

1 **A new potential nano-oncological therapy based on polyaminoacid nanocapsules**

2
3 Teresa Gonzalo ^{a,1,*}, Giovanna Lollo^{a,b,2,*}, Marcos Garcia-Fuentes^{a,b}, Dolores Torres^a, Juan Correa^c,
4 Ricardo Riguera^c, Eduardo Fernandez-Megia^c, Pilar Calvo^d, Pablo Avilés^d, Maria José Guillén^d,
5 Maria José Alonso^{a,b,†}

6
7 * Authors who equally contributed to this work; † Author for correspondence

8
9 *^aDepartment of Pharmaceutics and Pharmaceutical Technology, School of Pharmacy, Campus*
10 *Vida, University of Santiago de Compostela, 15782 Santiago de Compostela, Spain; ^bCenter for*
11 *Research in Molecular Medicine and Chronic Diseases (CIMUS) Campus Vida University of*
12 *Santiago de Compostela, 15782 Santiago de Compostela, Spain; ^cDepartment of Organic*
13 *Chemistry and Center for Research in Biological Chemistry and Molecular Materials, Campus*
14 *Vida, University of Santiago de Compostela, 15782 Santiago de Compostela, Spain; ^dPharmaMar*
15 *S.A., Avda de los Reyes, 1 Pol. Ind. La Mina 28770 Colmenar Viejo (Madrid) Spain.*

16
17 [€]Present address: Ambiox Biotech, Avda Buendia 11, 19005 Guadalajara (Madrid), Spain.

18 [‡] Present address: Université d'Angers, Angers F-49100, INSERM U1066 MINT, Micro et
19 Nanomédecines biomimétiques, IBS-CHU ANGERS, 4 rue Larrey, 49933 Angers Cedex 9,
20 France.

21
22 †Corresponding author: Maria J. Alonso

23 E-mail address: mariaj.alonso@usc.es
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49

50 **Abstract**

51 A critical objective in cancer therapy is to reduce the systemic toxicity through the modification of
52 the biodistribution of anticancer drugs. Herein, we disclose a new biodegradable nanocarrier,
53 polyglutamic acid (PGA) nanocapsules, and present the *in vivo* pharmacokinetics/toxicity proof-of-
54 concept for the anticancer drug plitidepsin. These novel nanocapsules were prepared using a
55 modified solvent displacement technique where the polyaminoacid was electrostatically deposited
56 onto the lipid core. The nanocapsules exhibited an average size of 200 nm, a negative zeta potential
57 and a great capacity for the encapsulation of plitidepsin (encapsulation efficiency above 90%). In
58 addition, the nanocapsules could be freeze-dried and showed an adequate stability profile upon
59 storage. Finally, the *in vivo* proof-of-concept studies performed in mice indicated that the
60 encapsulation provided the drug with a prolonged blood circulation and a significantly reduced
61 toxicity. In fact the maximum tolerated dose of the nanoencapsulated drug was more than 3 times
62 that of the reference formulation (Cremophor[®] EL plitidepsin solution). Overall, beyond the value
63 of this specific formulation, the work reported here represents the evidence of the potential of
64 polyaminoacid nanocapsules in nano-oncological therapy.

65
66
67 **Keywords:** nanomedicines, long-circulating nanocarriers, nanocapsules, polyglutamic acid, cancer.

68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90

91 **Introduction**

92 The clinical use of most anticancer drugs is associated to severe side effects and poor quality of life
93 for the patients. These effects are mainly related to the indiscriminate systemic biodistribution of
94 anticancer drugs and their accumulation in non-target tissues [1]. In addition, the excipients used for
95 formulating hydrophobic anticancer drugs may contribute to their systemic toxicity [2]. The
96 limitations of these conventional formulations have stimulated an intensive search for nanocarriers
97 capable of reducing the toxicity of anticancer drugs [3, 4]. The advances achieved so far are
98 illustrated by the commercialization and clinical development of a significant number of
99 formulations based on liposome [5], nanoparticle [6, 7], conjugates [8] and micelle technologies [9].
100 However, the need to further improve the efficacy/toxicity profile of anticancer drugs, and thus the
101 necessity to develop alternative delivery vehicles, persists.

102 Within this frame the design of polymer nanocapsules might, in our understanding, offer specific
103 advantages [10]. First, the oily core of polymeric nanocapsules is an ideal environment for the
104 encapsulation of hydrophobic antitumor drugs at high payload [11, 12]. Second, the polymeric shell
105 can be conveniently designed in order to improve the biodistribution profile of the nanocapsules,
106 either extending the half-life of the encapsulated drug or even targeting the drug to specific cells
107 [13]. Third, nanocapsules can be formulated to be stable over the time and also to be freeze-dried in
108 order to further prolong their stability profile [11]. Altogether these positive features render
109 nanocapsules as an attractive formulation strategy for improving the efficacy/toxicity balance of
110 anti-cancer drug candidates.

111 Among the different polymers described until now for the design of anticancer drug nanocarriers,
112 PEG and its derivatives have been the most widely investigated [14]. The PEG hydrophilic
113 character makes it a suitable material for preventing the uptake of nanocarriers by the mononuclear
114 phagocytic system (MPS) and enhancing the circulation time of associated drugs [15]. However,
115 current efforts are being conducted to the search for new polymers, which might provide the
116 nanocarriers with specific advantages beyond the long circulating properties. In this regard,
117 polyaminoacids, and in particular polyglutamic acid (PGA), are at the front line of attention because
118 of their attractive safety profile. PGA, an anionic polyaminoacid composed of naturally occurring
119 L-glutamic acid linked by peptide bonds, is known to be biocompatible and biodegradable [16, 17].
120 Moreover, its long-circulating behaviour and enhanced accumulation at the tumour site, has been
121 made evident for PGA-paclitaxel[®] conjugates, currently in phase III clinical trials [18, 19].
122 Alternatively, cisplatin loaded micelles consisting of PGA and methoxypoly(ethylene glycol) are
123 being investigated for NSCL lung cancer showing promising antitumor results [20].

