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1 **p722 ferrocifen loaded lipid nanocapsules improve survival of murine xenografted-**
2 **melanoma *via* a potentiation of apoptosis and an activation of CD8⁺ T lymphocytes**

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5

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17

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- 31 **Table of abbreviations**
- 32 **AUC** : Area under the curve
- 33 **Bax** : Bcl2-associated protein X
- 34 **BCL-2** : B-cell lymphoma 2
- 35 **CTLA-4** : Cytotoxic T-lymphocyte associated protein 4
- 36 **DMSO** : Dimethyl-sulfoxide
- 37 **DSPE-PEG** : 1, 2-Distearoyl-sn-glycero-3-phosphoethanolamine-Poly(ethylene glycol)
- 38 **HBSS** : Hank's Balanced Salt Solution
- 39 **LNC** : Lipid nanocapsules
- 40 **mAb** : Monoclonal antibody
- 41 **MeOH** : Methanol
- 42 **MEK** : Mitogen-activated extracellular signal-regulated protein kinase
- 43 **NaCl** : Sodium chloride
- 44 **PBS** : Phosphate buffered saline
- 45 **PD-1** : Programmed cell death protein 1
- 46 **PEG** : Polyethylene glycol
- 47 **PdI** : Polydispersity index
- 48 **ROS** : Reactive oxygen species
- 49 **RPMI** : Roswell Park Memorial Institute medium
- 50 **UPLC** : Ultra Performance Liquid Chromatography
- 51

52 **Highlights**

- 53 • p722-LNC induces a significant decrease of B16F10 melanoma cell viability
- 54 • p722-LNC slows tumor growth and improves the survival of treated mice
- 55 • Antitumor activities of p722-LNC are explained by a potentiation of intrinsic
56 apoptotic pathway and an activation of CD8⁺ T lymphocytes

57

58 **Abstract**

59 Metastatic melanoma is a malignant tumor with a poor prognosis. Recent new therapeutics
60 improved the survival of patients at a metastatic stage. However, the low response rate to
61 immunotherapy, explained in part by resistance to apoptosis, needs to develop new
62 strategies. The ferrocifen family represents promising bioorganometallic molecules for
63 melanoma treatment since they show potent anticancer properties. The aim of this study is
64 (i) to evaluate the benefits of a strategy involving encapsulated p722 in lipid nanocapsules
65 (LNC) in B16F10 melanoma mice models and (ii) to compare the beneficial effects with an
66 existing therapy such as anti-CTLA4 mAb. Interestingly, LNC-p722 induces a significant
67 decrease of melanoma cell viability. *In vivo* data shows a significant improvement in the
68 survival rate and a slower tumor growth with p722-loaded LNC in comparison with anti-
69 CTLA4 mAb. Western blots confirm that LNC-p722 potentiates intrinsic apoptotic pathway.
70 Treatment with LNC-p722 significantly activates CD8⁺ T lymphocytes compared to treatment
71 with anti-CTLA4 mAb. This study uncovers a new therapeutic strategy with encapsulated
72 p722 to prevent B16F10 melanoma growth and to improve survival of treated mice.

73 **Keywords:** Ferrocifen, metastatic melanoma, CD8 T lymphocytes, apoptosis

74

75

76 **Introduction**

77 Cutaneous cancers are among the most common cancers, with more than two to three
78 million new cases around the world each year. The incidence of melanoma increased
79 dramatically over the past 50 years [1]. Although it accounts for 5% of all skin cancers,
80 cutaneous melanoma is responsible for 75% of skin cancer deaths. The survival rate of
81 melanoma varies depending on the stage of the disease at the time of prognosis. Thus,
82 overall relative survival rate at any stage is 97% at one year, 85% at five years while, in
83 metastatic conditions, the median survival is six to nine months.

84 Where melanoma is concerned, the difficulties of early dissemination and pronounced
85 chemotherapy resistance remained completely unsolved for decades [2]. Since 2011, the
86 situation has improved due to the development of new targeted therapies such as BRAF or
87 MEK inhibitors and efficient immune-stimulating antibodies such as anti-CTLA4, anti-PD1 or
88 anti-PDL1. Several mechanisms have been linked to CTLA4-mediated activities [3]. During
89 native T cell activation, the expression of CTLA4 protein on the cell surface is upregulated
90 and directly competes with the CD28 protein in binding with the CD80 and CD86 molecules
91 [4]. The inhibitory signals from CTLA4 can also prevent the activation of self-reactive T cells
92 by attenuating T cell signaling. These strategies lead to a significant increase in metastatic
93 melanoma patients' overall survival time [5;6]. Nevertheless, for many patients, tumor
94 relapses and therapy resistance often follow within only a few months or years, after an
95 initial tumor reduction phase [7;8]. Thus, new therapeutic strategies are still needed, which
96 may further improve the clinical outcome. Many drug candidates have been investigated
97 and metal-based drugs, particularly ferrocifen-based drugs, represent a promising family of
98 anticancer compounds [9;10].

99 The bioorganometallic molecules are active molecules containing at least one carbon atom
100 directly bound to a metal or metalloid. In ferrocifens, the metal studied is iron and the
101 metallocene derivative is ferrocene chemically grafted onto a polyphenolic skeleton,
102 resulting in ferrocenyl phenol derivatives [11]. In recent years, our group has demonstrated,
103 **through tumor-xenografted mice model**, the beneficial effects of ferrociphenol on the tumor
104 cell apoptosis or senescence in triple negative breast cancer [12] or in melanoma [10].
105 Furthermore, cytotoxic properties of ferrocifens have been reported through an increase in
106 the production of reactive oxygen species (ROS) [13] and the generation of an active redox
107 metabolite that interact selectively with nucleophilic biomolecules such as glutathione or
108 the thioredoxin reductase. However, due to their hydrophobicity, the use of lipid core
109 vectors such as lipid nanocapsules (LNC), allows an optimal encapsulation and an
110 intravenous administration of these molecules [14].

111 Among the therapeutic strategies for melanoma, immunotherapy has a key role for several
112 years. Thus, a significant improvement of the median overall survival has been observed
113 after treatment with ipilimumab, a monoclonal antibody against cytotoxic T-lymphocyte
114 antigen-4 (CTLA-4), which is able to exploit the natural ability of the immune system to
115 eradicate primary cancer cells [7]. Furthermore, it has been reported the benefit of the
116 combination of anti-CTLA4 antibodies and radiotherapy in metastatic melanoma. Thus, a
117 median survival prolongation of 3 to 5 months and response rates of 25.7 vs 6.5% have been
118 observed in anti-CTLA4 groups [15]. Taken together, these studies confirm the interest of
119 using anti-CTLA4 antibodies to treat metastatic melanoma.

120 The aim of this study was to evaluate the benefits of a strategy involving encapsulated p722
121 in LNC in B16F10 melanoma mice models. For the first time, an increase in therapeutic
122 efficacy was observed in LNC-p722 treated mice in comparison with anti-CTLA mAb treated

123 mice. Interestingly, LNC p722 is able to improve survival of murine xenografted-melanoma
124 *via* a potentiation of apoptosis and an activation of CD8⁺ T lymphocytes

125

126 **Materials and methods**

127 **Chemical materials**

128 p722 (Fig. 1) was synthesized according to the protocol from Pigeon *et al.* [16]. The lipophilic
129 Labrafac[®] WL1349 (caprylic-capric acid triglycerides) was purchased from Gatefossé S.A.
130 (Saint-Priest, France). Lipoid[®] S75-3 (soybean lecithin at 69% of phosphatidylcholine came
131 from Lipoid GmbH (Ludwigshafen, Germany), Kolliphor[®]HS15 is a mixture of polyglycol
132 mono- and di-esters of 12-hydroxystearic acid and of about 30% of free polyethylene glycol
133 (BASF, Ludwigshafen, Germany), and NaCl from Prolabo (Fontenay-sous-bois, France).
134 Deionised water was acquired from Milli-Q system (Millipore, Paris, France). DSPE-
135 mPEG2000 (Mean Molecular Weight, MMw = 2805 g.mol⁻¹) was provided by Avanti Polar
136 Lipids (Alabaster, USA).

137

138 **Formulation of p722-loaded lipid nanocapsules (p722-LNC)**

139 The LNC vehicle was formulated in accordance with a phase inversion method, as previously
140 described[17]. Firstly, all the excipients were mixed (16.9% w/w Kolliphor[®]HS15 (BASF,
141 Ludwigshafen, Germany), 1.5% w/w Lipoid[®]S75-3 (Lipoid GmbH, Ludwigshafen, Germany),
142 20.6% w/w Labrafac[®] (caprylic-capric acid triglycerides, Gatefossé S.A. Saint-Priest, France),
143 1.8% w/w NaCl (Prolabo, Fontenay-sous-Bois, France) and 59.2% w/w water (obtained from
144 a Milli-Q system, Millipore, Paris, France)) under magnetic stirring and heated from room
145 temperature to 60°C for 15 min. Three temperature cycles between 60 and 90 °C were
146 performed to obtain phase inversions of the emulsion. Secondly, when the temperature was

147 in the phase inversion zone (73-78°C), ice-cold water was added (88.4%v/v) to induce
148 irreversible shock and dilution, and to form the LNC suspension. The nanocapsules were
149 stored at 4°C. To formulate p722-LNC, 0.5% w/w p722 powder (concentration at 2.35 mM)
150 was added at step I, with all the excipients and the formulation process was the same as for
151 empty LNC. To separate free p722 from p722-loaded lipid nanocapsules, the suspension was
152 passed through 0.2 µm cellulose acetate filter.

153

154 *Post-insertion of DSPE-PEG*

155 The polymer used for post-insertion was 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-
156 N- [methoxy(polyethyleneglycol)-2000] (DSPE-mPEG2000) (Avanti Polar Lipids, Inc,
157 Alabaster, USA). DSPE-mPEG2000 and LNC were co-incubated for 4h at 37 °C, with a final
158 polymer concentration adjusted to 5 mM. At this concentration, the post-insertion is total
159 [18] and the number of DSPE-mPEG2000 molecules per LNC is around 3 [19].

160

161 *Characterization of p722-LNC: size and zeta potential*

162 The mean diameter and polydispersity index (Pdl) of LNC were characterized by using
163 Dynamic Light Scattering (DLS) from a Malvern Zetasizer® apparatus (Nano Series ZS,
164 Malvern Instruments S.A., Worcestershire, UK) at 25 °C, after dilution by 100 with UPW. The
165 measured average values were calculated from 3 runs, with 10 measurements per run.

166 The zeta potential of the nanocapsules was determined by laser Doppler micro-
167 electrophoresis using a Zetasizer Nano ZS system (Nano Series ZS, Malvern Instruments S.A.,
168 Worcestershire, UK) [20].

