Comparison of antiproliferative effect of epigallocatechin gallate when loaded into cationic solid lipid nanoparticles against different cell lines

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Abstract

Several therapeutic properties have been attributed to epigallocatechin gallate (EGCG), a phytopharmaceutical polyphenol with antioxidant and antiproliferative activity. EGCG is however very prone to oxidation in aqueous solutions which changes its bioactive properties. Its loading in nanoparticles has been proposed to reduce its degradation while increasing its *in vivo* efficacy. The aim of this study was to compare the antiproliferative effect of EGCG before and after its loading in solid lipid nanoparticles (SLNs), against five different cell lines (Caco-2, HepG2, MCF-7, SV-80 and Y-79). EGCG produced concentration- and time-dependent antiproliferative effect, with efficacy dependent on the cell line. The order of potency was: MCF-7>SV-80>HepG2>Y-79>Caco-2, for 24h exposure (MCF-7 IC_{50}=58.60±3.29 µg/mL; Caco-2 IC_{50}>500.00 µg/mL). To the best of our knowledge this is the first study reporting EGCG antiproliferative effect in SV-80 and Y-79 cells. DDAB-SLN physicochemical properties (size ~134nm; PI~0.179; ZP ~+28mV) were only slightly modified with EGCG loading (EGCG-DDAB-SLN: ~144nm; PI~0.160; ZP ~+26mV). EGCG loadingin SLN, only slightly increases the EGCG antiproliferative effect in MCF-7 and SV-80 cells. SLN exhibited intrinsic toxicity, attributed to the surfactant used in its production. From the obtained results, the biocompatibility of blank SLN must be also considered when testing the efficacy of loaded phytopharmaceutics.

1. Introduction

Epigallocatechin gallate (EGCG) is the major polyphenol found in green tea (*Camellia sinensis* L.) which has been gaining special interest in food and pharmaceutical industries. EGCG has revealed several beneficial health effects, including anti-inflammatory (Cavet et al. 2011), anti-carcinogenic (Farabegoli et al. 2011; Santos et al. 2013; Radhakrishnan et al. 2016; Shin et al. 2016), antioxidant (Cavet et al. 2011; Zhou and Elias 2013), anti-angiogenic (Yamakawa et al. 2004; Piyaviriyakul et al. 2011), anti-diabetic (Wolfram et al. 2006; Chen N et al. 2009) and anti-bacterial (Lee S et al. 2017). It has also been reported its use as chondroprotective agent as it suppressed the inflammatory response in osteoarthritis models (Akhtar and Haqqi 2011; Min S-Y et al. 2015), as well as a cardiovascular protector (Wolfram 2007; Oyama et al. 2017) and neuroprotector (Lee JH et al. 2015; Ortiz-López et al. 2016). EGCG is the most powerful catechin present in the green tea, regarding the chemopreventive and apoptosis inducing molecules (Azam et al. 2004).

EGCG is known as a powerful antioxidant showing scavenging activity against reactive oxygen species (ROS), eliminating radicals (e.g., superoxide anion radical, peroxyl and hydroxyl radicals, singlet oxygen, nitric oxide and peroxynitrite, among others (Zhong and Shahidi 2012)), as the result of the transfer of hydrogen atom or single-electron transfer reactions, involving hydroxyl groups of the B and/or D rings (Min K-j and Kwon 2014). Oxidative stress has been considered in many pathological conditions, including cancer (Du et al. 2012; Doktorovová et al. 2014), giving EGCG a role as a chemoprotective compound. The anti-carcinogenic effects of EGCG are being studied and several works report that this polyphenol is able of (i) inhibiting tumorigenesis of the lung (by reducing oxidative DNA damage which produces 8-hydroxydeoxyguanosine) (Xu et al. 1992), of oral-digestive tract (Du et al. 2012; Santos et al. 2013), and of prostate (Khan et al. 2014); (ii) reducing

vascular endothelial growth factor (VEGF) expression, leading to the inhibition of tumor growth and/or angiogenesis (Braicu et al. 2013; Shankar et al. 2013); (iii) inhibiting tumor migration and invasion (Lim et al. 2008); and of (iv) inducing cell death through apoptosis or to improve lysosomal membrane permeation (Min K-j and Kwon 2014).

