EFFECT OF THREE MONOGLYCERIDE BASED CUBOSOMES SYSTEMS ON THE VIABILITY OF HUMAN KERATINOCYTES

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Abstract

Three lipid-based cubic liquid crystalline nanoparticles (or cubosomes) systems were formulated and prepared by emulsifying surfactants Brij® 30 and Brij®35 with aqueous solution of poly(ethylene) glycol derivatives and glyceryl monooleate: one formulation containing Monomuls® 90-O18 and Pluronic® PE 10500, another one containing Monomuls® 90-O18 and Brij®35, and the third one based on Pluronic® PE 6800, Brij®30 and Brij®35. Toxicity of formulations was evaluated by measuring the effect of those cubosomes on human keratinocytes (NCTC2544) viability. Cells were seeded at densities of 15,000 and 30,000 cells per well, respectively, into a 24-well tissue culture dish. The cell viability test was performed using 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay by measuring oxidative activity of mitochondrial succinate dehydrogenase enzyme. Effect on cell viability for each formulation was determined as function *v(t, c)* of time (24, 48 and 72 hours) and cubosomes concentrations (0, 5, 10, 20, 25 and 50 μg/mL).

Viability of cells decreased with increasing the concentration of cubosomes at all three measuring times but the results were highly variable and the dependence on concentration was far from being linear. Replacement of analysis of dependence of viability at the three independent times, with analysis of areas under time viability curves ($\mathcal{A}_t\mathcal{V}$) led to a much more statistically significant dependence on concentration. As a general rule, $\mathcal{A}_t\mathcal{V}$ dependence on concentration was linear. Decreasing of the number of cells or increasing of toxicity led to linear biphasic dependence: a rapid initial decrease followed by a slower final decline.

Classification of compounds as toxic and non-toxic was undertaken using the method of classification of plasma levels curves in bioequivalence studies. Based on comparison of time integral of viability ($\mathcal{A}_t\mathcal{V}$) - concentration of cubosomes curves, it was found that first formulation is not toxic; the second is at the frontier between toxic and non-toxic, the third is toxic. From comparison of formulations composition, appeared that toxicity is determined by the content of Brijs.

Rezumat

Au fost formulate şi preparate trei tipuri de nanoparticule cristale lichide cubice (cubozomi) pe bază de lipide prin emulsificarea surfactanţilor Brij® 30 şi Brij® 35 cu soluţii apoase de derivaţi de polietilen glicol şi monooleat de glicerină: o formulare conţinând Monomuls® 90-O18 şi Pluronic® PE 10500, alta conţinând Monomuls® 90-O18 şi Brij®35, şi a treia – pe bază de Pluronic® PE 6800, Brij®30 şi Brij®35. Toxicitatea acestor formulări a fost evaluată prin măsurarea efectelor asupra viabilităţii keratinocitelor (NCTC 2544) umane. Celulele au fost însămânţate la densităţile de 15,000 şi 30,000 celule per godeu, pe o placă de cultură celulară cu 24 de godeuri. Viabilitatea celulelor a fost testată folosind bromură de 3-(4,5-dimetiltiazol-2-il)-2,5-difenil tetrazoliu (MTT) prin măsurarea activităţii oxidative a succinat dehidrogenazei mitocondriale. Efectele fiecărei formulări asupra viabilităţii au fost determinate ca funcţie v(t, c) de timp (24, 48 şi 72 ore) şi concentraţiile de cubozomi (0, 5, 10, 20, 25 şi 50 µg/mL). Viabilitatea celulelor a scăzut odată cu creşterea concentraţiei cubozomilor la toţi cei trei timpi de măsurare, dar rezultatele au fost foarte variabile, iar dependenţa de concentraţie nu a fost liniară. Înlocuirea analizei dependenţei viabilităţii la intervale de timp independente cu analiza ariei de sub curbă viabilitate – timp, a condus la o dependenţă de concentraţie, statistic semnificativă. Ca regulă generală, dependenţa $\mathcal{A}_t\mathcal{V}$ a fost liniară. La un număr de celule mai mic şi la o toxicitate mai mare, s-a ajuns la o dependenţă liniară bifazică: o scădere iniţală rapidă, urmată de o scădere mai lentă în faza a doua. Clasificarea formulărilor ca toxice sau non-toxice a fost efectuată utilizând metode de clasificare a curbelor de concentraţie plasmatică în studiile de bioechivalenţă. Bazat pe compararea integralelor viabilităţii $\mathcal{A}_t\mathcal{V}$ ca funcţie de concentraţie de cubozomi, s-a găsit că prima formulare nu este toxică, a două este la graniţa între domeniul toxic şi non-toxic, şi a treia este non-toxică. Din compararea compoziţiilor formulărilor, s-a concluzionat că toxicitatea este determinată de conţinutul în surfactanţi de tip Brij®.

