1 **Dianthin-30 or gelonin versus monomethyl auristatin E, each configured with an**
2 **anti-calcitonin receptor antibody, are differentially potent *in vitro* in high grade**
3 **glioma cell lines derived from glioblastoma**

4

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35 **Abstract**

36 We have reported that calcitonin receptor (CTR) is widely expressed in biopsies from the
37 lethal brain tumour *glioblastoma* by malignant glioma and brain tumour initiating cells
38 (glioma stem cells) using anti-human CTR antibodies. A monoclonal antibody against an
39 epitope within the extracellular domain of CTR was raised (mAb2C4), and chemically
40 conjugated to either plant ribosome-inactivating proteins (RIPs) dianthin-30 or gelonin, or
41 the drug monomethyl auristatin E (MMAE), and purified. In the high grade glioma cell line
42 (HGG, representing glioma stem cells) SB2b, in the presence of the triterpene glycoside
43 SO1861, the EC₅₀ for mAb2C4:dianthin was 10.0 pM and for mAb2C4:MMAE (antibody
44 drug conjugate [ADC]) 2.5 nM, 250-fold less potent. With the cell line U87MG, in the
45 presence of SO1861, the EC₅₀ for mAb2C4:dianthin was 20 pM, mAb2C4:gelonin, 20 pM,
46 compared to the ADC (6.3 nM), which is >300 less potent. Several other HGG cell lines
47 that express CTR were tested and the efficacies of mAb2C4:RIP (dianthin or gelonin) were
48 similar. Co-administration of the enhancer SO1861 purified from plants enhances
49 lysosomal escape. Enhancement with SO1861 increased potency of the immunotoxin (>3
50 log values) compared to the ADC (1 log). The uptake of antibody was demonstrated with
51 the fluorescent conjugate mAb2C4:AlexaFluor 568 and the release of dianthin-
52 30:AlexaFluor488 into the cytosol following addition of SO1861 supports our model. These
53 data demonstrate that the immunotoxins are highly potent and that CTR is an effective
54 target expressed by a large proportion of HGG cell lines representative of glioma stem
55 cells and isolated from individual patients.

56

57 **Key Words:** Calcitonin receptor; immunotoxins; targeting; high grade glioma cell lines;
58 glioblastoma.

59

60 **Précis**

61 High grade glioma cell lines were exposed to immunotoxin (dianthin, gelonin conjugated to
62 an anti-calcitonin receptor antibody) and were more potent (EC_{50} 10 pM) than the
63 antibody:drug conjugate (MMAE) by 250-fold in the presence of enhancer SO1861.

64

65 **Abbreviations**

66	ADC	antibody-drug conjugate
67	BTIC	brain tumour initiating cell
68	CTR	calcitonin receptor
69	DTT	dithiothreitol
70	EDTA	ethylene diamine tetra acetate
71	EGF	epidermal growth factor
72	EGFR	epidermal growth factor receptor
73	FGF	fibroblast growth factor
74	GBM	glioblastoma multiforme
75	hCTR	human calcitonin receptor
76	HGG	high grade glioma
77	LDH	lactate dehydrogenase
78	MMAE	monomethyl auristatin E
79	NTA	nitritoltriacetate
80	RIP	ribosome-inactivating protein
81	VEGF	vascular endothelial growth factor.

82

83 **Introduction**

84 Successful treatment of recalcitrant tumours remains one of the great challenges of
85 cancer research. One such recalcitrant tumour, *glioblastoma* (GBM), is a deadly brain
86 tumour (grade 4 astrocytoma [1]). One of the underlying features of GBM is the invasive
87 potential of brain tumour initiating cells (BTIC) [2] which are regarded as clones of cells
88 with stem-like properties (glioma stem cells) [3, 4]. These cells are considered to have the
89 capacity to differentiate into a variety of cell types [5] including endothelial cells and
90 pericytes, which are essential for the maintenance and expansion of the solid tumour.
91 Thus it is considered that these stem-like cells provide the malignant basis of glioblastoma
92 and hence represent potentially important targets for treatment.

93 Conventional chemo- and radio-therapies, cyto-reductive surgery and trialled
94 treatment modalities including Avastin (anti-VEGF antibody) therapy, have had minimal
95 impact on the mean survival after diagnosis, which remains low at just 14-17 months [6].

96 The deployment of antibody drug conjugates (ADC) to treat recalcitrant tumours is a
97 relatively recent research strategy in which the number of lead ADCs entering clinical trials
98 has increased rapidly [7-9]. Successful treatment is partly dependent on the properties of
99 the antibody and its target, for efficient delivery of the drug into the tumour cell, as well as
100 the efficacy of the drug itself. Recently, an ADC based on an anti-EGFRvIII antibody (ABT-
101 806) conjugated to monomethyl auristatin F (MMAF) resulting in an ADC (ABT-414) has
102 been demonstrated to be effective in 22% of GBM patients in a phase I study [10].

103 Immunotoxins made from antibodies conjugated with either bacterial or plant toxins
104 have been investigated for many years and there are examples of the former that have
105 reached clinical trials. However, studies involving plant-based immunotoxins have been
106 less frequent and the development of ADCs for clinical trials is more advanced. However,
107 recent advances in immunotoxin research are beginning to change this preference. Firstly,
108 plant toxins such as the ribosome-inactivating proteins (RIP) dianthin-30 [11] and gelonin

109 [12] (originally extracted from seeds) are now synthesised as functional recombinant
110 proteins. Secondly, when administered in combination with enhancers of lysosomal
111 escape (non-toxic co-factors) such as the triterpene glycoside SO1861 [13] there is a
112 decrease by several orders of magnitude in the effective concentration (EC_{50}) for
113 cytotoxicity. This enhancer is a plant triterpene glycoside, purified from the plant *Saponaria*
114 *officinalis* L. [13], with specific structural features that enhance the cytotoxicity of
115 immunotoxins conjugated with RIPs. SO1861 has a triterpenoidal skeleton of oleanane
116 type and two sugar side chains (bisdesmosidic) attached to it at positions C-3 and C-28
117 [14]. Such enhancers interact at non-toxic concentrations with the immunotoxin within the
118 endosomal or lysosomal compartment of the target cells [14] and promote release into the
119 cytosol [15] where the toxin targets ribosomes.

