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Synthesis and Preclinical Fluorescence Imaging of Dually Functionalized Antibody Conjugates Targeting Endothelin Receptor-Positive Tumors

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31 led to homogenous and well-defined constructs that retained their high affinity and high
32 specificity for their respective target, as shown by flow cytometry and NIRF in vivo imaging
33 experiments in nude mice bearing CHO-ET_A and CHO-ET_B tumors. Ultimately, these bimodal
34 immunoconjugates could be used to improve outcomes of patients with ET⁺ tumors.

35

36 **KEYWORDS**

37 Endothelin axis, NIRF imaging, site-specific conjugation, monomolecular multimodal imaging
38 probe (MOMIP), Fluorescence-Guided Surgery.

39

40 **INTRODUCTION**

41

42 Comprising three 21-mer peptides (ET-1, ET-2 and ET-3) and two G-protein-coupled
43 receptors (GPCR) subtypes (ET_A and ET_B), the endothelin axis was first identified as a potent
44 endogenous vasoconstrictor and mediator of cardiovascular and renal disorders.¹ Since early
45 2000's, dysregulations of the ET axis have been linked to the growth and progression of
46 various tumors, inducing cell proliferation, survival, angiogenesis and metastatic spread.²⁻⁵

47 In total, aberrantly activation of endothelin-1 receptors seems to be involved in twelve of
48 the fourteen hallmarks of cancer described by Hanahan,⁶ emphasizing the potential of ET
49 axis as a therapeutic target. Several antagonists – targeting either ET_A (Zibotentan,
50 Atrasentan), ET_B (SPI 1620) or both (Bosentan, Macitentan) – have been developed and
51 pushed toward the clinics.^{5,7} However, despite promising preclinical data and many clinical
52 trials encompassing various cancers (such as melanoma, glioma, pancreatic cancer, prostate
53 cancer), none of the candidates has successfully reached the market. The elevated
54 concentration of ET-1 within the tumoral environment coupled with its strong affinity for its
55 receptor presents a significant challenge for an antagonist to compete, that could be
56 responsible for the discrepancy between preclinical and clinical results. To circumvent this
57 issue, two high-affinity antibodies – that do not compete with ET-1 – named Rendomab A63
58 (RA63) and Rendomab B49 (RB49), directed respectively against ET_A and ET_B receptors have
59 been patented.^{8,9} We have recently demonstrated that a chimeric version of RA63 called
60 xiRA63 could be randomly labeled with zirconium-89 (⁸⁹Zr, t_{1/2} = 3.3 days) and used to detect
61 ET_A⁺ glioblastoma in a mouse model xenografted orthotopically with patient derived Gli7
62 GSC, thanks to immunoPET imaging.¹⁰ Likewise, a chimeric version of the latter, xiRB49, was
63 modified with a cytotoxic molecule to obtain an antibody-drug conjugate showing a very

64 high efficacy to treat ET_B⁺ melanoma with no notable side effects on mice (Herbet et al., in
65 preparation). These promising results prompted us to develop new bimodal imaging
66 immunotracers that could help improving outcomes of patients with ET⁺ tumors.

67 In many types of cancer, surgery remains a key treatment option. For example, more than
68 50% of patients diagnosed with stage I or II breast, colorectum or lung cancer will undergo
69 surgery.¹¹ The extent of the resection is often linked to the prognosis. Indeed, the presence
70 of residual tumor cells, called positive margins, is a strong predictor of tumor recurrence.
71 Achieving a complete resection with the naked eye can be tricky. To obtain negative
72 margins, surgeon can turn to intraoperative histopathological analysis of frozen tumor
73 margins but the process is time-consuming. Non-optical imaging methods like intraoperative
74 CT and MRI can also be helpful but those methods are costly and their use disrupts the
75 surgical procedure, increasing operative and anesthesia times. In contrast, Fluorescence
76 Guided Surgery (FGS) is a low-cost technique that offers high sensitivity, ease of use and
77 safety to delineate tumor margins.^{12,13} Several fluorophores are already approved and used
78 in clinic, like indocyanine green (ICG), fluorescein or 5-ALA.¹² However, those imaging agents
79 do not specifically target cancer cells and the tumor-to-background ratio can be
80 disappointing. In addition to non-specific signal, tumors heterogeneity and the difficulty to
81 predict which patients could benefit from FGS may explain these mixed results. To overcome
82 some of those limitations, the fluorophore can be conjugated to a targeting vector such as a
83 monoclonal antibody.¹⁴ For example, the FDA-approved IRDye800CW has been conjugated
84 to various antibodies and is currently involved in promising clinical trials.¹⁵⁻¹⁸ Along with
85 specificity, stratification of patients could greatly improve FGS outcomes. Bimodal imaging,
86 combining a pre-operative imaging technique and real-time fluorescence imaging is an
87 attractive solution to increase the extent of tumor resection.^{19,20} Indeed, nuclear medicine
88 imaging – either single-photon emission computed tomography (SPECT) or positron emission
89 tomography (PET) – can be used to detect tumors and select patients that will be eligible to
90 FGS.²¹⁻²³ Several strategies can be employed for the synthesis of such bimodal
91 bioconjugates.^{24,25} Random and sequential introduction of the two probes leads to a highly
92 heterogeneous mixture of millions of conjugates, with different numbers of probes per
93 antibody, various ratios of the two probes, and a multitude of possible locations of these
94 probes on the protein. This may have deleterious consequences, including a poorly
95 reproducible synthesis, a potential effect on the photophysical properties of the dye, and,

96 more importantly, different pharmacokinetics and *in vivo* fate of the different bioconjugates,
97 or even a loss of affinity of the antibodies for their target.^{26–29} For these reasons, we focused
98 on a more elegant strategy involving: i) the preparation of a so-called MOmolecular
99 Multimodal Imaging Probe (MOMIP) bringing together the dye and the chelator on a
100 tetrazine platform, ii) the click conjugation of this MOMIP on the antibodies, previously
101 modified site-specifically with a *trans*-cyclooctene (TCO) group, through an inverse electron-
102 demand Diels-Alder (IEDDA) reaction. In this manuscript, we wish to report the synthesis of
103 two dually-functionalized immunoconjugates – containing both a NIR fluorophore
104 (IRDye800CW) for fluorescence-guided surgery and Desferrioxamine (DFO), the standard
105 chelating agent so far for ⁸⁹Zr-labeling of antibodies – and the *in vitro* validation of their
106 ability to bind ET receptors on CHO-ET_A and CHO-ET_B cells. We also conducted a preliminary
107 *in vivo* fluorescence imaging study to demonstrate the potential of the conjugates to
108 accumulate in ET_A⁺ and ET_B⁺ tumors implanted subcutaneously in mouse models.