169

170 *Characterization of p722-LNC: encapsulation efficiency (EE)*

171 The encapsulated p722 concentration was directly measured by quantifying p722 in the LNC
172 using a supplier recommended UPLC method (Waters, France). For this purpose, the LNCs
173 were broken via a dilution by 10 in methanol (VWR international, Fontenay-sous-bois,
174 France). A BEH C18 analytical column (2.1 x 50 mm, 1.7 μm, Waters, France) was used at
175 room temperature. DMSO (Thermofisher, Kandel, Germany) and MeOH were used as mobile
176 phases (isocratic mode: 10:90, 10 min). The flow rate was 0.2 mL.min⁻¹, injection volume was
177 2 μL and p722 was quantified by a UV detector at a wavelength of 450 nm. Analysis of the
178 data was performed by Empower 3 software (Waters). Calibration curves were established
179 by quantifying the area under the curves (AUCs) of 0-200 μg.mL⁻¹ solutions of p722 in DMSO
180 and MeOH 10:90.

181

182 Encapsulation efficiency, EE (%), was calculated using the following equation:

$$183 \quad EE (\%) = \frac{(\text{Encapsulated p722 conc. in LNC}) \times 100}{\text{Initial p722 conc. in LNC}}$$

184 Drug loading, DL (% w/w), was calculated using the following equation:

185 *Drug loading (% w/w)*

$$186 \quad = \frac{(\text{Mass of encapsulated p722 in 1 mL of LNC dispersion}) \times 100}{\text{Total mass of excipients and p722 in 1 mL of LNC dispersion}}$$

187

188 **Cell culture**

189 B16F10 mouse melanoma cell lines (gift from University of Brussels) were grown in RPMI-
190 1640 medium (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum
191 (Biowest, Nuaille, France), 1% non-essential amino acid (Lonza, Walkersville, USA), 10 units of
192 penicillin and 10 mg of streptomycin (Sigma-Aldrich, Saint Louis, USA). B16F10 was cultured
193 according to ATCC protocol and maintained at 37°C in a humidified atmosphere with 5% CO₂.

194 **Determination of cell viability**

195 B16F10 cells were seeded onto 96-well plates at a density of 5×10^4 cells/well and
196 precultured overnight. Cells were treated with p722, empty PEG-LNC (“blank”) and p722
197 PEG-LNC for 24 h at a concentration range from 10^{-4} to 100 mg/mL[10]. Cytotoxicity tests
198 were performed using the MTT assay. The OD was evaluated by Multiskan Ascent
199 (Labsystems, Fischer Scientific, Wilmington, USA) at 492 nm.

200

201 ***In vivo* tumor development**

202 *Ethical approval*

203 All procedures involving animals, including the breeding protocols, were conducted in
204 accordance with protocols approved by the ethical committee of the University of Angers
205 and the regional ethics committee on animal testing (APAFIS#6540-2017012017467029v1).
206 Furthermore, animal experiments were carried out in strict accordance with
207 recommendations in the guidelines of the Code for Methods and Welfare Considerations in
208 Behavioral Research with Animals (Directive 2010/63/UE).

209

210 *Orthotopic model of B16F10 melanoma*

211 C57BL/6 mice were housed at the university animal facility (Service Commun d’Animalerie
212 Hospitalo-Universitaire; Université d’Angers, France) and were distributed in cages (4
213 mice/cages). A syngenic, allograft model of melanoma was obtained by subcutaneously
214 injecting a suspension of 10^6 B16F10 melanoma cells in 100 μ L of HBSS with calcium and
215 magnesium (Lonza, Walkersvill, Etats-Unis) into the right flank of mice. Before injecting cells,
216 mice were anesthetized with isoflurane (isoflurane was chosen because it induced rapid and
217 reversible anesthesia). Tumor size and tumor volume were monitored until the end of the

218 protocol and have been estimated using the formula: $V=\pi/6 \times L \times W^2$ (L: length and W:
219 Width). When the tumor volume was approximately 100 mm³, mice were treated according
220 to the following protocol.

221 The treatment of mice was randomized and was administrated blind. When tumor volume
222 was around 100 mm³ (day 0), mice were treated, with intraperitoneal injections of PEG-
223 p722-LNC (7 mg/kg) – to limit the first-pass hepatic effect - or blank PEG-LNC, daily from day
224 1 to day 5 at the beginning of the afternoon. Otherwise, mice were treated with anti-CTLA4
225 monoclonal antibody (10 mg/kg) on days 2, 4 and 6. At the end of the protocol, mice were
226 sacrificed (animals were anesthetized with isoflurane and then, euthanized by CO₂), and
227 tumors were removed, frozen and stored at -80°C. In accordance with ethical rules, the
228 animals were sacrificed when the tumor volume was greater than 2500 mm³ or when the
229 animals showed signs of suffering.

230

231 **Western blot**

232 For the *in vitro* protocol, proteins were extracted from cells by scrapping with a cell lysis
233 buffer (10 mmol/L Tris-Base, 1 mmol/L Na₃O₄ and 1% SDS, pH 7.4). For the *in vivo* protocol,
234 mice tumors were grinded with Ultra-turrax in the same lysis buffer. Twenty micrograms of
235 proteins were resolved in 10% (v/v) SDS-PAGE gel and transferred onto a nitrocellulose
236 membrane (0.45 μm pore size) (Amersham GE Healthcare). Mouse anti-Bcl-2 (ab694,
237 Abcam) and rabbit anti-Bax (ab32503, Abcam), anti-Procaspase 9 (ab138412, Abcam), anti-
238 Caspase 9 (ab 202068, Abcam), anti-Procaspase 3 (ab 183179, Abcam) and anti-caspase 3 (ab
239 184787, Abcam) were used as primary antibodies. A second mouse (goat anti-mouse IgG,
240 secondary antibody, HRP conjugate, Life Technologies) and rabbit (61-6520, Invitrogen)
241 antibodies were used. Detection (using LAS4000, GE HealthCare) was performed using

242 enhanced chemoluminescence (ECL; Fisher Scientific, Pierce). In each case, protein levels
243 were normalized to the housekeeping protein actin and the protein expression fold
244 differences, between treated and control conditions, were plotted by ImageJ software.

245

246 **Immunophenotyping of mice lymphocytes**

247 To analyze the composition of mouse lymphocytes, blood samples were collected on days 1
248 and 9 (from the venous sinus) and at the end of the protocol (from a cardiac puncture). They
249 were diluted into a staining buffer (PBS, pH 7.0 plus 1% fetal calf serum) and cells were
250 stained with fluorescent-label CD3, CD8 and CD69 antibodies (BD Biosciences) for 45 min on
251 ice, followed by two washes in the staining buffer. Then, the cells were subjected to flow
252 cytometry analyses (PACEM platform, SFR ICAT, University of Angers, Angers, France).

253

254 **Statistical analysis**

255 Results were expressed as a median. For the *in vitro* study, statistical significance between
256 control and treated groups was evaluated using a Mann-Whitney test.

257 For the *in vivo* study, the statistical tests were performed with the R software. The tumoral
258 growth analysis was performed thanks to a linear mixt model (longitudinal data), including
259 the time influence (intra and inter day variability) and random intercept (intra and inter
260 individual variability). In order to obtain a reliable statistical model, the tumoral volume was
261 converted into a decimal logarithmic scale. Survival analysis was done using a log rang model
262 to compare survival curves and to calculate survival medians.

263 The significance threshold was defined at p value ≤ 0.05 for each statistical test.

264

265

266 **Results**

267 *Physico-chemical properties of lipid nanocapsules*

268 The p722 concentration into LNC was settled at 2.35 nM. At this concentration, the
269 encapsulation efficiency was 65% and the drug loading reached 0.7%. Hydrodynamic
270 diameter and zeta potential of the LNC suspensions are summarized in **Table 1**. The p722
271 entrapment into LNC did not modify the diameter compared to empty LNC. The size of
272 PEGylated LNC (unloaded or loaded with p722) showed a increase (of around 20 nm),
273 attributed to the surface decoration by the PEG shell [18]. The zeta potential represents the
274 apparent surface charge. The p722 entrapment did not modify the zeta potential compared
275 to blank LNC. LNC PEGylation decreased the zeta potential ~~from -5 mV to~~ (-29 mV and -13
276 mV) for unloaded ~~and from -6 mV to -13 mV for~~ loaded LNC (**Table 1**), respectively, due to
277 the electrical dipole of ethylene glycol moiety. The different suspensions have a
278 polydispersity index < 0.2, an important parameter illustrating the monodispersity of the
279 suspension. The stability of loaded LNC was assessed at 4°C (storage condition) for 3 weeks:
280 the hydrodynamic diameter, polydispersity index, zeta potential, entrapment encapsulation
281 and drug loading were not modified (**Fig.S1A-C**).

282

283 *Ferrocifen activity on melanoma cell viability and cell apoptosis*

284 Free p722 had a significant cytotoxicity with an IC₅₀ estimated at 0.09 mg/mL (corresponding
285 to 1.68 x 10⁻⁴ M). Encapsulated p722 (p722-LNC) had the same toxicity profile as the free
286 drug (IC₅₀ = 0.10 mg/mL corresponding to 1.87 x 10⁻⁴ M), evidencing the capacity of LNC to
287 deliver ferrocifen into B16F10 melanoma cells without additional toxicity. In addition, for
288 blank LNC, IC₅₀ was significantly increased (2.90 mg/mL *i.e.* 54.16 x 10⁻⁴ M) compared to free
289 or encapsulated p722 (**Fig. 2A**).

290 As shown in Fig. 2B, the Western blot analysis revealed that Bax, pro-caspase 9, cleaved
291 caspase 9 and pro-caspase 3 were found at higher levels in B16F10 cells under free p722
292 exposure compared with untreated cells. Furthermore, in these experimental conditions, no
293 difference in cleaved caspase 3 expression has been observed in cells treated with free p722
294 in comparison to untreated cells. This same experiment was carried out with p722-pegylated
295 LNC. No modification in the expression of Bcl2, Bax and cleaved caspase 3 was
296 demonstrated. However, an increase of pro-caspase 9, cleaved caspase 9 and pro-caspase 3
297 was found in comparison with untreated cells. ~~Furthermore, no modification of expression~~
298 ~~of the different proteins was shown in the presence of pegylated LNC without p722 (Fig. 3B).~~

299

300 *Anti-tumor efficacy of ferrocifens on a mouse model of melanoma*

301 Due to the absence of a significant difference in the volumes of tumors isolated from treated
302 mice, an analysis of the growth rate was considered; the final point chosen was the critical
303 tumor volume of 2,500 mm³. It was reported that this critical tumor volume was more
304 rapidly observed for mice treated with anti-CTLA4 mAb. Conversely, this critical tumor
305 volume was evolving more slowly in mice treated with p722-pegylated LNC compared to
306 mice treated with blank LNC (Fig. 3A).

