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Soft nanocarriers for new poorly soluble conjugate of pteridine and benzimidazole: Synthesis and cytotoxic activity against tumor cells

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ABSTRACT

New conjugated derivative of pteridine and benzimidazole, 7-(benzimidazol-2-yl)-6-(2,4-dichlorophenyl)-2-thioxo-2,3-dihydropteridin-4(1*H*)-one (BP) possessing antitumor activity was obtained and thoroughly characterized; its spectrophotometry control in solution was developed. To improve the solubility of the hydrophobic antitumor agent BP in aqueous solutions, amphiphilic systems (micellar solutions and modified liposomes) were fabricated, with their composition optimized. Stable 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)-based liposomes modified with cationic surfactants, namely cetyltrimetylammonium bromide and 1-methyl-3-hexadecylimidazolium bromide (IA-16), were obtained. It was shown that the use of cationic surfactants for the formation of hybrid drug carriers and varying of the surfactant/lipid molar ratios can be the key factor for obtaining the optimized particles with high efficiency of encapsulation, favorable loading efficacy and prolonged release of the drug. The most beneficial parameters in terms of stability and loading/release efficacy were obtained for IA-16/DPPC hybrid liposomes with the surfactant/lipid molar ratio of 1:50. Free BP demonstrated cytotoxicity toward M-Hela tumor cells, along with the nearly the same toxic effect toward normal Chang liver cells. Importantly, double effect occurred upon the encapsulation of BP into hybrid cationic liposomes, i.e. a sharp decrease in viability of diseased cells and increase of viability of normal cells. Therefore, liposomal BP formulation can be recommended as therapeutics with improved stability and high selective cytotoxicity toward M-Hela tumor cells at the level of commercial drug doxorubicin, in combination with much less toxicity (by 37 times) toward the normal Chang liver cell line.

1. Introduction

Pteridines and benzimidazoles are privileged nitrogen heterocycles of both natural and synthetic origin, known for a wide range of biological activities, which have been found in clinically accepted drugs as well [1–3]. Pteridines are described to be useful in biology as a base component of several nucleosides [4] or nucleotides [5], and in other biological activity such as anti-inflammatory [6], antimicrobial [7], antihepatitis [8], immunosuppressive [9], antitumor [10] and neurodegenerative agents [11] as well as α-tumor necrosis factor agents [12], adenosine kinase inhibitors [13], and nitric oxide synthase inhibitors [14,15]. On the other hand, benzimidazole and its derivatives are versatile molecules that occupy key positions in the synthesis of natural products and pharmaceutical materials and display significant activity toward several viruses such as human immunodeficiency virus (HIV) [16–18], human cytomegalovirus (HCMV) [17,19,20] herpes simplex virus (HSV-1) [19] and influenza [21], including anticancer activity [22]. Some of the potent hybrid molecules that have been recently developed as new anticancer agents are obtained by the combination of different pharmacophores [23,24]. In addition, it is critical to point out that all numerous tyrosine kinase inhibitors having *metha*-dichloroaryl fragment have proven to be very potent and hence using the fragment based approach, the *metha*-dichlorophenyl ring is appended at N1 in the core structure [25]. For example, 2-indolinone derivatives with the *metha*-dichloroaryl fragment at N1 and (hetero)aryl benzylidene at C3 have shown excellent cytotoxicity on a stomach cancer cell line and SW620 colon cancer cell line [26]. The promising biological activity exhibited by these conjugates prompted us to explore newer conjugates by combining pteridine, benzimidazole and *metha*-dichlorophenyl scaffolds with a view to enhance the cytotoxic activity. In this context, we have synthesized the conjugate of pteridine and benzimidazole and evaluated them for their cytotoxic potential.

When planning the synthesis and subsequent testing of potential biologically active heterocyclic compounds, researchers often face the problem of their low solubility in water, whereas this property is one

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of the most important criteria that determines the bioavailability of drugs. To solve these problems in pharmacology and medicine, lipid formulations are often used to solubilize hydrophobic compounds and increase their content in aquatic systems, thereby enhancing their bioavailability [27–30]. Lipid formulation is a broad term which includes systems based on synthetic surfactants (micellar solutions, nano- and microemulsions), as well as compositions based on natural surfactants, namely, lipids (liposomes, nanosomes, kerasomes). In pharmacology and medicine, the micellar solutions of low-toxic non-ionic surfactants (Tween 20, Tween 80, Triton X-100, Tiloxapol, etc.) are widely used as solubilizates for different drugs [31–36]. However, compositions containing ionic surfactants often exhibit a higher solubilizing effect, since the binding of drugs occurs not only due to hydrophobic interactions, but also due to electrostatic forces [37,38]. In addition, cationic surfactants are very effective agents for transdermal transfer of drugs [39–41], and also provide sufficient time to hold the drug on the eye surface, which determines their successful use in ophthalmology [42,43]. Thus, an important task in the application of cationic surfactants and, above all, in pharmacology when developing the dosage forms is the selection of cationic agents with an acceptable degree of safety, combining high efficiency with minimal toxic effects.

