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Original Article

SELECTION OF THE COMPOSITION OF A LIPOSOMAL DOSAGE FORM OF A RUSSIAN SOMATOSTATIN ANALOGUE WITH ANTITUMOR ACTIVITY

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ABSTRACT

Objective: Was to create the composition of the liposomal pharmaceutical form for injections of somatostatin analogue cyphetrylin using soybean phosphatidylcholine (SPC).

Methods: The cyphetrylin, active pharmaceutical ingredient (API), developed in the Chemical Synthesis Laboratory, the N. N. Blokhin National Medical Research Center of Oncology of the Ministry of Health of the Russian Federation; SPC and polyethylene glycol-2000-distearoylphosphatidylethanolamine (PEG-DSPE, Lipoid, Germany); cholesterol ≥99% (Sigma-Aldrich, Japan). The lipid film hydration method with subsequent liposomal dispersion filtration/extrusion through nylon membrane filters was used for the phospholipid vesicle production. Based on API and lipid components in different molar ratios, we studied over 15 model liposomal compositions and assessed each lipid's impact in use on quality attributes of resulting dispersions. Derived model samples of liposomal dispersion were estimated in terms of quality and efficiency of cyphetrylin encapsulation into vesicles, their average size and the surface charge (zeta potential), polydispersity index (PDI) and dispersion viscosity. We used spectral photometry, dispersion laser spectroscopy, electrophoretic particle mobility assay, and viscometry to assess these features.

Results: Pharmaceutical form components' desirable molar ratios determined: cyphetrylin/SPC at 1:60.0 and SPC/cholesterol/PEG-DSPE at 1:0.2:0.004, were determined. This composition allows cyphetrylin liposomal dispersion production with relatively stable vesicles of uniform size, 176 nm in diameter, and a 100% maximum rate of API encapsulation into the bilayer.

Conclusion: Technological and chemical/pharmaceutical studies resulted in selecting a preferable composition of an injectable liposomal pharmaceutical form model of somatostatin analog-based on the SPC.

Keywords: Somatostatin analogue, Liposomes, Soybean phosphatidylcholine, Pharmaceutical dosage form, Composition, The molar ratio

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INTRODUCTION

The concept of the liposomal system of active pharmaceutical ingredients (API) delivery revolutionized the pharmaceutical market. Liposomes, initially called "bangosomes", were discovered and first described in 1965 by the British biophysicist Alec Bangham and his colleagues at Babraham Institute in Cambridge. Since then, liposomes have evolved from a simple cellular membrane model into an actively studied and applied to a delivery system for API of a different origin. So far, the FDA has authorized the clinical use of 15 liposomal substances in various medicine fields: oncology, dermatology, anesthesiology, and treatment of infectious diseases. All these medicinal products resemble free API for their main clinical effect. However, their toxicological criteria compare favourably, which improves the tolerance of these medicinal products, the costs of eliminating the adverse toxic events, and, consequently, the patient's quality of life. Besides, ca. 30 liposome-based developments, mostly intended to treat cancers, are now in the clinical trials pipeline [1–3].

There are no alternative delivery systems with all properties inherent in liposomes now. These nanostructures are biocompatible and biodegradable, which allows for their parenteral administration. Liposomes protect the surrounding body tissues from API cytotoxic action, fortifying the active substance against enzymatic destruction and extending the effect of API introduced into the body, and are capable of targeting delivery of API to organs, tissues, and individual cells. The liposomal delivery system allows for the encapsulation of both water-retaining and water-repellent substances, facilitating the production of their water-soluble forms and thus improving their bioavailability. It is essential because the amount of water-insoluble and not readily water-soluble in API has increased recently [4–6].

We counted approximately 75% new candidate substances for therapeutic product development, which are not readily water-soluble; most are antitumor agents. Poor water solubility is the integral property of many antitumor drugs for two main reasons. Firstly, candidate drugs' physical and chemical properties rarely focus on the screening of substances for antitumor activity. Secondly, to achieve permeability, activity, and stability, an antitumor agent must contain certain hydrophobic structural features that make API not freely water-soluble [7].

