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3-hydroxycoumarin loaded vesicles for recombinant human tyrosinase inhibition in topical applications

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ABSTRACT

Tyrosinase is one of the key enzymes in mammalian melanin biosynthesis. Decreasing tyrosinase activity has been targeted for the prevention of conditions related to the hyperpigmentation of the skin, such as melasma and age spots. This paper is devoted to the engineering of vesicle formulations loaded with 3-hydroxycoumarin for topical pharmaceutical applications. At first, it was demonstrated the strong inhibiting ability of 3-hydroxycoumarin against recombinant human tyrosinase. Then, such a drug was effectively encapsulated within liquid or gel-like vesicle formulations, both based on monoolein and lauroylcholine chloride. In vitro skin penetration and permeation studies proved these formulations efficiently overcome the barrier represented by the *stratum corneum,* delivering 3-hydroxycoumarin to the deeper skin layers. The effect of applying for different times the liquid and the gel formulation was also evaluated. Results revealed that application of the gel formulation for 2 h favored the drug accumulation into the skin with low transdermal delivery, thus indicating this combination of administration time and formulation as ideal to locally inhibit tyrosinase activity with minimal systemic absorption. Moreover, when incubated with B16F10 melanoma cells, the liquid vesicle formulations did not show cytotoxic activity.

1. Introduction

Lipid-based nanostructured carriers have been extensively investigated in recent years for medicine applications because they present extremely appealing features as drug delivery tools [1–3]. Among these features we can list their biodegradability and biocompatibility, the possibility of carry and release poorly water-soluble drugs, and a morphological and topological diversity that manifests in the various typologies of self-assembled nanoaggregates that can be found in the literature, including the ubiquitous liposomes [4–6] as well as the more exotic hexosomes [7,8] and cubosomes [9–12]. Another important point in favor of this class of aggregates is the potential they have shown as drug nanocarriers useful for all the main administration routes: oral, transdermal, intravenous.

The concept of applying a medication on the skin to achieve either systemic or local effect is of considerable interest for pharmaceutical development. Indeed, dermal/transdermal administration offers the possibility to skip the harsh gastrointestinal environment and avoids the first pass metabolism, two processes which could severely impact on drug efficacy. Moreover, local administration could reduce the risk of side effects and increase patient acceptability through its intrinsic non-invasiveness and ease of application. However, the large majority of actives does not permeate efficiently within the skin, primarily because of the presence of the *stratum corneum* (SC), a barrier composed of keratinized, dead epithelial cells surrounded by a lipid rich matrix [13,14]. In the attempt to promote SC crossing of poorly diffusing molecules, a vast plethora of nanoparticles, based on different components and with different proposed mechanism of permeation, has been developed in the last decades [15]. More in detail, the topical use of liposomes as drug delivery system dates back to the eighties [16]. Since then, their nanostructure has been upgraded originating innovative nanocarriers, known as niosomes, ethosomes, and transferosomes [17–19] ensuring better performances in terms of dermal or transdermal release of the pharmaceutical payload [20]. Given their ability in transporting drugs through the SC, also hexosomes and cubosomes were proposed as transdermal nanocarriers [21,22], the latter having

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recently demonstrated their potential in photodynamic therapy of melanoma skin cancer [23].

Tyrosinase, (monophenol, *o*-diphenol:oxygen oxido-reductase, EC 1.14.18.1) is an enzyme widely distributed in nature [24], from microorganism [25] to humans, catalyzing both the ortho-hydroxylation of monophenols and the oxidation of ortho-catechols to the corresponding ortho-quinones. Tyrosinase substrates are not limited to mono-phenols and di-phenols, since *o*-aminophenols and aromatic diamines were proven to be substrates of the enzyme [26–28].

Tyrosinase is the key enzyme in the biosynthesis of melanin pigment in animals as the enzyme catalyzes the first two main steps of the melanogenesis: i) the hydroxylation of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA) and ii) the oxidation of L-DOPA to dopaquinone. Then, a cascade of enzymatic and non-enzymatic auto-polymerization reactions lead to the formation of melanin [29]. However, defects in tyrosinase activity could lead to some skin disorders that encompass hyperpigmentation, melasma, age spots, etc. So, tyrosinase activity has been targeted for the treatment of skin disorders related to hyperpigmentation [26].

The discovery of new tyrosinase inhibitors has been for many years the main goal of numerous investigations [30]. In most cases, in vitro studies were performed using mushroom tyrosinase (MT) from *Agaricus bisporus*, as this enzyme is easily commercially available although with a very low degree of purity [31]. Although MT and human tyrosinase (HT) share the same bi-cupric cluster, the structure of the two enzymes is quite different, being the first consisting of four subunits while the second is a monomer [30,32]. Despite the huge number of MT inhibitors described [33], very few were tested for their efficacy with the human enzyme [34–36]. Besides, an inhibitor, in addition of being effective at low concentrations, should also possess low or no cellular toxicity when used in humans.

Mono-hydroxycoumarins (hydroxy-1,2-benzopyrones) are common among higher plants, including edible vegetables and fruits, and may have the hydroxyl substituent either on the aromatic ring or on the pyrone ring. Recently, a new activity/relationship study of some hydroxycoumarins and MT was reported [37]. In that study, 3-hydroxycoumarin (3-HC) proved to be an effective MT inhibitor at very low concentration.

