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Original article

Improved anti-melanoma effect of a transdermal mitoxantrone ethosome gel

Xiang Yu [a,b,c], Lina Du [a], Yu Li [b], Guiying Fu [b,c], Yiguang Jin [a,c,*]

[a] Department of Pharmaceutical Sciences, Beijing Institute of Radiation Medicine, Beijing 100850, China
[b] Affiliated Hospital of Academy of Military Medical Sciences, Beijing 100071, China
[c] Anhui Medical University, Hefei 230001, China

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ABSTRACT

Melanomas are malignant tumors characterized by early metastasis, rapid development, poor prognosis and high mortality. A highly effective and convenient method is necessary for long-term treatment of melanomas. Mitoxantrone (MTO) was topically applied for melanoma therapy using an MTO ethosome gel. Firstly, an ethosome was prepared from MTO, phospholipids, ethanol and water followed by addition of hydroxypropyl methylcellulose to obtain an ethosome gel. The ethosome was characterized. The cytotoxicity on B_{16} melanoma cells was evaluated on an electrical cell-substrate impedance sensing system with a novel modified chip. In vivo anti-melanoma effect of the ethosome gel was explored. Immunohistochemical and flow cytometric investigations were done. The MTO ethosomes had the size of 78 nm and the zeta potential of −55 mV. The ethosomes were flexible vesicles and showed much higher in vitro permeability across the rat skin than MTO aqueous solutions. The ethosomes had significant cytotoxicity and higher in vivo anti-melanoma effect than MTO solutions. The calreticulin membrane translocation of B_{16} cells was improved by the MTO ethosomes and the cell uptake of MTO was confirmed. The MTO ethosome gel is a promising transdermal delivery system for melanoma therapy with the advantages of non-invasion and no significant side effects.

1. Introduction

The incidence of melanomas is increasing worldwide, particularly in the USA. Despite early detection, appropriate surgical resection and adjuvant therapy, the number of patients dying from this metastatic disease continues to rise. Approximately 80% of all skin cancer-related deaths are attributable to melanoma, although melanoma comprises only 5% of all skin cancers. Nearly 76,100 newly diagnosed cases of melanoma were reported in the USA in 2014 with an estimated 9710 expected deaths [1]. Melanoma is considered one of the most chemotherapy-resistant malignancies. Only three drugs for oral administration, including dacarbazine, hydroxyurea, and interleukin-2 (IL-2), were approved by the FDA for treatment of melanoma though severe side effects possibly happened [2].

Mitoxantrone (MTO) is a synthetic anthraquinone chemotherapeutic drug that can intercalate with DNA to inhibit their synthesis and transcription [3,4]. Additionally, MTO can increase calreticulin (CRT) expression on the cell surface to effectively induce the anticancer immune response [5,6]. MTO is generally administered via the intravenous (i.v.) route and causes some severe side effects that include myelo-suppression and cardiotoxicity [7,8]. Some nanoparticulate systems of MTO were studied, including MTO liposomes and nanoparticles, to target melanoma in vivo [9–11], though they were only administered via i.v. injection.

Ethosomes are the multilayer vesicles filled with much alcohol (20∼45%) [12]. Ethosomes can deliver both hydrophilic and lipophilic drugs through the stratum corneum (SC) into the deep layers of the skin more effectively than traditional liposomes [13,14]. Therefore, ethosomes become an effective transdermal formulation for skin diseases [15]. However, the application of ethosomes on skin-related cancers (e.g., melanoma) is few.

In this study, an MTO ethosome gel was prepared for transdermal melanoma therapy. The in vitro and in vivo studies were performed. Especially, a novel technique, modified electrical cell-substrate impedance sensing (ECIS) method, was firstly used for cytotoxicity evaluation of transdermal delivery systems. This study supplies a non-invasion melanoma therapeutic method without significant side effects.

* Corresponding author at: Department of Pharmaceutical Sciences, Beijing Institute of Radiation Medicine, 27, Taiping Road, Beijing 100850, China.
Tel.: +86 10 88215159; fax: +86 10 68214653.
E-mail address: jinyg@bmi.ac.cn (Y. Jin).

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2. Materials and methods

2.1. Materials

MTO chloride (98% purity) was purchased from Beijing Yikang Co., Ltd. (Beijing, China). Soybean phospholipid was purchased from Taisu Pharmaceutical Co., Ltd. (Shanghai, China). The anti-CRT antibody (primary antibody) was obtained from ABCAM (Boston, MA, USA). The secondary antibody labeled with biotin and streptavidin/horseradish peroxidase was obtained from Zhongshan Biotical Technology Development Co., Ltd. (Beijing, China). All of the other materials were of pharmaceutical or analytical grade.

2.2. Animals

Male six-week-old Balb/C nude mice (20 ± 3 g) and Wistar rats (190 ± 10 g) from the Laboratory Animal Center of the Beijing Institute of Radiation Medicine (BIRM) were used for the ex vivo and in vivo experiments. The abdominal skins were excised from the Wistar rats and the residual fat tissues were carefully removed. All of the animal handling and surgical procedures were conducted in strict accordance with the Guiding Principles for the Use of Laboratory Animals. The animal study was approved by the Animal Care Committee of BIRM. The mice were sacrificed to obtain tissues. All of the studies were conducted in accordance with the Declaration of Helsinki.