Keywords: monoglyceride liquid crystalline cubosomes, MTT assay area under viability curve, biphasic linear regression.

Introduction

Supramolecular nanotherapeutics were recently developed as a new frontiers in pharmaceutical science [1]. Based on self-assembling of biocompatible polymers and/or phospholipids, several applications in nanomedicine were found, thus providing an important revolution in therapy [2]. Organic and inorganic materials are generally used to self-assembling nanotherapeutics and to provide suitable size and shape for *in vivo* administration [3]. Some colloidal formulations are applied in therapeutic treatment for cancer and other pathologies [4].

Lipid-based cubic liquid crystalline nanoparticles (or cubosomes) consisting of "honeycombed (cavernous)" structure spontaneously formed when a certain concentration of amphiphilic lipids like glycerol monooleate (GMO) [5] dispersed in aqueous solution has curved bicontinuous lipid bilayer in three dimensions, separating two congruent networks of water channels. Its unique structure consists of internal double water channels and

large interfacial areas, which reveal great flexibility in encapsulation efficiency of various polarities and amount of drugs, and has variegated range of drugs encapsulated [6]. As a drug delivery vehicle, high drug payloads, stabilization of peptides or proteins and simple preparation process are also its advantages. The ability of cubic phase or cubosomes [7] to incorporate and control release of drugs of varying size and polar characteristics, and biodegradability of lipids make it an interesting drug delivery system for various routes of administration, including oral, topical (or mucosal) and intravenous administrations, with extensive application in a multitude of dosage forms [8]. Furthermore, a number of different proteins in cubic phase appear to retain their native conformation and bioactivity, and are protected against chemical and physical inactivation.

Nanotoxicology of colloidal carriers generally depends on materials self-assembling in their supramolecular architecture. Many of these materials are surfactants which are accumulated into membrane interfaces at high percentages [9]. Supramolecular rearrangement of surfactants can affect size and shape of colloidal nanocarriers, thus modifying their conventional structure. In particular, the increase of size can promote biological modifications, e.g. complement cascaded and inflammatory mediators, which can affect platelet and erythrocyte aggregations, thus generating potential thrombosis [10, 11].

In the last decade, toxic effects of liquid crystals were deeply investigated, efforts being focused mainly to investigate environmental impact of liquid crystals used in different technical fields [12-15].

The present paper concerns the formulation and toxicological evaluation of colloidal liquid crystals cubosomes containing monoglycerides and non-ionic surfactants, e.g. Pluronic® PE 6800, Pluronic® PE 10500, Brij® 30 and Brij® 35 proposed as potential supramolecular carriers in drug delivery and evaluation of their toxicity.

Toxic effects were carried out evaluating cell viability on human keratinocytes (NCTC2544) through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as function of exposure time and concentration of liquid crystals resulting surfaces of response. Commonly, either the exposure time is fixed and dose-response relationships are measured, or the concentration was fixed and time-response curves were determined.

Comparison between viability curves in order to classify studied compounds as function of their toxicity led further to problems of metrics on curves. These were defined in analogy with metrics in bioequivalence

studies, based on a model recently proposed [16] for comparison of pain curves. Most utilized metrics in bioequivalence studies, the difference between Areas Under Curves (AUC) was applied to comparison of viability curves in presence of cubosomes with control viability curves.

Materials and Methods

Materials.

Polyethylene glycol-polypropylene glycol-polyethylene glycol block copolymer 6800 (Pluronic® PE 6800), polyethylene glycol block copolymer 10500 (Pluronic® PE 10500), Brij® 30 and Brij® 35, were a kind gift of ACEF Spa (Piacenza, Italy). Glyceryl monooleate (Monomuls® 90-O18) was obtained from Cognis S.p.A. (Fino Mornasco (CO), Italy). Double-distilled pyrogen-free water was purchased from Sifra S.p.A. (Verona, Italy). Isotonic sterile saline solution (NaCl 0.9% w/v) was a product of Fresenius Kabi Potenza S.R.L. (Verona, Italy). Human keratinocytes (NCTC2544 cells) were purchased from Istituto Zooprofilattico della Lombardia e dell'Emilio Romagna (Brescia, Italy). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin solution and Trypsin/EDTA (1×) solution were obtained from GIBCO (Invitrogen Corporation, Giuliano Milanese (Mi), Italy). All other materials and solvents used during experiments were of analytical grade (Carlo Erba, Milan, Italy).

Preparation of cubosomes.

