This is the Post print version of a manuscript published in International Journal of Pharmaceutics at https://doi.org/10.1016/j.ijpharm.2020.119351.

PEGylated Solid Lipid Nanoparticles for Brain Delivery of Lipophilic Kiteplatin Pt(IV) Prodrugs: an In Vitro Study

Ilaria Arduino[1], Nicoletta Depalo[2], Francesca Re[3], Roberta Dal Magro[3], Annamaria Panniello[2], Nicola Margiotta[4], Elisabetta Fanizza[2,4], Antonio Lopalco[1], Valentino Laquintana[1], Annalisa Cutrignelli[1], Angela Assunta Lopedota[1], Massimo Franco[1] and Nunzio Denora[1,2,*]

[1]Department of Pharmacy - Pharmaceutical Sciences, University of Bari "Aldo Moro", Orabona St. 4, 70125, Bari, Italy.

[2]CNR-Institute for Physical and Chemical Processes SS, Bari, Italy.

[3]School of Medicine and Surgery, Nanomedicine Center NANOMIB, University of Milano-Bicocca, Vedano al Lambro, MB, Italy.

[4]Department of Chemistry, University of Bari Aldo Moro, Orabona St. 4, 70125, Bari, Italy.

*** Corresponding author:**

Department of Pharmacy – Pharmaceutical Sciences, University of Bari "Aldo Moro", Orabona St. 4, 70125 Bari, Italy
Tel: +39 080 544 2767.
E-mail address: nunzio.denora@uniba.it (Prof. N. Denora)

Abstract

Here, polyethylene glycol (PEG)-stabilized solid lipid nanoparticles (SLNs) containing Pt(IV) prodrugs derived from kiteplatin were designed and proposed as novel nanoformulations potentially useful for the treatment of glioblastoma multiforme. Four different Pt(IV) prodrugs were synthesized, starting from kiteplatin by the addition of two carboxylate ligands with different length of the alkyl chains and lipophilicity degree, and embedded in the core of PEG-stabilized SLNs composed of cetyl palmitate. The SLNs were extensively characterized by complementary optical and morphological techniques. The results proved the formation of SLNs characterized by average size under 100 nm and dependence of drug encapsulation efficiency on the lipophilicity degree of the tested Pt(IV) prodrugs. A monolayer of immortalized human cerebral microvascular endothelial cells (hCMEC/D3) was used as *in vitro* model of blood-brain barrier (BBB) to evaluate the ability of the SLNs to penetrate the BBB. For this purpose, optical traceable SLNs were achieved by co-incorporation of Pt(IV) prodrugs and luminescent carbon dots (C-Dots) in the SLNs. Finally, an *in vitro* study was performed by using a human glioblastoma cell line (U87), to investigate on the antitumor efficiency of the SLNs and on their improved ability to be cell internalized respect to the free Pt(IV) prodrugs.

Keywords: Solid lipid nanoparticles, Pt(IV) prodrugs, Luminescent carbon dots, *In vitro* BBB model, brain delivery, glioblastoma, hCMEC/D3.

1. Introduction

The development of efficient treatment for the malignant glial tumours affecting the central nervous system (CNS) remains an open challenge. Particularly, glioblastoma multiforme (GBM) is the most malignant and lethal form of gliomas in adults, causing death in patients just over 6 months after diagnosis without treatment. Actually, conventional GBM managements fail owing to the rapid growth and spread of tumour cells, which are able to rapidly migrate and invade healthy brain tissues (Stupp et al. 2005; Zhang et al. 2012). The current most effective treatment of GBM consists of combination of different therapies, namely surgical resection of the tumour followed by radiotherapy and chemotherapy often based on temozolomide, resulting in aggressive medical intervention and devastating neurological consequences with an average survival expectancy of patients less than 18 months. In this scenario, new therapeutic approaches are needed to ensure a better quality of life and longer survival times for patients affected by GMB.

At present, although only minimal success has been achieved by exploring the use of platinum (Pt) based drugs for the GBM treatment, new emerging experimental evidences are suggesting the exploitation of the still unexplored multi-faceted therapeutic effects of Pt based agents. Indeed, the therapeutic potential activity of the Pt based compounds seems to be related not only to cytotoxicity, due to DNA damaging effect that induces cell apoptosis, but also to concomitantly not-cytotoxic activities, such as inhibition of cancer cell invasion or angiogenesis and reverse immunosuppression (Calogero et al. 2011; Prasek Kutwin et al. 2017; Roberts et al. 2016; Sheleg et al. 2002). Furthermore, the combination of Pt-based drugs with current GBM therapies and radiation can also provide an enhanced therapeutic response (Rousseau et al. 2010; Shi et al. 2015; Zhou et al. 2013). Currently, the clinically approved Pt based anticancer complexes are only cisplatin, oxaliplatin and carboplatin. The use of these drugs in the management of GBM only marginally improves the overall survival of patients and concomitantly induces several and serious toxic side effects (Aoki et al. 2006; Brandes et al. 2003; Brandes et al. 2004; Buckner et al. 2006; Charest et al. 2012; Franceschi et al. 2004; Fruehauf et al. 2006; Galanis et al. 2005; Grossman et al. 1997; Haroun et al. 2002; Limentani et al.

2005; Peterson 2001; Prados et al. 2003; Scopece et al. 2006; Shi et al. 2015; Silvani et al. 2002; Silvani et al. 2004; Soffietti et al. 2004; Yung, Mechtler, and Gleason 1991). The main reasons of the limited success of Pt based agents for the GBM treatment is mainly ascribed to their limited accumulation to the specific tumor site, strictly related to the blood-brain barrier (BBB) if not damaged by the tumor (Denora et al. 2009; Provenzale, Mukundan, and Dewhirst 2005), and to the inherent or acquired resistance to this type of chemotherapy, along with severe side effects. In particular, the dose-limiting toxicity of Pt-based compounds for GBM treatment has prevented reaching the same level of success achieved for other cancers (Bernocchi et al. 2011). The search for more effective Pt anticancer drugs has led to the design, and pre-clinical testing of thousands of new Pt complexes. Among them a new promising Pt based drug is kiteplatin, which belongs to the category of anticancer compounds of Pt(II), such as cisplatin, carboplatin and oxaliplatin. The interest for this compound derives from its activity against sensitive and resistant cisplatin cell lines (Margiotta et al. 2012). Besides, Pt(IV) based drugs, considered prodrugs for Pt(II) compounds, are currently under investigation in order to find new more effective Pt based anticancer agents able to overcome side effects and limitations. In this contest, S. Shamsuddin et al. synthesized kiteplatin Pt(IV)-prodrugs with different degrees of lipophilicity by deriving them in axial position with two hydrophobic carboxylated ligands (Shamsuddin et al. 1998). Due to the increased lipophilicity, these prodrugs were subsequently loaded into polymeric micelles by Margiotta et al. evidencing, *in vivo,* to be effective anticancer agents endowed by toxicity lower than that shown by kiteplatin (Margiotta et al. 2016). Inspired from these findings and considering the promising studies reported in literature concerning drug delivery nanocarriers able to cross the BBB releasing the therapeutic cargo to the tumor region (Charest et al. 2012; Latronico et al. 2016; Shi et al. 2015; Zhou et al. 2013), the aim of this investigation was the development and characterization of solid lipid nanoparticles (SLNs) loaded with kiteplatin Pt(IV)-prodrugs potentially able to cross, *in vitro*, an endothelial cells monolayer simulating the BBB and to affect the cell vitality of glioblastoma cells.

In particular, four Pt(IV)-prodrugs derived from kiteplatin by the addition of two carboxylate ligands with different length of the alkyl chain and degree of lipophilicity were loaded into polyethylene glycol (PEG)-stabilized SLNs, which are appealing drug delivery nanovectors with an inherent ability to permeate the BBB even without any functionalization (Blasi et al. 2007; Chaichana et al. 2010; Tapeinos, Battaglini, and Ciofani 2017). The ability of SLNs to cross BBB was investigated *in vitro* on immortalized human cerebral microvascular endothelial cells (hCMEC/D3). For this purpose, optical traceable SLNs were prepared by means of the co-encapsulation, in the hydrophobic core, of Pt(IV)-prodrugs and luminescent carbon dots (C-Dots). Finally, the anticancer activity of the novel SLNs was investigated by performing an *in vitro* study on human glioblastoma cell line (U87), thus

highlighting an enhanced cellular uptake of the Pt(IV)-prodrugs when delivered by means of SLNs respect to free counterparts.

