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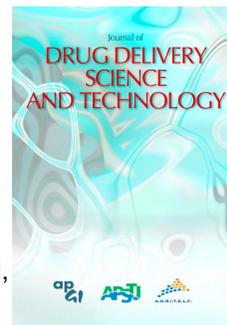
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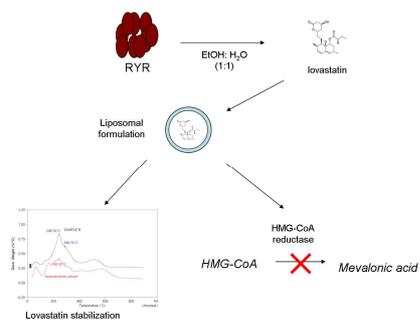
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Development of liposomal formulations to potentiate natural lovastatin inhibitory activity towards 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase

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Abstract

Liposomal formulations were obtained mixing 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) with synthetic lovastatin or lovastatin extracted from Red Yeast Rice (RYR) to prepare a vehicle able to overcome both the disadvantage of lovastatin, i.e its poor oral bioavailability and side effects. Liposomal formulation obtained combining DOPC, DOPE and hydro-alcoholic extract of RYR showed optimal physico-chemical, mechanical and thermal characteristics and the strongest inhibition activity versus 3-hydroxy-3-Methyl glutaryl coenzyme A (HMG-CoA) reductase.

Keywords: Liposomes; Lovastatin; Red Yeast Rice; HMG-CoA

1. Introduction

3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase is a key enzyme in cholesterol synthesis. Statins are inhibitors of cholesterol and are considered first-choice therapy for controlling dyslipidaemia reducing levels of low-density lipoproteins (LDL) [1]. Lovastatin can be considered as one of the most well tolerated statins, however, it shows some disadvantages, i.e. poor oral bioavailability (lower than 5%) because of its rapid metabolism in the gut and liver [2], and some side effects, as myalgia. Several polymeric formulations have been tested in order to increase its oral absorption [3,4]. However, the best strategy to circumvent the solubility limit consists in loading insoluble drugs into water soluble carriers able also to offer chemical and biological protection. Liposomes are biocompatible carriers which represent the first choice vehicle to enhance hydrophobic drug activity. Moreover, liposomes enable slow release at the target site over prolonged periods of time [5]. We developed a liposomal formulation for lovastatin and evaluated the effect of lipidic vesicles on lovastatin inhibitory activity. Moreover, to overcome statins side-effect, lovastatin from Red Yeast Rice (RYR) was also tested. RYR is a natural food, produced by fermenting the *Monascus Purpureus* fungus on steamed rice and it is well known that lovastatin by RYR is more effective in lowering cholesterol than the synthetic one (i.e. 5–6 mg/day of natural lovastatin has a comparable efficacy of 20–40 mg/day of synthetic lovastatin [1]) with a better side-effect profile as regards myalgia, and lack of drug-drug interactions [6]. The aim of this paper is the evaluation of effectiveness of liposomes on synthetic and RYR lovastatin inhibitory activity against HMG-CoA- reductase.

2. Materials and methods

2.1 Materials

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC -purity 99%) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE - purity 99%) were purchased from Avanti Polar Lipids, Inc., Alabaster (US) and used without further purification. All chemicals, solvents and HMG-CoA reductase assay kit were purchased from Sigma Aldrich. RYR was kindly provided by MediBase s.r.l. (Prato, Italy).

2.2 Methods

2.2.1 Lovastatin extraction and quantification

Lovastatin was extracted from RYR using two different solvents. 1 g of RYR was suspended in 10 mL of ethanol (sample A) and 10 mL of ethanol:H₂O 1:1 (sample B) by a vortex for 10 min. Then, it was subjected to agitation by an oscillation plate (15h) and magnetic stirrer (6h). Samples were centrifuged (2650 g for 3 min). The liquid phases were used to obtain liposomal formulations after quantifying lovastatin amount.

Lovastatin was quantified via HPLC-UV method already reported [3] using a R.P-C18 column and a mixture of acetonitrile, water and methanol (5:3:1 ratio v/v) as the mobile phase (flow rate: 1.0 mL/min; injection volume: 20 μ L), detector 230 nm. All the measurements were carried out in triplicate.