EGCG is a highly unstable molecule especially in water solution, in which it suffers oxidation and/or auto-oxidation, epimerization and hydrolysis (Wang et al. 2008; Fangueiro, Parra, et al. 2014; Krupkova et al. 2016). These reactions lead to the production of other molecules without the equivalent biological or pharmacological interest due to poor biological activity (Fangueiro, Parra, et al. 2014). The loading of EGCG has been described using lipid nanocapsules (LNC; (Barras et al. 2009)) and solid lipid nanoparticles (SLNs) (Fangueiro, Andreani, Fernandes, et al. 2014). The loading of EGCG was carried out to ensure EGCG stability in biological media and further improve its bioavailability (Barras et al. 2009; Fangueiro, Andreani, Fernandes, et al. 2014; Fangueiro, Parra, et al. 2014). The nanoencapsulation revealed promising results with excellent encapsulation efficiency (> 96%), controlled release of EGCG and safety profile (Fangueiro et al. 2016). The use of nanoparticles in drug delivery is a recognised strategy to improve drug's stability and enhance its biological effects. The use of SLN is therefore expected to strengthen the well-known effects of EGCG. EGCG seems to exhibit strong anticancer effects *in vitro*, as mentioned above. Thus the main aim of this work was to compare the antiproliferative effect of EGCG before and after its loading in SLNs, against five different cell lines, including Caco-2 (human epithelial colorectal adenocarcinoma), HepG2 (human liver hepatocellular carcinoma), SV-80 (human lung fibroblast), MCF-7 (human breast adenocarcinoma) and Y-79 (human retinoblastoma). The assessment of the effect of blank

SLN (without addition of EGCG, with the cationic lipid DDAB as surfactant), was also one objective of this work.

2. Materials and methods

2.1 Materials

Epigallocatechin gallate (EGCG, 98% purity, MW 458.375 g/mol, pKa 7.59–7.75; CAS Number: 989-51-5) and ascorbic acid (AA; MW 176.12 g/mol, CAS Number: 50-81-7) were acquired from Sigma-Aldrich (Sintra, Portugal). Dimethyldioctadecylammonium bromide (DDAB; MW 630.95 g/mol; CAS Number: 3700-67-2) was acquired from Avanti Polar Lipids (Alabama, USA). Softisan®100 (S100) was a free sample from Sasol Germany GmbH (Witten, Germany), Lipoid® S75, 75% soybean phosphatidylcholine was purchased from Lipoid GmbH (Ludwigshafen, Germany), Lutrol®F68 or Poloxamer 188 (P188) was a free sample from BASF (Ludwigshafen, Germany). Anhydrous glycerol was purchased from Acopharma (Barcelona, Spain). Ultra-purified water was obtained from a MiliQ Plus system (Millipore, Germany). All reagents were used without further treatment.

The cell lines Caco-2 (human epithelial colorectal adenocarcinoma), SV-80 (human lung fibroblast) and Y-79 (human retina retinoblastoma) were purchased from Cell Lines Service (CLS, Eppelheim, Germany), HepG2 (human liver hepatocellular carcinoma) and MCF-7 (human breast adenocarcinoma) were from ATCC (ATCC, Rockville, MD). Reagents for cell culture were from Gibco (Alfagene, Invitrogene, Portugal). Alamar Blue was from Invitrogen Alfagene, Portugal. Other reagents and salts not mentioned were of high purity and were acquired from Sigma-Aldrich (Sintra, Portugal).

2.2 Cationic SLN production

Cationic SLN dispersions were produced based on the multiple emulsion (w/o/w) technique as previously described by (Fangueiro, Andreani, Egea, et al. 2014; Fangueiro, Andreani, Fernandes, et al. 2014). In brief, the composition of lipid phase (wt%) was: glycerol (37.5), Sofitsan S100 (4.5), Lipoid S75 (0.5), cationic lipids (0.5 DDAB) and water. EGCG (15 mg; to a final 0.075 wt%) and ascorbic acid (1.25 mL; to a final 0.25 wt%) were dissolved in ultra-purified water, which was added to the lipid phase at same temperature (5 °C to 10 °C above the melting point of lipid S100) and homogenized for 60 s with a sonication probe (6 mm diameter) by means of an Ultrasonic processor VCX500 (Sonics, Switzerland). A power output with amplitude of 40% was applied. The poloxamer solution (1.0 wt%) was added and homogenized for additional 90 s. This pre-emulsion was poured in the total volume of poloxamer cooled solution under magnetic stirring for 15 min to allow the formation of the SLN (total volume of formulation was 20 mL). Empty SLNs followed the same procedure without adding EGCG. The obtained SLNs dispersions were used for subsequent studies.

2.3 Physicochemical characterization

The physicochemical parameters Z-Ave (mean particle size), PI (polydispersity index) and ZP (zeta potential) were determined by dynamic light scattering (DLS, Zetasizer Nano ZS, Malvern Instruments, Malvern, UK). All samples were diluted with ultra-purified water to suitable concentration and analyzed in triplicate. For the determination of the ZP ultra-purified water with conductivity adjusted to 50 µS/cm was used. Laser diffraction (LD) was performed for particle size analysis by a Mastersizer Hydro 2000 MU (Malvern Instruments, Malvern, UK).