124 Taking this information into account, the main goal of this work has been to design and develop a
125 new nanocapsule-type delivery platform based on the use of PGA. Besides the long circulating
126 properties, the specific advantages expected for PGA nanocapsules rely on their theoretical ability
127 to encapsulate significant amounts of hydrophobic drugs and also on their enhanced *in vitro* and *in*
128 *vivo* stability. We have also compared the behaviour of PGA nanocapsules with that of
129 polyethylenglycol-grafted PGA (PGA-PEG) nanocapsules in order to establish the effective value
130 of PGA as a material for the development of long-circulating nanocapsules [21, 22]. The potential
131 of this new nanotechnology platform has been assessed using the anticancer drug plitidepsin.
132 Plitidepsin is a highly hydrophobic cytostatic active ingredient originally isolated from the marine
133 tunicate Aplidium Albicans, and now manufactured synthetically by PharmaMar S.A. as a potential
134 treatment for a variety of cancers [23]. Following extensive *in vitro* characterization of the unloaded
135 and plitidepsin-loaded PGA and PGA-PEG nanocapsules, we assessed the *in vivo* proof-of-principle
136 for their capacity to improve the maximum tolerated dose and the pharmacokinetic parameters of
137 the drug.

138
139 **Materials and Methods**

140 **Chemicals**

141 Plitidepsin was kindly provided by PharmaMar S.A. (Spain). Poloxamer (Pluronic F-68®),
142 benzalkonium chloride (BKC) and poly-L-glutamic acid (PGA) (Mw 15-50 KDa) were purchased
143 from Sigma-Aldrich (Spain). Miglyol®812, which is a neutral oil formed by esters of caprylic and
144 capric fatty acids and glicerol, was donated by Sasol Germany GmbH (Germany).
145 Poly(ethyethylene glycol)-stearate of a degree of polymerization of 40 hence forth designated as
146 PEG 40-stearate [Simulsol M52] (Seppic, France). The surfactant Epikuron 170, which is a
147 phosphatidylcholine-enriched fraction of soybean Lecithin, was donated by Cargill (Spain).
148

149 **Synthesis of PGA-PEG**

150 PGA (100 mg, 0.662 mmol of repetition unit, M_n 10,900 by multi angle laser light scattering, degree
151 of polymerization 72) and MeO-PEG-NH₂ (43.6 mg, 8.4 μ mol, M_n 5,219, M_w 5,242 by MALDI-
152 TOF) were dissolved in H₂O (2 mL). 1-Hydroxy-benzotriazole (11 mg, 84 μ mol) and 1-ethyl-3-(3-
153 dimethylaminopropyl) carbodiimide hydrochloride (13 mg 84 μ mol) were added and the reaction
154 was allowed to stir overnight. The resulting product was purified by ultrafiltration (Amicon YM30,
155 15 x 50 mL H₂O) to obtain final PGA-PEG (degree of PEGylation 1.17 % by ¹H NMR, 87 % yield,
156 24% w/w of PEG). ¹H NMR (500 MHz, D₂O): δ 4.51-4.16 (m, 72 H), 3.89-3.57 (m, 523 H), 3.43
157 (s, 3.5H), 2.65-1.84 (m, 288H).
158

159 **Preparation of PGA and PGA-PEG nanocapsules**

160 PGA nanocapsules were prepared by the solvent displacement technique [24]. Briefly, the organic
161 phase composed of plitidepsin (1.2 mg), 0.125 mL Miglyol® 812, 7 mg of the cationic surfactant
162 BKC, 30 mg Epikuron 170 in 0.5 mL of ethanol and 9 mL acetone was added onto an aqueous
163 phase composed of the non-ionic surfactant Pluronic 188 (0.25% w/v) and the polymer PGA or
164 PGA-PEG (10 mg). Nanocapsules were formed immediately upon the mixture of both phases. The
165 organic solvents were evaporated under vacuum. Unloaded nanocapsules were prepared by the
166 same method, in absence of plitidepsin in the organic phase.

167 Nanoemulsions and PEG-coated nanoemulsions were also prepared and used as controls in order to
168 prove the value of the polymeric coating. They were obtained by the same technique as described
169 above, but without adding PGA or PGA-PEG to the external water phase. Instead, PEG-surface
170 modified nanoemulsions were formed by including 6.42 mg of PEG-stearate to the organic phase,
171 the calculated amount of modified lipid required for having the same amount of PEG than with the
172 PGA-PEG coating.
173

174 **Physicochemical Characterization of PGA and PEG-PGA nanocapsules**

175 PGA and PGA-PEG nanocapsules were characterized with regard to size, zeta potential and
176 morphology. Particle size and polydispersity index were determined by photon correlation
177 spectroscopy (PCS) after dilution with bi-distilled water. Analyses were carried out at 25°C with an
178 angle detection of 173°. The zeta potential values were calculated from the mean electrophoretic
179 mobility values, as determined by laser Doppler anemometry (LDA). For LDA measurements,
180 samples were diluted with KCl 1mM and placed in an electrophoretic cell. PCS and LDA analysis
181 were performed in triplicate using a NanoZS® (Malvern Instruments, Malvern, UK).

182 The morphology of nanocapsules was studied by Transmission Electron Microscopy (TEM) using a
183 Philips CM-12 (FEI Company, Eindhoven, The Netherlands), following negative staining with a
184 phosphotungstic acid solution (2%, w/v) and immobilization on copper grids with Formvar®.
185

186 **Plitidepsin encapsulation and release studies**

187 The encapsulation efficiency of plitidepsin in the nanocapsules was determined by the difference
188 between the amount of plitidepsin in the supernatant and the total amount in the nanocapsules.
189 Plitidepsin content in the supernatant was established upon isolation of the drug from the
190 nanocapsules by ultrafiltration in Amicon columns (Amicon Ultra-4, 100000MWCO, Millipore,

191 Spain). Then, samples of the supernatants or the nanocapsule suspension (for the total plitidepsin
192 content) were dissolved in acetonitrile and analysed by HPLC.