109

110 **EXPERIMENTAL PROCEDURES**

111 **Instruments and methods**

112 Purifications by semi-preparative HPLC were performed on an UltiMate 3000 system Dionex
113 (Thermo Scientific) equipped with an UV-visible detector, on the following columns:
114 BetaBasic-18 column (Thermo Scientific) (5 μm, 150 Å) at 20 mL/minutes, or Hypersil GOLD
115 column 100 x 10 (Thermo Scientific) (5 μm, 175 Å) at 3.5 mL/min, with HPLC grade eluents.
116 Unless specified otherwise, purifications were performed using acetonitrile and water, both
117 supplemented with 0.1% of trifluoroacetic acid. The fractions of interest were analyzed by
118 HPLC-MS, pooled, concentrated under reduced pressure to remove organic solvents, and
119 freeze-dried.

120 High performance liquid chromatography (HPLC) analyses were performed on an UltiMate
121 3000 system Dionex (Thermo Scientific) equipped with a DAD detector and coupled to a low-
122 resolution mass spectrometry detector MSQ Plus (Thermo Scientific) with an ESI source.
123 Separation was achieved using an RP Kinetex™ column (Phenomenex) (2.6 μm, 100 Å, 50 Å,
124 2.1 mm) with HPLC quality solvents: A: H₂O + 0.1% FA and B: MeCN + 0.1% FA. Analyses were
125 performed with the following gradient program: 5% to 100% of B in 5 min, 100% B for 1.5
126 min, 100% to 5% B in 0.1 min and 5% B for 1.9 min, at a flow rate of 0.5 mL/min. NMR
127 spectra were acquired on a Bruker NMR spectrometer 500 MHz Avance DRX. HRMS spectra

128 were recorded on a mass spectrometer LTQ Orbitrap XL (Thermo Scientific) using an ESI
129 source.

130 Bioconjugation reactions were followed by reverse phase HPLC (Vanquish, Thermo Fisher
131 Scientific) coupled to a high-resolution Orbitrap mass spectrometer (Exploris 240, Thermo
132 Fisher Scientific) using an ESI source (positive mode). Before analysis, a small sample was
133 digested with the IDES enzyme (Promega): 20 µg of conjugate were mixed with 20U of IDES
134 (10U/µL) and the volume was adjusted to 10 µL with deionized water. The reaction mixture
135 was incubated at 37°C for 60 min. HPLC: A MabPac RP column (2.1 mm x 100 mm, 4µm,
136 Thermo Fisher Scientific) was used at 80°C (denaturing conditions), with a flow rate of 0.5
137 mL/min and a gradient 20-45% B in 6 min (eluent A: H₂O + 0.02% TFA + 0.08% FA and B:
138 ACN + 0.02% TFA + 0.08% FA). Mass: Full scan data were acquired at resolving power 30000
139 at m/z 200 in the m/z range 1000 – 4000. Data were analyzed with FreeStyle and BioPharma
140 Finder 4.1 software (both Thermo Fisher Scientific). High-resolution mass spectra were
141 deconvoluted using the Xtract function for IDES fragments (≈ 25 kDa) and ReSpect function
142 for intact antibody (≈ 150 kDa). Degrees of labeling (DOL) were determined by comparison
143 of native and conjugated antibody masses, using BioPharma Finder 4.1 software.

144 FPLC purification of the conjugates was performed on an Äkta Pure 25 M chromatography
145 system (GE Healthcare Life Sciences) equipped with a HiTrap® MabSelect™ column
146 (MabSelect resin, Protein A, cross-linked agarose, column I.D. 7 mm, bed dimensions 7 x 25
147 mm, bed volume 1 mL). After deposition of the product, the conjugate was washed with 10
148 mL of PBS pH 7.4 (1 mL/min). The conjugate was then eluted with 5 mL of 25 mM acetic acid
149 (1 mL/min) and collected in 500 µL fractions. Purification was monitored at 280 and 700 nm.
150 UV-Vis absorbance spectra were recorded on a Varian Cary 50 scan equipped with a Hellma
151 Tray Cell or a UV5Nano spectrophotometer (Mettler Toledo).

152 NIRF imaging was performed using the KIS800 camera from Kaer Labs.

153 **Reagents**

154 All chemicals, unless otherwise noted, were acquired from Sigma-Aldrich, Acros Organics
155 and Alfa Aesar and used without further purification. All water used was ultra-pure (>18.2
156 MΩ.cm⁻¹).

157

158 **Synthesis of the MOMIP**

159 *DFO-NH-Tz-Cl 3*

160 Desferrioxamine mesylate salt **1** (1 equiv., 229 μmol , 150 mg) was added to a solution of 3,6-
161 dichloro-1,2,4,5-tetrazine **2** (1.2 equiv., 275 μmol , 41.3 mg) in dry DMSO (3 mL) with N,N-
162 diisopropylethylamine (4 equiv., 915 μmol , 160 μL). The reaction mixture was stirred at
163 room temperature for 1 hour and purified on semi-preparative HPLC on a BetaBasic-18
164 column (A: H₂O 0.1% TFA, B: MeCN 0.1% TFA; with the following gradient program: 2% of B
165 for 8 min, 2 to 30% in 5 min, 30 % to 60% of B in 60 min, at a flow rate of 20 mL/min) to
166 obtain an orange solid after lyophilization (m = 102 mg, y = 66%).

167 RP-HPLC-MS: tr = 4 min, m/z calculated for C₂₇H₄₈ClN₁₀O₈ [M+H]⁺ 675.3, found 675.6.

168 ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.63 (s, 3H), 8.84 (t, *J* = 5.7 Hz, 1H), 7.77 (m, 2H), 3.50-3.46
169 (m, 4H), 3.36 (q, *J* = 6.7 Hz, 4H), 2.99 (q, *J* = 6.6 Hz, 4H), 2.57 (t, *J* = 7.4 Hz, 4H), 2.26 (t, *J* = 7.3
170 Hz, 4H), 1.96 (s, 3H), 1.64-1.56 (m, 2H), 1.56-1.44 (s, 6H), 1.42-1.34 (m, 4H), 1.33-1.28 (m,
171 2H), 1.26-1.16 (m, 4H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 172.1 (C), 171.4 (C), 170.3 (C), 161.4
172 (C), 158.5 (C), 47.1 (CH₂), 46.9 (CH₂), 40.7 (CH₃), 38.5 (CH₂), 30.0 (CH₂), 28.9 (CH₂), 27.7 (CH₂),
173 27.6 (CH₂), 26.1 (CH₂), 23.5 (CH₂), 23.5 (CH₂), 20.4 (CH₂).