2.4 Encapsulation efficiency and loading capacity

Encapsulation efficiency (EE) and loading capacity (LC) of EGCG in SLNs was assessed indirectly using filtration/centrifugation followed by quantification of free EGCG (non-encapsulated) by reverse-phase high-performance liquid chromatography (RP-HPLC), using a method previously validated (Fangueiro, Parra, et al. 2014). A volume of 2.0 mL of each EGCG-SLNs was placed in centrifugal filter devices Ultracel 100K (100.000 MWCO, Amicon Millipore Corporation, Bedford, Massachusetts) and centrifuged at 3000 rpm, during 20 min (Sigma 4K10 cen-trifuge, Spain), to separate the lipid and aqueous phase. Free EGCG, present in the aqueous phase, was quantified by RP-HPLC, using the same method as described in (Fangueiro, Parra, et al. 2014), just after the separation. The parameters were quantifyed by applying the following equations:

$$EE\ (\%) = [(Total\ amount\ EGCG - Free\ EGCG)/(Total\ amount\ EGCG)] \times 100$$

$$LC\ (\%) = [(Total\ amount\ EGCG - Free\ EGCG)/(Total\ amount\ of\ lipid)] \times 100$$

2.5 Cell culture and viability assay

Cell culture and maintenance: Caco-2, HepG2 and SV-80 cells were maintained in DMEM (Dulbecco's Modified Eagle Medium), MCF-7 in DMEM/F12 (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12) and Y-79 in RPMI-1640 (Roswell Park Memorial Institute (RPMI) 1640 medium), supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics (100 U.mL^{-1} penicillin and 100 μg.mL^{-1} of streptomycin) in an atmosphere of 5% CO_2 in air at 37 ºC. Culture media was exchanged once or twice a week, depending on the cell growth, and passages were done once a week.

Cell manipulation for preparations of assays were as described before by us (Severino et al. 2014).

Cell manipulation to prepar the assays: Confluent cells were treated with trypsin (except Y-79 that does not requires enzymatic treatment as it grows in suspension), a procedure identical to that described by us (Severino et al. 2014). After trypsin treatment, cells were counted, diluted to a density of 5×10^4 cells.mL^{-1} and plated in 96-well plates (100 μL per well). Cells were left in culture for 24 hours for cell adherence.

Concerning to Y-79 cells, cells were removed from growing flasks, centrifuged (to eliminate the supernatant), re-suspended in FBS-free culture media (gently aspirations with the add of a Pasteur pipet permits the desagregation of cells from clusters), counted and, after appropriate dilution to 1×10^5 cells.mL^{-1}, cells were seeded in poly-L-lysine pre-coated 96-well plates (100 μL/well, i.e. 1×10^4 cells per well). Cells were left in culture for 24 hours for cell adherence.

Preparation of test solution and cytotoxicity/viability assay: In this study the different cell lines were assayed for cell viability to compare the effect of the following samples: i) EGCG in solution, ii) DDAB-SLNs, and iii) DDAB-loaded EGCG SLN. Each formulation was tested at four concentrations, calculated as EGCG concentration in solution or in formulation. When formulation was deprived of EGCG (blank SLN), identical volumes of SLNs formulation were taken and test solutions are presented as "equivalent concentrations". The concentrations used were: 10, 25, 50 and 100 μg.mL^{-1} (that is, in μM of EGCG,: 21.8, 54.5, 109.0 and 218.0, respectively). Test solutions were prepared just prior to cell application, using FBS-free culture media into which the required volume of respective formulations/solutions was added. To expose the cells to teste solutions, culture media was removed and replaced by FBS-free culture media containing the different test

samples. Exposure of 24 h and 48 h was performed, before assaying the cell viability with Alamar Blue.

Cell viability was assayed with Alamar Blue (Invitrogene, Alfagene, Portugal), as previously reported (Andreani et al. 2014). Briefly, after 24 h or 48 h of exposure, the incubating media was removed and replaced by FBS-free culture media supplemented with Alamar Blue 10% (v/v), 100 µL to each well. The absorbance at 570 nm (reduced form) and 620 nm (oxidative form) was read 5 h after addition of the Alamar Blue solution. Data were analysed by calculating the percentage of Alamar blue reduction (according to the manufacturer recommendation and as previously reported (Andreani et al. 2014; Fangueiro, Andreani, Egea, et al. 2014; Severino et al. 2014) and expressed as percentage of control (untreated cells).

Calculation of IC$_{50}$: The IC$_{50}$ (half maximal inhibitory concentration or the concentration that inhibits 50% of cell viability/proliferation) was calculated from each dose-response experiment using excel or GraphPad Prism tolls. For each cell line and for each exposure time (24 and 48 h), 3 independent experiments were done (each experiment in quadriplicate). For each condition, the IC$_{50}$' are indicated as the mean ± S.D. of the IC$_{50}$' calculated for the 3 independent experiments.

2.6. Statistical analysis

Statistical significance of cell viability data was performed using two-way analysis of variance (ANOVA), followed by Tukey multiple comparison test using 95% of confidence, i.e. significance of the difference between groups with a *p*-value < 0.05 was set as significant. The multiple t-test using the Bonferroni-Dunn method (95% confidence) was

also used when needed. Data were expressed as the mean value ± standard deviation (mean ± SD) of *n* experiments as indicated.