Monoglyceride colloidal liquid crystals were prepared by emulsifying non-ionic surfactants with aqueous solution of poly(ethylene) glycol derivatives herein reported. This procedure allows preparing formulations with different amount of surfactants self-assembling inside supramolecular structure. Briefly, glyceryl monooleate (90 mg) was dissolved in ethanol (2 mL); while Pluronic® PE 6800 or Pluronic® PE 10500 or Brij® 30 or Brij® 35 were dissolved into double-distilled pyrogen-free water NaCl 0.9% w/v (8 mL). Aqueous phase was dropped to ethanol phase under continuous stirring using a Ultraturrax T25 basic homogenizer (IKA® - Werke GmbH & Co. KG, Staufen, Germany) at a mixing speed of 18500 rpm (3 different cycles of 5 min). The procedure was carried out at room temperature. Cubosomes were obtained by incorporating Monomuls® 90-O18 to ternary phase made up from ethanol, monoolein and water, thus affecting final architecture and spatial arrangement of colloidal liquid crystals. Monoglyceride colloidal liquid crystals were maintained at room temperature for 48 h under continuous stirring (400 rpm) to remove traces of ethanol not self-assembling inside supramolecular structure. Unstructured

surfactants were further removed by dialyzing formulations using cellulose membrane dialysis tube with molecular cut-off 50,000 Dalton (Spectra/Por Membranes, Spectrum Laboratories, Inc., CA, USA). Dialysis was performed at room temperature for 48 h using a NaCl 0.9% w/v (200 mL) and under constant stirring (400 rpm). NaCl 0.9% w/v was replaced every 8 h during experiment.

Physicochemical characterization.

Photon correlation spectroscopy was performed to characterize monoglyceride colloidal liquid crystals. The average sizes, narrow size distribution and zeta potential of various formulations were carried out using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worchestershire, United Kingdom) as previously reported [17, 18]. Briefly, samples were suitable diluted with an isotonic aqueous solution previously filtered (pore size 0.22 mm) through polypropylene membranes (Whatman Inc., Clifton, NJ, USA). Sizes and zeta potential disposable cuvette (Malvern Instruments Ltd., Worchestershire, United Kingdom) were used to perform the analysis. The following set up for the instrument was applied during the analysis: 4.5m Wlaser diode operating at 670 nm as a light source; scattered photons detected at 173°. The third-order cumulant fitting autocorrelation function was applied to achieve average sizes and narrow size distribution from scattered photon patterns. The following instrumental parameters were used for the photon correlation analysis: real refractive index 1.59, imaginary refractive index 0.0, medium refractive index 1.330, medium viscosity 1.0 mPa· s and medium dielectric constant 80.4.

The zeta potential of monoglyceride colloidal liquid crystals were carried out using the Doppler laser anemometry and hence the electrophoretic mobility. A Smoluchowsky constant F (Ka) of 1.5 was applied during the analysis. The apparatus is equipped with a He/Ne laser doppler anemometry (633 nm) with a nominal power of 5.0 mW. Experimental data are the average of different experiments (n = 10 runs) ± standard deviation.

Viability of NCTC 2544 cells in presence of monoglyceride colloidal liquid crystals.

Human keratinocyte cells (NCTC 2544) were transferred from cryovial tubes to plastic culture dishes (100 mm diameter) and seeded (5% CO_2) for three days at 37°C using DMEM supplemented with penicillin (100 UI/mL), streptomycin (100 μg/mL), amphotericin B solution (1% v/v) and FBS solution (10% v/v). When NCTC 2544 cells had a confluence of 80%, cells were treated with trypsin-EDTA solution, washed with phosphate-buffered saline (PBS) and finally transferred into centrifuge

tubes. The obtained samples were centrifuged using a Megafuge 1.0 (Heraeus Sepatech) at 800×g for 10 min at 4°C. Supernatant was poured off and pellets dissolved into cell culture medium to obtain a final concentration of 3×10^4 cells/mL. NCTC 2544 cells (8×10^3 cells/cm^2) were seeded into 9 well plastic culture dishes before *in vitro* experiments.

After 24 h, cells were treated using different concentrations of supramolecular carriers (5, 10, 25 and 50 μg/mL). Untreated cells were used as control and blank, respectively. Different time points (24, 48 and 72 h) were evaluated during the experiment.

The cell viability was assessed based on the conversion of pale yellow MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to purple formazan crystals. MTT solution (5 mg/mL in PBS buffer) (10 μL) was added to each well and incubated for 3 h at 37°C. After 3 h the medium was removed and replaced with ethanol and DMSO (1:1 v/v) solution (100 μL) to dissolve crystal formazan that had precipitated inside the cells. Plates were then gently shaken at 230 rpm (IKA® KS 130 Control, IKA® WERKE GMBH & Co, Staufen, Germany) for 20 min. The sample absorbance was measured using an ELISA microplate reader (Labsystems mod. Multiskan MS, Midland, ON, Canada) at a wavelength of 570 nm. The percentage of viable cells was measured using the following equation:

$$\text{Cell viability (\%)} = [\text{AbsT}/\text{AbsC}] \times 100,$$

where AbsT is the absorbance of the treated cells and AbsC is the absorbance of control (untreated) cells.

Results and Discussion

The monoglyceride colloidal liquid crystals were obtained by combining of two or three components. Combinations with more than three components were avoided following a multitude of possible states and multiphase separations. Formulations which demonstrated the best physicochemical features, selected to be tested *in vitro* for their effect on cells viability are presented in Table I.

