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To cite this version:

Marie-Lynda Bouressam, Isabelle Lartaud, François Dupuis, Sandra Lecat. No answer to the lack of specificity: mouse monoclonal antibody targeting the angiotensin II type 1 receptor AT1 fails to recognize its target. Naunyn-Schmiedeberg's Archives of Pharmacology, 2018, 391 (8), pp.883-889. 10.1007/s00210-018-1522-4 . hal-01816221

HAL Id: hal-01816221

https://hal.univ-lorraine.fr/hal-01816221v1

Submitted on 15 Jun 2018

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Rapid Communication

Naunyn-Schmiedeberg's Archives of Pharmacology

NO ANSWER TO THE LACK OF SPECIFICITY: Mouse monoclonal antibody targeting the angiotensin II type 1 receptor AT_1 fails to recognize its target.

Marie-Lynda BOURESSAM,[1] MSc; Isabelle LARTAUD,[1] PharmD, PhD; François DUPUIS,[1] PharmD, PhD ; Sandra LECAT,[2] PhD

[1]: Université de Lorraine, CITHEFOR, F-54000 Nancy, France

[2]: BSC UMR7242 "GPCRs, pain and inflammation" team, CNRS, Université de Strasbourg Labex Medalis, 67412 Illkirch, France

Address for correspondence :

Dr Sandra LECAT

Institut de recherche de l'École Supérieure de Biotechnologie de Strasbourg : Biotechnologie et Signalisation Cellulaire, UMR7242

Phone: (33)3-68-85-47-38

Email: lecat@unistra.fr

Running title: Another non-specific AT_1 receptor mouse monoclonal antibody

Number of references: 24

Number of figures: 2

ABSTRACT

Purpose. Numerous antibodies targeting G protein-coupled receptors, GPCRs, have been described as non-specific among them polyclonal antibodies against angiotensin II type 1 receptor (AT_1). We have tested the newly developed AT_1 receptor mouse monoclonal antibody for its specificity.

Methods. Human Embryonic Kidney HEK293 cells, which do not endogenously express AT_1 receptor, were transfected in order to overexpress a fluorescently labelled EGFP-tagged human AT_1 receptor. Western blot and immunofluorescence assays were performed to test the specificity of the Santa Cruz monoclonal antibody sc-57036. These results were compared to the ones obtained with the polyclonal sc-1173 anti-AT_1 receptor antibodies that have already been described as non-specific.

Results. While the positive controls using GFP antibodies detected the EGFP-tagged AT_1 receptor, both polyclonal and monoclonal anti-AT_1 receptor antibodies failed to specifically recognize the corresponding band by western blot, as similar bands were revealed in either transfected or non-transfected cells. It also failed to detect AT_1 receptor in immunofluorescence experiments.

Conclusion. The lack of target recognition of the monoclonal AT_1 receptor antibody in our experimental conditions suggests that this antibody could give misleading results such as misidentification of the protein. To our knowledge, no specific antibodies targeting AT_1 receptors have been developed so far and the field is thus in need of new technical developments.

Key words: G protein-coupled receptors – Angiotensin II type 1 receptor –antibody – specificity

INTRODUCTION

G protein-coupled receptors (GPCR) antibodies raise a lot of questions concerning offtarget detections (Michel et al. 2009). Numerous publications criticize the non-specificity of different GPCR antibodies: histamine H4-receptors (Beermann et al. 2012) ; P2Y6 receptors (Yu and Hill 2013) ; cannabinoid receptors (Cécyre et al. 2014) ; dopamine receptors (Bodei et al. 2009).

Polyclonal antibodies targeting both angiotensin II (AngII) receptors type 1 (AT_1) and type 2 (AT_2) have been described as non-specific (Benicky et al. 2012; Herrera et al. 2013; Hafko et al. 2013). Despite these findings, antibodies directed against AT_1 receptors are still widely used in the literature with more than twenty publications in 2017 using the Santa Cruz aspecific sc-1173 polyclonal antibodies (Chao et al. 2017; Young et al. 2017; Liu et al. 2017) and already five publications in 2018 (Ahad et al. 2018; Ishikane et al. 2018; Rizzetti et al. 2018; Zhao et al. 2018; Ribeiro et al. 2018). In these papers, authors assume that they proved the specificity of these antibodies. However, in most cases, the control consisted of using the blocking peptide provided by the supplier to highlight the disappearance of the detected bands (Oestreicher et al. 2006). This type of experiment proves that the antibodies recognize the peptide used for immunization, however it is not sufficient to confirm that they recognize the targeted full-length protein. To overcome this issue, companies have developed monoclonal anti-AT_1 receptor antibodies presented as more specific.

In the present work, we evaluated the specificity of the Santa Cruz sc-57036 monoclonal anti-AT_1 receptor antibody.