193 The *in vitro* drug release from the nanocapsules was performed in PBS (0.01 M) with 4% bovine
194 serum albumin (BSA) under sink conditions. The concentration of plitidepsin in the medium was 1
195 µg/mL which corresponds to sink conditions [25]. Samples were incubated at 37°C and withdrawn
196 at appropriate time intervals (15 min, 1 h, 3 h, 6 h and 24 h). Total plitidepsin content was
197 determined by HPLC after dissolving a portion of each sample in acetonitrile, followed by mild
198 centrifugation (3 min, 4000 g) to precipitate suspended proteins. Released plitidepsin was calculated
199 upon isolation of the free drug by ultracentrifugation (27400 g, 1 h, 15°C). The supernatant was
200 then analysed by HPLC following the same treatment described above for total plitidepsin content.
201 The HPLC system consisted of an Agilent 1100 series instrument equipped with UV detector set at
202 225 nm. The analytic method for plitidepsin quantification has been previously reported by
203 PharmaMar S.A. [26].
204

205 **Stability of plitidepsin-loaded PGA nanocapsules during storage**

206 The stability of plitidepsin-loaded PGA nanocapsules was evaluated under storage conditions for 8
207 weeks at 4°C, room temperature and 37°C. Three parameters were assessed at different time points:
208 (i) macroscopic aspect (presence of aggregated, cream formation, changes in color, etc.); (ii)
209 particle size, polydispersity and zeta potential; (iii) plitidepsin concentration in the preparation and
210 encapsulation efficiency. All these characteristics were determined as described above.
211

212 **Freeze-drying studies of plitidepsin-loaded PGA nanocapsules**

213 Blank and plitidepsin-loaded PGA nanocapsules at different concentrations between 1 and 0.5%
214 w/v were freeze-dried by immersion in liquid nitrogen in the presence of trehalose (10% w/v). The
215 freeze-drying programme consisted in an initial drying step at -35°C, and secondary drying where
216 temperature was finally equilibrated at 20°C over a period of 60 h (Labconco Corp., USA). PGA
217 nanocapsules were resuspended by adding 1 mL of ultrapure water to the freeze-dried cake
218 followed by gentle agitation. The size and polydispersity of the resuspended nanocapsules was
219 evaluated by PCS.
220

221 ***In vivo* studies**

222 **Animals**

223 Studies were performed with CD-1 male mice (Harlan Interfauna Iberica S.L., Barcelona, Spain),
224 housed Makrolon cages (10 animals/cage). Animals were subjected to preliminary observation and
225 to an acclimatisation period. The animal house was maintained at 21-23°C, with 35-55% relative
226 humidity. Illumination was controlled to allow for 12 hours of light and 12 hours of darkness. All
227 animals were observed for morbidity/mortality throughout the whole assay.
228

229 **Pharmacokinetic evaluation**

230 Pharmacokinetic studies of plitidepsin were performed upon i.v. administration of different
231 formulations to CD-1 mice (n=36). The formulations tested were: PGA nanocapsules, PGA-PEG
232 nanocapsules, nanoemulsions and PEG-coated nanoemulsions. Mice of 20-25 g weight were
233 selected for these studies. A volume of 250 µl of the different plitidepsin formulations were injected
234 in the lateral vein of the tail. The injected plitidepsin dose was 0.1 mg/kg for nanoemulsion and
235 PEGylated nanoemulsion and 0.4 mg/kg for PGA and PGA-PEG nanocapsules. Blood samples
236 were collected in EDTA microtubes at the following times postinfusion: 5 min, 15 min, 30 min, 1 h,
237 3 h, 6 h, 24 h and 48 h. The samples were centrifuged at 4000 g for 15 minutes at approximately
238 5°C. The resulting plasma was frozen at -20°C until analysis by HPLC-MS/MS.

239 Plitidepsin concentrations were quantified by HPLC-MS/MS after solid-liquid extraction with a
240 mixture of tert-butyl methyl ether (TBME): hexane (1:1, v/v). The pharmacokinetic parameters of
241 plitidepsin were performed using a non-compartmental pharmacokinetic method with a

242 WinNonlin™ Professional Version 4.01 (Pharsight Corporation, Mountain View, CA, USA). The
243 AUC values given are normalized to the dose given.

244

245 **Toxicological evaluation**

246 The Maximum Tolerated Dose (MTD) of different formulations was evaluated after i.v.
247 administration. The formulations tested were: plitidepsin-loaded PGA nanocapsules, PGA-PEG
248 nanocapsules, nanoemulsions, PEG-coated nanoemulsions and the reference formulation
249 (Cremophor® EL/Ethanol/Water 15/15/70 w/w/w solution). The formulations at different plitidepsin
250 doses (0.2-1 mg/kg) were administered as a single i.v. bolus in the lateral vein of the tail. Groups of
251 8 animals were used for each dose level. A control group consisting of 8 animals was administered
252 with non-loaded nanocapsules, to evaluate potential toxicity. The animals were weighted at the start
253 of the study, twice a week, and before being sacrificed. Mortality checks were performed at least
254 once a day during the whole assay (14 days). Any mouse showing signs of extreme weakness,
255 toxicity or in a moribund state was sacrificed. The animals were monitored at least once a day
256 during the whole assay and any clinical responses were carefully noted. The observations included
257 changes in weight, skin and fur, eyes and mucous membranes, respiratory, circulatory, central
258 nervous and autonomic nervous systems, somatomotor activity and behavior.

259

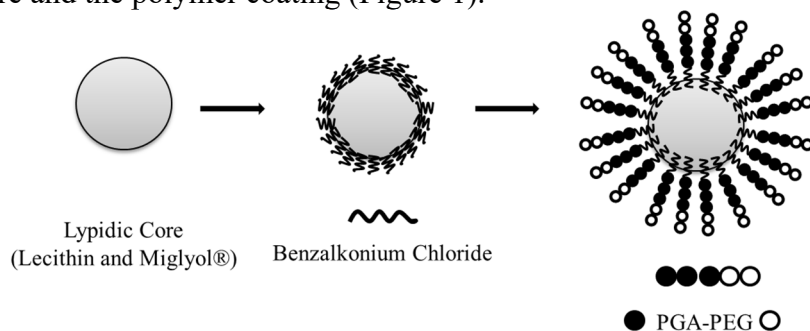
260 **Results and Discussion**

261 This article describes for the first time the design and development of a novel drug nanocarrier
262 based on PGA nanocapsules. The rationale for the selection of PGA for the formation of the
263 nanocapsule's shell was its biocompatibility and its potential ability to provide stealth properties to
264 the carrier [17, 27, 28]. As an alternative, a PEG-grafted PGA (PGA-PEG) copolymer was also
265 investigated. Herein, we discuss the development and physicochemical characterization of these
266 nanocarriers, their capacity for the encapsulation and delivery of the anticancer drug plitidepsin and,
267 finally, their ability to modify the toxicity and pharmacokinetics profiles of this drug.