Table I

Chemical composition of prepared cubosomes

	Monomuls® 90-O18	**Pluronic® PE 6800**	**Pluronic® PE 10500**	**Brij® 30**	**Brij® 35**
M - PL 10500	90 mg	–	50 mg	–	–
M - BJ35	90 mg	–	–	–	50 mg
PL - BJ30 & BJ35	–	50 mg	–	45 mg	45 mg

The average sizes (below 170 nm), narrow size distribution or polidispersity index or PDI (below 0.2), the zeta potential value (below −30 mV) (Table II) and surfactants used to make monoglyceride colloidal liquid crystals provided suitable physicochemical features for the potential administration of various formulations both *in vitro* and *in vivo* to treat different pathologies. Furthermore, these features seem to suggest a potential use of monoglyceride colloidal liquid crystals as supramolecular nanotherapeutics as previously reported for therapeutic liposomes [19-21].

Table II

Physicochemical characterization of monoglyceride colloidal liquid crystals

Formulations	Sizes (nm)	PDI[1]	Zeta Potential (mV)	EM[2] (μm × cm/Vs)
ML - 10500	165.3 ± 2.0	0.190 ± 0.05	-29.21 ± 2.0	-2.20 ± 0.6
M - BJ 35	160.2 ± 1.0	0.181 ± 0.06	-28.41 ± 2.5	-2.25 ± 0.2
M - BJ30 & BJ35	157.3 ± 2.4	0.179 ± 0.08	-27.25 ± 2.3	-2.04 ± 0.7

[1]Polidispersity Index, [2]Electrophoretic Mobility

Control time-viability curves.

Time course of viability in case of 30,000 cells control, is presented in figure 1. The curves were very similar, homogeneously distributed in space. Coefficient of variation of the set of four replicates, even in case of 30,000 cells was low: 6.7 % at 24 h, 2.4 % at 48 h and 6.2 % at 72 h.

Evaluation of time evolution put in evidence that their viability curves, for three days time interval, are rather increasing functions than "survival curves" (Figure 1). This is in agreement with reported results for longer periods by other authors [22, 23]. Viability of cells decreased from 24 to 48 h, remaining approximately constant from 48 h to 72 h.

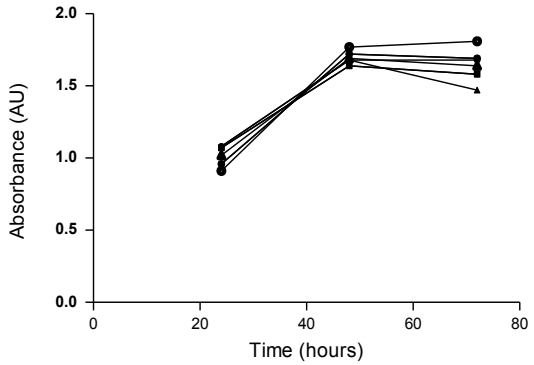

Figure 1

Five replicates control viability curves (culture of 30,000 cells/well)

As a rule, the results depended on the number of cells, variability being greater in case of 30,000 cells/well than in case of 15,000 cells/well.

Effect on viability of formulation M-PL10500.

A global inspection of sets of curves in case of 30,000/well reveals that viability, estimated at 24, 48 and 72 h, generally decreased with increasing of the concentration of cubosomes at 24 h, oscilated at 48 h and was rather constant at 72h (Figure 2).

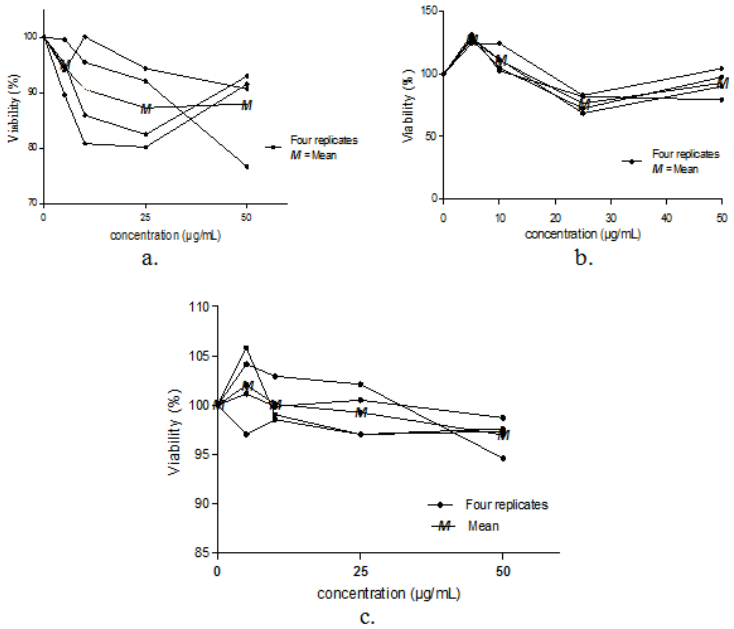

Figure 2

Dependence of viability of keratinocytes (30,000 cells/well) on the concentration of M-PL10500 formulation: a. 24h; b. 48 h and c.72h

It is to note that variability was much higher than in case of control curves, as can be seen in Table III.