2.2.2 Liposomes preparation

Appropriate amounts of DOPC and DOPE stock solution (4×10^{-2} M in CHCl₃) and alcoholic and hydro-alcoholic RYR extracts were mixed, dried and kept under vacuum overnight at 30°C, to remove traces of the organic solvent. The dried films were dissolved in H₂O, vortexed and multilamellar vesicles obtained. Subsequent treatment with nine freeze/thaw cycles and 27 extrusion passages through polycarbonate filters with 200 nm pore diameter allowed obtaining unilamellar vesicles of restricted size distribution [5]. To determine the exact total content of lovastatin, 100 μ L of liposomal suspension were mixed with 50 μ L of methanol to break down the liposomal structure. Then, 300 μ L of acetonitrile were added dropwise to precipitate the lipids. The solution was centrifuged (1490 g) for 15 min. The liquid phase was analyzed by HPLC-UV following the procedure previously reported [3].

2.2.3 DLS and ζ -potential measurements

DLS and ζ -potential measurements were performed on a Zetasizer Instrument Nano ZS 90, light source He-Ne laser 633 nm, Max 4 mW, (Malvern Instrument Ltd, UK). The liposomes were allowed to equilibrate for 2 min at 25 °C, and three measurements were performed for each sample.

The data were analyzed with the instrument software to generate the intensity-based size distribution and polydispersity index (PDI), and averaged based on the three measurements. ζ -potential was calculated from the electrophoretic mobility by means of the Helmholtz – Smoluchowski relation.

2.2.4 Rheological measurements

Flow curves and frequency-sweep experiments were performed using a AR2000 Rheometer (TA-Instruments, US) with a cone-plate measuring device of 40 mm diameter, employing a gap size of 0.1 mm. The temperature was maintained at 37°C. The dynamic viscosity η under a controlled shear rate ranging from 1 to 100 s⁻¹ and back from 100 to 1 s⁻¹ was measured. Once the linear viscoelastic region was identified by performing a strain sweep test, the elastic modulus G' , the viscous modulus G'' were determined by a dynamic time sweep test ($\omega = 10$ Hz; strain = 10%). All the measurements were carried out in triplicate [7,8].

2.2.5 Thermal Analysis

Thermogravimetric analysis (TGA) was performed using a Q600 thermogravimetric analyzer (TA Instruments). Liposomal formulations (10 mg) were inserted in a platinum crucible and heated from room temperature to 600 °C, with a rate of 10 °C/min, under nitrogen purge gas.

2.2.6 HMG-CoA reductase inhibition test

The in vitro inhibitory effect of liposomal formulations against HMG-CoA was determined. Samples were prepared as previously reported [3] 100 μ L of each sample were added to the assay kit. The rate of NADPH oxidation by HMG-CoA was monitored every 15 s at 340 nm for a period of 5min using a Ultraspec 200 UV (Biotech, USA) following the provided protocol.

2.2.7 Statistical Analysis

Multiple comparisons were performed by one-way ANOVA and individual differences tested by Fisher's test after the demonstration of significant intergroup differences by ANOVA. Differences with $p < 0.05$ were considered significant [9].

3. Results and discussion

3.1 Liposomes preparation

Several approaches have been tested to improve bioavailability of drugs with a low aqueous solubility and intestinal permeation, such as lovastatin. The most promising route to enhance the bioavailability of such a drug is the use of liposomes, or lipid nanovesicles since lovastatin is a lipophilic compound ($\log P = 3.91$) [10]. Similarly to synthetic lovastatin also lovastatin from RYR must be uploaded into a suitable carrier. In fact, RYR cannot be administered as it is or as a tablet, if a prolonged or delayed release must be achieved. Previous studies showed that lovastatin is quickly released from bare RYR, i.e. up to 42% of the total lovastatin amount was released within the very first hour and it increased to 54% (3h), 67% (5h) and 83% after 7h, reaching 100% after 24h [3].

Phospholipids like 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) are known for their propensity to form lamellar phases, as in biological membranes. Lipids such as 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), on the other hand, form non lamellar phases which are not common in biology. However, mixed phospholipid systems constitute biomembrane models that have stable lamellar structure, and moreover, phase changes between lamellar and non lamellar phases can influence certain major membrane functions, such as modulation of the action of membrane proteins [11].

