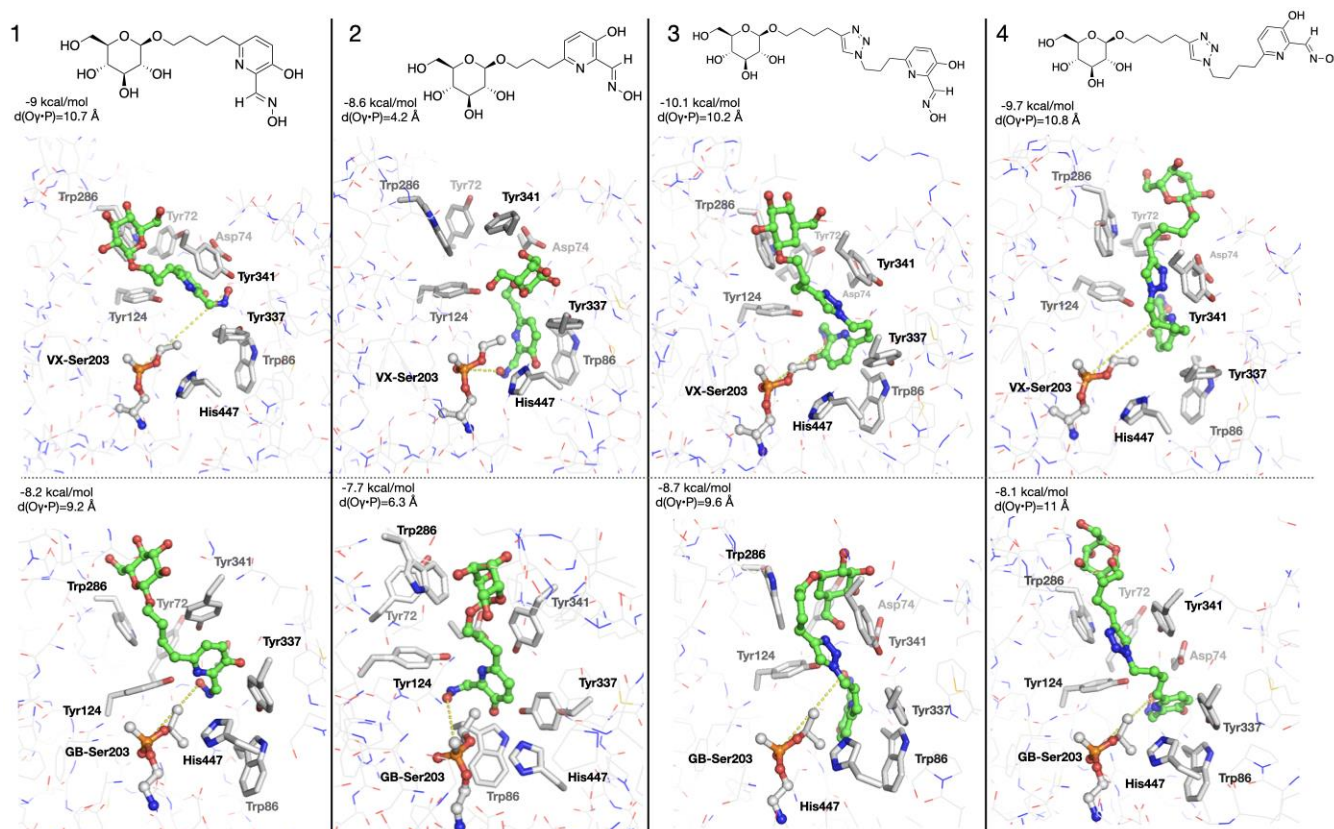


Figure 5: Molecular docking of selected oximes in the active site of VX and GB-inhibited human acetylcholinesterase (respectively top and bottom panel). The binding energy determined by the scoring function of Autodock Vina and the distance between the phosphorus Atom of VX or GB and the oxime oxygen atom are indicated in the top left corner each docking pose.

