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UNIVERSITÀ DEGLI STUDI DI TORINO

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30

31 **Abstract**

32 Paclitaxel loaded solid lipid nanoparticles (SLN) of behenic acid were prepared with the
33 coacervation technique. Generally, spherical shaped SLN with mean diameters in the range 300-600
34 nm were obtained. The introduction of charged molecules, such as stearylamine and glycol chitosan
35 into the formulation allowed to obtain positive SLN with Zeta potential in the 8-20 mV range and
36 encapsulation efficiency in the 25-90% range.

37 Blood-brain barrier (BBB) permeability, tested in vitro through hCMEC/D3 cells monolayer,
38 showed a significantly increase in the permeation of Coumarin-6, used as model drug, when
39 vehicled in SLN. Positive-charged SLN do not seem to enhance permeation although stearylamine-
40 positive SLN resulted the best permeable formulation after 24 h.

41 Cytotoxicity studies on NO3 glioblastoma cell line demonstrated the maintenance of cytotoxic
42 activity of all paclitaxel-loaded SLN that was always unmodified or greater compared with free
43 drug. No difference in cytotoxicity was noted between neutral and charged SLN.

44 Co-culture experiments with hCMEC/D3 and different glioblastoma cells evidenced that, when
45 delivered in SLN, paclitaxel increased its cytotoxicity towards glioblastoma cells.

46

47 **Abbreviations:**

48 PTX, paclitaxel; SLN, solid lipid nanoparticles; BA, behenic acid; ST, stearylamine; GCS, glycol
49 chitosan; BBB, blood-brain barrier; GBM, glioblastoma multiforme; CNS, central nervous system;
50 NP, nanoparticles; cationic bovine serum albumin, CBSA; NHEJ, non-homologous end joining;
51 PK, protein Kinase; CS, chitosan; Cou-6, coumarin 6; Na-BA, sodium behenate; CHOL,
52 cholesterol; FD, freeze-dried; DMEM, Dulbecco's modified Eagle's medium; NS, neurosphere;

53 AC, adherent cell; ATM, ataxia telangiectasia mutated; ChK2, checkpoint kinase; p-53BP1, 53
54 binding protein 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; IF,
55 immunofluorescence; HR, homologous recombination; TEM, transmission electron microscopy;
56 EE, entrapment efficiency; Pgp, P-glycoprotein; MRP1, multidrug resistance related protein 1.

57

58 Keywords: paclitaxel, SLN, coacervation, BBB permeability, cytotoxicity, glioblastoma

59

60 Chemical compounds studied in this article:

61 Paclitaxel (PubChem CID: 36314)

62 Coumarin-6 (PubChem CID: 100334)

63 Behenic acid (PubChem CID: 8215)

64 **1. Introduction**

65 Glioblastoma multiforme (GBM) is the most common form of primary brain tumor in the central
66 nervous system (CNS); its aggressive nature and evasiveness to treatments make it one of the most
67 lethal cancers [1]. Current treatments for GBM provide a tumor surgical resection followed by
68 pharmacotherapy and radiotherapy. Pharmacotherapy, directed by residual tumor cells elimination,
69 ranges from common chemotherapeutic agents such as temozolomide to more recent anti-
70 angiogenic agents and immunotherapeutic treatments [2]. However, anti-cancer therapeutic agents
71 have not significantly increased the median survival of GBM patients over the past 10 years. The 5-
72 year survival rate of GBM patients after treatment that includes surgical resection, radiation and
73 chemotherapy, is 9.8%.

74 The failure of chemotherapy is due to the inability of intravenously administered anticancer agents
75 to reach the brain parenchyma. An endothelial cell monolayer associated with pericytes and
76 astrocytes, known as the blood–brain barrier (BBB), separates blood from the cerebral parenchyma
77 and prevents the penetration of drugs into the CNS.

78 BBB is a functional unit composed by the peculiar endothelium of brain microvessels, the capillary
79 basal lamina and the surrounding astrocytes, neurons, microglial cells and pericytes, which
80 contribute to the maintenance of the barrier properties [3, Figure A.1]. The presence of tight
81 junctions and adherent junctions between adjacent endothelial cells [4], the lack of fenestrations and
82 pinocytotic vesicles [5], the abundance of efflux transporters belonging to the ATP binding cassette
83 family on the endothelium luminal side [6] account for the low delivery of drugs, such as
84 antineoplastic agents [7], from the bloodstream to the brain parenchyma.

85 Various invasive strategies have been developed to improve the penetration of drugs into the brain
86 [8]. Traditional approach to overcome brain drug delivery obstacles includes direct intracerebral
87 drug injection [9], which is associated with a high risk for the patient.

88 Less invasive strategies have also been investigated. One approach consists in generating a transient
89 disruption of BBB in conjunction with the systemic administration of anticancer agents. The

90 intracarotid administration of a hyperosmotic solution such as mannitol led to a rapid diffusion of
91 fluid across the cerebral endothelium, moving out of the endothelial cells into the vascular lumen
92 and inducing the opening of the tight junctions for a few hours [10].

93 Another approach concerns the modification of drugs in order to make them more lipophilic,
94 improving their penetration into the brain by passive diffusion. Lipophilic analogs and prodrugs
95 were thus developed [11].

96 A more recent strategy to deliver drugs to the brain is the use of colloidal polymers to form
97 nanometer sized carriers [12]. The basic reason of common acceptance of these vehicles is due to
98 their drug release controlled profile of as well as to their selected targeting mechanism. Targeting
99 action may be due to the steric hindrance created by nano-vectors: after parenteral administration,
100 due to steric phenomenon they conceal themselves from opsonization event induced by tissue
101 macrophages. By this way they achieve targeting ability to the brain and partially avoid other
102 reticuloendothelial system organs like liver, spleen, etc [13].

103 From the last few decades, nanoparticles (NP) have attracted considerable interest in targeting drug
104 molecules to the brain [14]. The correct mechanism of barrier opening by NP is not exactly known;
105 the delivered NP enter into the brain by crossing the BBB by various endocytotic mechanisms, as
106 polymeric albumin or poly(butylcyanoacrylate) NP are reported to enter into the brain by their small
107 size mediated endocytosis [15,16]. An increased drug retention in brain blood capillaries combined
108 with an adsorption to capillary can increase drug transport due to an enhanced concentration
109 gradient; an increase of BBB fluidization, an opening of tight junctions between endothelial, an
110 inhibition of the P-glycoprotein efflux system are other possible mechanisms that can increase brain
111 drug concentration [17].

112 Although NP may be designed to entrap high molecular weight or hydrophilic therapeutics, BBB
113 retardation of drug NP entrapped is based on NP characteristics and not on the therapeutic agent
114 [18].

115 Indeed, poly(butyl cyanoacrylate) NP overcoated with 1% polysorbate 80 have been experimentally
116 successful as brain drug delivery for doxorubicin [19] and dalargin [20], poly(lactic-co-glycolic
117 acid) and cetyl alcohol/polysorbate NP for paclitaxel brain delivery [21,22].

118 In addition to BBB functional characteristics limiting permeation, brain microvasculature
119 endothelia also present a luminal electrostatic barrier at physiologic pH. The negative electrostatic
120 charge is created by surface expression and adhesion of the glycocalyx residues: proteoglycans,
121 sulfated mucopolysaccharides, and sulfated and sialic acid-containing glycoproteins and glycolipids
122 [23]. Cationic molecules have been shown to occupy anionic areas at the BBB endothelium [24]
123 and increase BBB permeability via a presumed tight junction disruption [25].

124 Recent *in vitro* reports have demonstrated that positive-charged NP have an increased brain
125 distribution compared to anionic and neutral NP [26]. However, there is little data regarding brain
126 permeability of positive-charged NP. Lu et al. [27] developed and evaluated cationic bovine serum
127 albumin (CBSA) conjugated with poly(ethyleneglycol)–poly(lactide) NP (CBSA–NP). To evaluate
128 the effects of brain delivery, BSA conjugated with pegylated NP (BSA–NP) was used as the control
129 group and Coumarin-6 was incorporated into the NP as the fluorescent probe. The qualitative and
130 quantitative results of CBSA–NP uptake experiment compared with those of BSA–NP showed that
131 rat brain capillary endothelial cells took in much more CBSA–NP than BSA–NP at 37 °C, at
132 different concentrations and time incubations. After a dose of 60 mg/kg CBSA–NP or BSA–NP
133 injection in mice caudal vein, fluorescent microscopy of brain coronal sections showed a higher
134 accumulation of CBSA–NP in the lateral ventricle, third ventricle and periventricular region than
135 that of BSA–NP. In an experimental work Lockman et al. [15] evaluated the effect of neutral,
136 anionic and cationic charged NP on BBB integrity and NP brain permeability. Neutral NP and low
137 concentrations of anionic NP had no effect on BBB integrity, whereas, high concentrations of
138 anionic NP and cationic NP disrupted the BBB. The brain uptake rates of anionic NP at lower
139 concentrations were higher than of neutral or cationic formulations at the same concentrations.

140 In literature, many authors studied solid lipid nanoparticles (SLN) as drug delivery systems to
141 deliver drugs to the CNS [28,29]. SLN are disperse systems having size ranging from 1 to 1000 nm
142 which represent an alternative to polymeric particulate carriers and are composed of physiological
143 or biocompatible lipids or lipid molecules with a history of safe use in therapy and are generally
144 suitable for intravenous administration.

145 As few data are present in literature about positive-charged SLN for brain delivery, the purpose of
146 this work will be to prepare, characterize and evaluate *in vitro* the potential of positive-charged SLN
147 to vehicle paclitaxel (PTX) to the brain for GBM treatment.

148 PTX, a diterpene isolated from *Taxus brevifolia*, is one of the most active chemotherapeutic agents
149 against a wide panel of solid tumors including urothelial, breast, lung, and ovarian cancers. It has
150 been demonstrated that PTX is effective against glioblastoma cells *in vitro* [30,31], however its
151 clinical use is limited due to its poor BBB penetration capability and drug-resistance [32,33].