3. Results and discussion

3.1. SLN production and characteristics

The production of SLN was based on a previous 3^3 full factorial design (Fangueiro, Andreani, Egea, et al. 2014) and EGCG was loaded in SLN prepared by multiple emulsion technique (Fangueiro, Andreani, Fernandes, et al. 2014). The analysis by DLS revealed particles under 150 nm with a relatively narrow distribution (<0.25) (Table 1). The use of cationic lipids, such as DDAB, contributed to the high positive ZP values. This strategy was used to promote particles' stability due to repulsion between positive SLN surface charges, reducing particle aggregation and changes in particle size. During storage time, the positive charge at particles' surface, on the other hand, increases interaction with cells as these exhibit a negative surface charge. Such enhanced interaction between particles and cells would contribute to increase the delivery of the loaded drug but could also interfere with the cell membrane stability, leading to cytotoxicity. Indded, a previous work revelead that CTAB concentration is an important factor contributing to cell cytotoxicity (Fangueiro, Andreani, Egea, et al. 2014). Laser diffraction results also revealed a mean particle size within the nanometer range, varying between 115 nm and 120 nm (Table 1). The obtained results may anticipate the improvement of SLN cells' adhesion and their internalization to release the drug inside cells.

EGCG has been reported as a valuable natural polyphenol that interferes with several biochemical pathways and helps to reduce cancer proliferation (Du et al. 2012; Santos et al. 2013; Lewandowska et al. 2016). It has been proven that EGCG has high affinity for the lipid bilayer of cell membranes, which allows this molecule to enter the cells more easily and to exhibited pharmacological effects (Chen L et al. 2002), but the stability of this molecule in aqueous solution is poor (Wang et al. 2008), thus stratagies that protect EGCG from degradation and preserve its bioactivity are needed. In this work, we have chosen to produce SLN using DDAB as cationic surfactant, as a strategy to encapsulate EGCG in SLN aiming cell delivery and to protect EGCG from degradation while preserving its bioactivity. As shown in Table 1, the loading of EGCG slightly increases the particle size (less than 7.5% increase) and sligthy decreases the ZP (about 8 mV).

Concerning the cell viability, first the anti-proliferative effect of EGCG *per se* was tested (EGCG was dissolved in FBS-free culture media, see methods for details). Five different cell lines (Caco-2, HepG2, MCF-7, SV-80 and Y-79) were exposed to different concentrations of EGCG (10.0, 25.0, 50.0 and 100.0 μg/mL, that is 21.6, 54.0, 108.0 and 216.0 μM, respectively, as indicated in Table 2) for 24 and 48 h. In Figure 1, it is observed that EGCG dose- and time-dependently reduces cell viability/proliferation, and that the dose effect is not identical for the 5 cell lines, while Caco-2 cells were the least affected (the most tolerant). MCF-7 cells were those showing lower viability, which is confirmed by the IC_{50} values shown in Table 3. Considering the IC_{50} values obtained for each cell line (Table 3), the order of potency of EGCG in reducing cell viability was: MCF-7 > SV-80 > HepG2 > Y-79 > Caco-2, which is identical for 24 h as for 48 h exposure. We also can observe that the effect of exposure time is more evident for Caco-2 and for Y-79 cells (Table 3 and Figure 1) and has a lower effect in MCF-7 cells. Indeed, in Caco-2 cells

(Figure 1A) the viability after 48 h exposure was lower than at 24 h, for all tested concentrations ($p < 0.05$) while for MCF-7 cells (Figure 1C) statistically significant differences between time were only observed at the highest tested concentration ($p < 0.05$).

The antiproliferative effect of EGCG has been tested in several cell lines. There is a general concensus with the fact that cell lines having p53 mutation are more resistant to many chemotherapeutic agents (Du et al. 2012; He et al. 2017), as these cells may have enhanced drug efflux and metabolism, activated mechanisms that promote survival, inhibited apoptosis, among other characteristics (He et al. 2017). Among the five cell lines tested, Caco-2 has mutated p53 while HepG2, MCF-7 are p53 wild type; concerning Y-79 data indicate a mutation in *RB* and *p53* gene (Laurie et al. 2006) and SV-80 expresses two isoforms of p53 (Harris et al. 1986). In this study the cells with p53 mutation were the most resistant against the EGCG chemotherapeutic effect. Concerning EGCG effect in other colorectal cancer cell lines, Du et al., (2012) used HCT-116 (p53 wild type) and SW-480 (with a p53 mutation) and observed a stronger effect in HCT-116 that in SW-480, which is in line with the fact that p53 may play an important role in cell proliferation and in the modulation of polyphenols effects. Other authors also observed cell viability reduction upon EGCG exposure using Caco-2 (showing IC_{50} values lower than those reported in our work (Kang 2015)), MCF-7 (10 µM produced 70% viability (Kang 2015)), HepG2 (IC_{50} at 48 h exposure was 74.7 µg/mL (Shen et al. 2014) a value similar to that report by us) and to the best of our knowledge this is the first report testing different EGCG concentrations and incubation times in SV-80 and Y-79 cells (Figure 1D and Figure 1E, respectively).