174

175 *DFO-NH-Tz-S-NH₂* **5**

176 To a solution of DFO-NH-Tz-Cl **3** (1 equiv., 89 μmol , 60 mg) in DMF (2 mL) and DIPEA (8
177 equiv., 712 μmol , 124 μL) was added a solution of tert-butyl (2-mercaptoethyl)carbamate **4**
178 (1.2 equiv., 107 μmol , 19 mg) in DMF (2.5 mL). The resulting orange solution was stirred at
179 room temperature. After 2 h, the product was precipitated from diethyl ether, redissolved
180 into 2 mL of CH₂Cl₂ and 2 mL of TFA and the resulting solution was stirred at room
181 temperature for 1 h. After concentration under vacuum the mixture was purified by semi-
182 preparative HPLC on a BetaBasic-18 column (A: H₂O 0.1% TFA, B: MeCN 0.1% TFA; with the
183 following gradient program: 2% of B for 8 min, 2 to 20% in 5 min, 20 % to 55% of B in 40 min,
184 at a flow rate of 20 mL/min) to afford an orange powder (m = 58 mg, y = 90%).

185 RP-HPLC-MS: t_r = 3.75 min, m/z calculated for C₂₉H₅₄N₁₁O₈S [M+H]⁺ 716.4, found 716.6.

186 ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.67-9.61 (m, 3H), 8.48-8.40 (m, 1H), 7.91 (s, 3H), 7.75-7.66
187 (m, 2H), 3.43-3.37 (m, 8H), 3.31-3.25 (m, 2H), 3.14-3.04 (m, 2H), 2.96-2.87 (m, 4H), 2.50 (t, *J*
188 = 7.3 Hz, 4H), 2.19 (t, *J* = 7.3 Hz, 4H), 1.89 (s, 3H), 1.58-1.49 (m, 2H), 1.49-1.37 (s, 6H), 1.35-
189 1.26 (m, 4H), 1.25-1.20 (m, 2H), 1.19-1.09 (m, 4H). ¹³C-NMR (126 MHz, D₂O) δ = 172.0 (C),
190 171.4 (C), 170.2 (C), 163.2 (C), 161.0 (C), 47.1 (CH₂), 46.8 (CH₂), 40.4 (CH₃), 38.5 (CH₂), 38.3

191 (CH₂), 29.9 (CH₂), 29.9 (CH₂), 28.8 (CH₂), 27.9 (CH₂), 27.6 (CH₂), 27.6 (CH₂), 27.4 (CH₂), 26.1
192 (CH₂), 23.5 (CH₂), 20.4 (CH₂) ppm.

193

194 *DFO-NH-Tz-S-IR800 7*

195 To a solution of IRDye800CW **6** (1 equiv., 6.35 μmol, 6.9 mg) and HATU (1.1 equiv., 7.0 μmol,
196 2.7 mg) in DMF (250 μL) with DIPEA (1.1 equiv., 7.0 μmol, 1.2 μL) was added DFO-NH-Tz-S-
197 NH₂ **5** (1.1 equiv., 7.0 μmol, 5.0 mg) in DMF (250 μL). The green solution was stirred at room
198 temperature for 5 hours. After concentration under vacuum, the mixture was purified by
199 semi-preparative HPLC on an Hypersil Gold column (A: TEAB 50 mM, B: MeCN with the
200 following gradient program: 2% of B for 8 min, 2 to 20% in 5 min, 20 % to 55% of B in 50 min,
201 at a flow rate of 3.5 mL/min) to give a blue/green powder (m = 8.4 mg, γ = 78%). RP-HPLC-
202 MS: t_r = 3.6 min., m/z calculated for C₇₅H₁₀₆N₁₃O₂₂S₅ [M+H]⁺ 1700.6, found 1700.9, m/z
203 calculated for [M+2H]²⁺ 850.8, found 851.3. HRMS: m/z calculated for C₇₅H₁₀₆N₁₃O₂₂S₅
204 [M+H]⁺ 1700.61734, found 1700.62481, m/z calculated for [M+2H]²⁺ 850.81231, found
205 850.81535, m/z calculated for [M-H]⁻ 1698.60279, found 1698.60632, m/z calculated for [M-
206 2H]²⁻ 848.79776, found 848.80066 .

207

208 **Production of recombinant xiRA63 and xiRB49**

209 The two murine antibodies, Rendomab A63 and Rendomab B49, were chimerized in order to
210 anticipate and minimize the immunogenicity problems for their potential future use in the
211 human clinic. In this context, the variable heavy chain (VH) and variable light chain (VL)
212 regions were fused with the respective constant IgG1 and Kappa regions of the trastuzumab.
213 Plasmids were then co-transfected into ExpiCHO-S cells which were cultured accordingly to
214 the manufacturer's instructions (ThermoFisher-A29127). Twelve days post-transfection, the
215 production of the xiRA63 or xiRB49 were collected and purified using a HiTrap Protein A HP
216 column (GE Healthcare-17-0402-03) following the manufacturer's instructions.

217

218 **Synthesis of the immunoconjugates**

219 *Deglycosylation*

220 Deglycosylation of xiRA63 or xiRB49 (500 μg, 125 μL) was performed by adding 5 units of
221 recombinant PNGaseF enzyme (New England BioLabs, Ipswich, MA) per 1 μg of mAb (5 μL,
222 500 U/μL), 25 μL 50 mM sodium phosphate, pH 7.5 (GlycoBuffer 2 10x from New England

223 Biolabs) and water to make a total volume reaction of 250 μ L. The reaction was incubated at
224 37 °C for 3 h and then purified via size exclusion chromatography (Sephadex G-25 M, PD-10
225 column, GE Healthcare) and concentrated using centrifugal filter units with a 50,000 Da
226 molecular weight cut off (Amicon™ Ultra 2 mL Centrifugal Filtration Units, Millipore Corp.,
227 Billerica, MA) and phosphate buffered saline (PBS, pH 7.4) to obtain the deglycosylated
228 antibodies.