3.3 *In vitro* reactivation of OPNAs phosphylated *hAChE*

We first determined the half-maximal inhibitory concentration (IC₅₀) for compounds **1,2,3** and **4** (Table 2). Results showed a generally low affinity of the new sugar-oximes for recombinant uninhibited *hAChE*. Noteworthy, oximes **1** and **2**, which differ only by one carbon atom in the alkyl chain between the 3-hydroxypyridinaldoxime and the glucose, displayed a high binding affinity difference implying that the length of the linker (i.e., four carbons for oxime **1** versus 3 carbons for oxime **2**) is a key parameter for the binding of the reactivators to their target. The higher flexibility allowed by the four carbons alkyl chain of oxime **1** may prevent the molecule to bind tightly to the enzyme. Oximes **3** and **4** also differ by one carbon atom in the length of the alkyl chain between the oxime moiety and the triazole (3 carbons for oxime **3** and 4 for oxime **4**). The binding affinity of oxime **3** is approximatively three times lower than the affinity of oxime **4** which may indicate that the four carbons linker of compound **4** allows a more

effective binding of the molecule inside the active site of *hAChE*. Altogether, these results displaying a low affinity for native *hAChE*, clearly suggest that oximes **1-4** can be used at high concentration without inhibiting *hAChE*.

Table 2: Half maximal inhibitory concentration (IC₅₀) for *hAChE*.

Oxime	IC ₅₀ μ M
1	97% at 5 mM
2	140 \pm 20
3	1600 \pm 200
4	580 \pm 10
HI-6	55 \pm 5
2-PAM	560 \pm 30
Obidoxime	640 \pm 70

We then determined the reactivation kinetics constants for the selected oximes, as showed in table 3. The α anomer of compound **4** (compound **4'**) and the riboconjugate of compound **4**, (compound **5**) were also tested for their reactivation efficacy. Surrogates of VX (NEMP), sarin (NIMP) and tabun (NEDPA) as well as an organophosphorus pesticide (paraoxon) were used for *hAChE* inhibition, as they give the same phosphyl moiety on the serine residue as their corresponding OPNA.

Table 3: Reactivation kinetics of *hAChE* inhibited by nerve agent surrogated and paraoxon by selected oximes. *nd*: if [reactivator] \ll K_D, then there is a linear dependence between k_{obs} and [reactivator]: k_{obs} = (k_r/K_D)[reactivator]. In this case, K_r and K_D cannot be determined, but k_{r2}=k_r/K_D is the slope of the line and can be directly obtained by fitting.

OP	Oxime	k _r min ⁻¹	K _D mM	k _{r2} mM ⁻¹ .min ⁻¹
NEMP	1	0.4 \pm 0.1	4 \pm 1	0.1
	2	0.3 \pm 0.01	1 \pm 0.09	0.2
	3	0.5 \pm 0.07	1.5 \pm 0.3	0.3
	4	1 \pm 0.1	1 \pm 0.3	1
	4'	1 \pm 0.02	1 \pm 0.04	1
	5	2.5 \pm 0.2	2.4 \pm 0.4	1.0
	HI-6	0.65 \pm 0.03	0.07 \pm 0.008	9.3
	2-PAM	0.1 \pm 0.01	0.2 \pm 0.06	0.5
	Obidoxime	0.2 \pm 0.007	0.6 \pm 0.04	0.3
NIMP	1	0.2 \pm 0.006	1 \pm 0.08	0.2
	2	0.2 \pm 0.02	2 \pm 0.4	0.1
	3	0.2 \pm 0.01	1.3 \pm 0.2	0.15
	4	0.7 \pm 0.1	2 \pm 0.5	0.4
	4'	0.4 \pm 0.09	2 \pm 0.7	0.2

	5	nd	nd	0.2
	HI-6	1 ± 0.2	0.09 ± 0.03	11
	2-PAM	0.24 ± 0.02	0.2 ± 0.03	1.2
	Obidoxime	0.2 ± 0.02	0.5 ± 0.09	0.4
NEDPA	1	0.1 ± 0.01	1.3 ± 0.2	0.1
	2	0.2 ± 0.02	1.2 ± 0.3	0.2
	3	0.2 ± 0.08	3 ± 1.4	0.07
	4	0.6 ± 0.02	0.9 ± 0.1	0.6
	4'	0.5 ± 0.04	0.6 ± 0.1	0.8
	5	0.8 ± 0.2	3 ± 1	0.3
	HI-6	0.05 ± 0.005	0.2 ± 0.04	0.2
	2-PAM	0.15 ± 0.025	0.8 ± 0.2	0.2
	Obidoxime	0.6 ± 0.04	0.3 ± 0.07	2
PARAOXON	1	0.03 ± 0.005	0.5 ± 0.2	0.05
	2	nd	nd	0.2
	3	0.2 ± 0.08	2 ± 0.4	0.2
	4	0.6 ± 0.1	2 ± 0.7	0.3
	4'	nd	nd	0.8
	5	0.6 ± 0.1	2.7 ± 0.7	0.2
	HI-6	0.09 ± 0.009	0.8 ± 0.14	0.1
	2-PAM	0.05 ± 0.006	0.4 ± 0.08	0.1
	Obidoxime	0.3 ± 0.02	0.5 ± 0.09	0.6