268

269 **Preparation and characterization of unloaded and plitidepsin-loaded PGA and PGA-PEG 270 nanocapsules**

271 Nanocapsules were obtained according to a modified solvent displacement technique where the
272 coating polymer is deposited onto the oily core by electrostatic interaction. A similar approach has
273 been reported by our group for the formation of positively charged chitosan nanocapsules [24, 29].
274 However, in this case, it was necessary to define a technical approach that would facilitate the
275 interaction between the negatively charged oily core and the acidic polymer coating. This approach
276 was based on the use of cationic surfactants, which may potentially act as bridges between the oily
277 core and the polymer coating (Figure 1).



279 **Figure 1:** Illustration of the structure of PGA and PGA-PEG nanocapsules.

280

281 In a first instance, we explored a variety of poloxamines (Tetronic 908, Tetronic 904, Tetronic and
282 901, BASCOM, Brussels, Belgium, HLB = 2.5, 14.5 and 30.5, respectively) because of the
283 presence of amine groups in their polymer backbone, which could potentially become protonated.
284 However, irrespective of the amount of poloxamine, we found that the introduction of increasing

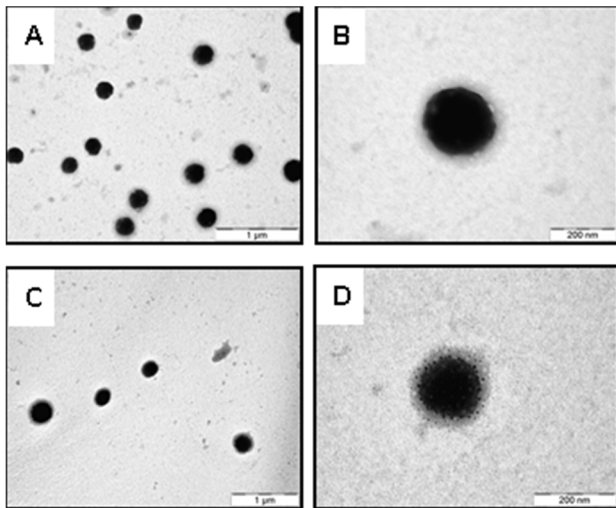
285 amounts of poloxamine in the system did not lead to a positive zeta potential nanoemulsions. In a
 286 second series of studies, we explored the utility of stearylamine, a cationic phospholipid extensively
 287 used in the formulations of liposomes and emulsions [30]. Unfortunately, under a number of
 288 different processing conditions (involving varying volumes of organic/aqueous phase, heating
 289 organic phase at 70°C, or using ethanol, ethyl acetate or dichloromethane as organic solvents) this
 290 surfactant led to the formation of aggregated particles rather than nanocapsules. Lastly, we studied
 291 the behavior of surfactants such as benzalkonium chloride and cetylpyridinium chloride, which had
 292 already been used in the formation of nanoparticles [31]. The introduction of these surfactants led to
 293 the formation of positively charged surfaces, which facilitated the further adhesion of the PGA shell
 294 while maintaining the small size and stability of the oily nanodroplets.

295 The mean particle size of unloaded PGA and PGA-PEG nanocapsules prepared using benzalkonium
 296 chloride was approximately 200 nm, corresponding to a monomodal and narrow size distribution
 297 (polydispersity index=0.1). Both unloaded PGA and PGA-PEG nanocapsules exhibited a negative
 298 charge (-40 mV and -28 mV respectively), whereas the control nanoemulsion had a high positive
 299 charge +38 mV). This charge inversion is an indication of the electrostatically-driven formation of
 300 the PGA shell. The reduced negative charge observed for the PEGylated nanocapsules could be
 301 associated to the known PEG shielding effect [32]. In addition to the nanocapsules and control
 302 nanoemulsion, a PEGylated nanoemulsion of the same composition as the control but containing
 303 PEG-stearate was also formulated for further comparative analysis. As expected, the introduction of
 304 PEG in the same amount as in the PGA backbone led to a reduction of the positive charge (+26
 305 mV), although in this case no charge inversion was observed. Plitidepsin-loaded nanocapsules
 306 showed similar physicochemical properties compared to the unloaded ones (Table 1).
 307

308 **Table 1:** Characterization of size and zeta potential of unloaded and plitidepsin-loaded
 309 nanocapsules and nanoemulsions (Mean \pm S.D.; n=3). E.E.: Encapsulation efficiency; P.I:
 310 Polydispersity Index, NCs: nanocapsules; NE: nanoemulsion.

Prototype	Plitidepsin conc. (mg/mL)	Size (nm)	P.I.	Zeta potential (mV)	E.E. (%)
NE	-	207 \pm 7	0.1	+38 \pm 1	-
	0.12	203 \pm 7	0.1	+40 \pm 1	95
PEG NE	-	200 \pm 3	0.1	+26 \pm 1	-
	0.12	203 \pm 5	0.1	+28 \pm 3	98
PGA NCs	-	202 \pm 5	0.1	-40 \pm 5	-
	0.12	183 \pm 6	0.1	-38 \pm 1	99
PEG-PGA NCs	-	191 \pm 4	0.1	-28 \pm 4	-
	0.12	201 \pm 5	0.1	-28 \pm 3	98

311 On the other hand, TEM images confirmed the values of particle size for PGA and PGA-PEG
 312 nanocapsules measured by PCS, and the homogeneity of the particle size distribution (Figure 2).
 313 Moreover, TEM images provided evidence of the rounded and regular morphology as well as the
 314 core-shell type of structure.
 315



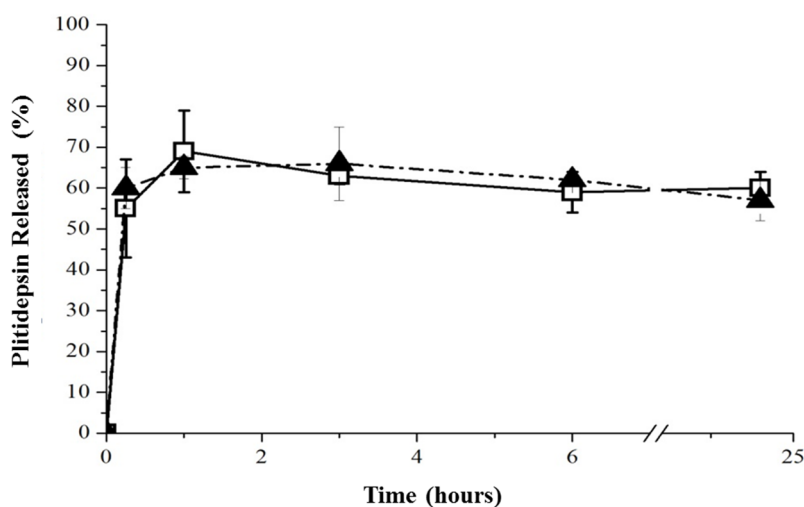
316

317 **Figure 2:** TEM images of PGA and PGA-PEG nanocapsules containing plitidepsin. PGA
 318 nanocapsules (A, B); PGA-PEG nanocapsules: (C, D).