Encapsulation of drugs in liposomes can be an alternative way to improve their delivery in biological systems. In recent years, liposomal drugs have gained particular popularity [44,45]. This is due to the fact that liposomes are biocompatible, biodegradable and considered as universal drug carriers. They have an aqueous core and a hydrophobic lipid bilayer; therefore, they are capable of enclosing both hydrophilic and hydrophobic substances [46,47]. The mechanism and degree of interaction between liposomes, cells and biological barriers largely depend on the sign and density of charge on the liposome surface [48]. Positively charged liposomes with zeta potential ranging between 30 mV and 60 mV have the advantage over the others [49,50]. Such carriers have proven to be effective and time-stable agents for delivery of various drugs in vitro and in vivo [51,52]. One of the ways to create a positive charge on liposome is its modification by cationic surfactants [53,54]. As a rule, this imbedding can provide a high charge on the particle, and as a result, increases its stability, as well as affinity for negatively charged parts of the cell.

The use of liposomal formulations in the fight against cancer is a topical and highly developed area of knowledge. Liposome is considered as promising carrier in drug delivery of antitumor agents. Analyzing the publications of the last two years devoted to the investigation of the anticancer drug doxorubicin, widely used in liposomal form in medical practice, it can be seen that the main trends of modern research are the search for opportunities to increase the drug effectiveness, the increase of its selectivity, the reduce of its negative effects, and the suppression of the immune response of the biosystem [55–67]. There are acute issues of regulating the drug release from the liposome into the external environment, as well as the stability of liposomes. The desired results were achieved by loading the liposome with doxorubicin in combination with other medicinal agents (lovastatin, celecoxib, irinotecan) [58–60], combining loaded liposomes with nanoscale metal particles [61], modifying the composition of the liposomes by surfactant [48,62], covering them with a polyelectrolyte shell [63,64], giving them the ability to respond to changes in pH and temperature [65–67]. However, despite a number of successful solutions, the problem of obtaining effective and selective agents, which destroy malignant cells, save healthy tissues and retain their useful properties for a long time, has not lost its relevance.

Taking into account the above-mentioned issues and basing on our own experience, we have set the task of obtaining new conjugate by combining pteridine and benzimidazole scaffolds namely 7-(benzimidazol-2-yl)-6-(2,4-dichlorophenyl)-2-thioxo-2,3-dihydropteridin-4(1*H*)-one (BP). The structure of this compound suggested the possibility to manifest antitumor activity. The synthetic approach to prepare this conjugate was based on the Mamedov rearrangement [68–72] of 3-(2,4-dichlorobenzoyl)quinoxalin-2(1*H*)-one when exposed to 5,6-diamino-2-mercaptopyrimidin-4-ol (Scheme 1).

Further stages of work included the investigation of the cytotoxic effect of this compound, the increasing of its bioavailability by incorporating into lipid formulations of various morphologies that include cationic surfactants, the determination and comparison of antitumor action.

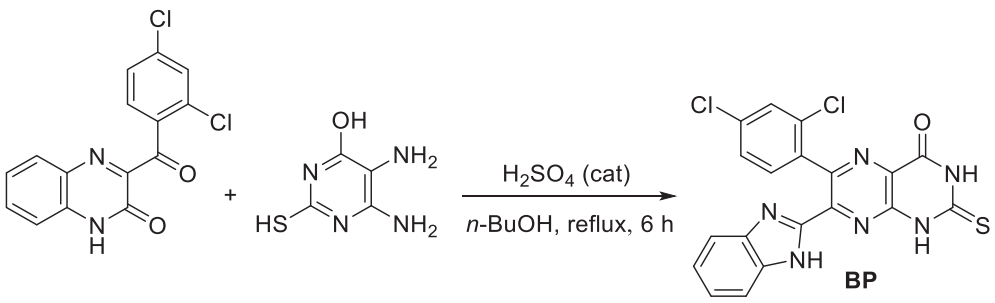
2. Material and methods

2.1. Chemicals

Lipoid PC 16:0/16:0 (1,2-dipalmitoyl-sn-glycero-3-phosphocholine, DPPC) was a gift from Lipoid GmbH (Ludwigshafen, Germany). Cetyltrimethylammonium bromide (CTAB) (Acros Organics, New Jersey, USA, 99%), Tween 80 (Acros Organics, New Jersey, USA, BioXtra) were used without preliminary purification. The surfactant aqueous solutions were obtained using water purified by a Milli-Q Water Purification System.

2.1.1. Synthesis of 7-(benzimidazol-2-yl)-6-(2,4-dichlorophenyl)-2-thioxo-2,3-dihydropteridin-4(1H)-one (BP)

To a stirred suspension of the quinoxalin-2(1*H*)-one (0.26 g, 0.82 mM) and 5,6-diamino-2-mercaptopyrimidin-4-ol (0.13 g, 0.82 mM) in *n*-BuOH (10 ml) was added 98% H_2SO_4 (2 drops) and the reaction mixture was heated at reflux. First, there was a gradual transition of the suspension into a solution and after 15 min heating at reflux a gradual precipitation occurred and stirring was continued at the same temperature for 6 h. After that the reaction mixture was cooled to rt. and left overnight. The precipitate formed was filtered off and washed with boiling water; the residue filtered off hot and dried in air to give analytically pure compound. Yield: 0.26 g (71%), bright yellow powder, mp > 350 °C. IR (KBr), ν, cm^{-1}: 3468, 3195, 3066, 2923, 1709, 1591, 1544, 1432, 1339, 1261, 1229, 1208, 1153, 1143, 1099. ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.23 (br s, 2H, H5/H6-BI), 7.53 (br s, 2H, H4/H7-BI), 7.56–7.60 (m, 1H, H6—Ar), 7.58 (br.s, 1H, H3—Ar), 7.62–7.66 (m, 1H, H5—Ar), 12.93 (s, 1H, NH3-Pt), 13.51 (s, 1H, NH1-Pt). ^{13}C{^{1}H} NMR (126 MHz, DMSO-d_6): δ 123.4 (C5/C6-BI), 127.4 (C3—Ar), 128.3 (C5—Ar), 128.5 (C4a—Pt), 132.7 (C6—Ar), 133.2 (C2—Ar), 133.9



Scheme 1. Synthesis of the conjugate BP.

