

1 **Receptor Mediated Delivery of Cas9-Nanobody Induces Cisplatin**

2 **Synthetic Dose Sensitivity**

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26 **Abstract:**

27 The CRISPR/Cas9 system has shown great potential for precisely editing genomic DNA
28 sequences by introducing site-specific DNA cuts that are subsequently repaired by the cell.
29 However, delivery of the CRISPR ribonucleoprotein remains an understudied area and hinders
30 realizing the full potential of the system. We prepared Cas9 ribonucleoprotein complexes
31 chemically conjugated to the 7D12 nanobody and demonstrate receptor-mediated transfection of
32 Cas9 into A549 non-small-cell lung cancer cells via binding to the epithelial growth factor receptor
33 for subsequent cell internalization. We further show that transfection with a Cas9
34 ribonucleoprotein targeting the BRCA2 gene results in an enhanced sensitivity to the
35 chemotherapeutic drug Cisplatin, and thereby induces a synthetic dose lethality in A549 cells.

36

37 **Introduction:**

38 CRISPR/Cas9 gene editing has opened therapeutic opportunities that were previously not
39 possible (1, 2). The first wave of CRISPR therapeutic companies (EDITAS, Caribou) and several
40 academic groups focused on *ex vivo* editing (i.e CTX001 for Thalassemia and Sickle cell (3)), and
41 localised injections (CRISPR-Gold in Fragile X syndrome and Duchenne's dystrophy (4)).
42 Systemic delivery methods of Cas9 include liposomal, cationic polymers, viral and viral-like
43 particles (5); and while Cas9 ribonucleoprotein (RNP) complexes assembled *in vitro* offer higher
44 editing efficiency and lower off-target cleavage than plasmid transfection (6), delivery is generally
45 achieved *ex vivo* by electroporation (7).

46

47 Receptor-mediated transfection offers an attractive means to achieve preferential accumulation
48 and increased efficacy of a therapeutic unit (8-11). Recently, small molecule ligands (12) and
49 aptamers (13) have been coupled to the Cas9 protein or a Cas9 containing nanoparticle,
50 respectively, for receptor-mediated uptake into cells. The potential to specifically deliver Cas9

51 RNP to cells overexpressing a particular receptor type offers many opportunities for targeting the
52 effects of gene editing specifically towards disease-causing cells.

53

54 The prospect of using Cas9 for gene knockdown/knockout is clearly understood and widely used,
55 however, applications such as homology directed repair (HDR) using donor DNA templates are
56 still in development in an *in vivo* setting. Having proved to be a powerful screening tool for
57 identifying gene essentiality, Cas9 may be used to overcome chemotherapy resistance (14) and
58 HDR precision would broaden the scope of this application. An ideal Cas9/chemotherapeutic
59 combination would involve targeted delivery through an overexpressed receptor on cancer cells
60 followed by knockout or correction of an oncogene, thereby maintaining or enhancing sensitivity
61 to a small molecule chemotherapeutic.

62

63 We chose lung cancer as a model system to evaluate potential therapeutic application. The
64 Epidermal Growth Factor Receptor (EGFR) is overexpressed in lung cancer cells providing an
65 alternative to CD133 for cancer cell identification (15). The Breast Cancer type 2 susceptibility
66 protein (BRCA2) is known to be essential for DNA repair in normal cells (16) and consequently,
67 loss of expression initiates tumorigenesis (17). However, in cancer patients subject to
68 chemotherapy, reversion mutations restoring the open reading frame of the BRCA2 gene can
69 occur and result in resistance to platinum-based chemotherapeutic drugs by maintenance of DNA
70 repair (18, 19). Additionally, it has been shown that knockdown of the BRCA2 gene product by
71 antisense oligonucleotides (ASO) limits cell proliferation in the human lung carcinoma cell line,
72 A549, when co-administered with the cytotoxic drug Cisplatin (20) validating the biological
73 potential for Cas9 knockout studies.

74

75 We hypothesize that Cas9 delivered via receptor-mediated transfection can be targeted to BRCA2
76 creating a synthetic dose lethality of Cisplatin without additional transfection agents. However,

77 the increased protein size with an antibody-targeted nanoparticle systems was shown to impair
78 biodistribution, thereby reducing efficacy, tumour penetration and retention (21, 22). Thus, we
79 chose to use a nanobody (Nb) receptor-mediated transfection system, which maintains target
80 specificity with the added benefit of its smaller size (15 kDa), which does not substantially increase
81 the Cas9 hydrodynamic radius. For our study, we selected two low-EGFR expressing cell lines
82 (A549 and 3T3) rather than A431 (high EGFR expression) as a stringent challenge to the process
83 of receptor mediated transfection (23). Binding of EGFR targeted nanobodies to 3T3 are
84 equivalent to HeLa and A549 cells (24). In this short report, we tested the concept of receptor-
85 mediated transfection of Cas9-Nb complexes leading to a synthetic Cisplatin dose lethality.

86

87 **Results:**

88 **Nanobodies can be conjugated to Cas9 via NHS/EDC chemistry**

89 To create a Cas9-Nb fusion, we applied amide coupling via NHS/EDC as the simplest method of
90 conjugation. In brief, Nb carboxylic acids were activated by EDC to form the o-acylisourea
91 intermediate that reacts with N-hydroxysulfosuccinimide (Fig. 1A). Sulfo-succinimide forms a
92 stable reactive group in the aqueous phase. Lastly, adjustment of pH to greater than 7.5 improves
93 amide bond formation with primary amines on Cas9, thereby linking the two proteins. The relative
94 simplicity of the chemistry and the few steps required offers flexibility in future applications by
95 allowing for selection of desired Cas9 and Nb variants.

96

97 To explore the potential for Cas9 nucleases to enter the cell by receptor-mediated transfection,
98 we developed two nuclease variants. The first was Cas9NLS fused to a monoavidin domain
99 (Cas9MAV). This nuclease is desirable for future homology directed repair (HDR) experiments
100 using biotinylated donor DNA. The second was Cas9NLS with 6 C-terminal cysteines (Cas9-
101 6Cys) to facilitate improved protein labeling. Bovine serum albumin (BSA) was included as a
102 control and all proteins were labelled with thiol coupling of the tetramethyl rhodamine (RHOD)

103 fluorophore. The above described Cas9 variants were chemically conjugated to the EGFR
104 nanobody, 7D12 (25) to form Cas9-6Cys-Nb and Cas9MAV-Nb, respectively.

105
106 Fig. 1B shows the comparison of three size exclusion chromatograms; Cas9-6Cys, activated
107 7D12 Nb and the resulting Cas9-6Cys-Nb conjugate. As the elution profiles of Cas9-6Cys-RHOD
108 and 7D12-conjugated Cas9-6Cys-RHOD did not change significantly, a dot blot was performed
109 to validate that the His-tagged Nb was indeed present in the Cas9 fractions upon conjugation. A
110 purple color demonstrates the presence of a His-tag and thus the 7D12 Nb. Control Cas9-6Cys-
111 RHOD (pink) and the unlabelled Cas9-6Cys (white) were negative for the His-tag.