2. Materials and Methods

2.1. Materials

All chemicals were of the highest purity available and were used as received without further purification or distillation. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy (poly (ethylene glycol)-2000] (16:0 PEG-2-PE) was purchased from Avanti Polar Lipids. Cetyl palmitate was purchased from Farmalabor. Nitric acid (HNO_3, 67%), hydrogen peroxide solution (H_2O_2, 30%), anhydrous citric acid (CA, ≥97%) and octadecene (ODE, 90%) were purchased from Sigma-Aldrich. 1-Hexadecylamine (HAD, 98%), was purchased by Fluka. All solvents used were of analytical grade and purchased from Aldrich. All aqueous solutions were prepared using water obtained from a Milli-Q gradient A-10 system (Millipore, 18.2 MΩ·cm, organic carbon content ≥ 4 μg/L). Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), penicillin (100 U/mL) and streptomycin (100 μg/mL) were purchased from EuroClone. Disposable culture flasks and Petri dishes were from Corning (Glassworks). 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich. EndoGRO basal medium and medium supplements were from Merck. Lucifer Yellow dilithium salt was purchased from Sigma Aldrich.

2.2. Synthesis of C-dots

Colloidal carbon quantum dots (C-Dots) were synthesized by the carbonization of carbon precursor (CA) in hot boiling solvent (ODE) in the presence of amphiphilic amine ligand (HDA). The synthesis was conducted by using a standard air-free technique. In particular, all reagents were dried and degassed in a vacuum-nitrogen line. Typically, a mixture of HDA (6 mmol) in ODE was degassed and successively heated to 200°C under nitrogen flux. Then, CA (5 mmol) was rapidly added to the ligand mixture. C-Dots were formed due to the CA decomposition, followed by the condensation among the carboxylic moieties of the carbon precursor and the amine and the subsequent carbonization step. The reaction was stopped after 180 min, by gradually cooling to room temperature. The obtained reaction mixture was purified by numerous cycles of non-solvent

precipitation and re-dispersion in acetone. Finally, a common weakly polar solvent, such as chloroform, was added to the NP pellet to obtain stable colloidal dispersion of C-Dots (Panniello et al. 2018).

2.3. Preparation of Solid Lipid Nanoparticles loaded with Pt-(IV)-Prodrugs

Four different Pt(IV)-prodrugs, namely *cis,trans,cis*[$PtCl_2\{O_2C(CH_2)_4CH_3\}_2$(*cis*-1,4-diaminocyclohexane)] (SMF 111), *cis,trans,cis*[$PtCl_2\{O_2CCH_3)_2CH_3\}_2$(*cis*-1,4-diaminocyclohexane)] (SMF 196), *cis,trans,cis*[$PtCl_2\{O_2C(CH_2)_2CH_3\}_2$(*cis*-1,4-diaminocyclohexane)] (SMF 200) and *cis,trans,cis*[$PtCl_2\{O_2C(CH_2)_8CH_3\}_2$(cis-1,4-diaminocyclohexane)] (SMF 144), were synthesized according to the experimental procedure reported elsewhere (Margiotta et al. 2016). An oil-in-water homogenization process at high temperature was used to prepare the SLNs loaded with the synthesized Pt(IV)-prodrugs or kiteplatin (Pt(IV)-Prodrug/SLNs or Kiteplatin/SLNs), according to a procedure reported in literature with some modifications (Grillone et al. 2015), more specifically, pertaining the evaporation process of the organic solvent and the SLNs purification method. In particular, for each type of chemotherapeutic agent, 1 mg of drug was dissolved in 500 μL of hot methanol (65 °C) and, 100 mg of cetyl palmitate and 12 mg of 16:0 PEG-2-PE were co-dissolved in chloroform (1 mL). Subsequently, the methanol and chloroform solutions were mixed and gently stirred to obtain an homogeneous mixture, that was then introduced drop by drop into a aqueous solution (ultrapure water, 3 mL) containing Tween 80 (3% p/V) at 65 °C and sonicated for 15 min by using a probe-tip ultrasonicator (0.27 W). The organic phase was rapidly evaporated at 65 °C by means of a rotary evaporator. In order to gain an insight into the stability of Pt(IV)-prodrugs during the formulation process, their chemical stability were checked by ^{1}H-NMR spectroscopy (Figure S1, Supplementary Material). The aqueous solution was left at room temperature for two hours to promote the complete evaporation of the organic solvents and, then, it was kept at 4 °C for 15 min to allow the SLNs formation. In order to remove the surfactant, solvent residuals and non-encapsulated drug, the produced SLNs were carefully purified by using ultrapure water and centrifugal concentrators (Centricon Centriplus *YM100*) at 800 g for 1 hour at 4°C. Luminescent SLNs loaded with Pt-(IV)-prodrug or kiteplatin and C-Dots (C-Dots/Pt(IV)-Prodrug/SLNs or C-Dots/Kiteplatin/SLNs) were prepared by following the same procedure and by adding the C-Dots (55 mg) in the organic phase. Stability studies of the SLNs were performed at 4 ° C for a period of 3 months evidencing no aggregates. All the SLNs were kept in ultrapure water at 4 °C.

2.4. Evaluation of Drug Encapsulation Efficiency

The encapsulation efficacy (EE%) values of drug loaded in hydrophobic core of SLNs, for each tested chemotherapeutic agent, were obtained by evaluating the platinum content in 100 µL of the SLNs aqueous dispersion. In particular, SLNs were digested by treatment with 2 mL of a mixture (1:1 (v/v)) containing HNO_3 (67%) and H_2O_2 (30%) solution, for 4 hours at 60 °C; the platinum content was determined by atomic absorption spectroscopy analysis (Varian Zeeman SpectrAA 880Z Spectrometer & GTA-100 Graphite Tube Atomizer). All atomic absorption measurements were carried out in triplicate at room temperature. The EE% values of drug were calculated according the following formula:

$$(EE\ \%) = (W_t/W_i) \times 100$$

where Wt is the total amount of drug in the SLNs dispersion and Wi is the total quantity of drug added initially during preparation.

2.5 In vitro drug release study

Release studies of Pt(IV)-prodrugs from SLNs were carried out with Franz cells, in presence and absence of human serum (Sigma Aldrich, Milan) in the donor compartment. Briefly, 500 µL of Pt(IV)-prodrug/SLNs dispersion was diluted with 500 µL of water or human serum and placed on the diffusion barrier (area of 0.6 cm^2) formed by an artificial cellulose acetate membrane (0.1-0.5 kDa, Fisher Scientific Milano), which separates donor and receptor cells. Phosphate buffer (PBS, 10 mM, pH 7.4) was selected as receptor medium. The receptor phase was continuously stirred and kept at a temperature of (37±0.5) °C. For each SLNs, at scheduled time the same volume (0.3 mL) of receptor solution was collected within 120 hours and the same amount of fresh PBS solution was added in the receptor cells to keep sink conditions. The collected fractions were analyzed by ICP-MS (Varian Zeeman SpectrAA 880Z Spectrometer & GTA-100 Graphite Tube Atomizer) to determine the Pt content. Each experiment was performed in triplicate and was carried out in three independent Franz cells using three different batches of SLNs.

2.6 Stability studies in human serum

The performance of the SLNs in biological conditions, in the case of i.v. administration, was tested by assessing the stability of the system in human serum, as described in Fontana et al (Fontana et al. 2018). A suspension of 200 µL of SLNs in 1.5 ml of human plasma was incubated at 37 ºC. Samples of 100 µL were withdrawn at 5, 15, 30, 60, 90, and 120 min and diluted in water (900 µL). Changes

on the size and PDI of the SLNs over time were checked. All the experiments were performed in triplicates.

2.7 In vitro model of Blood Brain Barrier (BBB)

hCMEC/D3 cells were provided by Pierre-Olivier Couraud (Université Paris Descartes, Paris, France). Cells between passages 25 and 35 were grown in EndoGRO medium supplemented with EndoGRO-MV supplement kit, basal FGF (200 ng/ml), penicillin-streptomycin (1%), lithium chloride (10 mM) and resveratrol (10 μM). To establish an *in vitro* model of BBB, cells (50000 cells/cm^2) were seeded on the apical side of transwell inserts (polyester 12-well, pore size 0.4 μm, translucent membrane inserts 1.12 cm^2; Costar) pre-coated with rat tail collagen type I (4 μg/cm^2) and grown for 14 days at 37 °C, 5% CO_2, and saturated humidity. Medium (500 μL in the apical compartment and 1 mL in the basolateral compartment) was changed every 2-3 days. The formation of tight junctions (TJ) was monitored by measuring the transendothelial electrical resistance (TEER) of the cell monolayer by using an STX2 electrode epithelial volt-ohm meter (World Precision Instruments, FL, USA).