307 Moreover, in mice with melanoma, treatment with p722-pegylated LNC significantly
308 improved their survival compared to mice treated with blank LNC (22 days *versus* 13 days of
309 survival, respectively) (Fig. 3B). No difference was observed for mice treated with anti-CTLA
310 mAb in comparison with blank LNC (Fig. 3C) while a significant difference was found in the
311 survival of mice treated with p722-pegylated LNC in comparison with anti-CTLA4 mAb (Fig.
312 3D).

313

314

315 *Ferrocifen loaded LNC potentiate apoptosis via the intrinsic pathway*

316 As shown in Fig. 4, no difference was found in the expression of the different proteins tested
317 both in tumors from mice treated with anti-CTLA4 mAb or anti-CTLA4 mAb and p722-
318 pegylated LNC compared to control mice. However, a significant increase in pro-caspase 9,
319 cleaved caspase 9, pro-caspase 3 and cleaved caspase 3 has been observed in tumors from
320 mice treated with p722-pegylated LNC in comparison with control mice.

321

322 *Ferrocifen loaded LNC potentiate the activation of CD₈⁺ T lymphocytes*

323 Quantification of CD₈⁺ T lymphocytes was conducted on mice blood samples at different
324 times (day 0, day 9 and at the end of the *in vivo* protocol). As described in Fig. 5A, no
325 difference in the percentage of CD₈⁺ T lymphocytes was observed whatever the time and
326 treatments. No difference was found at day 0 and at the end of the protocol in the
327 percentage of activated CD₈⁺ T lymphocytes. However, a significant increase of activated
328 CD₈⁺ T lymphocytes has been shown, at day 9, in blood samples from mice treated with
329 p722-pegylated LNC or p722-pegylated LNC and anti-CTLA4 mAb (Fig. 5B).

330

331

332 Discussion

333 Because numerous preclinical and clinical studies focused on the analysis of innovative
334 combinatory therapeutic [21];[22] in melanoma therapy, the main objective of the present
335 work is to evaluate the therapeutic properties of p722, a new promising molecule from the
336 ferrocifen family, encapsulated in LNC, in a mouse metastatic melanoma model.
337 Furthermore, the secondary objective is to compare the therapeutic benefits of p722-LNC
338 with a recognized therapeutic strategy using anti-CTLA4 antibody. Interestingly, a significant
339 improvement in the survival rate and a slower tumor growth were found with p722 loaded
340 LNC compared to anti-CLTA4 treatment. These data were supplemented by Western blot
341 analyzes which confirmed that p722-LNC potentiates apoptosis by enhancing the intrinsic
342 pathway. Furthermore, treatment with p722-LNC potentiates the activation of CD8⁺ T
343 lymphocytes suggesting new properties for these promising encapsulated antitumor
344 molecules.

345 In the ferrocifen family, the key motif is [ferrocenyl-ene-phenol] leading to the formation of
346 ferrocenyl quinone methides by oxidation on cancer cells. ~~Chemically, this new functional~~
347 ~~group behaves as a selective electrophile that can react with a variety of nucleophiles [23].~~
348 At the biological level, the entity offers some new perspectives, inducing very low IC₅₀
349 values, between 0.03 to 0.5 μM, on a range of cancer cells including some resistant to
350 coordination complexes of Pt²⁺ [16]. It operates by apoptosis and/or senescence. Inhibition
351 of pluripotent cancer stem-cells by ferrociphenols has also been found. ~~The potential for~~
352 ~~multisite activity shown by these species is of interest in precluding and/or sidestepping the~~
353 ~~phenomena of resistance. The role of the iron generating production of ROS, is initiate,~~
354 ~~control and modulate a series of novel molecular events within cancer cells. The system is~~
355 ~~not operative on healthy cells [23].~~ Amongst the synthesized ferrocifens, the succinimido-

356 ferrociphenol compound p722 shows a strongest *in vitro* antiproliferative effects [24] while
357 its *in vivo* behavior has not been evaluated so far. The exceptional antitumoral effect of this
358 new derivative is related to the succinimide function, allowing an unprecedented
359 stabilization of the transient quinone methide, *via* an interaction between the lone pair of
360 one carbonyl of the heterocycle and the center of the aromatic quinone methide ring
361 [16,24].

362 Considering the poor water-soluble property of ferrocifens, LNC represents a suitable drug
363 delivery system thanks to its oily core [25]. Furthermore, encapsulation of ferrocifens
364 enhances cellular and tumor delivery which represents an important challenge to treat
365 cancer. ~~Thus, previous studies on LNC encapsulating ferrocifens demonstrated the efficient~~
366 ~~encapsulation and the therapeutic benefits to treat.~~ Our results confirm and improve
367 ~~previous~~ findings ~~conducted on~~ glioblastoma [26] or melanoma [10] ~~cells~~, especially
368 concerning the characterization of nanovectors: as shown in **Table 1**, encapsulation
369 efficiency, mean size, zeta potential data are consistent with formerly cited studies and
370 these characteristics are stable over time, sustaining that these vectors are well adapted for
371 the protocols conducted on mice models.

372 Several studies evaluated the cytotoxicity of ferrocifen derivatives in various tumor cell
373 models. Thus, in a **previous study**, it was reported that P5 and DP1, two ferrociphenol
374 derivatives, exerted growth inhibitory effects, mainly *via* senescence, between 0.5 and 10
375 μM against Hs683 and U373 human glioma cells or B16F10 mice melanoma cells [9].
376 Furthermore, other experiments were conducted on triple-negative MDA-MB-231 breast
377 cancer cells for which an antiproliferative activity of ferrocifens was shown for an IC_{50} of 2.6
378 μM [27] and 2 μM [12]. Moreover, in a study conducted on SK-Mel28 human melanoma
379 cells, IC_{50} was 300 nM for ferrociphenol and 1.2 mM for ansa-ferrociphenol [28] while on 9L

380 rat gliosarcoma cells, the IC₅₀ was 0.4 and 0.6 μM for free ferrociphenol or ferrociphenol-
381 LNC, respectively [29]. Taken together, these findings confirm a better sensitivity for p722
382 ferrocifen derivatives since the present study shows lower IC₅₀ (168 and 187 nM for free
383 p722 and p722-LNC, respectively) compared to previous studies. This improved sensitivity
384 justifies the use of encapsulated p722 at lower doses, than those used in the previous
385 studies, which will ultimately limit the toxicity.

386 Both *in vitro* and *in vivo* studies confirm that encapsulated p722 is able to induce apoptosis
387 on B16F10 cells by potentiating the intrinsic pathway. Several *stimuli*, including ROS, are able
388 to regulate apoptosis of tumor cells. Thus, as previously described, ferrocifens increase the
389 levels of ROS in tumor cells [13] and then, could induce apoptosis. ~~Due to their reactive~~
390 ~~potential, ROS can oxidize bases, leading to DNA strand breaks, intra-strand adducts,~~
391 ~~crosslinks and mutations [30]. ROS can affect the integrity of proteins and/or lipids in~~
392 ~~membranes, decreasing the fluidity and increasing membrane permeability as well as in~~
393 ~~mitochondria [31].~~ Mitochondrial dysfunction, characterized by loss of transmembrane
394 potential and opening of mitochondrial permeability transition pores occurs *via* many
395 apoptotic *stimuli* [32]. Ferrocifen derivatives cause disruption of ΔΨ_{min} in breast cancer cells
396 [13]. In melanoma, the impairment of mitochondrial membrane permits the release of
397 cytochrome *c*, an event known to occur after externalization of phosphatidylserine [33],
398 triggering the apoptotic intrinsic pathway with activation of caspase 9 and 3 [31]. Thus,
399 taken together, these data suggest that the increase in caspase expression and activity,
400 showed in this study, could be explained by an enhancement of oxidative stress induced by
401 p722.

402 Numerous clinical studies [34] or pre-clinical models using several tumor cell lines of solid
403 cancers [35,36] reveal that the combination of chemotherapy and immunotherapy enhances

404 survival. In metastatic melanoma, it is manifest that the combination of radiotherapy with
405 ipilimumab (human anti-CTLA4) increases the median survival of 4 to 5 months and response
406 rates of 25.7% *versus* 6.5% (in patients treated with ipilimumab alone) [15]. Based on these
407 data, a part of this study aims to highlight the possible beneficial effects of a combination of
408 anti-CTLA4 mAb and p722-LNC. We purposely chose the B16F10 model and settings of high
409 tumor challenge ~~or delayed vaccination~~ in hopes of observing cooperative effects between
410 blockades of multiple pathways. Yet, we are surprised that, even with double blockade, we
411 are unable to observe any significant additive effects of these molecules in the context of
412 metastatic melanoma: anti-CTLA4 mAb is not active in this mice model while p722-LNC has a
413 significant therapeutic interest.

414 Interestingly, we confirm that p722-LNC is able to improve survival of treated mice by
415 inducing both antitumoral effect and activation of CD8⁺ T lymphocytes. While the pro-
416 apoptotic properties of many ferrocifens are known [23], we **identify for the first time** an
417 immune-preventative approach for p722-LNC, **as it has been previously proposed** ~~—A~~
418 ~~possible immunogenic cell death has been postulated with ferrocifens~~ [37]. Furthermore,
419 these properties have been suggested with hydroxytamoxifen for which an increase of
420 activation of Jurkat-T-cells has been shown. In this cellular model, an upregulation of cell
421 surface markers CD28, CD69 and CD5 has been reported in hydroxytamoxifen-treated cells
422 [38]. In the present study, a synergistic effect could explain the beneficial properties of p722.
423 After 9 days of treatment with p722-LNC, apoptosis induced by ferrocifens could enhance
424 the release of tumor antigens activating CD8⁺ T lymphocytes [39] *via* antigen presenting
425 cells. Thus, CD8⁺ T lymphocytes could either have a direct cytotoxic activity or could
426 constitute a pool of CD8⁺ T memory lymphocytes explaining the significant improvement in
427 mice survival treated with p722 [40]. Moreover, p722-LNC therapy could result in increasing

428 tumor infiltration of T cells and enhanced production of cytokines associated with cytotoxic
429 T-cell function [41]. However, the precise mechanisms associated with the synergistic effects
430 observed in the present study are not fully characterized, and mechanistic studies will be
431 required to identify the role of different cellular subsets in the generation of effective
432 antitumor immunity. Overall, our findings illustrate an original approach to treat metastatic
433 melanoma through a dual property: pro-apoptotic properties and CD8⁺ T lymphocytes
434 activation.

435 These properties are identified for p722-loaded-LNC, a promising antitumor strategy.
436 According to our data, p722 is able, on its own, to prevent the development of metastatic
437 melanoma and to improve the survival of treated mice. However, it is important to note that
438 the optimal schedule and dose to improve median survival in these murine models are not
439 directly transposable in studies in humans, herein suggesting further preclinical (in other
440 animal models) and clinical studies.