Human Embryonic Kidney (HEK293) cells, that lacks endogenous AT_1 receptor expression, were transfected with a plasmid containing the human AT_1 receptor tagged at its extracellular N-terminus with the enhanced green fluorescent protein (EGFP-

73 AT_1). First, we assessed the robustness of our model by verifying the functionality of
74 the fluorescent receptor and its localization. Second, we tested the specificity of the
75 monoclonal sc-57036 antibody by Western blot and immunofluorescence. We
76 compared our data to the ones obtained using the polyclonal sc-1173 antibodies that
77 have already been described as non-specific. Our results indicate that the Santa Cruz
78 anti-AT_1 receptor monoclonal antibody sc-57036 does not label human AT_1 receptor.
79 Moreover we confirmed the aspecificity of the polyclonal sc-1173 antibodies.

80 **MATERIALS AND METHODS**

81 ***Cell culture and transfection***

82 HEK 293 (Human Embryonic Kidney) cells were cultured to ~80% confluence in T-75-
83 cm^2 flasks in MEM with Earle's salt supplemented with 10% fetal calf serum, 2 mM
84 glutamine and 1% antibiotics (penicillin/streptomycin) and re-plated twice a week. The
85 expression vector pIRES (Clontech) encoding for the fluorescent EGFP-human AT_1
86 receptor was a gift from Prof Haiech and Prof Kilhoffer (University of Strasbourg) and
87 PCBIS UMS3286 CNRS-University of Strasbourg.
88 Cells stably expressing human EGFP-AT_1 receptors were, in addition, cultured with
89 600µg/ml G418 (Invitrogen)
90

91 ***Antibodies***

92 EGFP was detected with mouse monoclonal anti-GFP (A11120 Life technologies) and
93 goat polyclonal anti-GFP directly coupled to Horse Radish Peroxidase HRP (AB6663,
94 ABCAM). Tested antibodies against AT_1 receptors were the anti-AT_1 N-10 (Santa Cruz
95 sc-1173 batch number C2415) and the anti-AT_1 TONI-1 (Santa Cruz sc-57036 batch
96 number I2111). The anti-AT_1 N-10 is a rabbit polyclonal IgG with an epitope mapping
97 within the N-terminal extracellular domain of AT_1 receptor of human origin. It has just
98 been discontinued. The anti-AT_1 TONI-1 is a mouse monoclonal antibody raised
99 against a GST fusion protein corresponding to amino acids 297-356 of AT_1 receptor of
100 human origin. Both antibodies are recommended by the manufacturer for detection of
101 AT_1 receptor of mouse, rat and human origin by Western blot, immunoprecipitation,
102 immunofluorescence and ELISA.

103

104

105 ***Calcium mobilization assay***

106 Cells were cultured for three days in T10, then loaded with 2.5 µM Fluo-4 AM
107 (Molecular Probes) for 1 h. Cells were rinsed in PBS, collected in PBS-5mM EDTA and
108 suspended in 9 mL Hepes-BSA buffer (10 mM HEPES pH 7.4, 137.5 mM NaCl, 1.25
109 mM MgCl2, 1.25 mM CaCl2, 6 mM KCl, 5.6 mM glucose, 0.4 mM NaH2PO4, 0,1%
110 Bovine Serum Albumine). Cells were loaded in 96-well plates (Greiner 655090, 100
111 µL/well) and centrifuged for 3 min at 800 rpm. AngII-evoked increases in intracellular
112 calcium were recorded over time using a Flexstation III (Molecular Devices, Sunnyvale,
113 CA, USA) (3 sec intervals over 220 sec) at 37°C through fluorescence emission at 520
114 nm (excitation at 485 nm). Peak response amplitudes were normalized to basal
115 fluorescence levels. For each concentration of the agonist, we determined the maximal
116 value of the increase in calcium concentration in order to build concentration response
117 curves to AngII.

118

119 ***Cell surface expression of the fluorescent EGFP-AT_1 receptor***

120 Cells were cultured for two days in T10, then rinsed in PBS, collected in PBS-5mM
121 EDTA. Immunolabelling of EGFP-AT_1 receptors expressed at the cell surface was
122 done on intact cells (approximately 1 million cells) pre-incubated in 1 mL PBS-1% BSA
123 on ice for 1 hour, then labelled using mouse monoclonal anti-GFP (A11120 Life
124 technologies 1/100 dilution in PBS 1% BSA) for 30 min on ice and secondary labelling
125 done using a R-phycoerythrin-conjugated AffiniPure F(ab')2 fragment goat anti-mouse
126 IgG (1/100 dilution). EGFP fluorescence and phycoerythrin (PE) staining were
127 detected by flow cytometric analysis (20,000 cells per sample) on a cytometer
128 (FACSCalibur, Becton-Dickinson).