As all the IC_{50}' are above 30 µg/mL (i.e. 65.4 µM), the encapsulation in SLN should increase biological efficacy while reducing EGCG degradation, thus we would expect

lower IC_{50} values for encapsulated EGCG. EGCG was successfully encapsulated in SLN formulation, using DDAB as cationic lipid (Fangueiro, Andreani, Fernandes, et al. 2014), with an encapsulation efficiency (EE) of about 97 % (i.e. 96.86 ± 1.88% (Fangueiro, Andreani, Fernandes, et al. 2014)) and a LC of about 15%. In a previous study, we reported that CTAB concentrations compromise Y-79 cell viability at concentrations above 0.5% (wt%) (Fangueiro, Andreani, Egea, et al. 2014). CTAB concentration is however relevant to particle size which decreases as CTAB concentration increases (239.5 ± 0.61 nm to 144.7 ± 1.61 nm, at 0.25 and 1.0% (wt%), respectively) and to grant SLN high surface charge which increases with the concentration, from +24 mV, at 0.25%, to +48 mV, at 1.0% (wt% in formulation), which is needed to maintain size stability and to favour cellular interaction (Fangueiro, Andreani, Egea, et al. 2014). EGCG-DDAB-SLN were prepared, characterized (Table 1 and (Fangueiro, Andreani, Fernandes, et al. 2014)) and applied to the five cell lines. The test solutions were prepared by diluting the SLN volumes, in FBS-free culture media, yielding 10, 25, 50 and 100 µg/mL of EGCG.

Figure 2 shows the results obtained for cell viability after 24 h exposure to EGCG-DDAB-SLN (comparing with EGCG and blank SLN, DDAB-SLN, at 24 h) and Table 3 shows the calculated IC_{50}, for 24 and 48 h exposure. As shown in Figure 2 and Table 3, DDAB-SLN exhibited lower dose-dependent toxicity, being the cell viability above 90% of control (for all tested cell lines) at 165 μg/mL DDAB (or, 261.51 μM; i.e., at 25 μg/mL of EGCG equivalent; Table 2) and above 50% for twice this concentration. Also, the effect of DDAB-SLN was shown to be influenced by the cell line, showing a order of potency (at 24 h exposure): HepG2 > SV-80 > Y-79 > Caco-2 > MCF-7 (Table 3). Loading of EGCG in DDAB-SLN, enhanced the EGCG anti-proliferative effect only in SV-80 cells (Figure 2D),

while in the other cell lines the IC_{50} produced by EGCG-DDAB-SLN was comparable to the EGCG and to the blank SLN (Figure 2). In HepG2, MCF-7 and Caco-2 cell lines the encapsulated EGCG (EGCG-DDAB-SLN) protected the cells against toxicity, as it is lower than that of blank SLN, thus EGCG should be acting as an antioxidant rather than as antiproliferative agent. From the results obtained in this work, one should also bear in mind that the components of SLN may also exhibit some level of toxicity as the loaded phytochemicals/drugs, as that, if not targeted adequately, the site-unspecific damage might be higher then the desired therapeutic effect. Thus, convenient control assays should always be performed and demonstrated.

4. Conclusions

EGCG exhibits anti-proliferative effect which is time- and concentration dependent and also depends on the tested cell line (order of potency: MCF-7 > SV-80 > HepG2 > Y-79 > Caco-2, for 24 h exposure). As has been reported, encapsulation of EGCG improves its stability and should improve its efficacy as anti-proliferative agent, which was only observed for SV-80 cells. Production of SLN requires the use of surfactants both for stereochemical and electrostatic stabilization. Surfactants surrounding the particles may also play a relevant role in the as cell internalization of particles/drug. Some surfactants may also damage cells by direct interfering with cell membrane integrity. In this work we have shown that DDAB is tolerated by cells in concentrations that are compatible to SLN production. One must also bear in mind that these cells have no protection against membrane damaging agents which can be translated in a higher toxicity than the effect that may be observed *in vivo*.

Conflict of interest

The aurhors declare no conflicts of interest

Acknowledgements

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Figure captions:

Figure 1. Anti-proliferative effect of EGCG in Caco-2 (A), HepG2 (B), MCF-7 (C), SV-80 (D) and Y-79 (E) cells. Cells were exposed to different concentrations of EGCG (10, 25, 50 and 100 µg/mL (see methods for details)) for 24 and 48 h (as indicated). Cell viability was assessed with alamar blue indicator and data are presented as % of control (non-exposed cells), as mean ± SD (n = 3 independent experiments, see methods). Statistical significance ($p<0.05$) between control and different concentrations (at same incubation time) is indicated with an asterisk (*) and between different exposure times at same concentration is indicated by a delta (Δ).