The results, displayed in table 3, showed a generally low efficacy (k_{r2}) of the sugar oximes in the same range as 2-PAM in all cases, and in the same range of HI-6 (but lower than obidoxime), for NEDPA and paraoxon phosphylated *hAChE*. The low affinity (K_D), in the millimolar range, of these compounds for the phosphylated *hAChE* are clearly responsible for the poor reactivation efficiency. However, the compounds **4**, **4'** and **5** performed better than 2-PAM and obidoxime for the NEMP-inhibited *hAChE*, mainly due to the high reactivation rates observed. Regarding the NIMP-inhibited *hAChE*, the sugar oximes are outperformed by HI-6, 2-PAM and obidoxime, and they are slightly better than HI-6 and 2-PAM for NEDPA-inhibited enzyme. Compound **4'** appeared to reactivate the paraoxon inhibited *hAChE* better than HI-6, 2-PAM, and obidoxime.

3.4 X-ray structures of compounds **3** and **4** in native *hAChE*

In order to confirm the binding position of the sugar-oximes in the gorge of *hAChE*, we solved the structure of the complexes formed by the non-modified *hAChE* and compound **3** (PDB 7P1P) and **4** (PDB 7P1N) (Figure 6). Data collection and refinement statistics are presented in

table S2. An overall view of the binding of compounds **3** and **4** in the gorge of *hAChE* shows the binding of the oximes are in a favorable orientation. Indeed, the oxime moiety is located at the bottom of the gorge, close to the catalytic serine, and the sugar moiety can be observed outside the gorge not interacting with Trp286, thus not interfering with the reactivation reaction. A closer view (Figure 6 ; lower panel), illustrates the interactions of compounds **3** and **4** with the aromatic aminoacid lining the gorge of the *hAChE*. For both compounds, we can observe π -stacking interaction between the triazole and the Tyr341, as predicted by molecular docking.