129

130 ***Western blotting to detect the EGFP-tagged AT_1 receptor***

131 Cells were cultured for two days in 6-well plates. Cell lysates were performed in 250
132 µL RIPA buffer (20 mM de Tris-HCl pH 7.4, 150 mM de NaCl, 1% de NP40, 0.25% de
133 Na-deoxycholate, 0.1% SDS, Complete protease inhibitor EDTA-free (Roche)
134 according to Lecat et al. (2015). In order to concentrate the receptors, in some
135 experiments, cell lysates underwent immunoprecipitation. Briefly 450 µg of proteins
136 were exposed to mouse monoclonal anti-GFP (A11120 Life technologies, 1:100)
137 overnight on a wheel at 4°C before coupling to Protein A sepharose 4B (Life
138 technologies) for 2 hours at 4°C the following day. Protein A / antibodies complexes
139 were washed 3 times in 1 mL RIPA buffer by successive centrifugations at 2000 rpm
140 for 1 min at 4°C before being suspended in 2X Laemmli buffer.
141 Proteins were not heated and directly separated on 10 % acrylamide SDS-PAGE gel
142 electrophoresis. Proteins were then transferred onto PVDF polyvinyl difluoride
143 membranes for 2 hours and western blotting was performed according to Lecat et al.
144 (2015). Concentrations of antibodies were: polyclonal rabbit anti-AT_1 receptor (Santa
145 Cruz sc-1173) 1:500 and monoclonal mouse anti-AT_1 receptor (Santa Cruz sc-57036)
146 1:500. PVDF membranes were washed in acid buffer for desorbing the antibodies and
147 reprobed with goat polyclonal anti-GFP directly coupled to Horse Radish Peroxidase
148 HRP (AB6663, ABCAM) 1:25 0000. Secondary anti-rabbit HRP and anti-mouse HRP
149 antibodies were used at a dilution of 1:10 000 and 1:5 000 respectively (Immuno
150 Research Laboratories).
151

152 ***Immunofluorescence***

153 Cells were cultured for 24 hours in 24-well plates on 12-mm in diameter coverslips
154 coated with rat type I collagen. Cells were fixed in PBS containing 4%

155 paraformaldehyde, rinsed twice with PBS and exposed 15 min to 50 mM ammonium
156 chloride in order to quench remaining paraformaldehyde fluorescence. Cells were then
157 rinsed three additional times in PBS. Cells exposed to the monoclonal sc-57036
158 antibody were permeabilized 3 min in PBS containing 0,1% Triton X-100.
159 Immunolabelling was performed in PBS-0.2% BSA with either polyclonal anti-AT_1
160 receptor (sc-1173, 1:50), monoclonal anti-AT_1 receptor (sc-57036, 1:50) or monoclonal
161 anti-GFP (A11120, 1:100). Cells were washed 3 times in PBS-0.2% BSA and
162 incubated further with either secondary goat anti-rabbit or goat anti-mouse antibodies
163 coupled to Alexa 594 (Invitrogen ;1:200). After further washes in PBS, coverslips were
164 mounted onto slides using an anti-fading agent (Mowiol; Calbiochem). Image
165 acquisition was performed with a fluorescent microscope LEICA (motorized
166 microscope Leica DM5500B together with cold-camera Leica CTR 6000). Figures were
167 created with FigureJ.

168 **RESULTS**

169 ***Validation of the experimental model: in transfected cells, fluorescently-labelled***
170 ***AT_1 receptors are functional and localized at the plasma membrane***

171 In cells expressing the fluorescently labelled EGFP-AT_1, the receptor is fused at its
172 extracellular N-terminus with the fluorophore (Fig. 1A). We verified that the receptor
173 was active in these cells by measuring calcium responses to AngII. While the parental
174 HEK293 cells did not respond to the agonist, showing that the receptor is not
175 endogenously expressed, EGFP-AT_1 cells gave a nice AngII dose-response with an
176 EC50 around 2.10^{-8} mol/L AngII (Fig. 1B). Using fluorescence cell sorting analysis, we
177 have controlled that all the cells were expressing the fluorescent receptor (as detected
178 by the peak of EGFP fluorescence as compared to non-transfected cells, Fig. 1C, left)
179 and that the receptor was properly localized at the cell surface (as detected by surface
180 labelling with anti-GFP antibodies followed by red fluorescent secondary antibody
181 labelling, Fig. 1C, right).