2.8 Cytotoxicity of Pt(IV)-Prodrugs/SLNs formulations

Assessment of C-Dot/Pt(IV)-Prodrug/SLNs cytotoxicity on hCMEC/D3 cells was trialled by means of MTT assay. Namely, hCMEC/D3 cells were dispensed into 96-well plates at a density of 66000 cells/cm^2 and incubated with C-Dot/Pt(IV)Prodrug/SLNs or C-Dot/Kiteplatin/SLNs dispersed in cell medium at the final lipid concentrations of 0.03, 0.3 and 1.5 mg/mL (corresponding to drug concentration of 0.01, 0.1 and 0.5 μM respectively) at 37 °C, 5% CO_2. After 24 hours, medium was removed, and wells were filled with 100 μL of MTT solution (0.5 mg/mL in cell culture medium) and incubated at 37 °C. After 2 hours, the absorbance at 570 nm was measured by means of a spectrometer (SPECTROstar Nano, BMG Labtech). Control was represented by untreated cells.

2.9 Endothelial permeability of luminescent Solid Lipid Nanoparticles loaded with Pt-(IV)-Prodrugs across the in vitro BBB model

At day 14 of hCMEC/D3 culture in the transwell system, C-Dot/Pt(IV)Prodrug/SLNs or C-Dot/Kiteplatin/SLNs (0.1 μM of drug concentration, 0.2 mg/mL of C-Dot concentration) suspended in PBS were added to the apical compartment of the transwell. After 3 hours, samples from the basolateral compartments were collected and the C-Dots associated photoluminescence (PL) (λ_{ex}= 360 nm, λ_{em}= 450 nm) was measured using a spectrofluorometer FP-8500 (Jasco). Impact of the SLNs on the cell monolayer integrity was evaluated by estimating the paracellular permeability of Lucifer

Yellow (LY, 457 kDa). Briefly, 500 μL of LY (50 μM in PBS) were added to the apical compartment of the transwell system and incubated for up to 60 min at 37 °C. Samples from the basolateral compartment were collected every 30 min and the fluorescence of the probe (λ_{ex}= 425 nm, λ_{em}= 530 nm) was quantified. Calibration curves were previously calculated by reporting the fluorescence intensity values *versus* concentration of standard solutions for the both C-Dots and LY. The endothelial permeability (EP) of SLNs formulation and of LY were calculated as previously described (Sancini et al. 2016). MTT assay was finally performed to assess the cell viability of endothelial cells after incubation with the different SLNs.

2.10 Cytotoxicity assays and uptake study of Solid Lipid Nanoparticles loaded with Pt-(IV)-Prodrugs on U87-MG cells

Human glioblastoma U87-MG cell line were purchased from American Type Culture Collection (ATCC, VA, USA) and were cultured in DMEM medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 1% of MEM in a humidified incubator at 37°C with an atmosphere containing 5% CO_2.

Cytotoxicity of Pt(IV)-Prodrug/SLNs or Kiteplatin/SLNs was determined on glioblastoma cell lines U87-MG by using the MTT assay, as described before (Depalo et al. 2017). Briefly, cells were dispensed into 96 microtiter plates at a density of 5000 cell/well. After overnight incubation, cells were exposed to Pt(IV)-prodrugs/SLNs at four concentrations of lipids, ranging from 0.03-7.5 mg/mL in the nutrient medium. The concentrations of Pt(IV)-prodrugs tested were varied from 0.01-2.5 μM. After 24 and 72 hours of incubation, 10 μL of 0.5% w/v MTT was added to each well, and plates were incubated for an additional 3h at 37 °C. Finally, cells were lysed by addition of 100 μL of DMSO. The absorbance at 570 nm was determinate by using a PerkinElmer 2030 multilabel reader Victor TM X3. The viability of cells treated with Pt(IV)-prodrug/SLNs was compared with viability of cells treated with Pt(IV)-prodrugs in concentrations ranging from 0.01-2.5 μM.

The uptake study of Pt(IV)-Prodrug/SLNs or Kiteplatin/SLNs on U87-MG cells were performed by seeding the cells in 60 mm tissue culture dishes at a density of 500.000 cells/dish and incubated at 37°C in a humidified atmosphere with 5% CO_2. After 1 day, the culture medium was replaced with 3 mL of medium containing Pt(IV)-Prodrug/SLNs or Kiteplatin/SLNs at drug concentration of 0.1 μM and incubated for 4 and 24 h. After incubation, the cell monolayer was washed twice with ice-cold PBS, then treated with 2 mL of the freshly prepared mixture containing HNO_3/H_2O_2 (1:1, v/v), for 4h at 60 °C in a stove. Pt content was determinate by atomic absorption spectroscopy and by using Varian Zeeman SpectrAA 880Z Spectrometer & GTA-100 Graphite Tube Atomizer.

2.11 Spectroscopic investigation.

A Cary 5000 (Varian) UV/Vis/NIR spectrophotometer was used to perform the UV-Vis absorption measurements. Photoluminescence (PL) emission spectra were acquired by using a Fluorolog 3 spectrofluorimeter (HORIBA Jobin-Yvon), supplied with double grating excitation and emission monochromators, and employing a 450W Xe lamp as excitation source. The PL emission absolute quantum yield (QY) of C-Dots and luminescent SLNs dispersed in solution were obtained by using a ‘‘Quanta-phi’’ (HORIBA Jobin-Yvon) integrating spherecoated by Spectralons® and mounted along the optical path of the spectrofluorometer. All optical measurements were performed at room temperature on samples.

2.12 Particle size, size distribution and surface charge

The mean hydrodynamic diameter, size distribution, and ζ-potential values of the different SLNs were determined by using the Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, UK (DTS 5.00). In particular, size and size distribution were determined by means of dynamic light scattering (DLS), at room temperature, after sample dilution in demineralized water. Size distribution was described in terms of polydispersity index (PDI) and the average particle size was reported as intensity mean diameter. The ζ-potential measurements were carried out by using a laser Doppler velocimetry (LDV), at room temperature, after sample dilution in freshly prepared aqueous KCl solution (1 mM). All reported data are presented as means ± standard deviation of three replicates.

2.13 Morphological investigation

Morphological characterization of Pt(IV)-Prodrug/SLNs was performed by means Transmission Electron Microscopy (TEM) investigation, and by using a Jeol JEM-1011 microscope, working at an accelerating voltage of 100 kV and equipped by an Olympus Quemesa Camera (11 Mpx). The samples were prepared by dropping on the 400 mesh amorphous carbon-coated Cu grid a C-Dots chloroform dispersion or, alternatively, an aqueous SLNs suspension, and letting the solvent to evaporate. For the staining TEM observation, after the sample deposition, the grid was laid on top surface of a drop made of an aqueous phosphotungstic acid solution 2% (w/v) for 30 seconds and then removed and washed with ultrapure water. After complete drying of the sample, the grid was stored in a vacuum chamber until analysis.

3. Results and discussion

Novel nanoformulations based on polyethylene glycol (PEG)-stabilized SLNs and Pt(IV) prodrugs (Pt(IV)-prodrug/PEG-SLNs) derived from kiteplatin were prepared using the hot homogenization-evaporation technique. In particular, a series of four kiteplatin Pt(IV)-prodrugs, previously reported (Shamsuddin et al. 1998), were used for the preparation of the Pt(IV)-prodrug/PEG-SLNs. The four Pt(IV)-prodrugs, which are characterized by different lipophilicity degree, were synthesized by the addition of two hydrophobic *trans*-carboxylate ligands (with different length of the alkyl chains) in axial positions to kiteplatin, as reported before. (Margiotta et al. 2016).

The chemical structures of the four different Pt(IV)-prodrugs, namely SMF 111, SMF 196, SMF 200 and SMF 144 are reported in Figure 1B, along with a schematic picture illustrating the preparation of Pt(IV)-prodrug/PEG-SLNs (Figure 1 A).

<<Insert Figure 1>>

The SLNs were extensively characterized in terms of size, morphology, surface charge, drug encapsulation efficiency, and their capability to be cytotoxic toward U87-MG cells was also evaluated. Their ability to cross an *in vitro* BBB model was investigated using luminescent C-Dots to obtain optically traceable Pt(IV)-prodrug/PEG-SLNs.