112

113 **Non-specific cell penetration of Cas9 and 7D12 mediated Cas9 Transfection**

114 Next, we investigated whether Cas9-6Cys-Nb and Cas9MAV-Nb could target and thus facilitate
115 cellular uptake into A549 NSCLC cells and 3T3 murine cells (Fig. 2A). The experiment compared
116 unconjugated proteins to those with nanobody attachments, and were evaluated at 48 hours post
117 transfection by fluorescence microscopy (RFP channel, Fig. 2B) and fluorescence at 577 nm (Fig.
118 2A).

119

120 Cas9-6Cys-Nb and Cas9MAV-Nb showed significant cellular uptake in both cell lines compared
121 to the control BSA-RHOD as well as the unconjugated Cas9 variants. The increase was
122 concentration dependent with ~30-fold increased uptake at the highest concentration of 7D12-
123 conjugated protein in 3T3, and ~20- and ~35-fold increases for Cas9-6Cys-Nb and Cas9MAV-
124 Nb, respectively, in A549. Interestingly, a small non-specific dose response was also observed
125 for unconjugated Cas9-6Cys and Cas9MAV, though overall transfection level was low in
126 comparison to nanobody mediated transfection.

127

128

129 **Cisplatin Synthetic Dose Lethality Assay**

130 To explore the potential for synthetic dose lethality of Cisplatin, we pursued a BRCA2 knockout
131 in A549 cells to enhance the dose response to the drug (Fig. 3). The RNP tested was Cas9-6Cys-
132 Nb complexed to an sgRNA targeting BRCA2. Co-administration of Cas9-6Cys-Nb RNP and
133 Cisplatin was evaluated at a fixed protein concentration (8.3 pmol per well) and varying
134 concentrations of Cisplatin (0.2 - 8 μ M Cisplatin). Gene editing is most likely to demonstrate its
135 effect on cell viability post 48 hrs. This was validated at the 24 hr time point where Cisplatin-only
136 and RNP treated cells were indistinguishable (Fig. 3A).

137
138 Co-administration of RNP and Cisplatin dosage over 72 hrs exposure resulted in the most notable
139 improvement in Cisplatin sensitivity, however, similar significant trends were also observed after
140 only 48 hrs. We know from previous work that 48-72 hrs incubation is sufficient to establish the
141 desired gene edit in a substantial population of cells. Furthermore, Cisplatin-induced apoptosis
142 and cell cycle arrest happens within 8-11 hrs of treatment (26), which is also evident from our
143 results at 24 hrs. Fig. 3B summarizes the development over 72 hrs and shows that the largest
144 fold decrease in cell viability occurs at 0.2-1 μ M Cisplatin. Dose response curves were used to
145 calculate approximate IC₅₀ values at 48 and 72 hrs (Fig. 3C), which show that knockout of BRCA2
146 via Cas9-6Cys-Nb RNP transfection decreases the IC₅₀.

147
148 With a view towards future experiments with precision HDR and other targets, we explored
149 whether Cas9MAV behaves similarly and can be delivered to induce synthetic dose lethality. Fig.
150 3D shows that sensitisation of A549 cells by Cas9MAV-Nb RNP delivery has indeed occurred.

151
152 **Conclusion**
153 In this simple proof of concept study, we have demonstrated EGFR receptor-mediated delivery of
154 Cas9 nanobody conjugates and commensurate gene editing leading to synthetic Cisplatin dose

155 lethality. The 7D12 nanobody has a very tight binding affinity ($K_d \sim 0.29$ nM) for the EGFR receptor
156 (23). In both cell lines with low EGFR expression, the addition of nanobody resulted in greater
157 uptake of Cas9-Nb conjugates into cells compared to non-conjugated Cas9. Both Cas9-6Cys and
158 our HDR optimized Cas9MAV proteins were successfully delivered and achieved synthetic dose
159 lethality in A549 NSCLC cells. Testing precision HDR with Cas9MAV remains for future work. For
160 therapeutic Cas9 applications, the induction of synthetic dose lethality could be a means to reduce
161 the therapeutic dose and side effects of Cisplatin (27). Furthermore, the well-established
162 NHS/EDC conjugation technique used to fuse nanobody and Cas9 variants of interest, with
163 subsequent purification via gel filtration, brings receptor-mediated Cas9 delivery within the scope
164 of the basic research lab.

165

166 There are some limitations of this study: 1) only one sgRNA was evaluated for BRCA2 knockdown
167 where other guides may have generated a higher indel occurrence, 2) the degree of BRCA2
168 knockout was not characterised by Western Blot leaving the potential for further improvement in
169 lethality, 3) a wider NSCLC cell line screen would be a powerful predictor of the synthetic dose
170 lethality particularly in cells where Cisplatin resistance has developed, 4) recombinant Cas9-Nb
171 fusion proteins were not tested and compared to chemical conjugations and 5) a greater number
172 of biological replicates will eliminate 96-well plate to plate variance. The purpose of this paper is
173 to demonstrate that a biological effect occurs due to Cas9 receptor-mediated transfection. With
174 this limited objective, it is hoped that the principle will be taken by others and more widely applied
175 in enhancing combinatorial Cas9 RNP delivery and small molecule therapeutic studies.

176

177 Nanobodies can be generated by selection from recombinant library screening systems (28) or
178 purchased from commercial/academic suppliers with known binding characteristics, rather than
179 consuming significant effort and time in small molecule ligand screening. Additional advantages
180 of this system are the ease of combination with cell/tissue/disease specific sgRNA sequences;

181 the potential to combine multi-valent nanobodies that have enhanced tumour penetration (21)
182 with lower affinity constants (25). Delivery of canonical Cas9 and Cas9MAV make possible gene
183 knockout and high efficiency HDR, respectively, as potential therapeutic modalities to investigate
184 and receptor-mediated Cas9 RNP delivery offers therapeutic opportunities that could be
185 translated into animal models/preclinical evaluations (29, 30). In conclusion, the potential of
186 nanobody-conjugated Cas9 nucleases needs be explored in more depth *in vitro* and *in vivo*, as a
187 means to resolve Cas9 RNP delivery challenge.

188

189 **Methods:**

190 **Cell Culture and Transfection:**

191 3T3 and A549 cell lines were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin,
192 and 100 U/mL streptomycin and were maintained at 37°C and 5% CO₂. Media, trypsin and FBS
193 were supplied by Wisent. Cells were kept at low passage for experimentation, not exceeding 10
194 passages before starting fresh cultures from frozen stocks.