182

183 ***Polyclonal and monoclonal anti-AT_1 antibodies detected bands in Western blot***
184 ***whether the receptor was expressed or not***

185 The expected size of the fusion protein, EGFP-AT_1 receptor, estimated from the amino-
186 acid sequence, is 73 kDa. The estimation does not take into account post-translational
187 modifications such as *N*-glycosylation. In Western blot of total cell extracts (40 µg of
188 proteins), both monoclonal sc-57036 and polyclonal sc-1173 antibodies detected
189 bands whether the fluorescently labelled receptor was expressed or not (Fig. 2A; total
190 lysates). Reprobing of the membrane with monoclonal anti-GFP antibody specifically
191 revealed a major band in HEK-EGFP-AT_1 transfected cells (but not in parental cells)
192 around 90 kDa corresponding to the tagged-receptor. A smaller band around 40 kDa

193 is also detected with the anti-GFP, that could correspond to degraded receptors mainly
194 with the EGFP tag left.
195 In a second attempt to detect the receptors with anti-AT_1 receptor antibodies, we
196 performed immunoprecipitations of 450 µg of protein extracts with monoclonal anti-
197 GFP antibody in order to concentrate the receptors prior to Western blotting. Again,
198 polyclonal sc-1173 and monoclonal sc-57036 revealed unspecific bands in both non-
199 transfected and HEK-EGFP-AT_1 transfected cell lysates (Fig. 2A; anti-GFP-
200 immunoprecipitated lysates). Reprobing the membrane with anti-GFP-HRP antibody
201 specifically revealed the EGFP-AT_1 receptor bands only in cells expressing the
202 fluorescently-labelled receptor (Fig. 2A; immunoprecipitated lysates).
203

204 ***Both anti-AT_1 receptor antibodies failed to recognize the receptor by***
205 ***immunofluorescence***

206 Next, we tested the antibodies in immunofluorescence labelling experiments on EGFP-
207 tagged AT_1 receptor expressing cells. Cells cultured on coverslips were labelled either
208 with anti-GFP or anti-AT_1 receptors antibodies, followed by a red fluorescent
209 secondary antibody detection. Using epifluorescence microscopy, images collected in
210 the green channel to directly visualize the fluorescence of EGFP showed a localization
211 of the receptor principally at the cell plasma membrane and in some intracellular
212 perinuclear compartments (Fig. 2B-*a*, 2B-*d*, 2B-*g*).
213 Staining with the monoclonal GFP antibody on non-permeabilized cells revealed only
214 the plasma membrane localization of the receptor (Fig. 2B-*b)*.
215 When using the polyclonal anti-AT_1 receptor antibodies, no plasma membrane staining
216 was detected (Fig. 2B-*e*). We obtained similar results with the monoclonal anti-AT_1
217 receptor antibody. For this antibody, we performed permeabilization of the cells due to

218 the localization of the epitope in the C-terminus tail of the receptor. While, in the green
219 channel, the receptor could still be detected by the direct fluorescence of EGFP (Fig.
220 2B-*g*), no specific labelling with the monoclonal anti-AT_1 receptor antibody in the red
221 channel (Fig. 2B-*h*) and no colocalization in the overlay channel were visible (Fig. 2B-
222 *i*).
223 Therefore, in accordance with our results obtained by Western blot, monoclonal and
224 polyclonal anti-AT_1 receptors antibodies also failed to recognize AT_1 receptors by
225 immunofluorescence.

226 **DISCUSSION**

227 Here we bring the first evidence of the lack of specificity of the Santa Cruz monoclonal
228 anti-AT_1 antibody sc-57036 as it failed to specifically recognize the AT_1 receptor. In the
229 present work we used an heterologous model of human AT_1 receptors expression in
230 which the receptor is tagged with the EGFP fluorescent protein at its extracellular N-
231 terminal domain. Before assessing the specificity of anti-AT_1 receptor antibodies, we
232 first evaluated the robustness of our model. HEK cells stably expressed the EGFP-AT_1
233 receptors homogeneously in the cell population. The receptors were properly localized
234 at the cell surface and functional.

235

236 We confirm here the aspecificity of the AT_1 receptor polyclonal antibodies sc-1173,
237 since bands were revealed by Western blot in all lysates whether AT_1 receptor was
238 present or not. We obtained the same results with the AT_1 receptor monoclonal
239 antibody sc-57036. Moreover, both AT_1 receptor antibodies failed to recognize the
240 receptor in immunofluorescence labelling assays. To our knowledge, this is the first
241 report questioning the specificity of Santa Cruz monoclonal AT_1 receptor antibody sc-
242 57036. Already used in 10 publications (including three in 2017 and one in 2018) (Filice
243 et al. 2017; Yim et al. 2017; Ferrão et al. 2017; Sinphitukkul et al. 2018), this antibody
244 that lacks specificity could lead to false positive staining on tissue or primary cell culture
245 and therefore misinterpretations.