The phospholipids DOPC/DOPE were judged suitable precisely for their capacity to construct very fluid liposomes, with some loss of stability as well as capacity to retain the drug. In fact, membrane fluidity is a major factor affecting encapsulation efficiency of the drug [12] and unsaturated phospholipids increase membrane fluidity, which is expected to facilitate drug leakage [13]. This physicochemical property was exploited to favour insertion of both synthetic and natural lovastatin. The melting temperature (T_m) of the liquid crystalline phase transition can be used to know the thermodynamics of the lipid bilayer system of interest as a function of their composition. In particular, the T_m values for DOPC and DOPE (-17°C and -16°C , respectively) permit to prepare easily the fluid liposome. In fact, for processing (hydration and formulation) of phospholipids, the

temperature of the medium should be above the gel-liquid crystal transition temperature (T_m). Starting from DOPC and DOPE four liposomal formulations were obtained (Table 1). A significant difference was found according to the extraction solvent. Starting from 1 g of RYR, which corresponded to 50 mg of lovastatin, absolute ethanol extracted up to 35.4 mg of lovastatin (sample A) compared with 20.6mg of lovastatin extracted by the hydro-alcoholic solvent (sample B). Sample C was obtained uploading an intermediate amount of synthetic lovastatin (28 mg) using absolute ethanol as solvent. Despite the higher amount of extracted lovastatin using absolute ethanol, the highest amount of encapsulated lovastatin was obtained in sample B which was 6.4 mg, showing an encapsulating efficiency of about 31%, against 9% found for sample A and 14% for sample C. The higher amount of lovastatin uploaded using a hydro-alcoholic solvent can be explained considering that RYR contains several monacolins and their acidic forms with different solubility degree and affinity towards phospholipides, i.e. monacolins J, K (lovastatin), L, M and X and dihydromonacolin K [14]. However, among these, monacolin K and its dihydro-derivatives are the most active compounds [15].

3.2 DLS and ζ -potential measurements

The mean size of liposomal formulations significantly increased after encapsulation of the bioactive principle, as commonly observed in other liposomal formulations reported in literature [2,5,16-17]. The mean size of all the loaded liposomes was between 150 nm and 450 nm. As reported in table 1, the largest dimensions were found for sample B (450 ± 23 nm) whereas samples A and C showed a diameter of 155 ± 17 nm and 263 ± 15 nm, respectively. The larger diameter of sample C in comparison with sample A can be due to the higher amount of encapsulated lovastatin in the former (3.9 ± 0.1 mg vs 3.2 ± 0.1 mg). The significant difference found for sample B can be easily attributed to the presence of hydrophilic compounds inside the liposomal formulation due to the use of an hydro-alcoholic solvent. All liposomes were not perfectly monodispersed and showed similar polydispersity index (PDI). As reported in literature, when PDI increases a loss of stability of the system can be conjectured as a consequence of adding the drug [2]. In the developed

formulations all the loaded formulations showed an increased PDI. However, a significant difference was observed among the different samples. In particular, PDI increased only from 0.30 to 0.35 for sample B whereas the use of alcoholic solvent to load lovastatin (i.e. samples A and C) provoked a strongest effect, increasing the PDI from 0.30 (sample D, plain liposomes) to 0.47 for sample C and to 0.40 for sample A.

Liposomes did not change their surface charge as indicated by ζ -potential values when loaded. This demonstrated that the uploaded lovastatin did not affect their chemical structure. However, a significant difference ($p < 0.05$) was found for ζ potential. In fact, the presence of bioactive substances drastically reduced the ζ potential value. It reached, for all the loaded liposomal formulations, the critical value of -30mV , which can be considered as a threshold stability value. When ζ potential is higher than 30mV or lower than -30mV the analyzed system do not flocculate or aggregate [16]. The presence of the drug inside the liposomes increased the polydispersity index but also their stability as shown by the ζ potential value.

3.3 Rheological measurements

Several studies showed that the microstructure of lipidic formulation deeply affected the drug permeation. Incorporating a drug into liposomes can significantly alter their microstructure and rheology provides fundamental insights into microstructure of colloidal formulations [18].

Moreover, rheological measurements permitted to know the structural integrity of a sample by determining its structural response to deformation at longer and shorter oscillatory frequency, thus giving information about its workability. Oscillatory measurements, i.e. frequency sweep test permitted to determine the elastic modulus (G') and viscous modulus (G'') which describe the visco-elastic properties of a system. A significant difference was found among the liposomal formulations in terms of rheological parameters G' and G'' (Table 2). In particular, sample D, bare liposomal formulation, showed $G'' > G'$, or very high viscous character which was reflected in its incapability to recover after the removal of the stress. The observed behaviour was in accordance

with ζ potential value (i.e. -18mV) which is out of the threshold stability value. Accordingly with ζ potential values found for loaded liposomal formulations, the presence of bioactive substances increased their stability as evidenced by $G' > G''$. Sample B showed the highest difference between G' and G'' , i.e. about one order of magnitude. Measuring G' and G'' after the viscosity analysis, only sample B was able to maintain its original values of both G' and G'' (Table 2)