441

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448

449

450 **References**

- 451 1. Ossio, R.; Roldán-Marín, R.; Martínez-Said, H.; Adams, D.J.; Robles-Espinoza, C.D.
452 Melanoma: a global perspective. *Nat Rev Cancer* **2017**, *17*, 393-394,
453 doi:10.1038/nrc.2017.43.
- 454 2. Garbe, C.; Peris, K.; Hauschild, A.; Saiag, P.; Middleton, M.; Bastholt, L.; Grob, J.J.;
455 Malvey, J.; Newton-Bishop, J.; Stratigos, A.J., et al. Diagnosis and treatment of melanoma.
456 European consensus-based interdisciplinary guideline - Update 2016. *Eur J Cancer* **2016**, *63*,
457 201-217, doi:10.1016/j.ejca.2016.05.005.
- 458 3. Michot, J.M.; Bigenwald, C.; Champiat, S.; Collins, M.; Carbonnel, F.; Postel-Vinay, S.;
459 Berdelou, A.; Varga, A.; Bahleda, R.; Hollebecque, A., et al. Immune-related adverse events
460 with immune checkpoint blockade: a comprehensive review. *Eur J Cancer* **2016**, *54*, 139-148,
461 doi:10.1016/j.ejca.2015.11.016.
- 462 4. Tivol, E.A.; Borriello, F.; Schweitzer, A.N.; Lynch, W.P.; Bluestone, J.A.; Sharpe, A.H.
463 Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction,
464 revealing a critical negative regulatory role of CTLA-4. *Immunity* **1995**, *3*, 541-547,
465 doi:10.1016/1074-7613(95)90125-6.
- 466 5. Hughes, T.; Klairmont, M.; Sharfman, W.H.; Kaufman, H.L. Interleukin-2, Ipilimumab,
467 and Anti-PD-1: Clinical Management and the Evolving Role of Immunotherapy for the
468 Treatment of Patients With Metastatic Melanoma. *Cancer Biol Ther* **2015**, *0*,
469 doi:10.1080/15384047.2015.1095401.
- 470 6. Lorentzen, H.F. Targeted therapy for malignant melanoma. *Curr Opin Pharmacol*
471 **2019**, *46*, 116-121, doi:10.1016/j.coph.2019.05.010.
- 472 7. Hodi, F.S.; O'Day, S.J.; McDermott, D.F.; Weber, R.W.; Sosman, J.A.; Haanen, J.B.;
473 Gonzalez, R.; Robert, C.; Schadendorf, D.; Hassel, J.C., et al. Improved survival with

474 ipilimumab in patients with metastatic melanoma. *N Engl J Med* **2010**, *363*, 711-723,
475 doi:10.1056/NEJMoa1003466.

476 8. Paulson, K.G.; Lahman, M.C.; Chapuis, A.G.; Brownell, I. Immunotherapy for skin
477 cancer. *Int Immunol* **2019**, *31*, 465-475, doi:10.1093/intimm/dxz012.

478 9. Bruyère, C.; Mathieu, V.; Vessières, A.; Pigeon, P.; Top, S.; Jaouen, G.; Kiss, R.
479 Ferrocifen derivatives that induce senescence in cancer cells: selected examples. *J Inorg*
480 *Biochem* **2014**, *141*, 144-151, doi:10.1016/j.jinorgbio.2014.08.015.

481 10. Resnier, P.; Galopin, N.; Sibiril, Y.; Clavreul, A.; Cayon, J.; Briganti, A.; Legras, P.;
482 Vessières, A.; Montier, T.; Jaouen, G., et al. Efficient ferrocifen anticancer drug and Bcl-2
483 gene therapy using lipid nanocapsules on human melanoma xenograft in mouse. *Pharmacol*
484 *Res* **2017**, *126*, 54-65, doi:10.1016/j.phrs.2017.01.031.

485 11. Michard, Q.; Jaouen, G.; Vessieres, A.; Bernard, B.A. Evaluation of cytotoxic
486 properties of organometallic ferrocifens on melanocytes, primary and metastatic melanoma
487 cell lines. *J Inorg Biochem* **2008**, *102*, 1980-1985, doi:10.1016/j.jinorgbio.2008.07.014.

488 12. Lainé, A.L.; Adriaenssens, E.; Vessières, A.; Jaouen, G.; Corbet, C.; Desruelles, E.;
489 Pigeon, P.; Toillon, R.A.; Passirani, C. The in vivo performance of ferrocenyl tamoxifen lipid
490 nanocapsules in xenografted triple negative breast cancer. *Biomaterials* **2013**, *34*, 6949-
491 6956, doi:10.1016/j.biomaterials.2013.05.065.

492 13. Lu, C.; Heldt, J.M.; Guille-Collignon, M.; Lemaître, F.; Jaouen, G.; Vessières, A.;
493 Amatore, C. Quantitative analyses of ROS and RNS production in breast cancer cell lines
494 incubated with ferrocifens. *ChemMedChem* **2014**, *9*, 1286-1293,
495 doi:10.1002/cmdc.201402016.

- 496 14. Allard, E.; Huynh, N.T.; Vessières, A.; Pigeon, P.; Jaouen, G.; Benoit, J.P.; Passirani, C.
497 Dose effect activity of ferrocifen-loaded lipid nanocapsules on a 9L-glioma model. *Int J*
498 *Pharm* **2009**, *379*, 317-323, doi:10.1016/j.ijpharm.2009.05.031.
- 499 15. Koller, K.M.; Mackley, H.B.; Liu, J.; Wagner, H.; Talamo, G.; Schell, T.D.; Pameijer, C.;
500 Neves, R.I.; Anderson, B.; Kokolus, K.M., et al. Improved survival and complete response
501 rates in patients with advanced melanoma treated with concurrent ipilimumab and
502 radiotherapy versus ipilimumab alone. *Cancer Biol Ther* **2017**, *18*, 36-42,
503 doi:10.1080/15384047.2016.1264543.
- 504 16. Pigeon, P.; Wang, Y.; Top, S.; Najlaoui, F.; Garcia Alvarez, M.C.; Bignon, J.;
505 McGlinchey, M.J.; Jaouen, G. A New Series of Succinimido-ferrociphenols and Related
506 Heterocyclic Species Induce Strong Antiproliferative Effects, Especially against Ovarian
507 Cancer Cells Resistant to Cisplatin. *J Med Chem* **2017**, *60*, 8358-8368,
508 doi:10.1021/acs.jmedchem.7b00743.
- 509 17. Heurtault, B.; Saulnier, P.; Pech, B.; Proust, J.E.; Benoit, J.P. A novel phase inversion-
510 based process for the preparation of lipid nanocarriers. *Pharm Res* **2002**, *19*, 875-880.
- 511 18. Perrier, T.; Saulnier, P.; Fouchet, F.; Lautram, N.; Benoît, J.P. Post-insertion into Lipid
512 NanoCapsules (LNCs): From experimental aspects to mechanisms. *Int J Pharm* **2010**, *396*,
513 204-209, doi:10.1016/j.ijpharm.2010.06.019.
- 514 19. Morille, M.; Montier, T.; Legras, P.; Carmoy, N.; Brodin, P.; Pitard, B.; Benoît, J.P.;
515 Passirani, C. Long-circulating DNA lipid nanocapsules as new vector for passive tumor
516 targeting. *Biomaterials* **2010**, *31*, 321-329, doi:10.1016/j.biomaterials.2009.09.044.
- 517 20. Bastiat, G.; Pritz, C.O.; Roider, C.; Fouchet, F.; Lignières, E.; Jesacher, A.; Glueckert, R.;
518 Ritsch-Marte, M.; Schrott-Fischer, A.; Saulnier, P., et al. A new tool to ensure the fluorescent

519 dye labeling stability of nanocarriers: a real challenge for fluorescence imaging. *J Control*
520 *Release* **2013**, *170*, 334-342, doi:10.1016/j.jconrel.2013.06.014.

521 21. Zimmer, L.; Apuri, S.; Eroglu, Z.; Kottschade, L.A.; Forschner, A.; Gutzmer, R.; Schlaak,
522 M.; Heinzerling, L.; Krackhardt, A.M.; Loquai, C., et al. Ipilimumab alone or in combination
523 with nivolumab after progression on anti-PD-1 therapy in advanced melanoma. *Eur J Cancer*
524 **2017**, *75*, 47-55, doi:10.1016/j.ejca.2017.01.009.

525 22. Wolchok, J.D.; Chiarion-Sileni, V.; Gonzalez, R.; Rutkowski, P.; Grob, J.J.; Cowey, C.L.;
526 Lao, C.D.; Wagstaff, J.; Schadendorf, D.; Ferrucci, P.F., et al. Overall Survival with Combined
527 Nivolumab and Ipilimumab in Advanced Melanoma. *N Engl J Med* **2017**, *377*, 1345-1356,
528 doi:10.1056/NEJMoa1709684.

529 23. Jaouen, G.; Vessières, A.; Top, S. Ferrocifen type anti cancer drugs. *Chem Soc Rev*
530 **2015**, *44*, 8802-8817, doi:10.1039/c5cs00486a.

531 24. Wang, Y.; Pigeon, P.; Top, S.; Sanz García, J.; Troufflard, C.; Ciofini, I.; McGlinchey,
532 M.J.; Jaouen, G. Atypical Lone Pair- π Interaction with Quinone Methides in a Series of Imido-
533 Ferrociphenol Anticancer Drug Candidates. *Angew Chem Int Ed Engl* **2019**, *58*, 8421-8425,
534 doi:10.1002/anie.201902456.

535 25. Lainé, A.L.; Clavreul, A.; Rousseau, A.; Tétaud, C.; Vessieres, A.; Garcion, E.; Jaouen,
536 G.; Aubert, L.; Guilbert, M.; Benoit, J.P., et al. Inhibition of ectopic glioma tumor growth by a
537 potent ferrocenyl drug loaded into stealth lipid nanocapsules. *Nanomedicine* **2014**, *10*, 1667-
538 1677, doi:10.1016/j.nano.2014.05.002.

539 26. Karim, R.; Lepeltier, E.; Esnault, L.; Pigeon, P.; Lemaire, L.; Lépinoux-Chambaud, C.;
540 Clere, N.; Jaouen, G.; Eyer, J.; Piel, G., et al. Enhanced and preferential internalization of lipid
541 nanocapsules into human glioblastoma cells: effect of a surface-functionalizing NFL peptide.
542 *Nanoscale* **2018**, *10*, 13485-13501, doi:10.1039/c8nr02132e.