152 Due to its low water solubility, PTX is formulated in a mixture of Cremophor® EL and dehydrated
153 ethanol (50:50 v/v) a combination known as Taxol®. However, Taxol® has some severe side
154 effects related to Cremophor® EL and ethanol [34]. Therefore, there is an urgent need for the
155 development of alternative PTX formulations.

156 Recently, a new solvent-free technique, defined as “coacervation”, was developed to prepare fatty
157 acids-based SLN [35]. Briefly, a fatty acid alkaline salt micellar solution in the presence of an
158 appropriate polymeric stabilizer was prepared; when the pH is lowered by acidification, the fatty
159 acid precipitates as nanoparticles owing to proton exchange between the acid solution and the
160 sodium salt.

161 SLN were prepared using two different positive-charged substances: ST or GCS. ST possess a
162 hydrocarbonic chain that can probably be incorporated within the lipid matrix, while its positive
163 charge is exposed to the external surface. GCS, a chitosan derivative conjugated with ethylene
164 glycol branches, is a water soluble at a neutral/acidic pH values polymer whose pendant glycol
165 branches increase both the aqueous solubility of the native chitosan (CS) and provide steric

166 stabilization [36]. Mao et al [37] have established that CS is capable of opening the tight junctions
167 of epithelial cells and it can improve the uptake of hydrophilic drugs including peptides.
168 For the above mentioned purpose, PTX loaded cationic SLN were prepared and extensively
169 characterized *in vitro* with regard to their physicochemical properties, their capacity to load and
170 release PTX, their cytotoxicity and their permeability across hCMEC/D3 cell lines taken as an *in*
171 *vitro* model of BBB.

172 **2. Experimental**

173 *2.1 Chemicals*

174 Sodium Behenate (Na-BA) was purchased from Nu-Chek Prep, Inc. (Elysian, U.S.A.), Tween[®] 80,
175 sodium dodecyl sulfate, Triton X-100 and cholesterol from Fluka (Buchs, Switzerland), paclitaxel
176 (PTX) from Indena (Milan, Italy), citric acid from A.C.E.F. (Fiorenzuola d'Arda, Italy), 80%
177 hydrolyzed PVA 9000–10000 Mw (PVA 9000), Pluronic F68, stearylamine (ST), glycol chitosan
178 (GCS), coumarin 6 (Cou-6), triethanolamine phosphate, trehalose, penicilin-streptomycin,
179 hydrocortisone, ascorbic acid, Heses and b-FGF from Sigma (Dorset, UK), hydrochloric acid,
180 sodium hydroxide and sodium phosphate monobasic from Merck (Darmstadt, Germany),
181 Cremophor[®] EL from BASF (Ludwigshafen am Rhein, Germany), EBM-2 basal medium from
182 Lonza, (Basel, Switzerland), fetal bovine serum Gold from PAA The Cell Culture Company
183 (Pasching, Austria), rat collagen-I from Trevigen (Gaithersburg, Maryland, USA), chemically
184 defined lipid concentrate from Invitrogen Life technologies (Carlsbad, California, USA), methanol
185 and ethanol from Carlo Erba (Val De Reuil, France). Deionized water was obtained by a MilliQ
186 system (Millipore, Bedford, MO).

187

188 *2.2 hCMEC/D3 cells*

189 hCMEC/D3 cells, a primary human brain microvascular endothelial cell line that retains the
190 property of BBB *in vitro*, were cultured as reported [38]. Briefly, cells were maintained in Petri
191 dishes coated with 150 µg/ml rat collagen-I and cultured in EBM-2 basal medium supplemented
192 with 5% v/v fetal bovine serum Gold, 1% v/v penicilin-streptomycin, 1.4 µM hydrocortisone, 5
193 µg/ml ascorbic acid, 10 mM Heses, 1% v/v Chemically Defined Lipid Concentrate, 1 ng/ml b-
194 FGF. In all the experiments cells were used between passages 29 and 34. For the permeability
195 assays, cells were seeded at 50,000/cm² and grown for 7 days up to confluence in Petri dishes and
196 Transwell devices (0.4 µm diameter pores-size, Corning Life Sciences, Chorges, France), with 1.5

197 ml/cm² and 3 ml/cm² of the culture medium described above in the Transwell insert and in the
198 lower chamber, respectively.

199 Before each experiment we measured the transendothelial electrochemical resistance (TEER) and
200 the permeability coefficient of dextran-FITC, [¹⁴C]-sucrose and [¹⁴C]-inulin, taken as parameters
201 of paracellular transport across hCMEC/D3 monolayer, as described in [39]. TEER value was
202 between 30 and 40 Ω x cm², dextran-FITC permeability coefficient was 0.015 ± 0.003 x 10⁻³
203 cm/min, [¹⁴C]-sucrose permeability coefficient was 1.19 ± 0.21 x 10⁻³ cm/min, [¹⁴C]-inulin
204 permeability coefficient was 0.56 ± 0.09 x 10⁻³ cm/min. These values suggested the functional
205 integrity of BBB monolayer [38].

206

207 *2.3 GBM cell lines and culture conditions*

208 Primary human GBM cell lines NO3 and CV17 were obtained from primary human GBMs
209 surgically resected at the Department of Neuroscience, Neurosurgical Unit, University of Turin,
210 Italy. The histological diagnosis was performed according to World Health Organization (WHO)
211 guidelines. The study was in compliance with the local institutional review board and Committee on
212 Human Research and with the ethical human-subject standards of the World Medical Association
213 Declaration of Helsinki Research. Written informed consent was obtained from all patients.

214 010627 GBM cell line was a kind gift of Dr. Galli R, DIBIT San Raffaele, Milan, Italy). For
215 cytotoxicity studies in co-culture models U87-MG cells (ATCC, Rockville, MD) were also used.

216 By neurosphere assay, from a resected tumor tissue we obtained neurosphere (NS) or adherent cell
217 (AC) or both cell types [40]. NS lines were cultured on Dulbecco's modified Eagle's medium
218 (DMEM)/F-12 supplemented with 20 ng/ml epidermal growth factor (EGF) and 10 ng/ml basic
219 fibroblast growth factor (bFGF); AC lines were grown on DMEM supplemented with 10% fetal
220 bovine serum (FBS) for AC. Both cultures were maintained at 37°C and at 5% O₂/CO₂ in a
221 humidified multigas Sanyo MCO-18M incubator (Sanyo Scientific, Bensenville, IL).

222 All cell cultures were periodically checked for *Mycoplasma* contamination (e-Myco™
223 Mycoplasma PCR Detection kit, iNtRON Biotechnology, Korea).

224

225 *2.4 PTX-loaded SLN preparation*

226 Different SLN formulations were prepared (Table 1); non-charged SLN were prepared as reference
227 to evaluate the positive-charge influence.

228 Non-charged SLN: SLN were prepared by the coacervation method [35]. Briefly, appropriate
229 amounts of Na-BA and PVA 9000 were dispersed in 5 ml deionized water and the mixture was then
230 heated under 5 min-stirring (300 rpm) just above the Krafft point of Na-BA (75 °C) to obtain a clear
231 solution (micellar solution). A selected acidifying solution (100 µl 1M NaH₂PO₄ + 160 µl 1M HCl)
232 was then added drop-wise until pH 4.0 was reached. The obtained BA SLN suspension was then
233 cooled in a water bath under 10 min-stirring at 300 rpm until 15 °C temperature was reached. SLN
234 suspension was then again heated under stirring (300 rpm) just above the melting point of BA (80
235 °C). Different amounts of 30 mg/ml ethanol PTX solution, heated at the same temperature of SLN
236 suspension, were added to the warm mixture and then the sample was again cooled in a water bath
237 under stirring at 300 rpm until 15 °C was reached.

238 Charged SLN: SLN were prepared as described for non-charged SLN, introducing the positive-
239 charged agent as follows:

- 240 - ST-SLN: a fixed amount of 30 mg/ml 2-propanol ST solution (Table 1) was added to the warm
241 aqueous Na-BA solution just above the Krafft point of Na-BA.
- 242 - GCS-SLN: an appropriate amount of GCS (Table 1) was added at the beginning of the
243 preparation before obtaining the micellar solution.

244 All SLN formulations were also prepared in the presence of cholesterol (CHOL-SLN): 100 or 150
245 µl of 5 mg/ml ethanol CHOL solution were added to the micellar solution immediately before the
246 addition of the acidifying solution.

247 All SLN formulations were also prepared without drug (unloaded SLN) adding the same amount of
248 ethanol instead PTX-ethanolic solution.

249 Fluorescent SLN to be employed in *in vitro* experiments were prepared using Cou-6 as fluorescent
250 lipophilic model drug (Cou-6-CHOL-SLN). 5 ml SLN were prepared in aseptic conditions (under
251 laminar flow airfilter starting from materials sterilized with UV or in autoclave) adding 100 μ l
252 2mg/ml Cou-6 ethanol solution after SLN fusion. The samples were then cooled in a water bath
253 under stirring at 300 rpm until 15°C temperature was reached.SLN were lyophilized using a
254 programmable freeze-dryer (Shin PVTFD10R, Shinil Lab, Korea) without cryoprotectant addition.
255 Slow freezing was carried out on the shelves in the freeze dryer (shelf temperature -40 °C).
256 Samples were lyophilized for 24 h from -40 °C to 25 °C at a 5 °C/h. increasing rate. Lyophilized
257 products were reconstituted by magnetic stirring adding the same water amount.

258

259 *2.5 SLN characterization*

260 SLN shape and mean sizes were characterized by Transmission electron microscope (TEM, CM 10
261 Philips, The Netherlands) spraying the SLN suspension on the microscope grid by means of an
262 aerosol-sampling device.

263 Cou-6 localization into SLN dispersion was determined using optical microscopy equipped with a
264 fluorescent lamp (Leica DM 2500, Solms, Germany) at 1000x magnification.

