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**Bevacizumab loaded solid lipid nanoparticles prepared by the coacervation technique:
preliminary *in vitro* studies**

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Abstract

Glioblastoma is the commonest primary brain tumor in adults with an inauspicious prognosis, the major obstacle to the pharmacological treatment of brain tumors being the overcoming of the blood brain barrier. As neoangiogenesis plays a key role in glioblastoma growth, the Food and Drug Administration (FDA) approved bevacizumab (BVZ), an anti-VEGF antibody for the treatment of recurrent glioblastoma in patients to whom the initial therapy has failed.

In this experimental work BVZ was entrapped in solid lipid nanoparticles (SLN) prepared by the fatty acids coacervation technique, thanks to the formation of a hydrophobic ion pair. BVZ activity, evaluated by means of four different *in vitro* tests on HUVEC cells, was increased up to 100-200 fold, when delivered in SLN. Moreover SLN can enhance the permeation of fluorescently labelled BVZ through hCMEC/D3 cells monolayer, an *in vitro* model of the blood brain barrier. These results are promising, even if further *in vivo* studies are requested to evaluate the effective potential of BVZ-loaded SLN in glioblastoma treatment.

Keywords

Bevacizumab, solid lipid nanoparticles, coacervation, ion pair, anti-VEGF, HUVEC, BBB

Introduction

Glioblastoma (GBM) together with low and high grade gliomas, is the commonest primary brain tumor in adults with an inauspicious prognosis and a median survival of approximately 10 months [1]. The pharmacotherapy of GBM is adjuvant, subsequent to surgery and subsequent or contemporaneous with radiotherapy and is rarely applied in first instance. It is directed mainly towards the eradication of residual tumor cells after surgery or radiotherapy. Unfortunately, the current therapy is not satisfactory, because the prognosis remains fatal and the quality of life for patients is poor.

Today, pharmacological therapy is mainly based on orally administered temozolomide (TMZ), a drug able to overcome the blood brain barrier (BBB) and on intrathecally administered carmustine. Current standard GBM chemotherapy is based on Stupp et al. protocol. It consists in daily administration of TMZ for six weeks in combination with the radiation, followed by other six cycles of adjuvant TMZ therapy [2]. Up today, molecular "targeted therapies", anti-angiogenic therapy and gene therapy have a real benefit only in limited groups of patients [3].

Tumor growth depends on the availability of oxygen and nutrients in the environment close to the cells in fast expansion. Neoangiogenesis is necessary for tumor growth, a phenomenon that consists in the creation of new vascular network by pre-existent vascular vessels [4]. Vascular endothelial growth factor (VEGF) plays a key role in the regulation of angiogenesis and VEGF pathway is involved in the migration and proliferation of endothelial cells and in the development of cerebral oedema, which is caused by the high permeability of tumor blood vessel. In GBM there is a high density of new blood vessels and an alternative therapy can be based on the inhibition of angiogenesis using an anti-VEGF agent [5].

BVZ is an example of an anti-VEGF agent. It binds VEGF and prevents the interaction between VEGF and its receptor, placed on vascular endothelial cells surface, inhibiting endothelial cells proliferation and causing the decrease of tumor growth [6]. On 5 may 2009, basing on the results

obtained with the BRAIN Study and the NCI Study [2], FDA approved BVZ (Avastin®) as single agent for treatment of recurrent GBM in patients where the initial therapy has failed [4].

BBB is the barrier between brain parenchyma and blood vessels and it is constituted by endothelial cells joined by tight junctions [7]. BBB prevents brain access to pathogens, but also to drugs. Therefore, treating central nervous system (CNS) diseases is difficult, because drug permeation through the BBB depends on drug size and lipophilicity and by the presence of active carrier systems.

The difficulty of conventional cytotoxic drugs, which have high *in vitro* efficacy against GBM, to overcome BBB [8,9] is a major limitation to drug therapy. In addition to tight junctions, BBB is rich -on the luminal surface- of efflux membrane transporters like P-glycoprotein (Pgp) and multidrug-related proteins (MRP) [10], which recognise various anticancer drugs as substrates. Therefore, improving the current therapeutic approaches of GBM is an indispensable requirement.

Nanoparticles are colloidal systems constituted by polymeric, inorganic or lipidic solid matrix, with mean diameters lower than 1 µm. Nanoparticles have been extensively described in literature as potential vehicles for drug targeting to the CNS, due to their potential permeation through BBB [11]. SLN are consisting on biocompatible lipids, solid at room temperature; being made of physiological lipids [12] they can be proposed for controlled drug delivery. In literature several studies are reported on the entrapment of anti-neoplastic drugs in SLN and on the consequent increase of their both chemico-physical stability and cytotoxicity on tumor cell lines [13]. SLN can be internalized in cells by means of simple or carrier-mediated endocytosis or phagocytosis [14], also owing to particles surface charges. Recent studies show that SLN can be taken up by cells without altering their morphological and metabolic characteristics [15].

SLN can be produced with different technologies [12]: an innovative method is the fatty acid coacervation, which is based on precipitation of fatty acids SLN, starting from micellar solutions of their sodium salts by simple acidification [16]. Compared to most techniques described in literature [17], this preparation method allows to obtain SLN without using solvents or expensive machinery.

Moreover, the operating temperature can be adjusted as a function of the fatty acid used, to allow the entrapment of thermosensitive actives without damaging them.

Many antineoplastic drugs have been loaded into SLN produced by coacervation. Lipophilic drugs are directly loaded into the lipid matrix, whereas it is necessary to increase the lipophilicity of hydrophilic ones, by forming hydrophobic ion pairs or by synthesizing lipophilic prodrugs. Satisfactory results were obtained with doxorubicin, methotrexate, cisplatin, leuprolide and paclitaxel [18,19,20,21,22]. Nevertheless the incorporation of a monoclonal antibody like BVZ within SLN remains a difficult goal, because of the hydrophilicity and of the high molecular weight of the antibody. Incorporation of antibodies in SLN produced by coacervation has been already patented [23], thanks to hydrophobic ion pairing.

The aim of this work is the design, production and characterization of BVZ-loaded SLN and their *in vitro* preliminary evaluation for their potentiality in glioblastoma treatment: BVZ-loaded SLN are prepared and characterized *in vitro* for their antiproliferative and antiangiogenic activity, and their ability to permeate *in vitro* models of BBB is described

Materials

Avastin[®] 25 mg/ml was used as BVZ standard. Sodium dioctyl sulfosuccinate (AOT) and Fluorescein isothiocyanate (FITC) were from Merck (Darmstadt, Germany). Citric acid, lactic acid, sodium carbonate and sodium bicarbonate were from A.C.E.F. (Fiorenzuola d'Arda, Italy). 80% hydrolyzed polyvinyl alcohol (PVA) 9000–10000 Mw and trifluoroacetic acid (TFA) were from Sigma (Dorset, UK). Hydroxypropyl methylcellulose (HPMC) 15cP was a kind gift by Eigenmann and Veronelli. Sodium stearate (SS) and sodium palmitate (SP) were from Fluka (Buchs, Switzerland).