(C4—Ar), 136.7 (C1—Ar), 145.7 (C7—Pt), 146.0 (C6—Pt), 147.2 (C2-BI), 147.3 (C8a—Pt), 157.9 (C4—Pt), 176.2 (C2—Pt). The signals of C4/C7-BI, C3a-BI, C7a-BI have not been observed. ^{15}N NMR (51 MHz, DMSO-d_6): δ 152.3 (N1—Pt), 179.2 (N3—Pt). The signals of N1-BI, N3-BI, N5—Pt, N8—Pt have not been observed. HRMS (MALDI-TOF) *m/z*: $[M + H]^+$ Calcd for $C_{19}H_{11}Cl_2N_6OS$ 441.0087, 443.0060; Found 441.0077, 443.0065.

2.1.2. Synthesis of the surfactant

1-Methyl-3-hexadecylimidazolium bromide (IA-16) was synthesized in accordance with standard experimental procedure [73]. A mixture of 0.88 g (0.010 mol) of 1-methylimidazole and 3.60 g (0.011 mol) of 1-bromohexadecane in 15 ml of acetonitrile was refluxed for 24 h and cooled. Acetonitrile was then removed under vacuum, the dry residue was recrystallized from ethyl acetate and dried in vacuum to constant weight. After that 2.86 g (69%) of 1-methyl-3-hexadecylimidazolium bromide was obtained: Mp = 71°C; IR(KBr) cm^{-1}: 3480, 3431, 3084, 3063, 2916, 2851, 1631, 1574, 1474, 1177, 863, 792, 716, 624; ^{1}H NMR (400 MHz, $CDCl_3$, δ-ppm, *J*/Hz): 0.88 (t, 3H, J = 6.6, $\underline{CH_3}CH_2(CH_2)_{13}CH_2$), 1.25–1.35 (m, 26H, $CH_3CH_2(\underline{CH_2})_{13}CH_2$), 1.89–1.95 (m, 2H, $CH_3\underline{CH_2}(CH_2)_{13}CH_2$), 4.14 (s, 3H, CH_3), 4.32 (t, 2H, J = 7.3, $CH_3CH_2(CH_2)_{13}\underline{CH_2}$), 7.33 (br.s, 1H, H4 or H5), 7.46 (br.s, 1H, H5 or H4), 10.40 (br.s, 1H, H2). ESI MS, *m/z*: 307.3 $[M\text{-}Br]^+$.

2.2. Instruments and methods

Melting point was determined on a Boetius melting point apparatus. IR spectrum was recorded on a Tensor 27 (Bruker) FT-IR spectrometer with KBr pellets. NMR experiments were performed with Bruker spectrometer AVANCE(III)-500 (500.1 MHz for ^{1}H, 125.7 MHz for ^{13}C NMR and 50.6 MHz for ^{15}N, respectively) equipped with 5 mm diameter gradient inverse broad band probehead and a pulsed gradient unit capable of producing magnetic field pulse gradients in the z-direction of 53.5 G cm^{-1}, and were carried out at 303 K. Chemical shifts (δ in ppm) are referenced to the solvent DMSO-d_6 (δ 2.49 ppm for ^{1}H and 39.5 ppm for ^{13}C), to external CD_3NO_2 (δ 380.2 ppm) for ^{15}N. High resolution mass spectrum (HRMS) was obtained on a Bruker Ultraflex III MALDI-TOF/TOF mass spectrometer in the reflectron mode.

UV–Vis spectra of studied samples were recorded in 1 cm or 0.10 cm quartz cells using a Specord 250 Plus (Analytik Jena) spectrophotometer equipped with a thermostatic cell unit. The molar extinction coefficient (ε) of the Bi-Qx was determined from the optical density (D), measured at the wavelength corresponding to the absorption maximum from the relation $\varepsilon = D/LC$, where C is the concentration of the probe, and L is the path length. The average values of three to five independent measurements were taken into account, with the reproducibility being of ± 0.05. Solubilization effects toward BP in the micellar systems were determined as described elsewhere [74] by following the change in the absorbance of their saturated solutions with concentration of the surfactant added. The spectra were recorded in the range from 250 to 600 nm using the thermostated quartz cells of a 0.5–1.0 cm path length. The error of all experiments was <4%. The solubilization capacity of micelles (S) was calculated from eq. $S = B/(\varepsilon \times L)$ where B is the slope of the plot $D = f(C)$, D is the absorbance at 405 nm, L is the path length, and C is the concentration of surfactant above CMC.