- 543 27. Cázares-Marinero, J.e.J.; Top, S.; Vessières, A.; Jaouen, G. Synthesis and
544 antiproliferative activity of hydroxyferrocifen hybrids against triple-negative breast cancer
545 cells. *Dalton Trans* **2014**, *43*, 817-830, doi:10.1039/c3dt52070f.
- 546 28. Resnier, P.; LeQuinio, P.; Lautram, N.; André, E.; Gaillard, C.; Bastiat, G.; Benoit, J.P.;
547 Passirani, C. Efficient in vitro gene therapy with PEG siRNA lipid nanocapsules for passive
548 targeting strategy in melanoma. *Biotechnol J* **2014**, *9*, 1389-1401,
549 doi:10.1002/biot.201400162.
- 550 29. Laine, A.L.; Huynh, N.T.; Clavreul, A.; Balzeau, J.; Béjaud, J.; Vessieres, A.; Benoit, J.P.;
551 Eyer, J.; Passirani, C. Brain tumour targeting strategies via coated ferrociphenol lipid
552 nanocapsules. *Eur J Pharm Biopharm* **2012**, *81*, 690-693, doi:10.1016/j.ejpb.2012.04.012.
- 553 30. Murata, M. Inflammation and cancer. *Environ Health Prev Med* **2018**, *23*, 50,
554 doi:10.1186/s12199-018-0740-1.
- 555 31. Obrador, E.; Liu-Smith, F.; Dellinger, R.W.; Salvador, R.; Meyskens, F.L.; Estrela, J.M.
556 Oxidative stress and antioxidants in the pathophysiology of malignant melanoma. *Biol Chem*
557 **2019**, *400*, 589-612, doi:10.1515/hsz-2018-0327.
- 558 32. Kroemer, G.; Galluzzi, L.; Vandenabeele, P.; Abrams, J.; Alnemri, E.S.; Baehrecke, E.H.;
559 Blagosklonny, M.V.; El-Deiry, W.S.; Golstein, P.; Green, D.R., et al. Classification of cell death:
560 recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differ*
561 **2009**, *16*, 3-11, doi:10.1038/cdd.2008.150.
- 562 33. Ford, W.E.; Ren, W.; Blackmore, P.F.; Schoenbach, K.H.; Beebe, S.J. Nanosecond
563 pulsed electric fields stimulate apoptosis without release of pro-apoptotic factors from
564 mitochondria in B16f10 melanoma. *Arch Biochem Biophys* **2010**, *497*, 82-89,
565 doi:10.1016/j.abb.2010.03.008.

- 566 34. Arriola, E.; Wheeler, M.; Galea, I.; Cross, N.; Maishman, T.; Hamid, D.; Stanton, L.;
567 Cave, J.; Geldart, T.; Mulatero, C., et al. Outcome and Biomarker Analysis from a Multicenter
568 Phase 2 Study of Ipilimumab in Combination with Carboplatin and Etoposide as First-Line
569 Therapy for Extensive-Stage SCLC. *J Thorac Oncol* **2016**, *11*, 1511-1521,
570 doi:10.1016/j.jtho.2016.05.028.
- 571 35. Fritz, J.; Karakhanova, S.; Brecht, R.; Nachtigall, I.; Werner, J.; Bazhin, A.V. In vitro
572 immunomodulatory properties of gemcitabine alone and in combination with interferon-
573 alpha. *Immunol Lett* **2015**, *168*, 111-119, doi:10.1016/j.imlet.2015.09.017.
- 574 36. Jure-Kunkel, M.; Masters, G.; Girit, E.; Dito, G.; Lee, F.; Hunt, J.T.; Humphrey, R.
575 Synergy between chemotherapeutic agents and CTLA-4 blockade in preclinical tumor
576 models. *Cancer Immunol Immunother* **2013**, *62*, 1533-1545, doi:10.1007/s00262-013-1451-5.
- 577 37. Terenzi, A.; Pirker, C.; Keppler, B.K.; Berger, W. Anticancer metal drugs and
578 immunogenic cell death. *J Inorg Biochem* **2016**, *165*, 71-79,
579 doi:10.1016/j.jinorgbio.2016.06.021.
- 580 38. Chlichlia, K.; Moldenhauer, G.; Daniel, P.T.; Busslinger, M.; Gazzolo, L.; Schirmacher,
581 V.; Khazaie, K. Immediate effects of reversible HTLV-1 tax function: T-cell activation and
582 apoptosis. *Oncogene* **1995**, *10*, 269-277.
- 583 39. Palucka, A.K.; Coussens, L.M. The Basis of Oncoimmunology. *Cell* **2016**, *164*, 1233-
584 1247, doi:10.1016/j.cell.2016.01.049.
- 585 40. Ho, P.C.; Bihuniak, J.D.; Macintyre, A.N.; Staron, M.; Liu, X.; Amezquita, R.; Tsui, Y.C.;
586 Cui, G.; Micevic, G.; Perales, J.C., et al. Phosphoenolpyruvate Is a Metabolic Checkpoint of
587 Anti-tumor T Cell Responses. *Cell* **2015**, *162*, 1217-1228, doi:10.1016/j.cell.2015.08.012.
- 588 41. Wu, L.; Yun, Z.; Tagawa, T.; Rey-McIntyre, K.; de Perrot, M. CTLA-4 blockade expands
589 infiltrating T cells and inhibits cancer cell repopulation during the intervals of chemotherapy

590 in murine mesothelioma. *Mol Cancer Ther* **2012**, *11*, 1809-1819, doi:10.1158/1535-
591 7163.MCT-11-1014.

592 **Legends**

593 **Table 1**

594 Physico-chemical parameters and encapsulation yield of p722-LNC

595

596 **Figure 1**

597 **Chemical structure of p722**

598 **Figure 2**

599 Cytotoxicity of free p722, p722-loaded LNC on B16F10 melanoma cell line (A). Evaluation of
600 B16F10 cell viability by MTT assay, n=3. (B) Pro-apoptotic properties of free p722, blank LNC
601 and p722-LNC on B16F10 melanoma cell line. Evaluation of Bcl2, Bax, procaspase 9, cleaved
602 caspase 9, procaspase 3 and cleaved caspase 3, n=3 * p<0.05

603 **Figure 3**

604 Efficacy evaluation of p722-LNC and comparison with anti-CTLA4 mAb treatment on B16F10
605 mice-xenografted model. (A). Evaluation of the survival of mice treated with p722-LNC
606 compared to mice treated with blank LNC, n=6 *p=0.013 (B). Evaluation of the mice survival
607 treated with anti-CTLA4 mAb compared to mice treated with blank LNC, n=6 (C). Evaluation
608 of the survival of mice treated with p722-LNC compared to mice treated with anti-CTLA4
609 mAb, n=6 *p=0.012

610 **Figure 4**

611 Pro-apoptotic properties of p722-LNC. Western blot evaluation of Bcl2, Bax, procaspase 9,
612 cleaved caspase 9, procaspase 3, cleaved caspase 3 expression, n=3. Table represents the

613 ratio between the expression of studied proteins and the expression of β -actin. (a)
614 significant results compared to control mice ($p < 0.05$).

615 **Figure 5**

616 p722-LNC activates CD8⁺ T lymphocytes from mice tumor at day 9. (A) Evaluation of the
617 percentage expression of CD8⁺ T lymphocytes at day 0, day 9 and at the end of the protocol,
618 n=6. (B) Evaluation of the percentage expression of CD8⁺ activated T lymphocytes at D0, D9
619 and at the end of the protocol, n=6 * $p < 0.05$.

Figure 1

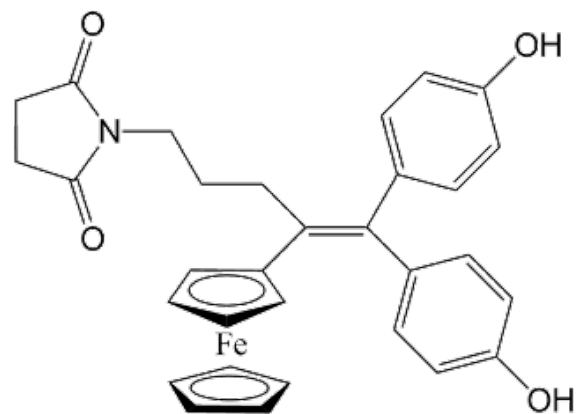
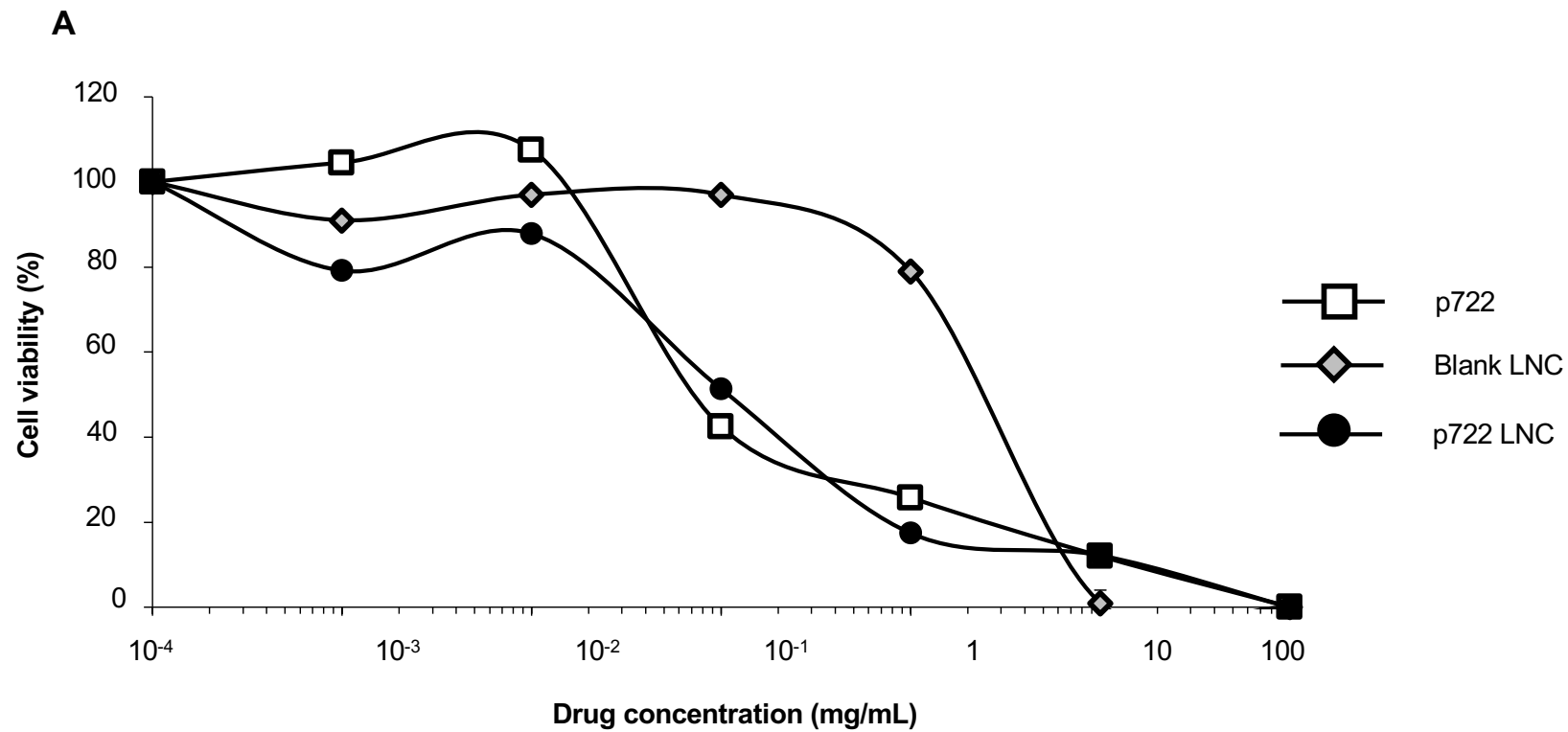