Deionized water was obtained by a MilliQ[®] system (Millipore, Bedford, MO). All other chemicals were analytical grade and used without any further purification.

M199 medium, heparin, MTT and VEGF- α , and crystal violet were from Sigma-Aldrich (St. Louis, MO); FCS was from Invitrogen (Burlington, ON, Canada); penicillin streptomycin and glutamine were from Hyclone Laboratories (South Logan, UT); Boyden chamber filters were from Neuro Probe (BIOMAP snc, Milan, Italy) and Matrigel, were from BD Biosciences

Methods

BVZ UV-Vis spectrophotometer analysis

BVZ was analyzed by UV-Vis spectrophotometer (Lambda 2 UV-Vis spectrophotometer, Perkin Elmer Waltham, MA, USA) at 280 nm. Calibration line was obtained by dilution of a solution of Avastin[®] 25 mg/ml BVZ with PBS buffer pH 7.4 in 0.10-1.0 mg/ml concentration range. In this concentration range there is a good linearity of response to the instrument and the extinction coefficient ($E_{280}^{0.1\%}$) was 1.54.

Ion pair preparation between BVZ and AOT

BVZ-AOT ion pair was prepared by mixing a 1mg/ml solution of BVZ and an AOT solution at 1:150 molar ratio: a suspension was formed, which was centrifuged at 20800 g (Eppendorf centrifuge, Hamburg, Germany). The supernatant was spectrophotometrically analyzed at 280 nm, while the precipitate was dissolved in pH 10.5 carbonate buffer, prior to spectrophotometric analysis.

Derivatization reaction between BVZ and FITC

BVZ was derivatized with FITC in order to obtain a fluorescently labelled molecule. Briefly, 250 μ l of a 1 mg/ml FITC solution in 0.1 M pH 10.5 Na₂CO₃/NaHCO₃ buffer was added to 1 ml of a 5 mg/ml BVZ solution in pH 10.5 buffer. The reaction mixture was stored under stirring at room temperature for 2 hours, protecting it from light with an aluminium foil [24]. BVZ-FITC was then

purified from free FITC which had not reacted by eluting the reaction mixture through a Bio-Gel P-6DG column .

BVZ-FITC UV-Vis spectrophotometric analysis

BVZ-FITC was analyzed by UV-Vis spectrophotometer (Lambda 2 UV-Vis spectrophotometer, Perkin Elmer Waltham, MA, USA) at 280 nm and 495 nm in a 0.0468-0.468 mg/ml concentration range. In this concentration range a good linearity of the instrument response was revealed and the extinction coefficient ($E_{495}^{0.1\%}$) was 1.02 at 495 nm. The extinction coefficient at 280 nm ($E_{280}^{0.1\%}$) was 1.54 (it was determined from calibration line of BVZ in PBS buffer) and it was assumed that it was not altered following the conjugation of BVZ with FITC. As a consequence, the following formula was utilized for calculation the concentration of BVZ-FITC:

$$\text{BVZ (mg/ml)} = [A_{280} - (0.35 \times A_{495}) / E_{280}^{0.1\%}] [24, 25].$$

Preparation of BVZ loaded SLN

SLN were prepared with the fatty acid coacervation method. Briefly, the sodium salt of the fatty acid is dispersed in water and the suspension is heated to a temperature above its Krafft point. Once a clear solution is obtained, it is acidified with a specific acidifying solution, depending on the fatty acid length and the precipitation of SLN is observed [16]. To complete the precipitation of SLN, the solution is placed in an ice bath. Different polymers may be used as stabilizers, such as PVA (added at temperature of about 50 °C) or HMPC (cold solubilized) [16]. Stearic (SA) and palmitic acid (PA) SLN were prepared starting from SS and SP. To facilitate the loading of BVZ into SLN, it is necessary to solubilize AOT in the water phase prior to acidification in order to form the lipophilic ion pair *in situ*.

Optical microscopy

SLN suspensions under study were observed by optical microscopy (DM2500 microscope, Leica Microsystems GmbH, Wetzlar, Germany) at x 630 magnification.

Scanning Electronic microscopy (SEM)

SLN were also observed by SEM (Stereoscan 410 Leica, Germany). BVZ-loaded SLN were diluted 1:50 in water and 15 μ l suspension were deposited on a copper stub and dried. Samples were then sputter coated with 15 nm gold layer (SCD 050 Balzers, Liechtenstein) for 60 seconds under vacuum at a current intensity of 40 mA. The gold-coated particle layer was scanned using the accelerating voltage scanning of 20 kV.

Particle size determination

SLN particle size distribution was determined by the dynamic light scattering technique (DLS - Brookhaven, New York, USA). The dispersions were diluted with water (1:1000) and measurements were done at an angle of 90° with a laser beam of 675 nm.

BVZ entrapment efficiency and loading

SLN suspension was diluted with an equal volume of water and centrifuged at 55,000 g (Allegra 64R centrifuge, Beckman Coulter, Brea, CA, USA) for 45 minutes. The supernatant was collected and spectrophotometrically analyzed. SLN were then washed twice with pH 10.5 carbonate buffer, in order to displace the ion pair adsorbed on their surface, and then they were centrifuged at 55,000 g for 15 minutes. The supernatant was then spectrophotometrically analyzed. The precipitate was treated with pure acetic acid to dissolve SLN; the solution, containing the drug which was incorporated in SLN, was spectrophotometrically analyzed.

The entrapment efficiency (EE%) is calculated as the ratio between the amount of BVZ entrapped into SLN compared to the total amount used in SLN preparation.

Drug loading is calculated as μ g BVZ entrapped into SLN/mg lipid used in the preparation of SLN.

Gel filtration of SLN

The stationary phase was a matrix of cross-linked of agarose (Sephacrose CL 4B). 1 ml SLN suspension was put at the head of the column and the sample was eluted by gravity, adding a hypertonic PBS buffer (NaCl and KCl in a 2:1 ratio). 12 fractions of 1 ml each were collected. The opalescent fractions containing the purified SLN were pooled concentrated by dialysis for concentration. A dialysis membrane (SERVAPOR® MWCO: 12.000–14.000; pore diameter ca 25 Å) and a PEG 4000 solution, hypertonic respect to the purified SLN dispersion, were used. When the volume of the suspension inside the dialysis membrane was almost 1 ml, the dialysis was stopped and the SLN were analyzed for particle size and shape. EE% was determined as previously described. It was not necessary to wash SLN with pH 10.5 buffer, as gel filtration should eliminate the drug adsorbed to the SLN surface.