3.1 Pt(IV)-Prodrug/PEG-SLNs: Fabrication and Characterization

The different PEG-SLNs samples loading SMF 196, SMF 200, SMF 111 or SMF 144 (SMF 196/SLNs, SMF 200/SLNs, SMF 111/SLNs and SMF 144/SLNs) were prepared by hot homogenization technique using cetyl palmitate as lipid matrix and Tween 80 and PEG-2-PE as surface-modifiers. For comparison purposes, PEG-SLNs loading kiteplatin (Kiteplatin/SLNs) were also prepared. Cetyl palmitate was selected as model wax, thanks to its *in vitro* degradation properties and low *in vivo* toxicity (Ruktanonchai et al. 2008), while the surfactant Tween 80 and the PEG modified phospholipid, PEG-2-PE, were used to promote the formation of SLNs characterized by good steric stabilization and "stealth" properties. For brain drug delivery, it is widely documented that SLNs functionalised by using surface modifiers, such as Tween 80 and PEG-lipids, can really enhance their brain uptake (Gastaldi et al. 2014). In fact, Tween 80 coated SLNs can mimic the low-density lipoproteins (LDL) when intravenously administered, since Tween 80 is able to absorb the apolipoproteins, in particular ApoE, which is involved in the delivery of LDL into the brain. Consequently, Tween 80 coated SLNs act as LDL, thus promoting their BBB passage by LDL receptor-mediated transcytosis without damaging the endothelium (Gao and Jiang 2006; Tosi, Duskey, and Kreuter 2020; Kreuter 2013; Wagner et al. 2012). Several studies have demonstrated that Tween 80 coated SLNs have an inherent ability, thanks to their lipid nature and average size

below 200 nm, to penetrate the BBB and, thus, to improve the brain bioavailability of delivered drugs, even without any further surface functionalization (Gao and Jiang 2006; Gastaldi et al. 2014; Martins et al. 2012; Tapeinos, Battaglini, and Ciofani 2017).

The morphology of Pt(IV)-prodrug/PEG-SLNs was investigated by TEM analysis, that revealed the formation of SLNs characterized by spherical shape and size ranging from 30 to 80 nm (Figure 2 A). Representative TEM micrographs of PEG coated SLNs loading SMF 144 (SMF 144/SLNs), obtained with staining, is reported in Figure 2A. No aggregation or agglomeration phenomena of SLNs were observed. Similar results were obtained for Kiteplatin/SLNs, SMF 196/SLNs, SMF 200/SLNs and SMF 111/SLNs (data not shown). In Figure 2B, representative close up of TEM micrograph, obtained without staining, of a single SLN loaded with SMF 144 clearly reveals the presence of small dark spots localized within the lipid matrix, that can be reasonably assigned to the Pt-based chemotherapeutic agent, successfully embedded in the lipid core of the SLN. The average hydrodynamic diameter and the corresponding polydispersity index (PDI) of the SLNs measured by DLS are reported in Figure 2 C. In Figure 2 D, a representative size distribution by intensity of SMF 144/SLNs is shown. Anyhow, the DLS investigation proved the formation of PEG-SLNs samples characterized by a homogenous and monomodal size distribution with an average hydrodynamic diameter below 100 nm, thus resulting in accordance with the TEM observations. Conversely, DLS analysis performed on Pt(IV)-prodrug/PEG-SLNs, before their purification, revealed a bimodal size distribution. Indeed, in addition to the peak attributable to Pt(IV)-prodrug/PEG-SLNs, a peak centred at about 20 nm appears in the size distribution, due to the formation of Tween 80 micelles that occurs since the non-ionic surfactant was added in concentration (42 mM) far above the critical micellar concentration (cmc, 0.012 mM) during SLNs preparation (Figure S2, Supplementary Material) (Heydenreich et al. 2003). The disappearance of this peak after SLNs purification proved the effectiveness of the centrifugal washing procedure, thus confirming the complete removal of the excess of Tween 80.

The ζ-Potential measurements highlighted the presence of an overall negative charge on the surface of all SLNs and provided values ranging from -15.1 to -28.1 mV that indicated their good colloidal stability in aqueous solution (Figure 2 C). A representative trend of ζ-Potential recorded for SMF 144/SLNs is reported in Figure 2 E.

The overall data obtained by TEM, DLS and ζ-Potential measurements suggested that the anionic Pt(IV)-Prodrug/PEG-SLNs, characterized by average size below 100 nm, are expected to be efficient nanovectors for the Pt-based brain delivery.

<<Insert Figure 2>>

For all the SLNs, the EE% values were quantified by atomic absorption spectroscopy analysis (Figure 2 C), where EE% represents the amount (w/w %) of the Pt-based drug incorporated in the lipid core of PEG-SLNs with respect to the starting drug amount employed for the preparation of SLNs. The highest EE% value was observed for the less lipophilic precursor of the Pt(IV)-prodrugs, namely kiteplatin. The differences in terms of EE% values observed for kiteplatin and the four Pt(IV)-prodrugs can be reasonably ascribed to a different equilibrium solubility of each tested drug in the aqueous and organic phases. The higher lipophilicity of the Pt(IV)-prodrugs respect to kiteplatin seems to favor a higher partitioning of each Pt(IV)-prodrugs in aqueous/surfactant phase respect to the kiteplatin, thus resulting in their lower concentration in organic/lipid phase and, ultimately, in the final nanoformulation. Indeed, it is fairly possible to presume that the Tween 80 micelles can encapsulate the Pt(IV)-prodrugs, which are supplied with alkyl chains, in their hydrophobic core in more consistent amounts respect to their pristine precursor. However, the EE% values recorded for the four Pt(IV)-Prodrug/PEG-SLNs with different lipophilicity degrees revealed a not negligible trend, although they were always lower than that one obtained for the kiteplatin. Even though the EE% values obtained for SMF 200/SLNs and SMF 111/SLNs were similar, the amount of Pt-prodrug embedded in SLNs resulted higher passing from SMF 196/SLNs to SMF144/SLNs. Therefore, increasing the chain length of the carboxylate ligand in the chemical structure of Pt(IV)-Prodrug/PEG-SLNs seems to enhance the prodrug affinity for the lipid matrix. As a result, the most lipophilic compound (SMF 144) exhibited the most elevated EE% value (27.68 %), among the tested Pt(IV)-prodrugs (Figure 2 C). Interestingly, the EE% values obtained for the Pt(IV)-Prodrug/PEG-SLNs are higher than those ones observed for the polymeric micelles already reported (Margiotta et al. 2016).

The *in vitro* drug release study, performed on the overall SLNs in PBS (pH 7.4) at 37 °C by using the Franz-diffusion cell, revealed no significant release of Pt(IV)-prodrugs (<0.5%) over a period of 120 hours, thus highlighting the high stability of the SLNs and finally suggesting that the release of the chemotherapeutic compounds could occur in the cells only after enzymatic degradation. In order to prove the release profile of the loaded Pt(IV)-prodrugs, we also performed the studies using human serum in the donor compartment instead of water. Results revealed that in presence of human serum the percentage of loaded drugs reached 100% in about 120 hours of incubation (Figure S3, Supplementary Material). Furthermore, as shown by the stability studies performed over 2 hours at 37 ºC in human serum (Figure 2F), SLNs are characterised by adequate colloidal stability without evidence of aggregation.

Optically traceable SLNs were purposely designed and prepared to investigate their ability to cross the *in vitro* model of BBB (Figure 3 A). Namely, optically traceable Pt(IV)-Prodrug/PEG-SLNs were achieved by using luminescent C-Dots (Figure 3 B), that are emerging as versatile imaging and/or delivery vehicles, thanks to their unique optical properties and nontoxic nature (Molaei 2019; Pardo, Peng, and Leblanc 2018).

In particular, oil soluble C-Dots were synthesised by exploiting a one-step procedure that promotes the carbonization of the CA as carbon source, in ODE as non-coordinating solvent and in the presence of HDA as surface coordinating agent (Panniello et al. 2018). After their accurate purification, the "as synthesized" C-Dots were morphological and optical characterized. The TEM investigation revealed the formation of nanostructures, appearing as small spherical dark spots of about 4 nm, as displayed in the inset of Figure 4 A. PL spectroscopic measurements were performed by exciting samples in the range between 300-540 nm. As reported in Figure 4A, the well-known excitation wavelength-dependent fluorescence of such organic soluble C-Dots was observed (Molaei 2019; Panniello et al. 2018). In particular, the recorded PL emission spectra clearly showed the presence of wide and asymmetric emission bands, and the maximum fluorescence intensity was observed for excitation at 380 nm (blue line, Figure 4A). The PL quantum yield (QY) obtained by exciting the "as synthesized" C-Dots in chloroform dispersion at 380 nm was 36%.