Figure 2



	IC50 (mg/mL)	IC50 (M)
p722	0.09	1.68×10^{-7}
Blank LNC	2.90	54.16×10^{-7}
p722 LNC	0.10	1.87×10^{-7}

Figure 2

B

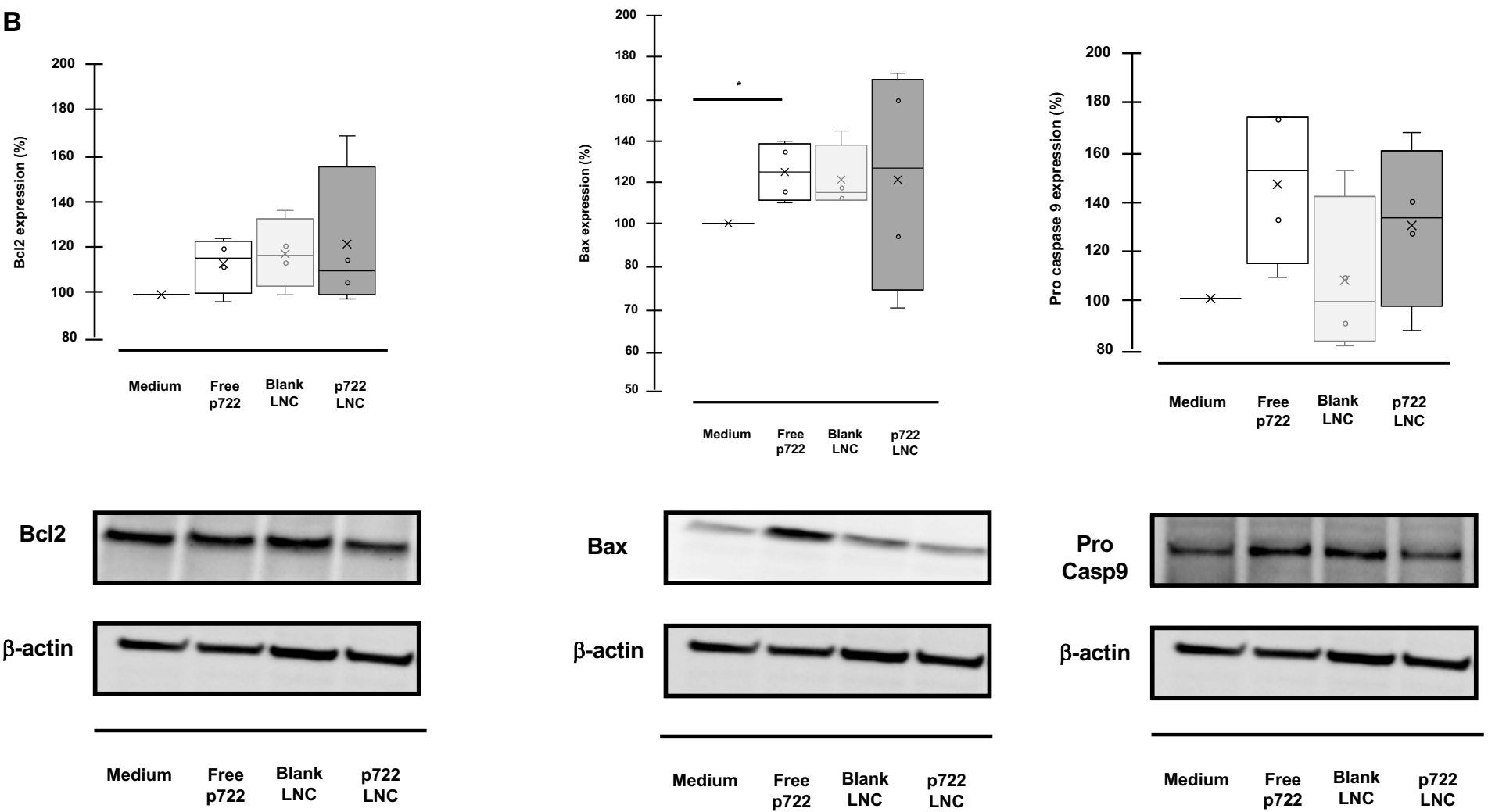


Figure 2

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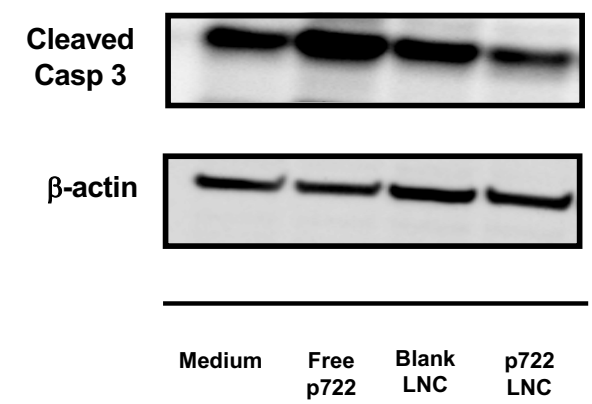
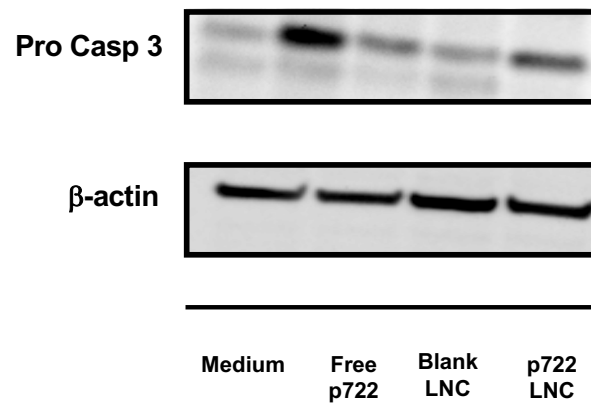
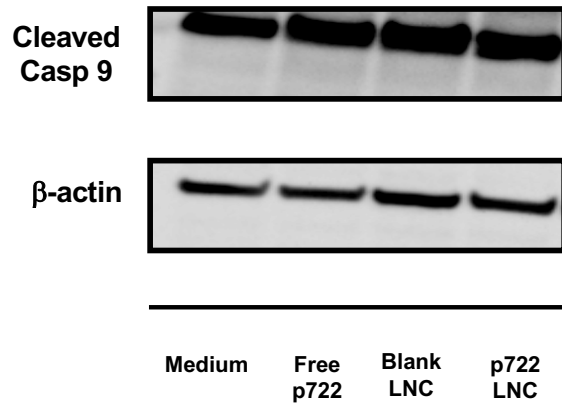
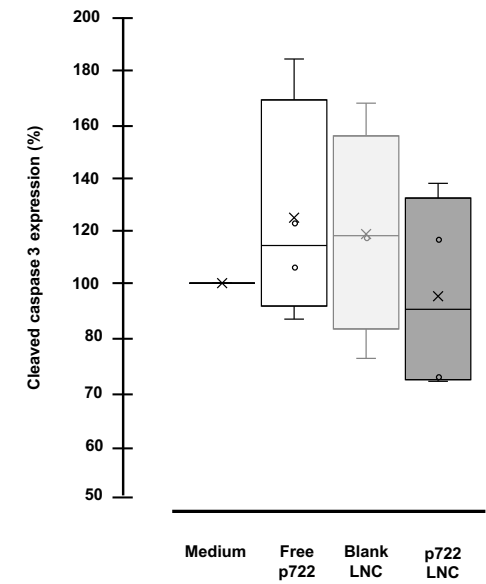
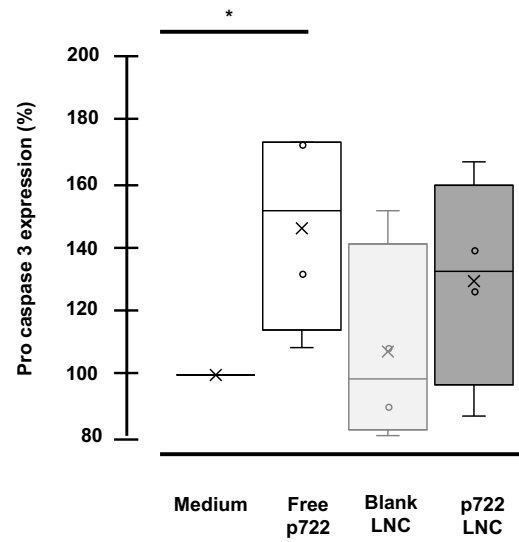
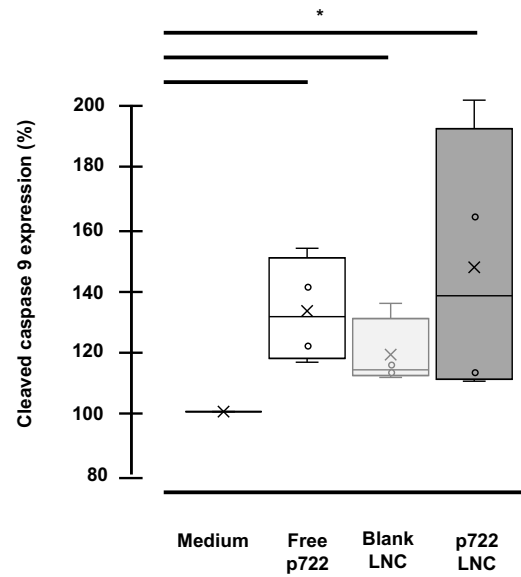