Table III

Coefficients of variation (%) of the set of replicates viabilities culture of 30,000 cells/well, M-PL

Contact time/concentration of cubosomes	**Control**	**5 µg/mL**	**10 µg/mL**	**25 µg/mL**	**50 µg/mL**
24h	6.7	4.4	9.7	8.0	8.6
48h	2.4	2.3	8.9	9.2	11.5
72h	6.2	3.8	**2.0**	2.5	8.6

As can be seen in Figure 3, mean curves didn't change the conclusion drawn after evaluation with the necked eye of the clusters of

replicate curves. Dependence of cells viability on PL concentration was rather confused.

Increasing of viability at 48 h, at 10 mg/mL in comparison with control viability at 24 h was obtained in all four replicates during the experiment and is difficult to explain. On the other hand, it is to note that in case of control curves the viability increased at 48 h in comparison with viability at 24h (Figure 3).

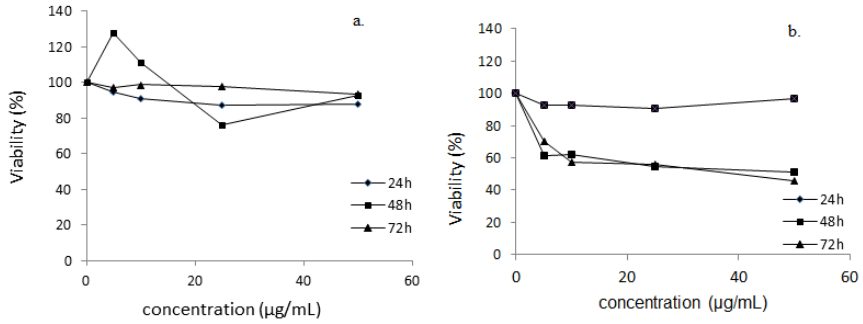

Figure 3

Mean viability curves of keratinocytes in presence of M- PL 10,500: a. 30,000 cells/well; b. 15,000 cells/well

Following the high registered variability induced by cubosomes, it was looked at another, supplementary endpoint to characterize their effect on viability. The most utilized endpoint in case of plasma levels in pharmacokinetics, used for evaluation of *in vitro – in vivo* correlations [24, 25], for predicting safety and efficacy of drugs starting from physiological models [26, 27] is the Area Under Curve (AUC). The most frequently used method for the calculation of AUC, is the trapezoid rule. Area under curve defined by a function *f(t)* in the time interval [0, T], if there are known the values of function in points

$$(t_i)_{i=1,n},$$

is approximated by the sum of areas of trapesis determined by segments

$$[0.f(t_{i-1})];\ [0.f(t_i)];\ [t_{i-1},t_i];\ [f(t_{i-1}).f(t_i)]$$

$$AUVC_o^T = \sum_{i=1}^{n} \frac{f(t_{i-1}) + f(t_i)}{2} * (t_i - t_{i-1})$$

In case of pharmacokinetics function *f(t)* becomes concentration *c(t)* and in our case was considered percent of viability in comparison with control curve and area calculated by time integration was denoted $\mathcal{A}_t\mathcal{V}(c)$.

Viability dependence on cubosome concentration in case of culture with lower number of cells, as can be seen in Figure 4 was a continuous decreasing.

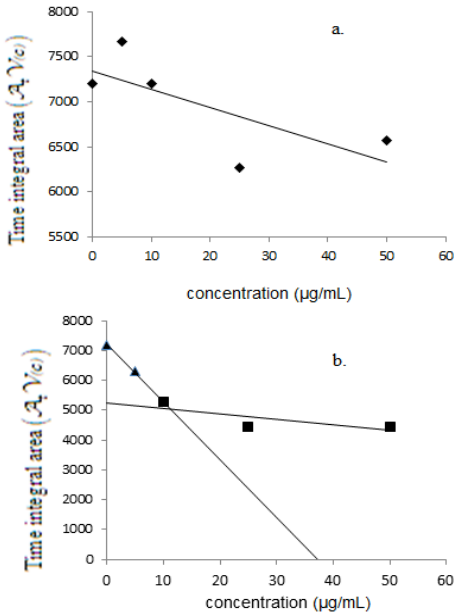

Figure 4

Time integral areas $\mathcal{A}_t\mathcal{V}(c)$ of viability curves as function of M- PL 10,500 concentration: a. 30,000 cells/well; b. 15,000 cells/well

On the contrary, in case of effect on 15.000 cells/well culture, it was obtained a clear, expected result: an increasing of toxicity measured by decrease of $\mathcal{A}_t\mathcal{V}(c)$ with the increasing of concentration. Dependence was biphasic linear: in the intervals 0 - 10 µg/mL and 10 – 25 µg/mL. The slope of the line of the toxicity was much greater in the first concentration interval (Figure 4).