319

320 **Plitidepsin encapsulation and release from PGA and PGA-PEG nanocapsules**

321 The encapsulation efficiency of plitidepsin into PGA and PEG-PGA nanocapsules was very high
 322 (98-99%, final loading 0.54 % weight of plitidepsin/weight of total components) (Table 1). This
 323 result was attributed to the great affinity of the drug for the selected oily core. The release rate of
 324 the drug was also monitored upon encapsulation of PGA and PGA-PEG nanocapsules in simulated
 325 biological media (PBS with BSA 4% w/w) at 37°C, under sink conditions. Despite de fact that
 326 nanocapsules were stable in this medium (data not shown), the results in Figure 3, indicate that
 327 PGA and PEG-PGA nanocapsules released 60% of their cargo during the first hour and no further
 328 release was observed for the remaining time of the experiment (24 h). This biphasic release profile,
 329 has been previously observed for other types of nanocapsules [11, 33]. The initial burst has been
 330 typically associated to the partition of the drug between the oily cores and the great volume of the
 331 external aqueous phase. Even though these results cannot be extrapolated to the *in vivo* situation,
 332 the fact that a significant fraction of the drug remained encapsulated despite the “sink conditions”,
 333 is an indication of the high affinity of plitidepsin for the oily core and/or the shell of the
 334 nanocapsules. Finally, the presence of PEG in the nanocapsules shell did not affect the release
 335 properties of the nanocarriers (Figure 3).

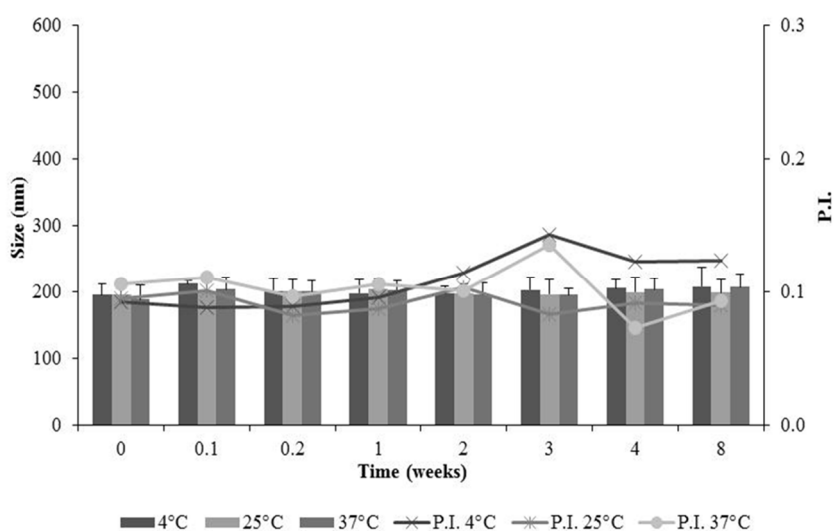


336

337 **Figure 3:** *In vitro* drug release from plitidepsin-loaded nanocapsules in PBS with BSA (4% w/w)
 338 medium. PGA nanocapsules (□); PGA-PEG nanocapsules (▲). (Mean ± S.D.; n=3).

339 **Stability of plitidepsin-loaded PGA nanocapsules upon storage.**

340 The stability of plitidepsin-loaded PGA nanocapsules was assessed upon storage at different
341 temperatures (4°C, room temperature and 37°C) for up to 8 weeks. The parameters determined at
342 different time points were: physicochemical properties, i.e., particle size, polydispersity and zeta
343 potential, and plitidepsin stability and encapsulation efficiency. The preservation of the
344 nanocapsules particle size and polydispersity is critical to ensure that the nanocarrier maintains the
345 biodistribution properties [34]. Finally, the stability of plitidepsin and its encapsulation efficiency
346 are important to ensure reproducible pharmacokinetics and pharmacological potency over time.
347 The particle size evolution of PGA nanocapsules over the time is shown in Figure 4. At 4°C and
348 room temperature, no significant differences on the mean particle size and polydispersity index of
349 plitidepsin-loaded PGA were observed during the 8-week study. Zeta potential determinations
350 performed in the different samples confirmed that the surface electrical charge of the nanocarriers
351 did not change over time, another indication of chemical and physicochemical stability of the
352 system (data not shown).



353 **Figure 4:** Stability upon storage of plitidepsin-loaded PGA nanocapsules at different temperatures.
354 (Mean ± S.D.; n=3) P.I: Polydispersity Index.
355
356

357 Finally, with regard to the stability of the encapsulated plitidepsin, the results showed that after an 8
358 week-storage period at 4°C, the drug content and the encapsulation efficiency of the formulation
359 were maintained. Overall these results indicate that PGA nanocapsules have excellent stability
360 characteristics.
361

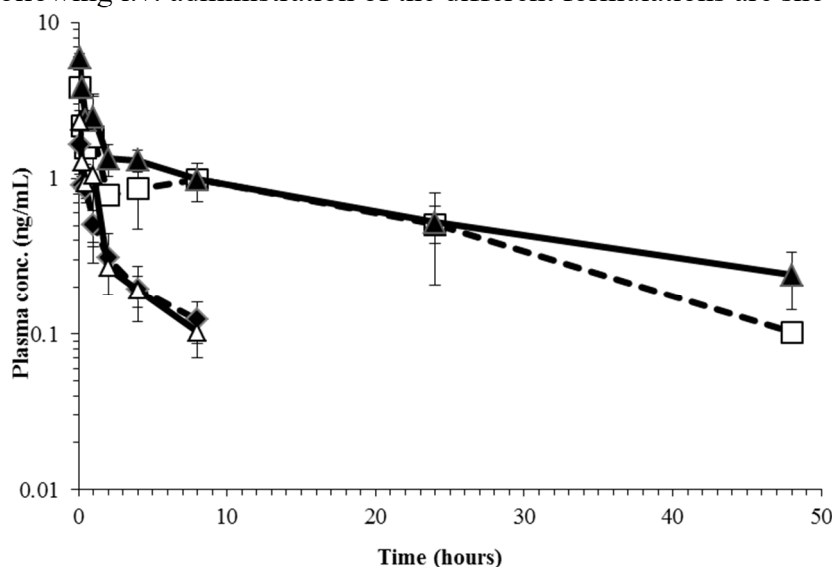
362 **Freeze-drying studies of plitidepsin-loaded PGA nanocapsules**