265 SLN particle sizes, polydispersity indexes (PDI) and Zeta potential were determined one hour after
266 preparation using laser light scattering technique-LLS (Brookhaven, New York, USA). Size
267 measurements were obtained at an angle of 90° at 25 °C using the number method. The dispersions
268 were diluted with water for size determination or with 0.01 M KCl for Zeta-potential determination,
269 in order to achieve the prescribed conductivity. Size measurements were also recorded diluting
270 samples with grown medium used to culture hCMEC/D3 cells to mime the conditions under which
271 SLN undergo *in vitro* experiments. For stability studies, the samples were stored at 4°C. All data
272 were determined in triplicate.

273 PTX entrapment efficiency (EE%) was calculated as the ratio between PTX amount in SLN and
274 that in the starting micellar solution $\times 100$. PTX EE% determination was performed as follows: 1
275 ml SLN suspension was centrifuged for 15 min at 62,084 g, the precipitate was washed twice with 1
276 ml ethanol:water 30:70 to eliminate adsorbed PTX. The solid residue was dissolved in 1 ml ethanol,
277 0.5 ml water were then added to precipitate the lipid matrix and the supernatant obtained was
278 injected in HPLC for PTX quantification.

279 EE% was also determined after 1:100 dilution in different media to mime the dilution that SLN can
280 undergo after administration. Briefly, 500 μ l SLN were introduced in a 50 ml flask and 49.5 ml
281 water or 0.1 M phosphate buffer pH 7.4 or 0.1 M citric acid buffer pH 5.5 were added. The
282 suspension was stirred for 2 hours. 8 ml suspension were centrifuged for 15 min at 62,084 g and
283 then treated as previously described.

284

285 *2.6 HPLC analysis*

286 HPLC analysis was performed using a LC9 pump (Shimadzu, Tokyo, Japan) with a
287 Chromosystem™ ODS 2.5 μ 125 \times 4.6mm column and a C-R5A integrator (Shimadzu, Tokyo,
288 Japan); mobile phase: CH₃CN:H₂O 60:40 (flow rate 1ml min⁻¹); detector: UV λ =227 nm
289 (Shimadzu, Tokyo, Japan). Retention time was 2.6 min.

290 The limit of quantification, defined as the lowest PTX concentration in the curve that can be
291 measured routinely with acceptable precision and accuracy, was 0.013 μ mol/ml; the limit of
292 determination, defined as the lowest detection limit, was 0.005 μ mol/ml (signal to noise>2.0).

293

294 *2.7 SLN and PTX stability*

295 SLN sizes and PTX EE% of samples stored at 4 °C were monitored for 90 days to study SLN
296 physical stability and PTX chemical stability. Mean sizes were determined by LLS and PTX EE%
297 over time was obtained as described by HPLC.

298 The same SLN suspensions were freeze-dried without adding any cryoprotectant (FD-SLN) using a
299 Modulyo Freeze Dryer (Edwards Alto Vuoto, Italy). The resulting samples, rehydrated with the
300 same water amount, were also characterized by size, Zeta potential and EE% determination.

301

302 *2.8 PTX in vitro release*

303 *In vitro* release of PTX was determined using the non-equilibrium dialysis method [41]. A
304 multicompartamental rotating cell system consisting of donor and receptor compartments of equal
305 volume (1.5 ml) separated by a dialysis membrane (cut-off 12,000 Da) was used. Receiving
306 medium was 0.1 M phosphate buffer (pH 7.4) containing 0.1% v/v Tween®80. Solution of PTX in
307 receiving medium and SLN suspensions were used as donor formulations. At fixed times, the
308 receptor solution was tipped out and used for HPLC analysis and the cell was refilled with fresh
309 receiving medium. Drug concentration was determined by HPLC. The results were evaluated as
310 PTX apparent permeability constant ($K_{d,app}$ [cm h^{-1}]) calculated from the slope of the straight line
311 obtained by plotting the amount of PTX diffused from the donor formulation versus time, assuming
312 pseudo zero-order kinetics.

313

314 *2.9 Permeability of SLN through hCMEC/D3 cell monolayer*

315 hCMEC/D3 cells, seeded as reported above in Transwell devices, were incubated at day 7 with
316 SLN 2, SLN 9, SLN 15 loaded with Cou-6, for 0.5, 3 and 24 h in the Transwell
317 insert. The amount of SLN added was 100 μl diluted with 900 μl of medium. This dilution of
318 SLN was the highest concentration devoid of cytotoxic effects on hCMEC/D3 cells, as previously
319 reported [42]. In parallel, a set of Transwell inserts were incubated with Cou-6 aqueous suspension
320 obtained with PVA 9000 as suspending agent, containing the same concentration of Cou-6
321 present in SLN. At the end of the incubation time, the medium in lower chamber was collected,
322 diluted 1: 20 into ethanol, sonicated with 10 bursts of 1 s to disrupt SLN, and centrifuged at 62,084
323 g for 5 min, to pellet debris. The dilution of the medium was necessary to obtain a fluorescence

324 falling in the detection range of the LS-5 spectrofluorimeter (PerkinElmer, Waltham, MA), used to
325 measure the fluorescence of Cou-6.

326 Excitation and emission wavelengths were 485 ± 20 nm and 528 ± 20 nm, respectively. The
327 fluorescence of Cou-6 at t_0 in the upper chamber, was considered 100%. The fluorescence of Cou-6
328 measured in the medium collected from the lower chamber was expressed as percentage of
329 fluorescence in the upper chamber at t_0 . The fluorescence of the medium without Cou-6 was
330 considered as blank and was subtracted from the fluorescence of all the other experimental
331 conditions.

332 To measure the amount of Cou-6 adsorbed on the Transwell membrane, Cou-6, either free or loaded
333 in SLN, was added in empty Transwells in the same experimental conditions of the Transwells
334 containing hCMEC/D3 cells, then the medium from the insert and the lower chamber was removed;
335 the Transwell membrane was washed with PBS and 0.5 ml ethanol was added to solubilize the
336 Cou-6 adsorbed on the membrane. The solution was collected and its fluorescence was read as
337 reported above. The fluorescence was always less than 4% of the fluorescence measured in the
338 lower chamber medium in all the experimental conditions, suggesting that such very low absorption
339 of Cou-6 on the Transwell insert did not influence the results of the permeability assays.

340 The level of significance was determined by a Student's *t* test. Statistical significance was defined
341 as $p < 0.001$.

342

343 *2.10 In vitro cytotoxicity assay*

344 For cytotoxicity studies, NO3 cells (both NS and AC) and CV17 NS were used at various passage
345 numbers between 20 and 30, whereas 010627 cells (both NS and AC) were used at passage
346 numbers between 160 and 180.

347 Free PTX (Sigma Aldrich Co., St. Louis, MO, USA) was dissolved in 100% DMSO for stock
348 solutions and dilutions for cell treatments were made extemporaneously in culture medium, so that
349 the final concentration of DMSO never exceeded 0.3% (v/v).

350 Cell lines were treated for 72 h with increasing doses (20, 100, 500, 1400 nM) of free PTX.

351 After exposure the cytotoxic effect of free Paclitaxel (PTX) and PTX-loaded SLN against tumor
352 cells was evaluated assessing the number of viable cells by the Trypan blue dye exclusion test,
353 using a TC20 automated cell counter (Bio-Rad, Berkeley, CA, USA). As for AC, results were
354 confirmed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay
355 (Roche, Diagnostic Corporation Indianapolis, IN, USA) [43].

356 For Trypan blue assay, cells were plated at a density of 200×10^3 cells in 5 ml medium in a 25 cm^2
357 flask and treated with PTX (free in solution or encapsulated in SLN2, SLN9 or SLN15) at 37°C in
358 5% O_2/CO_2 for different times (24 and 72 h). Stock solutions were diluted extemporaneously in
359 fresh medium at the desired concentration and administered to cells.

360 Cytotoxicity was expressed as number of surviving cells as percentage of control (untreated cells).

361 For MTT assay, cells were seeded in 96-well plates (1×10^4 cells/well in 100 μl medium) and
362 treated with the different concentrations and times above indicated of the drug (free or
363 encapsulated) at 37°C in 5% O_2/CO_2 . The medium with the SLN was replaced with fresh medium
364 and 10 μl of MTT solution (5mg/ml) were added to each well and incubated for 4 h at 37°C . The
365 extent of cell viability is indicated by mitochondrial conversion of yellow MTT, a tetrazole, to
366 purple formazan by the living cells. Formazan crystals were solubilized overnight in 10% sodium
367 dodecyl sulfate (SDS), 0.01 M HCl and optical density was measured at 570 nm (test wavelength)
368 and 660 nm (reference wavelength) using a microplate spectrophotometer (Synergy HT, BioTek
369 Instruments Inc., Winooski, VT, USA). Cell viability was expressed as a percentage of the
370 absorbance measured in the treated cells compared to the control (untreated cells). All experiments
371 were performed in quadruplicate.

372 The concentration of free PTX which caused a 50% cell growth inhibition (IC_{50}) compared with
373 untreated controls, was calculated by non-linear regression for each cell line.

374 To investigate the cytotoxic effect of PTX incorporated in SLN, a drug concentration of 100 nM
375 was chosen for the following *in vitro* experiments.

376 Cell viability was also measured after treatment with unloaded SLN (SLN2, SLN9 and SLN15) at
377 the highest SLN concentration used for the study, *i.e.* 0.1% w/v.

378 The level of significance was determined by a two-tailed Student's *t* test. Statistical significance
379 was defined as $p < 0.05$ or $p < 0.01$.

380

381 *2.11 DNA damage/repair study by Immunofluorescence (IF)*

382 The occurrence of a DNA damage after SLN treatment was investigated through the analysis by IF
383 of γ -H2AX histone, that localizes at sites of DNA fragmentation as subnuclear foci.

