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Chlorambucil labelled with the phenosafranin scaffold as a new chemotherapeutic for imaging and cancer treatment

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ABSTRACT

Here we report the first of the phenosafranin-chlorambucil conjugate as a new type of a chemotherapeutic agent suitable for dual detection methods (spectrophotometric and fluorescence) in imaging systems and cancer treatment. The synthetic cationic dye (3,7-diamino-5-phenylphenazinium chloride) is used as a fluorescent light-triggered scaffold that acts as a carrier for an anti-cancer drug. The chlorambucil was attached covalently *via* amide bonds to the bifunctional fluorophore, which facilitates tracking with visible light. Our studies revealed that the new photosensitive compound exhibits improved intrinsic activity *in vitro* in HeLa cells culture experiments; thus it could be a potential anti-cancer candidate in theranostic drug-delivery systems. In light of the urgent need for *in vivo* monitoring of the biodistribution of anti-cancer drugs, this strategy for the synthesis of innovative conjugates based on the phenosafranin backbone offers a promising possibility for drug control in anti-cancer therapy and diagnosis. This aspect makes the phenosafranin-chlorambucil conjugate unique among currently available biomarkers.

1. Introduction

Phenazine derivatives are an antibiotic drug possessing anti-malarial potency [1]. Moreover, phenazine dyes and their structural analogues can be optically induced to generate long-lived electronic excited states which make them ideal as photosensitizers in energy and electron transfer reactions [2]. Thus, phenosafranin (PSF, 3,7-diamino-5-phenyl phenazinum chloride) has potential for use as a photosensitizer in photodynamic therapy [3]. It is well known that PSF absorbs strongly in the region of visible light at 503–530 nm; the fluorescence spectrum appears in the 530–700 nm wavelength range with a maximum at λ_{Em} = 570 nm when excited at λ_{Ex} = 520 nm [4,5]. Additionally, this dye shows resistance to photobleaching by auto-oxidation and presents a good photodynamic effect, namely low toxicity in the dark and higher toxicity under optical excitation. Photosensitizers activated by light can induce the formation of singlet oxygen or other free radicals, which can irreversibly damage the treated tissue [6,7]. It is generally accepted that singlet oxygen is the primary cytotoxic agent responsible for photobiological activity [8]. This is the background for photodynamic therapy, which uses lasers, or other light sources, combined with a light-sensitive drug (as a photosensitizing agent) to destroy cancer cells. PSF has also been established as a DNA intercalator [9–12], facilitating an intercalative interaction by its planar tricyclic phenazinium structure with a positive charge on the nitrogen atom [13–16]. Most small-molecule anti-cancer drugs can interact with DNA [17,18] through noncovalent bonding interactions, including electrostatic interaction, groove binding and intercalation [19]. Thus these chemotherapeutic agents inhibit the synthesis of deoxyribonucleic acid [20,21]. In general, planarity and charge delocalization are important features needed for efficient intercalators [22]. The binding mode of PSF with DNA is intercalative with an electrostatic component [23]. Chlorambucil (Chl) is an anti-cancer drug that produces therapeutic effects in chronic lymphocytic leukemia. Moreover, Chl can be used to treat different types of cancer because it is an alkylating antineoplastic agent; it stops tumor growth by cross-linking guanine bases in DNA double-helix strands. This makes the strands unable to uncoil and separate, so the cells can no longer divide and it causes their death [24]. Unfortunately, its absorption maxima at λ = 258 nm and λ = 303 nm [25,26] are unsuited to pathway monitoring *in vitro* cell culture studies, because the spectral range of the Chl molecule is in the same region as autofluorescence of cells and tissues. In this report we offer a new DNA intercalating conjugate for anti-cancer drug design based on the substitution of Chl onto the cationic PSF fluorophore. This conjugate can cause pho-

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tosensitization involving energy transfer from the photoexcited molecule to cell tissue, including direct destruction of cancer tissues. This photoreactivity, together with the fluorescent properties of PSF, give PSF-Chl both therapeutic and diagnostic capabilities. Such compounds could enable simultaneous cancer treatment and imaging, enabling direct monitoring of the therapeutic effect. To the best of our knowledge only Singh at al. [27] described a fluorescent conjugate of Chl linked with the benzothiazole-coumarin analogue as a theranostic anti-cancer drug sensitive to photolysis by using soft UV irradiation at 365 nm and developed also acridin9-methanol/chlorambucil nanoparticles [28]. Shunmugam et al. considered Chl covalently attached to polymeric nanoaggregates embedded with the fluorescent pyrene motif [29]. They also described a multi-anticancer prodrug including doxorubicin with Chl attached to the backbone of a norbornene polymer [30]. In this report we present a new paradigm for the synthesis of fluorescent drugs based on the PSF scaffold. In our work we also investigated *in vitro* cytotoxic effects of PSF-Chl with the potential to be applied in photodynamic therapies. We demonstrate the PSF moiety as a "phototrigger" for delivery of Chl to help identify its location as well as facilitate its control within cells [31].

2. Materials and methods

2.1. Materials

Organic dye phenosafranin hydrochloride (PSF) (CAS No. 81-93-6, colour index number: 50200) (3,7-diamino-5-phenylphenazinum chloride), N-hydroxysuccinimide (NHS) (1-hydroxy-2,5-pyrrolidinedione), chlorambucil (Chl) (4-(4-[Bis(2-chloroethyl)amino]phenyl)butyric acid), *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC), Sephadex G-50, and all other chemicals as organic solvents *e.g.* acetone, dimethylformamide (DMF) used in this work were reagent grade quality, obtained from Sigma-Aldrich (St. Louis, MO), and used as received without further purification. Phospholipids (1,2-dimyristoyl-*sn-glycero*-3-phosphocholine (DMPC) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL., USA) as a dry powder.

