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Development and characterization of miltefosine-loaded polymeric micelles for cancer treatment

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ABSTRACT

Miltefosine presents antineoplastic activity but high hemolytic potential. Its use in cancer has been limited to treating cutaneous metastasis of breast cancer. To decrease hemolytic potential, we developed a formulation of miltefosine-loaded polymeric micelles (PM) of the copolymer Pluronic-F127. A central composite design was applied and the analysis of variance showed that the optimum level of hydrodynamic diameter and polydispersity index predicted by the model and experimentally confirmed were 29 nm and 0.105, respectively. Thermal analyses confirmed that miltefosine was molecularly dispersed within PM. Pluronic-F127 PM with miltefosine 80 μM presented a significant reduction of hemolytic effect (80%, $p < 0.05$) in comparison to free drug. *In vitro* assays against HeLa carcinoma cells demonstrated similar cytotoxicity to free miltefosine and PM. Our results suggest that, by lowering hemolytic potential, miltefosine-loaded Pluronic-F127 PM a promising alternative to broaden this drug use in cancer therapy, as well as of other alkylphosphocholines.

1. Introduction

Miltefosine (MTF) is an alkylphospholipid drug (APL) used orally for the treatment of leishmaniasis and topically to treat skin metastases of breast cancer [1]. This drug class also has potential to treat fungal and bacterial infections, as well as Chagas' disease [2]. Unlike other DNA-targeting anticancer agents, APL drugs are involved in phospholipid metabolism, non-vesicular cholesterol transport and homeostasis, biochemical survival pathways (for example, Akt-mTOR pathway), and interaction with membrane signal transduction proteins, such as phospholipase C, phospholipase D and protein kinase C. However, the precise mechanism of action has not been fully elucidated yet [3].

Miltefosine presents potent antitumor activity *in vitro* [4] and in experimental animal models. Nevertheless, clinical use is limited due to side effects associated with its amphiphilic nature [5–7]. More specifically, MTF is highly hemolytic when administered intravenously and orally it was associated with cumulative gastrointestinal toxicity. These side effects, correlated to the drug aggregation into micelles, have limited the maximum daily dose by these routes (maximum tolerated dose: 200 mg/day), preventing *in vivo* observation of antiproliferative effects [6–8]. One strategy to improve the efficacy and safety of oral and/or intravenous cancer treatment with MTF refers to nanotechnology. More specifically, MTF incorporation in nanocarriers may prevent its self-aggregation into micelles and, therefore, the above-mentioned side effects.

In the last decade, nanocarriers have been widely used in anticancer systems/formulations to avoid drug contact with healthy tissues and allow drug accumulation in tumor cells [8]. The incorporation of drugs in nanocarriers enhances solubility and blood half-life, promoting controlled and site-specific release. In addition, combined therapy can be achieved by incorporation of more than one drug [9–11]. The first application of nanocarriers for MTF delivery dates back to 1991 when it

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was incorporated in liposomes [12]. Thereafter, a series of formulations based on liposomes, pegylated or not, were developed with phospholipids and MTF as bilayer forming constituents [7,13–15]. However, poor stability of liposomal delivery systems limits their use in drug delivery; few liposome-based marketed products are available regardless of extensive and long research in this area [2]. On the other hand, polymeric micelles are an interesting alternative to prevent MTF side effects and, to the best of our knowledge, there are no previous reports using polymeric micelles for the delivery of MTF.

Polymeric micelles (PM) are unique core-shell nanostructures formed by amphiphilic copolymers aggregation that can incorporate poorly soluble drugs [16–20]. Amphiphilic block copolymers have the ability to self-assemble into PM in aqueous media and they have been widely studied in the field of nanomedicine, biomedicine and pharmacy [20–22]. The PM represent thermodynamic aggregations of multi amphiphilic macromolecules above their critical micelle concentration (CMC) [23].

An important property of PM is their size, which usually ranges from 10 to 80 nm, thereby closing the gap between individual macromolecule drug carriers (albumin, dextran, and antibodies) with sizes bellow 10 nm; and nanocarriers such as liposomes and nanocapsules with sizes of 100–200 nm [24]. Closing this gap is relevant for selected drug administration routes, such as percutaneous lymphatic delivery, or extravasation into solid tumors. Comparing to surfactant micelles (such as MTF micelles), PM are much less hemolytic and kinetically stable. They present slow dissociation that allows them to retain integrity and perhaps drug content in blood circulation above or even below the critical micelle concentration for some time, increasing the chances of reaching the target site before decaying into monomers [20].

One of the most commonly employed polymer class to prepare PM is the Pluronics [25], triblock copolymers of poly(ethylene oxide)–poly (propylene oxide)–poly(ethylene oxide) (PEO-PPO-PEO) with very low CMC values and a solution-gel transition behavior depending on the temperature [26]. Pluronic-based nanocarriers are safe, Food and Drug Administration (FDA) approved and undergo less opsonization than other nanocarriers (since they are sterically stabilized), preventing the subsequent recognition and uptake by macrophages of the reticuloendothelial system (RES). As a consequence, PM of Pluronics may have a reasonably longer half-life in circulation and may deliver the payload to desired sites of action more efficiently [27,28].

