Evaluation of AML-VAL Nanoparticles as Combined Therapy in Cardiovascular Disease

FAWZIA SHA'AT[1,2]*, RAMONA DANIELA PAVALOIU[1,2], DANIELA CRINA SALCEANU[1], CRISTINA HLEVCA[1], GHEORGHE NECHIFOR[2]*

[1] National Institute for Chemical-Pharmaceutical Research&Development ICCF, 112 Vitan Av., 031299, Bucharest, Romania

[2] University Politehnica of Bucharest, Faculty of Applied Chemistry and Materials Science, 1-7 Gheorghe Polizu Str., 011061, Bucharest

The main aim of this study was to investigate a mixture of two poorly water-soluble active pharmaceutical ingredients (APIs): an angiotensin II receptor antagonist (valsartan) and a calcium channel blocker (amlodipine besylate), chosen in a fixed-dose, in order to obtain new polymeric nanoparticles (NPs) for cardiovascular diseases treatment. NPs were prepared via nanoprecipitation method using poly (D,L-lactide-co-glycolide) (PLGA) as matrix and Pluronic F127 as stabilizer. Three formulations were investigated with different ratios of AML:VAL:PLGA (1:16:5, 1:16:7.5 and 1:16:10). Particle size, polydispersity index and zeta-potential analyses were performed to characterize and optimize the formulation. The in vitro drug release study was determined by using a dialysis membrane method under sink conditions. All NPs loaded with both APIs showed nano-size, negative potential, a high homogeneity and a slow drugs release in physiological environment.

Keywords: nanotechnology, cardiovascular, drug release, valsartan, amlodipine, PLGA

Cardiovascular diseases represent one of the most major cause of death worldwide and because its enormous impact on health, health-science and health-economy, biomedical research community needs to improve the technologies available nowadays or/and to develop new strategies for the cardiovascular disease's treatment [1-4].

Generally, almost all drugs, in addition to the beneficial therapeutic effects, have some unwanted side effects. In the process of obtaining a pharmaceutical product containing two or more active pharmaceutical ingredients (APIs) with complementary mechanisms of action, the main purpose is to achieve a cumulation of the therapeutic effects, without the presence of side effects.

The pharmaceutical industry, an important branch of the chemical industry, is in a continuous international development. This field is continuously enriched with new products of synthesis, semi-synthesis or extraction even if the number of pharmaceuticals is very large. Nowadays pharmaceutical industry has set out to focus on cutting edge research areas that includes nanotechnology [5, 6].

The purpose of this research is to achieve and develop nano-therapeutic systems since the use of nanoparticles (NPs) as Drug Delivery Systems (DDS) presents some important advantages [1, 2, 6-8]: both active and passive drug targeting can be achieved by easily manipulating the particle size and surface characteristics of NPs; the systems obtained can be used for various routes of administration such as oral, nasal, parenteral, intraocular, etc.; improves stability and therapeutic index and reduces the side/toxic effects; by attaching specific ligands on their surfaces, NPs can be used for directing the drug to specific target cells; drug release and particle degradation can be controlled by a judicious selection of the matrix constituents; APIs loading is relatively high and could be incorporated into systems without a chemical reaction, which is very important in terms of preserving the activity of the active entity studied.

The oral delivery of cardiovascular drugs has as obstacles: low bioavailability and instability. The NPs protect the unstable drugs against the harsh environment of gastrointestinal tract, particularly the highly content of the hydrochloric acid of the gastric juice, the presence of an active aspartic protease (pepsin) in the stomach and pancreatic and small intestinal brush-border enzymes [1, 9].

Moreover, the combination therapy produced a statistically significantly greater reduction in patients with cardiovascular disease than the corresponding monotherapy [6].

This work is geared toward the study of systems that contain two APIs: an angiotensin II receptor antagonist drug (valsartan) and a calcium channel blocker (amlodipine besylate), in order to obtain polymeric formulations with slow release forms, increased bioavailability, a long-lasting action and a good stability.