384 The cell response to DNA insults was studied monitoring the activation of p-ATM, p-Chk2 and p-
385 53BP1. HR or NHEJ repair system activities were analyzed through RAD51 or Ku70/Ku80 and
386 DNA-PKcs proteins respectively. After 72 h treatment with unloaded SLN (SLN9) or with 100 nM
387 PTX-loaded SLN9, cells were fixed for 20 minutes with 4% paraformaldehyde at room
388 temperature, rinsed three times with PBS, blocked/permeabilized with PBS containing 2% of the
389 appropriate serum and 0.1% Triton X-100 for 30 minutes and stained with the following primary
390 antibodies: mouse anti- γ -H2AX (Ser139), mouse anti-p-ATM (Ser1981) (both from Millipore),
391 rabbit anti-p-Chk2 (Thr68) (Cell Signaling Technology), rabbit anti-p-53BP1 (Ser25) (Bethyl
392 Laboratories), mouse anti-RAD51, mouse anti-DNA-PKcs and mouse anti-Ku70/Ku80 (all from
393 NeoMarkers). Negative controls were obtained by omitting the primary antibody. Alexa Fluor®
394 488-AffiniPure goat anti-rabbit IgG and Alexa Fluor® 594-AffiniPure rabbit anti-mouse IgG
395 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) were used as secondary
396 antibodies. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and
397 observations were made under a Zeiss Axioskop fluorescence microscope (Karl Zeiss, Oberkochen,
398 Germany) equipped with an AxioCam5MR5c and coupled to an Imaging system (AxioVision
399 Release 4.5; Karl Zeiss).

400

401 *2.12 Specimen preparation for transmission electron microscopy (TEM)*

402 Analysis by TEM was employed in order to get information on the possible ultrastructural
403 modifications of cells treated with empty and PTX-loaded SLN. NO3 NS and AC lines were used
404 for the investigation.

405 For transmission electron microscopy, untreated cells, cells treated with unloaded SLN15 and cells
406 treated with 100 nM PTX-loaded SLN15 for 24 h were collected, pelleted by centrifugation and
407 then fixed with 2.5% glutaraldehyde prepared in 0.1 M phosphate buffer at 4 °C. Samples were
408 post-fixed with 1% osmium tetroxide for 2 h, dehydrated in a graded acetone series and embedded
409 in the Epon 812 epoxy resin. Ultrathin sections were prepared using an ultracut microtome
410 (Reichert-Jung, Germany), collected on copper grids, stained with both lead citrate and uranyl
411 acetate, and then observed with a Philips CM-12 transmission electron microscope.

412

413 *2.13 Glioblastoma cell cytotoxicity in co-culture models*

414 In co-culture experiments, 500,000 glioblastoma cells (i.e. the U87-MG cell line and the primary
415 human glioblastoma cells CV17 and 01010627) were added in the lower chamber of Transwells 4
416 days after seeding hCMEC/D3 cells in the upper chamber of Transwells. After 3 days of co-culture
417 the medium of the upper chamber was replaced with fresh medium, with or without free PTX or
418 PTX-loaded SLN (SLN 2) at different concentration (20, 50, 100, 200, 500 nM) for 24 h, as
419 detailed under results.

420 To measure the viability of glioblastoma cells co-cultured with hCMEC/D3 cells, extracellular
421 medium of glioblastoma cells was collected and centrifuged at 12,000 g for 15 min to pellet cellular
422 debris, whereas cells were washed with fresh medium, detached with trypsin/EDTA (0.05/0.02%
423 v/v), re-suspended in 0.2 ml of 82.3 mmol/l triethanolamine phosphate-HCl (pH 7.6) and sonicated
424 on ice with two 10 s bursts (Labsonic sonicator, 100 W). LDH activity was measured in the
425 extracellular medium and in the cell lysate: 50 µl of supernatant from extracellular medium or 5 µl
426 of cell lysate were incubated at 37 °C with 5 mmol/l NADH. The reaction was started by adding 20
427 mmol/l pyruvic acid and was followed for 6 min, measuring absorbance at 340 nm with Packard

428 EL340 microplate reader (Bio-Tek Instruments, Winooski, VT). The reaction kinetics was linear
429 throughout the time of measurement. Both intracellular and extracellular enzyme activity was
430 expressed in $\mu\text{mol NADH oxidized}/\text{min}/\text{dish}$, then extracellular LDH activity was calculated as
431 percentage of the total LDH activity in the dish.

432 The level of significance was determined by a Student's t test. Statistical significance was defined
433 as $p < 0.05$, $p < 0.02$, $p < 0.01$, $p < 0.005$ or $p < 0.001$.

434 **3. Results and discussion**

435 As described in the introduction chapter, BBB is a physical barrier characterized by tight
436 intracellular junctions and by the absence of fenestrations that limit permeability for therapeutic
437 molecules with no exception for molecules in the glioblastoma treatment. The use of drug-loaded
438 nanoparticles could be a winning strategy if an active targeting and a suitable drug concentration to
439 the brain could be obtained, provided small-sized hydrophilic vehicles are achieved to prevent rapid
440 degradation by reticuloendothelial system and carriers with proper superficial characteristics are
441 coupled.

442 Moving from this background, SLN were prepared using the coacervation method which uses
443 hydrophilic polymeric as stabilizers able to confer hydrophilic surface to SLN. Further to a
444 cytotoxicity study against hCMEC/D3 cells relating to different fatty acids [40], BA was chosen as
445 lipid matrix. In order to take advantages of possible interaction between BBB negative charges and
446 surface positive-charged SLN, two positive charged molecules currently used in literature were
447 introduced in the SLN preparation: ST [44] and GCS [36]. As antineoplastic model drug PTX was
448 chosen, a lipophilic molecule suitable to be entrapped in SLN matrix.

449 Preliminary formulation studies (data not reported) demonstrated the need to modify the preparation
450 procedure to obtain higher drug EE% by introducing PTX after SLN melting instead of adding it to
451 the micellar solution; probably the high PTX lipophilicity does not allow its distribution within
452 micelles, while promoting its solubilization in the molten lipid.

453

454 *3.1 SLN characterization*

455 In Table 1 compositions of PTX-loaded SLN are reported. SLN composition was the result of a
456 preliminary screening carried out in a previous work [45].

457 In Table 1 ST-SLN and GCS-SLN compositions are also reported. Different formulations were
458 obtained varying the amount of PVA 9000, ST, GCS or PTX in order to obtain the most suitable
459 PTX vehicle as concerns Zeta potential, mean sizes, EE%, over time stability. In order to improve

460 SLN stability, CHOL was also added. As reported in literature [46] high CHOL/lipid ratio matrix
461 reduced mean diameters of SLN prepared from emulsion. This led to a rigid surfactant layer with
462 decrement in van der Waals attraction and promotion of net repulsion forces between oil droplets;
463 therefore, agglomeration and coalescence of small oil droplets were reduced. In addition, the
464 interaction between CHOL and surface active agent might increase the surface curvature of oil
465 droplets consequently leading to small size of SLN after cooling down. A similar behavior can be
466 hypothesized also in SLN obtained by coacervation. In the present experimental conditions, CHOL
467 probably might interact with Na-BA at the interface of micelles.

468 In Table 1 SLN, ST-SLN and GCS-SLN mean diameter and Zeta potential are reported. Most SLN
469 tested had sizes below 600 nm. In the neutral SLN series (SLN 0-3), increasing the amount of PTX
470 no changes in particles dimension were noted; different behavior was noted in the case of charged
471 SLN. In the ST-SLN series (SLN 4-10) an increase in diameter sizes can be noted when ST or PTX
472 amounts were increased or when CHOL was introduced in the formulation. Nevertheless the
473 presence of CHOL was essential to SLN stability: in the absence of CHOL, ST-SLN and GCS-SLN
474 showed a sudden increase in mean size immediately after preparation. Instead, in the GCS-SLN
475 (SLN 11-17) series a slightly decrease in SLN size was obtained increasing the GCS amount and a
476 more marked increase was noted when a great amount of stabilizer (PVA) was used. Also in this
477 case the presence of CHOL was necessary to formulations stability; the addition of this component
478 led to a decrease in mean diameter and the smallest SLN were obtained using 50 mg (1% w/w)
479 PVA (SLN 17).

480 The dissimilar influence of ST and GCS on ST-SLN and GCS-SLN sizes might be due to different
481 positioning of both charged molecules: probably, lipophilic ST chain was incorporated in the lipid
482 matrix while GCS was mainly adsorbed to the external surface of nanoparticles.

483 Size measurements were employed also diluting the samples with growth medium used in the in
484 vitro experiments in order to verify SLN sizes when they are tested in vitro. The growth medium
485 does not seem to influence SLN dimensions: all SLN have maintained their size (data not reported).

486 In Figures 1a and 1b TEM PTX-SLN2 images at different magnifications were reported. The SLN
487 have spherical shape and sizes between 300 and 500 nm. The drug introduction does not seem to
488 affect shape and size (data not reported). Similar characteristics were obtained also with SLN9 and
489 SLN15.

490 The observation of Cou-6-SLN nanosuspensions under optical microscope confirmed both the
491 spherical shape and the dimensional range of the particles. The use of fluorescent light allowed to
492 locate Cou-6 in SLN nanosuspensions: homogeneous dispersions of fluorescent nanoparticles were
493 observed with all formulations (data not shown).

494 In Table 1 also Zeta potential values are reported. All GC or ST-containing formulations showed
495 positive charge with values in the 7.6 - 23.8 mV range. ST-SLN presented Zeta potential ranging
496 from 7 to 15 mV (depending on ST amount used); these values are smaller than those of GCS-SLN
497 and those of SLN containing the same ST percentages but prepared with methods other than
498 coacervation [41]; probably PVA 9000 used as stabilizer locates itself externally thus partially
499 screening the ST positive charge.

500 In GCS-SLN series, Zeta potential values confirmed the external positioning of the polymer:
501 probably positively-charged GCS interacted with carboxylate groups on SLN surface, conferring
502 them a positive charge. The values were influenced by GCS amount: by increasing the polymer an
503 increase of Zeta potential from 16.9 mV to 23.8 mV was noted; the presence of CHOL and the PTX
504 amount variation did not seem to influence this parameter.