Viscosity of the systems was determined to evaluate flow behaviour of liposomal formulations and the effect of loaded lovastatin. The pattern of formulations viscosity vs shear rate was reported in figure 1. Sample D (plain liposomes), sample C (liposomes loaded with synthetic lovastatin using alcoholic solvent) and sample A (liposomes loaded with natural lovastatin extracted from RYR using alcoholic solvent) exhibited shear thinning property, since their viscosity decreased as increasing shear rate. Increasing shear rate from 1 s^{-1} to 100 s^{-1} their viscosity decreased from $6.2 \cdot 10^{-2} \text{ Pa.s}$ to $1.7 \cdot 10^{-3} \text{ Pa.s}$, from $2.4 \cdot 10^{-3} \text{ Pa.s}$ to $1.6 \cdot 10^{-3} \text{ Pa.s}$ and from $1.4 \cdot 10^{-3} \text{ Pa.s}$ to $9.9 \cdot 10^{-4} \text{ Pa.s}$, respectively. On the contrary, liposomal formulation (sample B) loaded with lovastatin extracted from RYR using hydro-alcoholic solvent showed a Newtonian behaviour with a viscosity not dependent on shear rate ($1.5 \cdot 10^{-3} \text{ Pa.s}$) assuming a behaviour close to that of water (0.001 Pa.s) (Figure 1) [19].

3.4 Thermal Analysis

Thermal analysis permitted to evaluate both the thermal stability of the formulations and the effect of their composition and dimensions on liposomal structure. The weight loss in 30-120°C, related to free water, was similar for all the loaded formulations and slightly higher than bare formulation according to the use of the alcoholic solvent. Some differences were found in other temperature ranges. In particular, a significant difference was found in 120-200°C range, comparing sample B and C with sample A. The use of RYR as source for lovastatin increased the amount of bound water. However, all the preparations showed the main loss in the 200-400°C where sample B showed the lowest loss (58%). On the contrary, on the following range it showed the highest loss. This evidence can be used to further confirm its superior stability as shown by R, defined as the

ratio between the weight loss in 400-600°C and in 200-400°C. The higher R the higher the stability of the system. From a qualitative point of view, as shown in figure 2, thermographs of sample D and C were superimposable, thus suggesting that lovastatin did not affect the liposomal structure. On the contrary, sample A and B thermographs were very similar. The different behaviour cannot be ascribed to lovastatin itself but to different substances which derived by RYR since in sample C a higher amount of lovastatin was detected in comparison with sample A. This was confirmed analyzing the temperatures at which the main losses were observed. In particular, the band centred at 229°C (sample C and D) shifted to 237°C (sample A) and 245°C for sample B. The higher the temperature at which the main loss is detected the higher the stability of the system. Bare lovastatin showed this band at 215°C.

3.5 HMG-CoA reductase inhibition test

Previous studies showed that both components of the developed formulation, i.e. the zwitterionic DOPC/DOPE liposomes as well as RYR were well tolerated by different cell lines [3,5, 20].

As regards as the inhibitory activity towards HMG-CoA reductase the use of a lipidic vehicle for lovastatin did not alter its activity. On the contrary, driving lovastatin with a liposomal formulation drastically enhanced its inhibiting activity. It was sufficient a very low amount of sample C to drastically inhibit the enzyme in comparison with free lovastatin. Moreover, a significant difference was observed using lovastatin by RYR.

A reduction of about 40% of the active dose was observed using sample A in comparison with sample C. Sample B showed the strongest inhibition activity thanks to the highest amount of loaded lovastatin (Table 3).

Conclusion

Liposomes were an efficient vehicle for lovastatin. Lovastatin from RYR is more efficient than synthetic lovastatin. The best extraction solvent was the hydro-alcoholic one which, starting from 1,000 mg of RYR, guaranteed the encapsulation of about 6 mg which had a similar effect than the 20 mg/day dose of synthetic lovastatin [1], actually the most used daily dose, since myalgia

incidence is proportional to the dose (ranging from 0.1% at 20 mg/day to 1.5% at 80 mg/day) [21]. Consequently, the amount of lovastatin from RYR uploaded in liposomal formulation (sample B) is sufficient to exert the same effect of the common dose of lovastatin (20 mg/day). However, it is possible to slightly increase the amount of lovastatin uploaded by the liposomes reaching 10 mg which is the recommended daily dose of lovastatin from RYR to gain the claimed effect, increasing the RYR amount to 1,800 mg [22] or even higher, i.e. 2,400mg [23], which are common tested doses of RYR.

Declaration of interest

Conflicts of interest: none.