Formulation M – BJ 35.

As can be seen in Figure 5 toxicity increased with concentration. $\mathcal{A}_t\mathcal{V}(c)$ was approximately linear dependent of concentration ($R^2 = 0.86$) in case of 30,000 cells/well. Concerning the case of 15,000 cells/well dependence between $\mathcal{A}_t\mathcal{V}(c)$ and concentration was linear biphasic. It is to note that behaviour, at least qualitatively, is similar to the case of M-PL10500 formulation.

Formulation PL-BJ 30& BJ35.

Addition of a new quantity of Brijs led to an increase of toxicity. Regression line had a greater slope and correlation coefficient was significantly higher than in case of the other two formulations. It concerns the effect on the two types of cell cultures, the behaviour was practically the

same as in the case of PL-BJ 35 formulation: $\mathcal{A}_t\mathcal{V}(c)$ – concentration dependence was linear in case of high number of cells and biphasic linear in the case of poorer culture (Figure 5).

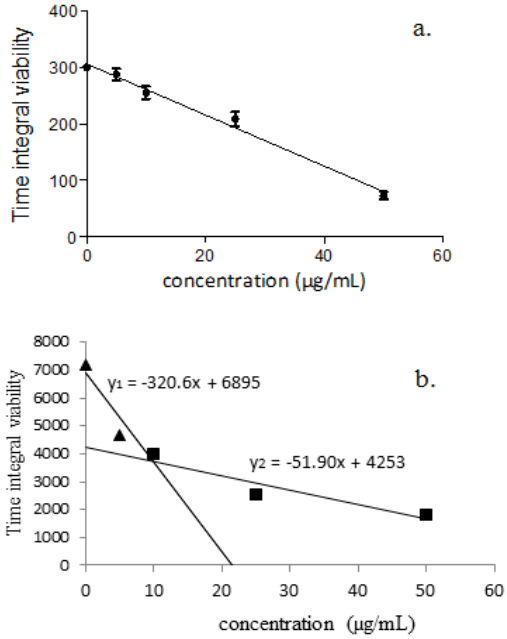

Figure 5

Dependence of viability on concentration of PL-BJ30 &BJ35 formulation: a. 30,000 cells/well; b.15,000 cells/well

Intercomparison of effects on viability of the three formulations.

Dependence of the time integrals of viability on the concentrations of cubosomes is presented in Figure 6.

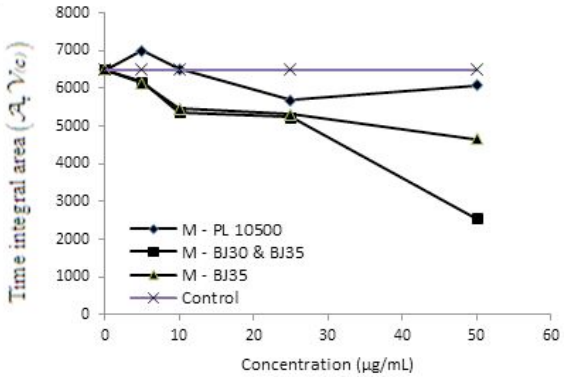

Figure 6

Dependence of the $\mathcal{A}_t\mathcal{V}(c)$ on the concentrations of cubosomes

For analysing the viability curves, the methods for comparison of plasma levels of drugs in bioequivalence studies were applied. A first type of metrics in biopharmaceutical studies compares a reference drug *R* and a tested one *T*, being based on differences between matched points. In particular, it can be considered a single point, for example the maximum concentration

$$d(R,T) = \left| C_{\max}^{R} - C_{\max}^{T} \right|,$$

or the sum of differences

$$d\left(R,T\right) = \sum \left| c_R\left(t_i\right) - c_T\left(t_i\right) \right|,$$

where $c(t_i)$ is the concentration of drug in blood at time t_i.