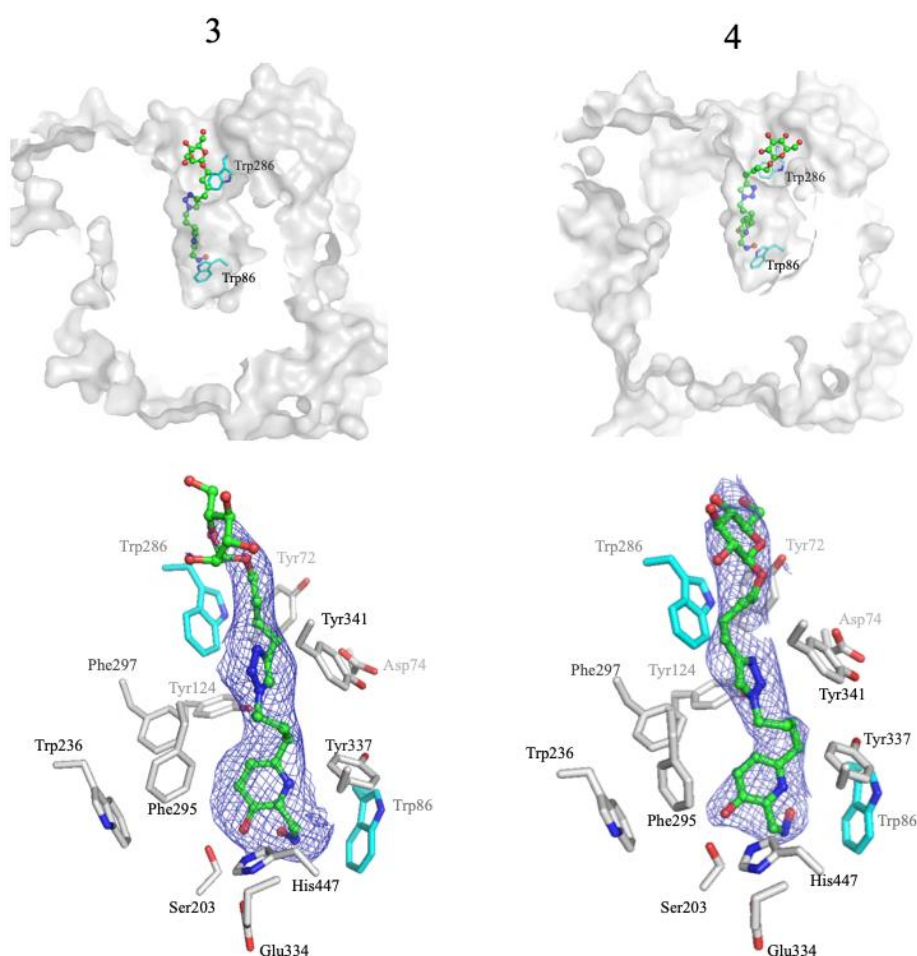


Figure 6 : Views of oxime **3** (PDB 7P1P) and **4** (PDB 7P1N) in complex with *hAChE*. Top : Overall view of oximes **3** and **4** location inside the gorge of *hAChE* defined by the solvent accessible surface, showing the sugar moiety outside the gorge. Bottom : Closer view of oximes **3** and **4** inside the gorge of *hAChE*. A 1- σ feature-enhanced map is represented as a blue mesh. Key peripheral (Trp286) and active site (Trp86) tryptophan residues are represented in sticks with carbons in cyan.

3.5 *In vivo* protective index assessment

In order to determine the *in vivo* efficacy of the new oximes, protective indexes (PI) were determined for compounds **4** and **4'** at 100 $\mu\text{mol/kg}$ and compared to 2-PAM, HI-6, obidoxime. Experiments of up-and-down procedure performed on paraoxon, NIMP and NEMP exposure are presented in Table S3, S4 and S5 respectively and summed up in Figure 7. LD₅₀ of paraoxon, NIMP and NEMP were respectively established to 818, 605 and 350 $\mu\text{g/kg}$. PI of a 100 $\mu\text{mol/kg}$ i.p. 2-PAM treatment 1 min after paraoxon exposure was previously assessed to 2.58³⁷. Alone, 100 $\mu\text{mol/kg}$ of obidoxime gave the most interesting protective spectrum against the three studied OPs even if HI-6 showed a higher PI than obidoxime in the case of treatment of NEMP exposure (3.00 vs. 2.06), the lowest protective efficacy of HI-6 against paraoxon exposure (PI = 1.6) was crippling. It should be noted that oximes **4** and **4'** presented an almost identical and moderate *in vivo* protection against the three OP exposures.

