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A New Class of Bi- and Trifunctional Sugar Oximes as Antidotes against Organophosphorus Poisoning

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

35 **1. Introduction**

36 The recent use of organophosphorus nerve agents (OPNAs) such as VX against Kim Jong-Nam
37 in Malaysia in 2017¹ or Novichoks in march 2018 against a former Russian spy, Sergei Skripal
38 and his daughter Yulia² demonstrates that these compounds are still a real threat for the civilian
39 and military populations. Commonly, nerve agents affect the cholinergic neurotransmission by
40 phosphorylation of the catalytic serine residue of acetylcholinesterase (AChE, EC 3.1.1.7), a key
41 enzyme for nerve impulse termination, located in the central nervous system (CNS) and at the
42 neuromuscular junctions. This covalent binding leads to the irreversible inhibition of the
43 enzyme, accumulation of the neurotransmitter acetylcholine, and subsequently to a cholinergic
44 crisis characterized by nausea, dyspnea, seizure and death if not treated rapidly³. The current
45 therapy for OPNAs poisoning associates a muscarinic antagonist drug (e.g. atropine), an
46 anticonvulsant drug (e.g. diazepam) and a pyridinium aldoxime reactivator (pralidoxime,
47 trimedoxime, obidoxime, HI-6)⁴ able to remove the phosphyl group attached to the catalytic
48 serine residue. These permanently charged quaternary oximes present an adequate pKa for the
49 nucleophilic oxime residue increasing its nucleophilic character, and an electron deficient
50 heteroaromatic moiety responsible for their binding affinity, and their positioning close to the
51 phosphorylated serine residue in the enzyme catalytic site. However, these charged oximes are
52 known cross the blood-brain barrier (BBB) poorly. Thus, if this medical countermeasure can
53 limit the deadly peripheral cholinergic crisis, they do not reactivate cholinesterases of the CNS,
54 causing long-lasting neurological disorders and side effects⁵. Only 4 to 10 % of the oxime
55 present in the plasma cross the BBB and are subsequently available for brain protection against
56 OP poisoning⁶. Moreover, despite the synthesis and evaluation of numerous new oximes over

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

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

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

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

101 **2. Materials and Methods**

102 **2.1 Chemicals**

103 2-PAM and HI-6 were obtained from Pharmacie Centrale des Armées (Orléans, France), NIMP
104 (4-nitrophenyl isopropyl methylphosphonate), NEMP (4-nitrophenyl ethyl
105 methylphosphonate) and NEDPA (4-nitrophenyl ethyl dimethylphosphoramidate) from UMR
106 CNRS 7515 ICPEES (Strasbourg, France). Obidoxime, heparine, DTNB, acetylthiocholine

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

110 **2.2 Chemistry**

111 **General.** Solvents were purified by a dry solvent station MB-SPS-800 (MBraun, Garching,
112 Germany) immediately prior to use. Triethylamine was distilled from KOH. All reagents were
113 obtained from commercial suppliers (Sigma-Aldrich, St Quentin Fallavier, France; Acros
114 Illkirch, France; TCI Europe, Paris, France) unless otherwise stated. The melting points were
115 recorded on a Stuart SMP30 apparatus (Stuart, Staffordshire, UK). Column chromatography
116 purifications were performed with prepacked SI-HP (30 μ m) or SI-HC (15 μ m) columns from
117 Interchim (Montluçon, France). Preparative normal phase chromatography was carried out on
118 an automated flash purification apparatus, either Biotage Isolera One (Biotage, Uppsala,
119 Sweden) or Interchim 420 PuriFlash. Thin-layer chromatography (TLC) was carried out on
120 Merck DC Kieselgel 60F-254 aluminum sheets (Merck, Darmstadt, Germany). Compounds
121 were visualized by UV irradiation and/or spraying with a solution of vanillin, followed by
122 smooth heating. ^1H and ^{13}C NMR spectra were recorded with a Bruker DPX 300 spectrometer
123 (Bruker, Wissembourg, France) and are presented in the Supporting Information (S9, NMR
124 spectra). Chemical shifts are expressed in parts per million (ppm) from CDCl_3 ($\delta\text{H} = 7.26$ ppm,
125 $\delta\text{C} = 77.16$ ppm). *J* values are expressed in hertz. Mass spectra were obtained with a Finnigan
126 LCQAdvantage MAX (ion trap) apparatus equipped with an electrospray source (Thermo
127 Electron Corporation, Waltham, MA). High-resolution mass spectra were obtained with a
128 Varian MAT 311 spectrometer (Varian MAT, Bremen, Germany) using electrospray analysis.
129 HPLC quality grade acetonitrile and Milli-Q purified water were used for analytical and
130 preparative HPLC. Preparative HPLC run was carried out with an Interchim 4250 apparatus
131 (Interchim, Montluçon, France) with an Interchim puriflash C18 column (Interchim,

132 Montluçon, France), 30 × 250 mm, C18AQ-5 μm. Analytical HPLC was performed on a
133 ThermoFisher UHPLC Ultimate 3000 instrument (ThermoFisher, Waltham, MA) equipped with
134 a PDA detector under the following conditions: Synchronis C18 column (3 μm, 3 × 100 mm)
135 with MeCN and 20 mM NH₄OAc as eluents [using a gradient from 100% 20 mM NH₄OAc to
136 100% MeCN over 30 min] at a flow rate of 0.5 mL/min with UV detection at 254 nm. The
137 synthesis of oxime 1, 2, 3, 4, 4', 5 and 15 are described below. The general chemistry,
138 experimental information, and syntheses of all other compounds are supplied in the Supporting
139 Information. The purity of all final compounds as determined by HPLC analysis is ≥ 95 %. The
140 canonical SMILES of all compounds tested are provided in Table S1.

141 **(*E*)-3-hydroxy-6-(4-(((2*R*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-**
142 **2*H*-pyran-2-yl)oxy)butyl)picolinaldehyde oxime 1**

143 General procedure of oxime formation/deacetylation applied on aldehyde **47** (496 mg, 0.94
144 mmol). The residue was purified by preparative HPLC (0 % for 5 min then 0 % to 30% MeCN
145 in H₂O over 25 min then 30% to 100% over 5 min, 40 mL/min, Interchim puriflash prep
146 C18AQ, 30x250 mm, 5 micro, PF5C18AQ-250/300) to afford the title compound as a solid
147 (137 mg, 39%). mp = 47-53 °C. $[\alpha]_D^{20} = -22.8$ (c 0,50 MeOH). ¹H NMR (300 MHz, MeOD):
148 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
149 (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,
150 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 –
151 1.73 (m, 2H), 1.73 – 1.61 (m, 2H). ¹³C NMR (75 MHz, MeOD): δ 153.3, 152.4, 151.4, 134.8,
152 124.7, 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*
153 calculated for [C₁₆H₂₅N₂O₈]⁺ 373.1611, found 373.1608.

154 **(*E*)-3-hydroxy-6-(3-(((2*R*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-**
155 **2*H*-pyran-2-yl)oxy)propyl)picolinaldehyde oxime 2**

156 General procedure of oxime formation/deacetylation applied on aldehyde **46** (336 mg, 0.98
157 mmol). The residue was purified by preparative HPLC (0% for 5 min then 0 % to 40% MeCN
158 in H₂O over 25 min then 30% to 100% over 5 min, 40 mL/min, Interchim puriflash prep
159 C18AQ, 30x250 mm, 5 micro, PF5C18AQ-250/300) to afford the title compound as an oil (143
160 mg, 41%). $[\alpha]_D^{20} = -18.0$ (*c* 0,50 MeOH). ¹H NMR (300 MHz, MeOD): δ 8.32 (s, 1H), 7.29
161 (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,
162 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 –
163 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).
164 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,
165 70.3, 68.4, 61.4, 32.8, 29.9. HRMS (ESI⁺): *m/z* calculated for [C₁₅H₂₃N₂O₈]⁺ 359.1454, found
166 359.1458.