120 Our understanding of the role of calcitonin receptor (CTR) in cell physiology has
121 been evolving over the last 20 years with new knowledge of the mechanisms of agonist
122 bias [16] and an increasing appreciation of the broad range of tissues in specific
123 physiological states, in which CTR is expressed. For instance, CTR expression may be
124 transitory in wound healing [17] and in neurons of the gut around birth [18]. It is expressed
125 by activated lymphocytes [19, 20] and during foetal development [21-23]. Recently, we
126 have proposed that CTR is externalised during the pre-apoptotic cell stress response [24].

127 CTR is a target of interest because it is expressed by malignant tumor cells of the
128 brain tumor *glioblastoma* [25]. Furthermore, CTR is also expressed in other malignancies
129 including multiple myeloma [26], leukemia [17], lymphoma [27], bone tumors [28, 29],
130 breast [30] and prostate cancers [31, 32], and medullary thyroid cancers [33]. The
131 function(s) of CTR in the contexts of these different cancers is largely unknown, however
132 an anti-apoptotic or survival activity has been proposed together with metabolic
133 reprogramming [27].

134 Important considerations in the design of immunotoxins for the treatment of cancers
135 includes the sufficient expression of the target receptor on the surface of BTICs compared
136 to other exposed tissues [34] and internalisation of receptors with the immunotoxin
137 attached. High grade glioma (HGG) cell lines [35] derived from GBM, represent BTICs and
138 include the subtypes, pro-neural, neural, classical and mesenchymal, classified according
139 to gene profiling. Five HGG cell lines (BAH1, JK2, PB1, SB2b & WK1 [36]) out of the
140 twelve originally tested (~40%) were shown to express human CTR (hCTR) on
141 immunoblots and four of these plus two others from a separate source, were included in
142 this study. CTR-positive HGG cell lines represent each of the subtypes listed above.
143 These HGG cell lines, when injected intracranially into immuno-deficient mice, form GBM-
144 like tumours [37].

145 In this study CTR is evaluated as a potential target expressed by HGG cell lines
146 and as an uptake mechanism for internalisation of immunotoxins. For comparison, the
147 efficacies of two anti-CTR immunotoxins based on an anti-CTR antibody conjugated to the
148 plant toxins dianthin-30 and gelonin, and an ADC with monomethyl auristatin E [38], are
149 reported here with and without SO1861. Monomethyl auristatin is included in this study as
150 it is frequently incorporated into ADCs tested in clinical trials and reported in the literature
151 [7-9] due to its toxicity as an anti-microtubule agent.

152 **Materials and Methods**

153 **High Grade Glioma cell lines**

154 In total seven HGG cell lines out of 14 screened expressed detectable levels of
155 calcitonin receptor as determined by immunoblot. These include four (JK2 [39], PB1 [40],
156 SB2b [41] & WK1 [36, 41]) from the QIMR-Berghofer Medical Research Institute
157 (Brisbane, Australia). Two further cell lines GBM-4 and GBM-L2 [42] were obtained from
158 the Monash Institute of Medical Research (Professor TG Johns). These lines were all
159 grown on Matrigel (Corning, US)-coated plastic surfaces under serum-free conditions in
160 StemPro® NSC SFM - Serum-Free Human Neural Stem Cell Culture Medium (Thermo
161 Fisher Scientific, US) with EGF, FGF2, glutaMAX plus penicillin/streptomycin (Thermo
162 Fisher Scientific) and cultured at 37°C in a 95% humidified air, 5% CO₂ atmosphere [37].

163 The cell line U87MG, described extensively in the literature and derived from
164 glioblastoma, was also included to provide a reference point for other published studies.
165 U87MG was maintained in DMEM-F12/10% FBS plus penicillin/streptomycin (Thermo
166 Fisher Scientific) grown at 37°C in an atmosphere of 5% CO₂. For efficacy studies in the
167 96-well format and growth in chamber slides for confocal analysis, U87MG cells were
168 cultured on Matrigel under identical serum-free conditions used for HGG cell lines, in
169 StemPro® NSC SFM.

170

171 **Ribosome-inactivating proteins (RIP) and the drug monomethyl auristatin E (MMAE)**

172 The RIP dianthin-30 [His⁶] was synthesised as a recombinant protein in *E. coli* and
173 purified by affinity (nickel-nitrilotriacetic acid [NTA] agarose) chromatography [43]. rGelonin
174 was a gift from Professor Michael G Rosenblum (MD Anderson Cancer Center, Texas)
175 which was synthesised and purified from *E. coli* [44]. MMAE (OSu-Glu-VC-PAB-MMAE)
176 was purchased from Concortis Biosystems Corp., (US).

177

178 **The enhancer triterpene glycoside SO1861**

179 The extraction and isolation of the enhancer SO1861 from plant roots of *Saponaria*
180 *officinalis* L. Caryophyllacea has been described [13]. The chemical structure of SO1861
181 has been solved and will be published in detail elsewhere (Dr Alexander Weng, personal
182 communication). At 3 µg/mL used in the growth assays, SO1861 is slightly toxic resulting
183 in 10% retardation of cell proliferation. For U87MG cells the concentration used was 1
184 µg/mL.