Experimental part

Materials

Poly (D,L-lactide-co-glicolide) (PLGA, 50:50, MW = 30,000 - 60,000 Da) was purchased from Sigma Aldrich (USA). The two active pharmaceutical ingredients (APIs): amlodipine besylate ($C_{26}H_{31}ClN_2O_8S$, 2-[(2-aminoethoxy)-methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylic acid 3-ethyl 5-methyl ester benzene sulfonate) and valsartan ($C_{24}H_{29}N_5O_3$, N-(1-Oxopentyl)-N-[[22 -(2H-tetrazol-5-yl)[1,12 -biphenyl]-4-yl]methyl]-L-valine) were obtained from Sigma Aldrich (USA). As surfactant was used Poloxamer 407, known as Pluronic F127 (poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol)) obtained from Sigma-Aldrich (USA). Acetone (AdraChim SRL, Bucharest, Romania), analytical grade, was used in the precipitation process. The water used for all experiments was distilled. The *in vitro* drug release studies were performed in phosphate buffer 0.1 M at *p*H 7.4. All other chemicals were of analytical

*email: fawzya.shaat@gmail.com; doru.nechifor@yahoo.com

grade obtained from standard sources and used without further purification.

Methods

Preparation of PLGA NPs with AML-VAL encapsulated

NPs were prepared according to nanoprecipitation method [10, 11]. The experimental procedure was as follows. PLGA polymer at three different concentrations (5, 7.5, 10 mg) was dissolved in acetone (5 mL). The both drugs in a fixed-dose combination (AML:VAL - 1:16 mg) were accurately weighed and solved in PLGA/acetone solution. Pluronic-F127 (10 mg) was dissolved in distilled water (15 mL). The organic phase was added dropwise into the aqueous phase solution and stirred magnetically at 1500 rpm at room temperature (25^{0}C) until complete evaporation of the organic solvent (table 1). All samples were prepared in triplicate. The final nanosuspension was centrifuged at 10 000 rpm (Universal 320R Hettich, Germany) for 30 minutes at 3^{0}C to separate the drug polymeric aggregates and then it was filtered through 0.22 µm Millex®filter membrane (Low Protein Binding Hydrophilic LCR Membrane, Merck Millipore Ltd., Carrigtwohill Co. Cork Ireland).

Evaluation of drug encapsulation efficiency

The AML-VAL NPs formulation was centrifuged and the supernatant was separated. The amount of drug encapsulated in the polymeric NPs was determined as the difference between the initial amount of APIs used for NPs preparation and the amount of APIs present in supernatant. The percentage of encapsulated drugs was determined by using UV-Vis spectrophotometer at 365 nm for AML and 250 nm for VAL (JASCO V-630 Spectrophotometer, Jasco International Co., Ltd., Japan). Drug encapsulation efficiency was expressed as Encapsulation Efficiency (EE, %) and was calculated using the following equation (1):

$$EE(\%) = \frac{\text{Initial amount of APIs - Amount of APIs in supernatant}}{\text{Initial amount of APIs}} \times 100 \quad (1)$$

Measurement of particle size, polydispersity index and ζ-potential

Particle size, polydispersity index (PDI) and ζ-potential were determined by Dynamic Light Scattering (DLS) technique using a particle size analyzer - Malvern Zetasizer (Malvern Instruments Ltd, UK) with measurement range between 3 nm and 3 µm. Particle size and PDI were measured on samples appropriately diluted with distilled water (1:80) and the analyses of ζ-potential were performed on undiluted samples. All the measurements were performed at a scattering angle of 90^0, temperature of 25^{0}C, solvent refractive index of 1.458 and solvent viscosity of 0.8872 cP. For each sample the mean value ± standard deviation of ten determinations were established. Values reported are the mean value ± standard deviation for three replicate samples.