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

170 General procedure of oxime formation/deacetylation applied on aldehyde **48** (677 mg, 1.07
171 mmol). The residue was purified by normal phase flash chromatography (10% to 30% MeOH
172 in DCM over 30 min, 12G SIHC) then reversed-phase flash chromatography (5 to 60% MeCN
173 in H₂O over 30 min, 80 G, C18-15 micro, Interchim) to afford an off-white solid (384 mg,
174 75%). *R_f* = 0.12 (DCM/MeOH 90/10, v/v). $[\alpha]_D^{20} = -15.3$ (*c* 0,53 MeOH). ¹H NMR (300 MHz,
175 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,
176 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,
177 *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
178 (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
179 (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,
180 2H). ¹³C NMR (75 MHz, MeOD): δ 152.5, 151.6, 151.4, 147.7, 135.2, 124.6, 124.0, 121.9,

181 103.0, 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
182 calculated for [C₂₁H₃₁N₅O₈Na]⁺ 504.2070, found 504.2072.

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

186 General procedure of oxime formation/deacetylation applied on aldehyde **49** (146 mg, 0.22
187 mmol). The residue was purified by normal phase flash chromatography (10% to 30% MeOH
188 in DCM over 30 min, 12G SIHC) then reversed-phase flash chromatography (5 to 100 % MeCN
189 in H₂O over 30 min, 80 G, C18-15 micro, Interchim) to afford an oil (112 mg, 100%). R_f = 0.14
190 (DCM/MeOH 9/1, v/v). [α]_D²³ = -14.5 (c 0.43 MeOH). ¹H NMR (300 MHz, MeOD): δ 8.26
191 (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),
192 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
193 (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
194 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,
195 6H). ¹³C NMR (75 MHz, MeOD): δ 153.9, 153.8, 152.7, 149.1, 136.3, 126.1, 125.4, 123.2,
196 104.3, 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⁺):
197 m/z calculated for [C₂₂H₃₄N₅O₈]⁺ 496.2407 found 496.2413.

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

201 General procedure of oxime formation/deacetylation applied on aldehyde **50** (369 mg, 0.57
202 mmol). The residue was purified by normal phase flash chromatography (10 to 30% MeOH in
203 DCM over 30 min, 12G SIHC) then reversed-phase flash chromatography (5 to 100 % MeCN
204 in H₂O over 30 min, 80 G, C18-15 micro, Interchim) to afford an oil (225 mg, 80%). [α]_D²⁶ =
205 +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**

231 General procedure of oxime formation/deacetylation applied on aldehyde **52** (318 mg, 0.66
232 mmol). The residue was purified by normal phase flash chromatography (5 to 30% MeOH in
233 DCM over 30 min, 25G SIHC), then purified by preparative HPLC (5 to 50% MeCN in H₂O
234 over 30 min, puriflash C18, 30x250 mm, C18AQ-5 micro) to afford the title compound as an
235 oil (128 mg, 52%). $R_f = 0.22$ (90/10 DCM/MeOH, v/v). $[\alpha]_D^{20} = -26.3$ (c 0.51 MeOH). ¹H
236 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),
237 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,
238 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
239 (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, $J = 5.5, 4.5$
240 Hz, 4H). ¹³C NMR (75 MHz, CDCl₃): δ 154.8, 153.8, 152.8, 136.2, 126.1, 125.3, 108.7, 84.7,
241 76.3, 72.8, 68.8, 65.2, 37.8, 31.3, 30.6, 30.1, 27.1. HRMS (ESI⁺): m/z calculated for
242 [C₁₇H₂₇N₂O₇]⁺ 371.1818, found 371.1824.

243 **2.3 Molecular docking**

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

250 **2.4 Recombinant human acetylcholinesterase**

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

252 **2.5 Phosphylation of recombinant human acetylcholinesterase**

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

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

262 **2.6 IC₅₀ measurements**

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

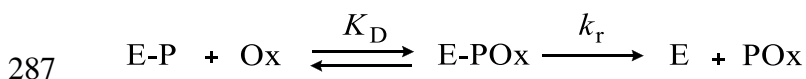
$$272 \quad \% \text{ activity} = \frac{100 \times IC_{50}}{(IC_{50} + [Cp])}$$

273
274

275 **2.7 *In vitro* reactivation of phosphylated human acetylcholinesterase**

276
277 The reactivation of OPNAs-inhibited *hAChE* was performed as previously described¹⁵. Briefly,
278 the phosphylated enzymes were incubated at 37°C with various concentrations of oximes in 0,1
279 % BSA / 0,1 M phosphate buffer pH 7.4. Final concentration of methanol was kept below 2 %
280 and had no effect on enzyme activity. Aliquots of the reactivation mixture were transferred at
281 different time interval to cuvettes containing 1 mM ATC in 1 mL of Ellman's buffer (0.5 mM
282 DTNB in 0.1 M sodium phosphate buffer, pH 7.4) to measure the *hAChE* activity through the

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



$$288 \quad k_{obs} = \frac{k_r [Ox]}{K_D + [Ox]} \quad \text{and} \quad k_{r2} = \frac{k_r}{K_D}$$

289

290 **2.8 Human Acetylcholinesterase crystallization**

291 Recombinant human acetylcholinesterase crystals were grown using the hanging drop vapor
292 diffusion method as described previously²³. Crystals were soaked 60 min in the mother liquor
293 containing 2 mM of each compound. Crystals were then washed with a cryoprotectant solution
294 (1.6 M lithium sulfate, 100 mM HEPES pH 7.0, 60 mM magnesium sulfate and 18 % glycerol)
295 and flash-cooled in liquid nitrogen.

296 **2.9 Data collection, reduction and refinement**

297 Diffraction data were collected at the European Synchrotron Radiation Facility (ESRF,
298 Grenoble, France) at the ID23-2 beam line ($\lambda = 0.873 \text{ \AA}$) and processed with XDS²⁶ and scaled
299 with XSCALE. The structure was solved by molecular replacement with PHASER²⁷ using pdb
300 4EY4 as starting model, iterative cycles of model building using Coot²⁸ and refinement using
301 Phenix²⁹. Data collection and refinement statistics are presented in supporting information
302 (table S2).

303 **2.10 Animals**

304 We used 9-week-old male Swiss mice (Janvier Labs, Le Genest-Saint-Isle, France), weighing
305 35-45 g at the experimentation time. The animals (3-4/cage) were housed for 14-18 days before
306 the experiments in an environment maintained at $22 \pm 1^\circ\text{C}$ with controlled humidity on a 12 h

307 dark/light cycle with light provided between 7 a.m. and 7 p.m. They were given food and tap
308 water *ad libitum*. All experiments were carried out in compliance with the European Directive
309 on the protection of animals used for scientific purposes (2010/63/UE) and were approved by
310 our Institutional Animal Care and Research Advisory Committee (approval n°239 of 10-09-
311 2018).

312 **2.11 Plasma test protocols: monitoring of the reactivability of blood samples**

313 This protocol has been previously fully described ¹⁴.

314 *Standard reactivation curves for pharmacokinetics*

315 Naive mice plasma from Janvier Labs (Le Genest-Saint-Isle, France) was heated at 56°C for 30
316 min to inactivate endogenous cholinesterases. In a 96-well Greiner plate, VX-phosphylated
317 hAChE solution in sodium phosphate buffer was incubated for 30 min at 37°C in presence of
318 different concentrations of oximes diluted in heat-inactivated mice plasma (0, 1, 5, 10, 25, 50,
319 75, 100, 150, 200 and 500 µM). A mix of 2 mM acetylthiocholine and Ellman's buffer (0.5 mM
320 DTNB in 0.1 M sodium phosphate buffer, pH 7.4, 25°C) was then simultaneously added in all
321 wells for measurement of resulting hAChE activity at 412 nm every 5 s for 30 min with a
322 SAFAS spectrophotometer (Monaco).