185

186 **Antibodies**

187 These studies involved the use of a mouse monoclonal anti-human CTR antibody
188 (mAb 46/08-2C4-2-2-4 [IgG1]) directed against an extracellular epitope (Welcome
189 Receptor Antibodies [WRA, Melbourne) [24]. MAb1H10 (IgG_{2A}) was raised against a
190 cytoplasmic epitope (mAb 31/01-1H10, WRA, Melbourne; also distributed as MCA 2191 by
191 BioRad, UK). These antibodies have been previously validated (in the supplementary
192 materials linked to [24]) and further data published elsewhere [25].

193

194 **Conjugation of toxins to mAb2C4**

195 Dianthin-30[His⁶] or gelonin was conjugated to mAb2C4 using succinimidyl 3-(2-
196 pyridyldithio) propionate (SPDP, Thermo Fisher Scientific). This cross-linking procedure
197 introduced a covalent disulphide bridge between the toxins and the monoclonal antibody.
198 In the case of dianthin-30[His⁶] and mAb2C4, both proteins (molar ratio 1:1) were modified
199 by SPDP and the molar ratios that were considered for their chemical reaction were
200 protein:cross-linker of 1:3, 1:6 and 1:12. In the case of gelonin and mAb2C4, in a first
201 attempt both proteins (molar ratio 1:1) were modified by SPDP at a molar ratio
202 protein:cross-linker of 1:6. In a second and third attempt, only mAb2C4 (molar ratio

203 gelonin:mAb2C4 of 3:1) was modified by SPDP at a molar ratio of protein:cross-linker of
204 1:3 and 1:6.

205 In brief, proteins (dianthin-30[His⁶], gelonin and mAb2C4) were equilibrated in PBS-
206 EDTA buffer (20 mM sodium phosphate, 150 mM sodium chloride, 1 mM EDTA, 0.02%
207 sodium azide, pH 7.5) using a 5 mL Zeba polyacrylamide de-salting columns (Thermo
208 Fisher Scientific). Proteins were modified by addition of SPDP (20 mM in DMSO) for 60
209 min at room temperature. Proteins were desalted again using 5 mL Zeba de-salting
210 columns and then SPDP-modified toxin was reduced with the addition of dithiothreitol
211 (DTT, final concentration of 50 mM) for 30 min at room temperature. DTT was removed
212 using a Zeba de-salting column. Monoclonal antibody and reduced toxins (or non-modified
213 gelonin containing one available cysteine) were allowed to react for 18 h at room
214 temperature. The conjugates were stored at 4°C pending further purification.

215 Monomethyl auristatin E (MMAE) was conjugated to mAb2C4 via the cross-linker
216 OSu-Glu-VC-PAB as indicated by the manufacturer (Concortis Biosystems Corp, US). The
217 chemical reaction was performed with a molar ratio mAb2C4:OSu-Glu-VC-PAB-MMAE of
218 1:7. The antibody was equilibrated in the conjugation buffer 1 (CB1, 50 mM potassium
219 phosphate, 50 mM sodium chloride, 2 mM EDTA, pH 6.5) using a 5 mL Zeba de-salting
220 column. Antibody was modified by addition of OSu-Glu-VC-PAB-MMAE (10mM in DMA).
221 Further organic solvent was added up to 10% (v/v) DMA and cross-linking reaction run for
222 18 h at room temperature. The reaction was stopped by exchanging the CB1 with the
223 conjugation buffer 2 (CB2, 50 mM sodium succinate, pH 5.0) using a 5 mL Zeba de-salting
224 column. Antibody-drug conjugate was stored at 4°C pending further purification steps.

225

226 **Purification of immunotoxins and the ADC**

227 *MAb2C4:dianthin-30[His⁶]*. The first step in purification involved chromatography with a
228 nickel-NTA agarose matrix (Life Technologies).

229 In brief, 20 mM imidazole in buffer (50 mM sodium phosphate, 300 mM sodium
230 chloride [pH 8.0]) was added to the crude cross-linked reaction mix, mixed with nickel-NTA
231 agarose (3 mL bed volume) and equilibrated with inversion at 25°C for 60 min. The bound
232 matrix was poured into a 10 mL column, with gravity feed and washed with 3 column
233 volumes of binding buffer (as above). The bound products were eluted with 250 mM
234 imidazole in buffer. The eluted proteins (mAb2C4:dianthin-30[His⁶] and dianthin-30[His⁶])
235 were dialysed against PBS overnight at 6°C.

236 The second step of purification was achieved with chromatography on a peptide
237 affinity column (thiopropyl sepharose 6B, prepared by Mimotopes, Clayton, Australia)
238 using the peptide sequence (CSGS-PSEKVTKYCDEKGVWFK, synthesised by
239 Mimotopes) equivalent to the extracellular epitope of human CTR that binds mAb2C4. This
240 step excluded antibody products in which the binding determinants for the peptide
241 sequence had been compromised during the conjugation step. The dialysed material was
242 passed down the affinity column, washed in 3 volumes of 20mM sodium phosphate (pH
243 7.0), eluted with 100 mM glycine (pH 2.2) and the fractions neutralised with 10% of the
244 collected volume with 1 M TRIS (pH 9.0). These fractions were dialysed overnight in PBS
245 and concentrated using Amicon Ultra-15 centrifugal filters (10 kD cut off). The yield of
246 conjugated material after purification was approximately 8%, compared to the initial
247 amounts of dianthin-30[His⁶] and mAb2C4 used in the conjugation.