2.3. Preparation of BP-loaded liposomes

The preparation of surfactant-modified liposomes with loading of BP compound was carried out by the lipid film hydration method [75,76]. The dry amphiphile and lipid were taken in sufficient quantities to obtain the surfactant/DPPC molar ratio of 1:50 (0.16 mg/ml, 15.4 mg/ml) and 1:25 (0.32 mg/ml, 15.4 mg/ml) and dissolved in 100 μl of chloroform. The amount of BP in both systems was 0.9 mg/ml. The lipid concentration in all systems was 21 mM. The system was stored at room temperature for 16 h for complete removal of the solvent. The formed thin lipid film was suspended in water by stirring at 55–60 °C for 30 min. The resulting coarse dispersion was frozen in liquid nitrogen five times and thawed on water bath at 60 °C. Next, the suspension was extruded through an Avanti carbohydrate filter with a pore size of 100 nm using a LiposoFast Basic extruder (Avestin) to obtain monolamellar liposomes.

2.4. Characterization of liposomes

The mean particle size, zeta potential and polydispersity index of liposomes were determined by dynamic light scattering (DLS) measurements using a Malvern Instrument Zetasizer Nano (Worcestershire, UK). The measured autocorrelation functions were analyzed by Malvern DTS software, applying the second-order cumulant expansion methods. The effective hydrodynamic radius (R_H) was calculated according to the Einstein-Stokes equation $D = k_BT/6\pi\eta R_H$, in which D is the diffusion coefficient, k_B is the Boltzmann's constant, T is the absolute temperature, and η is the viscosity. The diffusion coefficient was measured at least in triplicate for each sample.

Three measurements of electrophoretic mobilities for each sample were converted into zeta potential by using the Smoluchowski equation [77]: $\zeta = \mu\eta/\varepsilon$, where ζ is the zeta potential, η is the dynamic viscosity of the fluid, μ is the particle mobility and ε is the dielectric constant.

The average error of measurements was approximately 4%. All the samples were diluted with ultra-purified water to suitable concentration and analyzed in triplicate.

The transmission electron microscopy (TEM) images were obtained with Hitachi HT7700, Japan. The images were acquired at an accelerating voltage of 100 kV. Samples were dispersed on 300 mesh copper grid with continuous carbon-formvar support films.

2.5. Encapsulation efficiency and loading capacity

Encapsulation efficiency (EE, %) and loading capacity (LC, %) were assessed for samples containing BP. These parameters were determined indirectly by centrifugation and measurement of free BP (non-encapsulated) by spectrophotometry. 1 ml of BP-loaded liposomes was placed in 2 ml Eppendorf tube and centrifuged (Eppendorf AG, Hamburg, Germany) for 10 min at 10000 rpm to remove precipitated free BP compound. Then a solution with BP-loaded liposomes was taken from the system, without touching the precipitated drug. Next, 1 ml of water was added, centrifugation was performed again and water was removed. The precipitated BP was dissolved in alcohol and measured spectrophotometrically using a Specord 250 Plus (Analytik Jena AG, Germany). For accuracy, this procedure was repeated three times. The extinction coefficient in alcohol for BP is 15,428 at λ = 401 nm.

The encapsulation parameters were calculated from appropriate calibration curve using the following equations:

$$EE(\%) = \frac{Total\ amount\ of\ BP - Free\ BP}{Total\ amount\ of\ BP} \times 100\% \quad (1)$$

$$LC(\%) = \frac{Total\ amount\ of\ BP - Free\ BP}{Total\ amount\ of\ lipid} \times 100\% \quad (2)$$

2.6. In vitro BP release profile

The release of BP from liposomes was performed using the dialysis bag diffusion method. Dialysis bags retain liposomes and allow the released drug to diffuse into the medium. The bags with pore size of 2–4 kDa were soaked in Milli-Q water for 12 h before use. 2 ml of BP-liposomes was poured into the dialysis bag. The bag was then placed in a vessel containing 30 ml ethanol and 20 ml of 0.025 M sodium phosphate buffer (PBS) adjusted to pH 7 with a minor volume of 10 M NaOH as the receiving phase. Dialysis was performed on a magnetic stirrer Heidolph

MR Hei-Standart at 37 °C at a stirring rate of 250 rpm. At predetermined time intervals, 2 ml of solution was withdrawn and analyzed by determining the absorbance at 401 nm. Then the samples were returned to the system. All the samples were analyzed in triplicate.

2.7. Cytotoxic effects of the BP-liposomes on human cell lines

The liposomes were evaluated for their cytotoxic effects against human cancer cell line M-Hela (carcinoma of the cervix) and human normal liver cells (Chang liver). M-Hela was obtained from the Collection of the Institute of Cytology of the Russian Academy of Science (St-Petersburg, Russia). Chang liver was acquired from the Collection of the Research Institute of Virology RAMS. Cells were cultured in a standard Eagle's nutrient medium manufactured at the Chumakov Institute of Poliomyelitis and Virus Encephalitis (PanEco) and supplemented with 10% fetal bovine serum and 1% nonessential amino acids. Cells were seeded in 96-well plates at a density of 100,000 cells per well and they were incubated at 37 °C with 5% CO_2 for 24 h. In each well, 150 μl of the investigated compound was added to the culture medium. Dilutions of the compound were prepared directly in culture medium. The cytotoxic activity was determined by calculating the viability of human cancer and normal cell cultures by the Cytell Cell Imaging (GE Healthcare Life Science, Sweden) multifunctional system, using the Cell Viability BioApp application, which makes it possible to precisely count the number of cells and estimate their viability from the fluorescence intensity.