246

247 Concerning the sc-1173 antibodies, our results are in accordance with the ones
248 obtained by Benicky et al. (2012). They tested 6 commercially available anti-AT_1
249 receptor antibodies (including sc-1173) by Western blot and immunofluorescence. All
250 of them failed to recognize specifically the receptor. First, they assessed the

251 robustness of their models (AT_{1A} knock-out mice and rat hypothalamic 4B cells) by
252 verifying the lack of expression of AT_1 receptor in both models using radioligand
253 binding assays. Second, they compared the immunoreactivity of the antibodies in
254 knock-out tissues and in cells transfected or not with AT_1 receptor. The six antibodies
255 (including sc-1173) displayed very different pattern and intensity of immunoreactivity.
256 But all tested antibodies gave signals in tissues from AT_{1A} knock-out mice and non-
257 transfected 4B cells. Despite this first report of aspecificity, the sc-1173 antibodies
258 were still widely used in the literature up to now (Rizzetti et al. 2018; Zhao et al. 2018;
259 Ribeiro et al. 2018) and it has just been discontinued.
260

261 Since antibodies directed against GPCR have been widely criticized for their lack of
262 specificity, a correct validation of the used antibodies should be provided by the
263 authors and requested by journals editors. Use of blocking peptide is a weak proof of
264 specificity and using tagged receptors is a more reliable alternative method. Authors
265 could also rely on one of the four criteria proposed by Michel et al. (2009) to
266 demonstrate specific immunolabelling of receptors in tissues: a) disappearance of
267 staining in knock-out animals of the target receptor, b) reduction of staining upon
268 knock-down approaches such as siRNA treatment, c) selectivity of staining in
269 immunoblots or immunocytochemistry for the target receptor *vs* related subtypes when
270 expressed in the same cell line and/or d) antibodies raised against multiple distinct
271 epitopes of a receptor yielding very similar staining patterns.
272

273 Given the issues concerning GPCR antibody specificity, the use of new tools to detect
274 these membrane proteins is thus essential. Single-domain antibody (or nanobody)
275 arouse great interest in the scientific community. Indeed, they show a promising

276 potential for research or therapeutic (Rasmussen et al. 2011; Maussang et al. 2013).
277 They have smaller sizes (around 15 kDa *vs* 150 kDa for a classical antibody), they can
278 be expressed intracellularly, they can display higher affinity and specificity and they
279 have lower cost of production. Hence, the emergence of nanobodies is likely to replace
280 actual existing polyclonal and monoclonal antibodies to ensure a reliable detection of
281 GPCR proteins.
282 In conclusion, we demonstrate here that the monoclonal AT_1 receptor antibody sc-
283 57036 should be added to the long list of non-specific antibodies targeting GPCRs.

284 **ABBREVIATIONS**

285 AngII: angiotensin II

286 AT_1: angiotensin II type 1 receptor

287 AT_2: angiotensin II type 2 receptor

288 EGFP: enhanced green fluorescent protein

289 HEK293: human embryonic kidney cell

290 GPCR: G protein-coupled receptor

291 PE: phycoerythrin

292 PBS: phosphate buffered saline

AUTHORS CONTRIBUTION

SL conceived and designed the experiments. MLB and SL conducted the experiments. MLB, IL, FD, SL analyzed data and wrote the manuscript. All the authors read and approved the manuscript.

REFERENCES

Ahad A, Raish M, Al-Jenoobi FI, Al-Mohizea AM (2018) Sorbitane Monostearate and Cholesterol based Niosomes for Oral Delivery of Telmisartan. Curr Drug Deliv 15:260–266. doi: 10.2174/1567201814666170518131934

Beermann S, Seifert R, Neumann D (2012) Commercially available antibodies against human and murine histamine H-receptor lack specificity. Naunyn Schmiedebergs Arch Pharmacol 385:125–135. doi: 10.1007/s00210-011-0700-4

Benicky J, Hafko R, Sanchez-Lemus E, et al (2012) Six commercially available angiotensin II AT1 receptor antibodies are non-specific. Cell Mol Neurobiol 32:1353–1365. doi: 10.1007/s10571-012-9862-y

Bodei S, Arrighi N, Spano P, Sigala S (2009) Should we be cautious on the use of commercially available antibodies to dopamine receptors? Naunyn Schmiedebergs Arch Pharmacol 379:413–415. doi: 10.1007/s00210-008-0384-6

Cécyre B, Thomas S, Ptito M, et al (2014) Evaluation of the specificity of antibodies raised against cannabinoid receptor type 2 in the mouse retina. Naunyn Schmiedebergs Arch Pharmacol 387:175–184. doi: 10.1007/s00210-013-0930-8

Chao Y, Zhu L, Qu X, et al (2017) Inhibition of angiotension II type 1 receptor reduced human endothelial inflammation induced by low shear stress. Exp Cell Res 360:94–104. doi: 10.1016/j.yexcr.2017.08.030

Ferrão FM, Cardoso LHD, Drummond HA, et al (2017) Luminal ANG II is internalized as a complex with AT1R/AT2R heterodimers to target endoplasmic reticulum in LLC-PK1cells. Am J Physiol Renal Physiol 313:F440–F449. doi: 10.1152/ajprenal.00261.2016

Filice M, Amelio D, Garofalo F, et al (2017) Angiotensin II dependent cardiac remodeling in the eel Anguilla anguilla involves the NOS/NO system. Nitric Oxide Biol Chem 65:50–59. doi: 10.1016/j.niox.2017.02.007