248 *MAb2C4:gelonin* The conjugated crude product mAb2C4:gelonin was purified in a two-
249 step process, firstly with the affinity column described above. The second chromatographic
250 step was dependent on the binding of the product to HiTrap Blue HP matrix (GE
251 Healthcare) [44, 45] and elution with sodium chloride. The yield of the final product
252 (mAb2C4:gelonin) was similar to that of mAb2C4:dianthin-30[His⁶].

253 *MAb2C4:MMAE* The conjugated crude solution of mAb2C4:MMAE was also purified by
254 two chromatographic steps, firstly with the peptide affinity column described above. This

255 purification step eliminates inactive binding products of mAb2C4:MMAE that might have
256 been generated during the conjugation as they pass through within the void volume. The
257 second step in purification was chromatography on a hydrophobic interactive matrix
258 (HiTrap Phenyl HP, GE Healthcare) and the active fraction was eluted with a reverse
259 ammonium chloride gradient.

260

261 **Growth/cytotoxicity assays in the 96-well format**

262 For efficacy studies in the 96-well format all cell lines were grown on Matrigel
263 (Corning, US) under serum-free conditions in StemPro® NSC SFM (Life
264 Technologies/Thermo Fisher Scientific) with growth factors and seeded at 2,500 cells per
265 well. Two to three hours after plating the cells, additions such as SO1861 and
266 immunotoxins (mAb 2C4:dianthin-30[His⁶], mAb2C4:gelonin) or ADC (mAb2C4:MMAE)
267 were made. Control plates included mAb2C4 with SO1861 or immunotoxin without
268 SO1861. Cells were incubated at 37°C for 4 days (U87MG) or 6 days (HGG cell lines).
269 Total lactate dehydrogenase (LDH, lysed cells) and residual LDH (non-lysed samples)
270 were performed in triplicate. LDH measured using an LDH cytotoxicity detection kit
271 (Roche) and read on a FLUOstar OPTIMA (BMG Labtech).

272

273 **Confocal microscopy of HGG cell lines and immuno-fluorescence with multi-** 274 **channel detection**

275 The confocal analysis of the uptake of mAb2C4 into HGG cell lines was
276 accomplished with mAb2C4 labelled with AlexaFluor 568 (AF568, Thermo Fisher
277 Scientific) using conjugation of lysines with NHS succinimidyl esters of AF568 [24].

278 HGG cell lines were cultured (described above) to 50-80% confluence on 4-well
279 glass slides (Lab Tek II chamber slides, NUNC) coated with Matrigel (Corning, US) in a
280 humidified incubator (5% CO₂) at 37°C then washed with media without growth factors.

281 MAb2C4:AF568 (1 µg/mL) was added to 1 mL of fresh growth medium and the chamber
282 slides incubated for 24 h in the incubator.

283 Cells were washed with PBS, fixed with 4% paraformaldehyde/PBS for 30 min and
284 then washed twice with PBS. Cells were blocked with 5% normal goat serum/1% bovine
285 serum albumin for 1 h.

286 Slides of fixed cells were incubated overnight at 6°C with rabbit anti-LAMP1
287 antibody (Cell Signalling) that detects lysosomes. The secondary antibody used was goat
288 anti-rabbit AlexaFluor 488 (4µg/mL, Thermo Fisher Scientific). The samples were then
289 mounted using DAPI aqueous mount (Prolong Gold, Thermo Fisher Scientific) to visualise
290 the nuclei and dried at RT for several days in the dark. The samples were imaged by
291 confocal microscopy (Objectives x20 and x63) on a Zeiss Imager Z1/LSM 510 Meta
292 confocal laser scanning system using Zen software. Images (LSM format) were captured
293 in a single focal plane (optical sections of 0.7 µm nominal thickness) or using the Z-series
294 feature where ~ 15 optical sections were compressed (devolution using LSM Image
295 Browser, Zeiss) to create a single plane image (TIFF format) equivalent to approximately
296 10 µm tissue thickness.

297

298 **Live cell imaging**

299 U-87 MG (ATCC® HTB-14™) cells were seeded (2000/dish) in Ibidi µ-Dishes (35
300 mm, low, Martinsried, Germany) and cultured in 2 mL Dulbecco's MEM supplemented with
301 10% fetal bovine serum and 1% penicillin/streptomycin. After 92 h, the medium was
302 removed and 1 mL fresh culture medium was added. Dianthin:AF488 (100nM) was added
303 and cells were incubated over night for an incubation period of 18 h. Dianthin was labelled
304 beforehand with Alexa-Fluor 488 5-TFP ester (Life Technologies) as described previously
305 [46].

306 The live cell imaging marker pHrodo™ Red Dextran (final concentration 40 µg/ mL,
307 Life Technologies) was added 6 h before the end of the incubation period. pHrodo™ Red
308 Dextran is a live cell imaging marker for acidic intracellular compartments such as
309 lysosomes. Hoechst 33342 (Life Technologies) was added 30 min before the end of the
310 incubation period. Cells were washed with PBS (3×), covered with 1 mL live cell imaging
311 solution (Life Technologies), supplemented with 1% FBS and 20 mM D-glucose and
312 analyzed using a LSM780 laser scanning microscope, Axio Observer Z1 with a Plan-
313 Apochromat 63×/1.4 Oil Objective (Carl Zeiss, Jena, Germany). Cells were maintained at
314 37°C throughout the experiment. Cells were continuously monitored (supplementary video)
315 and 36 s after the start of the analyses SO1861 was added at a final concentration of 5
316 µg/mL. To determine the SO1861 induced endosomal escape of dianthin:AF488 into the
317 cytosol, regions of interest were assigned for individual cells and the increase of intensity
318 was determined offline using ZEN 2.3 lite software (Carl Zeiss).
319

320 **Results**

321 The principal aim of this study was to synthesize conjugates of mAb2C4 antibody
322 including the immunotoxins mAb2C4:dianthin-30, mAb2C4:gelonin and the antibody:drug
323 conjugate (ADC) mAb2C4:MMAE, for comparison of their efficacies to promote cell death
324 in high grade glioma cell lines and U87MG. The validation of mAb2C4, which binds to an
325 extracellular linear epitope (epitope 4) of human CTR, has been presented elsewhere [24].