229 *Enzymatic bioconjugation with MTGase*

230 To a solution of deglycosylated antibody in PBS was added TCO-PEG₄-NH₂ **8** (60 equiv. at 60
231 mM in PBS), MTGase (5 U/mg of antibody, Zedira) and phosphate buffered saline (PBS, pH
232 7.4) to adjust the antibody concentration to 7.5 g/L. The reaction was incubated at 25 °C for
233 2 h and then purified via size exclusion chromatography (Sephadex G-25 M, PD-10 column,
234 GE Healthcare) and concentrated using centrifugal filter units with a 50,000 Da molecular
235 weight cut off (Amicon™ Ultra 2 mL Centrifugal Filtration Units, Millipore Corp., Billerica,
236 MA) and phosphate buffered saline (PBS, pH 7.4) to give the TCO-bearing antibodies.
237 Degrees of labeling were estimated by mass spectrometry (Fig S1-2).

238 *Inverse electron demand Diels-Alder reaction*

239 To a solution of TCO-bearing antibody in PBS was added the MOMIP DFO-NH-Tz-S-IR800 **7**
240 (20 equiv. *per* accessible TCO at 30 mM in DMSO) and the antibody concentration was
241 adjusted to 5 g/L with phosphate buffered saline (PBS, pH 7.4). The reaction mixture was
242 stirred at room temperature overnight and purified by Protein A affinity chromatography
243 (Hitrap Mabselect Xtra column, Cytiva) to give the immunoconjugates **9** and **10**. Degrees of
244 labeling were measured by UV spectroscopy and mass spectrometry (Fig S1-2).

245

246 **Flow cytometry**

247 We used the protocol of Herbet et al.³⁰ Briefly, three cell lines were used to assess the
248 apparent affinity of xiRA63 or xiRB49: Chinese hamster ovary wild-type (CHO-WT) cells and
249 CHO cells stably transfected either with human ET_A (CHO-ET_A) or human ET_B (CHO-ET_B).
250 Apparent affinity was determined with a dilution range from 0.001 nM to 50 nM for xiRA63
251 and 0.01 nM to 125 nM for xiRB49. The binding was revealed by an Alexa-Fluor 488™
252 conjugated with F(ab')₂-goat anti-human IgG Fc (Invitrogen) and incubated for 4 hours at
253 4°C. Samples were washed three times with PBS between each step. Sample fluorescence
254 was measured at 488 nm with the FACSCalibur flow cytometer (BD Biosciences) and

255 expressed as a percentage of the mean fluorescence intensity per sample. The “one site
256 specific binding” function of GraphPad was applied to fit the binding curve and determine
257 the B_{max} and the apparent K_D values. To compared two data group, a statistical Student t test
258 (two-tailed) was carried out (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

259

260 **Animals**

261 All animal experiments were performed according to the European Directive 2010/63/EU.
262 The experimental protocol was approved by a local ethics committee for animal use (CETEA)
263 and by the French ministry of agriculture (authorization APAFIS#34522-
264 2022010412087915v1)/ethics committee n°44). Twelve female 4 weeks old NMRI nude mice
265 (Janvier Labs) were subcutaneously implanted with $5 \cdot 10^6$ CHO-ET_A, CHO-ET_B or CHO-WT cells
266 in the shoulder. Animal anesthesia was performed with 2.5 % of isoflurane in oxygen
267 associated with local xylocaine anesthesia.

268 When the tumor volume reached about 65 mm^3 , mice were injected intravenously in the tail
269 vein with either xiRA63-MOMIP or xiRB49-MOMIP (100 μg in a volume of 100 μL in PBS (pH
270 7.4)).

271

272 **NIRF imaging**

273 At 4 h, 24 h, 48 h, 72 h, and 168 h after injection, animals were imaged using the Kaer
274 Imaging System 800 camera from Kaer Labs (exposure time = 300 ms for xiRA63 and 100 ms
275 for xiRB49). 7 days post-injection, animals were euthanized and fluorescence guided
276 resection of tumors was performed. After the surgery, key organs were collected (liver,
277 spleen, stomach, intestine, heart, kidney, muscle and bones) and imaged to obtain an *ex vivo*
278 biodistribution (exposure time = 800 ms for xiRA63 and 400 ms for xiRB49).

279

280 **Statistics**

281 GraphPad Prism software (v9.0.1) was used for statistical analyzes. Student t test (two-
282 tailed) were carried out to compare two data groups (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$,
283 **** $P < 0.0001$).