Here, for the first time, we describe the interaction of 3-HC with recombinant human tyrosinase (RHT) in vitro, and discuss the potential use of a vesicular system endowed of low toxicity and constituted by two penetration enhancers (namely, monoolein and lauroylcholin chloride) [38] for the delivery of 3-HC in the deeper strata of the skin.

2. Materials and methods

2.1. Materials

Monoolein (MO, 1-monooleoylglycerol, RYLO MG 90-glycerolmonooleate; 98.1 wt% monoglyceride) was kindly provided by Danisco Ingredients, Brabrand, Denmark. Lauroylcholine chloride (LCh) was from TCI Europe. Distilled water, passed through a Milli-Q water purification system (Millipore), was used to prepare the samples. 3-Hydroxycoumarine (3−HC) was purchased from Sigma-Aldrich (Milan, Italy). All substances were used without further purification.

In this study RHT from two suppliers was tested because of RHT is described as inherently inefficient enzyme, with typical small changes in optical density (O. D.) and uncertain degree of purity [39]. RHT (product number: T0206) was purchased by Sigma-Aldrich (Milan, Italy). A second sample of RHT (product number: BML-SE535) was from Enzo (3V-Chimica, Rome, Italy). L-DOPA (3,4-dihydroxy-L-phenylalanine), MBTH (3-methyl-2-benzothiazolinone hydrazone), 3-HC (3-hydroxycoumarin) and all salts used for preparing the buffer solutions were purchased from Sigma-Aldrich and used without any further purification.

2.2. Enzyme assay and kinetics

The enzymatic activity of tyrosinase can be followed spectrophotometrically using L-tyrosine or L-DOPA as the substrate. When the enzyme acts on L-tyrosine, it generally shows a lag phase that can cause a considerable lengthening of the measurement times [27]. Therefore, we have chosen to follow the activity of the enzyme with L-DOPA.

Spectrophotometric enzyme assays were based on the reaction of dopaquinone, the product of L-DOPA oxidation, with Besthorn's hydrazone (MBTH) to form a pink adduct, and were carried out as previously described [40] with slight modifications. Briefly, RHT activity was spectrophotometrically determined measuring the amount of adduct formed at 37 °C using 3 mM L-DOPA, 0.5 mM MBTH, in 100 mM sodium phosphate buffer pH 7.3, and monitoring formation of dopaquinone-MBTH adduct at 505 nm ($\varepsilon 505 = 22.300\ M^{-1}\,cm^{-1}$).

IC_{50} value was determined through a series of measurements of RHT enzyme activity. For all experiments, a high and constant concentration of L-DOPA was used. In each experiment, the amount of inhibitor was steadily increased, and the observed rate of the reaction decreased accordingly. Control experiments were carried out in the absence of tyrosinase. All spectrophotometric data were plotted with Origin 2016 software (Origin Corporation, Northampton, MA, USA). Lineweaver-Burk data and IC_{50} calculations were analyzed with Grafit 7.0 (Erithacus Software ltd, UK).

2.3. Vesicle and vesicle gels preparation

The nanocarriers, empty or loaded with 3-hydroxycoumarine, were prepared dispersing the weighted amount of MO in a solution of LCh in Milli-Q water using an Ultra-Turrax T10 (IKA) device, equipped with a S10N-5 G dispersing tool working at 30,000 rpm. The mixture was then sonicated for 15 min (usually in three cycles of 5 min) obtaining a bluish and transparent solution in case of vesicle or a homogeneous gel. To obtain drug-loaded vesicles, 3-HC was dissolved in melted MO before adding the aqueous solution of LCh. For all samples prepared the MO : LCh ratio (wt/wt) was kept equal to 10 : 1, while throughout the article sample compositions are indicated by the volume fraction of the dispersed phase (DP) in terms of MO + LCh content. Samples composition is given in Table 1.

All samples were analyzed at least 48 h after their preparation.

2.4. Drug content

The quantification of 3-HC was performed through a chromatograph Alliance 2690 (Waters, Italy) equipped with a photodiode array detector and an integrating software (Empower 3). The column was a XSelect C18 (3.5 µm, 4.6 × 150 mm, Waters), and the mobile phase was a mixture of water and acetonitrile (45:55) acidified with 0.017% (v/v) acetic acid, eluted at a flow rate of 0.8. mL/min. 3−HC was detected at 306.5 nm wavelength. A standard calibration curve was built up by using standard solutions, and plotted according to the linear regression analysis (correlation coefficient value $R^2 = 0.999$).

Table 1
Samples composition (wt%).

Sample	Monoolein	Lauroylcholin chloride	Water	3-Hydroxycoumarin
DP8	7.0	0.6	92.4	–
DP8 + 3-HC	7.0	0.6	92.3	0.1
DP16	14.5	1.5	84.0	–
DP16 + 3-HC	14.5	1.5	83.9	0.1
DP20	18.2	1.8	80.0	–
DP20 + 3-HC	18.3	1.8	79.3	0.6
DP22	20.0	2.0	78.0	–
DP22 + 3-HC	19.9	2.0	77.3	0.8