Figure 3

A

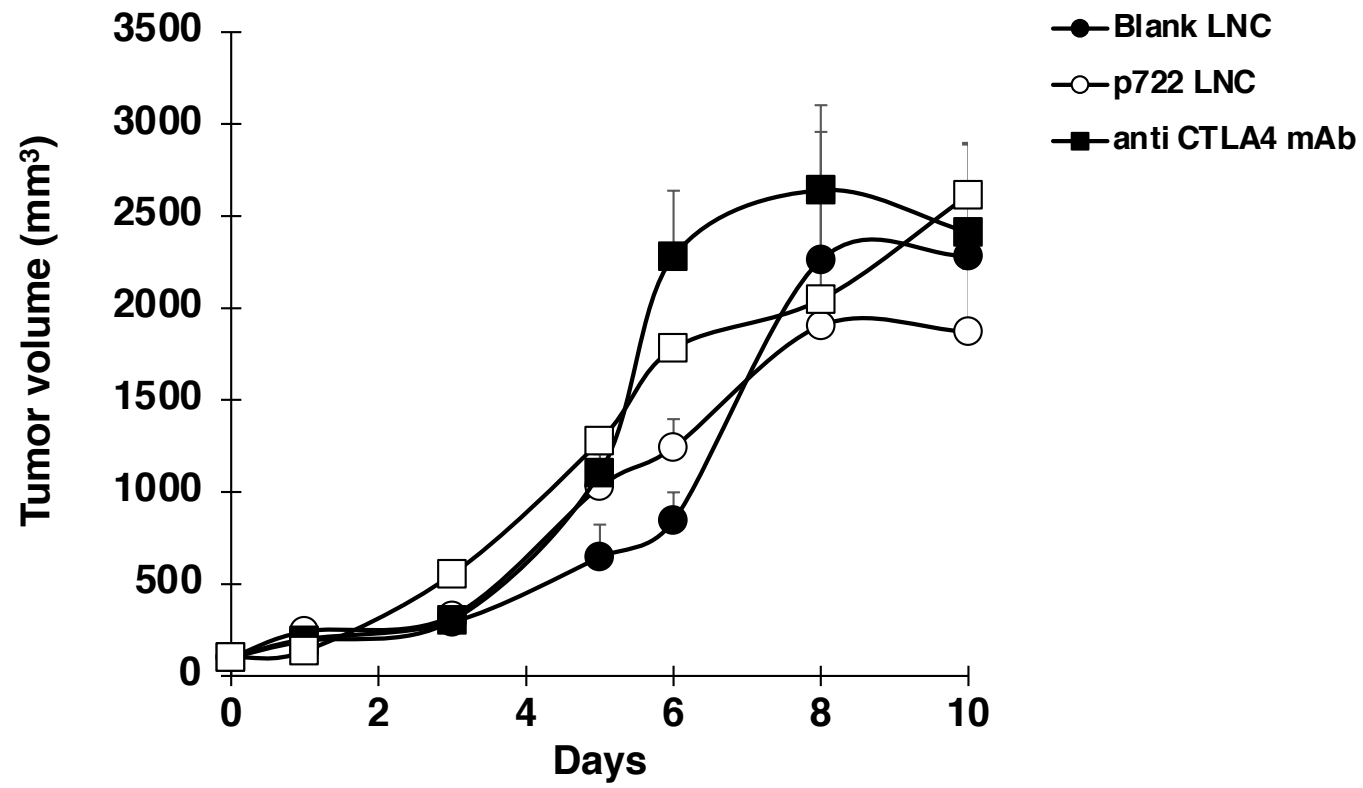
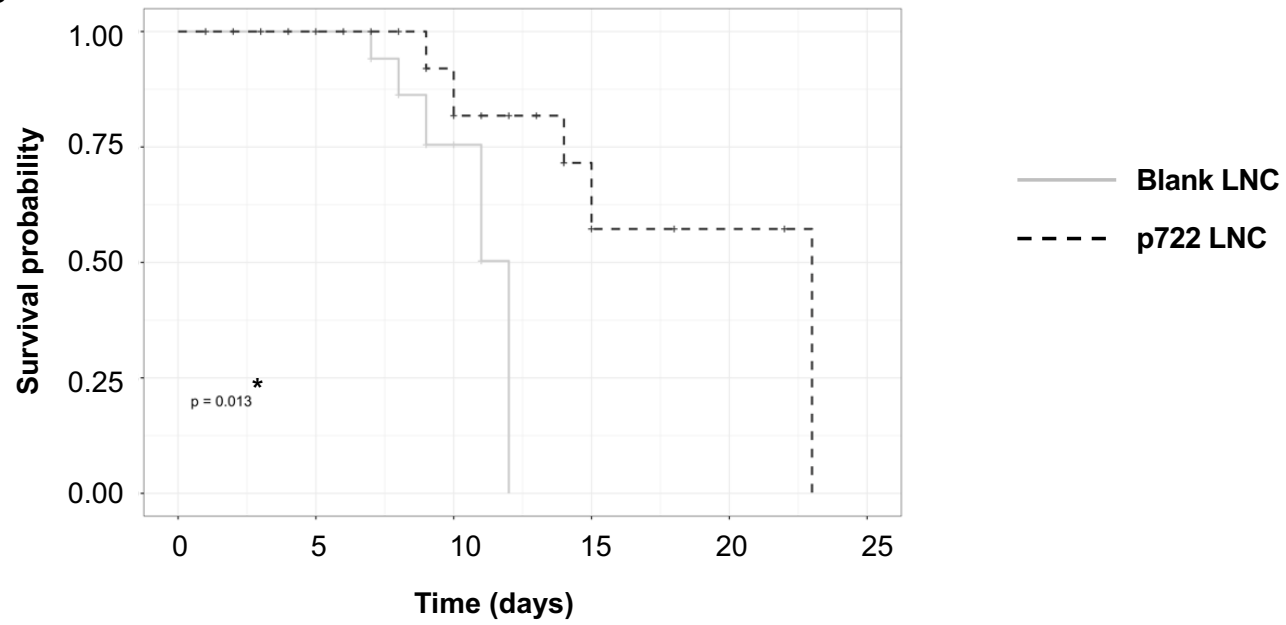


Figure 3

B



C

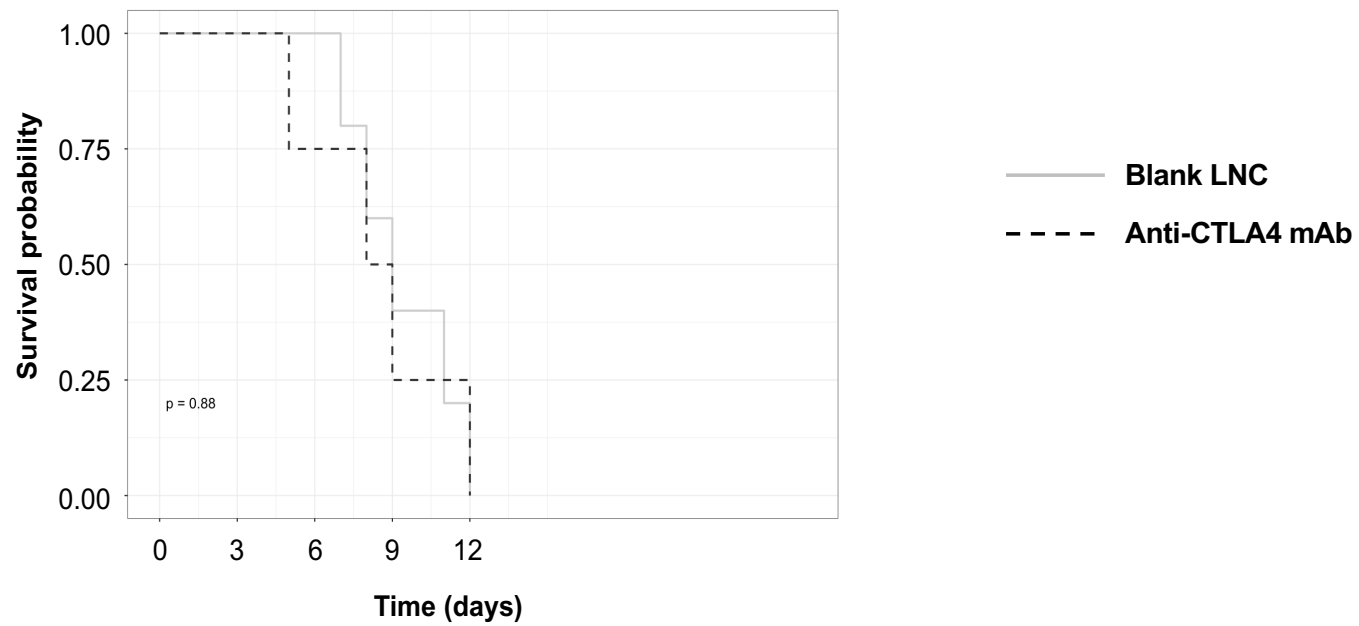


Figure 3

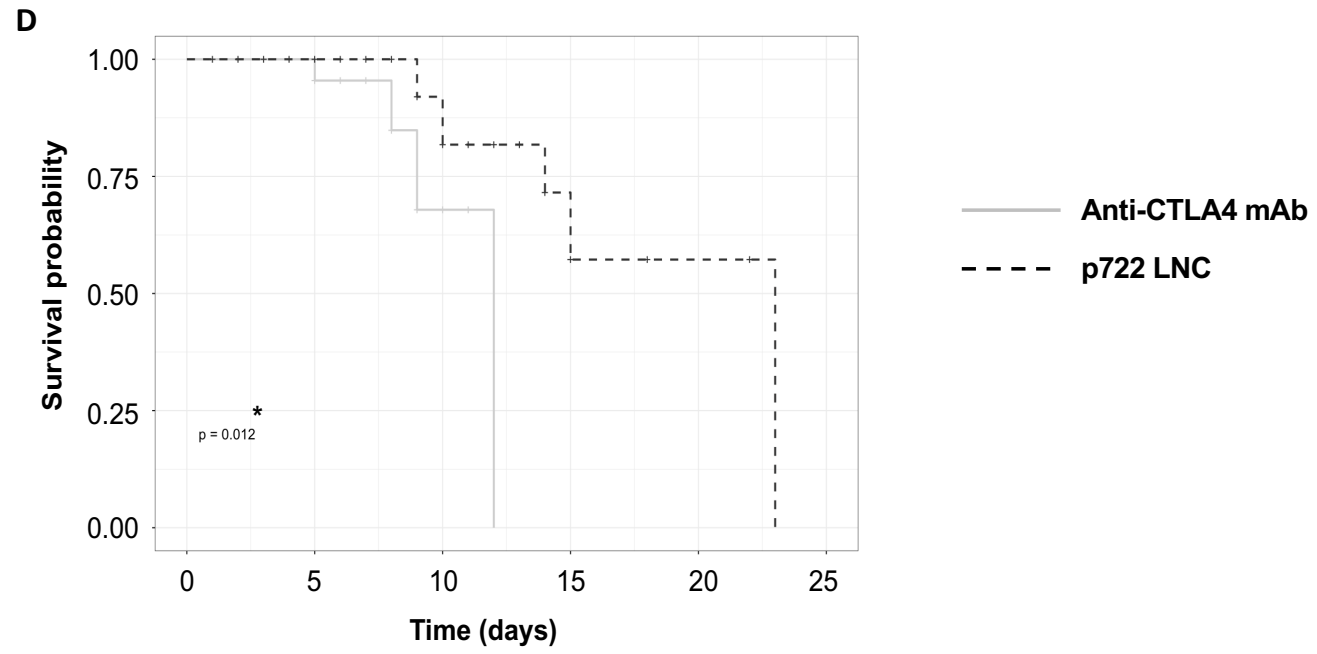
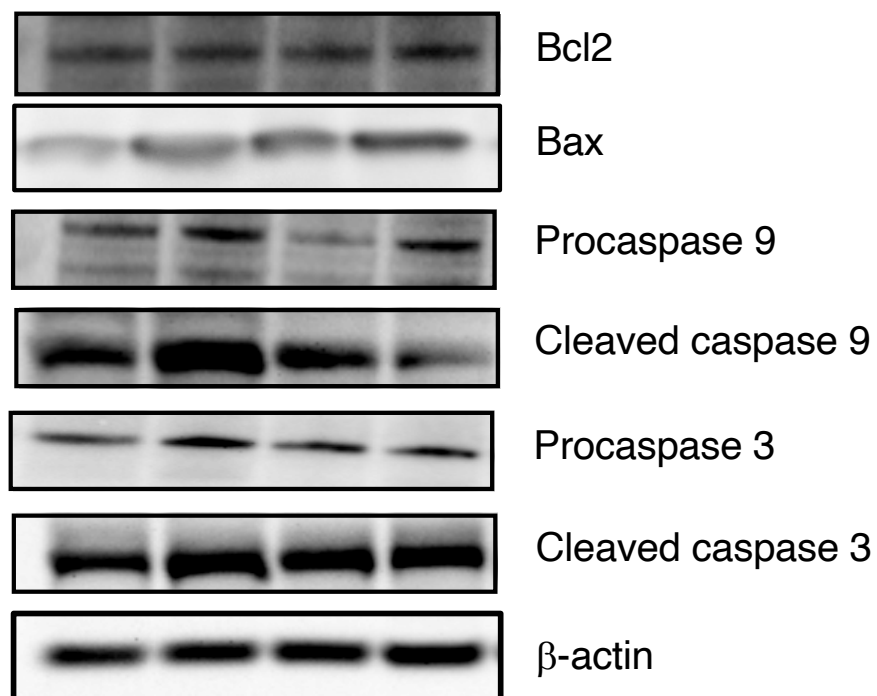