3. Results and discussion

A new conjugate of pteridine and benzimidazole 7-(benzimidazol-2-yl)-6-(2,4-dichlorophenyl)-2-thioxo-2,3-dihydropteridin-4(1*H*)-one (BP) with potential cytotoxic activity was synthesized. The structure of this compound was confirmed by a number of physicochemical methods (see experimental part). The estimation of its cytotoxic effects against human normal and human cancer cell lines was carried out. The experiments were performed using DMSO due to the very the concentration values at which 50% of viable cells die (IC_{50}) were 54.0 ± 4.1 μM and 61 ± 5.2 μM for human cancer cell line M-Hela and human normal liver cells (Chang liver), respectively. However, in order to realize the full potential of using this compound in fight against the malignant cells, it was necessary to convert this compound into water-soluble and, therefore, more bioavailable forms.

3.1. Determination of BP solubility in water and micellar solutions

To investigate the solubility of BP in solutions, a spectrophotometric method of analytical control over its content in various systems was developed. The position of the absorption bands in the spectra of BP depends on the pH of the medium, since this molecule has several centers involved in acid-base interactions, which leads to the appearance of various forms having their own spectral characteristics. These are weakly basic amino groups of the pteridine and benzimidazole cycles protonated in acidic media, as well as N—H groups capable of deprotonation in alkaline media. It was not possible to deter mine and correlate the particular pK values for BP, but we have used as reference points reported pK values of 4.79 and 11.21 for pteridine [78], as well as 5.6 and 12.8 for benzimidazole [79]. The combination of these two cyclic fragments in one BP molecule, the introduction of substituents into the molecule, as well as the transition from water to micellar solutions, should affect the acid-base equilibrium of this compound. The variety of possible forms can be reflected in its spectral characteristics. In this regard, the constant monitoring of pH in tested solutions during the experiment is requested. The effect of pH on the maximum position and the absorption intensity of BP aqueous solutions with and without surfactant additives is shown in Figs. 1, S1–S3 and Table S1.

The obtained data indicates that there are two intense absorption bands in the region of 280–330 nm and 385–430 nm in the spectra of BP solutions. When going from acidic to neutral solutions, a bathochromic shift of the maximum absorption for these bands is observed, and deprotonation of BP in strongly basic solutions leads to hypsochromic shift. The largest shift is typical for the short-wave band up to 30 nm, while the shift of the long-wave band does not exceed 10 nm. The trends in absorption observed in aqueous and micellar solutions of BP are the same, but the ranges of existence of various forms are slightly different. Based on analysis of obtained data, it can be concluded that the spectral characteristics of BP remain unchanged at pH from 4.5 to 9 and it can be assumed that the compound exists predominantly in a neutral form in this range. The values of maximum absorption and the corresponding molecular extinction coefficients of BP in different media at pH 6.86 (phosphate buffer) are given in Table S1. When determining the content of this compound in solutions, a longer wavelength absorption band that is less sensitive to the state of BP molecule was used as an analytical signal.

To increase the BP content in aqueous solutions, we have used the ability of supramolecular systems based on surfactants to solubilize low-polar organic compounds. The solubilization process is the distribution of hydrophobic compounds between the dispersed phase and the dispersion medium, which leads to an increase of their solubility due to the localization in hydrophobic domains of supramolecular systems, particularly in the lipid bilayer, non-polar core of micelles or microemulsions [80–83]. The ability of supramolecular systems to act as nanocontainers immobilizing the target compound and ensure its controlled release from the aggregate underlies the action of a large number of drug delivery systems that provide a high therapeutic effect when the loaded nanocontainer interacts with biotarget [84–88].

We have investigated low toxic non-ionic surfactant, Tween 80, widely used in medicine and pharmacology as solubilizer, as well as cationic surfactants: cetyltrimethylammonium bromide (CTAB) and its analogue cetylmethylimidazolium bromide (IA-16). To determine the solubilization activity of micellar systems, the electron spectroscopy was used, which makes it possible to simply and reliably determine the BP content in solutions. Considering that this compound is practically insoluble in water (the maximum attainable content is ~$2.5 \cdot 10^{-6}$ M), the significant increase in the optical density (D) of its saturated solutions with the addition of surfactants at the concentration greater than CMC reflects the micellar solubilization of this hydrophobic substance. As an example, Fig. S4 shows the BP spectra in IA-16 solutions. At a concentration three times greater than CMC in the case of Tween 80, the BP solubility doubles, and in the case of CTAB it increases sixfold.

The dependences $D/l = f(C_{surf})$ (Fig. S5) formed the basis for determination of solubilization capacity (S) of the micellar system: $S = b/\varepsilon$,

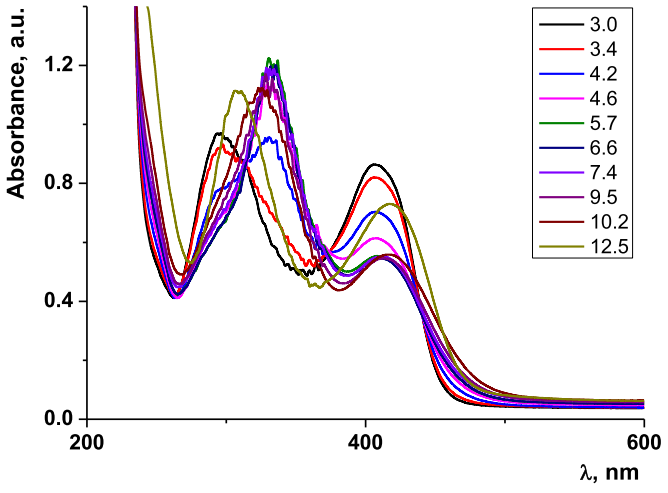


Fig. 1. BP absorption spectra in IA-16 solution (3 mM) at different pH.