SA amount was determined in SLN suspension both before and after gel filtration by HPLC method. Analysis was performed using a LC9 pump (Shimadzu, Japan) with a Chromsystem™ ODS 2.5 μ 150×4.6 mm column and a C-R5A integrator (Shimadzu, Japan); mobile phase: CH₃OH:H₂O:TFA 90:10:0.05 (flow rate 1ml min⁻¹); detector: ELSD Sedex 75 (Sedere, France). Retention time was 6.1 min.

BVZ release from SLN

2 ml BVZ-loaded SLN were diluted with 2 ml water and centrifuged. The supernatant was discarded, while the precipitate was dispersed in 10 ml PBS. The obtained suspension was kept at 37°C on stirring and at scheduled times a sample was withdrawn from the suspension and centrifuged: the clear supernatant was spectrophotometrically analyzed at 280 nm. The experiment was performed up to 48 hours.

HUVEC cell preparation

HUVEC were isolated from human umbilical veins by trypsin treatment (1%) and cultured in M199 medium with the addition of 20% fetal calf serum (FCS) and 100 U/ml penicillin, 100 µg/ml streptomycin, 5 UI/ml heparin, 12 µg/ml bovine brain extract and 200 mM glutamine. HUVEC were grown to confluence in flasks and used at the 2nd –5th passage. The use of HUVEC was approved by the Ethics Committee of the ‘‘Presidio Ospedaliero Martini’’ of Turin and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all donors.

All the described experiments on different cell cultures were performed in aseptic conditions working in a biological safety cabinet – Bio Air Top Safe ®1.2 (Laftech, Australia)

Cell growth assays

In the MTT assay, HUVEC were normalized at 3,000 cells/100 µl in 96-well plates. After an overnight incubation, the medium was replaced with 100 µl culture medium with BVZ (0.5-5 µM), BVZ-loaded SLN (0.05-0.025-0.01 µM) or free SLN (used at the same dilution used to obtain BVZ-loaded SLN (0.05-0.025-0.01 µM). After 72-hour incubation, the viable cells were detected by 2,3-bis[2-methoxy-4-nitro-5sulphophenyl]-2H-tetrazolium-5carboxanilide inner salt reagent at 570 nm, as described by the manufacturer’s protocol. The absorbance of controls (i.e. cells that had received no drug) were normalized to 100%, and the readings from BVZ and BVZ-loaded SLN treated cells were expressed as % of controls. Eight replicate wells were used to determine each data point, and five different experiments were performed.

Cell motility assays

In the Boyden chamber migration assay, cells (8,000) were plated onto the apical side of 50 µg/ml Matrigel-coated filters (8.2 mm diameter and 0.5 µm pore size) in serum-free medium with or without BVZ (0.5-5 µM), BVZ-loaded SLN (0.05-0.025-0.01 µM) or free SLN. Medium containing 10 ng/ml VEGF-α was placed in the basolateral chamber as a chemoattractant. The chamber was

incubated at 37°C under 5% CO₂. After 18 hours, the cells on the apical side were wiped off with Q-tips. Cells on the bottom of the filter were stained with crystal-violet and counted (five fields of each triplicate filter) with an inverted microscope (magnification X100). The results are expressed as number of migrated cells. The control migration was 45±4 cells per microscope field (n=5).

In the wound healing assay, after being starved for 18-24 hours in 2% serum medium (to stop cell proliferation), cells were plated onto six-well plates (10⁶ cell/well) and grown to confluence. Cell monolayers were wounded by scratching with pipette tip along the diameter of the well, and they were washed twice with serum-free medium before their incubation with culture medium in the absence or presence of BVZ (0.5-5 µM), BVZ-loaded SLN (0.05-0.025-0.01 µM) or free SLN. In order to monitor cell movement into the wounded area, five fields of each of the three wounds analysed per condition were photographed immediately after the scratch (0 hour) and 24 hours later.

***In vitro* angiogenesis assays**

In the tube-formation assay, HUVEC cells were seeded onto 24-well plates (5x10⁴/well) previously coated with 150 µl of growth factor-reduced Matrigel, in the absence or presence of BVZ (5 µM), BVZ-loaded SLN (0.05-0.025-0.01 µM) or free SLN. The morphology of the capillary-like structures formed by the HUVECs was analysed after 18 hours of culture by an inverted microscope, and photographed with a digital camera. Tube formation was analyzed with an imaging system (Image Pro Plus Software for micro-imaging, Media Cybernetics, version 5.0, Bethesda, MD, USA).

hCMEC/D3 cell culture

hCMEC/D3 cells, a primary human brain microvascular endothelial cell line that retains the property of BBB *in vitro*, were cultured as reported [26, 27] and were seeded at 50,000/cm² and grown for 7 days up to confluence in Petri dishes and Transwell devices (0.4 µm diameter pores-size, Corning Life Sciences, Chorges, France).

Permeability of BVZ through hCMEC/D3 cell monolayer

hCMEC/D3 cells, seeded as reported above in Transwell devices, were incubated at day 7 with free BVZ-FITC or BVZ-FITC loaded SLN, at the experimental conditions described in the Results section. The medium in lower chamber was then collected and BVZ-FITC amount was measured by LS-5 spectrofluorometer (PerkinElmer, Waltham, MA). Excitation and emission wavelengths were 490 nm and 520 nm, respectively. Fluorescence was converted in nmol BVZ/cm², using a calibration curve previously set.

Before each experiment, the transendothelial electrochemical resistance (TEER) and the permeability coefficient of dextran-FITC, [¹⁴C]-sucrose and [¹⁴C]-inulin were measured, taken as parameters of paracellular transport across hCMEC/D3 monolayer, as described in [28]. TEER value was between 30 and 40 Ω cm², dextran-FITC permeability coefficient was $0.015 \pm 0.003 \times 10^{-3}$ cm/min, [¹⁴C]-sucrose permeability coefficient was $1.19 \pm 0.21 \times 10^{-3}$ cm/min, [¹⁴C]-inulin permeability coefficient was $0.56 \pm 0.09 \times 10^{-3}$ cm/min. These values supported the functional integrity of BBB monolayer [29].

Data analysis.

Data are shown as mean±SEM. Statistical analyses were performed with GraphPad Prism 3.0 software using the one-way ANOVA and the Dunnett's test.

Results

Ion pair and SLN preparation and characterization

Antibodies are hydrophilic molecules and it is necessary to increase their lipophilicity to facilitate their incorporation into SLN. Therefore, a BVZ lipophilic ion pair was prepared with AOT, by exploiting the ionic interactions that are established between residues of the basic amino acids of

BVZ (protonable amino groups) and the sulfonate group of AOT molecule. It was calculated that there are 150 protonable amino acids in the BVZ molecule, therefore the molar ratio used to form the ion pair between BVZ and AOT was 1:150. When AOT and BVZ water solutions are mixed together as described in method section, a precipitate spontaneously forms, which is constituted by BVZ-AOT ion pair. Such ion pair is stable over a wide range of pH and can be displaced only at pH>10. 100% of BVZ was recovered in the precipitate.