The Chemical Synthesis Laboratory of N. N. Blokhin National Medical Research Center of Oncology of the Ministry of Health of the Russian Federation derived a hydrophobic somatostatin analogue, cyphetrylin, with antitumor activity. Unlike other somatostatin equivalents, cyphetrylin is a non-cyclical peptide derived with classical peptide chemistry methods. By its chemical structure, cyphetrylin is a methyl ether of N^{α}-tret-butyloxycarbonyl-S-tetrahydropyranyl, cysteine phenyl, alanyl-D-tryptophyl-N^{ε}-carbobenzoxylysyl threonine (fig. 1). All lateral reactive groups in its molecule are protected with tert-butyloxycarbonyl, benzyloxycarbonyl, and tetrahydropynal groups. *In vivo* studies, cyphetrylin inhibits the secretion of growth hormone, prolactin and insulin secretion. Cyphetrylin antitumor activity shown in continuous cell lines of mouse tumors: adenocarcinoma of the breast Ca755, human breast cancer BrC1, cervical cancer CC5, Lewis lung carcinoma LLC, and those of rat tumors: prostatic adenocarcinoma R-3327-H and DMBA-induced breast tumors [8–10].

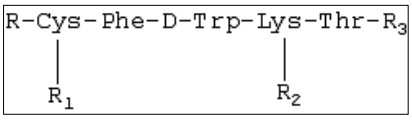

Fig. 1: Cyphetrylin structural formula. Legend: R–tret-butyloxycarbonyl, R_1–tetrahydropyranyl, R_2–carbobenzyloxycarbonyl, R_3–methyl

With the view to hydrophobic properties of cyphetrylin, the Pharmaceutical Form Development Laboratory of N. N. Blokhin National Medical Research Center of Oncology of the Ministry of Health of Russian Federation derived cyphetrylin a solid pharmaceutical dosage form of tablets, which has already passed Stage I clinical trials in patients with neuroendocrine tumors [11–14]. It was proposed to develop the liposome-based injectable pharmaceutical form as an alternative to cyphetrylin tablets, to improve its efficacy and mitigate adverse events.

MATERIALS AND METHODS

Materials

Cyphetrylin substance (N. N. Blokhin National Medical Research Center of Oncology of the Ministry of Health of Russian Federation), soybean phosphatidylcholine (SPC, Lipoid, Germany), cholesterol ≥99% (Sigma-Aldrich, Japan), polyethylene glycol-2000-distearoylphosphatidylethanolamine (PEG-DSPE, Lipoid, Germany), chloroform stabilized with ethanol (Chimmed, Russia), 95% ethanol (Flora Kavkaza OSJC, Russia), water for injections; 1.2 um, 0.45 um and 0.22 um Pall nylon membrane filters, N66, d 25 mm (Pall Eurasia LLC, Russia).

Methods

Production of liposomes with cyphetrylin

To produce cyphetrylin liposomes, we used the Bangham method [15]. Cyphetrylin substance and lipid components (SPC, cholesterol, and PEG-DSPE) were dissolved in chloroform. We transferred the resulting solution into a round-bottom flask and removed the organic solvents by vacuuming a Heidolph Hei-VAP Advantage rotary evaporator (Heidolph, Germany) at low-pressure at+(40±1) °C water bath until the film became evenly distributed on the formed flask walls. The lipid film was terminally dried in a vacuum (120–125 mbar) until the organic solvent residues were removed and then hydrated with water for injection to produce large multi-layer liposomes dispersion with the theoretical active substance concentration of 1.0 mg/ml. To reduce cyphetrylin liposomes size, the dispersion was successively screened through 1.2 um, 0.45 um, and 0.22 um nylon membranes in the Lipex™ extruder (Northern Lipids, Canada). "Empty" liposomes were generated in the same way as those with an active substance, but without adding cyphetrylin to the drug components organic solution. Using cyphetrylin, SPC, cholesterol, and PEG-DSPE, we generated and tested 15 model compositions of cyphetrylin in the liposomal pharmaceutical dosage form with different components molar ratios (table 1) [16].