195

196 For transfections, seeding density was 50000 cells per well (96 well plate) the day before
197 transfection. At a confluency of 60-70%, transfection of RNP was accomplished by addition of
198 12µl of RNP solution (8.3pmol Cas9 per well), followed by gentle agitation of the plate.

199

200 **sgRNA:**

201 A single piece sgRNA guide was used in this study. The BRCA2 sgRNA (protospacer sequence
202 GCAGGUUCAGAAUUAUAGGG) was designed using Synthego sgRNA designer and
203 synthesised from Synthego with 5'/3' 2-O-Me ribose and phosphorothioate backbone
204 modifications.

205

206

207 **Nanobody Expression and Purification**

208 The 7D12 nanobody was expressed in BL21 (DE3) cells, induced with 0.5mM IPTG at OD600 =
209 0.9 and grown ON at 18°C. Cells were either subjected to complete cell lysis by sonication and
210 cleared by centrifugation, or partial lysis to obtain the protein from the periplasmic space, both
211 with similar low yields of 1mg/2L culture. The 7D12 Nb was subjected to Ni affinity
212 chromatography in buffer A (50 mM Tris pH8, 500 mL NaCl, 5% glycerol, 1 mM PMSF, 20 mM
213 imidazole) and eluted with 500 mM imidazole before being purified by gel filtration in 20 mM
214 HEPES pH 7.5, 150 mM NaCl.

215

216 **Purification of Cas9 proteins**

217 SpCas9 fusion constructs were expressed in BL21(DE3) Rosetta2 cells grown in LB media at
218 18°C for 16 h following induction with 0.2 mM IPTG at OD600 = 0.8. The cell pellet was lysed in
219 500 mM NaCl, 5 mM imidazole, 20 mM Tris-HCl pH 8, 1 mM PMSF and 2 mM B-me, and disrupted
220 by sonication. The cleared lysate was subjected to Ni affinity chromatography using two
221 prepacked 5 mL HisTrap columns/3 L cell culture. The columns were extensively washed first in
222 20 mM Tris pH 8.0, 500 mM NaCl, 5 mM imidazole pH 8.0, 2 mM B-me, followed by 20 mM
223 HEPES pH 7.5, 200 mM KCl, 10 % glycerol, 0.5 mM DTT, before elution with 250 mM imidazole.
224 The His-MBP tag was removed by overnight TEV protease cleavage w/o dialysis. The cleaved
225 Cas9 protein was separated from the tag and co-purifying nucleic acids on a 5 mL Heparin HiTrap
226 column eluting with a linear gradient from 200 mM - 1 M KCl over 12 CV.

227

228 Gel filtration of Cas9 proteins and Nb conjugates were performed on a Superdex 200 increase
229 column in 5% glycerol, 250 mM KCl, 20 mM HEPES pH 7.5. Eluted proteins were concentrated
230 and stored at -80°C.

231

232

233 **Fluorescent Cas9-Nb Conjugations and Nanobody Biotinylation:**

234 Cas9 proteins used in nanobody conjugates were fluorescently labelled using maliamide-
235 tetramethylrhodamine, where 4µl of tetramethylrhodamine maleimide (Anaspec, 10mg/ml, 100x
236 molar excess) was added to a 200µl of protein (8-10mg/ml) in degassed Cas9 buffer and reacted
237 overnight at 4°C. The reaction conjugates dye via thiol ester formation between dye and cysteines.
238 Purification was achieved using a Pierce dye removal kit (Thermofisher) following manufacturer's
239 protocol.

240

241 7D12 nanobodies and Cas9 proteins were conjugated by a two-step reaction. 7D12 was diluted
242 in 0.1M MES buffer pH 5.5 to 1mg/ml concentration (final volume 500µl) and COOH R-groups
243 were activated using 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC, 0.5mM final,
244 Geobiosciences) forming O-acylisourea intermediates and the more stable amine reactive
245 intermediate N-hydroxysulfosuccinimide (sulfoNHS, 4mM final, (Geobiosciences).The reaction
246 was allowed to proceed for 4-12 hrs at 22°C. The sample was cleaned up by a G-50 micro spin
247 column (Amersham). Amide bond formation occurring between sulfo-NHS and primary amine R-
248 groups of the Cas9 proteins was conducted at 4°C overnight (Nanobody and Cas9 variants in 4:1
249 molar ratio), with pH adjustment to 7.5 using 10x PBS buffer. Complexes were separated from
250 unconjugated Nb and excess NHS/EDC reagents by purification on a Superdex 200 increase
251 column.

252

253 **Dot blotting**

254 Briefly, peak fractions from Cas9-6Cys alone, 7D12 Nb alone and Cas9-6Cys-Nb conjugations
255 were dotted onto a nitrocellulose membrane, blocked in 5% low-fat milk, incubated with mouse
256 anti-His tag antibody (1:2000, Biobasic), washed with TBS-T and incubated with anti-mouse IgG,
257 AP Conjugate (1:2000, Promega) before additional washing steps and development with
258 Sigmafast BCIP/NBT (Sigma).

259

260 **RNP formation for transfection:**

261 20µl of 1x phosphate buffered saline (sterile and 0.22µm filtered), 20µl of Cas9 proteins or
262 Cas9Nb conjugates (25 pmol per 3 wells), sgRNA (concentration varied with respect to Cas9
263 molarity to maintain 1:1 ratio) were combined in a sterile PCR tube, vortexed gently and incubated
264 for 20 minutes at 25°C. 180µl of DMEM was added to each tube and mixed by pipetting, followed
265 by incubation at 37°C for 10 minutes. Serial dilutions were made of the RNP stock for receptor
266 mediated transfection assay.

267

268 **Receptor mediated Transfection Assay**

269 96 well plates were seeded with 3T3 and A549 cells. Four RNP concentrations (1 to 8.3 pmol) of
270 each protein (Cas9-6Cys, Cas9MAV and BSA) were prepared from RNP stocks. Each protein
271 was assigned a block of 3 columns (25 pmol total) and each cell line received the 4 concentrations
272 from the RNP serial dilution in triplicates. 7D12-conjugated RNP was introduced and incubated
273 for 48hrs, at which point DMEM media was removed, cells washed with warmed PBS and
274 visualized by fluorescent microscopy (RFP channel) and then fluorescent was read using a
275 molecular dynamics SpectraMax M5 plate reader (Emission 577nm).

276

277 **Receptor Mediated Cisplatin Synthetic Dose Lethality and MTT Assay**

278 A549 Cells were plated to 50-75,000 cells per well overnight. For co-administration (RNP +
279 cisplatin), RNP was introduced at 8.3 pmol per well for each cas9-Nb conjugate, followed by
280 immediate Cisplatin serial dilution administration (8 to 0.2µM + DMSO control), with time points
281 of 24, 48 and 72 hrs incubation. Plates were measured at 590nm to be normalised for background.
282 20 µl of 5 mg/ml MTT was added to each well and incubated for 2 hrs at 37°C. Media was carefully
283 removed and 150 µl MTT solubilisation buffer (40% DMF, 16% SDS, 2% glacial acetic acid, pH

284 4.7) was added followed by agitation for 1 hour. MTT absorbance was read at 590 nm using
285 SpectraMax M5 plate reader.