Protective index without atropine

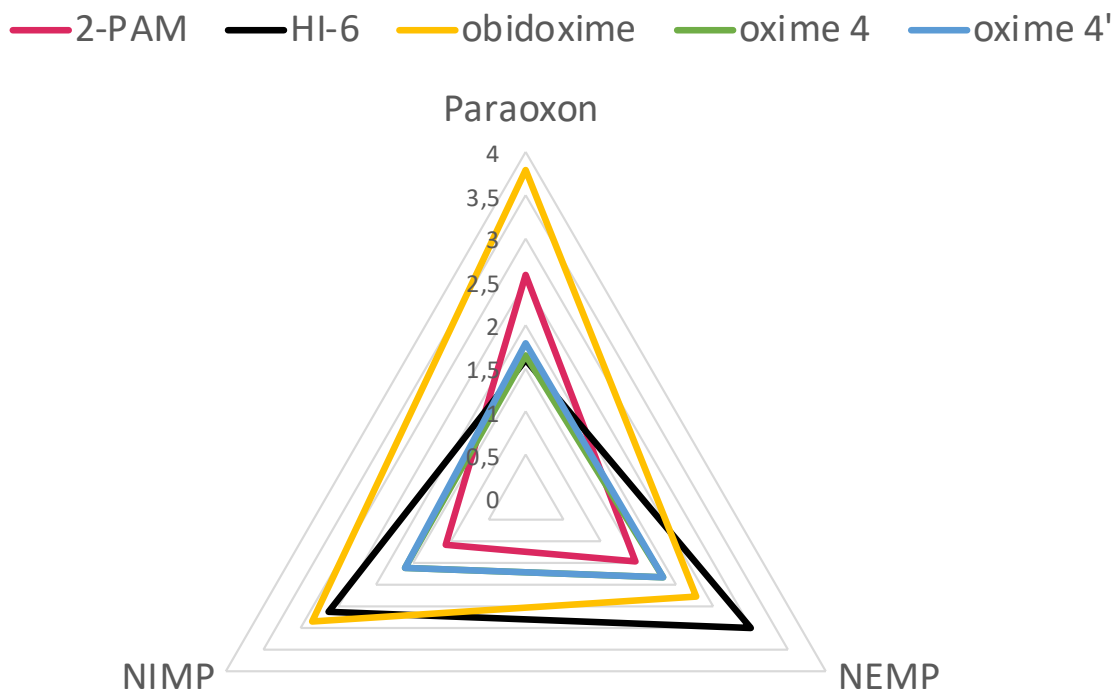


Figure 7. Radar representation of PI values of 100 $\mu\text{mol/kg}$ intraperitoneally treatment of 2-PAM, HI-6, obidoxime, oxime **4** and **4'** one minute after paraoxon, NIMP or NEMP subcutaneous exposure. PI were determined by the up-and-down method.

3.6 Pharmacokinetic study

Both oximes (**4** and **4'**) exhibited a similar enzymatic reactivation profile with a low reactivation percentage at the peak (React max = $2,5 \pm 0,8$ % and $3,3 \pm 0,7$ % respectively). The peak is reached more rapidly for oxime **4** than for oxime **4'** ($T_{\max} = 7,0 \pm 0,0$ vs $10,0 \pm 0,0$ min after injection) as shown in Fig. 8. Oxime **4** also persisted for a shorter time than oxime **4'** in mice plasma as demonstrated by the MRT value ($15,0 \pm 0,0$ vs $20,0 \pm 0,0$ min).

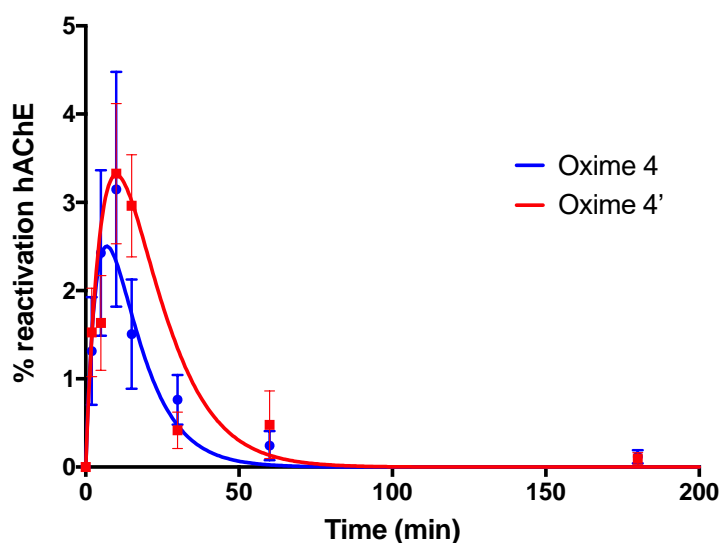


Figure 8. reactivation of VX-inhibited *hAChE* by oximes **4** and **4'** in mice plasma. The same dose of 100 $\mu\text{mol/kg}$ of oxime **4** and **4'** was administered intraperitoneally to mice ($n = 7$). Blood samples were drawn at various time points (0, 2, 5, 10, 15, 30, 60 and 180 min) after treatment, and the levels of reactivation of VX-phosphorylated *hAChE* were determined. Values are presented as percentages of maximum reactivation and points are means \pm SEM. Fitting was performed on GraphPad Prism software.

Table 4. Pharmacokinetic data of oximes **4** and **4'**. MRT: mean residence time, T_{\max} and React max respectively x and y coordinates of the peak of reactivation of VX-phosphorylated *hAChE* by oximes **4** and **4'** in mice plasma presented in Figure 8, and C_{\max} the peak concentration of oximes **4** and **4'** calculated from standard reactivation curves (Figure S1).