326

327 **Expression of calcitonin receptor protein was determined by immunoblot**

328 In Fig. 1a is shown expression of CTR protein by HGG cell lines and the cell line
329 U87MG, probed with the antibody mAb1H10 which recognises an intracellular epitope
330 (band 'a'). In U87MG cells probed with mAb2C4 (band 'b') is shown the target of MW~55
331 kD. Band 'c' is a product of degradation of CTR.

332

333 **Syntheses and purification steps for immunotoxins and ADC**

334 Chromatography with the peptide affinity column constructed with the peptide
335 sequence used for immunization and the cloning/expression of mAb2C4, was used for
336 purification of each conjugate (immunotoxin or ADC), and mAb2C4:AF568 (see Fig. 3) as
337 the bound material retains the capacity to bind to epitope 4 of CTR. Any antibody with
338 compromised binding capacity passes through in the flowthrough during chromatography.

339 In Fig. 1 b-e are images of SDS-PAGE gels which represent the stages of synthesis
340 (Fig. 1b, crude product, lane 1 and lane 2 [reduced] compared to mAb2C4 alone) and the
341 purified mAb2C4:dianthin-30[His⁶] (Fig. 1c), following chromatography on nickel-NTA
342 column and the peptide affinity column. In Fig. 1d is shown the profile of purified
343 mAb2C4:gelonin following chromatography on the peptide affinity and HiTrap Blue HP
344 columns, and in Fig. 1e, purified mAb2C4:MMAE following chromatography on a peptide
345 affinity column and HiTrap Phenyl HP. Further details of the purifications of

346 mAb2C4:dianthin, mAb2C4:gelonin and mAb2C4:MMAE can be found supplementary
347 figures 1-3.

348

349 **The comparative potency of immunotoxin versus ADC in HGG cell line SB2b and** 350 **cell line U87MG**

351 In Fig. 2a, using the HGG cell line SB2b, are the graphical displays from the results
352 of multiple experiments with mAb2C4:dianthin (n=4) with and without SO1861, compared
353 to mAb2C4:MMAE (n=4) [Fig. 2b with and without SO1861]. With SO1861 the negative
354 logarithm of the half maximal effective concentration (pEC_{50}) for mAb2C4:dianthin was
355 calculated, using a 3 parameter logistic equation, as -11.0 ± 0.14 (EC_{50} , 10.0 pM) and for
356 mAb2C4:MMAE as -8.6 ± 0.20 (EC_{50} , 2.5 nM) which is approximately 250-fold less potent
357 (Table 1).

358 In the HGG cell line SB2b, SO1861 increased the potency of mAb2C4:dianthin-30
359 by 3 log values (from supplementary figure 7) and mAb2C4:MMAE by 1 log value (Fig.
360 2b). With the HGG cell line JK2 treated with mAb2C4:dianthin and SO1861
361 (supplementary figure 5) the EC_{50} was 10.0 pM (Table 1), similar to the value calculated
362 for SB2b.

363 We tested the biological activity of mAb2C4:dianthin-30 and mAb2C4:MMAE in the
364 cell line U87MG as a reference cell line for comparison with other studies reported in the
365 literature. In the presence of SO1861 (Fig. 2c) the EC_{50} was 20.0 pM and 6.3 nM (Table 1)
366 respectively, which is 300-fold less potent. The EC_{50} for mAb2C4:gelonin was similar to
367 that for mAb2C4:dianthin-30, namely 20 pM (Table 1).

368

369 **The potency of each immunotoxin vs ADC in a range of GBM cell lines**

370 The EC_{50} values in several high grade glioma cell lines are listed in supplementary
371 table 1 after one experimental determination. The values for mAb2C4:dianthin-30 in the

372 presence of SO1861 are close to 10 pM for SB2b, JK2, GBM-4 and GBM-L2,
373 approximately 50 pM for WK1, but PB1 is quite resistant to both mAb2C4:dianthin-30 and
374 mAb2C4:gelonin (supplementary figures 6, 7 and supplementary table 1).

375 376 **The uptake of mAb2C4:AF568 into HGG cell lines and U87MG**

377 In Fig. 3 is shown images of the uptake of mAb2C4:AF568 by cell lines JK2 (low
378 [x20] magnification), and at higher [x63] magnification, SB2b, PB1 and U87MG.
379 Fluorescence associated with mAb2C4:AF568 is present on the cell membranes as well
380 as concentrated in the perinuclear region. Some of the latter fluorescence coincides with
381 LAMP1 staining (green in Fig. 3 d and f) as shown in yellow [overlap], although the extent
382 of LAMP1-positivity varies considerably between the cell lines. LAMP1 is commonly used
383 to identify the late endosomal/lysosomal compartments.

384 Of note PB1, which is relatively resistant to mAb2C4:dianthin-30 showed uptake of
385 mAb2C4:AF568 following live staining for one hour.

386 387 **Triterpene glycoside SO1861 promotes the release of dianthin into the cytosol**

388 The release of toxin with enhancer SO1861 from intracellular compartments was
389 tested by pre-loading the U87MG cells with Alexa Fluor 488-labelled dianthin
390 (dianthin:AF488) prior to real time imaging. In Fig. 4 a-d, U87MG cells were incubated with
391 the nuclear stain Hoechst 33342 (Fig. 4a), pHrodo™ Red Dextran (Fig. 4b), which is a
392 marker for endosomes and lysosomes, and dianthin:AF488 (Fig. 4c; merged image Fig.
393 4d). Dianthin:AF488 was detected in intracellular vesicles (Fig. 4c) and co-localisation with
394 pHrodo™ Red Dextran was observed (white arrows), indicating that dianthin:AF488 was
395 transported into endosomes/lysosomes.