Hafko R, Villapol S, Nostramo R, et al (2013) Commercially Available Angiotensin II At2 Receptor Antibodies Are Nonspecific. PLoS ONE 8:. doi: 10.1371/journal.pone.0069234

Herrera M, Sparks MA, Alfonso-Pecchio AR, et al (2013) Lack of specificity of commercial antibodies leads to misidentification of angiotensin type 1 receptor protein. Hypertens Dallas Tex 1979 61:253–258. doi: 10.1161/HYPERTENSIONAHA.112.203679

Ishikane S, Hosoda H, Nojiri T, et al (2018) Angiotensin II promotes pulmonary metastasis of melanoma through the activation of adhesion molecules in vascular endothelial cells. Biochem Pharmacol. doi: 10.1016/j.bcp.2018.04.012

Lecat S, Belemnaba L, Galzi J-L, Bucher B (2015) Neuropeptide Y receptor mediates activation of ERK1/2 via transactivation of the IGF receptor. Cell Signal 27:1297–1304. doi: 10.1016/j.cellsig.2015.03.016

Liu C-H, Gong Z, Liang Z-L, et al (2017) Arrestin-biased AT1R agonism induces acute catecholamine secretion through TRPC3 coupling. Nat Commun 8:14335. doi: 10.1038/ncomms14335

Maussang D, Mujić-Delić A, Descamps FJ, et al (2013) Llama-derived single variable

341 domains (nanobodies) directed against chemokine receptor CXCR7 reduce head and
342 neck cancer cell growth in vivo. J Biol Chem 288:29562–29572. doi:
343 10.1074/jbc.M113.498436

344 Michel MC, Wieland T, Tsujimoto G (2009) How reliable are G-protein-coupled
345 receptor antibodies? Naunyn Schmiedebergs Arch Pharmacol 379:385–388. doi:
346 10.1007/s00210-009-0395-y

347 Oestreicher EM, Guo C, Seely EW, et al (2006) Estradiol increases proteinuria and
348 angiotensin II type 1 receptor in kidneys of rats receiving L-NAME and angiotensin II.
349 Kidney Int 70:1759–1768. doi: 10.1038/sj.ki.5001897

350 Rasmussen SGF, Choi HJ, Fung JJ, et al (2011) Structure of a nanobody-stabilized
351 active state of the β2 adrenoceptor. Nature 469:175–181. doi: 10.1038/nature09648

352 Ribeiro VS, Cabral EV, Vieira LD, et al (2018) Perinatal α-tocopherol overload
353 programs alterations in kidney development and renal angiotensin II signaling
354 pathways at birth and at juvenile age: Mechanisms underlying the development of
355 elevated blood pressure. Biochim Biophys Acta. doi: 10.1016/j.bbadis.2018.04.007

356 Rizzetti DA, da Silva TM, Escobar AG, et al (2018) Mercury-induced vascular
357 dysfunction is mediated by angiotensin II AT-1 receptor upregulation. Environ Res
358 162:287–296. doi: 10.1016/j.envres.2018.01.026

359 Sinphitukkul K, Manotham K, Eiam-Ong S, Eiam-Ong S (2018) Nongenomic action of
360 aldosterone on colocalization of angiotensin II type 1 and type 2 receptors in rat
361 kidney. J Histotechnol 0:1–8. doi: 10.1080/01478885.2018.1438756

362 Yim HE, Yoo KH, Bae IS, Hong YS (2017) Early Treatment With Enalapril and Later
363 Renal Injury in Programmed Obese Adult Rats. J Cell Physiol 232:447–455. doi:
364 10.1002/jcp.25444