286

287 **Statistical Tests:**

288 The significance of the improvement in dose-response of Cisplatin between Cas9-6Cys-Nb and
289 no RNP was calculated by two-way ANOVA grouped analysis with GraphPad Prism 6 and R.

290

291 **Figures:**

292 Figure 1. *Confirmation of successful Cas9-6Cys-Nb conjugation.* **A.** Graphic of the NHS/EDC
293 coupling chemistry **B.** Example of size exclusion chromatograms of Cas9-6Cys, Nb 7D12 and
294 Cas9-6Cys-Nb conjugation. **Inset.** Dot blot was performed to validate that the His-tagged Nb was
295 present in the untagged Cas9-Nb conjugated fractions. Purple color demonstrates the presence
296 of a His-tag and thus the 7D12 Nb. The Cas9-6Cys-Nb conjugated preps give a positive signal,
297 while the control Cas9-6Cys-RHOD (pink) and unlabelled Cas9-6Cys (white) are negative for the
298 His-tag.

299

300 Figure 2. *Transfection with 7D12-conjugated Cas9 increases specific cellular uptake of Cas9MAV*
301 *and Cas9-6Cys.* **A.** Dose-response assay of cell penetrating properties of unconjugated and Nb-
302 conjugated protein variants in 3t3 cells. Cells were washed with PBS to remove non-associated
303 labeled protein before cellular uptake was measured in the 96 well plate at 577nm in a
304 SpectraMax M5 plate reader. **B.** Dose response assay in A549 cells. **C.** Examples of transfected
305 cells visualized using fluorescent microscopy.

306

307 Figure 3. *Cas9-Nb conjugates targeted to BRCA2 increase synthetic dose lethality of Cisplatin in*
308 *A549 cells.* **A.** MTT assay was used to measure dose-response of Cisplatin over the course of 72
309 hours. Percentages cell viability were calculated relative to control Cas9-6Cys-Nb-only (no

310 sgRNA) and untransfected controls. All experiments were conducted in triplicates and significance
311 is denoted by a star, as calculated using GraphPad Prism 6 paired two-way ANOVA. **B.** Summary
312 of mean cell viability percentages at 24, 48 and 72 hours. Cisplatin dose is indicated by colors,
313 while squares denote Cisplatin only and circles denote co-administration with Cas9-6Cys-Nb. **C.**
314 IC50 values calculated on the basis of log(inhibitor) vs. response (three parameters) fitting in
315 GraphPad Prism 6 for 48 and 72 hours, respectively. **D.** MTT assay of Cas9MAV-NB at 48 hours.

316

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321

322 **References:**

- 323 1. Cox DB, Platt RJ, Zhang F. Therapeutic genome editing: prospects and challenges. *Nat*
324 *Med.* 2015;21(2):121-31.
- 325 2. Luo J. CRISPR/Cas9: From Genome Engineering to Cancer Drug Discovery. *Trends*
326 *Cancer.* 2016;2(6):313-24.
- 327 3. Mullard A. First in vivo gene-editing drugs enter the clinic. *Nat Rev Drug Discov.*
328 2017;17(1):7.
- 329 4. Lee B, Lee K, Panda S, Gonzales-Rojas R, Chong A, Bugay V, et al. Nanoparticle delivery
330 of CRISPR into the brain rescues a mouse model of fragile X syndrome from exaggerated
331 repetitive behaviours. *Nature Biomedical Engineering.* 2018;2(7):497-507.
- 332 5. Hindriksen S, Bramer AJ, Truong MA, Vromans MJM, Post JB, Verlaan-Klink I, et al.
333 Baculoviral delivery of CRISPR/Cas9 facilitates efficient genome editing in human cells. *PloS one.*
334 2017;12(6):e0179514.

- 335 6. Mout R, Ray M, Lee YW, Scaletti F, Rotello VM. In Vivo Delivery of CRISPR/Cas9 for
336 Therapeutic Gene Editing: Progress and Challenges. *Bioconjug Chem.* 2017;28(4):880-4.
- 337 7. Seki A, Rutz S. Optimized RNP transfection for highly efficient CRISPR/Cas9-mediated
338 gene knockout in primary T cells. *J Exp Med.* 2018;215(3):985-97.
- 339 8. Georgieva VJ, Hoekstra D, Zuhorn SI. Smuggling Drugs into the Brain: An Overview of
340 Ligands Targeting Transcytosis for Drug Delivery across the Blood–Brain Barrier. *Pharmaceutics.*
341 2014;6(4).
- 342 9. Prakash TP, Graham MJ, Yu J, Carty R, Low A, Chappell A, et al. Targeted delivery of
343 antisense oligonucleotides to hepatocytes using triantennary N-acetyl galactosamine improves
344 potency 10-fold in mice. *Nucleic Acids Research.* 2014;42(13):8796-807.
- 345 10. Molas M, Gomez-Valades AG, Vidal-Alabro A, Miguel-Turu M, Bermudez J, Bartrons R,
346 et al. Receptor-mediated gene transfer vectors: progress towards genetic pharmaceuticals. *Curr*
347 *Gene Ther.* 2003;3(5):468-85.
- 348 11. Tanowitz M, Hettrick L, Revenko A, Kinberger GA, Prakash TP, Seth PP.
349 Asialoglycoprotein receptor 1 mediates productive uptake of N-acetylgalactosamine-conjugated
350 and unconjugated phosphorothioate antisense oligonucleotides into liver hepatocytes. *Nucleic*
351 *Acids Res.* 2017;45(21):12388-400.
- 352 12. Rouet R, Thuma BA, Roy MD, Lintner NG, Rubitski DM, Finley JE, et al. Receptor-
353 Mediated Delivery of CRISPR-Cas9 Endonuclease for Cell-Type-Specific Gene Editing. *J Am*
354 *Chem Soc.* 2018;140(21):6596-603.
- 355 13. Liang C, Li F, Wang L, Zhang ZK, Wang C, He B, et al. Tumor cell-targeted delivery of
356 CRISPR/Cas9 by aptamer-functionalized lipopolymer for therapeutic genome editing of VEGFA
357 in osteosarcoma. *Biomaterials.* 2017;147:68-85.
- 358 14. Chen Y, Zhang Y. Application of the CRISPR/Cas9 System to Drug Resistance in Breast
359 Cancer. *Adv Sci (Weinh).* 2018;5(6):1700964.