505 In Table 2 the amount of PTX in SLN is expressed as EE% and as loading (mg PTX/mg BA).
506 Almost all tested SLN showed a good PTX loading capacity; in particular, in neutral SLN series
507 (SLN 0-3) PTX EE% did not significantly vary increasing the drug amount, while drug loading was
508 increased over twice folds. In the ST-SLN series (SLN 4-10), PTX EE% was significantly
509 influenced by several factors. Increasing ST amounts determined a reduction of PTX EE% (SLN 5)
510 which was partially counteracted by the presence of CHOL (SLN 6) probably as a consequence of a

511 hardened structure of SLN surface. Also PVA amount seemed to be relevant in influencing PTX
512 EE%, as higher PVA concentrations determined a reduction in PTX EE%.

513 In GCS-SLN series (SLN 11-17), EE% and PTX loading ranged from 24% to 83% and from 1.4 to
514 5.0 mg/mg BA respectively. Also in this series PTX EE% was strongly influenced by PVA 9000
515 amount: when the highest stabilizer amount was used (SLN 14), a dramatic decrease in drug EE%
516 up to 24% can be noted; probably PVA 9000 formed micelles in aqueous phase able to solubilize
517 PTX. CHOL introduction enhanced PTX EE% from 52% to 75% particularly in SLN prepared with
518 50 mg PVA 9000. On the contrary, GCS amount variations did not seem to affect drug EE%.

519 To mime SLN behavior after administration when they are diluted in physiological fluids, drug
520 EE% was determined also after 1:100 dilutions. When water was used as dilution medium, EE%
521 remained almost unvaried in all ST-SLN ($\pm 1\%$), except in SLN 15 in which a 10% decrease was
522 registered. Probably, in GCS-SLN, PTX is partially trapped in GCS chains located on SLN surface
523 and therefore it might be released upon high dilutions. To evaluate the influence of different
524 dilution media, SLN 9, which were chosen as those having the best properties for our experimental
525 purposes, were diluted also by pH 7.4 PBS and pH 5.5 citric buffer. No significant variation was
526 noted upon dilution by pH 7.4 PBS ($\pm 1\%$), while a certain PTX amount was released when 0.1
527 citric pH 5.5 buffer was used, that mimed the lysosomal environment: the EE% decreased from
528 92% to 77% and this could be due to a PTX slow release.

529

530 *3.2 SLN and PTX stability*

531 The studies were carried out only on SLN giving the best results concerning sizes and EE%. The
532 chosen formulations were analyzed to verify their stability overtime. The results are reported in
533 Table 3. SLN 9 showed a great increase in mean diameter (up to 1500 nm) after 30 days, while only
534 a slight decrease occurred in EE%. The poor dimensional stability of SLN 9 could be due to their
535 low Zeta potential (about 9 mV) which is probably not sufficient to prevent SLN aggregation. On
536 the other hand, GCS-SLN (SLN 15, 16, 17) showed a higher dimensional stability although a

537 gradual increase in mean diameter and a gradual decrease in Zeta potential were registered due
538 probably to a slow solubilization of the external GCS coating in the aqueous phase.

539 To overcome the poor stability of SLN suspension and to find an optimal storage method, they were
540 submitted to freeze drying. In Table 4 SLN sizes after freeze-drying were reported. The freeze-
541 drying process was carried out without adding any cryoprotectant; in fact in a previous work [45]
542 PVA 9000 was found to be able to protect SLN during freeze-drying as well as 2% threalose.

543 The characterization of resulting SLN suspensions showed a mean diameter increase of all SLN
544 analyzed probably due to a partial SLN aggregation. This increase was about 300 nm in SLN with
545 ST while only a slight variation was registered in SLN with GCS; probably also GCS, positioning
546 externally, could behave as cryoprotectant preventing nanoparticles aggregation. The smallest
547 increase (about 40 nm) was noted when higher amounts of stabilizer were used. EE% was kept
548 constant in almost all preparations; an increase in EE% (from 75% up to 89%) was registered in
549 SLN 17 that presented a higher amount of free PTX: probably during freeze-drying a part of free
550 PTX was internalized or absorbed onto nanoparticles.

551

552 *3.3 Determination of PTX release*

553 In Figure 2 PTX release plots from SLN using the dialysis method were reported. In this experiment
554 drug diffusion through the dialysis membrane was influenced by drug release rate from SLN. As it
555 can be seen, PTX was released very slowly from SLN: less than 1% PTX was released from SLN
556 formulations in 12 hours while, at the same time, about 40% PTX diffused from PTX solution. This
557 slow release could confirm PTX incorporation into SLN. From the K_d app data it could be seen as
558 PTX release from GCS-SLN was still slower than from ST-GCS; these results could be the
559 confirmation that GCS formed an external coating that further limited drug release.

560

561 *3.4 Permeability of SLN across BBB*

562 As shown in Figure 3, the permeability of free Cou-6 across the hCMEC/D3 monolayer
563 remained very low at all the time points considered; the permeability of Cou-6-loaded SLN was
564 similar to that of free Cou-6 after 0,5 h, but became significantly higher after 3 h and 24 h.
565 Apparently there were no differences in the permeability of uncharged SLN (SLN 2) versus
566 positively charged SLN (SLN 9 and SLN 15), although SLN 9 resulted the best permeable
567 formulation after 24 h.

568

569 *3.5 In vitro cytotoxicity studies*

570 To assess the sensitivity of the five malignant glioma cell lines to PTX, we treated previously the
571 cells with Cremophor® EL:ethanol 50:50 drug solution (free PTX) at concentrations ranging from
572 20 nM to 1.4 μ M for 72 h.

573 After different exposure times both MTT and trypan blue assays were performed. PTX was able to
574 block *in vitro* cellular proliferation in a dose-dependent manner; it inhibited clonogenic growth of
575 NS (Figure 4a–4d) and hindered adhesion process in AC (Figure 4e–4i).

576 The growth inhibitory effect was more evident on AC than on NS.

577 The IC₅₀ values of free PTX at 72 h for the three NS cell lines studied were < 20 nM; for the two
578 AC lines were < 15 nM (Figure 5). Consequently, drug concentration of 100 nM was employed for
579 the following experiments with PTX incorporated in SLN. At this dosage, a statistically significant
580 decrease of cell viability was caused by PTX compared with untreated cells, both as free drug and
581 as SLN-carried, and this effect increased prolonging drug exposure time from 24 to 72 h. It was
582 observed that also PTX encapsulated in SLN caused inhibition of sphere formation in the NS
583 cultures (Figure 4e) and interfered with cell adhesion in AC cultures (Figure 4i).

584 Graphs in Figure 7 show cell viability of NO3 line after the treatments: as regards NS, the viable
585 cell number decreased to 60-70% after 24 h treatment with the different PTX-SLN (Figure 6a) and
586 to 30-40% after 72 h treatment (Figure 6b). At 24 h free PTX and all PTX-SLN gave a cytotoxicity
587 statistically different from control, while only SLN 2 produced an increase in cytotoxicity

588 significantly different from that of empty SLN 2. Prolonging the incubation time, also SLN 9 were
589 significantly different from the corresponding empty SLN.

590 The reduction of viability of AC was about 10-15% higher than the one of NS (Figure 7). An
591 analogous behavior of PTX-SLN 2 and PTX-SLN 9 was noted already after 24 h incubation, while
592 after 72 h all SLN under study produced a cytotoxicities significantly different from those produced
593 by the corresponding empty SLN. The cytotoxicity of PTX incorporated in SLN appeared similar
594 or even slightly higher in comparison with the one of free drug solution. In particular, SLN2 seemed
595 to enhance growth inhibitory activity of PTX on NS lines, while SLN 9 did it on AC lines.

596 The results slightly varied in the other cell lines (data not shown), but the cytotoxic activity of PTX-
597 loaded SLN remained always equal or greater compared with free drug. SLN 2 and SLN 9 appeared
598 as the most effective carrier formulations.

599 Control experiments demonstrated that the exposure of the cells with the unloaded SLN at the
600 highest SLN concentration used for the study determined a mild reduction of cell viability too,
601 mostly on AC. Unloaded SLN 2 formulation displayed the smallest cytotoxic effect, while unloaded
602 SLN15 had a more accentuated action on cell proliferation.

603 Treatment with PTX vehicle (50:50 v/v mixture of Cremophor® EL and ethanol) at the same
604 concentrations used for drug solutions showed a slight cytotoxicity too (data not reported).

605 Our results suggest that, first of all, incorporation of PTX in SLN did not alter its cytotoxic
606 capability and that these carriers, thanks to a good cell membrane permeation, are able to efficiently
607 transport the drug inside the cells. Encapsulation into SLN appeared even to enhance the growth
608 inhibitory effect of PTX on GBM cell lines.

609 In conclusion, loading of PTX in SLN seems to sensitize cells and to decrease their resistance to the
610 drug.

611

612 *3.6 Study of PTX-SLN cytotoxic effect by TEM*

613 The observation by TEM enabled to evaluate the ultrastructural alterations of cells after exposure to
614 SLN. The analysis revealed that the administration of unloaded SLN 15 didn't cause modifications
615 to the cell morphology both in NS and in AC. Cells treated with empty SLN maintained unchanged
616 nucleus, ergastoplasma, Golgi apparatus and mitochondria (Figure 8a).

617 On the contrary, 24 h exposure to SLN loaded with 100 nM PTX induced severe alterations of the
618 cellular morphology that included cytoplasmic accumulation of vacuoles, loss of mitochondrial
619 ridges and finally mitochondrial lysis (Figure 8b-8f). The vacuoles became gradually larger and
620 confluent and in some cases they blended and formed autophagolysosomes surrounded by a
621 membrane. Cytoplasmic rarefaction, glycogen accumulation and destruction of all organelles
622 suggested a typical regressive apoptotic pattern. Only in some areas there was evidence of SLN
623 residues. These results indicate an evident cell damage caused by the PTX vehicled in the SLN.