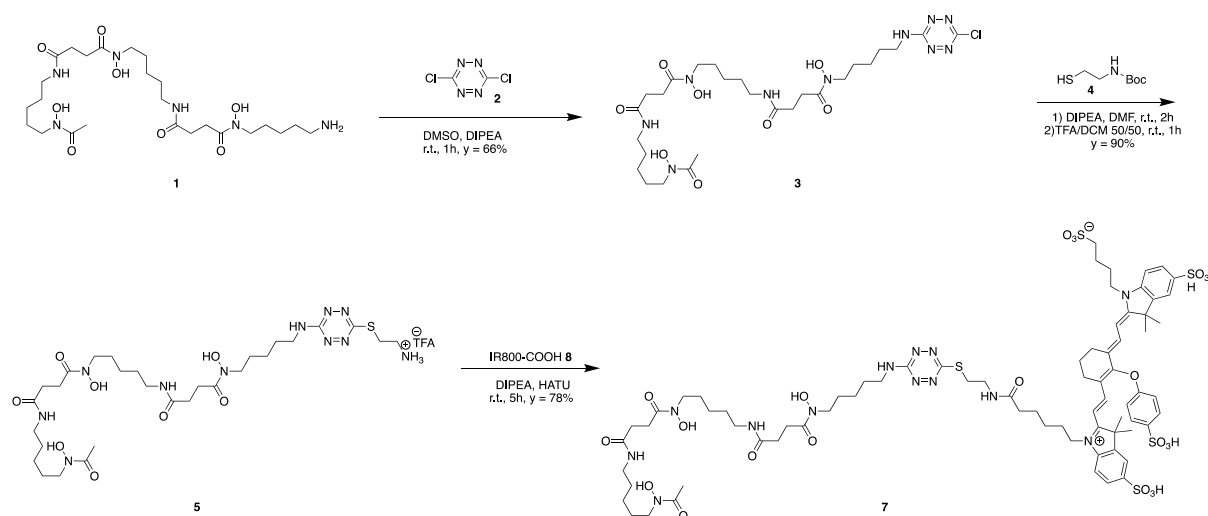
284

285 **RESULTS AND DISCUSSION**

286 **Synthesis of the immunoconjugates**

287 The first step of the synthesis of the immunoconjugates consists in the preparation of a
 288 MOnomolecular Multimodal Imaging Probe (MOMIP) containing both a fluorophore and a
 289 chelating agent for further ^{89}Zr radiolabeling. This so-called “multifunctional, single
 290 attachment point” (MSAP) design has proven to be the most elegant way to introduce two
 291 different functionalities on a biovector, since it allows a perfect control of the ratio between
 292 the two functional groups and a better reproducibility of the synthesis when compared to
 293 sequential introduction of the two functionalities.^{23,28,31,32} We have chosen IRDye800CW as a
 294 fluorophore since it is a water-soluble, near-infrared cyanine-based dye (excitation 775 nm,
 295 emission 795 nm) that has been extensively studied in clinical trials for Fluorescence Guided
 296 Surgery (FGS).^{33–36} This dye has been shown to be safe and capable of providing robust
 297 tumor-to-background contrast during surgery. To allow further ^{89}Zr radiolabeling,
 298 Desferrioxamine (DFO) was chosen as chelating agent. Although not ideal for ^{89}Zr chelation,
 299 DFO is by far the most commonly used chelator in ^{89}Zr immunoPET clinical trials³⁷. In order
 300 to bring together IRDye800CW and DFO in a single molecule, ready to be conjugated, we
 301 used a tetrazine platform that was recently developed in our group.^{31,32} The MOMIP DFO-
 302 NH-Tz-S-IR800 **7** was obtained in three steps (Scheme 1). In the first step, DFO-NH₂ **1** was
 303 reacted with 3,6-dichloro-1,2,4,5-tetrazine **2** to yield the monosubstituted tetrazine **3** in 66%
 304 yield. The first functionalization of the tetrazine ring deactivates the reactivity of the second
 305 chlorine atom towards $\text{S}_{\text{N}}\text{Ar}$ reaction. Thus, a stronger nucleophile, the thiol **4**, was used to
 306 prepare the difunctionalized tetrazine **5** in 90% yield. In the last step, **5** was reacted with the
 307 commercially available IRDye800-COOH **6** to give the aimed compound **7** in 78% yield.

308

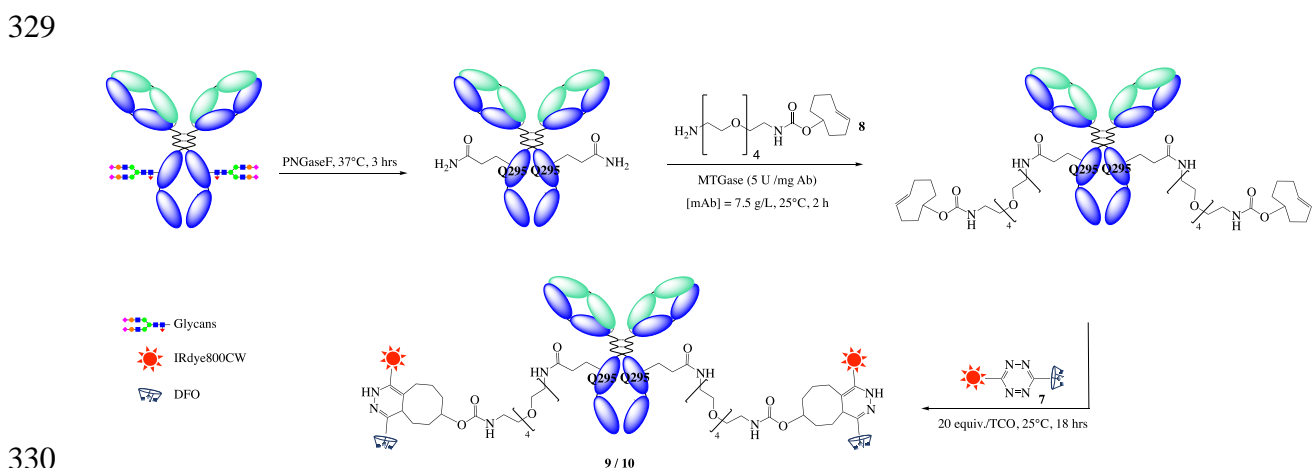


309

310

Scheme 1: Synthesis of the MOMIP DFO-NH-Tz-S-IR800

311
 312 The conjugation of DFO-NH-Tz-S-IR800 **7** to xiRA63 and xiRB49 requires the prior
 313 introduction on the antibodies of a click partner for iEDDA reaction. *Trans*-cyclooctene (TCO)
 314 / tetrazine ligation has been extensively studied^{38–41} and we decided to functionalize the
 315 antibodies with TCO moieties in a site-specific way. Indeed, the superiority of site-specific
 316 conjugation vs. random conjugation on lysine residues has been well established, leading to
 317 much more chemically defined and homogeneous conjugates.^{28,29} Among the existing site-
 318 specific conjugation methods, we have chosen the efficient enzymatic ligation of amine
 319 derivatives on the heavy chain of IgGs catalyzed by transglutaminase (MTGase) (Scheme
 320 2).^{42,43} Besides being affordable, this technique requires to remove the glycan chains located
 321 in positions N297, which could help reducing off-target uptake in the liver and spleen thanks
 322 to an impaired Fc γ RI binding.^{44,45} This feature might be interesting as fluorophores have a
 323 tendency to increase the liver uptake due to their hydrophobicity. After quantitative
 324 deglycosylation of xiRA63 and xiRB49 using PNGaseF enzyme (Fig S2), the coupling of TCO-
 325 PEG₄-NH₂ **8** to the antibodies in positions Q295 was performed using transglutaminase.
 326 Finally, the iEDDA reaction of DFO-NH-Tz-S-IR800 **7** with the TCO-modified antibodies was
 327 performed prior to the injection to yield the aimed bimodal antibody conjugates xiRA63-
 328 MOMIP **9** and xiRB49-MOMIP **10**.



Scheme 2: Synthesis of the immunoconjugates xiRA63-MOMIP **9** and xiRB49-MOMIP **10**

334 The number of MOMIP per antibody, called Degree of Labeling (DOL), was determined by
 335 UV-spectroscopy, using the integral method described by Grabolle *et al.*,⁴⁶ and by mass

336 spectrometry. Briefly, the immunoconjugates were digested with the IDES enzyme and a
337 detailed mass spectrometry characterization was performed by liquid chromatography–mass
338 spectrometry middle-down analyses of Fc/2 fragments (Fig S3-4). The values obtained for
339 xiRA63-MOMIP **9** and xiRB49-MOMIP **10** were $1.6 \leq \text{DOL} \leq 1.9$ and $1.6 \leq \text{DOL} \leq 1.7$,
340 respectively, which are closed to the theoretically expected value of 2 (Table 1). Besides, as
341 shown on the UV chromatogram at 780nm, we confirmed that the conjugation only took
342 place on the Fc fragment.