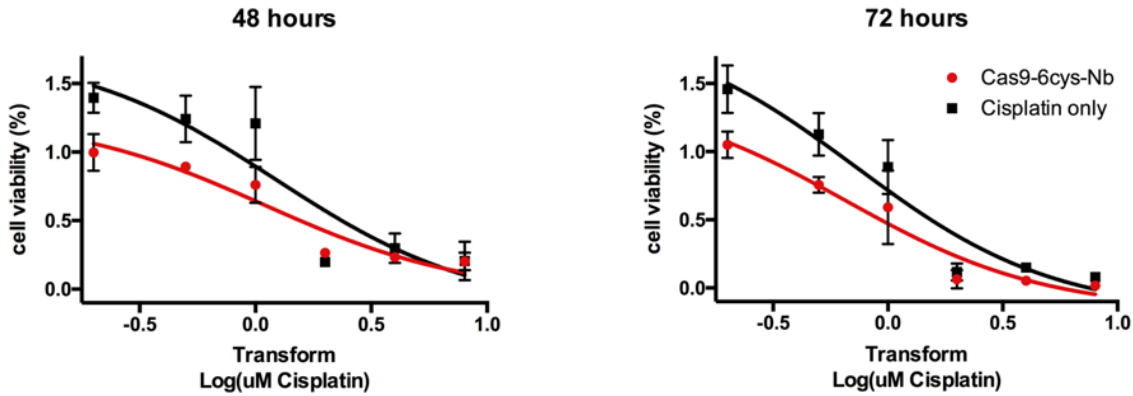
- 360 15. Alama A, Gangemi R, Ferrini S, Barisione G, Orengo AM, Truini M, et al. CD133-Positive
361 Cells from Non-Small Cell Lung Cancer Show Distinct Sensitivity to Cisplatin and Afatinib. Arch
362 Immunol Ther Exp (Warsz). 2015;63(3):207-14.
- 363 16. Yoshida K, Miki Y. Role of BRCA1 and BRCA2 as regulators of DNA repair, transcription,
364 and cell cycle in response to DNA damage. Cancer Sci. 2004;95(11):866-71.
- 365 17. Ganesan S. Tumor Suppressor Tolerance: Reversion Mutations in BRCA1 and BRCA2
366 and Resistance to PARP Inhibitors and Platinum. JCO Precision Oncology. 2018(2):1-4.
- 367 18. Cheng HH, Salipante SJ, Nelson PS, Montgomery B, Pritchard CC. Polyclonal BRCA2
368 Reversion Mutations Detected in Circulating Tumor DNA After Platinum Chemotherapy in a
369 Patient With Metastatic Prostate Cancer. JCO Precision Oncology. 2018(2):1-5.
- 370 19. Weigelt B, Comino-Mendez I, de Bruijn I, Tian L, Meisel JL, Garcia-Murillas I, et al. Diverse
371 BRCA1 and BRCA2 Reversion Mutations in Circulating Cell-Free DNA of Therapy-Resistant
372 Breast or Ovarian Cancer. Clin Cancer Res. 2017;23(21):6708-20.
- 373 20. Rytelwski M, Tong JG, Buensuceso A, Leong HS, Maleki Vareki S, Figueredo R, et al.
374 BRCA2 inhibition enhances cisplatin-mediated alterations in tumor cell proliferation, metabolism,
375 and metastasis. Mol Oncol. 2014;8(8):1429-40.
- 376 21. Mucchekehu R, Liu D, Horn M, Campbell L, Del Rosario J, Bacica M, et al. The Effect of
377 Molecular Weight, PK, and Valency on Tumor Biodistribution and Efficacy of Antibody-Based
378 Drugs. Transl Oncol. 2013;6(5):562-72.
- 379 22. Wilhelm S, Tavares AJ, Dai Q, Ohta S, Audet J, Dvorak HF, et al. Analysis of nanoparticle
380 delivery to tumours. Nature Reviews Materials. 2016;1:16014.
- 381 23. Zhang F, Wang S, Yin L, Yang Y, Guan Y, Wang W, et al. Quantification of epidermal
382 growth factor receptor expression level and binding kinetics on cell surfaces by surface plasmon
383 resonance imaging. Anal Chem. 2015;87(19):9960-5.
- 384 24. Heukers R, van Bergen en Henegouwen PM, Oliveira S. Nanobody-photosensitizer
385 conjugates for targeted photodynamic therapy. Nanomedicine. 2014;10(7):1441-51.

- 386 25. Schmitz KR, Bagchi A, Roovers RC, van Bergen en Henegouwen PM, Ferguson KM.
387 Structural evaluation of EGFR inhibition mechanisms for nanobodies/VHH domains. *Structure*.
388 2013;21(7):1214-24.
- 389 26. Alborzinia H, Can S, Holenya P, Scholl C, Lederer E, Kitanovic I, et al. Real-time
390 monitoring of cisplatin-induced cell death. *PloS one*. 2011;6(5):e19714.
- 391 27. Astolfi L, Ghiselli S, Guaran V, Chicca M, Simoni E, Olivetto E, et al. Correlation of adverse
392 effects of cisplatin administration in patients affected by solid tumours: a retrospective evaluation.
393 *Oncol Rep*. 2013;29(4):1285-92.
- 394 28. Hu Y, Liu C, Muyldermans S. Nanobody-Based Delivery Systems for Diagnosis and
395 Targeted Tumor Therapy. *Front Immunol*. 2017;8:1442.
- 396 29. Anderson KR, Haeussler M, Watanabe C, Janakiraman V, Lund J, Modrusan Z, et al.
397 CRISPR off-target analysis in genetically engineered rats and mice. *Nature methods*.
398 2018;15(7):512-4.
- 399 30. Kosicki M, Tomberg K, Bradley A. Repair of double-strand breaks induced by CRISPR-
400 Cas9 leads to large deletions and complex rearrangements. *Nature biotechnology*. 2018.
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Supplemental information