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

328 *Blood sampling*

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

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

344 **2.12 LD₅₀ estimation and protective index using the up-and-down method**

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

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

361 **2.13 Blood-brain barrier permeability tests**

362 *Human Blood-brain barrier model setting up*

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

380 *Endothelial permeability coefficient evaluation*

381 The permeability of the BBB to the different oximes and ^{14}C D-glucose was evaluated by
382 measuring the endothelial permeability coefficient (Pe) which represents the speed of diffusion
383 through the BLECs monolayer ^{38, 39}. Human BLECs monolayers developed after 6 days of
384 coculture were transferred into new plates containing 1.5 mL per well (abluminal compartment)
385 of HEPES buffered-Ringer's solution (RH; 150mM NaCl, 5.2mM KCl, 2.2mM NaCl₂, 0.2mM
386 MgCl₂, 6mM NaHCO₃, 2.8mM glucose, 5mM HEPES). Medium in apical chambers (luminal
387 compartment) was replaced by 0.5 mL of RH containing either one glycoconjugate oximes or
388 2-PAM or HI-6 or obidoxime used as oximes control. All compounds were tested at 50 μM
389 (dose checked as non-toxic for the human BLECs) for a diffusion duration of an hour at 37°C.
390 Then the amount of each oxime in the luminal and abluminal compartments was measured by
391 mass spectrometry with a TripleTOF 5600+ System (AB SCIEX, Concord, ON, Canada).
392 Percentages of recovery were checked between 86 and 106%. The quantification of
393 radiolabeled ^{14}C D-glucose (PerkinElmer, Boston, MA, USA) was performed using a
394 scintillation counter TriCarb 2100TR (PerkinElmer, USA).

395 The clearance principle was used to calculate a concentration-independent permeability
396 coefficient. The mean compound cleared volume was plotted against time, and the slope was
397 estimated by linear regression. The permeability values of the inserts (PSf for inserts with a
398 Matrigel™ coating only) and the inserts with hBLECs (PSt, Matrigel™-coated inserts +
399 endothelial cells) were taken into consideration by applying the following equation: $1/P_{\text{Se}} =$
400 $1/P_{\text{St}} - 1/P_{\text{Sf}}$. To obtain the endothelial permeability coefficient (Pe expressed in cm/min), the
401 permeability value (PSe) was divided by the insert's membrane surface area (1.13 cm²).

402 **3 Results**

403 **3.1 Chemistry**

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

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

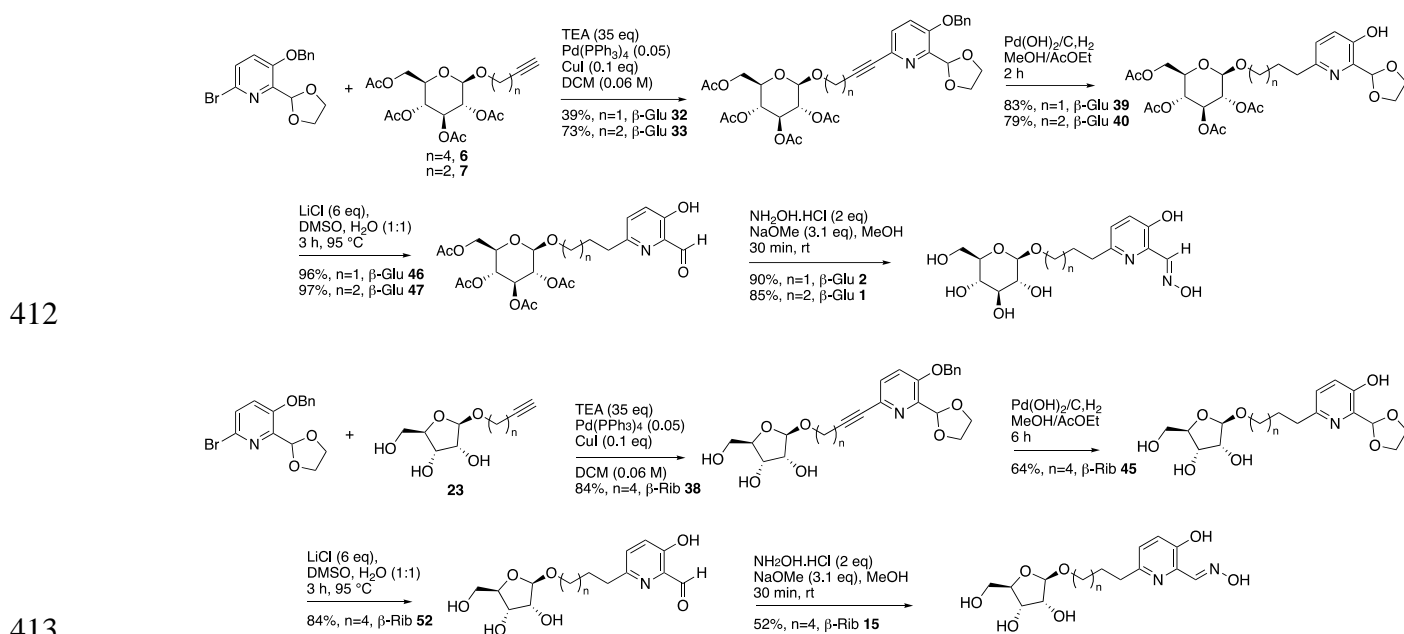
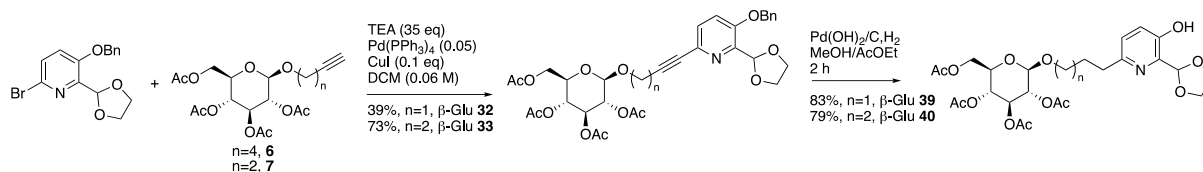
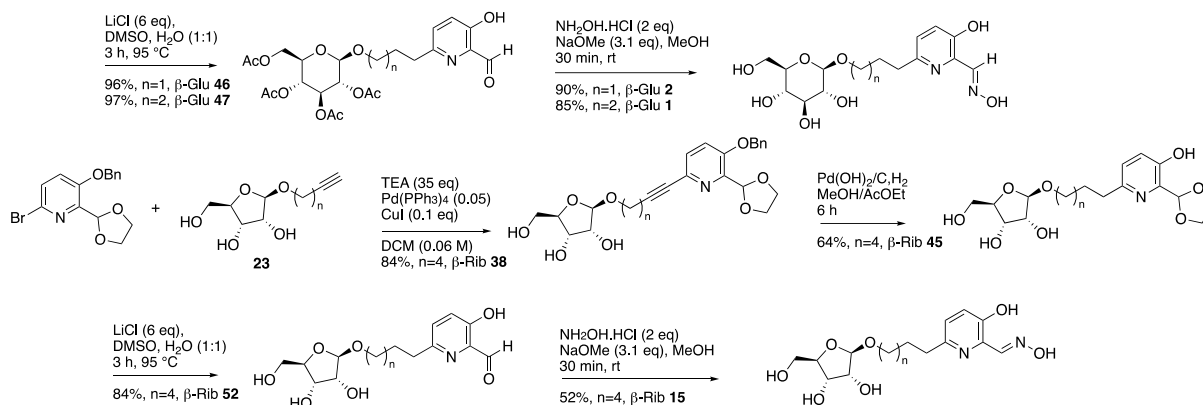


Figure 1). Peracylated glucose bearing a terminal alkyne moiety at its anomeric position of
 different lengths was obtained as previously described⁴⁰. A Sonogashira cross-coupling
 reaction was used to link the glycosyl moiety and a pre-functionalized pyridine ring using
 standard procedure (CuI, Pd(PPh₃)₄) 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.