Figure 2. Dose-dependent effect of DDAB-SLN (∇); EGCG (▼), EGCG-DDAB-SLN (∎) in Caco-2 (A), HepG2 (B), MCF-7 (C), SV-80 (D) and Y-79 (E) cell viability, at 24 h exposure. Cells were exposed to different concentrations of EGCG and to concentrations of EGCG-DDAB-SLN and blank SLN (DDAB-SLN) that give the same amount of EGCG, as denoted (10, 25, 50 and 100 µg/mL (see methods for details)), for 24 h. Cell viability is presented as % of control (non-exposed cells), as mean ± SD (n = 3 independent experiments, see methods). For each condition, statistical significance ($p<0.05$) between control and different concentrations is indicated with an asterisk (*, set above the line for DDAB-SLNs and below the line for EGCG-DDAB-SLNs; for EGCG please see Figure 1). Statistical significance ($p<0.05$) between the effect of EGCG in solution and encapsulated (EGCG-DDAB-SLNs), at same concentration, is indicated by a delta (Δ).

Table captions:

Table 1. Analysis by dynamic light scattering (DLS) indicating the mean particle size (Z-Ave), polydispersity index (PI) and zeta potential (ZP) and by laser diffraction (LD) of Lipid Nanoparticles dispersions. The results are expressed as mean $\pm$ S.D. (n = 3).

Table 2. Composition of test solutions regarding to EGCG and DDAB, in µg/mL and µM. The molecular weight (g/mol) of EGCG and DDAB is 458.37and 630.95, respectively. The wt% (g/100 mL) of each compound in formulation was 0.075and 0.5, respectively, as indicated in methods.
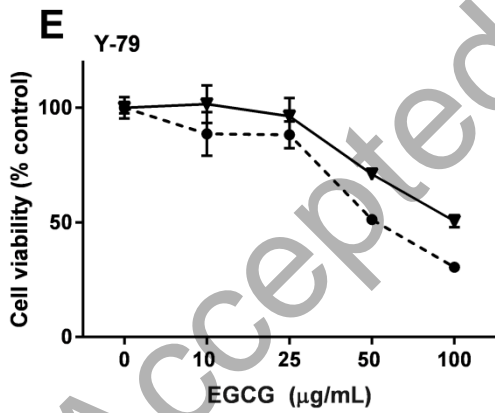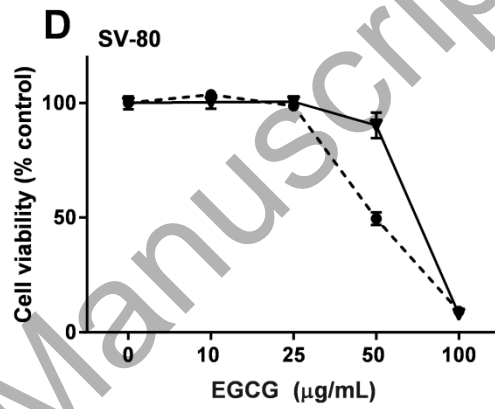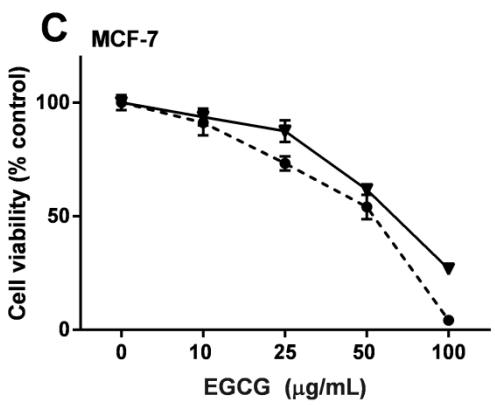
Table 3. Values of IC_{50} calculated for each cell line exposed to EGCG, EGCG-DDAB-SLN, and DDAB-SLN for 24 or 48 h (as indicated). Values represent the mean $\pm$ S.D., as indicated in methods.

Table 1. Analysis by dynamic light scattering (DLS) indicating the mean particle size (Z-Ave), polydispersity index (PI) and zeta potential (ZP) and by laser diffraction (LD) of Solid lipid Nanoparticle dispersions. The results are expressed as mean ± S.D. (n = 3).