Table 1
Solubilization capacity of micellar solutions toward BP.

Surfactant	b	Maximum achievable BP concentration at $C_{surf} = 3$ CMC, M	S, mole $_{BP}$/mole $_{surf}$
Tween 80	140	$6.5 \cdot 10^{-6}$	0.009
CTAB	240	$4.8 \cdot 10^{-5}$	0.016
IA-16	332	$7.4 \cdot 10^{-5}$	0.021

where b is the slope of the linear part of the dependence, ε is the molecular extinction coefficient.

According to the obtained data (Table 1) the solubility of BP increases significantly when going from non-ionic surfactant to cationic surfactant. The S value in solutions is almost an order of magnitude higher than for Tween 80, which may be due to electrostatic interaction between the solubilizate and the micelle. However, in this case it was not possible to achieve sufficient concentration to obtain a cytotoxic effect.

3.2. Synthesis and properties of hybrid liposomes

As an alternative carrier for BP drug, the liposomes based on 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) modified by cationic surfactant additives and loaded with BP were obtained and characterized (Table 2). Cetyltrimethylammonium bromide and cetylmethylimidazolium bromide were used as cationic surfactants. The choice of the imidazolium amphiphile was due to the fact that it proved to be a surfactant exhibiting unusual behavior with biological systems [72,74,89,90]. The molar ratio of surfactant/lipid was 1:50; 1:25; 1:10. The resulting hybrid liposomes were characterized by a positive electrokinetic potential, which ranged from +30 to +70 mV and differed from the individual DPPC liposomes in high time stability (Fig. S6). It was shown by the dynamic light scattering method that the hydrodynamic diameter of modified liposomes is at the level of 80–100 nm and has a narrow size distribution (PDI < 0.2). These values varied slightly for at least 4 months (Fig. 2), whereas unmodified liposomes were destroyed in two weeks.

Prepared liposomes were visualized using transmission electron microscopy technique (Fig. 3). As could be seen, diameter of particles fabricated reaches approximately 150 nm, that is 1.5-fold higher than hydrodynamic diameter obtained by DLS technique. This contradiction in data obtained by two various techniques could be due to conditions of sample preparation for TEM images registration: drying of liposomes at room temperature could be a cause of its agglomeration and increase of its size.

Encapsulation efficiency and loading efficiency of BP in liposomes were characterized quantitatively using spectrophotometric method by varying the surfactant/lipid ratio (Table 2). It was found that a factor contributing to an increase in BP encapsulation parameters is a growth of the lipid component content (Table 2). The highest parameters are achieved for IA-16/lipid system with 1:50 molar ratio. The encapsulation efficiency and the load efficiency calculated for this system are 88% and 5%, respectively. Such a big difference between these parameters is due to the fact that the hydrophobic layer of the liposome has a limited load, so the lipid mass was much greater than substance mass. For comparison, BP was loaded into liposomes modified with conventional ammonium surfactant CTAB with the same molar ratio of components. It was shown that the encapsulation characteristics of hybrid liposomes containing IA-16 are higher than that of liposomes containing CTAB (Table 2).

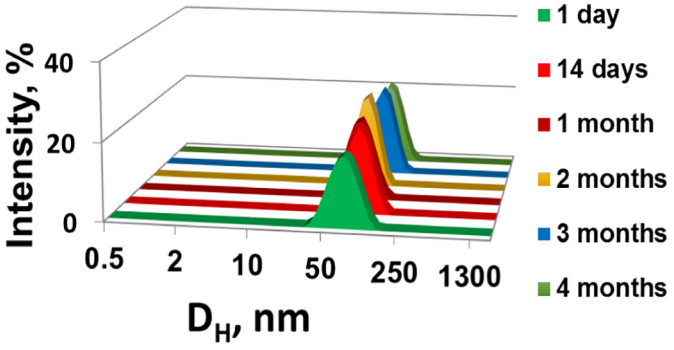


Fig. 2. Intensity-averaged particle size distribution for IA-16/DPPC/BP systems (amphiphile/lipid molar ratio is 1:50) at different storage time, 25 °C.

Evaluation of the release of the investigated compound from liposomes was carried out by the method of dialysis, which has proven effective for the investigation of the release of drugs from nanoscale systems in vitro. Due to the fact that BP is practically insoluble in water, the mixed solvent ethanol/phosphate buffer (pH 7.0) at the ratio of 3:2 (vol.) was used as the medium. The amount of released BP was estimated using the spectrophotometric method by recording electronic absorption spectra at the certain time intervals (Fig. 4).