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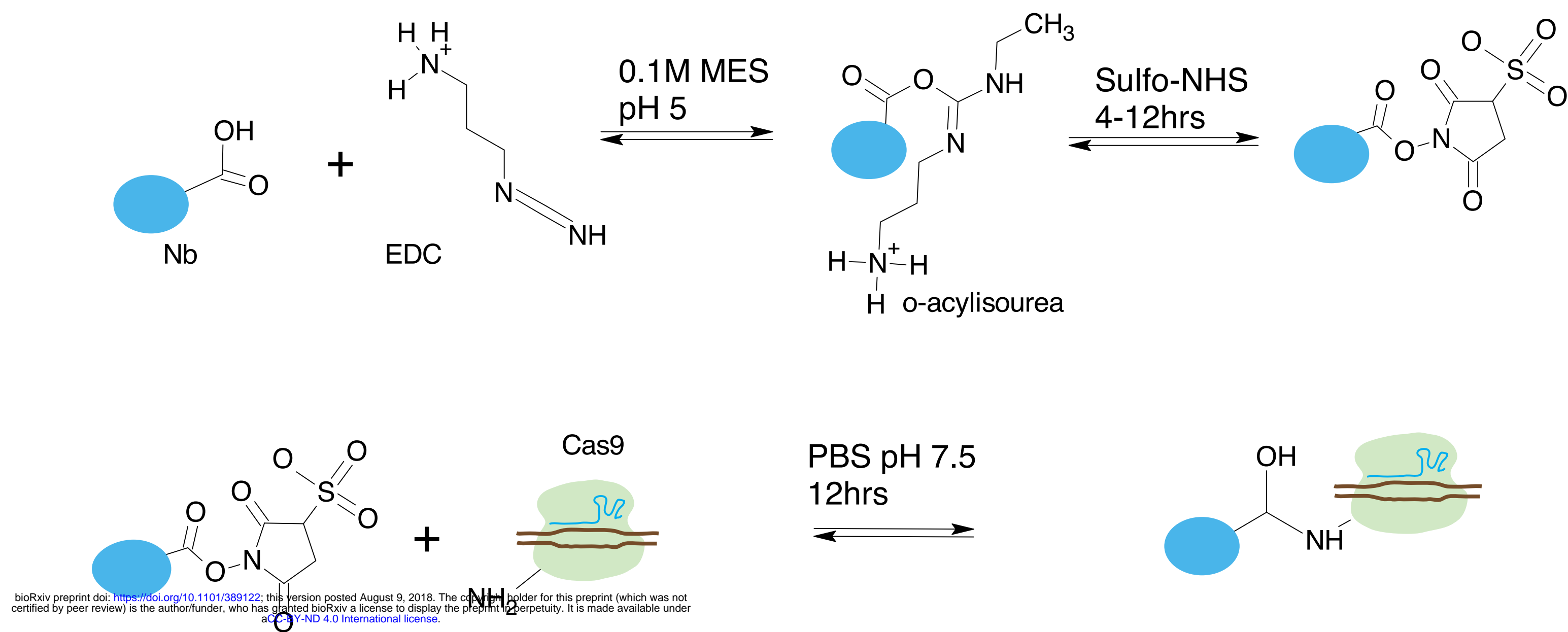
412

413 **S1. Calculation of approximate IC₅₀ values of Cisplatin.** Nonlinear fit of transformed Cisplatin

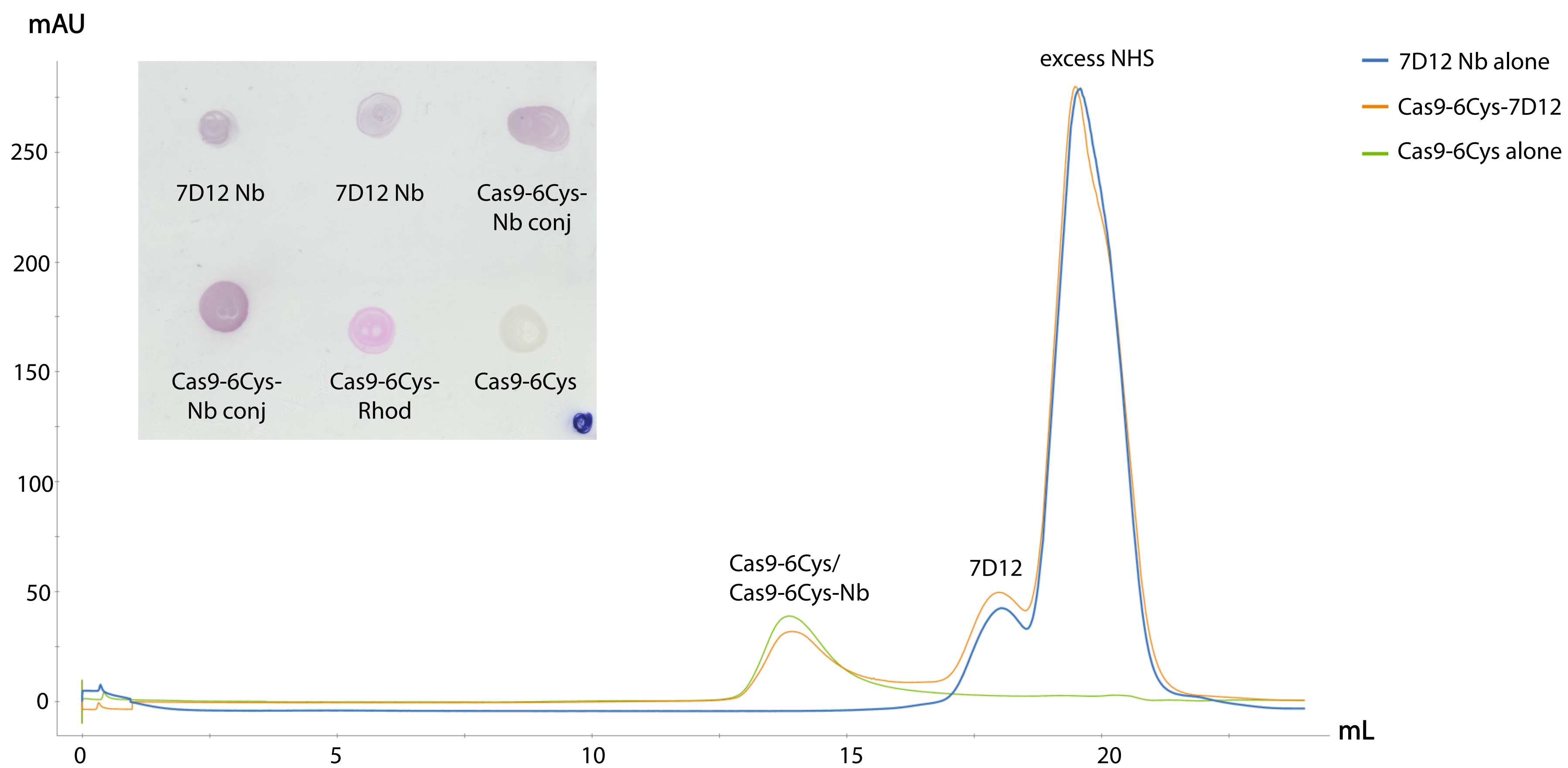
414 concentrations for determination of IC₅₀ at 48 and 74 hrs, respectively. Analysis was performed