363 For better handling and for storing for prolonged periods of time, a freeze-dried formulation of PGA
364 nanocapsules was developed. Non-loaded and plitidepsin-loaded PGA nanocapsules suspensions at
365 different concentrations (1, 0.75, 0.5 % w/v final concentration) were mixed with trehalose (10%
366 w/v final concentration) and freeze-dried. The results indicated that PGA nanocapsules could be
367 freeze-dried at any of the concentrations tested with minimum changes in their particle size. The
368 same behaviour was observed for the formulations containing plitidepsin (data not shown).
369

370 **Pharmacokinetic evaluation**

371 As indicated, in this work plitidepsin was encapsulated into PGA and PGA-PEG nanocapsules with
372 the final objective of enhancing the plasma residence time of the drug, a necessary step in
373 promoting its passive targeting to solid tumors [35]. To assess this, the pharmacokinetic profile of
374 plitidepsin administered in the PGA and PGA-PEG nanocapsules formulations in comparison to

375 that of the control emulsions was studied in healthy mice. The plasmatic drug concentrations
 376 following i.v. administration of the different formulations are shown in Figure 5.



377
 378 **Figure 5:** Pharmacokinetic profiles of plitidepsin after i.v. administration to mice of plitidepsin-
 379 loaded PGA nanocapsules (\square), PGA-PEG nanocapsules (\blacktriangle), PEGylated nanoemulsion (\blacklozenge) and
 380 control anionic nanoemulsion (\triangle). (Mean \pm S.D.; n=3).

381
 382 It can be noted that plitidepsin plasmatic levels achieved after the administration of drug-loaded
 383 nanocapsules are much higher than those corresponding to the control emulsions, which were below
 384 the level of detection (0.1 ng/mL) after 8 hours of administration. Similar conclusions can be drawn
 385 from Table 2, which shows the pharmacokinetic parameters associated to all formulation groups.
 386 PGA and PGA-PEG nanocapsules showed higher half-life times, lower clearance and significantly
 387 higher mean residence time (MRT) than the control emulsions (nanoemulsion or PEGylated
 388 nanoemulsion). Besides this effect in the disposition parameters, it could also be noted that the
 389 volume of distribution of the nanocapsules was larger than that of the nanoemulsions. This increase
 390 in the volume of distribution has been associated to a prolonged drug plasmatic residence time and,
 391 thus, to a facilitated access to peripheral tissues and notably to those that are hypervascularized.
 392 This is critical in order to enhance the concentration of the drug in the tumoral areas [36].

393
 394 **Table 2:** Pharmacokinetic parameters of plitidepsin-loaded PGA, PGA-PEG nanocapsules,
 395 nanoemulsion and PEGylated nanoemulsion after a single i.v. administration to mice.

Prototype	$t_{1/2\beta}$ (h)	$AUC_{0 \rightarrow t}/Dose$ (ng·h/mL/mg)	CL_p (mL/min/kg)	$V_{d\beta}$ (L/kg)	MRT (h)
NCs PGA	13.3	69.32	224.8	258.7	17.0
NCs PGA-PEG	18.4	84.1	166.5	265.8	24.1
NE	4.5	25.0	507.5	197.9	5.2
PEG NE	4.2	29.9	462.8	168.5	3.9

396
 397 Overall, these parameters point out to a clear improvement in the pharmacokinetic behaviour of
 398 plitidepsin when encapsulated into PGA nanocapsules. This improvement in pharmacokinetics has
 399 already been observed for a variety of drug nanocarriers [7, 37-39] and has been related to the
 400 stealth properties of nanocarriers [35].
 401

402 **Toxicity study**

403 As pointed out in the introduction, improving the efficacy/toxicity ratio of current therapies is the
404 ultimate goal of anticancer nanomedicines. In this study, the toxicity of plitidepsin-loaded PGA and
405 PGA-PEG nanocapsules was evaluated upon i.v. administration to healthy mice. Plitidepsin-loaded
406 nanoemulsion and plitidepsin-loaded PEGylated nanoemulsion were also studied to compare with
407 PGA and PGA-PEG coated systems. Plitidepsin in the reference formulation (Cremophor[®] EL/
408 ethanol/ water 15/15/70 w/w/w) was studied as a benchmark. The toxicity for all groups was
409 quantified by comparing the maximum tolerated dose (MTD), defined as the maximum plitidepsin
410 dose resulting in less than 15% loss in body weight and that does not cause lethality.

411 The results indicated that the MTD for plitidepsin in the reference formulation was 0.3 mg/kg. In
412 contrast, the MTD for plitidepsin in PGA nanocapsules was above the maximum dose administered
413 in this study (1 mg/kg), whereas for PGA-PEG nanocapsules, nanoemulsion and PEGylated
414 nanoemulsion, the MTD was 0.9, 0.9 and 0.95 mg/kg, respectively. Furthermore, no toxicity was
415 observed upon administration of the unloaded nanocapsules. From these results it can be inferred
416 that the maximum toxicity reduction was achieved for plitidepsin-loaded PGA nanocapsules (MTD
417 more than 3 times higher than that of the reference formulation) and that the PEGylated
418 formulations behaved similarly to the reference nanoemulsion. The limited efficacy of the
419 PEGylated formulations as compared to a reference nanoemulsion could be seen as a controversy
420 with the pharmacokinetic profiles and requires further investigation. A hypothesis for the reduced
421 toxicity of the nanoemulsion formulation despite its rapid blood clearance could be related to the
422 potential slow release properties of the nanoemulsion. Despite of this, overall these data evidence
423 the reduced toxicity of the nano-oncological compositions developed as compared to the standard
424 formulation approaches [40].

426 **Conclusions**

427 Herein we report for the first time a novel nanocarrier consisting of polyaminoacid nanocapsules
428 and the pharmacokinetics/toxicity proof-of-principle for the anticancer drug plitidepsin. Besides
429 their optimum pharmaceutical properties (easy production and stability), these nanocapsules
430 exhibited highly improved biodistribution and toxicity profiles compared to the plitidepsin
431 conventional formulation. Overall, this study highlights the promising potential of PGA
432 nanocapsules as delivery carriers systems for anticancer drugs.