422

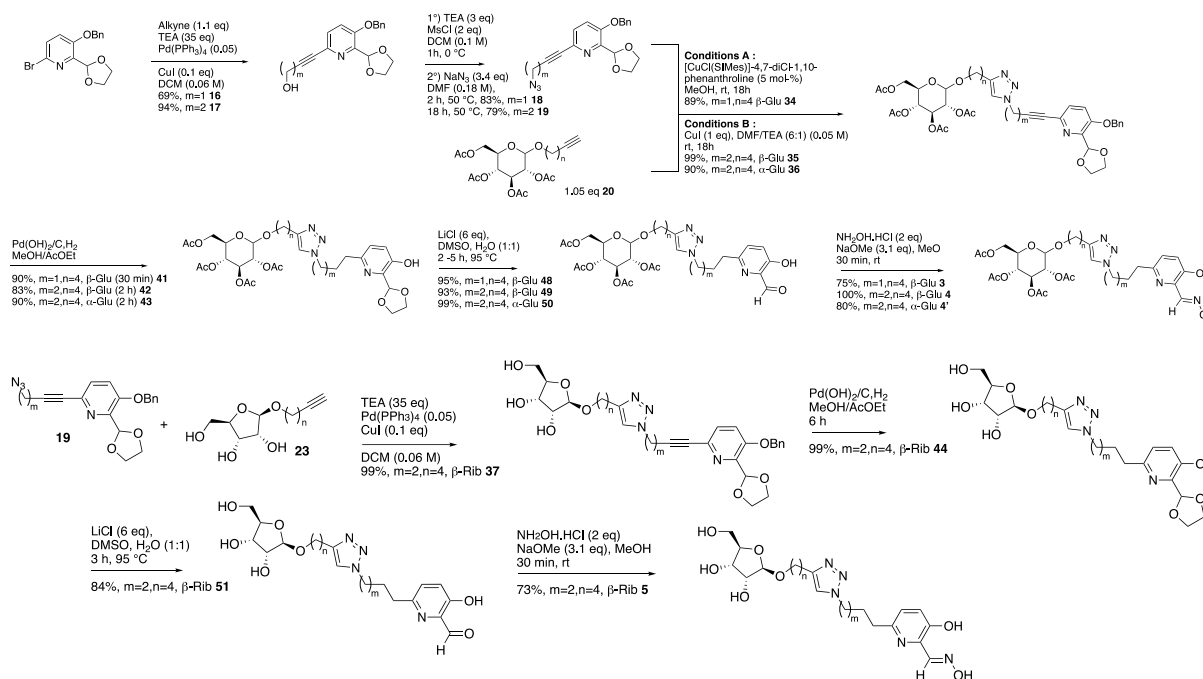


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424

Figure 1. Alkylchain-containing aldoxime synthetic pathway

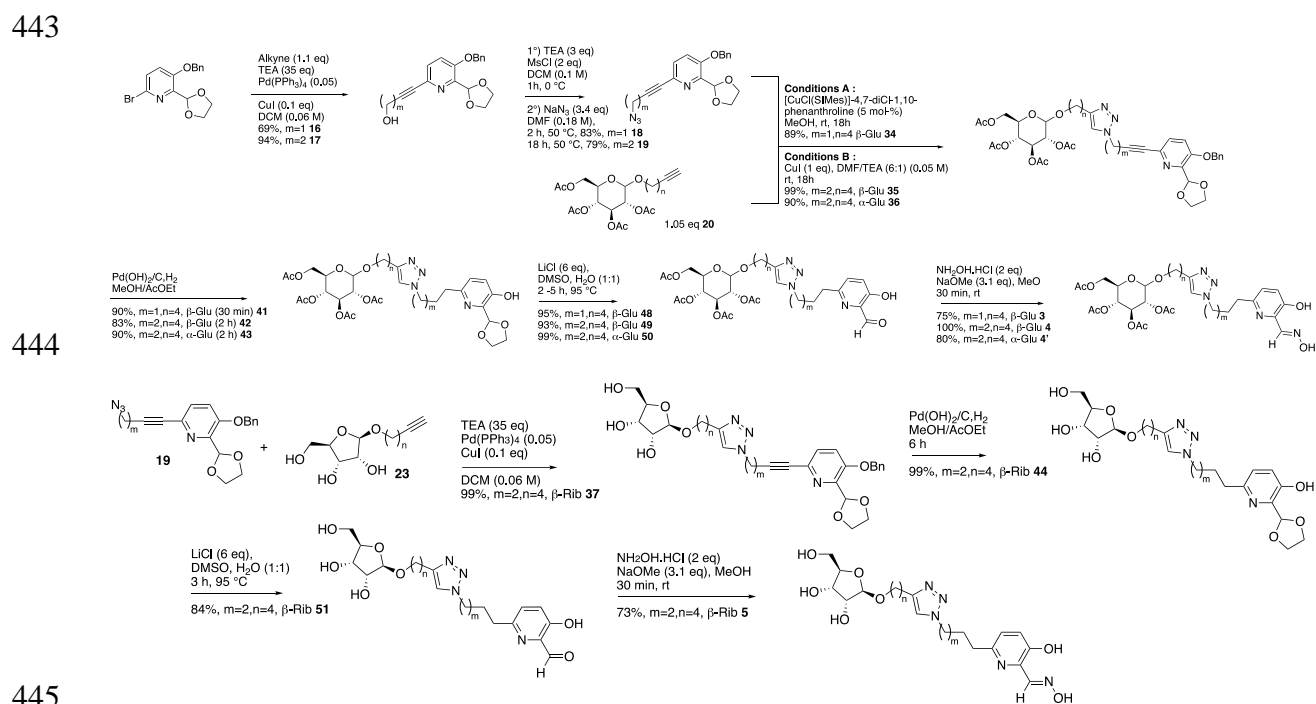
426 Aldoximes **3**, **4**, **4'** and **5** (

427

428

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

434 the best binding affinities were obtained with a 3 or 4 carbon atoms distance between the oxime
 435 and the triazole ring, thus the β -glucoconjugate **3** and **4** were first targeted. In addition, α -
 436 glucoconjugate **4'** and β -riboconjugate **5** were also synthesized to evaluate the role of the sugar
 437 moiety (glucose *vs.* ribose) and of the stereochemistry at the anomeric position (α -glucose *vs.*
 438 β -glucose) in the hexose series. Particular attention was paid to the Huisgen cycloaddition with
 439 the azidopropynylpyridine which required the use of 5-mol-% of [CuCl(SiMes)]-4,7-dichloro-
 440 1,10-phenanthroline⁴³ to avoid by-product formation. The same sequence was used to form the
 441 oxime moiety (overall yields over the four final steps: 57%, 76%, 64% and 60% respectively
 442 for **3**, **4**, **4'** and **5**).



445
 446 Figure 2. Triazole-containing aldoxime synthetic pathway

447
 448 The glycosylation steps were carried out in the presence of either 3-butyn-1-ol, 5-hexyn-1-ol
 449 as a glycol acceptor and the corresponding glycosyl donor in the form of peracetylated
 450 glucopyranose or ribofuranose (

451

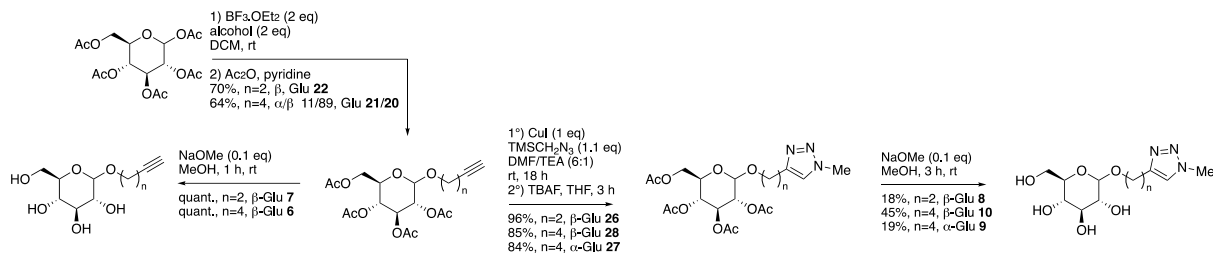
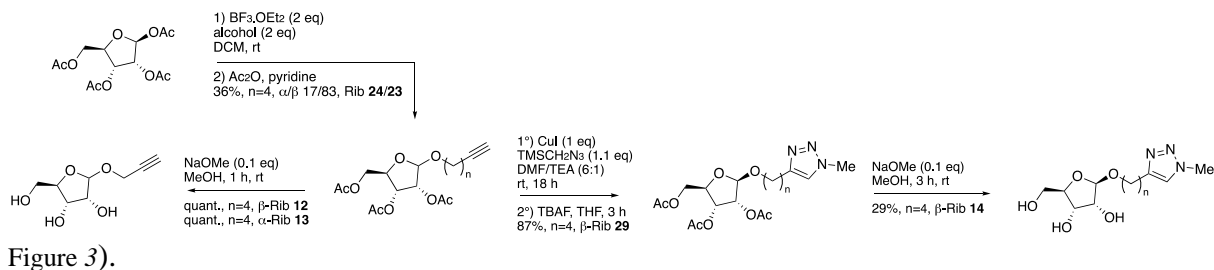
452
453

Figure 3).