Table 1: Model compositions of the liposomal dosage form of cyphetrylin

Formula	Molar ratio	
	Cyphetrylin/SPC	SPC/cholesterol/PEG-DSPE
F1	1:70.0	1:0.2:0.004
F2	1:70.0	1:0.1:0.004
F3	1:67.5	1:0.2:0.004
F4	1:67.5	1:0.1:0.004
F5	1:65.0	1:0.3:0.004
F6	1:65.0	1:0.2:0.004
F7	1:65.0	1:0.1:0.004
F8	1:60.0	1:0.3:0.004
F9	1:60.0	1:0.2:0.005
F10	1:60.0	1:0.2:0.004
F11	1:60.0	1:0.2:0.003
F12	1:57.5	1:0.3:0.004
F13	1:57.5	1:0.2:0.004
F14	1:55.0	1:0.3:0.004
F15	1:55.0	1:0.2:0.004

Cyphetrylin inclusion in liposomes

Cyphetrylin content in liposomes was performed using the working reference standard (RS) at 282±2 nm wavelength in Cary 100 spectrophotometer (Agilent Technologies, Australia). As liposomes lipid components absorb in this spectrum, too, measurements were conducted in comparison with the "empty" liposomes solution.

Cyphetrylin RS preparation: add about 15 ml 95% ethanol to 2.5 mg cyphetrylin. The resulting ethanol solution is transferred into a 25 ml volumetric flask and diluted with ethanol. The solution to be used when fresh.

Comparison solution preparation: 2 ml liposomal dispersion of "empty" liposomes is dissolved with ethanol in a 25 ml volumetric flask and diluted to the volume with ethanol. The solution is used when fresh.

Test solution preparation: dissolve 2 ml cyphetrylin liposomal dispersion into a 25 ml volumetric flask. Add a small quantity of 95% alcohol. Mix and dilute to the volume with alcohol.

Measure the test solution's optical density in trays with 10 mm optical layer "thickness", in maximum absorption compared with the comparison solution. Simultaneously, measure the optical density of the cyphetrylin RS solution vs that of the comparison solution. Using the formula (the dilution is identical), calculate the cyphetrylin concentration in liposomes (X, mg/ml):

$$X = \frac{A \times a_0}{A_0 \times 2}$$

A and A_0–optical densities of the liposomal sample solutions and cyphetrylin RS, respectively; a_0–weight of cyphetrylin RS, mg; 2–volume of cyphetrylin liposomal sample, ml [16].

Determination of cyphetrylin liposomal encapsulation efficiency

As cyphetrylin is a hydrophobic substance and directly included in the lipid bilayer during liposome production, the encapsulation efficiency (EE) was determined as the ratio of API concentration in post-extrusion liposomal dispersion to cyphetrylin concentration in the dispersion after the lipid film hydration. The indicator expressed as a percentage:

$$\mathrm{EE} = \frac{\mathrm{Ce}}{\mathrm{Ch}} \times 100\%$$

Ce–cyphetrylin concentration in dispersion after extrusion, mg/ml; Ch–cyphetrylin concentration in dispersion after hydration, mg/ml [16].

Determination of average diameter, polydispersity index (PDI) and the zeta potential for cyphetrylin liposomes

The liposomal test by size and PDI was conducted using the correlation light scattering spectroscopy. Zeta potential of vesicles

was determined by measuring their electrophoretic mobility. All measurements used zeta sizer Nano-ZS 3600 (Malvern, U. K.). 100 µl test sample of freshly made liposomal dispersion was measured with an automatic pipette, poured into a 100 ml volumetric flask, and diluted in volume with water for injection. A 1 ml diluted sample was transferred to a polystyrene tray, placed into the zetasizer cell, and the above indicators were measured [17, 18].

Determination for cyphetrylin liposomal dispersion viscosity

Dynamic viscosity of cyphetrylin liposomal dispersion samples was determined using Vibro Viscometer SV-10 (AND Company Limited, Japan). All measurements were conducted at a temperature ranging from 21 °C to 25 °C. To this end, 10 ml of liposomal dispersion was poured onto the device's tray and measured [19].