<<Insert Figures 3 and 4>>

Optically traceable SLNs were prepared by simultaneous co-encapsulation of drug and C-Dots within the hydrophobic core of PEG-SLNs. In particular, only the two Pt(IV)-prodrugs were selected for the preparation of luminescent SLNs, namely SMF200 and SMF144, as they ensured the higher EE% when incorporated in the lipid matrix of PEG-SLNs, if compared with SMF 111 and SMF 196 (Figure 2C). Furthermore, C-Dot/Kiteplatin/SLNs were also obtained to be subsequently tested, as a comparison, in the BBB passage and cell uptake studies.

A representative TEM micrograph of SLNs loaded with C-Dots and SMF 144 (C-Dot/SMF 144/SLNs), obtained after staining procedure, is shown in Figure 4 B. Similar results were obtained for C-Dot/Kiteplatin/SLNs and C-Dot/SMF 200/SLNs (data not shown). Similarly, to what observed in TEM micrographs of the "as synthesized" C-Dots, the TEM analysis performed on luminescent SLNs revealed the presence of nanostructures with spherical shape and diameter values ranging from 30 to 90 nm. DLS measurements performed on aqueous dispersion of purified SLNs highlighted the homogenous and monomodal size distribution of nanoobjects exhibiting an average hydrodynamic diameter smaller than or equal to 100 nm (Figure 4 E). Size distribution by intensity of C-Dot/SMF

144/SLNs is reported in Figure 4 D as representative trend. The data obtained by DLS investigations resulted in good accordance to those obtained by TEM analysis, thus indicating that the presence C-Dots encapsulated within the core of PEG-SLNs induced the formation of luminescent SLNs with average size slightly larger than those ones recorded for the corresponding SLNs loaded with only the therapeutic agent (Figure 2 C and 4 E). Again, the ζ-Potential measurements proved the good colloidal stability in aqueous solution of luminescent SLNs (Figure 4 E).

For all the luminescent SLNs the EE% values (Figure 4 E) resulted higher than those ones obtained for their not luminescent counterparts (Figure 2C). This achievement can be reasonably justified by assuming that the incorporation of C-Dots into the lipid matrix of SLNs can cause the occurrence of lattice defects in the crystal structure of the cetyl palimitate based PEG-SLNs, thus causing a disorder that can promote an enhanced entrapment of therapeutic agents (kitelaptin or Pt(IV)-prodrugs) (Lukowski et al. 2000; Neves et al. 2015).

The PL emission spectroscopic measurements performed by exciting at 380 nm the luminescent SLNs dispersed in aqueous solution finally proved that the peculiar PL emitting properties of the C-Dots were retained in the SLNs (Figure 4 C). However, an unavoidable loss of fluorescence intensity was observed (QYs of about 10 and 15 % for the C-Dot/Kiteplatin/SLNs and C-Dot/SMF 144/SLNs or C-Dot/SMF 200/SLNs, respectively), due to changes occurring at C-Dots interface. Indeed, such relative reduction in C-Dots QYs can be ascribed to the encapsulation of C-Dots in the SLNs and the consequent transfer in aqueous medium, as PL emission has demonstrated to be highly sensitive to altered surface states and/or chemical environment of luminescent NPs (Panniello et al. 2015). Nevertheless, the emitting properties of C-Dots, encapsulated within the SLNs and coupled with the therapeutic agents (kitelaptin or Pt(IV)-prodrugs) demonstrated as effective probes to test the ability of the developed nanoformulation in crossing the *in vitro* BBB model.

3.2 Endothelial cell viability in presence of SLNs

Preliminary to the evaluation of the SLN ability to cross the *in vitro* BBB model, cell toxicity induced by SMF 144/SLNs, Kiteplatin/SLNs and SMF 200/SLNs on hCMEC/D3 cells was evaluated by MTT assay. hCMEC/D3 cells were incubated with increasing concentration of drug-loaded SLNs (drug concentration range varying from 0.01 to 0.5 μM) and the cell viability was assessed 24 h after treatment. Results showed that the viability of endothelial cells decreased by increasing the concentration of drug/SLNs. A reduction of 41-53% of the mitochondrial activity was observed when cells were incubated with SLNs loaded with drug concentration of 0.1 μM, while treatment with 0.5 μM of drug-loaded SLNs was associated with 57-74% decrease of cell viability. For all the tested

concentration, a lower toxicity was associated with the treatment with SMF 144/SLNs, compared to the other formulations (Figure 5 A).

3.3 Cellular uptake and permeability of luminescent SLNs through the in vitro BBB model

To investigate the capacity of Pt(IV)-Prodrug/PEG-SLNs to permeate the BBB, an *in vitro* BBB transwell model composed by a monolayer of polarized endothelial cells seeded on a porous membrane was set up, to allow the formation of an apical compartment ('blood' side) physically separated from the basolateral one ('brain' side) (Figure 3 A). In particular, immortalized human brain capillary endothelial cells (hCMEC/D3) were used as representative of human BBB model. Indeed, they represent an easily grown model of *in vivo* human BBB that is amenable to cellular and molecular studies on pathological and drug transport mechanisms with relevance to the CNS. Monolayer of hCMEC/D3 cells retains the expression of most transporters and receptors expressed *in vivo* at the human BBB (Sancini et al. 2016).

Trans-electrical endothelial resistance (TEER) was monitored during the formation of the hCMEC/D3 monolayer and its value gradually increased from 28.1 ± 3.7 $\Omega \cdot cm^2$ (5 days after seeding) to 40.7 ± 1.9 $\Omega \cdot cm^2$ (on the 14th day after seeding), according to the literature (Weksler, Romero, and Couraud 2013).

Permeation experiments through hCMEC/D3 cells monolayer were performed by using concentration of drug loaded into SLNs of 0.1 μM, corresponding to 0.3 mg/mL and 0.18 mg/mL in terms of lipid and C-Dots concentration, respectively. The ability of SLNs to cross the *in vitro* BBB model was assessed at 3 hours by measuring the C-Dots-associated PL in the basolateral compartment of the transwell (Figure 5 B). The EP values recorded with C-Dot/Kiteplatin/SLNs, C-Dot/SMF 144/SLNs, and C-Dot/SMF 200/SLNs were $2.9\pm0.9\times10^{-4}$ cm/min, $2.7\pm0.2\times10^{-4}$ cm/min and $1.7\pm0.1\times10^{-4}$ cm/min respectively. As reported in the literature, the main mechanism involved in the SLN uptake by hCMEC/D3 was found to be clathrin-mediated endocytosis. NPs may partially follow lysosomal and endosomal trafficking inside cells. In fact, the lysosomal pathway is responsible for particle degradation and release of drug content inside cells, while the endosomal trafficking may be involved in the transport of intact drug loaded NPs from one side to the other of the cell barrier (Graverini et al. 2018).

The transport rates of LY were evaluated in the absence and after treatment with the SLNs formulations, to evaluate the tight junction integrity. In untreated cells, the EP of the paracellular tracer was found to be $1.4\pm0.3\times10^{-3}$ cm/min, which is in agreement with previously reported values (Cox et al. 2018; Graverini et al. 2018). Incubation with C-Dot/drug/SLNs increased permeability of

LY to some extent, which is possibly associated with the 35-40% reduction of cell viability observed after treatment (Figure 5 C and D).

<<Insert Figures 5 and 6>>

In order to exclude that during the permeability studies the SLNs released the loaded C-dots the integrity of the luminescent SLNs, after evaluation of their ability to cross the *in vitro* BBB model for 3 hours, was investigated by TEM analyses. TEM representative micrographs, obtained with staining, of C-Dot/SM144/SLNs in the medium recovered from the both apical and basolateral compartments at the end of the BBB permeability experiment, reported in Figure 6, clearly revealed the presence of stable and intact SLNs characterized by retained morphology also after their transport from one side to the other of the cell monolayers, thus suggesting that LDL receptor or clathrin-mediated endocytosis, transition through the cytoplasm, and exocytic release can represent the main transport mechanisms involved in the SLN uptake by hCMEC/D3 cells (Graverini et al. 2018; Neves et al. 2015).

3.4 Cytotoxicity evaluation of SLNs on U87cell line

After the BBB crossing, the SLNs should be able to reach the brain tissue where the glioblastoma is present. In this perspective, the cytotoxic effect of kiteplatin and Pt(IV)-prodrugs loaded PEG-SLNs was assessed on the U87 human glioblastoma cell line, that was selected as a valid *in vitro* model of human glioblastoma. Namely, *in vitro* cytotoxicity study was performed on U87 cells by incubating them with free drugs (kiteplatin, SMF 200 and SMF 144) or the corresponding SLNs (Kiteplatin/SLNs, SMF 200/SLNs and SMF 144/SLNs) for 24 hours. The drug concentration was ranged from 0.01 to 0.25 μM, values that correspond to 0.03 and 0.75 mg/mL in terms of lipid concentration for all the SLNs. Untreated cells were used as control. The effect of the different tested compounds on cell viability was evaluated by MTT assay (Figure 7 A).