624

625 *3.7 DNA damage and repair response induced by PTX-SLN treatment*

626 In response to DNA damage caused by genotoxic agents, the eukaryotic cell activates a complex
627 checkpoint/repair pathway in order to repair the lesion before replication. The signalling involves
628 the recruitment of the sensor protein kinase ataxia telangiectasia mutated (ATM), that initiates a
629 transduction cascade phosphorylating downstream effectors, including H2AX histone, 53 binding
630 protein 1 (53BP1) and the checkpoint kinases 1 and 2 (Chk1 and Chk2). When checkpoints are
631 activated, the cell cycle is halted to allow DNA repair. If the damage is too severe, repair fails and
632 the cell enters into apoptosis [47]. Two are the major pathways used by mammalian cells to repair
633 double strand break damage: the homologous recombination (HR), which takes place during the S
634 and G2 phases and is driven mainly by RAD51 protein, and the non-homologous end joining
635 (NHEJ), which occurs during G0 and G1 phases. The key NHEJ effector is DNA-dependent protein
636 kinase (DNA-PK), which consists of a regulatory subunit (Ku70/Ku80 heterodimer) and a catalytic
637 subunit (DNA-PKcs) [48]. In glioma cells, increased DNA damage response and repair ability
638 contribute to determine the radio- and chemoresistant phenotype of these cells [49].

639 After glioma cell treatment for 72 h with relatively high concentration (100 nM) of free PTX we
640 observed a moderate activation of checkpoint and repair proteins. A mild expression of all markers,
641 was found both in NS and in AC (data not shown), indicating that this drug is able to induce
642 genotoxic lesions.

643 Even after NO3 cell line treatment with PTX carried by SLN, we found the presence of γ -H2AX
644 positive-nuclei and a moderate expression of all sensors and effectors of checkpoint/repair pathway,
645 both in NS (Figure 9a-9g) and in AC (Figure 9l-9q), demonstrating that the encapsulated drug
646 maintains the ability to induce a DNA strand break damage in the glioma cell lines. As already
647 observed for the free drug, mostly on AC lines, several cells treated with PTX-SLN appeared
648 blocked in metaphasis (Figure 9i) and presenting a strong expression of the checkpoint/repair
649 proteins (p-ATM, p-Chk2, p-53BP1, γ -H2AX, DNA-PKcs). It seemed from these findings that cells
650 try to repair the genotoxic lesions mainly through the NHEJ system. Untreated cells were negative
651 for all markers (inserts in Figure 9a and 9i are examples for p-ATM expression).

652 After exposure of cells to unloaded SLN for 72 h we did not observe positivity of any
653 checkpoint/repair protein (panels h and r of Figure 9 are examples for p-ATM expression).

654

655 *3.8 Effective cytotoxicity of paclitaxel-loaded SLN against glioblastoma cells in co-culture models.*

656 The cytotoxicity of PTX-loaded SLN on glioblastoma cells was finally compared with that of free
657 PTX in co-culture models of hCMEC/D3 cells and glioblastoma cells. As shown in Figures 10,
658 11 and 12, free PTX induced a dose-dependent increase of cytotoxicity in all the glioblastoma cell
659 lines; PTX-loaded SLN followed a similar trend and showed – at the same concentration – higher
660 efficacy than the free drug. Also the toxicity on hCMEC/D3 cells increased dose-dependently,
661 without significant differences between free PTX and PTX-loaded SLN (Figure 13).

662 By comparing the cytotoxicity profile on glioblastoma cells and hCMEC/D3 cells, we can state that
663 PTX-loaded SLN achieves significant toxicity against tumor cells at concentrations that are not
664 toxic for BBB cells. To obtain the same degree of cytotoxicity on glioblastoma cells, free PTX

665 should be used at concentration that are unacceptably toxic for brain microvascular endothelial
666 cells. The different efficacy of free versus SLN-loaded PTX can be explained by the increased
667 permeability of the latter through the BBB monolayer. PTX is a known substrate of P-glycoprotein
668 (Pgp) [50], which is abundant on the luminal side of BBB cells [51]; therefore, it is a poorly
669 delivered drug into the brain parenchima. Its loading within SLN may represent a valid strategy to
670 overcome the efflux by Pgp at BBB levels.

671 In addition, also glioblastoma has been reported to be rich of Pgp [52] and multidrug resistance
672 related protein 1 (MRP1) [53], a second protein that actively effluxes PTX [50], limiting its
673 intracellular accumulation and toxicity. Therefore, a lower accumulation and toxicity of paclitaxel is
674 expected within glioblastoma cells. The loading within SLN may promote a faster uptake and/or a
675 lower efflux by Pgp and MRP1 from glioblastoma cells as well. Therefore, the loading in SLN may
676 represent a valid strategy to simultaneously bypass the drug efflux at BBB and glioblastoma levels.

677 **4. Conclusion**

678 The fatty acid coacervation technique, a solvent free method, was used to prepare PTX-loaded SLN.

679 The use of positive-charged molecules, such as ST and GCS, allowed to obtain positive SLN with

680 sizes in the 300-600 nm range and PTX EE% in the 25-90% range.

681 Permeability studies through hCMEC/D3 cells monolayer, assumed as *in vitro* model of the BBB,

682 showed a significantly increase in the permeation of Cou-6, used as model drug, when vehicled in

683 SLN; no significantly differences in the permeability of uncharged SLN versus positively charged

684 SLN were noted after 24 h.

685 Cytotoxicity studies on NO3 glioblastoma cell line demonstrated the absence of toxicity of

686 unloaded SLN 2 and 9 and the preservation of cytotoxic activity of PTX-loaded SLN that remained

687 always equal or greater compared with free drug.

688 In co-culture experiments with hCMEC/D3 and different glioblastoma cells, it was demonstrated

689 that, when delivered in SLN, PTX increased its cytotoxicity towards glioblastoma cells, because of

690 its increased permeation through the endothelial cell monolayer.

691 According to these *in vitro* results, the positive charge did not seem essential to enhance PTX brain

692 uptake but fundamental seem to be the PTX encapsulation into SLN.

693 Further i.v. administration of PTX-loaded SLN in rats are in progress to confirm the suitability of

694 SLN in enhancing PTX overcoming of the BBB.

695

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834 **FIGURE CAPTIONS**

835

836 Figure A.1: Anatomical structures composing the BBB. AJs: adherent junctions. TJs: tight
837 junctions. ABC: ATP binding cassette transporters.

838

839 Figure 1: TEM images. a) 2200X magnification; b) 8900X magnification.

840

841 Figure 2: PTX release plots from nanoparticles using the dialysis method

842

843 Figure 3: Cou-6 permeability 0.1% dilution. * $p < 0.001$

844

845 Figure 4: Effect of 72 h treatment with increasing doses (100, 500, 1400 nM) of free and 100 nM
846 SLN10-encapsulated PTX on NO3 NS (b–e) and NO3 AC (g–k) growth. Control cells (a, f) did not
847 receive any treatment. All 200x magnification.

848

849 Figure 5: Cytotoxicity of free PTX on NS (A) and AC (B) of NO3 glioma cell line at various
850 dosages of drug (20, 100, 500, 1400 nM) after 72 h treatment.

851

852 Figure 6: a) cell viability of NO3 NS after 24 h treatment; b) cell viability of NO3 NS after 72 h
853 treatment + $p < 0.05$ vs CTRL; * $p < 0.05$ PTX-SLN vs SLN; ++ $p < 0.01$ vs CTRL; ** $p < 0.01$
854 PTX-SLN vs SLN

855

856 Figure 7: a) cell viability of NO3 AC after 24 h treatment; b) cell viability of NO3 AC after 72 h
857 treatment ++ $p < 0.01$ vs CTRL; * $p < 0.05$ PTX-SLN vs SLN;

858

859 Figure 8: TEM of NS after treatment with SLN15 at 15000 x. (a) After 24 h treatment with
860 unloaded SLN15. (b) After 6 h treatment with 100 nM PTX-loaded SLN15 (c) After 24 h treatment
861 with 100 nM PTX-loaded SLN15 (d) Id, large vacuoles surrounded by a membrane, (e) Id,
862 autophagolysosomes, 15000 x. (f) Possible remnants of SLN (20000 x).

863

864 Figure 9: Expression by IF of checkpoint/repair proteins in NO3 glioma cells after 72 h treatment
865 with 100 nM PTX-loaded SLN10: p-ATM, 200x (a), γ -H2AX, 200x (b), p-Chk2, 200x (c), p-
866 53BP1, 200x (d), DNA-PKcs, 200x (e), Ku70/80, 200x (f), RAD51, 200x (g) in NS; AC arrested in
867 metaphases, DAPI, 200x (j); p-ATM, 200x (k), γ -H2AX foci, 400x (i), p-Chk2, 200x (l), p-53BP1,
868 200x (m), DNA-PKcs, 200x (n), RAD51, 200x (o) in AC; nuclei counterstained with DAPI.
869 Negative reaction for p-ATM in untreated NS and AC, respectively (upper and lower left inserts of
870 panels a and i) and in NS and AC treated with unloaded SLN for 72 h (h and p); nuclei
871 counterstained DAPI, 200x.

872

873 Figure 10: cytotoxicity against CV17 cells. + $p < 0.05$ vs CTRL; ++ $p < 0.02$ vs CTRL;
874 +++ $p < 0.01$ vs CTRL; ++++ $p < 0.005$ vs CTRL; +++++ $p < 0.001$ vs CTRL; * $p < 0.05$ SLN vs
875 PTX; ** $p < 0.02$ SLN vs PTX; *** $p < 0.001$ SLN vs PTX.