2.2. Synthesis of chlorambucil-N-hydroxysuccinimidyl ester (Chl-NHS)

The esterification reaction between the carboxylic group of Chl (0.1 g, 3.29×10^{-4} M) and the hydroxyl group of *N*-hydroxysuccinimide (NHS, 0.151 g, 1.315×10^{-3} M) was made by using water-soluble carbodiimide: *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC, 0.204 g, 1.31×10^{-3} M) in DMF (5 ml) as solvent (pH = 5.5–6 adjusted by 1N HCl) with addition of a small amount of water to dissolve EDC. The solution was stirred overnight at 5 °C then DMF was evaporated under vacuum and the obtained chlorambucil-*N*-hydroxysuccinimidyl ester (Chl-NHS) was washed using 0.1 M phosphate buffer (pH = 6) to remove urea. The product isolated by recrystallization in isopropanol gave Chl-NHS in 74% yield. [^{1}H]-NMR 6.85, ^{4}H, 2 doublets, arom.; 3,65, ^{8}H, s,(Cl-CH_2-CH_2-)$_2$; 2.85, ^{4}H, s, −ON-(CH_2CO_2)$_2$; 1.5–2.7, m ^{6}H, (CH_2)$_3$.

2.3. Synthesis of phenosafranin-chlorambucil conjugate (PSF-Chl)

The reaction of obtained Chl-NHS (m = 0.094 g, 2.344×10^{-4} M) with amino groups of PSF (m = 0.04 g, 1.239×10^{-4} M) was carried out with magnetic stirring for 24 h in DMF (pH = 8–9 adjusted by NaOH) at 15 °C. At pH 6–7, primary amines like PSF are mostly protonated (*i.e.* positively charged) and are not reactive for the nucleophilic acyl substitution reaction with NHS ester, whereas at pH 8–9 the primary amine retains sufficient electron density to react rapidly [32]. The reaction scheme is illustrated in Fig. 1. The PSF-Chl conjugate was precipitated in deionized water and separated by centrifugation. Unbound dyes were removed by washing. This procedure was repeated several times using centrifugation until the supernatant showed no absorbance in the region of visible light. Additionally, the product dissolved in acetone was passed through a Sephadex G-50 resin, then dried under vacuum. We established the molecular weight of PSF-Chl using the matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) technique with application of 2,5-dihydroxybenzoic acid (DHB) as a matrix. The PSF-Chl shows a mass spectral molecular ion for the cation (m/z 860) implying that both amino groups of PSF reacted with the Chl-succinimidyl ester. The experiment was prepared using Shimadzu Biotech AXIMA Performance (Kyoto, Japan).

2.4. Liposomes preparation

The lipid solution was prepared following the same procedure as described in our previous work [33–35]. A defined amount of DMPC lipids (42.8 mg, 6.313×10^{-5} M) in chloroform solution with addition of PSF-Chl (6.0 mg, 6.9×10^{-6} M), Chl (2.1 mg, 6.9×10^{-6} M) or PSF (2,4 mg, 7.4×10^{-6} M) was dried under a stream of nitrogen, then under vacuum. The dry film was hydrated by using 2 ml of deionized water, swelled and vortexed rigorously to result in a dispersion of multilamellar vesicles. The dispersion has undergone a freeze-thaw process (10-fold) then extruded (30 times) at 30 °C through a polycarbonate nucleopore track-etch membrane (Whatman, with 0.1 μm pore size) using a Lipex stainless steel extruder (Northen Lipids). Non-entrapped compounds were separated using size-exclusion chromatography with applications of Sephadex G-50. The concentration of PSF-Chl solution was determined by UV absorbance measurements in acetone. The total concentration of the PSF-Chl conjugate (c_t) is determined as $c_t = (A_{486})/(\varepsilon_{486})$. (The molar extinction coefficient (ε) of PSF-Chl is 5500 L(M cm)$^{-1}$ at $\lambda_{Ab.max}$<span class=ẍps_thinspaceẍ = 486 nm, and A is the measured absorbance). The concentration of entrapped ingredients in liposomes were estimated from the calibration curves as follows: PSF-Chl = 2.4×10^{-3} M L^{-1}, PSF = $3,4 \times 10^{-3}$ M L^{-1} [36], and Chl = $3,3 \times 10^{-3}$ M L^{-1} [24].

2.5. Determination of the liposomes size distribution and zeta potential

The size, size distribution and the zeta potential of the lipid vesicles were determined using dynamic light scattering (DLS) measurements. The zeta potential of the liposomes was determined from electrophoretic mobility measurements with the Zetasizer-Nano-Z (Malvern Instrument, UK). Analyses of liposomes dispersed in 1.0×10^{-3} mol/L aqueous NaCl were prepared at 25 °C. Data were analyzed using a cumulative method from five measurements for each sample. The hydrodynamic diameter of liposomes was evaluated from the diffusion coefficient of spherical aggregates (vesicles) calculated according to the Einstein-Stokes equation.

2.6. Microscopy measurements

Transmission electron microscopy (TEM) was performed using a TESLA BS500 electron microscope operating at 90 KV. A dispersion of liposomes in water was collected using carbon-film-covered copper grids for analysis.