<<Insert Figure 7>>

The data revealed that the overall tested drugs (kiteplatin and Pt(IV)-prodrugs) were able to induce a more significant reduction of the cell viability when encapsulated in the PEG-SLNs than their free counterparts, for each explored drug concentration, thus finally resulting characterized by an enhanced antitumor efficacy. The PEG-SLNs without drug were found to be not cytotoxic in the tested lipid concentration range (0.03 and 0.75 mg/mL, data not shown). Therefore, the therapeutic efficacy of Kiteplatin/SLNs and of the two different Pt(IV)-Prodrug/SLNs was determined exclusively by the embedded drug. Furthermore, it is also possible to observe (Figure 7 A) that the

two tested Pt(IV)-Prodrugs/SLNs, namely SMF 200/SLNs and SMF 144/SLNs, resulted to affect the cell viability more significantly than the kiteplatin/SLNs at the higher explored drug concentrations (0.1 and 0.25 μM).

The cellular uptake of SLNs in U87 cells was evaluated as platinum content by means of ICP-MS analysis, after cells incubation with Kiteplatin/SLNs, SMF 200/SLNs and SMF 144/SLNs at drug concentration of 0.1 μM, that was the same concentration value tested for the BBB permeability study, at 4 and 24 hours. Cells treated under the same experimental conditions, containing the U87 cells incubated with free kiteplatin, SMF 200 and SML 144 were used as references, while the controls were represented by untreated cells. The data reported in Figure 7 B clearly show that the uptake in U87 cells of each tested Pt-based drugs was greatly increased when delivered by PEG-SLNs if compared to the corresponding free counterparts, already after 4 h incubation. The cell uptake of SMF 200/SLNs reached its maximum values already at 4 hours, while the cell uptake of Kiteplatin/SLNs and SMF 144/SLNs increased at incubation time of 24 hours. These results can be attributed to an enhanced ability of the all SLNs to be internalized by tumour cells if compared to the free therapeutic agents. On the other hand, SLNs are able to bypass drug efflux pumps expressed on human cancer cells, and consequently to increase intracellular drug concentration (Grillone et al. 2019). Among the explored SLNs, the SMF 200/SLNs resulted characterized by higher cell uptake. The trend of the data observed for the uptake of the SLNs is in agreement with that one recorded for the cell viability (Figure 7 A). Indeed, the antitumor efficacy of SMF 200/SLNs resulted higher than that one of SMF 144/SLNs and Kiteplatin /SLNs, since cell viability passing from 59% to 65 and 81% for SMF 200/SLNs, SMF 144/SLNs and Kiteplatin /SLNs, respectively. This achievement can be justified by the higher cell uptake of SMF 200/SLNs if compared with SMF 144/SLNs and Kiteplatin /SLNs.

Conclusions

This study concerned the fabrication and characterization of SLNs containing Pt(IV) prodrugs that were purposely designed as therapeutic compounds potentially useful for the treatment of GBM. The incorporation of the synthesized Pt(IV)-prodrugs in PEG stabilized SLNs was explored to obtain nanovectors with enhanced ability to cross the BBB and to accumulate the therapeutic cargo into the brain respect to the free Pt(IV)-prodrugs. The obtained data revealed the formation of anionic Pt(IV)-Prodrug/PEG-SLNs with good stability in aqueous media and characterized by average hydrodynamic diameters below 100 nm. Among the tested Pt(IV)-prodrugs, the most elevated EE% value was obtained for the most lipophilic compound (SMF 144). Luminescent C-Dots emitting in the UV-Vis region were co-encapsulated in the SLNs with Pt(IV)-prodrugs to obtain optical traceable

SLNs that were used to prove their ability to permeate the *in vitro* BBB model based on a hCMEC/D3 monolayer. Finally, the anticancer activity of the Pt(IV)-Prodrug/PEG-SLNs was investigated by performing an *in vitro* study on human glioblastoma cell line (U87) cell line and an improved cellular uptake of the Pt(IV)-prodrugs was demonstrated when delivered in the SLNs. *In vivo* pharmacokinetic and pharmacodynamic studies will be performed in the near future, but formulative and *in vitro* data of this study highlight SMF144 loaded PEG-SLNs as a noteworthy nanoformulation candidate for further pharmacological studies in a suitable brain tumor animal model.

Acknowledgments

Authors acknowledge the University of Bari "Aldo Moro" (Italy), Angelini A.C.R.A.F. R&D and the Inter-University Consortium for Research on the Chemistry of Metal Ions in Biological Systems (C.I.R.C.M.S.B.) for their financial and technical support.

References

Aoki, T., J. A. Takahashi, T. Ueba, N. Oya, M. Hiraoka, K. Matsui, T. Fukui, Y. Nakashima, M. Ishikawa, and N. Hashimoto. 2006. 'Phase II study of nimustine, carboplatin, vincristine, and interferon-beta with radiotherapy for glioblastoma multiforme: experience of the Kyoto Neuro-Oncology Group', *J Neurosurg*, 105: 385-91.

Bernocchi, G., M. G. Bottone, V. M. Piccolini, V. Dal Bo, G. Santin, S. A. De Pascali, D. Migoni, and F. P. Fanizzi. 2011. 'Developing central nervous system and vulnerability to platinum compounds', *Chemother Res Pract*, 2011: 315418.

Blasi, Paolo, Stefano Giovagnoli, Aurélie Schoubben, Maurizio Ricci, and Carlo Rossi. 2007. 'Solid lipid nanoparticles for targeted brain drug delivery', *Advanced Drug Delivery Reviews*, 59: 454-77.

Brandes, A. A., U. Basso, F. Vastola, A. Tosoni, L. M. Pasetto, A. Jirillo, S. Lonardi, M. K. Paris, H. Koussis, S. Monfardini, and M. Ermani. 2003. 'Carboplatin and teniposide as third-line chemotherapy in patients with recurrent oligodendroglioma or oligoastrocytoma: a phase II study', *Ann Oncol*, 14: 1727-31.

Brandes, A. A., A. Tosoni, U. Basso, M. Reni, F. Valduga, S. Monfardini, P. Amista, L. Nicolardi, G. Sotti, and M. Ermani. 2004. 'Second-line chemotherapy with irinotecan plus carmustine in glioblastoma recurrent or progressive after first-line temozolomide chemotherapy: a phase II study of the Gruppo Italiano Cooperativo di Neuro-Oncologia (GICNO)', *J Clin Oncol*, 22: 4779-86.

Buckner, J. C., K. V. Ballman, J. C. Michalak, G. V. Burton, T. L. Cascino, P. J. Schomberg, R. B. Hawkins, B. W. Scheithauer, H. M. Sandler, R. S. Marks, and J. R. O'Fallon. 2006. 'Phase III trial of carmustine and cisplatin compared with carmustine alone and standard radiation therapy or accelerated radiation therapy in patients with glioblastoma multiforme: North Central Cancer Treatment Group 93-72-52 and Southwest Oncology Group 9503 Trials', *J Clin Oncol*, 24: 3871-9.

Calogero, Antonella, Antonio Porcellini, Vincenza Lombari, Cinzia Fabbiano, Antonietta Arcella, Massimo Miscusi, Donatella Ponti, and Giuseppe Ragona. 2011. 'Sensitivity to cisplatin in primary cell lines derived from human glioma correlates with levels of EGR-1 expression', *Cancer Cell International*, 11: 5.

Chaichana, K., S. Parker, A. Olivi, and A. Quinones-Hinojosa. 2010. 'A proposed classification system that projects outcomes based on preoperative variables for adult patients with glioblastoma multiforme', *J Neurosurg*, 112: 997-1004.

Charest, G., L. Sanche, D. Fortin, D. Mathieu, and B. Paquette. 2012. 'Glioblastoma treatment: bypassing the toxicity of platinum compounds by using liposomal formulation and increasing treatment efficiency with concomitant radiotherapy', *Int J Radiat Oncol Biol Phys*, 84: 244-9.

Cox, Alysia, Patrizia Andreozzi, Roberta Dal Magro, Fabio Fiordaliso, Alessandro Corbelli, Laura Talamini, Clizia Chinello, Francesca Raimondo, Fulvio Magni, Maria Tringali, Silke Krol,

Paulo Jacob Silva, Francesco Stellacci, Massimo Masserini, and Francesca Re. 2018. 'Evolution of Nanoparticle Protein Corona across the Blood–Brain Barrier', *ACS nano*, 12: 7292-300.