For the preparation of SLN, BVZ ion pair was prepared *in situ*, by dissolving BVZ and AOT in SS or SP micellar solution prior to acidification. BVZ is a thermosensitive monoclonal antibody: it begins to degrade at temperatures higher than 60 °C. For this reason, a fatty acid with a Krafft point below this temperature must be used. Therefore, stearic (SA) and palmitic acid (PA) SLN were prepared starting from SS and SP as described in methods section.

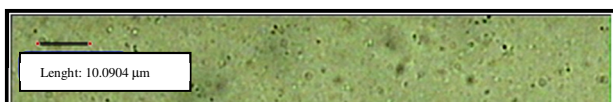
The compositions and mean particle sizes (DLS) of BVZ-loaded SLN are shown in table 1.

	BVZ-loaded SA SLN	BVZ-loaded PA SLN
0.5 mg/ml AOT solution (ml)	2.4	2.4
SS (mg)	27 (25 mg SA)	--
SP (mg)	--	27 (25 mg PA)
HMPC (mg)	25	25
1M Lactic Acid (μl)	125	--
1M Citric Acid (μl)	--	50
Avastin® 25 mg/ml (μl)	100 (2.5 mg BVZ)	100 (2.5 mg BVZ)
Particle size (nm)	515.6 ± 113.6	1213.3 ± 251.4
Polydispersity	0.191	0.120

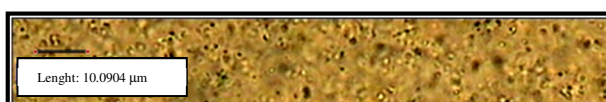
Table 1: BVZ-loaded SLN composition and mean particle size

The observation of SLN by optical microscopy was aimed to visualize their shape and to exclude the presence of aggregates and crystals or needles in the suspension. Meanwhile it cannot give any exhaustive information about actual size and shape of SLN. Microphotographs of BVZ-loaded PA SLN and BVZ-loaded SA SLN are shown in figure 1.

13
a



b



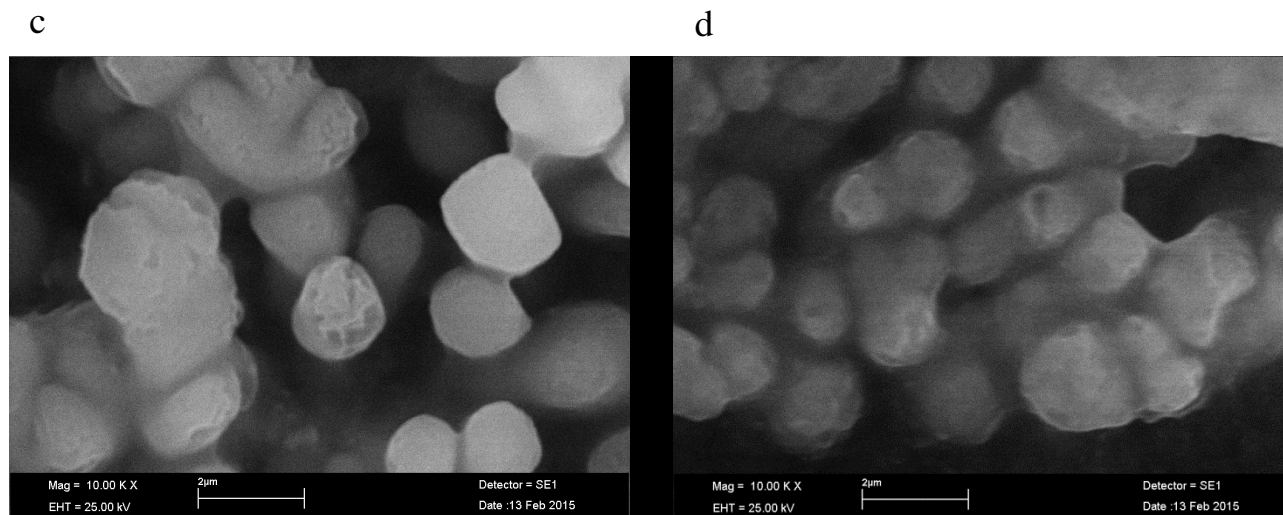


Figure 1: BVZ-loaded SLN microphotographs

- a) BVZ-loaded SA SLN by optical microscopy b) BVZ-loaded PA SLN by optical microscopy
c) BVZ-loaded SA SLN by SEM d) BVZ-loaded PA SLN by SEM

As it can be noted by optical microscopy observation, SLN suspensions are homogeneous and no aggregate can be detected. However BVZ PA SLN (reported in Figure 1b) have mean size, determined by LLS, larger than $1\ \mu\text{m}$, while BVZ SA SLN (reported in Figure 1a) are submicron sized.

The observation with SEM (Figure 1c-d) shows that both BVZ SA SLN and BVZ PA SLN have spherical shape and rugose surface but BVZ PA SLN seem to have an external film (probably due to the stabilizer PVA9000) which aggregates more particles together. This observation might confirm the different sizes of PA and SA SLN.

In perspective of an *in vivo* i.v. administration, submicron sized SLN are preferred: therefore, further studies were performed only on BVZ SA SLN.

BVZ-FITC SA SLN were also prepared using BVZ-FITC to be used for permeation studies; particle size was $420.0 \pm 30\ \text{nm}$ (0.204 polydispersity). SLN were observed with optical microscopy in normal light and fluorescence: microphotographs are shown in figure 2. Several fluorescent dots were observed, indicating the localization of BVZ-FITC within the SLN