In Fig. 5 BP release profiles for liposomes modified with IA-16 at the molar ratio of 1:50 surfactant/DPPC are presented. The figure shows that the use of a hybrid liposomal carrier can significantly prolong the time of drug release. Thus, the release time of non-encapsulated BP is approximately 1 h, whereas its encapsulation in IA-16/DPPC liposomes at the molar ratio of 1:25 increases the time of complete drug release up to 3 days, and in the case of the molar ratio of 1:50 this time increases up to 5 days.

Further, the investigation of cytotoxic effect of BP loaded into liposomes on the human normal and tumor cell lines was carried out. Cytotoxicity was studied by taking the various concentrations suitable for screening of new antitumor agents (100–1 μM). The dilutions of the BP was implemented immediately in nutrient media; for this purpose, 5% DMSO was added for better solubility that does not induce the inhibition of cells. While single BP solution in DMSO exhibits cytotoxicity on the M-Hela tumor line at a concentration of 54.0 ± 4.1 μM and more than half of healthy cells die, the encapsulation of BP in modified liposomes significantly changes this effect. Firstly, cytotoxicity with respect to the M-Hela tumor line is significantly increased, with the IC_{50} values of BP in liposomes being comparable with the data for the doxorubicin used in cancer treatment. Secondly, BP in liposomes is less toxic to the normal Chang liver cell line (Table 3), which is beneficial result over the commercial drug doxorubicin.

To obtain additional information, cytotoxicity of BP liposomal formulation versus micellar BP solution was tested (Table 3). Single IA-16 systems are revealed to show high cytotoxicity toward both tumor and normal cells: IC50 equals 4.4 μM for M-Hela and 3.3 μM for Chang liver cell, whereas some selectivity appeared in the presence of BP.

Table 2
Physicochemical characteristics of liposomes based on DPPC modified by cationic surfactants and containing BP loaded in them: hydrodynamic diameter (D_H), polydispersity index (PdI), zeta potential (ζ), encapsulation efficiency (EE), loading efficiency (LE).

System	Molar ratio of the components	D_H, nm	PdI	ζ, mV	EE, %	LE, %
DPPC	–	80 ± 1	0.094 ± 0.014	5.0 ± 1		
IA-16/DPPC	1:50	92 ± 3	0.126 ± 0.020	32 ± 1	88%	5.6%
	1:25	105 ± 5	0.088 ± 0.015	60 ± 2	40%	2.3%
	1:10	102 ± 3	0.135 ± 0.018	71 ± 2	28%	1.6%
CTAB/DPPC	1:50	91 ± 4	0.143 ± 0.010	43 ± 1	49%	2.8%

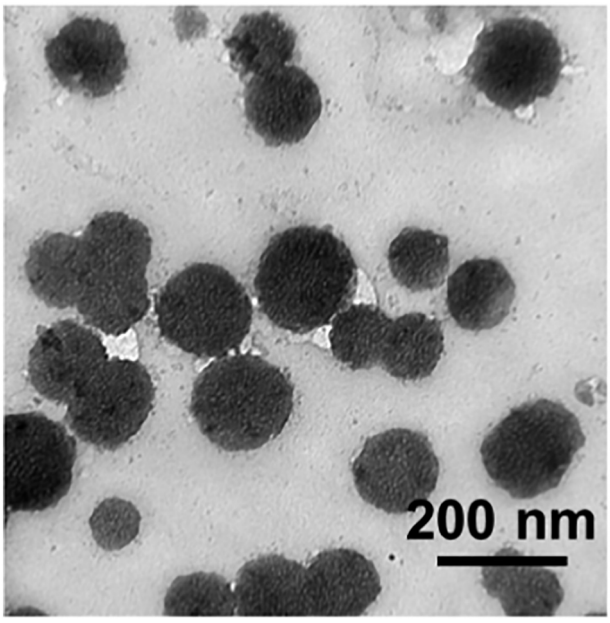


Fig. 3. Transmission electron microscopy image for IA-16/DPPC liposomes (amphiphile/lipid molar ratio is 1:50), 25 °C.

This is evident from the tests of two samples of micellar systems loaded with BP. One sample is prepared at the same IA-16/BP ratio as in the case of liposomes, while another sample contained IA-16 in a 3 cmc concentration and the greatest possible BP amount. Since both components show cytotoxic effect, values of IC_{50} are annotated in the table with the corresponding concentrations of surfactant and BP. Data in Table 3 testify that an increase in the fraction of BP results in an enhancement of the selectivity of toxic effect. Noteworthy, in the course of the assay initial micellar systems underwent the multiple dilutions, so that micelle-to-monomer transition can occur. Therefore, both aggregated and free surfactant molecules can be responsible for the IC_{50} values determined. Liposomes loaded with BP are characterized by lower IC_{50} values compared to micellar systems, with no toxic effect revealed toward normal cells.

Thus, liposomal BP formulations demonstrate obvious advantages over free and micellar bound BP, which is testified by higher stability of liposomes in time and under dilution, selectivity of toxic effect, and wider concentration range of BP loading.

