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7-nitroindazol-loaded nanoemulsions: Preparation, characterization and its improved inhibitory effect on nitric oxide synthase-1

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ABSTRACT

Nitric oxide (NO) participates in several physiological processes such as maintenance of blood pressure, host defense, neurotransmission, inhibition of platelet aggregation and learning and memory. NO is also involved in several diseases or dysfunctions in the cardiovascular, central nervous and musculoskeletal systems. NO also has been shown to be a major player in sepsis. NOS-1-derived NO has been shown to be a relevant species in physiology but also is an important element in pathology. There exist some NOS-1 inhibitors and among of them, 7-nitroindazole has been used for its *in vivo* selectivity. However, 7-NI has a very short half-life (~2 h) and a poor water solubility. In this study, we describe the preparation and characterization of 7-NI-loaded nanoemulsions ($NE_{7\text{-NI}}$). The chemical stability of 7-NI was greatly increased and the drug release rate could be controlled after nanoemulsification. $NE_{7\text{-NI}}$ reduced NO production in a long-lasting manner in vascular smooth muscle cells and skeletal muscle, without cytotoxicity. Our results evidenced that nanoemulsification approach increases the effective action time of 7-NI, rendering a suitable dosage form, which may be an interesting tool to study the role of NOS-1 in physiology and disease.

1. Introduction

Nitric oxide (NO) is produced by a family of enzymes called nitric oxide synthases (NOS) of which are known three isoforms, namely the neuronal or NOS-1, the endothelial or NOS-3 both of them constitutively expressed, and the inducible isoform or NOS-2 which is induced upon and injurious stimulus (for review see Förstermann & Sessa, 2012) [1].

Nitric oxide participates in several physiological processes such as maintenance of blood pressure, host defense, neurotransmission, inhibition of platelet aggregation and learning and memory [2]. However, NO also has a prominent role in several diseases or dysfunctions such as in the cardiovascular [3], central nervous [4] and musculoskeletal [5] systems. NO also has been shown to be a major player in sepsis [6].

Part of the work done studying NO and diseases or dysfunctions revealed that NOS-1 is an important element in pathology. For instance, NOS-1-derived NO has been implicated in neurological disorders [7], cardiac pathophysiology [8] and muscular dystrophy [9], to give only a few examples.

Although not so well studied as NOS-2, data from our and other [10,11] laboratories implicate NOS-1 as a relevant player in sepsis. For instance, NO produced by NOS-1 in the early phase of sepsis plays a critical role as signaling element in the triggering systemic inflammatory response [12]. Following the temporal course of sepsis, there is an increase in NOS-1 expression which also interacts protein:protein with soluble guanylate ciclase. This interaction is critical for the late vascular dysfunction [13].

The two reports above and others [14,15] have used 7-nitroindazole (7-NI) to inhibit NOS-1. 7-NI is a lipophilic compound of the indazole family and inhibits the activity of the neuronal isoform with *in vivo* high selectivity [16,17]. Its mechanism of action involves reversible binding to the heme group of the enzyme, preventing the binding of the cofactor tetrahydrobiopterin ($BH4$)[17] as well as a competition with the substrate L-arginine [18]. 7-NI has a short half-life that is dose-dependent and even in very high doses does not exceed 2 h. In addition, 7-NI has a very low solubility in aqueous solutions, which prevents its administration

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by intravenous route as an aqueous solution [18].

Nanoemulsions are kinetically stable oil-in-water dispersions displaying droplet size in the nanometer range [19]. Besides their capacity to dissolve large quantities of hydrophobic drugs, their compatibility with the physiological environment and ability to protect drugs from hydrolysis and enzymatic degradation make them suitable vehicles for parenteral administration. Nanoemulsions provide favorable drug pharmacokinetic profiles, reducing the toxicity and improving the pharmacological activity of drugs dissolved in the oil phase [20]. In this study we describe the preparation and characterization of a 7-NI-loaded nanoemulsion aiming to improve the drug duration time and potency. The effect of 7-NI-loaded NE on the NO production is evaluated.

2. Materials and methods

2.1. Materials

Castor oil, 7-NI and polysorbate 80 were purchased from Sigma Chemical (USA). Soy bean lecithin was purchased from Lipoid S100 (Germany). Glycerol and ethanol were obtained from Vetec (Brazil).

2.2. Preparation of the 7-NI-loaded nanoemulsion

Nanoemulsions containing 7-NI were prepared using the spontaneous emulsification method described by Bouchemal et al. [21]. Briefly, an organic phase containing 700 mg of castor oil, 7 mg of soy bean lecithin and 10 mg of 7- NI in 20 mL of ethanol (≥99.8%, HPLC grade) was slowly added to an aqueous phase (40 mL) containing 0.5% of polysorbate 80 and 2.25% of glycerol, under vigorous magnetic stirring. After, the organic solvent was removed by evaporation under reduced pressure and the final volume of the colloidal dispersions was concentrated to 20 mL. The final nanoemulsion was filtered through 8 μm pore-sized filter paper. Unloaded nanoemulsions (NE_{blank}) were prepared using the same procedure. All formulations were prepared in triplicate.