RESULTS AND DISCUSSION

In 2014–2017, the N. N. Blokhin National Medical Research Center of Oncology of the Ministry of Health of the Russian Federation researched on the development of a liposomal drug form of cyphetrylin. The components for cyphetrylin liposomal pharmaceutical dosage form were selected based on their functional purpose. Phosphatidylcholine of animal origin, derived from egg yolk (EPC), was used as the principal liposomal bilayer-forming excipient because of its properties. It has an acceptable phase-transition temperature (+37 °C), good solubility in organic solvents, biocompatibility and natural origin. Natural phospholipids are preferred in pharmaceutical technology compared with their synthetic counterparts because they are available on a large scale, at a reproducible quality with low production costs, and manufactured with smaller quantities of chemical agents and solvents a higher yield of the finished product [20]. To enhance the bilayer rigidity and improve the phospholipid vesicle stability in the blood flow, cholesterol was added to liposomes. To protect the vesicles from being captured by reticuloendothelial cells after their introduction into the body and to slow down their clearance from the blood flow, we developed the stealth-liposomes with cyphetrylin. To this end; we injected the phospholipid "sewed" with PEG-DSPE into the bilipid layer of the pharmaceutical form, which increases osmotic pressure near the liposomes and prevents their discovery by macrophages. Using these components, we derived the model of the liposomal pharmaceutical dosage form with molar ratios of cyphetrylin/EPC at 1:70.0 and those of EPC/cholesterol/PEG-DSPE at 1:0.2:0.003. The created cyphetrylin liposomal dispersion has 98% drug encapsulation rate and with 168±6 nm vesicle size [16].

To improve the injectable liposomal form of cyphetrylin, it was proposed to modify cyphetrylin liposomal composition by replacing EPC to SPC. Egg lecithin contains less unsaturated lipids than soybean lecithin (table 2) [19]. However, by forming different bonds with phospholipids, the hydrophobic active substance is encapsulated into the lipid bilayer. Based on this data, we suggested that the phosphatidylcholine (PC) origin may significantly impact the API encapsulation rate and that a large number of unsaturated bonds would lead to a lower lipid concentration in the drug and a higher API/PC ratio.

Table 2: Egg and soybean lecithin lipid composition

Chain composition	Egg lecithin	Soybean lecithin
16:0	32 (33)	15 (14)
16:1	5 (1)	4
16:2	1	0
18:0	14 (13)	1 (4)
18:1	19 (32)	14 (11)
18:2	13 (16)	59 (64)
20:1	4	0
20:2	6	0
20:3	2	1
20:4	2 (3)	4
other	2 (5)	2 (3)
saturated	46	16
unsaturated	52	82

The test was conducted by reducing SPC content in cyphetrylin/SPC from the initial 1:70.0 established for liposomes with EPC, as described above, and by varying the lipid composition ratio, with monitoring of such indicators as EE, average post-extrusion vesicle size and their fraction distribution index (PDI), zeta potential of liposomes and the resulting dispersion viscosity (table 3).

Table 3: Characteristics of SPC-based investigatory compositions of cyphetrylin liposomal pharmaceutical form