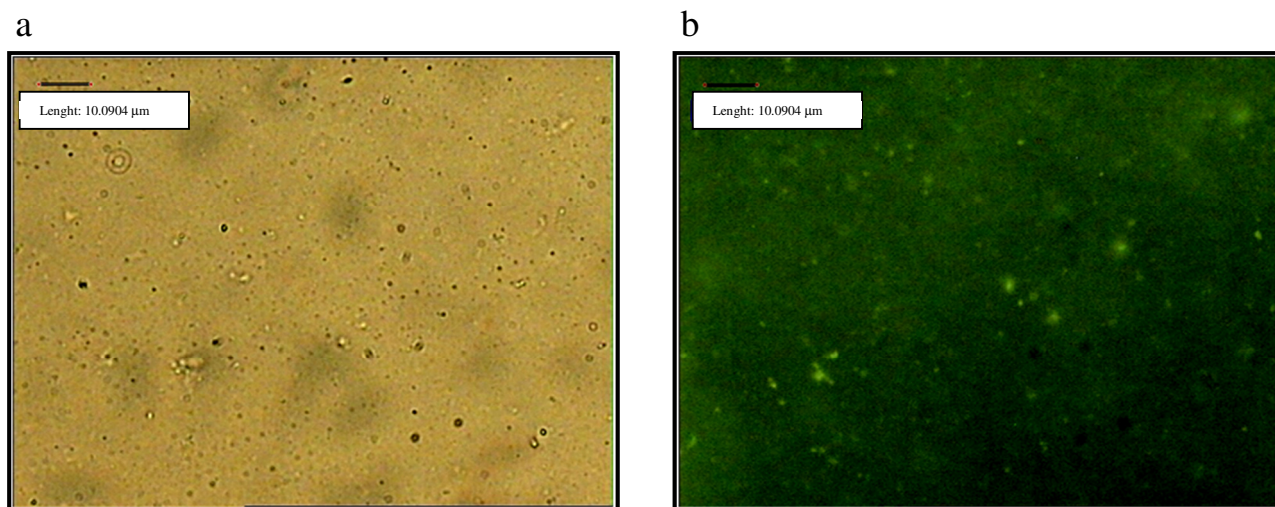


Figure 2: BVZ-FITC loaded SA SLN microphotographs

a) BVZ-FITC loaded SA SLN (normal light)

b) BVZ-FITC loaded SA SLN (Fluorescence)

EE% was calculated both after SLN centrifugation and washing with pH 10.5 carbonate buffer, and after gel filtration, as described in the methods section. Obtained results are shown in table 2, together with calculated drug loading.

	BVZ loaded SA SLN	BVZ-FITC loaded SA SLN
EE% (after centrifugation)	29.8 ± 4.4	31.0 ± 5.3
% BVZ in supernatant	--	--
% BVZ in washing	71.8 ± 6.1	72.7 ± 3.5
% EE (after gel filtration)	29.7 ± 7.0	43.3 ± 5.1
Drug loading ($\mu\text{g}/\text{mg}$)	30.0 ± 5.0	29.3 ± 4.9

Table 2: EE% and drug loading

After centrifugation and washing with pH 10 carbonate buffer, resulting EE% both for BVZ and BVZ-FITC was lower than 30%, since most of the drug (almost 70%) was recovered in the washing medium (carbonate buffer pH 10.5), which is able to displace the ion pair which was adsorbed onto SLN surface. Data were confirmed by EE% obtained after gel filtration. Gel filtration was

performed to eliminate BVZ which was adsorbed onto SLN surface. However it should be noted that no drug is found in the supernatant of SLN suspension after gel filtration: this is interesting, because it shows that, even if only 30% of the drug is effectively entrapped in the inner lipid matrix of SLN, most BVZ (70%) is not free in the outer water phase, but probably it is weakly bound, or adsorbed on SLN surface.

SA determination performed by HPLC analysis on SLN suspensions before and after gel filtration revealed that only $5\pm 1\%$ of SA was lost during gel filtration (data not shown).

BVZ release from SLN

In physiologic pH conditions, no BVZ release from SLN can be revealed upon 48 hours (data not shown): this means that the drug amount, which is weakly bound to SLN surface, cannot be released in physiologic conditions, but remains associated with SLN. According to release results, in further *in vitro* studies on cells cultures with BVZ SA SLN (hereafter named SLN BVZ) and BVZ-FITC SA SLN (hereafter named SLN BVZ-FITC), both entrapped and surface-adsorbed BVZ was considered as effectively delivered in SLN.

SLN BVZ inhibit endothelial cell growth

Initially, the ability of BVZ, SLN BVZ and free SLN to inhibit the growth of HUVEC was compared *in vitro*. Cells were cultured in the absence or presence of BVZ (0.5-5 μM), SLN BVZ (0.05-0.025-0.01 μM) or free SLN; the cells were then assessed by the MTT assay. Figure 3 shows the inhibition of cell proliferation induced by BVZ and SLN BVZ. The effect was concentration-dependent. BVZ was able to inhibit cell proliferation, showing a respective growth inhibition of 50% and 30% at 5 and 0.5 μM , after 72 h of treatment. By contrast, HUVEC were more sensitive to SLN BVZ, in fact the maximal inhibition reached approximately 80% at the highest concentration used (0.05 μM), which was 100 times lower than the higher used with free BVZ. 50% of growth inhibition was reached using 0.025 μM SLN BVZ, being 200 times more active than that obtained with free BVZ. To investigate the possibility that free SLN may exert cell toxicity not related to

BVZ, the effect of free SLN on cell growth was evaluated. Results showed that free SLN did not affect cell growth even at the highest concentrations (data not shown).

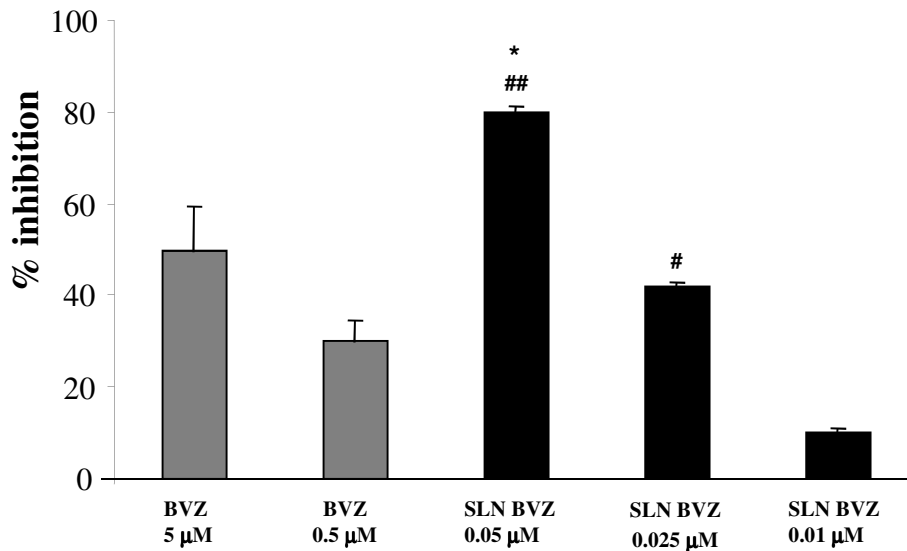


Figure 3: MTT test

* $p < 0.05$ vs BVZ 5 μ M - # $p < 0.05$ vs BVZ 0.5 μ M - ## $p < 0.01$ vs BVZ 0.5 μ M

SLN BVZ inhibit cell migration

To evaluate the SLN BVZ effect on cell migration, the endothelial cells were seeded in the upper chamber of a Boyden chamber in serum-free medium in the absence or presence of BVZ (0.5-5 μ M), SLN BVZ (0.05-0.025-0.01 μ M) or free SLN and allowed to migrate for 18 h toward the lower chamber containing medium supplemented with VEGF- α (10 ng/ml), used as chemoattractants. The results showed that BVZ significantly inhibited cell migration by about 30-50% at 0.5-5 μ M respectively, while SLN BVZ induced the same inhibitory effect at concentrations that were 100-50 times lower (figure 4). By contrast, no significant inhibition of migration was detected using free SLN at any concentration (data not shown).