Denora, Nunzio, Adriana Trapani, Valentino Laquintana, Angela Lopedota, and Giuseppe Trapani. 2009. 'Recent Advances in Medicinal Chemistry and Pharmaceutical Technology- Strategies for Drug Delivery to the Brain', *Current topics in medicinal chemistry*, 9: 182-96.

Depalo, Nicoletta, Rosa Iacobazzi, Gianpiero Valente, Ilaria Arduino, Silvia Villa, Fabio Canepa, Valentino Laquintana, Elisabetta Fanizza, Marinella Striccoli, Cutrignelli Annalisa, Angela Lopedota, Letizia Porcelli, Amalia Azzariti, Massimo Franco, Maria Curri, and Nunzio Denora. 2017. 'Sorafenib delivery nanoplatform based on superparamagnetic iron oxide nanoparticles magnetically targets hepatocellular carcinoma', *Nano Research*, 10.

Fontana, Flavia, Silvia Albertini, Alexandra Correia, Marianna Kemell, Rici Lindgren, Ermei Mäkilä, Jarno Salonen, Jouni T. Hirvonen, Franca Ferrari, and Hélder A. Santos. 2018. 'Bioengineered Porous Silicon Nanoparticles@Macrophages Cell Membrane as Composite Platforms for Rheumatoid Arthritis', *Advanced Functional Materials*, 28: 1801355.

Franceschi, E., G. Cavallo, L. Scopece, A. Paioli, A. Pession, E. Magrini, R. Conforti, E. Palmerini, S. Bartolini, S. Rimondini, R. D. Esposti, and L. Crino. 2004. 'Phase II trial of carboplatin and etoposide for patients with recurrent high-grade glioma', *Br J Cancer*, 91: 1038-44.

Fruehauf, J. P., H. Brem, S. Brem, A. Sloan, G. Barger, W. Huang, and R. Parker. 2006. 'In vitro drug response and molecular markers associated with drug resistance in malignant gliomas', *Clin Cancer Res*, 12: 4523-32.

Galanis, E., J. C. Buckner, M. J. Maurer, J. M. Reid, M. J. Kuffel, M. M. Ames, B. W. Scheithauer, J. E. Hammack, G. Pipoly, and S. A. Kuross. 2005. 'Phase I/II trial of pyrazoloacridine and carboplatin in patients with recurrent glioma: a North Central Cancer Treatment Group trial', *Invest New Drugs*, 23: 495-503.

Gao, Kepan, and Xinguo Jiang. 2006. 'Influence of particle size on transport of methotrexate across blood brain barrier by polysorbate 80-coated polybutylcyanoacrylate nanoparticles', *International Journal of Pharmaceutics $V 310*: 213-19.

Gastaldi, Lucia, Luigi Battaglia, Elena Peira, Daniela Chirio, Elisabetta Muntoni, Ilaria Solazzi, Marina Gallarate, and Franco Dosio. 2014. 'Solid lipid nanoparticles as vehicles of drugs to the brain: Current state of the art', *European Journal of Pharmaceutics and Biopharmaceutics*, 87: 433-44.

Graverini, Giulia, Vieri Piazzini, Elisa Landucci, Daniela Pantano, Pamela Nardiello, Fiorella Casamenti, Domenico E. Pellegrini-Giampietro, Anna Rita Bilia, and Maria Camilla Bergonzi. 2018. 'Solid lipid nanoparticles for delivery of andrographolide across the blood-brain barrier: in vitro and in vivo evaluation', *Colloids and Surfaces B: Biointerfaces*, 161: 302-13.

Grillone, Agostina, Matteo Battaglini, Stefania Moscato, Letizia Mattii, César De Julián Fernández, Alice Scarpellini, Mario Giorgi, Edoardo Sinibaldi, and Gianni Ciofani. 2019. 'Nutlin-loaded magnetic solid lipid nanoparticles for targeted glioblastoma treatment', *Nanomedicine*, 14: 727-52.

Grillone, Agostina, Eugenio Redolfi Riva, Alessio Mondini, Claudia Forte, Lucia Calucci, Claudia Innocenti, Cesar de Julian Fernandez, Valentina Cappello, Mauro Gemmi, Stefania Moscato, Francesca Ronca, Rodolfo Sacco, Virgilio Mattoli, and Gianni Ciofani. 2015. 'Active Targeting of Sorafenib: Preparation, Characterization, and In Vitro Testing of Drug-Loaded Magnetic Solid Lipid Nanoparticles', *Advanced Healthcare Materials*, 4: 1681-90.

Grossman, S. A., M. Wharam, V. Sheidler, L. Kleinberg, M. Zeltzman, N. Yue, and S. Piantadosi. 1997. 'Phase II study of continuous infusion carmustine and cisplatin followed by cranial irradiation in adults with newly diagnosed high-grade astrocytoma', *J Clin Oncol*, 15: 2596-603.

Haroun, R. I., R. E. Clatterbuck, M. C. Gibbons, P. C. Burger, R. Parker, J. P. Fruehauf, and H. Brem. 2002. 'Extreme drug resistance in primary brain tumors: in vitro analysis of 64 resection specimens', *J Neurooncol*, 58: 115-23.

Heydenreich, A. V., R. Westmeier, N. Pedersen, H. S. Poulsen, and H. G. Kristensen. 2003. 'Preparation and purification of cationic solid lipid nanospheres—effects on particle size, physical stability and cell toxicity', *International Journal of Pharmaceutics*, 254: 83-87.

Kreuter, Jörg. 2013. 'Mechanism of polymeric nanoparticle-based drug transport across the blood-brain barrier (BBB)', *Journal of microencapsulation*, 30: 49-54.

Latronico, Tiziana, Nicoletta Depalo, Gianpiero Valente, Elisabetta Fanizza, Valentino Laquintana, Nunzio Denora, Anna Fasano, Marinella Striccoli, Matilde Colella, Angela Agostiano, M. Lucia Curri, and Grazia Maria Liuzzi. 2016. 'Cytotoxicity Study on Luminescent Nanocrystals Containing Phospholipid Micelles in Primary Cultures of Rat Astrocytes', *PloS one*, 11: e0153451.

Limentani, S. A., A. Asher, M. Heafner, J. W. Kim, and R. Fraser. 2005. 'A phase I trial of surgery, Gliadel wafer implantation, and immediate postoperative carboplatin in combination with radiation therapy for primary anaplastic astrocytoma or glioblastoma multiforme', *J Neurooncol*, 72: 241-4.

Lukowski, G., J. Kasbohm, P. Pflegel, A. Illing, and H. Wulff. 2000. 'Crystallographic investigation of cetylpalmitate solid lipid nanoparticles', *International Journal of Pharmaceutics*, 196: 201-05.

Margiotta, Nicola, Cristina Marzano, Valentina Gandin, Domenico Osella, Mauro Ravera, Elisabetta Gabano, James A. Platts, Emanuele Petruzzella, James D. Hoeschele, and Giovanni Natile. 2012. 'Revisiting [PtCl2(cis-1,4-DACH)]: An Underestimated Antitumor Drug with Potential Application to the Treatment of Oxaliplatin-Refractory Colorectal Cancer', *Journal of Medicinal Chemistry*, 55: 7182-92.

Margiotta, Nicola, Salvatore Savino, Nunzio Denora, Cristina Marzano, Valentino Laquintana, Annalisa Cutrignelli, James D. Hoeschele, Valentina Gandin, and Giovanni Natile. 2016. 'Encapsulation of lipophilic kiteplatin Pt(iv) prodrugs in PLGA-PEG micelles', *Dalton Transactions*, 45: 13070-81.

Martins, Susana, Ingunn Tho, Isolde Reimold, Gert Fricker, Eliana B. Souto, Domingos Ferreira, and Martin Brandl. 2012. 'Brain delivery of camptothecin by means of solid lipid nanoparticles: Formulation design, in vitro and in vivo studies', *International Journal of Pharmaceutics*, 439.

Molaei, Mohammad Jafar. 2019. 'Carbon quantum dots and their biomedical and therapeutic applications: a review', *RSC Advances*, 9: 6460-81.

Neves, Ana Rute, Joana Fontes Queiroz, Babette Weksler, Ignacio A. Romero, Pierre-Olivier Couraud, and Salette Reis. 2015. 'Solid lipid nanoparticles as a vehicle for brain-targeted drug delivery: two new strategies of functionalization with apolipoprotein E', *Nanotechnology*, 26: 495103.