No	EE, %	Vesicle size, nm	PDI	Zeta potential, mV	Viscosity, mPa·s
F1	100.0±0.62	171±6.94	0.170±0.01	-13.2±0.75	9.8±1.19
F2	100.0±1.51	167±6.05	0.282±0.01	-19.3±1.31	8.4±1.24
F3	100.0±0.59	179±7.11	0.231±0.02	-16.4±1.10	5.6±0.33
F4	99.0±0.82	173±7.01	0.177±0.01	-14.6±0.77	8.8±0.50
F5	100.0±0.70	173±6.50	0.290±0.01	-18.1±0.95	10.8±0.62
F6	100.0±0.33	174±5.91	0.195±0.01	-19.6±0.92	8.5±1.01
F7	100.0±0.73	210±8.22	0.213±0.01	-19.3±1.02	6.3±0.74
F8	100.0±0.97	174±7.15	0.230±0.01	-20.0±1.07	6.7±0.55
F9	99.6±0.80	170±7.23	0.168±0.01	-19.2±0.72	10.7±0.43
F10	100.0±0.54	176±6.02	0.212±0.01	-18.3±0.90	7.5±0.75
F11	99.0±0.50	183±6.55	0.256±0.02	-18.0±1.25	6.5±0.67
F12	95.6±0.88	185±7.30/11±0.02	0.195±0.01	-17.5±1.18	7.9±0.35
F13	90.8±1.23	211±7.89	0.162±0.01	-17.3±1.30	6.2±0.49
F14	93.1±1.04	152±6.70/11±0.10	0.099±0.01	-18.0±0.95	8.6±0.58
F15	88.1±1.10	182±8.05	0.110±0.01	-19.7±0.85	6.6±0.71

Note: Data given in mean±SD, n=3

As a result, we established that the content of all lipid components influenced cyphetrylin liposomal EE. When the overall lipid concentration decreased, the 100% maximum encapsulation was achieved in a liposomal composition No. 10 with the molar ratio of cyphetrylin/SPC at 1:60.0 and those of SPC/cholesterol/PEG-DSPE at 1:0.2:0.004. Further increase in cyphetrylin/SPC ratio resulted in a significant reduction of the encapsulation rate to ~91%, which may have increased to 95.6% at a higher cholesterol content (F12). A more significant increase in its concentration is not appropriate due to the deterioration of the dispersion technological properties resulting from the rigid liposomal structure. We also noted that both the decrease and the increase in pegylated phospholipid content contribute to a slight reduction in cyphetrylin vesicular EE.

When reviewing other quality parameters, we did not establish any correlation between their values and the liposomal lipid composition. The size distribution curves review suggested that virtually all model liposomes' diameter did not exceed the preferable one for therapeutic use, 220 nm, and PDI are below 0.3, which testifies to the narrow size distribution of the particles [21]. The only exception is being F8: the vesicle diameter amounted to 243 nm. F12 and F14 displayed the bimodal size distribution, which points to two vesicle fractions with different sizes. Vesicle size reduction as the cholesterol level increases may be accounted for by a higher packing density. The electrophoretic mobility measurement analysis showed that cyphetrylin liposomes charge of the prepared compositions ranges from-13.2 mV to-20.0 mV, and dispersion dynamic viscosity range from 5.6–10.8 mPa·s.

The comparative data analysis by quality attributes of the investigated compositions allowed to select the preferable model for the production of cyphetrylin liposomal pharmaceutical dosage form based on soybean lecithin with the following molar ratios of components: cyphetrylin/SPC at 1:60.0 and SPC/cholesterol/PEG-DSPE at 1:0.2:0.004.

The conducted study suggests that phospholipids derived from different sources may influence active hydrophobic substance loading into a liposomal membrane. If cyphetrylin was included, the SPC selected as the primary, bilayer-forming component in liposomal pharmaceutical dosage form for further research had the advantage.

CONCLUSION

Cyphetrylin, the hypothalamic hormone somatostatin analogue with antitumor activity, is a promising drug to treat many cancers, including neuroendocrine tumors. Cyphetrylin liposomal encapsulation was proposed for the injectable pharmaceutical dosage form creation to overcome the active substance's low water solubility. The conducted research helped select the desirable model composition of the cyphetrylin SPC-based liposomal pharmaceutical dosage form.

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Nil

AUTHORS CONTRIBUTIONS

Maria Dmitrieva: Research design, obtaining and analyzing data, writing the text of the manuscript. Zoya Shprakh: Research design, preparation of the manuscript, translation. Olga Orlova, Ivan Krasnyuk: Research design, data analysis, verification of the final version of the manuscript. Elena Ignatyeva: Obtaining and analyzing data. Anna Lantsova, Ludmila Nikolaeva: Review of publications, design of the manuscript.

CONFLICT OF INTERESTS

The authors declare no conflict of interest

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