343

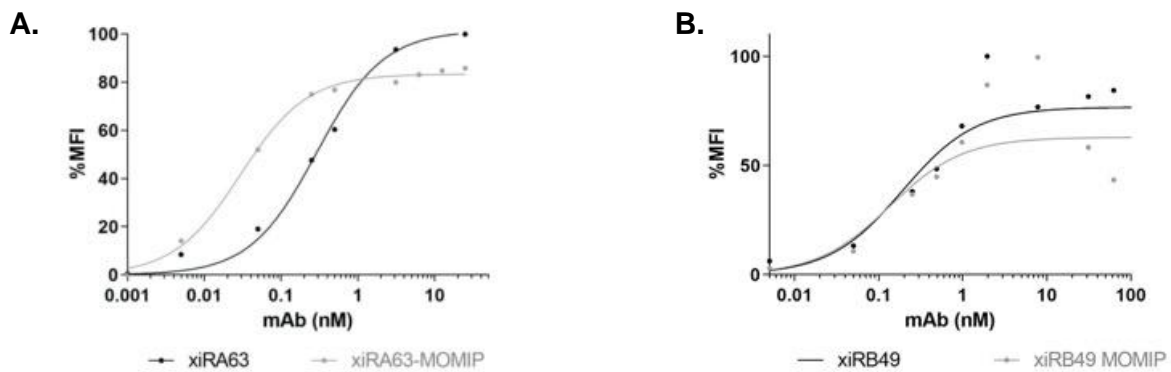
344 *Table 1: Degree of labeling of xiRA63-MOMIP **9** and xiRB49-MOMIP **10** determined by UV-*
345 *spectroscopy and mass spectrometry.*

Degree of labeling	UV	Mass spectrometry
<i>xiRA63-MOMIP 9</i>	1.9 ± 0.1	$0.8 \times 2 = 1.6$
<i>xiRB49-MOMIP 10</i>	1.7 ± 0.1	$0.8 \times 2 = 1.6$

346

347 ***In vitro* studies**

348 The next step was to assess the binding affinity of xiRA63-MOMIP **9** and xiRB49-MOMIP **10**
349 to CHO-ET_A and CHO-ET_B cells, respectively. This was performed by flow cytometry. The
350 binding curves as well as the binding affinity constants (K_{DAPP} and Bmax) are reported on an
351 indicative basis in Fig. 1. No loss of binding potential was observed between the MOMIP
352 conjugates and the native antibodies (subnanomolar range), confirming that site-specific
353 conjugation of the MOMIP to the antibodies did not affect the functionality of both xiRA63
354 and xiRB49 for their target.



356

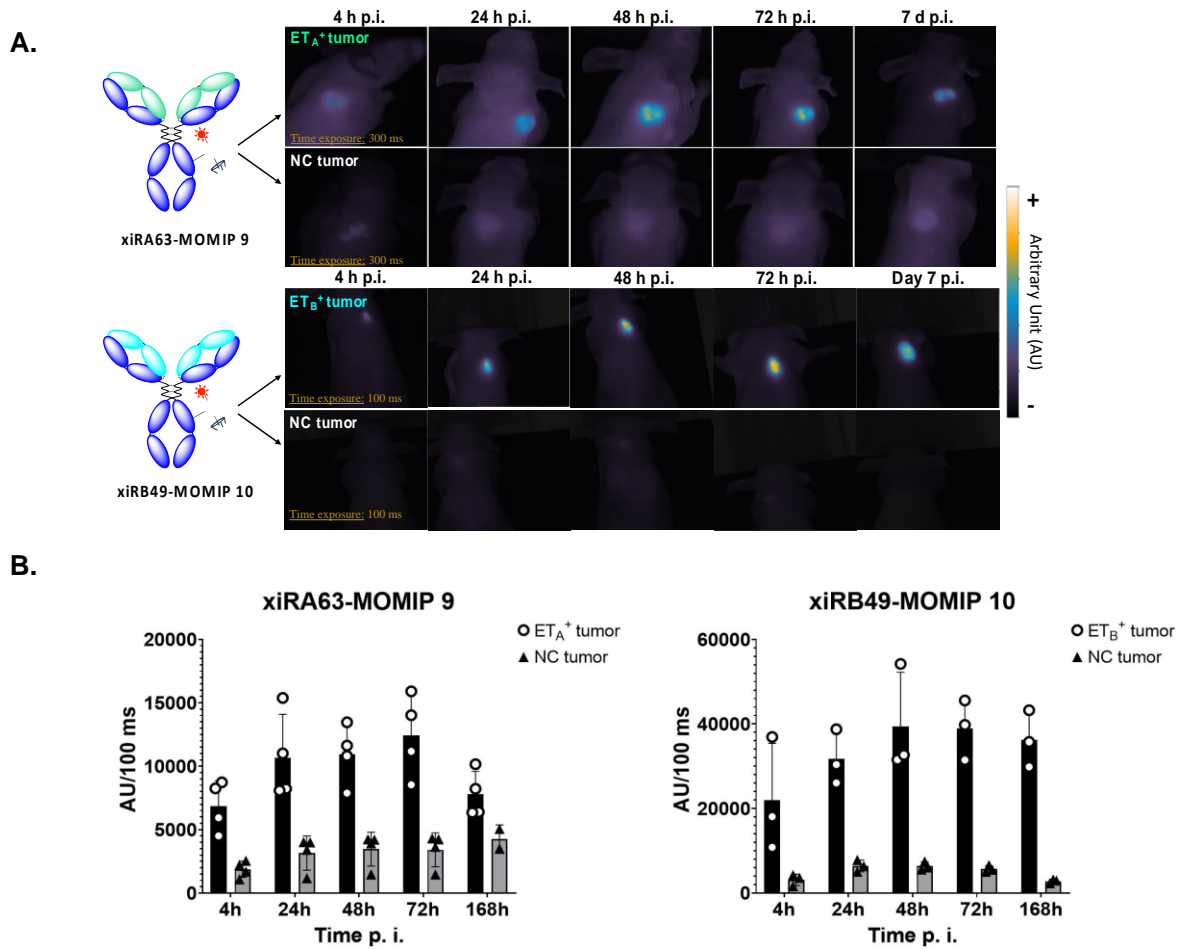
357 *Figure 1: Characterization of the MOMIP conjugation impact onto the xiRA63 and the xiRB49.*
 358 **A.** Binding curve comparison of the xiRA63 (black curve) and the xiRA63-MOMIP 9 (grey
 359 curve) by flow cytometry. **B.** Binding curve comparison of the xiRB49 (black curve) and the
 360 xiRB49-MOMIP 10 (grey curve) by flow cytometry. **C.** Apparent binding affinities and B_{max} of
 361 xiRA63, xiRB49 and the corresponding MOMIP conjugates 9 and 10. ~~Data are presented as~~
 362 ~~mean ± SEM. A two-tailed paired Student t test was used for data comparison.~~ MFI: Median
 363 Fluorescence Intensity, mAb: concentration of the monoclonal antibody.