In case of continuous curves defined in the time interval [0, τ], the metric becomes difference between Areas Under plasma level Curves ($AUC_{0-\tau}$) measured by integration.

$$d_{AUC_{0-\tau}}\left(R,T\right) = \int_0^{\tau} \left| c_R\left(t\right) - c_T\left(t\right) \right| dt = \left| \int_0^{\tau} c_R\left(t\right) dt - \int_0^{\tau} c_T\left(t\right) dt \right| = \left| AUC_{0-\tau}^{R} - AUC_{0-\tau}^{T} \right|,$$

which becomes when we compare the effect on cell viability of two formulations (F1 and F2):

$$d_{\mathcal{A}_t \mathcal{V}}\left(\mathrm{F1},\mathrm{F2}\right) = \int_0^{\tau} \left| \mathcal{V}_{F1}\left(t\right) - \mathcal{V}_{F2}\left(t\right) \right| dt = \left| \int_0^{\tau} \mathcal{V}_{F1}\left(t\right) dt - \int_0^{\tau} \mathcal{V}_{F2}\left(t\right) dt \right| = \left| \mathcal{A}_t \mathcal{V}_{0-\tau}^{F1} - \mathcal{A}_t \mathcal{V}_{0-\tau}^{F2} \right|,$$

As further development, similar with drug classification as bioequivalent and non-bioequivalent, could be compared toxicity of two formulations or of the same formulations at different concentrations. In case of comparison of sets of plasma level curves or effect curves [16, 28] or toxicity curves, it is possible to extend the rules for calculating average bioequivalence of drug formulations. For example we can say that two formulations are toxic equivalent if:

$$\mathcal{P}(0.8 < \frac{\mu_{\mathcal{A}_t \mathcal{V}}^{F1}}{\mu_{\mathcal{A}_t \mathcal{V}}^{F2}} < 1.25) \geq 0.90,$$

where $\mu_{\mathcal{A}_t \mathcal{V}}^{F1}$ and $\mu_{\mathcal{A}_t \mathcal{V}}^{F2}$ are the true means of the concentration integral toxicity for the compared formulations. In case when viability is measured at a single time, metric reduces to comparison of areas under curves.

Conclusions

Cell viability decreased with increasing concentration of cubosomes at all three measuring times (24, 48 and 72 hours) but the results were

highly variable and the concentration dependence didn't follow a well-defined model.

Replacement of analysis of dependence of viabilities at the three independent times with analysis of areas under time viability curves.

($\mathcal{A}_t\mathcal{V}$) led to a significant decrease of variability and a more smooth dependence on concentration. As a general rule, dependence on concentration was linear. Decreasing of the number of cells as well as increasing of toxicity led to linear biphasic dependence: a more rapid initial decrease followed by a slower final decline.

Comparative examination of dependence of mean $\mathcal{A}_t\mathcal{V}$ on concentration of tested cubosomes leads to the conclusion that all formulations have not a significant toxicity. Results put in evidence the fact that toxicity depends on the composition and concentration of cubosomes as well as incubation time. In appeared that formulation without Brijs is not toxic and successive addition of Brijs surfactants increased the toxicity.

References

1. Nanotherapeutics. Drug delivery concepts in nanoscience. Alf Lamprecht (Edt.). Pan Stanford Publishing Pte. Ltd. 2009.
2. Venditto V.J., Szoka F.C.Jr., Cancer nanomedicines: so many papers and so few drugs! *Adv. Drug. Deliv. Rev*., 2013; 65(1): 80-88.
3. Adriani G., de Tullio M.D., Ferrari M., Hussain F., Pascazio G., Liu X., Decuzzi P., The preferential targeting of the diseased microvasculature by disk-like particles. *Biomaterials*, 2012 Aug; 3(22): 5504-5513.
4. Di Paolo A., Bocci G., Drug distribution in tumors: mechanisms, role in drug resistance, and methods for modification. *Curr. Oncol. Rep*., 2007; 9: 109-114.
5. Faivre V., Rosilio V., Interest of glycolipids in drug delivery: from physicochemical properties to drug targeting. *Expert Opin. Drug. Deliv*., 2010; 7(9): 1031-1048.
6. Gan L., Han S., Shen J., Zhu J., Zhu C., Zhang X., Gan Y., Self-assembled liquid crystalline nanoparticles as a novel ophthalmic delivery system for dexamethasone: Improving preocular retention and ocular bioavailability. *Int. J. Pharm*., 2010; 396(1-2): 179-187.
7. Pan X., Han K., Peng X., Yang Z., Qin L., Zhu C., Huang X., Shi X., Dian L., Lu M., Wu C., Nanostructured cubosomes as advanced drug delivery system. *Curr. Pharm. Des*., 2013; 19(35): 6290-6297.
8. Gunjan Verma and P. A. Hassan., Self assembled materials: design strategies and drug delivery perspectives. *Phys. Chem. Chem. Phys*., 2013; 15: 17016-17028.
9. Voicu V, Mircioiu C., Mecanisme farmacologice la interfete membranare. Ed. Academiei Romane, Bucuresti, 1994.
10. Voicu V.A., Mircioiu C., Jiquidi M., Gref R., Olteanu M., Studies concerning some effects of drugs, colloid vectors for drugs and decorporators on some physicochemical parameters of blood. in Sohns T.,Voicu V. (edts): NBC Risks. Current Capabilities and Future Perspectives for Protection, Kluwer Academic, Amsterdam, 1999; 311-330.
11. Olteanu M., Cinteza O., Dudău M., Mircioiu C., Investigation on the embedding of decorporators in microemulsions, in Sohns T., Voicu V. (edts): NBC Risks. Current Capabilities and Future Perspectives for Protection, Kluwer Academic, Amsterdam. 1999; 389-400.
12. Simon-Hettich B., Becker W., "Toxicological Investigations of Liquid Crystals", 28th Freiburger Arbeitstagung "Flüssigkristalle", Freiburg March 24 – 26, 1999.