396 In further real time experiments, SO1861 was added to the cells in order to
397 investigate the SO1861 mediated intracellular release of dianthin:AF488 into the cytosol.

398 This is quantified in Fig. 4e. The complete video sequence can be found in the
399 supplementary video. The cytosolic fluorescence indicates the release of dianthin:AF488
400 from intracellular compartments, a process that is mediated by SO1861. In Fig. 4 (f-i) are
401 shown images taken from the supplementary video 1: panel (f) at 36 s when 5 µg/mL
402 SO1861 was added, panel (g) at 136 s (no change), panel (h) at 180 s when
403 dianthin:AF488 was released into the cytosol of cell 1 and panel (i) at 500 s when
404 dianthin:AF488 was released into the cytosol of cell 2.

405 Discussion

406 CTR was found expressed in a high percentage of human biopsies of glioblastoma
407 [25] and as reported here, by approximately 50% of HGG cell lines that were available to
408 us. These cell lines are considered to represent glioma stem cells. In view of their role in
409 the rapid expansion of the tumour and the relative resistance glioma stem cells confer to
410 conventional therapies, we chose to target CTR and compare the efficacies of
411 antibody:conjugates representing two immunotoxins and an ADC. Part of the reasoning
412 was the current popularity of ADCs being tested in clinical trials and our own interest in the
413 potency of enhancers to improve the efficacy of RIP-based immunotoxins. Thus the
414 question we addressed concerned the comparison of the efficacies of immunotoxins and a
415 related ADC tested on HGG cell lines *in vitro*.

416 The opportunity for this study began with our identification of the expression of CTR in
417 glioblastoma and the validation of the antibody mAb2C4 which specifically binds an
418 extracellular epitope of hCTR [24].

419 The first step was the synthesis of immunotoxins from mAb2C4 and the RIPs, diathin-
420 30 [43] and gelonin [44], and the drug MMAE [38] with a linker, together known as vedotin.
421 A range of protein (antibody or toxin) and cross-linker SPDP molar ratios were tested and
422 the optimal 1:6 for both were conjugated and further purified as described. One important
423 step in purification for each synthesis employed chromatography using a peptide affinity
424 column which selected moieties that were capable of specifically binding the target
425 sequence of hCTR located in the extracellular N-terminal domain and excluded modified
426 antibody conjugates that no longer retained affinity for CTR.

427 Following the purification steps we determined with statistical accuracy the efficacy of
428 treatment with mAb2C4:dianthin compared to mAb2C4:MMAE which involved 96-well
429 assays to determine net LDH and these were repeated four times (data shown in Fig. 2a -
430 c). With the HGG cell line SB2b, in the presence of SO1861, the EC₅₀ for

431 mAb2C4:dianthin is 10.0 pM and for mAb2C4:MMAE 2.5 nM, which is approximately 250-
432 fold less potent. Similarly with the cell line U87MG, in the presence of SO1861, the EC₅₀
433 for mAb2C4:dianthin is 20.0 pM and for mAb2C4:MMAE 6.3 nM, which is approximately
434 300-fold less potent.

435 To further explore the underlying biological variation that might be expected between
436 patients we pooled the data for all HGG cell lines tested (data not shown) and U87MG
437 treated with either mAb2C4:dianthin or mAb2C4:gelonin (EC₅₀ for both, 8 pM), but
438 excluded the data derived with the HGG cell line PB1, which was resistant to both the
439 immunotoxins. A comparison with the pooled for the ADC (EC₅₀, 10 nM) demonstrated the
440 immunotoxins were greater than 3 logs more potent. These pooled data demonstrate a
441 remarkably similar sensitivity to both immunotoxins in a range of HGG cell lines. This
442 observation adds weight to the claim that CTR is a potential target for the treatment of
443 glioblastoma as a high proportion of lines that express CTR were sensitive to the
444 immunotoxins and were isolated from individual biopsies.

445 MAb2C4:MMAE was consistently less potent, with EC₅₀ in the nM range for both SB2b
446 and U87MG, which is 250-300 times less potent than mAb2C4:dianthin-30 and
447 mAb2C4:gelonin in the presence of SO1861. Controls with unconjugated antibody (in the
448 higher nM range) showed little effect on cell death. In the absence of SO1861
449 mAb2C4:MMAE is 10 fold less potent and mAb2C4:RIPs were relatively ineffective (1000
450 fold less potent) such that their potencies are similar at approximately 10-20 nM (Table 1
451 and supplementary figure 6). These data demonstrate the enhancer SO1861 has a high
452 degree of specificity for the RIP. The EC₅₀ data for these cell lines with mAb2C4:RIPs as
453 described above is consistent with data published recently for cetuximab:dianthin-30 (5.3
454 pM), panitumumab:dianthin-30 (1.5 pM) and trastuzumab:diathin-30 (23 pM) co-
455 administered with SO1861 [43].

456 The HGG cell line PB1 was resistant to mAb2C4:RIPs although it expressed CTR as
457 determined by immunoblot (Fig. 1a) and accumulated mAb2C4:AF568 (Fig. 3). This
458 demonstration of resistance by PB1 alone compared to other cell lines, supports a
459 mechanism in which uptake of immunotoxin is receptor-mediated rather than some non-
460 specific mechanism of internalisation. Possible explanations for resistance in this cell line
461 include that CTR is not efficiently internalised to reach the lysosomal compartment or the
462 immunotoxin is not cleaved in the lysosomal compartment or that the RIP is not released
463 into the cytosol. We note the significant difference in the intracellular distribution of
464 mAb2C4:AF568 in PB1 compared with SB2b and U87MG. The large concentration of
465 antibody suggests stalling and localisation in a peri-nuclear structure, perhaps the
466 microtubule organising centre, with little access to the lysosomal compartment.