454

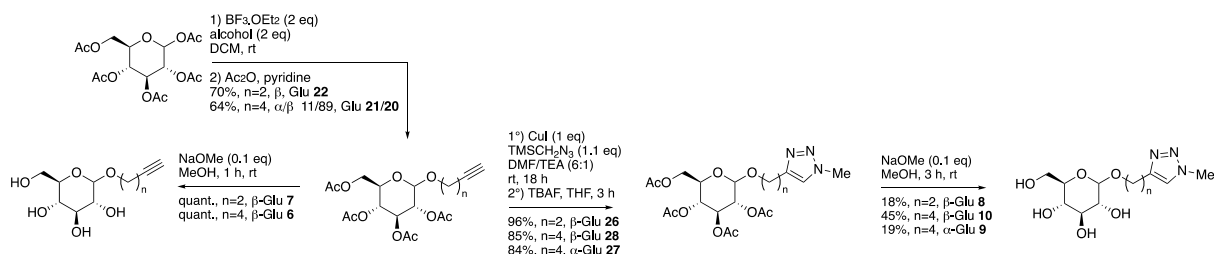
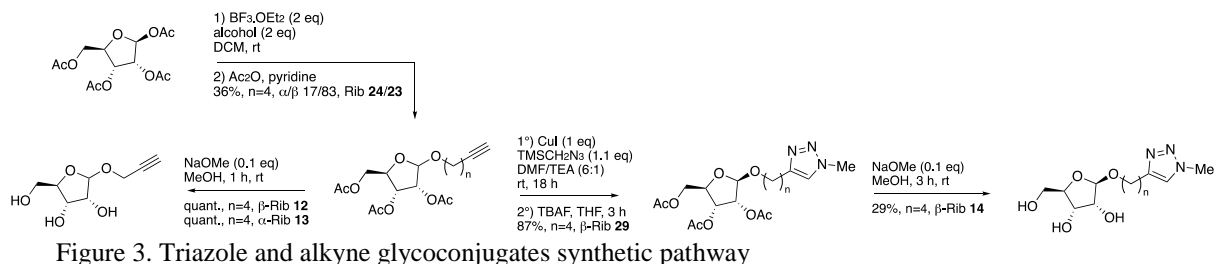
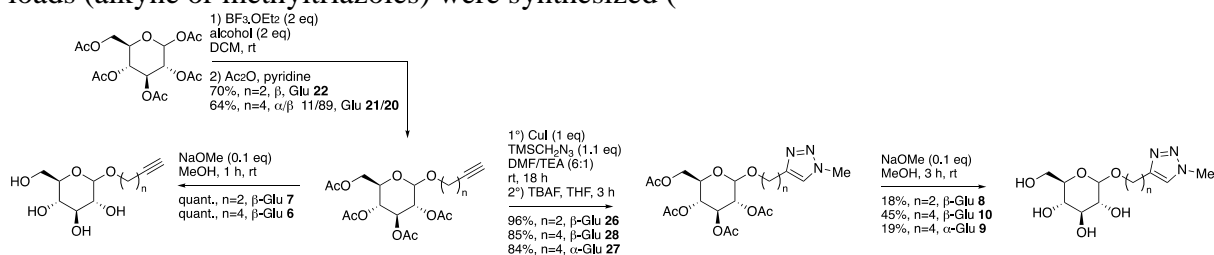
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Figure 3. Triazole and alkyne glycoconjugates synthetic pathway

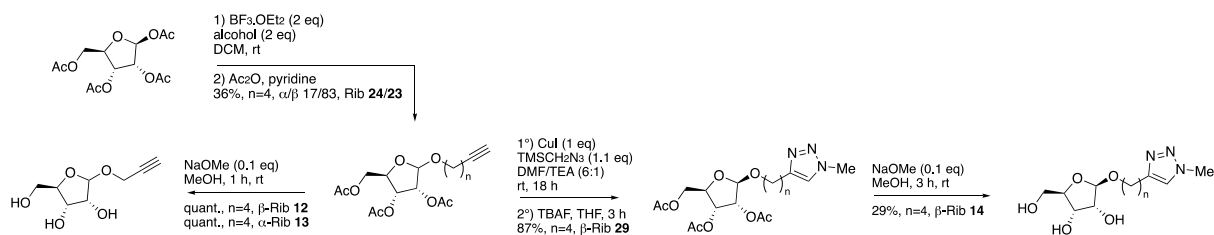
457

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 (

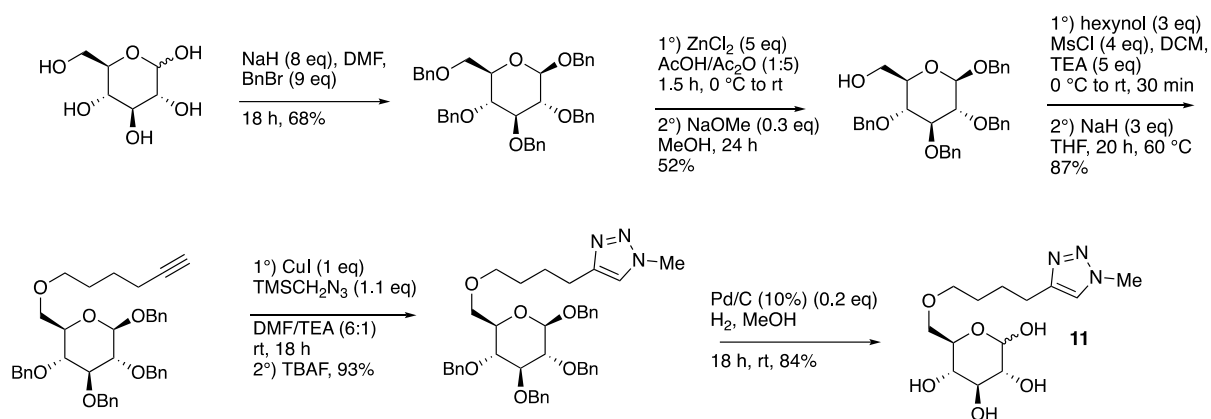
461



462



463 Figure 3). The alkyne chain glycoconjugates **6**, **7**, **12** and **13** were synthesized as a single
 464 diastereoisomer in two steps from the corresponding peracetylated sugar in respectively 57%,
 465 70%, 36% and 6% yield by a glycosylation reaction followed by Zemplén procedure to study
 466 the influence of possible inhibiting interaction. The triazole glycoconjugates, **8**, **9**, **10** and **14**
 467 were synthesized, as pure diastereoisomer, in 3 steps and respectively in 12%, 1%, 22 % and
 468 8% overall yields using Huisgen cycloaddition with 1.1 equivalent of trimethylsilylmethyl
 469 azide followed by TBAF treatment and Zemplén transesterification procedure to uncover the
 470 *N*-methyltriazole glycoconjugates as free hydroxyl groups.
 471 Compound **11**, bearing the triazole alkyl chain on position 6 of the sugar, was also synthesized
 472 to investigate the impact of the position of the triazole-comprising chain on the BBB
 473 permeability. Compound **11** was obtained in 5 steps and with 24 % overall yield from glucose.
 474