Panniello, Annamaria, Michela Corricelli, Roberto Comparelli, Maria Lucia Curri, Angela Agostiano, Raffaele Tommasi, and Marinella Striccoli. 2015. 'Recombination Dynamics of Colloidal Nanocrystals in Functionalized-Poly-Methylmethacrylate Nanocomposites', *Nanoscience and Nanotechnology Letters*, 7: 67-73.

Panniello, Annamaria, A. Evelyn Di Mauro, Elisabetta Fanizza, Nicoletta Depalo, Angela Agostiano, M. Lucia Curri, and Marinella Striccoli. 2018. 'Luminescent Oil-Soluble Carbon Dots toward White Light Emission: A Spectroscopic Study', *The Journal of Physical Chemistry C*, 122: 839-49.

Pardo, Joel, Zhili Peng, and Roger Leblanc. 2018. 'Cancer Targeting and Drug Delivery Using Carbon-Based Quantum Dots and Nanotubes', *Molecules*, 23: 378.

Peterson, K. 2001. 'Brain tumors', *Neurol Clin*, 19: 887-902.

Prados, M. D., S. C. Schold, Jr., H. A. Fine, K. Jaeckle, F. Hochberg, L. Mechtler, M. R. Fetell, S. Phuphanich, L. Feun, T. J. Janus, K. Ford, and W. Graney. 2003. 'A randomized, double-blind, placebo-controlled, phase 2 study of RMP-7 in combination with carboplatin administered intravenously for the treatment of recurrent malignant glioma', *Neuro Oncol*, 5: 96-103.

Prasek Kutwin, Marta, Ewa Sawosz, Sławomir Jaworski, Mateusz Wierzbicki, Barbara Strojny, Marta Grodzik, and André Chwalibog. 2017. 'Assessment of the proliferation status of glioblastoma cell and tumour tissue after nanoplatinum treatment', *PloS one*, 12.

Provenzale, James, Srini Mukundan, and Mark Dewhirst. 2005. 'The Role of Blood-Brain Barrier Permeability in Brain Tumor Imaging and Therapeutics', *AJR. American journal of roentgenology*, 185: 763-7.

Roberts, Nathan B., Aniket S. Wadajkar, Jeffrey A. Winkles, Eduardo Davila, Anthony J. Kim, and Graeme F. Woodworth. 2016. 'Repurposing platinum-based chemotherapies for multi-modal treatment of glioblastoma', *OncoImmunology*, 5: e1208876.

Rousseau, J., R. F. Barth, M. Fernandez, J. F. Adam, J. Balosso, F. Esteve, and H. Elleaume. 2010. 'Efficacy of intracerebral delivery of cisplatin in combination with photon irradiation for treatment of brain tumors', *J Neurooncol*, 98: 287-95.

Ruktanonchai, Uracha, Surachai Limpakdee, Siwaporn Meejoo, Usawadee Sakulkhu, Nuntavan Bunyapraphatsara, Varaporn Junyaprasert, and Satit Puttipipatkhachorn. 2008. 'The effect of cetyl palmitate crystallinity on physical properties of gamma-oryzanol encapsulated in solid lipid nanoparticles', *Nanotechnology*, 19: 095701.

Sancini, Giulio, Roberta Dal Magro, Francesca Ornaghi, Claudia Balducci, Gianluigi Forloni, Marco Gobbi, Mario Salmona, and Francesca Re. 2016. 'Pulmonary administration of functionalized nanoparticles significantly reduces beta-amyloid in the brain of an Alzheimer's disease murine model', *Nano Research*, 9: 2190-201.

Scopece, L., E. Franceschi, G. Cavallo, A. Paioli, G. Paioli, R. Conforti, E. Palmerini, C. Berzioli, F. Spagnolli, R. D. Esposti, and L. Crino. 2006. 'Carboplatin and etoposide (CE) chemotherapy in patients with recurrent or progressive oligodendroglial tumors', *J Neurooncol*, 79: 299-305.

Shamsuddin, Shaikh, Claudia C. Santillan, Joseph L. Stark, Kenton H. Whitmire, Zahid H. Siddik, and Abdul R. Khokhar. 1998. 'Synthesis, characterization, and antitumor activity of new platinum(IV) trans-carboxylate complexes: Crystal structure of [Pt(cis-1,4-DACH)trans-(acetate)2Cl2]', *Journal of Inorganic Biochemistry*, 71: 29-35.

Sheleg, Sergey, Eugeny Korotkevich, Edvard Zhavrid, Galina Muravskaya, Arnold Smeyanovich, Yury Shanko, Tatsiana Yurkshtovich, Pavel Bychkovsky, and Sergey Belyaev. 2002. 'Local Chemotherapy with Cisplatin-depot for Glioblastoma Multiforme', *Journal of neuro-oncology*, 60: 53-9.

Shi, Minghan, David Fortin, Léon Sanche, and Benoit Paquette. 2015. 'Convection-enhancement delivery of platinum-based drugs and LipoplatinTM to optimize the concomitant effect with radiotherapy in F98 glioma rat model', *Investigational new drugs*, 33.

Silvani, A., M. Eoli, A. Salmaggi, A. Erbetta, L. Fariselli, and A. Boiardi. 2002. 'Intra-arterial ACNU and carboplatin versus intravenous chemotherapy with cisplatin and BCNU in newly diagnosed patients with glioblastoma', *Neurol Sci*, 23: 219-24.

Silvani, A., M. Eoli, A. Salmaggi, E. Lamperti, E. Maccagnano, G. Broggi, and A. Boiardi. 2004. 'Phase II trial of cisplatin plus temozolomide, in recurrent and progressive malignant glioma patients', *J Neurooncol*, 66: 203-8.

Soffietti, R., M. Nobile, R. Ruda, M. Borgognone, A. Costanza, E. Laguzzi, and R. Mutani. 2004. 'Second-line treatment with carboplatin for recurrent or progressive oligodendroglial tumors after PCV (procarbazine, lomustine, and vincristine) chemotherapy: a phase II study', *Cancer*, 100: 807-13.

Stupp, Roger, Warren P. Mason, Martin J. van den Bent, Michael Weller, Barbara Fisher, Martin J.B. Taphoorn, Karl Belanger, Alba A. Brandes, Christine Marosi, Ulrich Bogdahn, Jürgen Curschmann, Robert C. Janzer, Samuel K. Ludwin, Thierry Gorlia, Anouk Allgeier, Denis Lacombe, J. Gregory Cairncross, Elizabeth Eisenhauer, and René O. Mirimanoff. 2005. 'Radiotherapy plus Concomitant and Adjuvant Temozolomide for Glioblastoma', *New England Journal of Medicine*, 352: 987-96.

Tapeinos, Christos, Matteo Battaglini, and Gianni Ciofani. 2017. 'Advances in the design of solid lipid nanoparticles and nanostructured lipid carriers for targeting brain diseases', *Journal of Controlled Release*, 264: 306-32.

Tosi, Giovanni, J. T. Duskey, and Jörg Kreuter. 2020. 'Nanoparticles as carriers for drug delivery of macromolecules across the blood-brain barrier', *Expert opinion on drug delivery*, 17: 23-32.

Wagner, Sylvia, Anja Zensi, Sascha Wien, Sabrina Tschickardt, Wladislaw Maier, Tikva Vogel, Franz Worek, Claus Pietrzik, Jörg Kreuter, and Hagen v. Briesen. 2012. 'Uptake Mechanism of ApoE-Modified Nanoparticles on Brain Capillary Endothelial Cells as a Blood-Brain Barrier Model', *PloS one*, 7: e32568.

Weksler, Babette, Ignacio A. Romero, and Pierre-Olivier Couraud. 2013. 'The hCMEC/D3 cell line as a model of the human blood brain barrier', *Fluids and Barriers of the CNS*, 10: 16.

Yung, W. K., L. Mechtler, and M. J. Gleason. 1991. 'Intravenous carboplatin for recurrent malignant glioma: a phase II study', *J Clin Oncol*, 9: 860-4.

Zhang, X., W. Zhang, W. D. Cao, G. Cheng, and Y. Q. Zhang. 2012. 'Glioblastoma multiforme: Molecular characterization and current treatment strategy (Review)', *Exp Ther Med*, 3: 9-14.

Zhou, Jiangbing, Toral R. Patel, Rachael W. Sirianni, Garth Strohbehn, Ming-Qiang Zheng, Nha Duong, Thomas Schafbauer, Anita J. Huttner, Yiyun Huang, Richard E. Carson, Ying Zhang, David J. Sullivan, Joseph M. Piepmeier, and W. Mark Saltzman. 2013. 'Highly penetrative, drug-loaded nanocarriers improve treatment of glioblastoma', *Proceedings of the National Academy of Sciences*, 110: 11751-56.