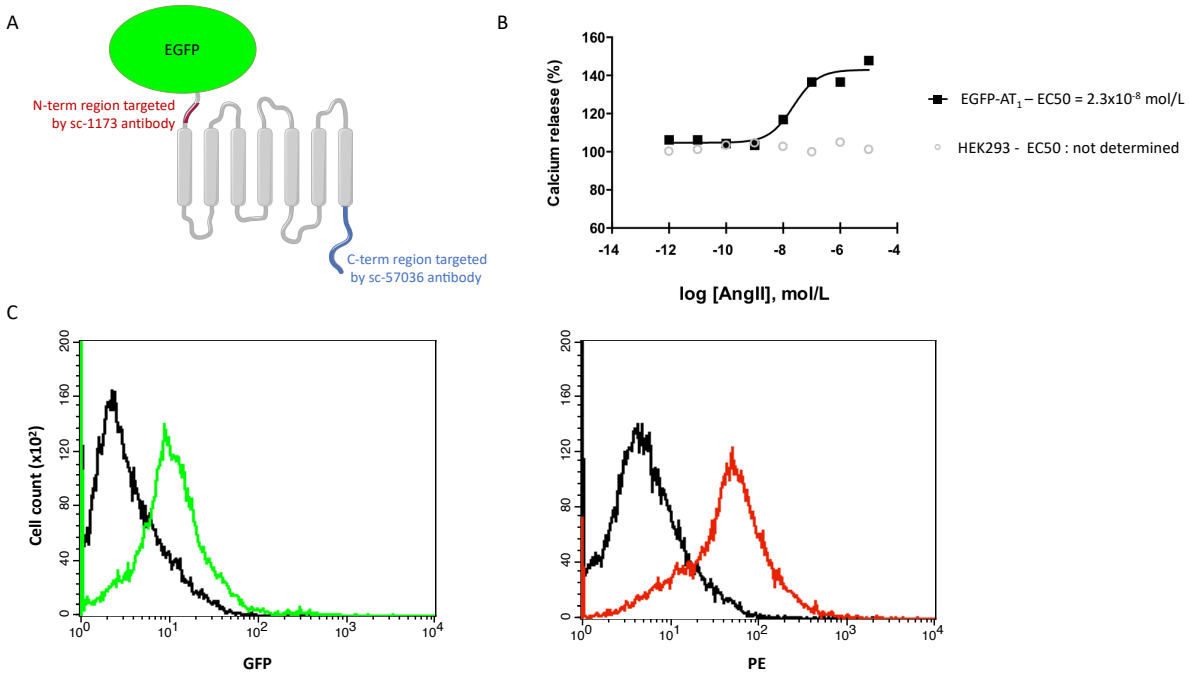
365 Young BM, Nguyen E, Chedrawe MAJ, et al (2017) Differential Contribution of
366 Transmembrane Domains IV, V, VI, and VII to Human Angiotensin II Type 1
367 Receptor Homomer Formation. J Biol Chem 292:3341–3350. doi:
368 10.1074/jbc.M116.750380

369 Yu W, Hill WG (2013) Lack of specificity shown by P2Y6 receptor antibodies. Naunyn
370 Schmiedebergs Arch Pharmacol 386:885–891. doi: 10.1007/s00210-013-0894-8

371 Zhao Y, Zhu Q, Sun S, et al (2018) Renal transplantation increases angiotensin II
372 receptor-mediated vascular contractility associated with changes of epigenetic
373 mechanisms. Int J Mol Med 41:2375–2388. doi: 10.3892/ijmm.2018.3435

374

375 **FIGURES**



376

377 **Figure 1. In EGFP-AT_1 transfected cells, AT_1 receptor is properly localized at the**

378 **cell surface**

379 A - Schematic view of the fusion protein EGFP-AT_1 receptor. The EGFP fluorophore is

380 localized in the extracellular N-terminal part of the receptor. The sc-1173 polyclonal

381 anti-AT_1 receptor targets the short N-terminal domain of the receptor while the epitope

382 for the sc-57036 monoclonal anti-AT_1 receptor encompassed the whole C-terminal tail

383 of the receptor.

384 B – HEK293 and EGFP-AT_1 transfected cells were exposed to Angiotensin II (AngII ;

385 10^{-12} to 10^{-4} mol/L). AngII-evoked increases in intracellular calcium were recorded over

386 time using a Flexstation III (3 sec intervals over 220 sec) at 37°C through fluorescence

387 emission at 520 nm (excitation at 485 nm). Peak response amplitudes were normalized

388 to basal fluorescence levels. Each point represents the maximal value at 520 nm for a

389 given AngII concentration.

390 C - The cell population expressing the EGFP-AT_1 receptors was compared to non-

391 transfected cells by fluorescence-activated cell sorting. Left panel: Histogram of cells
392 expressing the GFP fluorescence (green line: cells expressing stably the EGFP-AT_1
393 receptors, black line: non-transfected HEK293 cells). Right panel: Histogram of cells
394 labelled with phycoerythrin (PE): intact cells were stained with anti-GFP antibodies and
395 secondary phycoerythrin-labeled antibodies (PE). Fluorescence intensities of non-
396 transfected HEK cells (black line) and cells expressing stably the EGFP-AT_1 receptors
397 (red line).