2.3. Preparation of MTO ethosome gels

Ethosomes were prepared using a thin film dispersion method [16]. MTO chloride (200 mg), triethylamine (1.0 ml), and soybean phospholipid (250 mg) were dissolved in ethanol (35 ml) in a round flask. Ethanol was removed on a rotary evaporator under vacuum. A homogenous and thin film was formed on the flask wall. An ethanol aqueous solution (5 ml, 20% v/v) was added to the flask followed by water sonication for 15 min at 4 °C to hydrate the film. A homogenous MTO ethosome suspension was obtained. A 10% (w/v) solution of hydroxypropyl methylcellulose (HPMC, RG4T, International Specific Product Co., USA) in a 20% (v/v) ethanol solution was prepared as the gel matrix. The above ethosome suspension was mixed with the HPMC solution (1:1, v/v) followed by slowly stirring until a homogeneous ethosome gel containing 2% MTO was obtained.

2.4. Characterization of ethosomes

Ethosomes were observed on a HITACHI H-7650 (HITACHI, 80 kv, Japan) transmission electron microscope (TEM). The samples were negatively stained with a 2% sodium phosphotungstate solution. A dynamic light scattering method was used to measure size distribution and zeta potentials of ethosomes on Zetasizer Nano ZS (Malvern, UK) at 25 °C.

Entrapment efficiencies of ethosomes were determined with a dialysis method. An ethosome suspension (0.1 ml) was filled into a dialysis bag (cut MW, 3000) that was then immersed in a bottle full of saline (100 ml) and oscillated (120 rpm) at 37 °C for 5 h. MTO in the dialysis solution (10 ml) was determined on an ultraviolet-visible (UV-Vis) spectrophotometer (TU-1901, Beijing Purkinje General Instrument Co., Ltd., China) at 609 nm. The dialysis solution was replaced with a fresh saline solution (100 ml) and continually oscillated for 1 h followed by MTO measurement. The accumulated amount of free MTO was recorded as D_f. Additionally, an MTO ethosome suspension (0.1 ml) was mixed with Triton X-100 (0.1 ml) followed by dilution with saline (100 ml) to release total MTO that was determined as D_t. The entrapment efficiencies of ethosomes were equal to $(D_t - D_f)/D_t \times 100\%$.

2.5. In vitro permeation study

Permeation experiments were performed on the vertical Franz-type diffusion cells (Tianjin Xinzhou Technical Co., Ltd., Tianjin, China) with the diffusion surface area of 1.96 cm^2 and the receptor volume of 17 ml. A piece of rat skin was placed between the donor compartment and the receptor compartment with the SC face in the donor. The receptor compartment was filled with saline (17 ml), maintained at 32 °C, and stirred at 200 rpm. An MTO solution and an MTO ethosome gel (1.5 g), containing 20 mg MTO chloride/g, were added into the donor compartments, respectively. At the predetermined time points (3, 6, 9, 12, 15, 18, 21, and 24 h), a 5-ml aliquot of the solutions in the receptor compartments were withdrawn, and the compartments were refilled with the equivalent volume of saline at the same temperature. The collected solutions were filtered through a 0.45-μm filter and assayed using the spectrometry at 609 nm [17,18].

The steady-state flux (J_{ss}, $\mu g \cdot cm^{-2} \cdot h^{-1}$) at time t represented the slope of the linear plots of the cumulative drug permeation amount (Q_n, $\mu g \cdot cm^{-2}$) as a function of time (t, h) [19]. Q_n was the cumulative MTO permeation amount in the receptor compartment, and calculated according to the following equation:

$$Q_n = \frac{\left[C_n \cdot V + V_0 \sum_{i=1}^{n-1} C_i\right]}{A}$$

where V (ml) was the volume of saline in the receptor compartment, C_n (mg/ml) was the concentration of sample n, C_i (mg/ml) was the concentration of sample i, V_0 (ml) was the withdrawn volume, and A (cm^2) was the permeation area [20].

2.6. In vitro cellular electrical impedance study

An ECIS system (ACEA, USA) consisted of an ECIS device and a chip. The chip has ten holes and cells may grow on the bottoms. Few cells lead to high electric impedance. However, the primitive chip only has one chamber in a hole, not suitable for cytotoxicity investigation of transdermal formulations. We designed a modified chip that included two linked individual chambers in a hole, i.e., a cell chamber and a drug chamber, and they may be separated by a piece of abdominal rat skin. The skins were immersed in a penicillin/streptomycin solution (100 μg/ml penicillin and 100 units/ml streptomycin) and preserved at −20 °C for two days. Both sides of the skins were sterilized using UV light before use. A DMEM solution supplemented with 10% (v/v) fetal bovine serum and the penicillin/streptomycin solution (culture Medium A, 100 μl, 37 °C) was added in each cell chamber for 1 min to achieve baseline equilibrium. A B_{16} cell line from mouse melanoma was used. A B_{16} cell suspension (300 μl, 8×10^3 cells, 37 °C) was added to the cell chambers. The ECIS device was placed in a humidified incubator (37 °C, 5% CO_2). Electric impedance data were recorded every 25 min. The Medium A in the cell chambers was refreshed after 10 h. Subsequently, the aliquots (1.5 g) of Medium A, the MTO ethosome gels, and the blank ethosome gels were added to the drug chambers, respectively. The ECIS device was placed in the incubator again and impedance data were continually recorded.

2.7. Pharmacodynamic study on melanoma-bearing animals

The nude mice were subcutaneously injected in the hips with the cell suspension (0.2 ml for each mouse, 1×10^6 B_{16} cells/ml). The volume (V) of xenografts was measured with a caliper every day and calculated as $0.5L \times W^2$, where L was the largest superficial diameter and W was the smallest superficial diameter of the xenografts [21]. The MTO formulations were applied to the