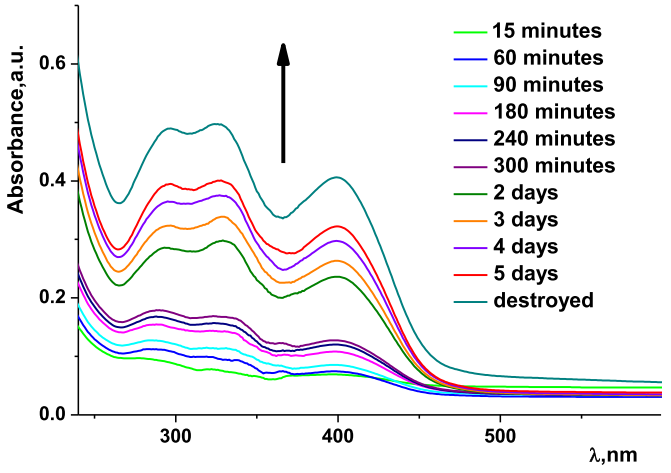


Fig. 4. Electronic absorption spectra of BP released from the modified IA-16/DPPC liposomes, with a molar ratio of 1:50 and different dialysis time; 37 °C (arrow indicates the direction of increase in the dialysis duration).

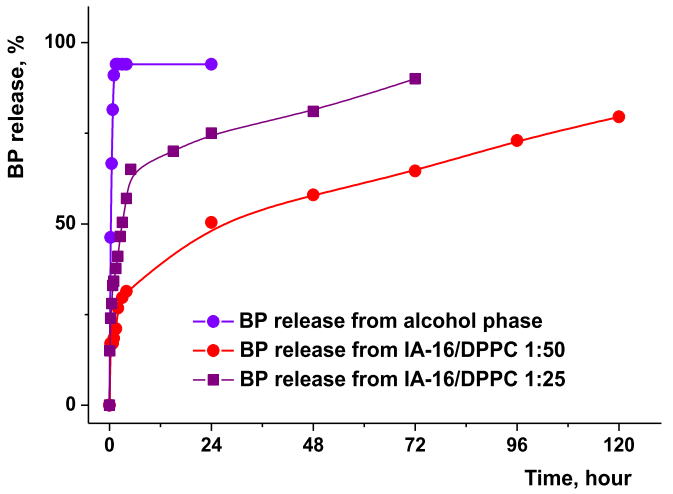


Fig. 5. Dependence of BP release degree on time in the presence and in the absence of IA-16/DPPC modified liposomes at the molar ratio of surfactant/DPPC 1:50 and 1:25, pH 7, 37°C.

4. Conclusion

In summary, new conjugated derivative of pteridine and benzimidazole, 7-(benzimidazol-2-yl)-6-(2,4-dichlorophenyl)-2-thioxo-2,3-dihydropteridin-4(1*H*)-one having antitumor activity, was obtained and characterized. Two types of soft nanocarriers based on synthetic and natural amphiphilic building blocks, i.e. micelles and liposomes, were fabricated to increase the solubility and loading efficacy of hydrophobic newly synthesized antitumor therapeutics, BP. Superior results from the viewpoint of long-term stability, encapsulation efficacy and prolonged release of the load were obtained for the hybrid liposomes based on DPPC modified by cationic surfactants, with the optimized surfactant/lipid molar ratios equaling 1:50. It was shown that liposomes with encapsulated BP have cytotoxicity toward M-Hela tumor cells at the level of commercial doxorubicin drug, but are less toxic (37 times) to the normal Chang liver cell line.

CRediT authorship contribution statement

Alla B. Mirgorodskaya - systematization of the majority of data obtained, analysis of literature data and preparation of manuscript for publication.

Darya A. Kuznetsova - the formation of hybrid liposomes, determination of their efficiency of loading and encapsulation, study of the release of the drug.

Rushana A. Kushnasarova - performing of experiments on conjugate BP solubility in water and micellar solutions.

Dinar R. Gabdrahmanov - performing of experiments on dynamic and electrophoretic light scattering for IA-16/DPPC hybrid liposomes.

Table 3
Cytotoxic effect of BP and IA-16 in different compositions toward the normal and tumor human cell lines.

N	Compound	IC_{50} (μM)	
		Tumor cell lines	Normal cell lines
		M-Hela	Chang liver
1	BP in DMSO	54.0 ± 4.1	61 ± 5.2
2	BP in liposomes	7.9 ± 0.7	>100
3	Doxorubicin	3.0 ± 0.2	3.0 ± 0.1
4	IA-16 in water	4.4 ± 0.3	3.3 ± 0.2
5	IA-16 (0.42 mM)/BP (0.02 mM)	9 ± 0.7/0.42 ± 0.03	90 ± 7.3/4 ± 0.3
6	IA-16 (2 mM)/BP (0.06 mM)	9 ± 0.8/0.26 ± 0.02	50 ± 5.8/1.5 ± 0.2

Nataliya A. Zhukova - synthesis of new conjugated derivative of pteridine and benzimidazole.

Svetlana S. Lukashenko - synthesis and characterization of methyl-3-hexadecylimidazolium bromide.

Anastasia S. Sapunova - obtaining of experimental data on cytotoxicity of systems.

Alexandra D. Voloshina - systematization of data and preparation of the part of the manuscript dedicated to cytotoxicity.

Oleg G. Sinyashin - valuable recommendations on the synthesis of organic compounds used in the work.

Vakhid A. Mamedov - development of a methodology for the synthesis of heterocyclic compounds with antitumor properties.

Lucia Y. Zakharova - systematization of data, analysis of literature data and preparation of the part of the manuscript dedicated to physicochemical characteristics of liposomes.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molliq.2020.114007.

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