476 Figure 4. Position 6-substituted alkyl-triazole glycoconjugate

477 3.2 Molecular Docking

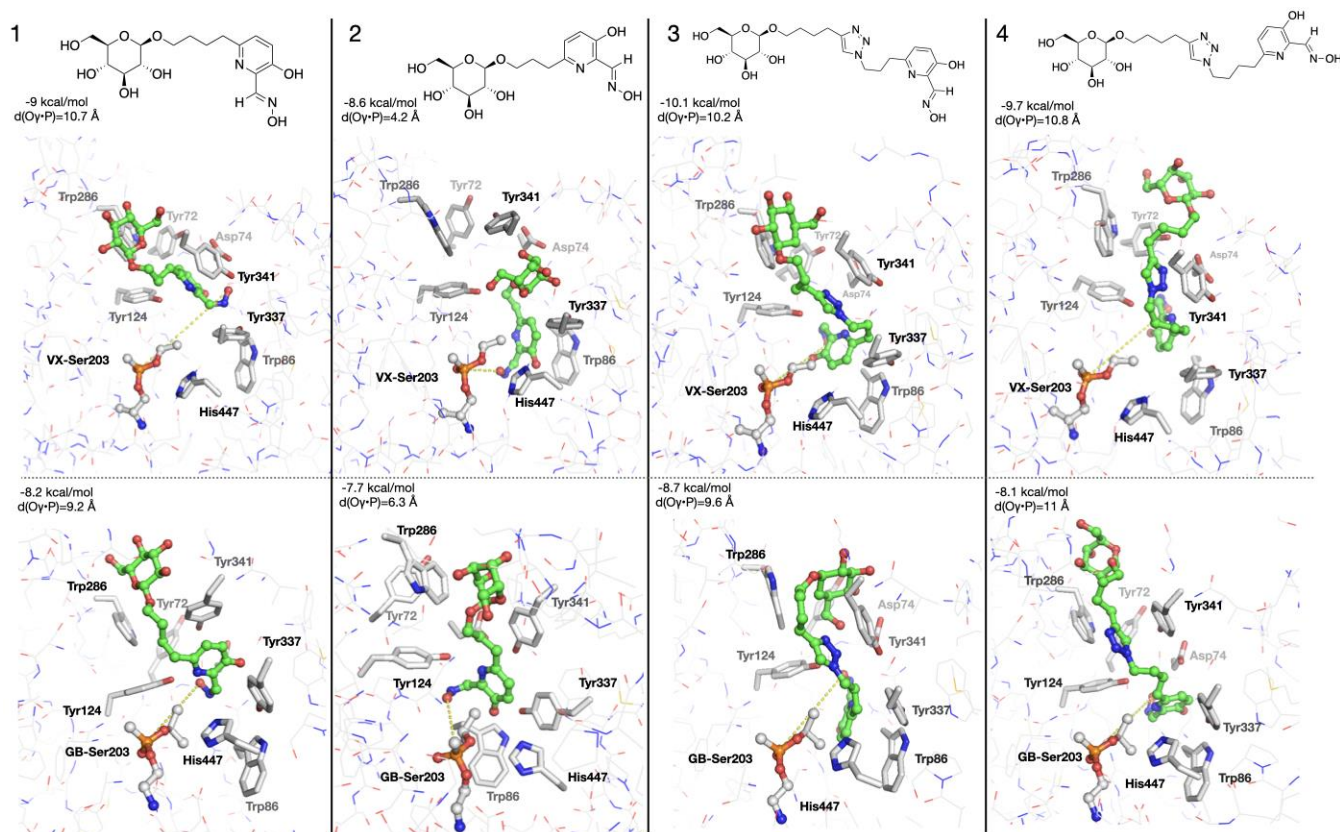
478 To determine if the candidates oximes **1,2,3** and **4** can bind in a productive way to the OPNAs
 479 phosphorylated *hAChE* close to the catalytic OP-serine adduct, we performed flexible molecular
 480 docking on *hAChE* phosphorylated by either VX or GB (Sarin) as previously described²². The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

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

486 Table 1: Binding affinity and distances between the phosphorus atom of VX and GB-inhibited *hAChE* and the
487 oxime oxygen atom of the best molecular docking pose of selected oximes. Binding affinity in kcal/mol and P-O
488 distances in angstrom.
489

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

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



512 Figure 5: Molecular docking of selected oximes in the active site of VX and GB-inhibited human
 513 acetylcholinesterase (respectively top and bottom panel). The binding energy determined by the scoring function
 514 of Autodock Vina and the distance between the phosphorus Atom of VX or GB and the oxime oxygen atom are
 515 indicated in the top left corner each docking pose.
 516

517 3.3 *In vitro* reactivation of OPNAs phosphorylated *hAChE*

518 We first determined the half-maximal inhibitory concentration (IC_{50}) for compounds **1,2,3** and
 519 **4** (Table 2). Results showed a generally low affinity of the new sugar-oximes for recombinant
 520 uninhibited *hAChE*. Noteworthy, oximes **1** and **2**, which differ only by one carbon atom in the
 521 alkyl chain between the 3-hydroxypyridinaldoxime and the glucose, displayed a high binding
 522 affinity difference implying that the length of the linker (i.e., four carbons for oxime **1** versus 3
 523 carbons for oxime **2**) is a key parameter for the binding of the reactivators to their target. The
 524 higher flexibility allowed by the four carbons alkyl chain of oxime **1** may prevent the molecule
 525 to bind tightly to the enzyme. Oximes **3** and **4** also differ by one carbon atom in the length of
 526 the alkyl chain between the oxime moiety and the triazole (3 carbons for oxime **3** and 4 for
 527 oxime **4**). The binding affinity of oxime **3** is approximately three times lower than the affinity
 528 of oxime **4** which may indicate that the four carbons linker of compound **4** allows a more

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

532
 533 Table 2: Half maximal inhibitory concentration (IC₅₀) for *hAChE*.
 534

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

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

547 Table 3: Reactivation kinetics of *hAChE* inhibited by nerve agent surrogated and paraoxon by selected oximes.
 548 *nd*: if [reactivator] $\ll K_D$, then there is a linear dependence between k_{obs} and [reactivator]: $k_{obs} =$
 549 $(k_r/K_D)[reactivator]$. 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
 550 be directly obtained by fitting.
 551