876

877 Figure 11: cytotoxicity against O625 cells. + $p < 0.05$ vs CTRL; ++ $p < 0.02$ vs CTRL; +++ $p <$
878 0.01 vs CTRL; ++++ $p < 0.005$ vs CTRL; +++++ $p < 0.001$ vs CTRL; * $p < 0.05$ SLN vs PTX.

879

880 Figure 12: cytotoxicity against U87 cells. + $p < 0.01$ vs CTRL; ++ $p < 0.002$ vs CTRL; +++ $p <$
881 0.005 vs CTRL; * $p < 0.05$ SLN vs PTX; ** $p < 0.02$ SLN vs PTX; *** $p < 0.001$ SLN vs PTX.

882

883 Figure 13: cytotoxicity against hCMEC/D3 cells. + $p < 0.01$ vs CTRL; ++ $p < 0.005$ vs CTRL
884 * $p < 0.05$ SLN vs PTX; ** $p < 0.002$ SLN vs PTX.

SLN	Na-BA* (mg)	PVA 9000 (mg)	PTX (mg)	CHOL (mg)	ST (mg)	GCS (mg)	Mean diameter (nm) (PDI)	Zeta Potential (mV)
0	53	37.5	3	-	-	-	299.2 ± 0.3 (0.053)	- 3.04 ± 0.56
1	53	37.5	3	0.5	-	-	381.3 ± 20.4 (0.152)	- 3.30 ± 0.47
2	53	37.5	4.5	0.5	-	-	386.4 ± 12.3 (0.167)	- 3.34 ± 0.79
3	53	37.5	6	0.5	-	-	383.8 ± 23.2 (0.085)	- 3.41 ± 0.60
4	53	37.5	3	-	3	-	345.6 ± 9.7 (0.089)	7.85 ± 0.91
5	53	37.5	3	-	6	-	537.7 ± 13.5 (0.105)	14.37 ± 1.52
6	53	37.5	3	0.5	3	-	581.0 ± 61.1 (0.201)	8.03 ± 0.70
7	53	25	3	0.5	3	-	507.0 ± 10.5 (0.099)	10.19 ± 0.69
8	53	37.5	3	0.75	3	-	579.6 ± 7.7 (0.115)	10.40 ± 1.14
9	53	37.5	4.5	0.5	3	-	548.6 ± 20.2 (0.130)	9.07 ± 2.45
10	53	37.5	6	0.5	3	-	984.9 ± 93.6 (0.103)	7.65 ± 0.85
11	53	37.5	6	-	-	10	553.5 ± 5.7 (0.196)	16.90 ± 4.13
12	53	37.5	6	-	-	16	488.4 ± 19.3 (0.213)	23.79 ± 1.81
13	53	50	6	-	-	10	469.6 ± 3.8 (0.202)	15.46 ± 2.32
14	53	100	6	-	-	10	624.3 ± 16.1 (0.205)	15.64 ± 3.12
15	53	37.5	4.5	0.5	-	10	462.7 ± 32.3 (0.227)	18.21 ± 1.67
16	53	37.5	6	0.5	-	10	476.4 ± 8.4 (0.196)	20.28 ± 1.10
17	53	50	6	0.5	-	10	374.5 ± 4.8 (0.232)	19.56 ± 1.73

886

887 Table 1

888

SLN	PTX EE%	PTX LOADING (μg PTX/mg BA)
0	78 \pm 4	47 \pm 2
1	85 \pm 5	50 \pm 3
2	89 \pm 3	80 \pm 3
3	90 \pm 3	108 \pm 4
4	70 \pm 6	42 \pm 4
5	25 \pm 3	16 \pm 2
6	87 \pm 4	52 \pm 2
7	98 \pm 2	60 \pm 1
8	65 \pm 6	40 \pm 4
9	92 \pm 4	82 \pm 3
11	77 \pm 2	92 \pm 2
12	81 \pm 4	98 \pm 5
13	52 \pm 5	62 \pm 6
14	24 \pm 3	28 \pm 3
15	83 \pm 4	74 \pm 3
16	83 \pm 3	100 \pm 4
17	75 \pm 5	90 \pm 6

889

890 Table 2

891

		SLN 9	SLN 15	SLN 16	SLN 17
7 days	Size (nm)	682.4 ± 15.1	783.2 ± 74.3	554.2 ± 9.5	500.1 ± 6.4
	%EE	92 ± 3	81 ± 2	80 ± 2	74 ± 3
	Zeta potential (mV)	9.15 ± 0.85	13.4 ± 0.9	15.9 ± 2.8	18.3 ± 1.6
30 days	Size (nm)	1500.0 ± 21.6	799.7 ± 63.2	598.1 ± 14.6	599.2 ± 9.5
	%EE	91 ± 4	80 ± 3	79 ± 3	73 ± 2
	Zeta potential (mV)	9.05 ± 0.54	11.6 ± 1.4	14.5 ± 3.3	14.4 ± 3.1
60 days	Size (nm)	1536.8 ± 39.2	933.8 ± 17.9	669.8 ± 27.3	686.1 ± 12.4
	%EE	89 ± 4	80 ± 3	71 ± 2	73 ± 2
	Zeta potential (mV)	9.07 ± 0.65	10.7 ± 2.1	13.9 ± 3.4	12.9 ± 4.1

892

893 Table 3

894

	SLN 9	SLN 15	SLN 16	SLN 17
Mean diameter (nm)	842.0 ± 17.9	632.4 ± 18.4	592.3 ± 18.0	408.4 ± 5.8
Zeta potential (mV)	8.66 ± 1.02	18.56 ± 1.34	19.68 ± 1.98	19.87 ± 1.45
PTX EE%	88 ± 3	81 ± 2	83 ± 2	89 ± 3

895 Table 4

896

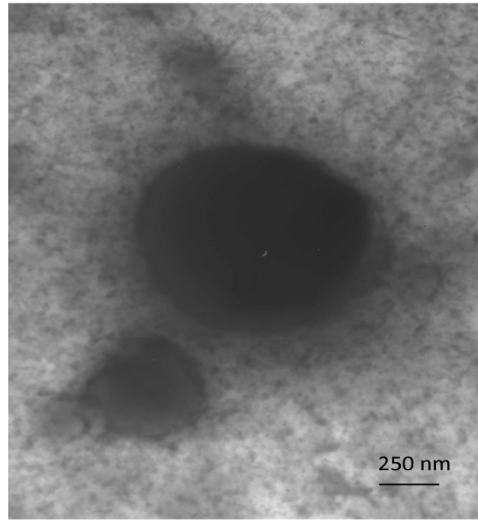
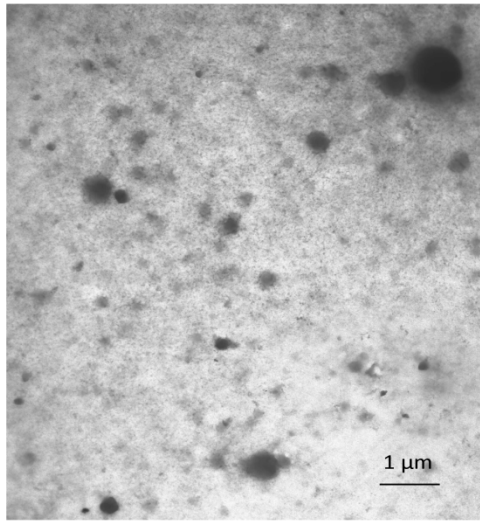


Figure 1a and 1b

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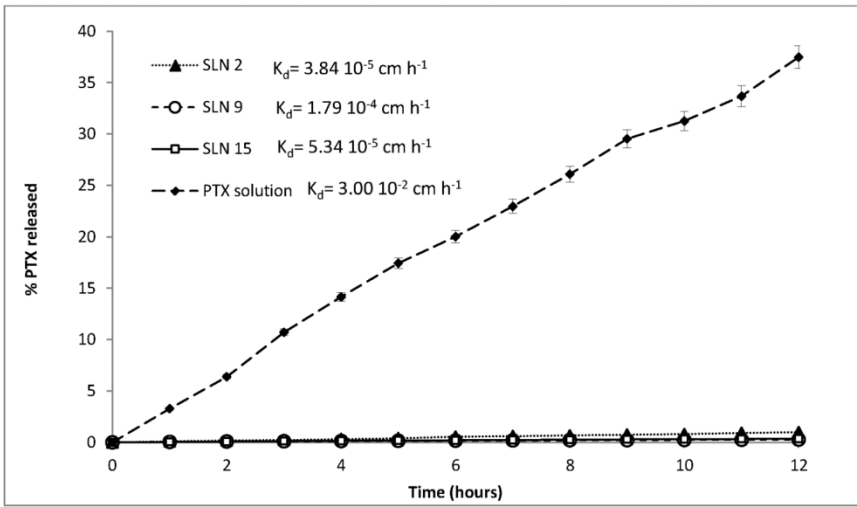


Figure 2

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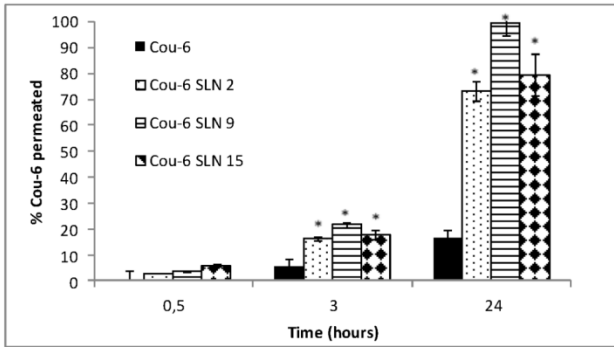