Formulation	DLS			LD
	Z-Ave (nm)	PI	ZP (mV)	d50 (nm)
DDAB-SLN	134.2 ± 1.120	0.179 ± 0.067	+28.20 ± 2.290	119.8 ± 0.01
EGCG DDAB-SLN	143.7 ± 0.450	0.160 ± 0.015	+25.70 ± 1.420	115.0 ± 0.00

Table 2. Composition of test solutions regarding to EGCG and DDAB, in µg/mL and µM. The molecular weight (g/mol) of EGCG and DDAB is 458.37and 630.95, respectively. The wt% (g/100 mL) of each compound in formulation was 0.075and 0.5, respectively, as indicated in methods.
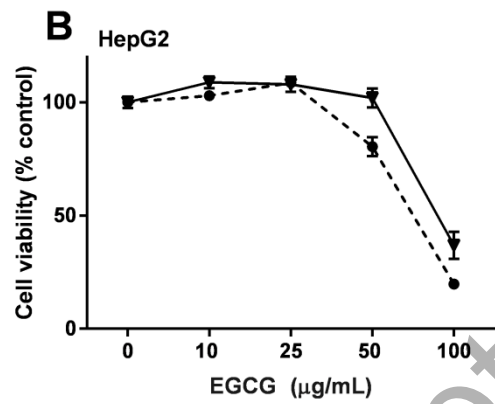
		Test solution concentration (EGCG equivalent (µg/mL))			
Compound	**Concentration**	**10**	**25**	**50**	**100**
EGCG (*)	µg/mL	10.00	25.00	50.00	100.00
	µM	21.60	54.00	108.00	216.00
DDAB (*)	µg/mL	66.00	165.00	330.00	660.00
	µM	104.60	261.51	523.02	1046.04

(*) Test solutions are identified regarding these compounds (see figure legends).

Table 3. Values of IC_{50} calculated for each cell line exposed to EGCG, EGCG-DDAB-SLN, DDAB-SLN for 24 or 48 h (as indicated). Values represent the mean ± S.D., as indicated in methods.
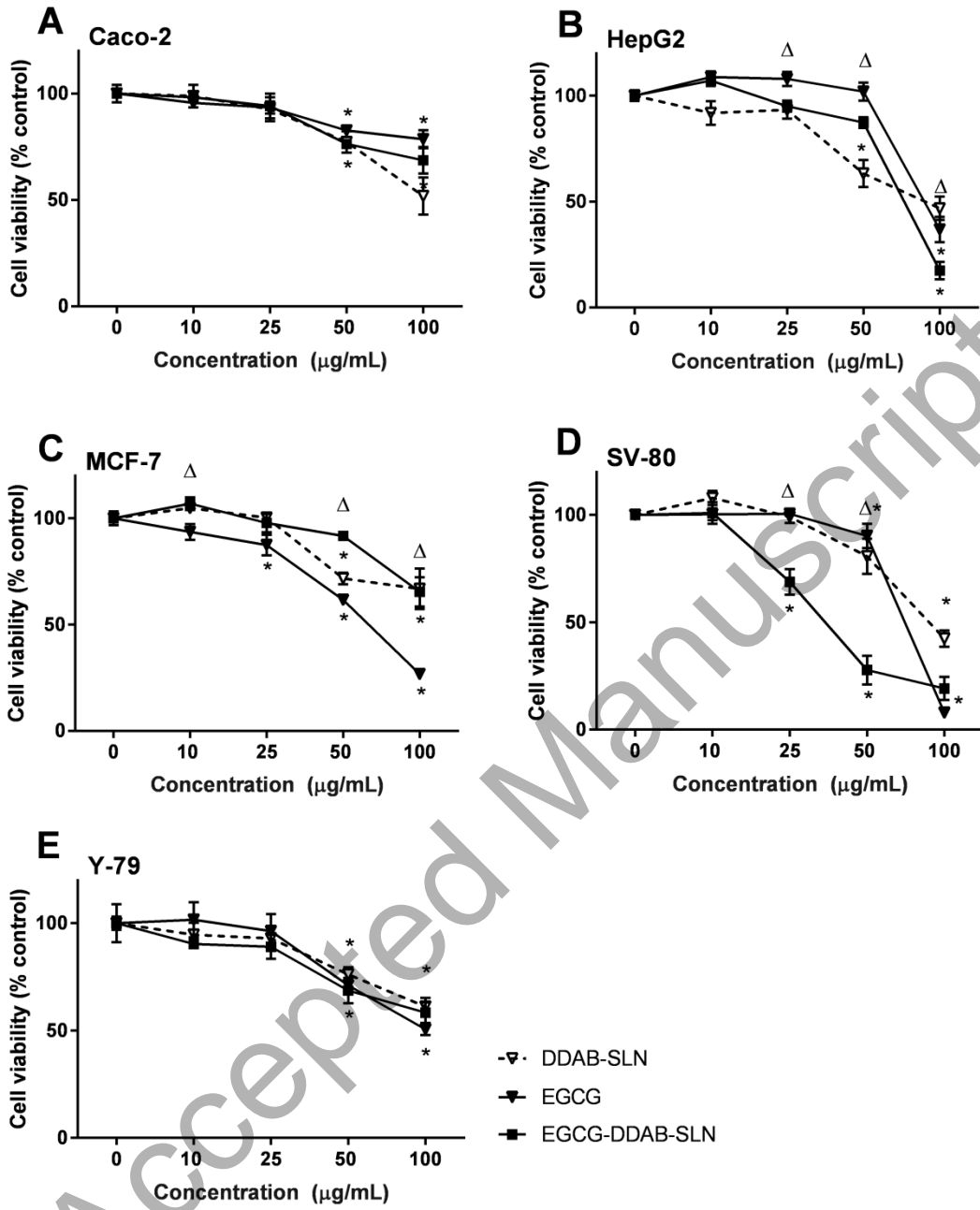
		IC_{50} (µg/mL)*		
Cell line	**Time**	**EGCG**	**EGCG-DDAB-SLN**	**DDAB-SLN**
Caco-2	24 h	> 500.00	482.54 ± 41.46	189.09 ± 29.41
	48 h	298.61 ± 2.39	412.99 ± 41.98	168.06 ± 16.36
HepG2	24 h	83.47 ± 7.53	91.17 ± 14.87	77.31 ± 5.47
	48 h	82.23 ± 4.31	114.68 ± 36.17	52.05 ± 1.60
MCF-7	24 h	58.60 ± 3.29	145.15 ± 45.93	233.87 ± 31.60
	48 h	35.67 ± 3.34	127.72 ± 20.39	176.38 ± 18.93
SV-80	24 h	62.13 ± 2.69	91.67 ± 6.007	106.67 ± 4.98
	48 h	46.12 ± 1.41	31.35 ± 3.15	43.04 ± 5.17
Y-79	24 h	133.33 ± 10.56	181.59 ± 30.29	153.83 ± 11.97
	48 h	57.41 ± 8.45	85.09 ± 8.92	81.85 ± 3.18