434 **Acknowledgements**

435 Financial support from CENIT-NANOFAR XS53 project, PharmaMar, Spain, the Ministry of
436 Sciences and Innovation (CTQ2009-10963 and CTQ2009-14146-C02-02) and the Xunta de Galicia
437 (Competitive Reference Groups-FEDER funds Ref. 2010/18 and 10CSA209021PR, CN2011/037)
438 and European Commission FP7 EraNet - EuroNanoMed Program-Instituto Carlos III (Lymphotarg
439 proyect, Ref. PS09/02670). Giovanna Lollo has a fellowship from the Ministry of Education of
440 Spain. Marcos Garcia Fuentes acknowledges an Isidro Parga Pondal Fellowship from Xunta de
441 Galicia.

443 **References**

- 445 1. J.H. Park, S. Lee, J.-H. Kim, K. Park, K. Kim, I.C. Kwon, Polymeric nanomedicine for
446 cancer therapy, *Progress in Polymer Science*, 33 (2008) 113-137.
- 447 2. H. Gelderblom, J. Verweij, K. Nooter, A. Sparreboom, Cremophor EL: the drawbacks and
448 advantages of vehicle selection for drug formulation, *European Journal of Cancer*, 37 (2001)
449 1590-1598.
- 450 3. E. Blanco, A. Hsiao, A.P. Mann, M.G. Landry, F. Meric-Bernstam, M. Ferrari,
451 *Nanomedicine in cancer therapy: Innovative trends and prospects*, *Cancer Sci.*, 102 (2011)
452 1247-1252.

- 453 4. G. Lollo, G. Rivera-Rodriguez, D. Torres, M.J. Alonso, Oncologic nanotherapies: current
454 applications and future perspectives, *An. Real Acad. Nac. Farm.*, 77 (2011) 76-98.
- 455 5. S.M. Moghimi, J. Szebeni, Stealth liposomes and long circulating nanoparticles: critical
456 issues in pharmacokinetics, opsonization and protein-binding properties, *Progress in Lipid*
457 *Research*, 42 (2003) 463-478.
- 458 6. Y. Malam, M. Loizidou, A.M. Seifalian, Liposomes and nanoparticles: nanosized vehicles
459 for drug delivery in cancer, *Trends in Pharmacological Sciences*, 30 (2009) 592-599.
- 460 7. J. Hrkach, D. Von Hoff, M.M. Ali, E. Andrianova, J. Auer, T. Campbell, D. De Witt, M.
461 Figa, M. Figueiredo, A. Horhota, S. Low, K. McDonnell, E. Peeke, B. Retnarajan, A.
462 Sabnis, E. Schnipper, J.J. Song, Y.H. Song, J. Summa, D. Tompsett, G. Troiano, T. Van
463 Geen Hoven, J. Wright, P. LoRusso, P.W. Kantoff, N.H. Bander, C. Sweeney, O.C.
464 Farokhzad, R. Langer, S. Zale, Preclinical Development and Clinical Translation of a
465 PSMA-Targeted Docetaxel Nanoparticle with a Differentiated Pharmacological Profile,
466 *Science Translational Medicine*, 4 (2012) 128-139.
- 467 8. F. Canal, J. Sanchis, M.J. Vicent, Polymer–drug conjugates as nano-sized medicines,
468 *Current Opinion in Biotechnology*, 22 (2011) 894-900.
- 469 9. C. Oerlemans, W. Bult, M. Bos, G. Storm, J.F.W. Nijssen, W.E. Hennink, Polymeric
470 Micelles in Anticancer Therapy: Targeting, Imaging and Triggered Release, *Pharm. Res.*, 27
471 (2010) 2569-2589.
- 472 10. P. Hervella, G. Lollo, F. Oyarzun-Ampuero, G. Rivera-Rodriguez, D. Torres, M.J. Alonso,
473 Nanocapsules as Carriers for the Transport and Targeted Delivery of Bioactive Molecules,
474 in: Trindade T, Daniel A.L., (Ed.) *Nanocomposite particles for bio-aplications: Materials*
475 *and bio-interfaces*, Pan Stanford Publishing, Singapore, 2011, pp. 350.
- 476 11. M.V. Lozano, H. Esteban, J. Brea, M.I. Loza, D. Torres, M.J. Alonso, Intracellular delivery
477 of docetaxel using freeze-dried polysaccharide nanocapsules, *Journal of*
478 *Microencapsulation*, 30 (2013) 181-188.
- 479 12. N.T. Huynh, C. Passirani, P. Saulnier, J.P. Benoit, Lipid nanocapsules: A new platform for
480 nanomedicine, *International Journal of Pharmaceutics*, 379 (2009) 201-209.
- 481 13. M.J. Alonso, P. Couvreur, Historical view of the design and development of nanocarriers for
482 overcoming biological barriers, in: A.M.J.a.C. N. (Ed.) *Nanostructured Biomaterials for*
483 *Overcoming Biological Barriers*, The Royal Society of Chemistry Cambridge UK, (2012) pp
484 3-36.
- 485 14. N.T. Huynh, E. Roger, N. Lautram, J.-P. Benoît, C. Passirani, The rise and rise of stealth
486 nanocarriers for cancer therapy: passive versus active targeting, *Nanomedicine*, 5 (2010)
487 1415-1433.
- 488 15. D.E. Owens III, N.A. Peppas, Opsonization, biodistribution, and pharmacokinetics of
489 polymeric nanoparticles, *International Journal of Pharmaceutics*, 307 (2006) 93-102.
- 490 16. J.V. González-Aramundiz, M.V. Lozano, A. Sousa-Herves, E. Fernandez-Megia, N. Csaba,
491 Polypeptides and polyaminoacids in drug delivery, *Expert Opinion on Drug Delivery*, 9
492 (2012) 183-201.
- 493 17. B.C. Dash, G. Réthoré, M. Monaghan, K. Fitzgerald, W. Gallagher, A. Pandit, The influence
494 of size and charge of chitosan/polyglutamic acid hollow spheres on cellular internalization,
495 viability and blood compatibility, *Biomaterials*, 31 (2010) 8188-8197.
- 496 18. J.W. Singer, Paclitaxel poliglumex (XYOTAX™, CT-2103): A macromolecular taxane,
497 *Journal of Controlled Release*, 109 (2005) 120-126.
- 498 19. C. Li, S. Wallace, Polymer-drug conjugates: Recent development in clinical oncology,
499 *Advanced Drug Delivery Reviews*, 60 (2008) 886-898.
- 500 20. W. Song, M. Li, Z. Tang, Q. Li, Y. Yang, H. Liu, T. Duan, H. Hong, X. Chen,
501 Methoxypoly(ethylene glycol)-block-Poly(L-glutamic acid)-Loaded Cisplatin and a
502 Combination With iRGD for the Treatment of Non-Small-Cell Lung Cancers,
503 *Macromolecular Bioscience*, 12 (2012) 1514-1523.