Figure 3

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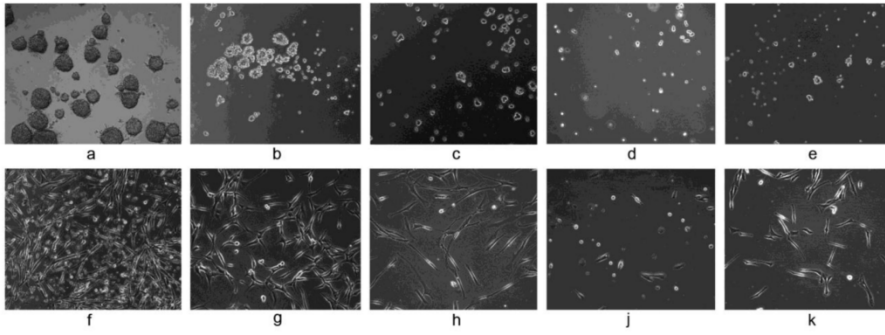


Figure 4

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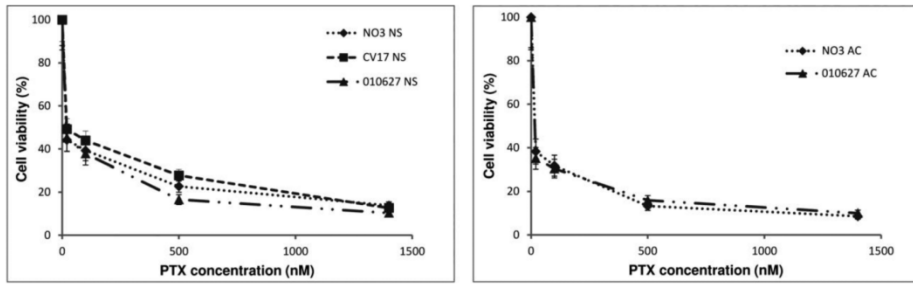


Figure 5

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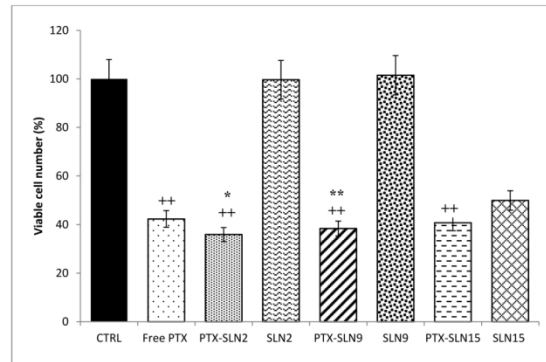
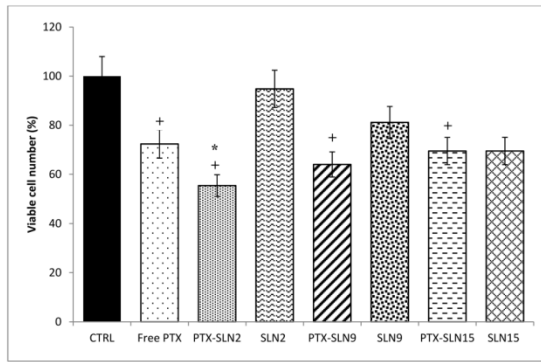


Figure 6

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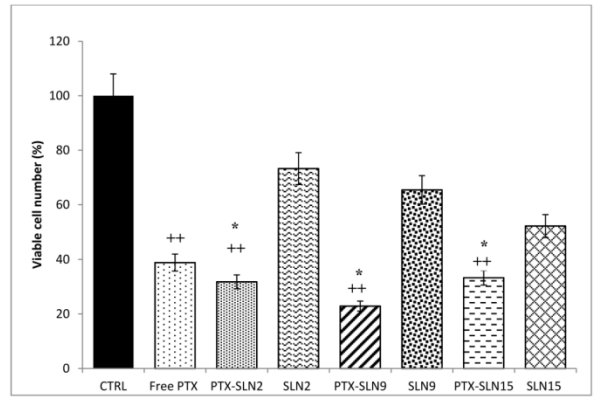
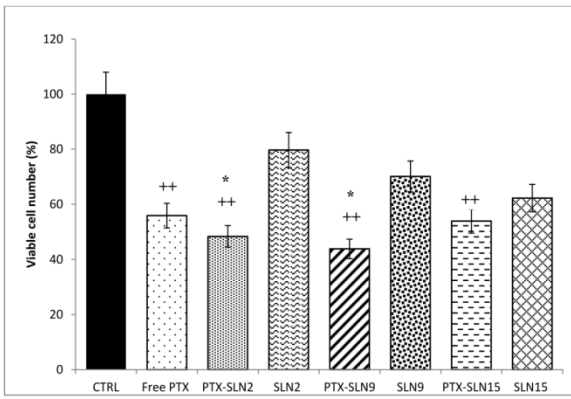


Figure 7

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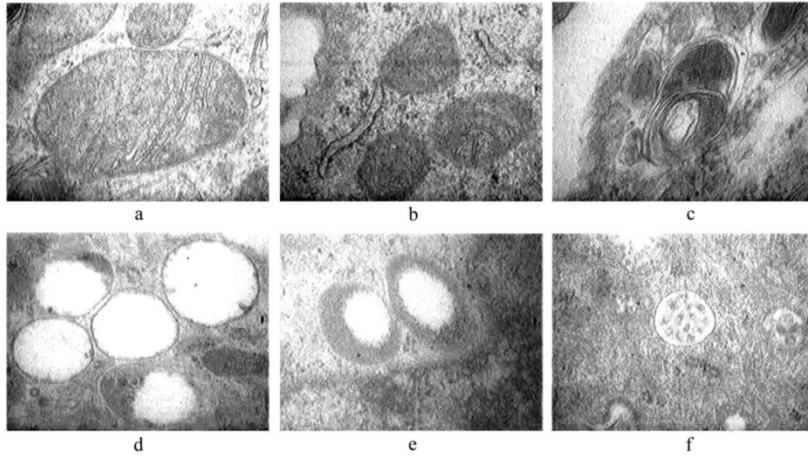


Figure 8

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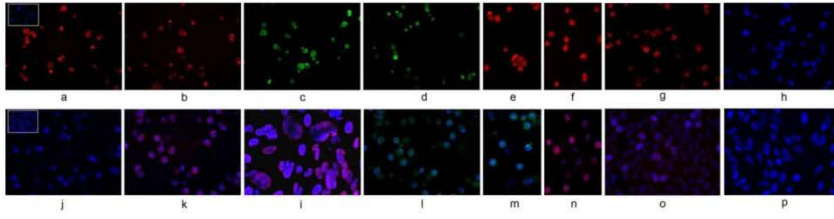


Figure 9

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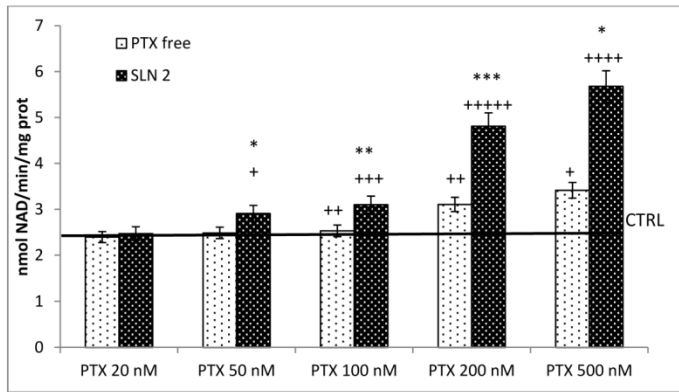


Figure 10

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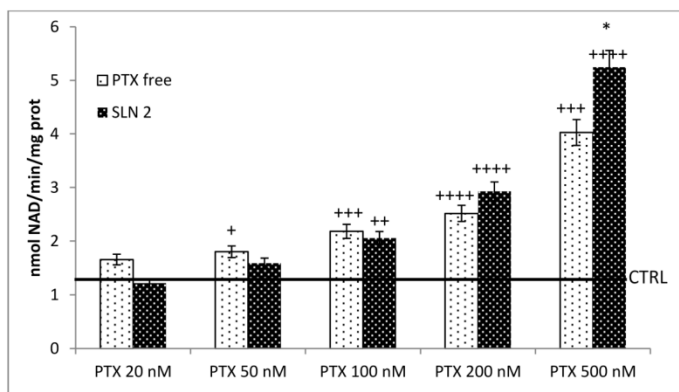


Figure 11

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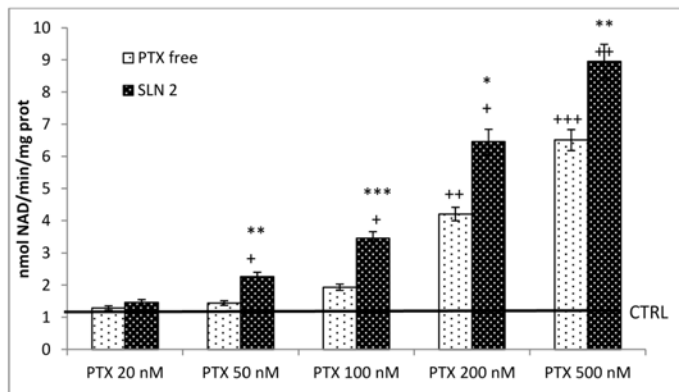


Figure 12

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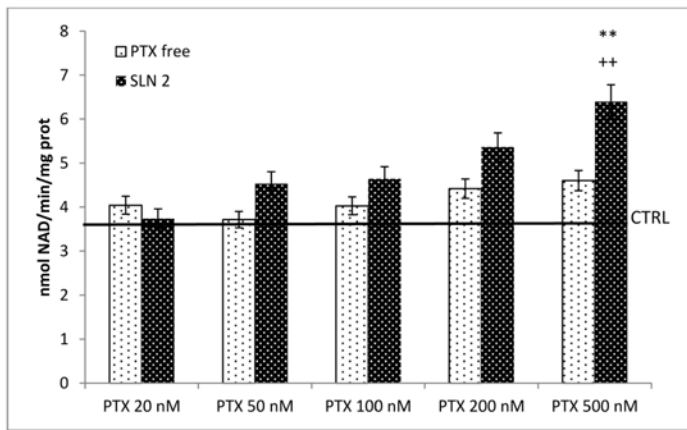


Figure 13

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