2.3. Physicochemical and morphological characterization of nanoemulsions

The mean droplet size and polydispersity index were determined by dynamic light scattering and the zeta potential by laser-Doppler anemometry, both using Zetasizer Nano Series (Malvern Instruments, UK). The measurements were carried out at 25 °C after appropriate dilution of the samples with ultrapure water (MilliQ, Millipore, USA). Each size analysis lasted 100 s and was performed at detection angle of 173°. For measurement of zeta potential, the diluted samples were placed in the electrophoretic cell, where a potential of ±150 mV was established. The zeta potential values were calculated as mean of electrophoretic mobility values using the Smoluchowski's equation.

The morphology of the nanoemulsions was examined using a JEM-1011 transmission electron microscope (TEM) operating at 100 kV. $NE_{7\text{-NI}}$ and NE_{blank} were firstly diluted in water (1:250). Then, a drop of each sample was directly deposited on carbon-coated copper grids and negatively stained with 1% (w/v) fosfotungistic acid solution. The pH of the colloidal dispersions was determined using a pHmeter previously calibrated with pH 4.0 and 7.0 buffer solutions.

2.4. Determination of 7-NI content, entrapment efficiency and drug recovery

7-NI was determined by a HPLC method in the whole 7-NI-loaded nanoemulsions after dissolving the sample in buffer ammonium acetate:acetonitrile solution (50:50) and in the supernatant obtained after ultrafiltration/centrifugation procedure using an ultrafiltration device (Amicon Ultra with Ultracel-100 membrane, 100 kDa molecular weight limit, Millipore, USA). A liquid chromatographic method was developed (see Supplementary Material and Fig. S1) based on the method developed by Bush and Pollack [17] and validated according to the parameters of linearity, quantification and detection limits, accuracy, and precision in accordance with ICH (International Conference on Harmonization) guidelines [22]. The entrapment efficiency (%) was estimated as the difference between the total concentration of 7-NI found in the NE and the concentration of drug in the supernatant. Drug recovery (%) was estimated by comparing the total amount of 7-NI found in the NE formulation with the initial amount added to the formulations. The drug content was expressed as μg of 7-NI per mL of colloidal dispersion.

2.5. Release assays

In vitro release studies were carried out using the dialysis bag method in a USP dissolution apparatus 2. The study was conducted at 37±1 °C under mechanical stirring (75 rpm) using pH 7.0 phosphate buffer solution containing 0.5% (w/v) dodecyl sulfate as release medium to attain *sink* conditions. For the experiment, 15 mL of the $NE_{7\text{-NI}}$ or a 7-NI solution in pH 7.0 PBS containing 5% of dimethyl sulfoxide were placed in dialysis bags. At specified intervals of time, aliquots of the release medium (3 mL) were withdrawn and the 7-NI content was determined by HPLC using the same chromatography conditions described in Supplementary Methods. The release medium was immediately replaced with fresh medium. The experiments were carried out in triplicate. The cumulative amounts of 7-NI released (%) were plotted against time (h).

2.6. Stability studies

The stability of 7-NI-loaded and unloaded nanoemulsions was evaluated after exposing the colloidal dispersions to an artificial UV lamp (254 nm). Twenty mL of the $NE_{7\text{-NI}}$ (500 μg/mL), NE_{blank}, and a 7-NI aqueous solution were packaged into clear glass vials and irradiated in a UV chamber for 30 days. Each sample was also packaged into amber glass vials and kept at 4 or 25 °C during the same period. Aliquots of samples were withdrawn at predetermined time intervals (0, 5, 10, 15, and 30 days) and assayed for 7-NI content, mean size, polydispersity index and zeta potential, as described above.

2.7. Biological characterization of 7-NI-loaded nanoemulsion

2.7.1. Cell culture

Rat aorta vascular smooth cell line A7r5 (ATCC® CRL-1444) was purchased from Rio de Janeiro Cell Bank and cultured in DMEM with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM glutamine and 10 mM HEPES, pH 7.4 (complete DMEM). Cells were maintained at 37 °C in humidified atmosphere with 5% CO_2, detached with trypsin and used from passages 3–9.

2.7.2. NO detection in cultured cells

Intracellular NO production was detected using the highly selective and sensitive fluorescent probe, DAF-FM DA [23]. Briefly, cells were plated in black 96-well plates with clear bottom (4×10^4 cells/well) in complete DMEM and kept overnight at 37 °C and 5% CO_2. Cultures were pretreated for 4 h with 7-NI (0.2% DMSO final) (100 μM) or $NE_{7\text{-NI}}$ (100 μM) in different times (24, 8 and 4 h before probe loading) and then washed 3 times with HBSS (in mM:

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