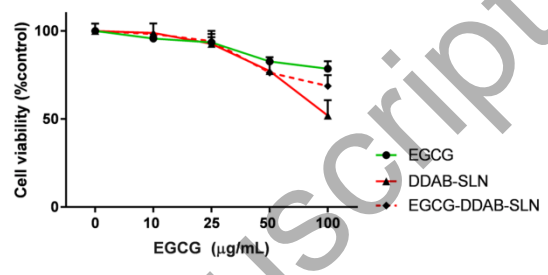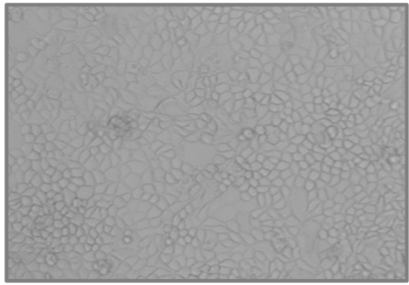* Values are expressed as the amount of EGCG equivalent in formulation.

A Caco-2

B HepG2

C MCF-7

D SV-80

E Y-79

▼ 24 h
● 48 h

SLNs

DDAB

EGCG
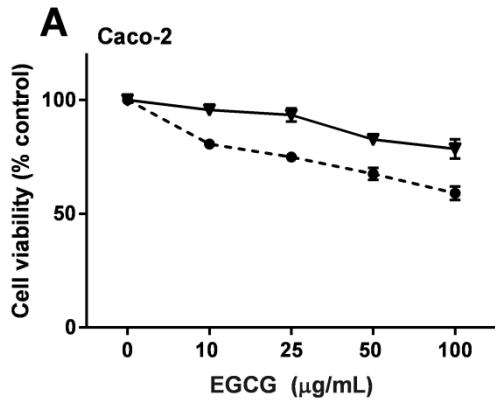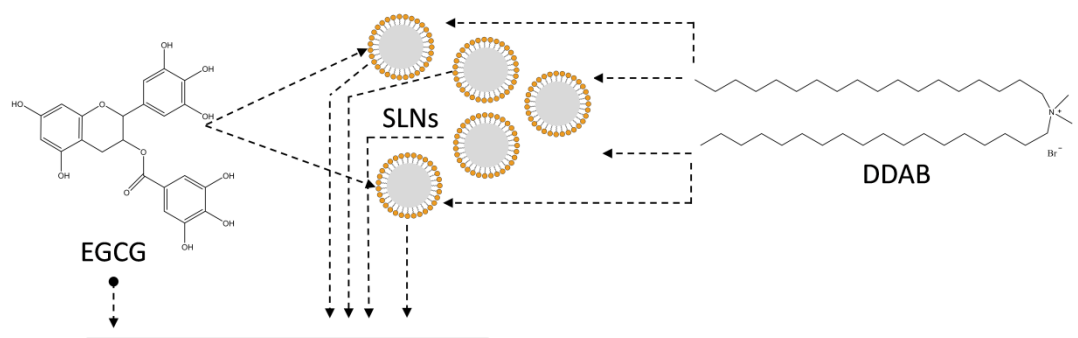
Cell culture: cytotoxicity assay

Cell viability (%control)

EGCG
DDAB-SLN
EGCG-DDAB-SLN

EGCG (μg/mL)