364

365 ***In vivo* and *ex vivo* evaluation**

366 CHO-ET_A and CHO-ET_B cells were subcutaneously implanted in the right shoulder of nude
 367 mice. A third group of animals was implanted with CHO cells which present no expression of
 368 ET human receptors (CHO-WT). After 5 to 7 days after implantation, xiRA63-MOMIP 9 was
 369 injected intravenously in a group of mice bearing ET_A⁺ tumors (n=3), xiRB49-MOMIP 10 was
 370 injected in a group of mice bearing ET_B⁺ tumors (n=3), and both conjugates were injected in
 371 mice bearing CHO-WT tumors (2 groups of 3 mice) as a negative control (NC). Fluorescence
 372 images were recorded at 4h, 24h, 48h, 72h and 7 days post injection (Figure 2).

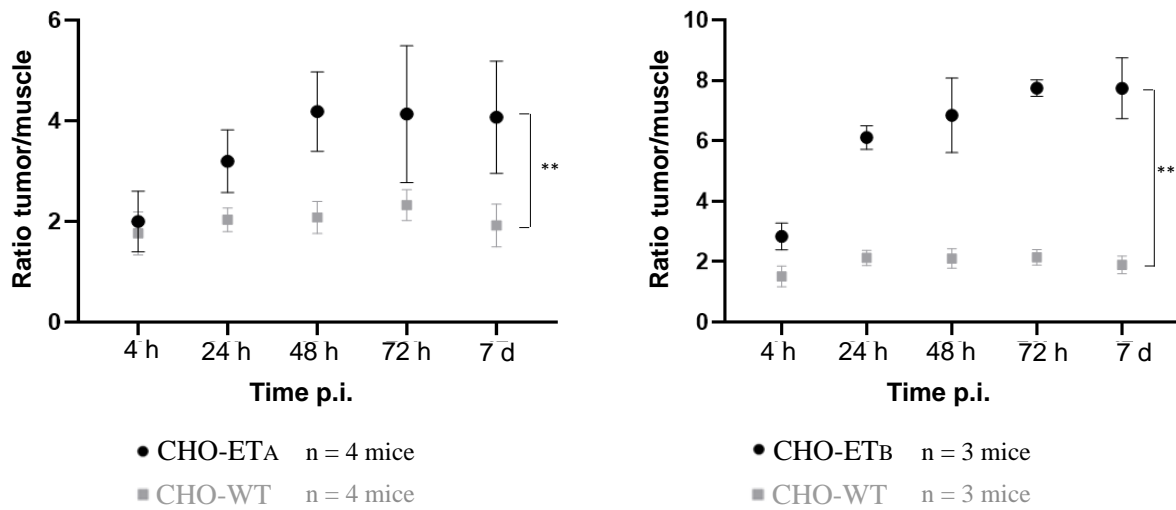
373 The images show a clear fluorescent signal in the CHO-ET_A and CHO-ET_B tumors 24h post-
 374 injection, while the signal is barely distinguishable in CHO-WT tumors, demonstrating that
 375 the high affinity and specificity of the conjugates for their target is maintained *in vivo*. It has
 376 to be noted that the background is a bit brighter in the images of mice injected with xiRA63-
 377 MOMIP 9 because of a longer exposure time (300 ms vs. 100 ms for the mice injected with
 378 xiRB49-MOMIP 10).



379
 380 **Figure 2: NIRF experiments. A.** Longitudinal study of the emitted fluorescence after i.v.
 381 injection of xiRA63-MOMIP 9 (top) and xiRB49-MOMIP 10 (bottom) conjugates in ET_A⁺, ET_B⁺
 382 and NC tumors bearing mice. **B.** Tumor mean fluorescence intensity over the 7 days.

383
 384 A relative quantification of the fluorescence intensity was estimated at different time points
 385 (Figure 3, Table S1). These data illustrate the specific uptake of xiRA63-MOMIP 9 and xiRB49-
 386 MOMIP 10 in mice bearing CHO-ET_A and CHO-ET_B tumors, respectively, and prove that the
 387 accumulation of the conjugates in the tumors is not due to passive targeting as no signal was
 388 observed in NC tumors. It can also be seen that maximum fluorescence intensity is obtained
 389 around 48h post-injection of the conjugates and maintained up to 7 days post-injection.

390

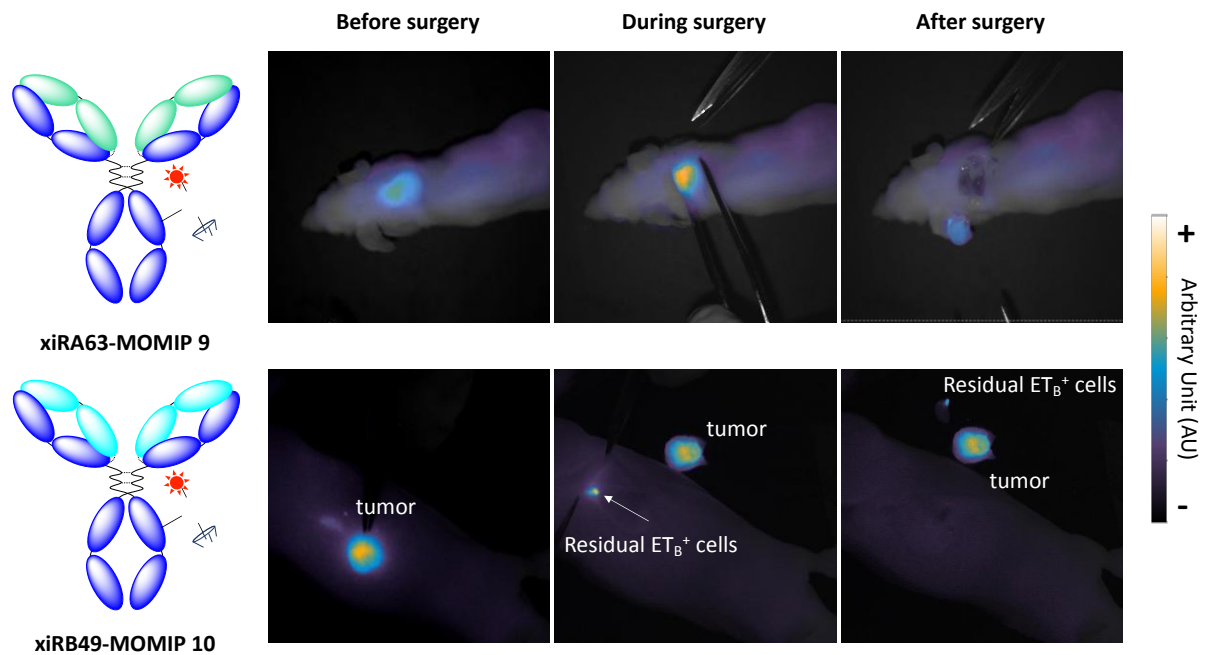


391

392 *Figure 3: Tumor/background ratio of the fluorescence intensity for xiRA63-MOMIP 9 (left)*
393 *and xiRB49-MOMIP 10 (right)*