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

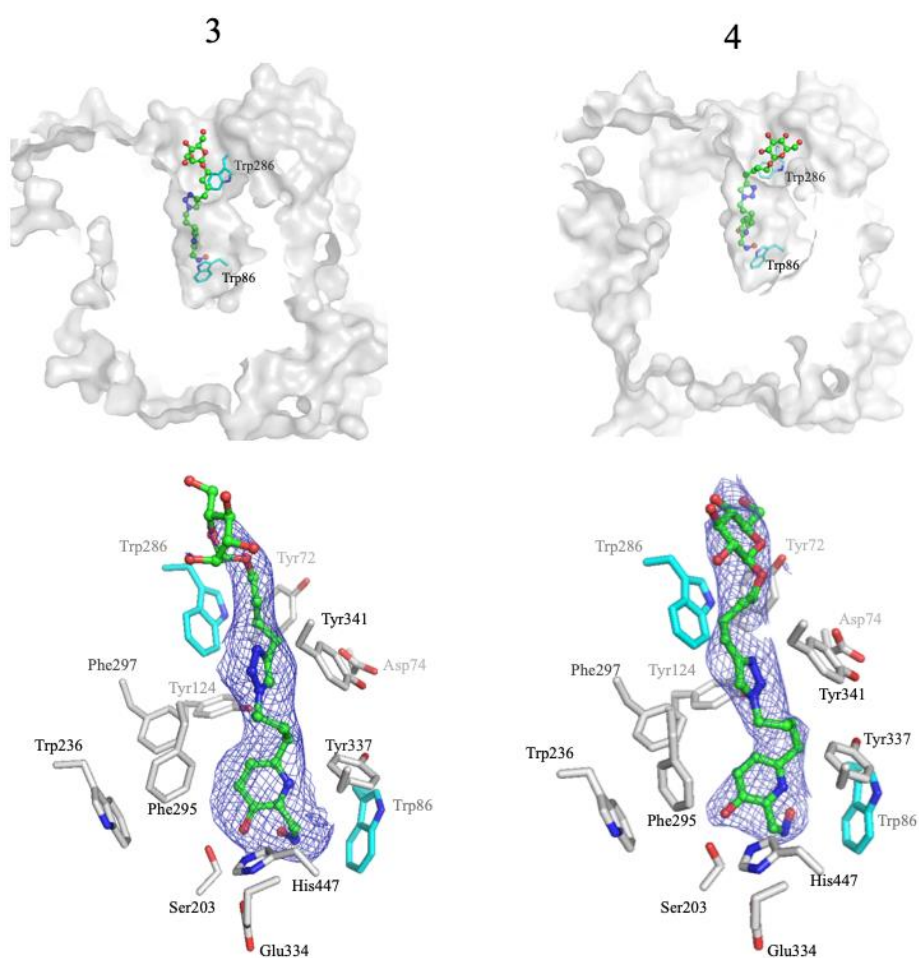
	5	nd	nd	0.2
	HI-6	1 ± 0.2	0.09 ± 0.03	11
	2-PAM	0.24 ± 0.02	0.2 ± 0.03	1.2
	Obidoxime	0.2 ± 0.02	0.5 ± 0.09	0.4
NEDPA	1	0.1 ± 0.01	1.3 ± 0.2	0.1
	2	0.2 ± 0.02	1.2 ± 0.3	0.2
	3	0.2 ± 0.08	3 ± 1.4	0.07
	4	0.6 ± 0.02	0.9 ± 0.1	0.6
	4'	0.5 ± 0.04	0.6 ± 0.1	0.8
	5	0.8 ± 0.2	3 ± 1	0.3
	HI-6	0.05 ± 0.005	0.2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

552
553 The results, displayed in table 3, showed a generally low efficacy (k_{r2}) of the sugar oximes in
554 the same range as 2-PAM in all cases, and in the same range of HI-6 (but lower than
555 obidoxime), for NEDPA and paraoxon phosphylated *hAChE*. The low affinity (K_D), in the
556 millimolar range, of these compounds for the phosphylated *hAChE* are clearly responsible for
557 the poor reactivation efficiency. However, the compounds **4**, **4'** and **5** performed better than 2-
558 PAM and obidoxime for the NEMP-inhibited *hAChE*, mainly due to the high reactivation rates
559 observed. Regarding the NIMP-inhibited *hAChE*, the sugar oximes are outperformed by HI-6,
560 2-PAM and obidoxime, and they are slightly better than HI-6 and 2-PAM for NEDPA-inhibited
561 enzyme. Compound **4'** appeared to reactivate the paraoxon inhibited *hAChE* better than HI-6,
562 2-PAM, and obidoxime.

563 3.4 X-ray structures of compounds **3** and **4** in native *hAChE*

564 In order to confirm the binding position of the sugar-oximes in the gorge of *hAChE*, we solved
565 the structure of the complexes formed by the non-modified *hAChE* and compound **3** (PDB
566 7P1P) and **4** (PDB 7P1N) (Figure 6). Data collection and refinement statistics are presented in

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



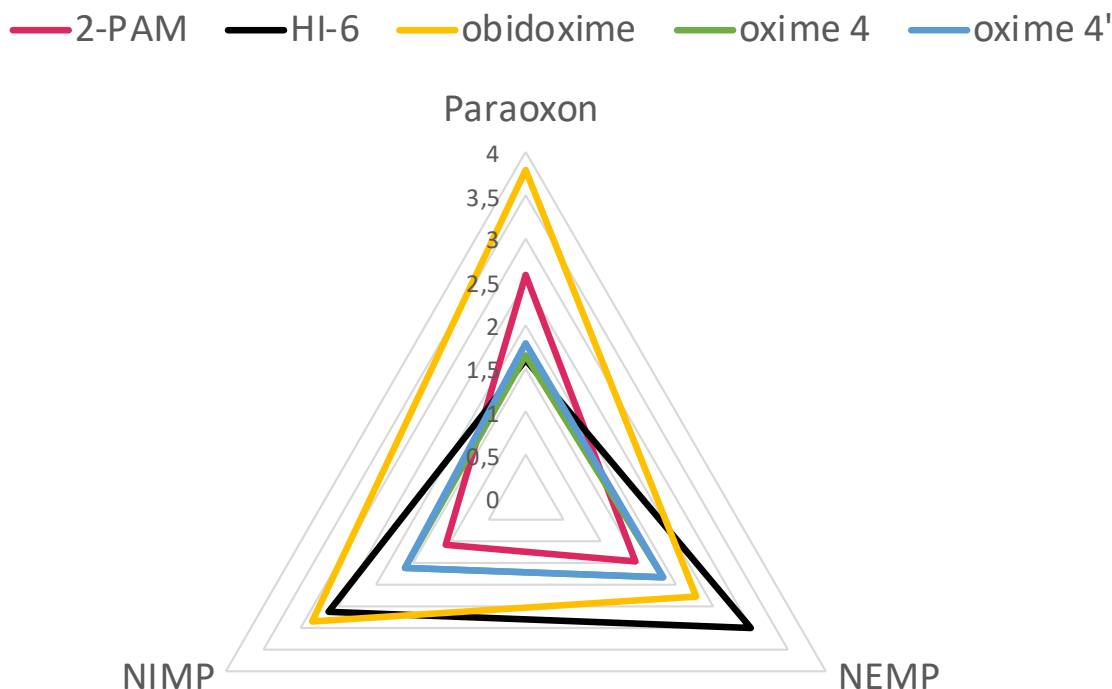
574
575 Figure 6 : Views of oxime **3** (PDB 7P1P) and **4** (PDB 7P1N) in complex with *hAChE*.
576 Top : Overall view of oximes **3** and **4** location inside the gorge of *hAChE* defined by the solvent accessible surface,
577 showing the sugar moiety outside the gorge. Bottom : Closer view of oximes **3** and **4** inside the gorge of *hAChE*.
578 A 1- σ feature-enhanced map is represented as a blue mesh. Key peripheral (Trp286) and active site (Trp86)
579 tryptophan residues are represented in sticks with carbons in cyan.

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585 3.5 ***In vivo* protective index assessment**

586 In order to determine the *in vivo* efficacy of the new oximes, protective indexes (PI) were
587 determined for compounds **4** and **4'** at 100 µmol/kg and compared to 2-PAM, HI-6, obidoxime.
588 Experiments of up-and-down procedure performed on paraoxon, NIMP and NEMP exposure
589 are presented in Table S3, S4 and S5 respectively and summed up in Figure 7. LD₅₀ of paraoxon,
590 NIMP and NEMP were respectively established to 818, 605 and 350 µg/kg. PI of a 100 µmol/kg
591 i.p. 2-PAM treatment 1 min after paraoxon exposure was previously assessed to 2.58³⁷. Alone,
592 100 µmol/kg of obidoxime gave the most interesting protective spectrum against the three
593 studied OPs even if HI-6 showed a higher PI than obidoxime in the case of treatment of NEMP
594 exposure (3.00 vs. 2.06), the lowest protective efficacy of HI-6 against paraoxon exposure (PI
595 = 1.6) was crippling. It should be noted that oximes **4** and **4'** presented an almost identical and
596 moderate *in vivo* protection against the three OP exposures.