- 504 21. Y. Bae, K. Kataoka, Intelligent polymeric micelles from functional poly(ethylene glycol)-
505 poly(amino acid) block copolymers, *Advanced Drug Delivery Reviews*, 61 (2009) 768-784.
- 506 22. M. Morille, T. Montier, P. Legras, N. Carmoy, P. Brodin, B. Pitard, J.P. Benoit, C.
507 Passirani, Long-circulating DNA lipid nanocapsules as new vector for passive tumor
508 targeting, *Biomaterials*, 31 (2010) 321-329.
- 509 23. G.-S.L. Muñoz-Alonso MJ, Martínez T, Losada A, Galmarini CM, Muñoz A., The
510 mechanism of action of plitidepsin, *Curr Opin Investig Drugs*, 10 (2009) 536-542.
- 511 24. P. Calvo, C. Remuñán-López, J. Vila-Jato, M. Alonso, Development of positively charged
512 colloidal drug carriers: Chitosan-coated polyester nanocapsules and submicron-emulsions,
513 *Colloid & Polymer Science*, 275 (1997) 46-53-53.
- 514 25. P. Avilés, M.J. Guillén, P. Calvo, M.J. Alonso, D. Torres, M. García Fuentes, T. Gonzalo,
515 Lollo G., Nanocapsules for use in pharmaceutical compositions (*EP11382003.9*)
- 516 26. E.F.A. Brandon, R.D. van Ooijen, R.W. Sparidans, L.L. Lázaro, A.J.R. Heck, J.H. Beijnen,
517 J.H.M. Schellens, Structure elucidation of aplidine metabolites formed in vitro by human
518 liver microsomes using triple quadrupole mass spectrometry, *Journal of Mass Spectrometry*,
519 40 (2005) 821-831.
- 520 27. B. Romberg, J.M. Metselaar, L. Baranyi, C.J. Snel, R. Bünger, W.E. Hennink, J. Szebeni, G.
521 Storm, Poly(amino acid)s: Promising enzymatically degradable stealth coatings for
522 liposomes, *International Journal of Pharmaceutics*, 331 (2007) 186-189.
- 523 28. K. Knop, R. Hoogenboom, D. Fischer, U.S. Schubert, Poly(ethylene glycol) in Drug
524 Delivery: Pros and Cons as Well as Potential Alternatives, *Angewandte Chemie*
525 *International Edition*, 49 (2010) 6288-6308.
- 526 29. M.V. Lozano, D. Torrecilla, D. Torres, A. Vidal, F. Dominguez, M.J. Alonso, Highly
527 efficient system to deliver taxanes into tumor cells: Docetaxel-loaded chitosan oligomer
528 colloidal carriers, *Biomacromolecules*, 9 (2008) 2186-2193.
- 529 30. C. Kusunwiriawong, K. Atuah, O.H. Alpar, H.P. Merkle, E. Walter, Cationic stearylamine-
530 containing biodegradable microparticles for DNA delivery, *Journal of Microencapsulation*,
531 21 (2004) 25-36.
- 532 31. S. Barbault-Foucher, R. Gref, P. Russo, J. Guechot, A. Bochot, Design of poly-ε-
533 caprolactone nanospheres coated with bioadhesive hyaluronic acid for ocular delivery, *J*
534 *Control Release*, 83 (2002) 365-375.
- 535 32. M. Garcia-Fuentes, D. Torres, M. Martín-Pastor, M.J. Alonso, Application of NMR
536 Spectroscopy to the Characterization of PEG-Stabilized Lipid Nanoparticles, *Langmuir*, 20
537 (2004) 8839-8845.
- 538 33. C.E. Mora-Huertas, H. Fessi, A. Elaissari, Polymer-based nanocapsules for drug delivery,
539 *International Journal of Pharmaceutics*, 385 (2010) 113-142.
- 540 34. R. Gref, A. Domb, P. Quellec, T. Blunk, R.H. Müller, J.M. Verbavatz, R. Langer, The
541 controlled intravenous delivery of drugs using PEG-coated sterically stabilized nanospheres,
542 *Advanced Drug Delivery Reviews*, 16 (1995) 215-233.
- 543 35. V. Torchilin, Tumor delivery of macromolecular drugs based on the EPR effect, *Adv Drug*
544 *Deliv Rev*, 63 (2011) 131-135.
- 545 36. A.O. Nornoo, D.S.L. Chow, Cremophor-free intravenous microemulsions for paclitaxel: II.
546 Stability, in vitro release and pharmacokinetics, *International Journal of Pharmaceutics*, 349
547 (2008) 117-123.
- 548 37. S.-W. Lee, M.-H. Yun, S.W. Jeong, C.-H. In, J.-Y. Kim, M.-H. Seo, C.-M. Pai, S.-O. Kim,
549 Development of docetaxel-loaded intravenous formulation, Nanoxel-PM™ using polymer-
550 based delivery system, *J Control Release*, 155 (2011) 262-271.
- 551 38. L. Wang, Z. Liu, D. Liu, C. Liu, Z. Juan, N. Zhang, Docetaxel-loaded-lipid-based-
552 nanosuspensions (DTX-LNS): Preparation, pharmacokinetics, tissue distribution and
553 antitumor activity, *International Journal of Pharmaceutics*, 413 (2011) 194-201.

- 554 39. L. Zhang, Y. He, G. Ma, C. Song, H. Sun, Paclitaxel-loaded polymeric micelles based on
555 poly(ϵ -caprolactone)-poly(ethylene glycol)-poly(ϵ -caprolactone) triblock copolymers: in
556 vitro and in vivo evaluation, *Nanomedicine: Nanotechnology, Biology and Medicine*, 8
557 (2012) 925-934.
- 558 40. F.K. Engels, R.A.A. Mathot, J. Verweij, Alternative drug formulations of docetaxel: a
559 review, *Anti-Cancer Drugs*, 18 (2007) 95-103.