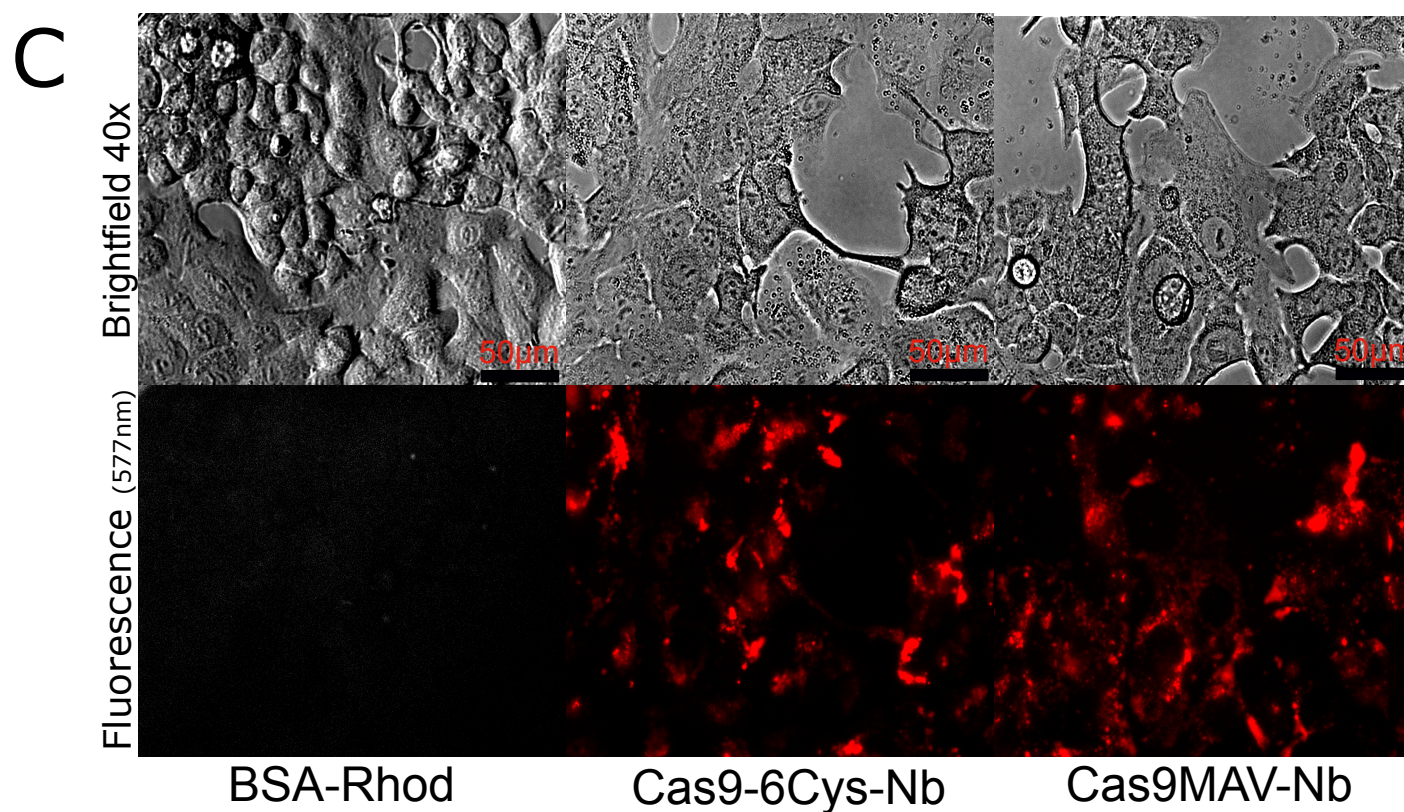
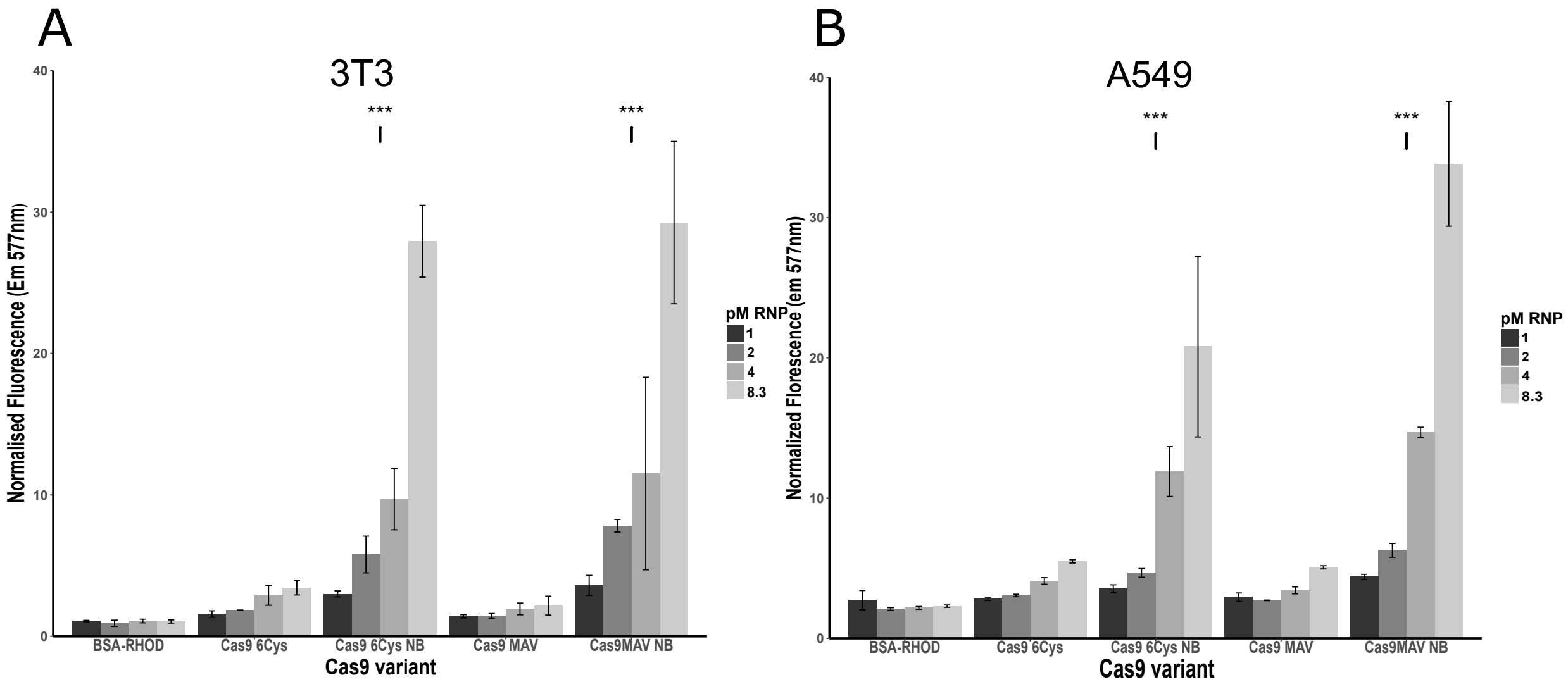
415 with GraphPad Prism 6.