Oxime	MRT (min)	T_{\max} (min)	React max (%)	C_{\max} (μM)
Oxime 4	$15,0 \pm 0,0$	$7,0 \pm 0,0$	$2,5 \pm 0,8$	96,7
Oxime 4'	$20,0 \pm 0,0$	$10,0 \pm 0,0$	$3,3 \pm 0,7$	145,7

3.7 Blood-brain barrier permeability tests

One aim was to improve the transport of the oximes across the blood-brain barrier (BBB) endothelial cells to reactivate the central AChE. In order to determine if our sugar oximes are able to reach the CNS, we evaluated the endothelial permeability coefficients (Pe) of some gluco- and riboconjugate oximes (compounds **1**, **2**, **3**, **4**, **4'**, **5**, **15**) as well as representative simpler glycoconjugate molecules (compounds **6**, **7**, **8**, **9**, **10**, **11**, **12**, **13**, **14**) using the human BBB *in vitro* model. No toxicity was observed in our culture conditions (data not shown).

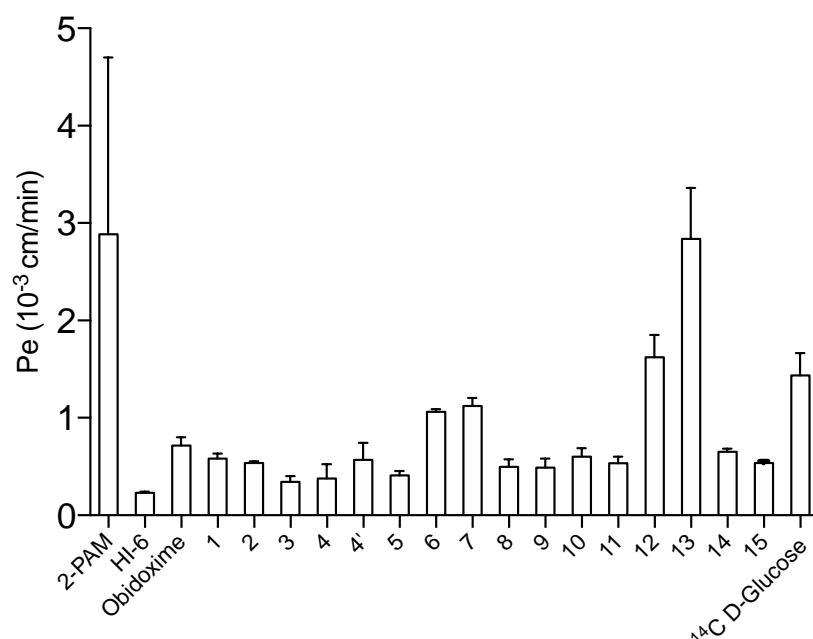


Figure 9: Endothelial permeability coefficients (Pe) of control oximes (2-PAM, HI-6, obidoxime), glycoconjugate oximes (compounds **1**, **2**, **3**, **4**, **4'**, **5**, **15**), glycoconjugate intermediates (compounds **6**, **7**, **8**, **9**, **10**, **11**, **12**, **13**, **14**) and ¹⁴C D-glucose. Values are means ± SD, n=3-9.

Results showed that all the compounds, the conjugate oximes or the simpler glycoconjugate molecules, had a lower rate of transport through BBB endothelial cells than 2-PAM ($Pe_{2-PAM} = 2.89 \pm 1.81 \times 10^{-3} \text{ cm} \cdot \text{min}^{-1}$) (Figure 9). Only one glycoconjugate without oxime moiety (compound **13**) exhibited a permeability coefficient in the same range ($Pe_{13} = 2.83 \pm 0.52 \times 10^{-3} \text{ cm} \cdot \text{min}^{-1}$). Most of the molecules showed Pe values between those of HI-6 ($Pe = 0.23 \pm 0.01 \times 10^{-3} \text{ cm} \cdot \text{min}^{-1}$) and obidoxime ($Pe = 0.72 \pm 0.08 \times 10^{-3} \text{ cm} \cdot \text{min}^{-1}$) that are described to slowly cross the BBB which is confirmed in our *in vitro* model study (Figure 9).