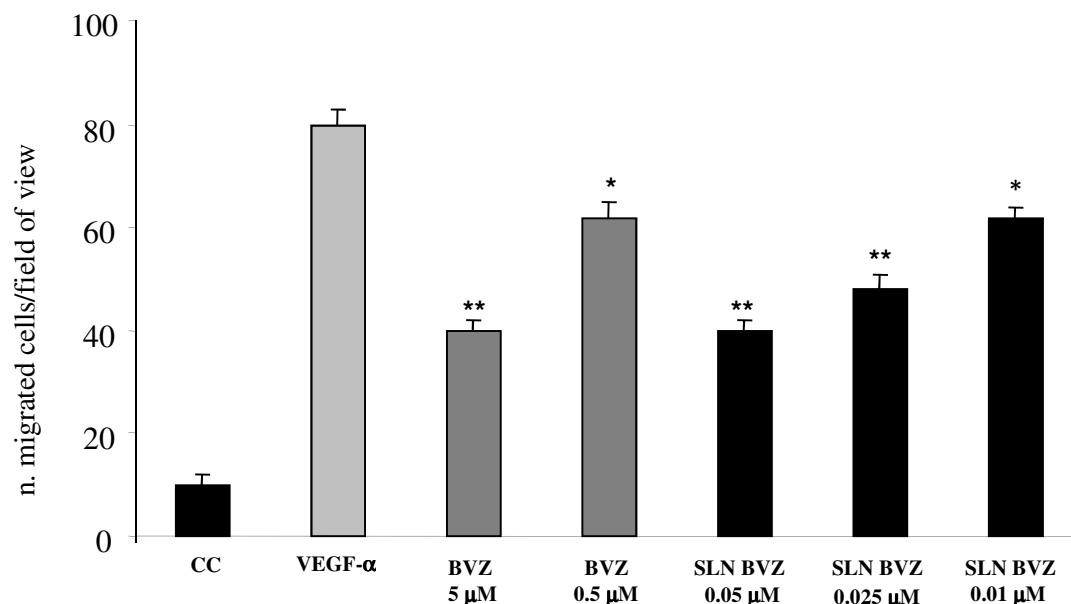


Figure 4: Boyden chamber migration assay

* $p < 0.05$ vs VEGF- α - ** $p < 0.01$ vs VEGF- α

To confirm the effect of BVZ and SLN BVZ on directional cell migration, the scratch assay was performed, an *in vitro* “wound healing” assay. A linear scratch was performed on a confluent monolayer of endothelial cell, and they were then cultured in 2% FCS medium, to minimise cell proliferation, in the absence or presence of BVZ (0.5-5 μ M), SLN BVZ (0.05-0.025-0.01 μ M) or empty SLN. Microscopic analysis evaluating cell capacity to migrate and fill the empty areas at different times showed that, in the presence of BVZ substantial cell migration was detectable in the wound area and that it was partially inhibited only by the higher concentration used (figure 5). This inhibition was also detectable for SLN BVZ, which displayed an almost 100 times higher. By contrast, empty SLN were ineffective.

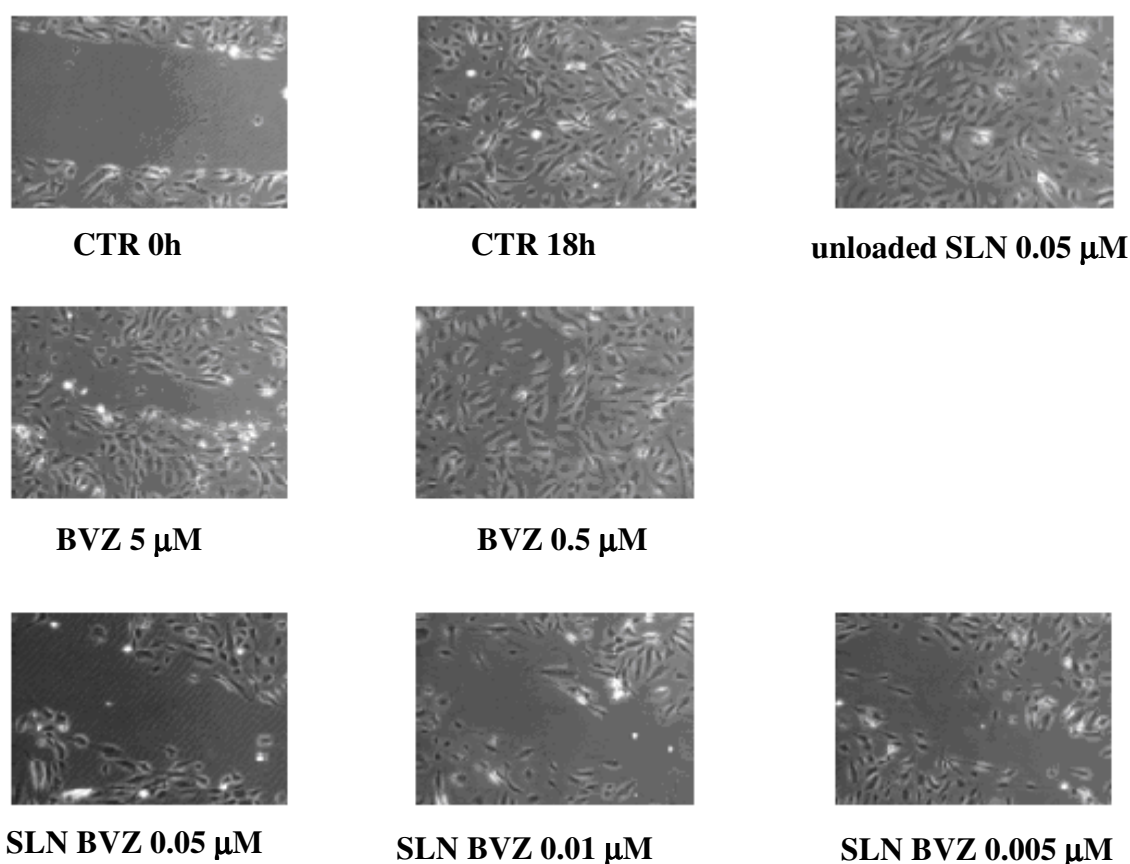


Figure 5: wound healing assay

SLN BVZ inhibit *in vitro* angiogenesis

Tumor growth is favored by tumor angiogenesis, which is continuously activated in cancer resulting in the accumulation of immature and chaotic blood vessels.