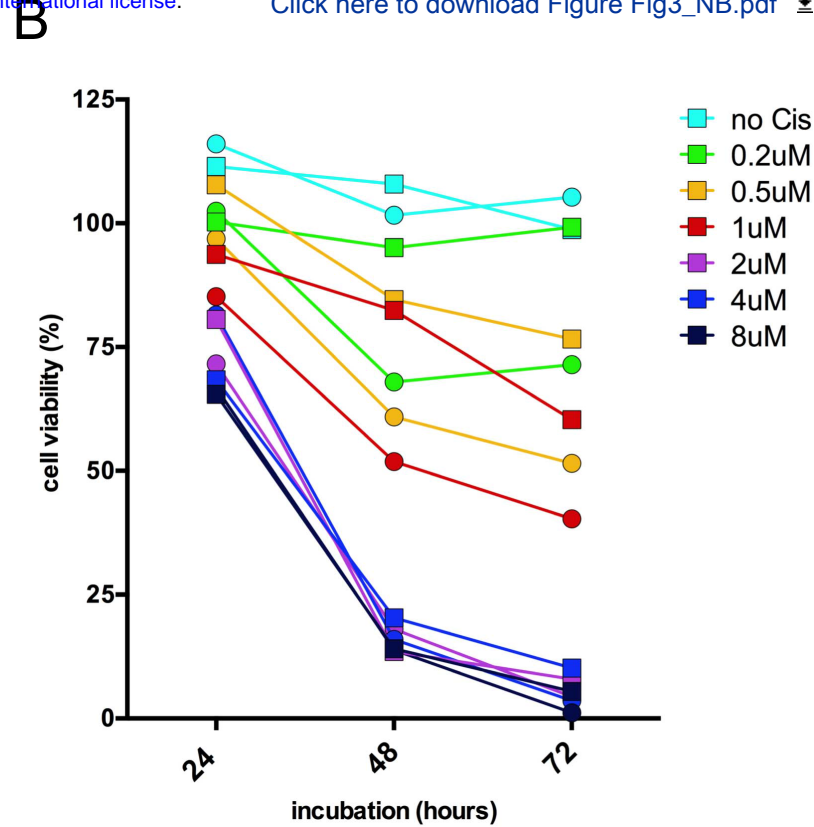
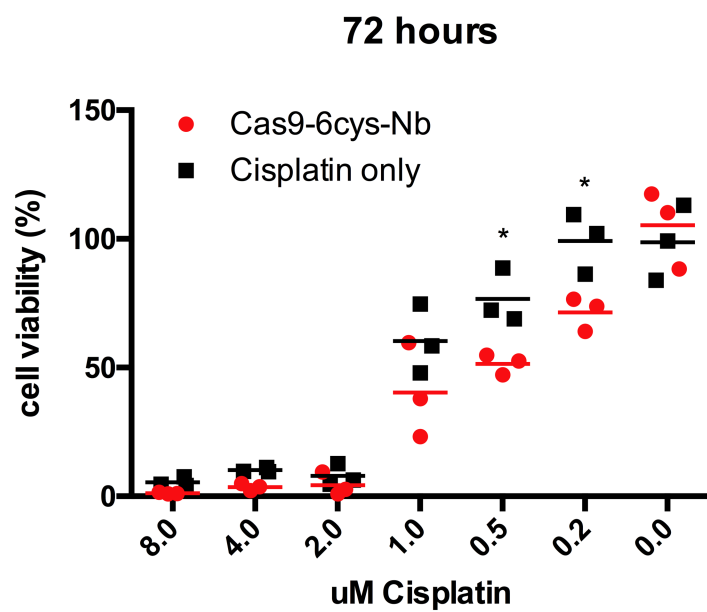
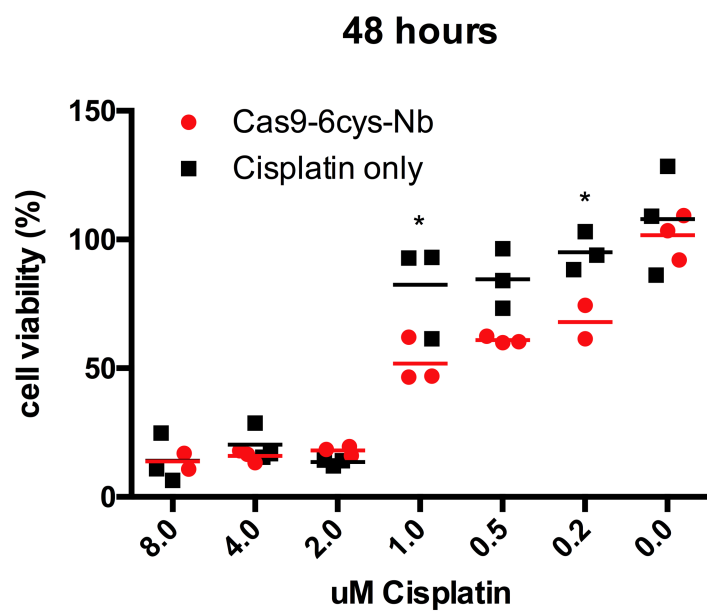
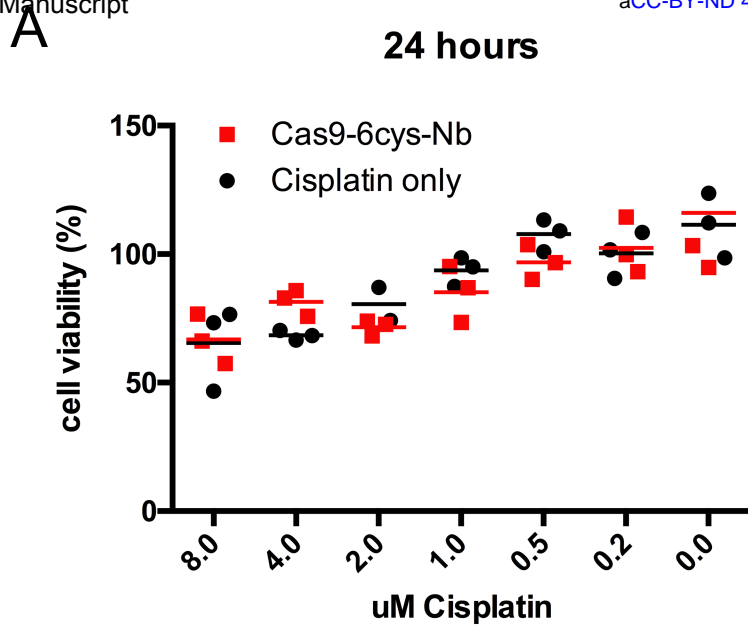
A



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C

Time point (hours)	IC50 (μM)	
	Cisplatin	Cisplatin/Cas9-Nb
48	1.224	1.096
72	0.7222	0.6581