The rate of transport of glycoconjugate oximes (compounds **1, 2, 3, 4, 4', 5, 15**) has also been compared to that of ^{14}C D-glucose which is taken up by a transporter (GLUT-1) so as to facilitate its crossing through membranes of the endothelial cells. The Pe values of glycoconjugate oximes (Pe from 0.34 to $0.58 \times 10^{-3} \text{ cm.min}^{-1}$, table S6) were two to three-fold lower than that of glucose ($\text{Pe}_{\text{glucose}} = 1.43 \pm 0.23 \times 10^{-3} \text{ cm.min}^{-1}$). At this stage, to understand at which level of the oxime glycoconjugates were no longer supported by the facilitated diffusion, the Pe values of the simpler glycoconjugate molecules were also evaluated and compared to that of glucose. None or slight differences were observed between the endothelial permeability coefficients of the ^{14}C D-glucose and those of the ribose (compounds **12, 13**) or glucose (compounds **6,7**) connected with an additional alkyne moiety bearing carbon chain (3 or 5 additional carbons branched at the anomeric oxygen atom) showing that these molecules crossed the BBB endothelium in the same way as glucose. The strategy used here, is a first step to improve the transport of molecules through the BBB. However, when a triazole moiety has been connected to the additional carbon chain of ribose (compound **14**) or of glucose (compounds **8, 9, 10, 11, 12**) Pe values showed a three- and two-fold decrease, respectively demonstrating that the rate of transport through the endothelial cells was slowed down in the same range as for the gluco- and riboconjugate oximes (Figure 9, Table S6). These results showed that in the process of the glycoconjugates synthesis, the triazole moiety, required for the binding of the oximes to *hAChE*, has a detrimental effect on BBB crossing ability, as this moiety has been shown to restrain the facilitated diffusion through glucose transporters.

4 Discussion/conclusion

Reactivation of the central OP-phosphylated AChE is one of the aims of the newly synthesized oximes which have been described lately in the literature. An efficient crossing of the BBB appears to be one major limitation for these numerous new designed oxime reactivators. If efficient reactivation of peripheral phosphylated *hAChE* is the key for survival after OPNA

665 exposure, inhibition of central AChE may induce long-term side effects and detrimental
666 neurological disorders. Many design strategies, among which the use of uncharged or
667 glucoconjugated reactivators have been evaluated to overcome this limitation. In this study, we
668 intended to evaluate the combination of these two strategies, and evaluated the ability of a new
669 family of uncharged glucoconjugated 3-hydroxypyridinaldoxime to reactivate inhibited *hAChE*
670 with the goal in mind to take advantage of the glucose transporter system located at the BBB
671 in order to reach the centrally OP-phosphorylated AChE. We thus synthesized simple bi-
672 functional glycoconjugated uncharged reactivators **1** and **2**, which displayed, as expected, low
673 affinity for the phosphylated *hAChE* and a poor reactivation profile. We also synthesized tri-
674 functional glycoconjugated uncharged oximes **3**, **4**, **4'** and **5** bearing an additional triazole
675 moiety in order to increase the affinity of these oximes for the phosphylated AChE through
676 binding of the triazole moiety to the peripheral site of the enzyme. An *in-silico* study by
677 molecular docking of bi-functional oximes **1** and **2** and tri-functional oximes **3** and **4** showed a
678 general low binding affinity between -7,7 and -10,1 kcal/mol for VX or GB inhibited *hAChE*.
679 Except for compound **2**, the results showed P-O distances between 9,2 and 11 Å. All together,
680 these *in-silico* results suggested that oximes **1**, **2**, **3** and **4** may reactivate, yet with moderate
681 efficiency the VX or GB-phosphorylated *hAChE*. We then evaluated the reactivation efficiency
682 of oximes **1**, **2**, **3**, **4**, and **4'** (the α anomer of compound **4**) and oxime **5** (the riboconjugated
683 analogue of oxime **4**). The results show a general low binding affinity (K_D) in the millimolar
684 range of the sugar oximes for the phosphylated *hAChE*. This low binding affinity negatively
685 affected the overall reactivation efficacy (k_{r2}). It may be due to the presence of the sugar moiety
686 on the oximes, which does not allow the compound to penetrate deep enough inside the gorge
687 of the phosphylated enzyme for an efficient reactivation. Crystal structures of compounds **3** and
688 **4** have been solved in complex with *hAChE* (PDB 7P1P and 7P1N). Data show that both oximes
689 were able to enter the gorge of the *hAChE* with the 3-hydroxypyridinaldoxime moiety located