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Captions

Figure 1: Formulations rheograms: Viscosity (Pa.s) vs shear strain (s^{-1}): (sample A: ■; Sample B: ■; sample C: ●; Sample D: ▼)

Figure 2: Thermographs of the liposomal formulations in the heating range 30-900 °C.
(*:lovastatin decomposition).

Table 1: Physico-chemical characterization of liposomal formulations (A: liposomes obtained mixing DOPC, DOPE and RYR alcoholic extract; B: liposomes obtained mixing DOPC, DOPE and RYR hydro-alcoholic extract; C: liposomes obtained mixing DOPC DOPE and standard lovastatin in EtOH; D: empty liposomes) by particle size, zeta potential and lovastatin encapsulation efficiency (EC: encapsulation efficiency; D: mean diameter \pm standard deviation; PI: polydispersity index; ζ :-potential).

Composition	mg [#]	mg [§]	E.C	D (nm)	PI	ζ (mV)
A:DOPC-DOPE+R EtOH	20.6	3.2 \pm 0.1	9%	155 \pm 17	0.40	-37 \pm 7
B:DOPC-DOPE+R(EtOH:H ₂ O)	35.4	6.4 \pm 0.2	31%	450 \pm 23	0.35	-32 \pm 7
C:DOPC-DOPE+Lov	28	3.9 \pm 0.1	14%	263 \pm 15	0.47	-29 \pm 5
D: DOPC-DOPE	---	----	---	85 \pm 11	0.30	-18 \pm 5

[#]: mg of extracted lovastatin

[§]: mg of encapsulated lovastatin

Table 2: Rheological and thermal characteristics of liposomal formulations (A: liposomes obtained mixing DOPC, DOPE and RYR alcoholic extract; B: liposomes obtained mixing DOPC, DOPE and RYR hydro-alcoholic extract; C: liposomes obtained mixing DOPC DOPE and standard lovastatin in EtOH; D: plain liposomes)

	<u>Rheological parameters</u>				R
	^a G' (Pa)	^a G'' (Pa)	^b G' (Pa)	^b G'' (Pa)	
A:DOPC-DOPE+R EtOH	$(4.5\pm 0.2)E^{-2}$	$(1.1\pm 0.2)E^{-2}$	$(4.4\pm 0.2)E^{-2}$	$(3.2\pm 0.2)E^{-2}$	
B:DOPC-DOPE+R(EtOH:H ₂ O)	$(1.5\pm 0.1)E^{-3}$	$(4.4\pm 0.2)E^{-4}$	$(1.6\pm 0.2)E^{-3}$	$(4.5\pm 0.2)E^{-2}$	
C:DOPC-DOPE+Lov	$(1.7\pm 0.1)E^{-3}$	$(1.3\pm 0.1)E^{-3}$	$(1.8\pm 0.2)E^{-3}$	$(8.3\pm 0.2)E^{-4}$	
D:DOPC-DOPE	$(3.6\pm 0.2)E^{-2}$	$(8.1\pm 0.4)E^{-2}$	$(3.1\pm 0.2)E^{-3}$	$(1.4\pm 0.2)E^{-3}$	
	<u>Weight loss per cent</u>				
	30-120°C	120-200°C	200-400°C	400-600°C	
A:DOPC-DOPE+R EtOH	5.6±0.3%	9.0±0.1%	69±2%	13±1%	0.19
B:DOPC-DOPE+R(EtOH:H ₂ O)	5.2±0.2%	11±1%	58±3%	18±2%	0.31
C:DOPC-DOPE+Lov	6.1±0.2%	5.1±0.3%	80±2%	5.3±1%	0.07
D:DOPC-DOPE	9.0±0.5%	6.7±0.4%	66±3%	5.6±0.4%	0.08

^a: G' and G'' obtained before viscosity analysis

^b: G' and G'' obtained after viscosity analysis

Table 3: Inhibitory activity versus HMG-CoA reductase of liposomal formulations (A: liposomes obtained mixing DOPC, DOPE and RYR alcoholic extract; B: liposomes obtained mixing DOPC, DOPE and RYR hydro-alcoholic extract; C: liposomes obtained mixing DOPC DOPE and standard lovastatin in EtOH; D: plain liposomes)

	<u>Inhibitory activity</u> Units/mgP*±SD
A:DOPC-DOPE+R EtOH	0.30±0.08
B:DOPC-DOPE+R(EtOH:H ₂ O)	0.13±0.02
C: DOPC-DOPE+Lov	0.51±0.08
D: DOPC-DOPE	-----
Lovastatin	6.4±0.2

*One unit will convert 1.0 μ mole of NADPH to NADP⁺. The unit specific activity is defined as μ mol/min/mg-protein (Units/mgP).