Considering the advantages of PM as drug carriers and the potential of MTF as a chemotherapeutic agent for cancer therapy, we developed MTF-loaded PM of Pluronic F127 that showed to be significantly less hemolytic than the free drug. To develop the formulation, a factorial design was employed to evaluate the effect of hydration temperature, stirring speed and stirring time on nanostructure mean size (D_h) and polydispersity index (PI). Our results of *in vitro* activity show that MTF-loaded PM are less hemolytic than the free drug while preserving the antiproliferative activity against tumor cell lines.

2. Materials and methods

2.1. Materials

The miltefosine (MTF) was purchased from Avanti® Polar Lipids, Inc. (Alabama, USA). Pluronic® F127 ($(PEO)_{100}$-$(PPO)_{65}$-$(PEO)_{100}$; Mw: 12,600 Da), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and phosphotungstic acid were supplied by Sigma-Aldrich® (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) was supplied by Gibco® Laboratories (USA). Defibrinated sheep blood was obtained from New Prov® (Pinhais, Brazil). The human cervical carcinoma cell line (HeLa) and the human bronchioalveolar carcinoma cell line (H-358) were obtained from ATCC® (Manassas, VA, USA). All other chemicals were of analytical grade and used without further purification. All experiments were carried out with ultra-purified water (Milli-Q, Millipore®, Bedford, MA, USA).

2.2. Preparation of miltefosine-loaded polymeric micelles

MTF-loaded PM were prepared by thin-film hydration method [29]. Briefly, 3.6 mg of MTF and 90.7 mg of Pluronic F127 were dissolved in 5 mL of chloroform in a round-bottom flask. The solvent was extracted by rotary evaporation (Buchi Rotavapor R-210/215, Buchi®, Switzerland) at 50 °C, 150 rpm, and 100 mBar for 30 min to obtain MTF:polymer matrix forming a thin film. Then, the film was hydrated with phosphate buffer saline (PBS), 10 mM, pH 7.4, at different temperatures, stirring rates and stirring times according to the experimental design detailed in the following section. The micellar dispersions were filtered through a 0.22 μm membrane to remove aggregates. After defining the optimal micellar dispersion, prepared according to the experimental design, the system was freeze-dried (24 h at 0.120 mBar, after freezing at −70 °C overnight) in a Liotop® L101 equipment (Liobras, Brazil).

2.3. Central composite design (CCD)

Preliminary experiments at a fixed Pluronic F127 concentration (7.2 mM) and varying MTF concentration were conducted to define the maximum drug concentration to be incorporated in the polymeric micelles. Then, a central composite design (CCD) was used for the optimization of three independent variables, namely hydration temperature (X_1), stirring speed (X_2) and stirring time (X_3). The micelles mean hydrodynamic diameter (D_h) and polydispersity index (PI) were selected as dependent variables, according to a 2^3 full factorial design matrix generated by Minitab® software (Trial version 17). Response surface methodology (RSM) was used in the optimization of response variables of D_h and PI. For the estimation of the significance and validity of the model, analysis of variance (ANOVA) was applied with 5% significance level, and regression coefficients were calculated. Fisher's *F*-test was performed to test the adequacy of the model.

The D_h and PI values were measured by dynamic light scattering (DLS) in a Zetasizer® Nano ZS (Malvern Instruments, Worcestershire, UK). Analyses were performed at a scattering angle of 90° at 25 °C. Previous to measurement, each freshly prepared sample was diluted two times in PBS to avoid multi-scattering phenomena. The results were expressed as a mean size ± SD for three separate experiments.

2.4. Surface morphology by transmission electronic microscopy (TEM)

The morphology of the MTF-loaded PM obtained from the optimized formulation was evaluated by TEM (JEM-2100, Jeol® Tokyo, Japan). Samples were dropped onto copper coated carbon grids, then stained with phosphotungstic acid solution 2% (w/v) and the excess was wiped by filter paper [30].

2.5. Thermal analysis

Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) were carried out using TA-Instruments® (New Castle, DE, USA) calibrated with indium. Freeze-dried MTF-loaded PM were weighed to place 4 mg for DSC and 3 mg for TGA, and the samples were retained in hermetically sealed aluminum pans. As a control, a pan containing the same amount of free MTF was prepared. Thermal analysis of isolated copolymer Pluronic F127 and the physical mixture of MT and Pluronic F127 (a blend of both solids without interaction media) were also carried out. The dynamic scans were taken in N_2 atmosphere at the heating rate of 10 °C/min.

2.6. Hemolytic potential

The effect of the MTF-loaded PM on erythrocyte membranes integrity was investigated by *in vitro* hemolysis assay [31]. The systems were prepared with MTF-loaded PM and free MT at different drug