394

395 After the last *in vivo* imaging 7 days post-injection, mice were sacrificed and tumors
396 resection was successfully performed under the Kaer Imaging System 800, an open
397 fluorescence imaging system adapted to intraoperative imaging on small animals (Fig. 4,
398 Video S1-2). Thanks to the fluorescence signal, tumor margins were clearly delineated and
399 we were able to spot and remove residual ET_B^+ cells that would have been missed with white
400 light.



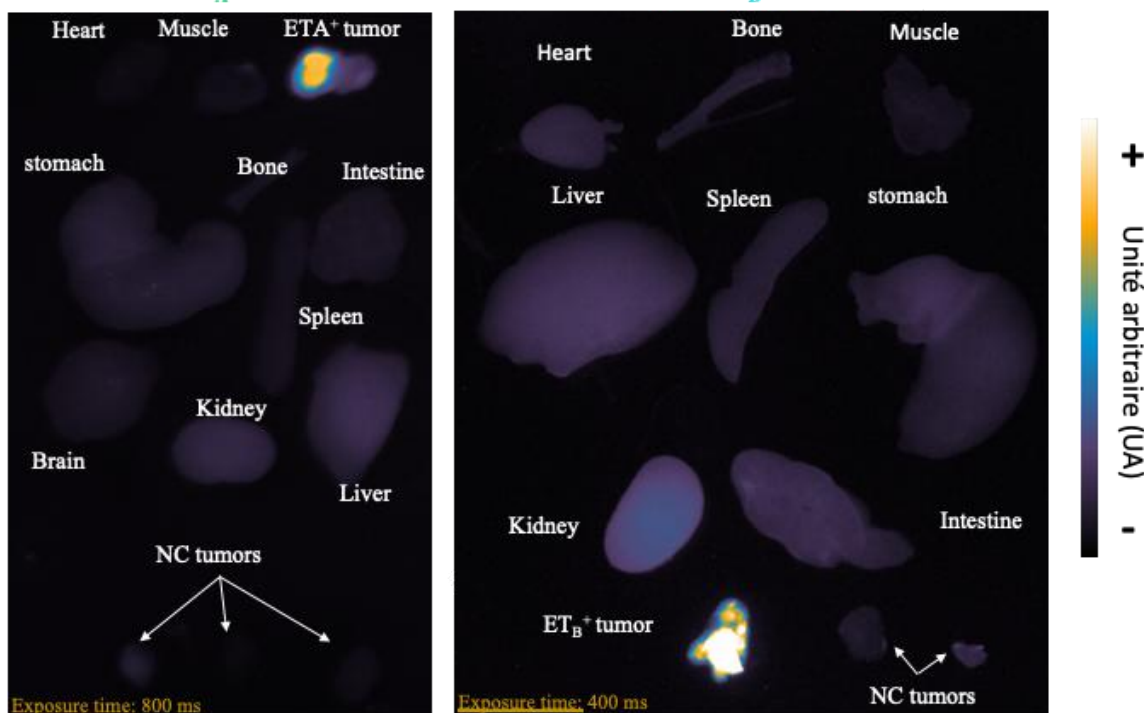
401
 402 *Figure 4: Fluorescence guided surgery of ET_A^+ (top) and ET_B^+ (bottom) tumors in nude mice.*
 403 *Images were recorded with a Kaer Imaging system 800 (excitation 785 nm – emission: high*
 404 *pass filter > 810).*

405 Major organs were also collected and fluorescence images were recorded (Fig. 5, Table S2).
 406 It is clear from these images that while the tumors overexpressing ET receptors are very
 407 bright, no or very weak fluorescence is observed in organs or NC tumors. In particular, no
 408 significant signal can be observed in the liver, which can be the case when using organic
 409 fluorescent dyes. These data are consistent with PET imaging data obtained with a ^{89}Zr -DFO-
 410 xiRA63 conjugate, showing that the enzymatic conjugation of the fluorescent dye did not
 411 affect the biodistribution of the antibody.¹⁰

412

**xiRA63-MOMIP biodistribution in mice
with ET_A⁺ tumor**

**xiRB49-MOMIP biodistribution in mice
with ET_B⁺ tumor**



413
414 *Figure 5: Fluorescence imaging of the different organs and tumors. ET_A⁺ tumor bearing*
415 *mouse injected with xiRA63-MOMIP 9 (left) and ET_B⁺ tumor bearing mouse injected with*
416 *xiRB49-MOMIP 10 (right). In both cases, a NCT tumor was added.*

417
418 **CONCLUSION**
419

420 Two bimodal immunoconjugates targeting endothelin receptors ET_A and ET_B were prepared.
421 Site-specific conjugation of a multifunctional monomolecular imaging probe (MOMIP)
422 allowed the synthesis of chemically defined and homogenous conjugates. IRDye800CW and
423 DFO were brought together into a tetrazine platform that is highly versatile and could enable
424 the use of any other dye/chelator combination. The careful control of the modification of
425 the antibodies resulted in immunoconjugates that retain their affinity and specificity for
426 their target, as shown by *in vitro* and *in vivo* experiments. In particular, fluorescence images
427 of mice with subcutaneously implanted ET_A⁺ and ET_B⁺ tumors showed a strong and selective
428 accumulation of the immunoconjugates in the tumors. These preliminary results are very
429 promising and we now wish to investigate these new bimodal conjugates in an orthotopic
430 mouse model of glioblastoma to demonstrate their potential as i) a tool for the diagnosis
431 and stratification of patients thanks to PET imaging and ii) a support to help visualizing
432 tumor margins during surgery thanks to the fluorescence.

433

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439

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446

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