467 Seck et al [47] originally proposed that CTR is embedded in the endosomal membrane
468 and that this structure is bound to the cytoskeleton through the intermediate filament
469 filamin. We have extended this idea [24] in which carboxyl terminal arginines together with
470 a PDZ docking site are identified in all species and are predicted to be important sites of
471 binding to tubulin. We proposed that the mechanism of uptake of mAb2C4 conjugates was
472 driven by receptor-mediated endocytosis with endosomes trafficked along the
473 microtubules driven by molecular motors as reviewed [48]. When immunotoxins are
474 transported by these endosomes, which subsequently become transformed into
475 lysosomes (at low pH), the enhancers induce the release of cleaved RIPs as illustrated in
476 Fig. 4 and supplementary video 1. During this release, the RIP does not breach the
477 plasma membrane in significant amounts but remains concentrated within the cytosol,
478 consistent with the actions of enhancers in the release of RIP into the cytosol.

479 The relatively recent description of the powerful additional effects of enhancers and
480 prior inefficient escape of RIPs from intracellular compartments in their absence, might be

481 a major reason why plant immunotoxins have not yet achieved acceptance for clinical
482 studies. With successful animal studies this situation is likely to change.

483 Recent unpublished studies in mice by our group have investigated the toxicity of
484 mAb2C4:dianthin with SO1861. The mice tolerate the highest serum concentration tested,
485 namely 300 pM immunotoxin, with no apparent effects on health (unpublished results).
486 Given the EC_{50} *in vitro* is approximately 10 pM this provides a potential therapeutic
487 window. From these observations of tolerance it seems that CTR-neural circuits, the
488 expression in distal tubules, thyroid and elsewhere, may escape exposure to the
489 immunotoxin. Any depleted populations, perhaps osteoclasts, might be replenished from
490 bone marrow progenitors. These results are also consistent with the lack of significant
491 penetration of the vasculature in which endothelial cells are CTR-negative and supports
492 the view that uptake of this immunotoxin into cells is a receptor-mediated process.

493 As far as we are aware this is the first published study in which an anti-G protein-
494 coupled receptor antibody has been employed to target cancer stem cells. In view of the
495 expression of CTR by a range of tumour types it seems likely that this technology could be
496 included in strategies for the treatment of other cancers (or subtypes) including breast,
497 bone, prostate cancers and others (see Introduction). We ask the question, is there a link
498 between expression of CTR in cancer stem cells and expression in the pre-apoptotic cell
499 stress response as proposed elsewhere [24]? In other words do some cancer cells exist in
500 a state equivalent to the pre-apoptotic cell stress response?

501 In clinical trials immunogenicity associated with RIPs has been overcome with T-cell
502 epitope depletion, for instance in the case of de-bouganin [49]. When combined with
503 improved potency afforded with triterpene glycosides this is likely to create a powerful and
504 effective combinational therapy for recalcitrant tumours.

505 In summary we have demonstrated that an anti-CTR antibody conjugated to an RIP
506 can be deployed to target glioma stem cells (HGG cell lines). These cells form the basis

507 for resistance to chemotherapy and expansion of the deadly tumour glioblastoma. The
508 immunotoxins studied here are 250-300 times more potent than an equivalent ADC in a
509 range of HGG cell lines and suggests more potent, aggressive treatment regimens can be
510 configured than are currently being investigated in clinical trials. This improved efficacy
511 may be important for the successful treatment of recalcitrant tumours such as GBM and
512 such strategies should be investigated with clinical trials as the next step to improve
513 patient outcomes.

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525 the anti-CTR antibodies.

526

527 **Conflict of Interest**

528 Dr Wookey is a Director of Welcome Receptor Antibodies Pty Ltd (Australia) which
529 developed the anti-CTR antibodies. All other authors declare no conflict of interest.

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690

691

692 **Figure legends**

693 **Figure 1**

694 Expression of CTR on different cell lines, gel electrophoresis of samples taken during
695 conjugation of mAb2C4 to dianthin-30[His⁶] and final products for the syntheses of
696 mAb2C4:dianthin-30[His⁶], mAb2C4:gelonin and mAb2C4:MMAE.

697 (a) Immunoblot of whole cell lysates prepared from SB2b (lane 1), PB1 (lane 2), U87MG
698 (lane 3) WK1 (lane 4) and JK2 (lane 5) stained with mAb1H10 anti-human CTR antibody
699 and U87 stained with mAb2C4 (lane 6); U87MG stained with anti- β -tubulin antibody (lane
700 7). It is likely that band 'b' represents the full length hCTR protein without glycosylation and
701 band 'a' the N-terminal truncated form with the absence of epitope 4 to which mAb2C4
702 binds. Band 'c' represents the extracellular domain (~14 kD) cleaved from the 40 kD
703 protein.

704 (b) Samples were assayed following the conjugation reaction (the molar ratio of
705 proteins:crosslinker SPDP was 1:6 and the molar ratio of activated dianthin-30[His⁶] and
706 mAb2C4 was 1:1). The sample was non-reduced (lane 1) or reduced (lane 2) and for
707 controls, mAb2C4 was non-reduced (lane 3) or reduced (lane 4). The mAb2C4:dianthin-
708 30[His⁶] conjugates are indicated with an arrowhead and dianthin-30 with arrows.