In vitro drug release study of AML-VAL from the nanoparticulate formulation

The APIs release from the PLGA NPs was determined by a dialysis membrane method under sink conditions [12-14]. 1.0 mL of the nanoparticle suspension was placed in a dialysis tubing cellulose membrane (dialysis bag) with 14 000 molecular weight cut-off (Sigma Aldrich, USA), the ends of the dialysis bag were tightened and then immersed into a 200 mL previously prepared of 0.1 M sodium phosphate buffer *p*H 7.4 at 37°C. The whole system was under stirring at 100 rpm. Samples were taken at predetermined intervals (15, 30, 45, 60, 120, 180, 240, 300, 360, 420 min and 24 h) from the receiver solution. The released drugs in each time point were determined by spectrophotometry using a UV-Vis spectrophotometer. During the release process, a dialysis medium of 5.0 mL was removed at a predetermined time point while adding the same volume of fresh medium (0.1 M sodium phosphate buffer, *p*H 7.4). The release studies were performed in triplicate.

Statistical analysis

Statistical analysis of the data was performed with SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Values are represented as mean ± standard deviation (SD). Differences were considered significant at $p < 0.05$.

Results and discussions

The nanoprecipitation method demonstrated the efficient capability of encapsulating such APIs inside PLGA NPs. In a previous study [15], formulations with AML-VAL loaded in a polymeric matrix of higher concentrations of PLGA were assessed and for which were demonstrated good EE(%). In this study we investigate formulations with a smaller amount of PLGA matrix in order to obtain better characteristics. In order to prove this, we calculated the EE (%). The NPs characteristics are reported in table 2. All formulations (F1, F2 and F3) showed high EE (%) for both APIs, ranged from 79.38 ± 0.10 % to 91.98 ± 0.18 % for VAL and from 73.93 ± 0.10 % to 82.54 ± 0.12 % for AML. By increasing the amount of PLGA (5, 7.5 and 10 mg), the nanoparticle suspension became more and more turbid, the EE (%) and the stability were also increased. As literature have shown, PLGA can be processed into any shape and size, and can encapsulate biomolecules of any size [16]. Increased amount of PLGA polymer may result in encapsulation of hydrophobic drug to a greater extent.

Based on the physicochemical characterization such as mean particle size, PDI and ζ-potential, the performance of PLGA NPs with cardiovascular APIs encapsulated was recognized. As shown in table 2, PLGA NPs with mean volume diameters of 124.7 ± 1.15, 135.6 ± 1.30 and 140.4 ± 1.34 nm respectively, were prepared. As the content of PLGA was increased, the particle size of NPs was also increased from F1 (PLGA 5 mg) 124.7 ± 1.15 nm to F3 (PLGA 10 mg) 140.4 ± 1.34 nm. As the concentration of PLGA increases, the viscosity of the nanosuspension increases proportionally and efficiency of stirring

Formulation cod	$m_{AML:mVAL}$ (mg)	m_{PLGA} (mg)	m_{F127} (mg)	$V_{CH3-CO-CH3}$ (mL)	V_{H2O} (mL)	Drop rate (mL/min)	Stirring speed (rpm)	Stirring time (min)
F1	1:16	5	10	5	15	0.5	1500	25
F2	1:16	7.5	10	5	15	0.5		
F3	1:16	10	10	5	15	0.5		

Table 1
FORMULATION OF PLGA NPS WITH AML-VAL ENCAPSULATED

Formulation code	Mean particle size (nm)	ζ-potential (mV)	PDI	EE (%)
F1	124.7 ± 1.15	-11.86 ± 0.31	0.095 ± 0.05	79.38 ± 0.10 for VAL 73.93 ± 0.10 for AML
F2	135.6 ± 1.30	-18.07 ± 0.52	0.089 ± 0.02	80.18 ± 0.15 for VAL 77.62 ± 0.12 for AML
F3	140.4 ± 1.34	-25.34 ± 0.21	0.108 ± 0.03	**91.98 ± 0.18 for VAL** 82.54 ± 0.12 for AML

Table 2
CHARACTERISTICS OF PLGA NPS WITH AML-VAL ENCAPSULATED.

decreases; thereby these phenomena lead to an increase in the size of the nanoparticles [17]. Also, particle size analysis showed a narrow range of variability in dispersion (PDI, 0.089 ± 0.02 – 0.108 ± 0.03). All samples showed a PDI less than 0.15, which means a significantly higher homogeneity of the systems.