Figure 4



p722 LNC - + - +
_manti-CTLA4 - - + +

	Control	p722 LNC	_m anti-CTLA4	p722 LNC _m anti-CTLA4
BCI2	99.29 +/- 4.27	120.60 +/- 1.17	117.16 +/- 6.32	114.00 +/- 19.03
Bax	99.57 +/- 7.25	127.00 +/- 18.61	102.28 +/- 19.77	91.85 +/- 30.23
Procaspase 9	107.75 +/- 17.57	126.28 +/- 5.52 ^a	115.71 +/- 23.22	103.5 +/- 25.81
Cleaved caspase 9	115.25 +/- 20.41	171.00 +/- 17.30 ^a	95.00 +/- 19.00	63.66 +/- 22.81
Procaspase 3	86.61 +/- 6.54	115.00 +/- 7.22 ^a	86.66 +/- 10.92	98.66 +/- 16.48
Cleaved caspase 3	98.07 +/- 7.71	236.57 +/- 66.95 ^a	166.28 +/- 48.72	199.28 +/- 99.22

Figure 5

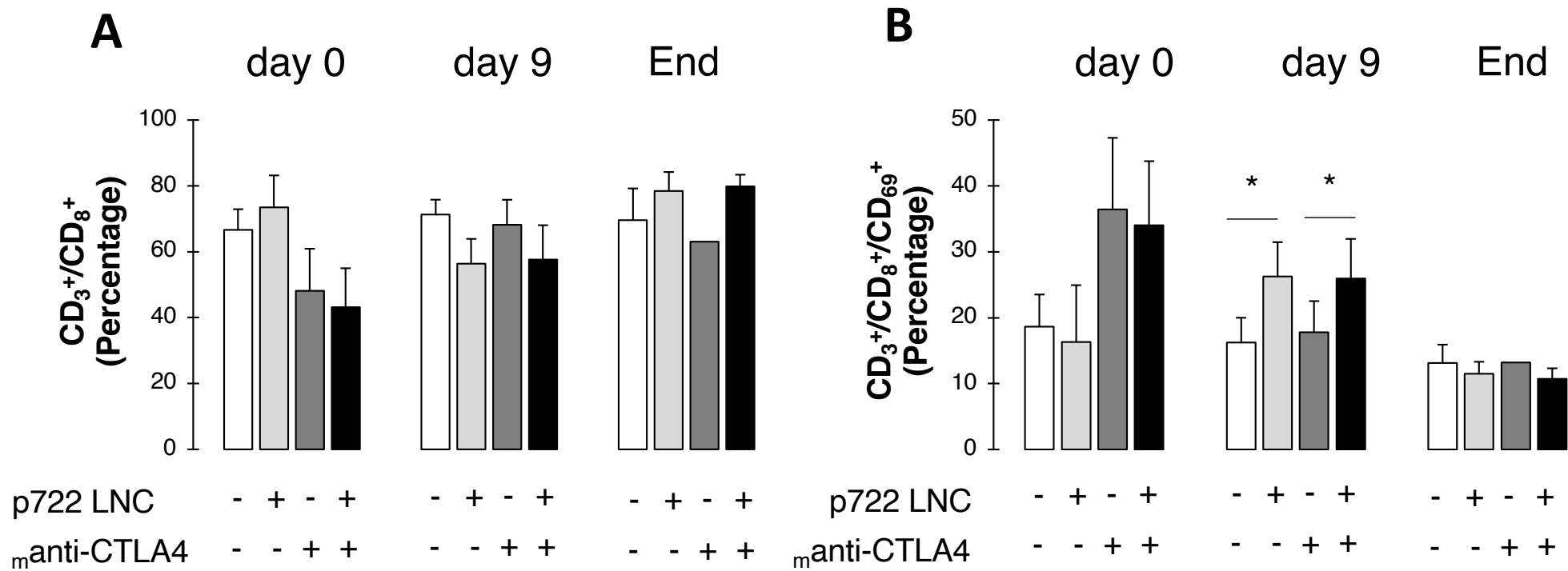


Figure S1

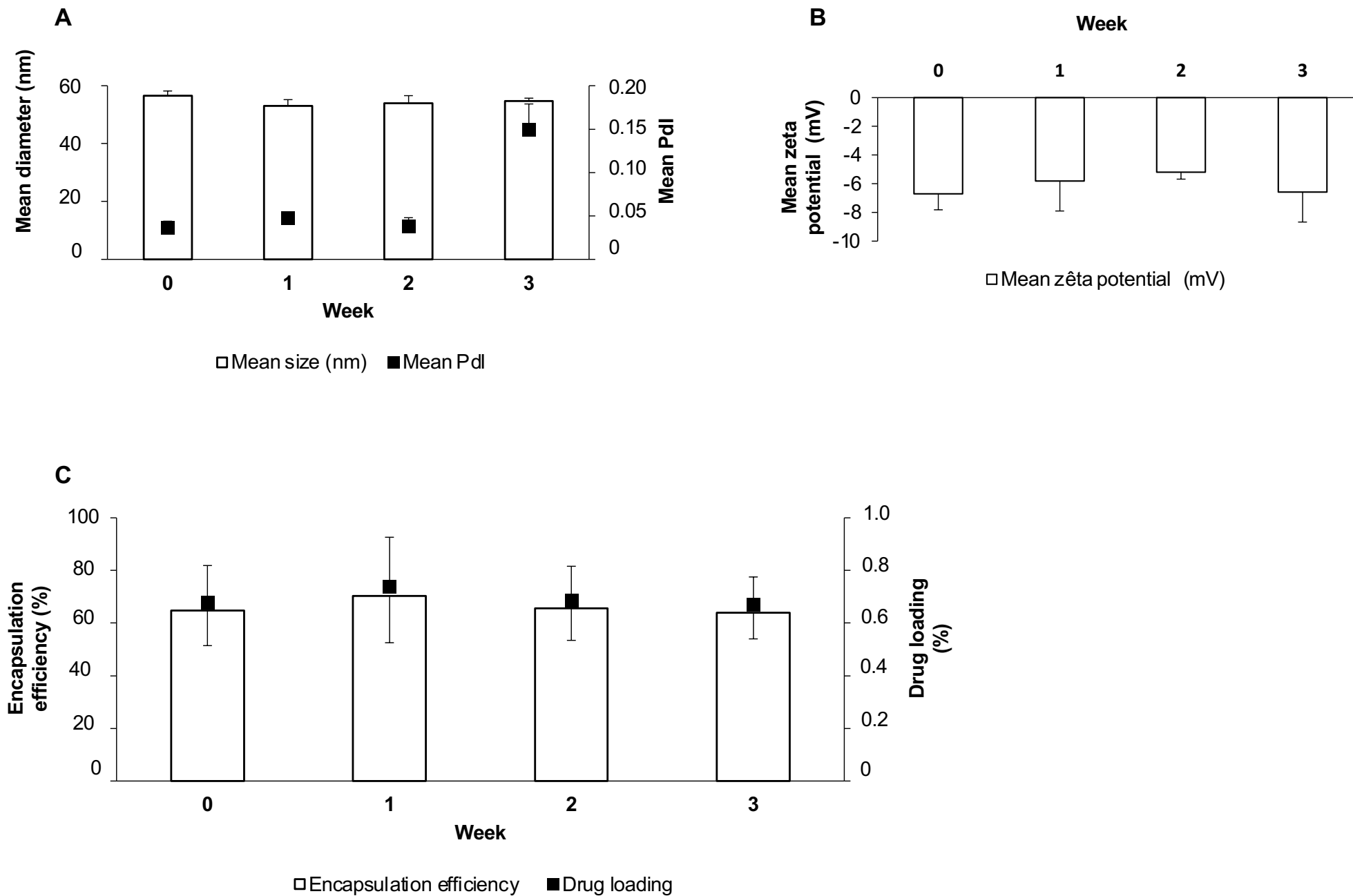
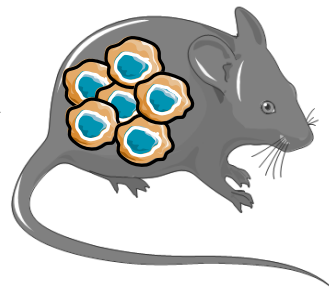
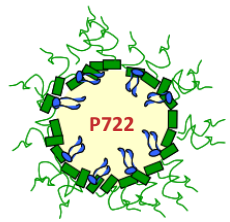


Table 1

	Mean diameter (nm)	Mean Pdl	Mean zeta potential (mV)	EE (%)	Drug loading (%)
Blank pegylated-LNC	72.4 +/- 0.8	0.12 +/- 0.01	-29.0 +/- 1.2	/	/
p722 pegylated-LNC [2.25 mM]	70.0 +/- 1.0	0.15 +/- 0.01	-13.5 +/- 0.5	64.8 +/- 13.4	0.7 +/- 0.1



Improved mice
survival



Potentiation of intrinsic
apoptotic pathway

Activation of CD₈⁺ T
lymphocytes