To assess the effect of BVZ and SLN BVZ triggering on angiogenesis, the effect of both formulations was evaluated on endothelial tube-formation assay, which is able to estimate the formation of three-dimensional vessels *in vitro*. HUVEC were seeded onto 24-well plates (5×10^4 /well) previously coated with 150 μ l of growth factor-reduced Matrigel (BD Biosciences), in the absence or presence of BVZ (0.5-5 μ M), SLN BVZ (0.05-0.025-0.01 μ M) or empty SLN. The morphology of capillary-like structures formed by HUVEC was analyzed 18 hours after culturing. The results showed that SLN BVZ dose-dependently inhibit endothelial tube-formation, being 100-

200 times more active than the free drug (figure 6). Also in this experimental condition, free SLN were ineffective.

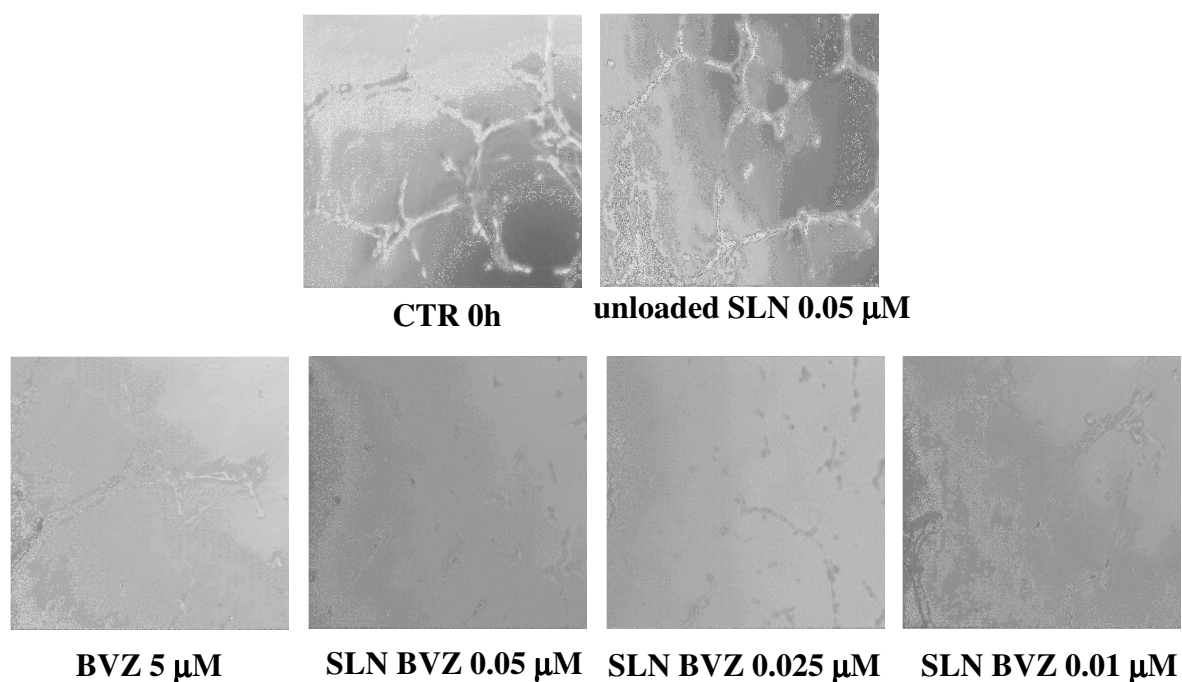
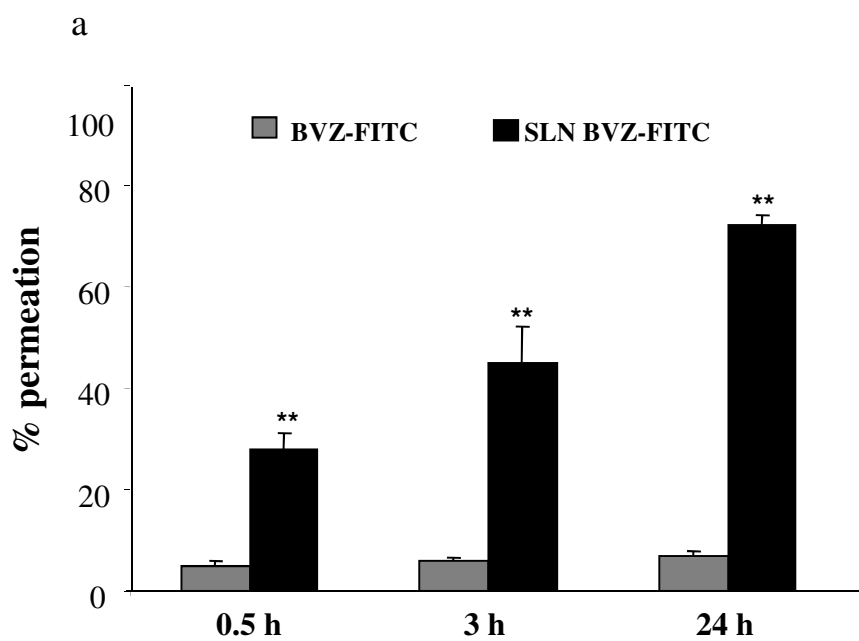