709 (c) Purified mAb2C4:dianthin-30[His⁶] (lane 1) compared to mAb2C4 (lane 2) and dianthin-
710 30[His⁶] (lane 3).

711 (d) Purified mAb2C4:gelonin (lane 1) compared to gelonin (lane 2) and mAb2C4 (lane 3).

712 (e) Purified mAb2C4:MMAE (lane 1) compared to mAb2C4 (lane 2).

713

714

715 **Figure 2**

716 (a) The comparative toxicity profiles of the immunotoxin mAb2C4:dianthin-30 with (●, 3
717 µg/mL, n=4) and without (■) SO1861 (n=4), and (b) the ADC, mAb2C4:MMAE with (●,
718 n=3) and without (■, n=4) SO1861 in the HGG cell line SB2b. (c) The comparative toxicity
719 profiles of the immunotoxin mAb2C4:dianthin-30 (■, n=3) and the ADC, mAb2C4:MMAE
720 (●, n=3) in the cell line U87MG with 1 µg/mL SO1861. In any one 96-well experiment to
721 measure lactate dehydrogenase (LDH) activity, each data point represents the nett LDH
722 activity in which the nett LDH is calculated from the difference between the mean total
723 LDH (3 lysed cell samples) minus mean residual LDH (3 un-lysed cell samples). Each
724 experiment was repeated n=3 or n=4 as indicated above. Values were normalised against
725 the mean of LDH activity in untreated cells that results in % maximal LDH activity. The
726 EC₅₀ values are listed in Table 1.

727

728 **Figure 3**

729 Typical uptake of mAb2C4:AF568 (red) by HGG cell lines and intracellular localisation in
730 relation to the distribution of LAMP1-positive (green) lysosomes within the cytoplasmic
731 domain and to nuclei stained with DAPI (blue).

732 (a) Merged image following the uptake of mAb2C4:AF568 (red) by cell line JK2.

733 (b) Merged image following the uptake of mAb2C4:AF568 by the cell lines SB2b.

734 (c) Image of PB1 (red channel).

735 (d) Merged image of PB1.

736 (e) Image of U87MG (red channel).

737 (f) Merged image of U87MG.

738 A subset of fluorescence in the red channel coincides with LAMP1 staining (green in the
739 merged images in panels e & f) as shown in yellow [overlap]. The calibration bar in (f)
740 represents 40 μm in (a), 6 μm in (b) and 10 μm in (c-f).

741

742 **Figure 4**

743 Intracellular localisation of dianthin:AF488 and release into the cytosol in the presence of
744 SO1861.

745 (a) U87MG cells were stained with the membrane permeable dye Hoechst 33342 (blue
746 nuclei),

747 (b) pHrodo™ Red Dextran (red),

748 (c) dianthin:AF488 (green) and

749 (d) merged image. Co-localization with pHrodo™ Red Dextran and dianthin-30:AF488 was
750 partly observed (white arrows), indicating accumulation in endosomes/lysosomes.

751 (e) shows the increase in the fluorescence intensity with the release of dianthin:AF488 into
752 the cytosol in cell 1 (green trace) and cell 2 (blue trace). Regions of interest were defined
753 for affected cells and the SO1861 mediated intracellular release of dianthin:AF488 was
754 analysed off-line by Carl Zeiss ZEN 2.3 lite software. The sudden increase of the intensity
755 indicates the release of dianthin:AF488 into the cytosol.

756 (f-i) images taken from the supplementary video, show the time series of SO1861
757 mediated intracellular release of dianthin:AF488 from two cells in a field, in which merged
758 images at different time points are shown, of U87MG cells loaded with dianthin:AF488.

759 (f) t=36 s, 5 $\mu\text{g/mL}$ SO1861 was added.

760 (g) t= 136 s, no change.

761 (h) $t=180$ s, high intensity green fluorescence is evident in cell 1, which indicates the
762 SO1861 mediated intracellular release of dianthin:AF488 into the cytosol. At 500 s, the
763 leakage of dianthin:AF488 into the supernatant was restricted by the plasma membrane
764 and is approximately 25% over 5 minutes (green trace).

765 (i) $t=504$ s, high intensity green fluorescence is evident in cell 2, which indicates the
766 SO1861 mediated intracellular release of dianthin:AF488 into the cytosol.

767 The calibration bar in (a) represents 20 μm .

769

771

772 **Table 1:** EC₅₀ values derived from Fig. 2 are listed with different cell lines using
 773 immunotoxins mAb2C4:dianthin-30[His⁶], mAb2C4:gelonin, and the ADC mAb2C4:MMAE
 774 in the LDH release assay as described for Fig. 2. Each determination represents data from
 775 n independent experiments. Also included are data derived from supplementary figure 4.
 776 ND = No curve could be determined from data over the tested range 1-100pM. ND* the
 777 EC₅₀ could be estimated from supplementary figure 6 as approximately 10-20 nM.

Cell line	Immunotoxin/ADC	Addition of SO1861	Independent experiments (n)	Log EC ₅₀	EC ₅₀
SB2b	mAb2C4:dianthin	nil	4	ND	ND*
	mAb 2C4:dianthin	3 µg/mL	4	-11.0±0.14	10.0 pM
	mAb 2C4:MMAE	nil	4	-7.6±0.23	25.1 nM
	mAb 2C4:MMAE	3 µg/mL	3	-8.6±0.20	2.5 nM
U87MG	mAb 2C4:dianthin	1 µg/mL	3	-10.7±0.11	20.0 pM
	mAb 2C4:MMAE	1 µg/mL	3	-8.2±0.02	6.3 nM
JK2	mAb 2C4:gelonin	1 µg/mL	3	-10.7±0.1	20 pM
	mAb 2C4:dianthin	3 µg/mL	4	-11.0±0.33	10.0 pM