13. Takatsu H., "Investigation Activity and Data on Safety of Liquid Crystal Materials", 18th International Liquid Crystal Conference, Sendai, Japan. July 24 – 28, 2000.
14. Simon-Hettich B., Broschard T. H., Becker W. Exotoxicological properties of liquid-crystal compounds, *Journal of the SID* 2001; 9/4: 307 – 312.
15. Becker W., Liquid Crystals in Liquid Crystal Displays (LCDs), Basics, Safety and End-of-Life Aspects, Take-Back Conference in Prague, Czech Republic, available at www.e-bookspdf.org
16. Mircioiu C., Borisova S.A., Voicu V.A., Biopharmaceutic Metrics Applied in Comparison of Clusters of time Courses of Effect in Clinical Trials. *Journal of Applied Biopharmaceutics and Pharmacokinetics*, 2013; 1(1): 37- 44.
17. Paolino D., Licciardi M., Celia C., Giammona G., Fresta M., Cavallaro G., Folate-targeted supramolecular vesicular aggregates as a new frontier for effective anticancer treatment in *in vivo* model. *Eur. J. Pharm. Biopharm*., 2012; 82(1): 94-102.
18. Licciardi M., Paolino D., Celia C., Giammona G., Cavallaro G., Fresta M., Folate-targeted supramolecular vesicular aggregates based on polyaspartyl-hydrazide copolymers for the selective delivery of antitumoral drugs. *Biomaterials*, 2010; 31(28): 7340-7354.
19. Paolino D., Cosco D., Racanicchi L., Trapasso E., Celia C., Iannone M., Puxeddu E., Costante G., Filetti S., Russo D., Fresta M., Gemcitabine-loaded PEGylated unilamellar liposomes vs GEMZAR: biodistribution, pharmacokinetic features and *in vivo* antitumor activity. *J. Control Release*., 2010; 144(2): 144-150.
20. Cosco D., Bulotta A., Ventura M., Celia C., Calimeri T., Perri G., Paolino D., Costa N., Neri P., Tagliaferri P., Tassone P., Fresta M., *In vivo* activity of gemcitabine-loaded PEGylated small unilamellar liposomes against pancreatic cancer. *Cancer Chemother. Pharmacol*., 2009; 64(5): 1009-1020.
21. Celia C., Malara N., Terracciano R., Cosco D., Paolino D., Fresta M., Savino R., Liposomal delivery improves the growth-inhibitory and apoptotic activity of low doses of gemcitabine in multiple myeloma cancer cells. *Nanomedicine*, 2008; 4(2): 155-166.
22. Kirker K.R., Secor P.R., Garth A.J., Fleckman P., Olerud J.E., Stewart P.S., Loss of viability and induction of apoptosis in human keratinocytes exposed to *Staphylococcus aureus* biofilms *in vitro*. *Wound Repair Regen*., 2009; 17(5): 690–699.
23. Majid F.A.A., Basic *in vitro* studies of human skin culture: melastoma malabathricum extract on fibroblasts growth. Department of Bioprocess Engineering Faculty of Chemical Engineering and Natural Resources Engineering University Technology of Malaysia, research vote No: 75127. 2007.
24. Mircioiu C., Mircioiu I., Voicu V., Miron D., Dissolution-bioequivalence non-correlations. *Basic Clin. Pharmacol. Toxicol*., 2005 Mar; 96(3): 262-264.
25. Mircioiu C., Voicu V., Miron D., Mircioiu I., Non-standard correlations: *in vitro in vivo* correlations for immediate release products: comparison of different bioequivalence experiments. *Basic. Clin. Pharmacol. Toxicol*., 2005 Mar; 96(3): 265-267.
26. Tvrdonova M., Chrenova J., Rausova Z., Miklovicova D., Durisova M., Mircioiu C., Dedik L., Novel approach to bioequivalence assessment on based physiologically motivated model. *Int. J. Pharm*., 2009 Oct 1; 380(1-2): 89-95.
27. Mircioiu C., Ionica G., Danilceac A., Miron D., Mircioiu I., Radulescu F., Pharmacokinetic and mathematical outliers for drugs with active metabolites. Model independent analyses for pentoxifylline. *Farmacia*, 2009; 58(3): 264-278.
28. Borisova S., Mormenschi D., Sarbu I., Mircioiu C., New methods for comparison of erythrocyte sedimentation curves in estimationof aggregant and antiaggregant effects of grugs. *Therapeutics, Pharmacology & Clinical Toxicology*, 2012; 16(3): 190-196.

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