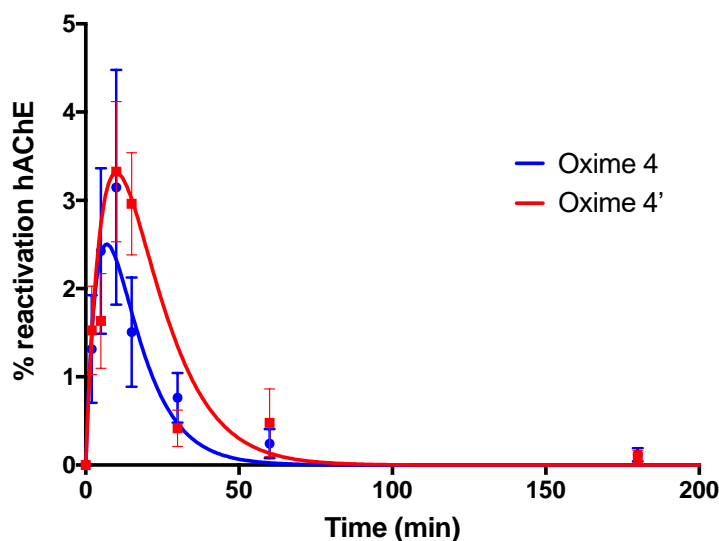
Protective index without atropine



597 **Figure 7.** Radar representation of PI values of 100 µmol/kg intraperitoneally treatment of 2-PAM, HI-6,
598 obidoxime, oxime **4** and **4'** one minute after paraoxon, NIMP or NEMP subcutaneous exposure. PI were
599 determined by the up-and-down method.

600 **3.6 Pharmacokinetic study**

601 Both oximes (**4** and **4'**) exhibited a similar enzymatic reactivation profile with a low
 602 reactivation percentage at the peak (React max = $2,5 \pm 0,8$ % and $3,3 \pm 0,7$ % respectively).
 603 The peak is reached more rapidly for oxime **4** than for oxime **4'** ($T_{max} = 7,0 \pm 0,0$ vs $10,0 \pm$
 604 $0,0$ min after injection) as shown in Fig. 8. Oxime **4** also persisted for a shorter time than
 605 oxime **4'** in mice plasma as demonstrated by the MRT value ($15,0 \pm 0,0$ vs $20,0 \pm 0,0$ min).



606 **Figure 8.** reactivation of VX-inhibited *hAChE* by oximes **4** and **4'** in mice plasma. The same dose of 100
 607 $\mu\text{mol/kg}$ of oxime **4** and **4'** was administered intraperitoneally to mice ($n = 7$). Blood samples were drawn at
 608 various time points (0, 2, 5, 10, 15, 30, 60 and 180 min) after treatment, and the levels of reactivation of VX-
 609 phosphorylated *hAChE* were determined. Values are presented as percentages of maximum reactivation and
 610 points are means \pm SEM. Fitting was performed on GraphPad Prism software.

611

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

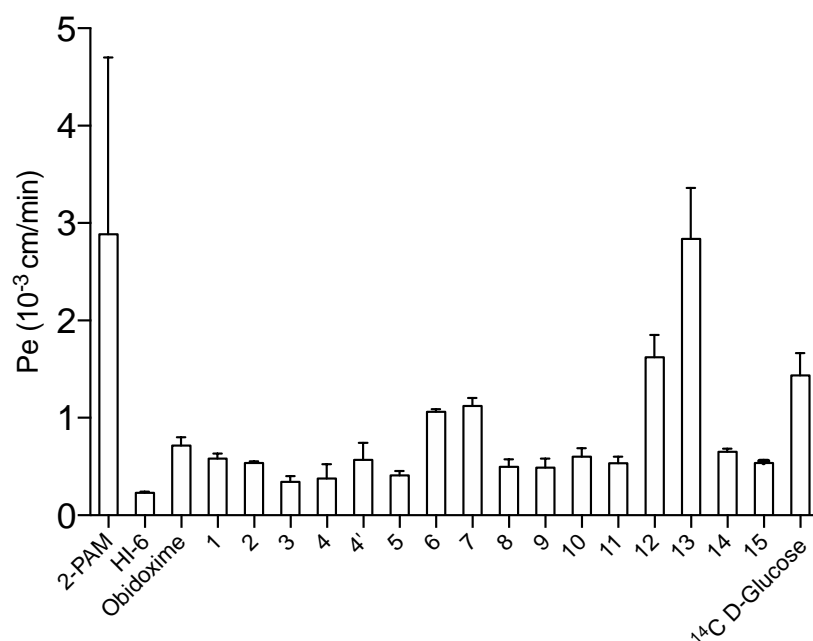
Oxime	MRT (min)	T_{max} (min)	React max (%)	C_{max} (μM)
Oxime 4	$15,0 \pm 0,0$	$7,0 \pm 0,0$	$2,5 \pm 0,8$	96,7
Oxime 4'	$20,0 \pm 0,0$	$10,0 \pm 0,0$	$3,3 \pm 0,7$	145,7

620

621

622 3.7 Blood-brain barrier permeability tests

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



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

632
633 Results showed that all the compounds, the conjugate oximes or the simpler glycoconjugate
634 molecules, had a lower rate of transport through BBB endothelial cells than 2-PAM ($Pe_{2-PAM} =$
635 $2.89 \pm 1.81 \times 10^{-3} \text{ cm}\cdot\text{min}^{-1}$) (Figure 9). Only one glycoconjugate without oxime moiety
636 (compound **13**) exhibited a permeability coefficient in the same range ($Pe_{13} = 2.83 \pm 0.52 \times 10^{-3}$
637 $\text{ cm}\cdot\text{min}^{-1}$). Most of the molecules showed Pe values between those of HI-6 ($Pe = 0.23 \pm 0.01$
638 $\times 10^{-3} \text{ cm}\cdot\text{min}^{-1}$) and obidoxime ($Pe = 0.72 \pm 0.08 \times 10^{-3} \text{ cm}\cdot\text{min}^{-1}$) that are described to slowly
639 cross the BBB which is confirmed in our *in vitro* model study (Figure 9).

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

660 **4 Discussion/conclusion**

661 Reactivation of the central OP-phosphylated AChE is one of the aims of the newly synthesized
662 oximes which have been described lately in the literature. An efficient crossing of the BBB
663 appears to be one major limitation for these numerous new designed oxime reactivators. If
664 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-phosphylated 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-phosphylated *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

690 near the catalytic site, the triazole moiety interacting by π -stacking interactions with Tyr341
691 and the glucose moiety spanning outside the gorge without unfavorable interactions with amino
692 acids of the peripheral site. These results showed that compounds **3** and **4** were orientated inside
693 the gorge in a productive way that may reactivate the inhibited *hAChE*. The presence of the
694 triazole moiety on oximes **3**, **4**, **4'** and **5** was predicted to improve the affinity of the sugar
695 oximes for the inhibited enzymes, but results showed a similar low binding affinity in the same
696 range as compounds **1** and **2**. However, a high enough reactivation kinetics k_r allows oximes **4**,
697 **4'** and **5** to be more efficient than 2-PAM and obidoxime to reactivate the NEMP-phosphylated
698 *hAChE* as well as oximes **4** and **4'** to reactivate NEDPA-phosphylated *hAChE*. The α anomer
699 of the oxime **4** (named **4'**) is the only compound to perform better than 2-PAM, HI-6 and
700 obidoxime for paraoxon phosphylated *hAChE*.

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

715 dose.

716 To evaluate the ability of our sugar oximes conjugates to cross the BBB we determined the
717 endothelial permeability coefficients of gluco- and riboconjugate oximes (**1, 2, 3, 4, 4', 5, 15**)
718 as well as simpler glycoconjugated molecules (compounds **6, 7, 8, 9, 10, 11, 12, 13, 14**) using
719 the human BBB *in vitro* model (Figure 9). Our results showed that the designed sugar oximes
720 do not cross the BBB at the same transport rate as glucose, implying a structural restraint from
721 our compounds. 2-PAM has the highest transport rate than any other evaluated oximes, but the
722 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
723 obidoxime. However, we were disappointed to observe that the presence of the triazole moiety
724 on tri-functional oximes **3, 4, 4', 5**, incorporated to increase the affinity of these compounds for
725 phosphorylated *hAChE*, as well as its presence in simpler glycoconjugated molecules such as **8,**
726 **9, 10, 11** and **14** reduces the transport rate through the BBB which may negatively impact the
727 protective index determined in our *in vivo* experiments.

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

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754

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