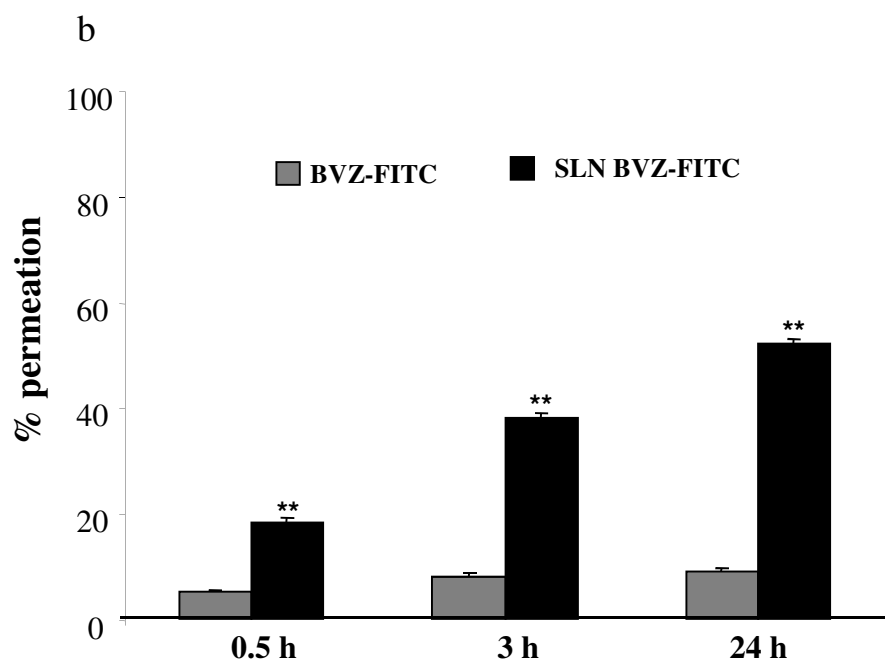
Figure 6: tube-formation assay

SLN BVZ-FITC enhance permeability through hCMEC/D3 cell monolayer

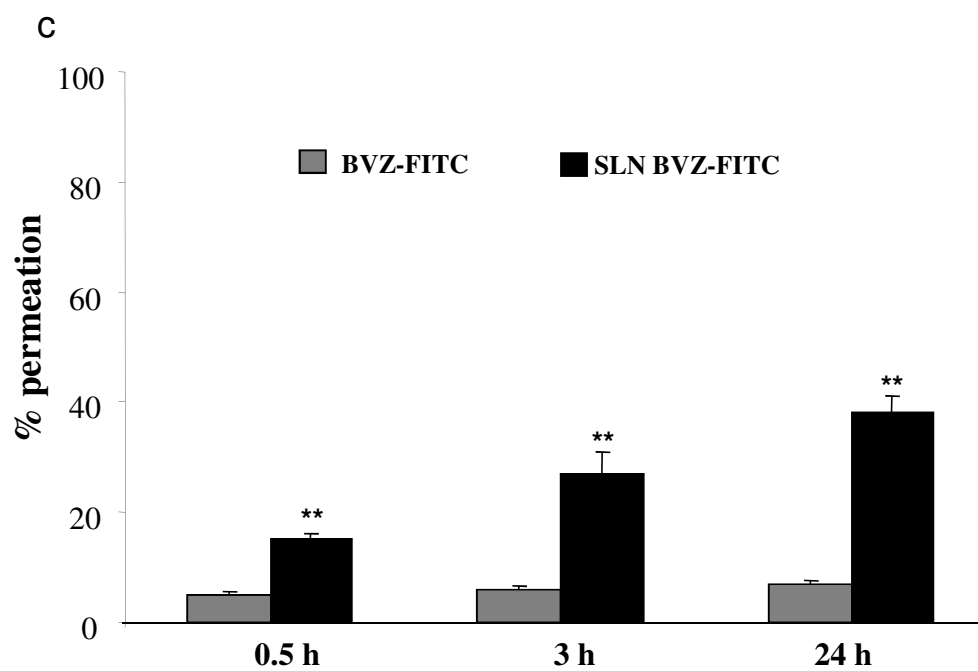
In order to evaluate *in vitro* the suitability of SLN as vehicles for BVZ delivery across the BBB, permeation of SLN BVZ-FITC and BVZ-FITC through hCMEC/D3 cells monolayer were compared at three different BVZ-FITC concentrations (0.53-0.0053 μ M). As it can be observed by figure 7, BVZ-FITC permeation is increased when loaded in SLN at the three concentrations considered.



a) 0.53 μ M BVZ-FITC



b) 0.053 μ M BVZ-FITC



c) 0.0053 μ M BVZ-FITC

Figure 7: BVZ-FITC and SLN BVZ-FITC permeation through hCMEC/D3 cells monolayer

** $p < 0.01$ vs BVZ-FITC

Discussion

New angiogenesis plays a key role for tumor growth. It was shown that BVZ, a monoclonal antibody with anti-VEGF activity, can cause a dramatic suppression of tumor growth *in vivo* [30]. BVZ-loaded nanoparticles allow to target the drug to tumor sites, especially where biological barriers, like the BBB, are a limiting step for therapeutic efficacy [9]. SLN are a safe vehicle, which has been proposed as drug delivery system for different cytotoxic drugs, and have been recently proposed for the treatment of glioblastoma, a CNS primary tumor [17]. However, BVZ high molecular weight and hydrophilic nature hamper the entrapment of such molecule in a lipid nanoparticulate system.

Recently, a new technology has been developed for SLN preparation, named coacervation [16]: through this technique different hydrophilic drugs have been loaded in SLN by using the hydrophobic ion pairing technique [18, 20, 21]. In particular in this technique operating temperatures are relatively mild, allowing the entrapment of thermolabile drugs, like protein and peptides [19].

In this experimental work BVZ was entrapped in SLN produced by coacervation technique, thanks to ion pair formation with AOT: BVZ. EE% was measured both after selective displacement of the ion pair at alkaline pH and after gel filtration. EE% recovered was not very high, since most part of the drug was adsorbed or weakly bound to SLN surface, but no drug was detected in the supernatant of SLN and the drug was not released from SLN at physiologic pH. This means that in physiologic conditions 100% of the drug remains associated with SLN, thus being effective as anti-angiogenic drug. To investigate whether the ion pair formation and the entrapment of the same in the lipid matrix could cause a loss of BVZ activity, 4 different *in vitro* assays on cells were chosen (proliferation assay, migration assay, wound healing assay and tube-formation assay), aiming to evaluate anti-angiogenic activity of SLN BVZ [31]. Surprisingly, BVZ activity, when loaded in SLN, was not only maintained, but increased up to 100-200 fold compared to free BVZ, while free SLN were inactive. Despite the reasons of this phenomenon remain uncertain and need further

investigation, it is noteworthy that the activity of a protein macromolecule can be enhanced so much through the delivery within a colloidal system. Moreover, the entrapment of BVZ in SLN allows the use of the same as targeting agents towards specific tumor sites. One of the tumors with the poorest prognosis is glioblastoma, a CNS primary tumor, the major obstacle to the pharmacological treatment of brain tumors being the overcoming of the BBB. The abundance of tight and adherent junctions, the lack of fenestrations, the presence of several drug efflux transporters on the endothelial cells constituting the BBB are majorly responsible for poor drug delivery across BBB [32]. SLN can be considered a valid strategy to overcome the BBB, since they can be phagocytosed by the endothelial cells, favoring the delivery of their cargo within the brain parenchyma. Therefore, the permeation of SLN BVZ-FITC was evaluated across a hCMEC/D3 cells monolayer: these cells are known to have tight junctions and express several drug efflux transporters, as well as primary human BBB cells [33] and consequently they are a good model of human BBB. Interestingly SLN BVZ-FITC cross hCMEC/D3 cells monolayer in a dose dependent manner, while BVZ-FITC, a protein macromolecule, is almost unable to overcome this barrier. This is promising in perspective to use SLN BVZ for glioblastoma treatment.

Conclusions

BVZ, a protein macromolecule with anti-VEGF activity, was entrapped in SLN prepared through the fatty acid coacervation technique, thanks to the ion pairing with AOT. BVZ activity, evaluated through four different *in vitro* tests on HUVEC cells, was increased 100-200 fold when delivered in SLN. Moreover SLN can enhance the permeation of a fluorescently labelled BVZ (BVZ-FITC) through hCMEC/D3 cells monolayer, an *in vitro* model of the BBB. These results are promising, since by encapsulating BVZ in SLN, the biological activity of the macromolecule is enhanced and SLN can be used as targeting agent to the brain. Further *in vivo* studies are needed to evaluate the effective potential of SLN BVZ in glioblastoma treatment.

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