By increasing the amount of PLGA, the NPs suspensions had became more and more turbid (PLGA NPs formation) and the ζ-potential decreased, becoming more and more negative. According to literature data, the dispersion stability is high for the range (-) 30mV- (+) 30mV, in terms of zeta potential value [18]. In all three samples, particles had a negative ζ-potential ranging between -11.86 ± 0.31 mV and -25.34 ± 0.21 mV, indicating a moderate stability.

In vitro release data showed that all formulations had a biphasic profile characterized by an initial *burst effect* of both drugs during the first half hour followed by a slower release reaching a maximum after 24h. The results were presented in figure 1. Approximately 78.47 ± 0.02% of the total loaded AML and 80.54 ± 0.2 of the total loaded VAL was released from NPs with 10 mg PLGA content (F3) after 24 h while the NPs with lower PLGA amount reached higher values than 90% for both drugs in the same timeframe, respectively 94.06 ± 0.01% for AML and 94.97 ± 0.16% for VAL (F2) and 96.37 ± 0.02% for AML and 96.60 ± 0.26% for VAL (F1). Also, the formulation with a higher PLGA amount (F3) presented an attenuated *burst effect* than the formulation with lower PLGA amount (F1 and F2). The initial burst release of both drugs may be attributed to the diffusion of AML and VAL crystals adhered to the surface of the NPs easy accessible after the hydration of the polymer. The biphasic pattern of AML and VAL release from PLGA NPs was consistent with the findings of other studies [18, 19].

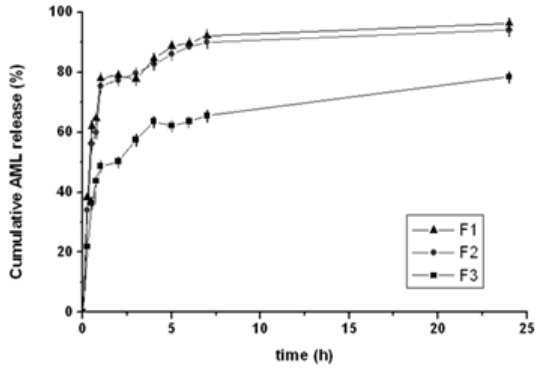

Fig. 1. *In vitro* AML release profile of F1, F2 and F3 nanoformulations

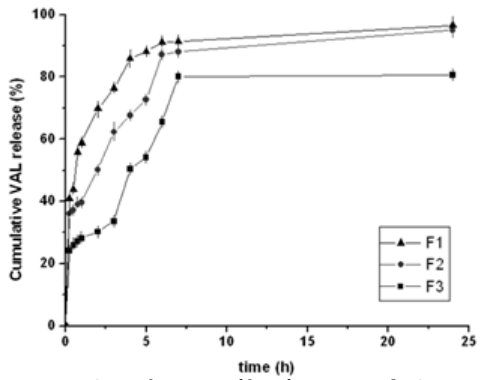

Fig. 2. *In vitro* VAL release profile of F1, F2 and F3 nanoformulations

In order to evaluate the *in vitro* drug release data, four kinetics models were used to describe AML and VAL release kinetics from PLGA NPs. Zero-order, First order, Higuchi and Hixson-Crowell, kinetics models were applied and, on the basis of best fit with the highest correlation value (R^2), it was concluded that AML and VAL release from all samples follows the Higuchi model (R^2>0.91). The Higuchi model describes the drug release from a matrix system by using the equation 2 (the simplified form):

$$Q = K_H \cdot \sqrt{t} \quad (2)$$

where Q is the concentration of drug in the drug matrix at time t and K_H is the Higuchi dissolution constant. This model considers the degradation effect of polymer matrices on the drug release rate negligible. This hypothesis was proved to be valid for PLGA NPs since PLGA degradates under sinking in similar conditions after approximately 2–6 weeks [12, 20].

Conclusions

In this paper, we have shown that encapsulating amlodipine and valsartan into a single formulation, to get the fixed-dose combination as NPs is a feasible strategy which aims to decrease pill burden. The encouraging results suggest that the AML-VAL-PLGA NPs may represent a promising alternative as combined therapy in cardiovascular disease. However, further *in vitro* studies on various cell lines are needed to demonstrate the therapeutic effect.

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