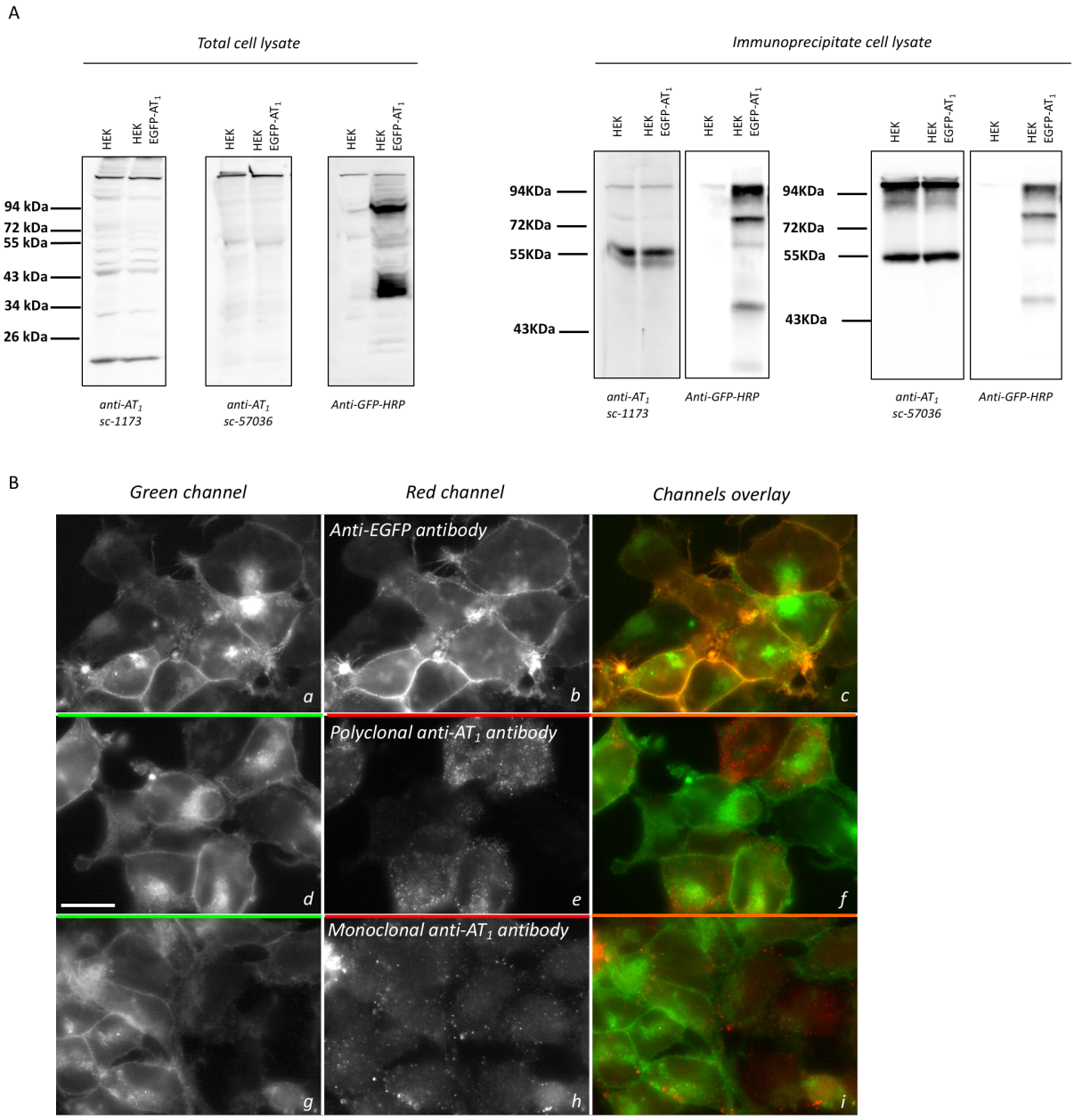


Figure 2. Monoclonal and polyclonal anti-AT_1 receptor antibodies failed to recognize EGFP-AT_1 receptor in both Western blot and immunofluorescence assays

A - Immunoblot of total cell lysates (left – 40 µg of proteins) or of anti-GFP immunoprecipitated material (right – 450 µg of proteins) of non-transfected HEK293 cells and of cells expressing stably the EGFP-AT_1 receptors. Proteins were revealed with either polyclonal anti-AT_1 sc-1173, monoclonal anti-AT_1 sc-57036 or anti-GFP-

406 HRP antibodies. Anti-GFP antibody revealed bands only in cells expressing the EGFP-
407 AT_1 receptor whereas sc-1173 and sc-57036 anti-AT_1 antibodies revealed unspecific
408 bands whether the receptor had been transfected or not.
409 B - Antibodies immunolabelling on EGFP-tagged AT_1 cells. (*a*, *d*, *g*) Images collected
410 in the green channel using epifluorescence microscopy showing the fluorescence of
411 the EGFP-tagged receptor which is localized principally at the cell plasma membrane
412 and in some perinuclear compartments. (*b*, *e*, *h*) Images collected in the red channel,
413 stained with primary antibodies directed against GFP epitope in *b*, or AT_1 receptor (*e*,
414 sc-1173, *h*, sc-57036) followed by a red secondary antibody. Plasma membrane
415 localization of the receptor and colocalization with the GFP signal (displayed in the
416 channel overlay) is only detected with the anti-GFP labelling (*b* and *c* respectively),
417 whereas no plasma membrane signal (*e* and *h*) or colocalization (*g* and *i*) could be
418 detected when using anti-AT_1 antibodies. Scale bar is 10 μM.