near the catalytic site, the triazole moiety interacting by π -stacking interactions with Tyr341 and the glucose moiety spanning outside the gorge without unfavorable interactions with amino acids of the peripheral site. These results showed that compounds **3** and **4** were orientated inside the gorge in a productive way that may reactivate the inhibited *hAChE*. The presence of the triazole moiety on oximes **3**, **4**, **4'** and **5** was predicted to improve the affinity of the sugar oximes for the inhibited enzymes, but results showed a similar low binding affinity in the same range as compounds **1** and **2**. However, a high enough reactivation kinetics k_r allows oximes **4**, **4'** and **5** to be more efficient than 2-PAM and obidoxime to reactivate the NEMP-phosphylated *hAChE* as well as oximes **4** and **4'** to reactivate NEDPA-phosphylated *hAChE*. The α anomer of the oxime **4** (named **4'**) is the only compound to perform better than 2-PAM, HI-6 and obidoxime for paraoxon phosphylated *hAChE*.

Our goal was to design new uncharged oximes that could reach the CNS more efficiently through the active glucose transporter located at the BBB. In order to evaluate our hypothesis we determined protective indexes for compounds **4** and **4'** on mice exposed to NIMP, NEMP and paraoxon. Our results showed that oximes **4** and **4'** display a similar protection profile. The use of the α anomer of compound **4** did not improve the protective index. *In vivo* results showed that both oxime protect as well as HI-6 in mice exposed to paraoxon and appeared to be more effective than 2-PAM against NIMP and NEMP. Pharmacokinetic studies demonstrated, after an intraperitoneal injection, the presence of compounds **4** and **4'** in blood during the experiments but showed that only a low percentage of VX-phosphylated enzymes could be reactivated. *In-vitro* reactivation studies have shown the low affinity of the sugar-oximes for the phosphylated *hAChE* and, therefore, their moderate reactivation capabilities. We can hypothesize that the low protective indexes recorded in this study may be improved by administering a higher dose of oxime in adequacy with the compound properties : interestingly, contrary to HI-6, these oximes poorly inhibit native *hAChE* and can thus be used at a higher

dose.

To evaluate the ability of our sugar oximes conjugates to cross the BBB we determined the endothelial permeability coefficients of gluco- and riboconjugate oximes (**1, 2, 3, 4, 4', 5, 15**) as well as simpler glycoconjugated molecules (compounds **6, 7, 8, 9, 10, 11, 12, 13, 14**) using the human BBB *in vitro* model (Figure 9). Our results showed that the designed sugar oximes do not cross the BBB at the same transport rate as glucose, implying a structural restraint from our compounds. 2-PAM has the highest transport rate than any other evaluated oximes, but the Pe for oxime **1, 2, 3, 4, 4'** and **5** is higher than the Pe for HI-6 and slightly lower than the Pe for obidoxime. However, we were disappointed to observe that the presence of the triazole moiety on tri-functional oximes **3, 4, 4', 5**, incorporated to increase the affinity of these compounds for phosphylated *hAChE*, as well as its presence in simpler glycoconjugated molecules such as **8, 9, 10, 11** and **14** reduces the transport rate through the BBB which may negatively impact the protective index determined in our *in vivo* experiments.

In summary, we designed a new family of uncharged oximes glycoconjugates to combine two strategies to reach the CNS more efficiently through the use of non-permanently charges oximes and taking advantage of an active glucose transport system to improve the protection of oxime-based reactivators against neurotoxic organophosphorus poisoning. Our study showed that our compounds perform roughly as the oximes currently used across the world but highlights the structural restraints which apply to oximes targeting the glucose transport system: addition of an additional aromatic moiety to improve binding of the oximes to phosphylated *hAChE* negatively impacts their BBB crossing ability. Based on this work, new sugar-oximes could be designed using other moieties than triazoles to bind efficiently phosphylated *hAChE*, and probably longer side chains between the first two components of these tri-functional reactivators (sugar / AChE binding moiety / oxime), synthesized in order to use efficiently this glucose transport system of the BBB to reach the central OP-phosphylated AChE.

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