Calixarene-based nanoformulation for ocular delivery of curcumin:

in vitro and *in vivo* anti-inflammatory activity evaluation

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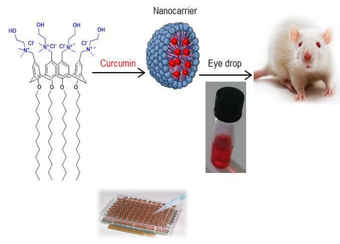
PURPOSE

Curcumin is a natural polyphenolic compound with multiple molecular targets and biological activities. There are many scientific papers on anti-oxidant,[1] anti-inflammatory,[2] anti-antimicrobial, anti-cancer, anti-Alzheimer's, [3] and anti-cystic fibrosis effects of curcumin. Despite significant medical efficacy and bio-safety profiles of curcumin, poor systemic bioavailability originating from poor water solubility (Log P = 2.5), chemical instability, sub-optimal absorption, active binding with serum proteins, rapid metabolism and fast systemic elimination, are impeding factors retarding its clinical success.[4]

Calix[n]arene are polyphenolic macrocycles with high interest in supramolecular chemistry due to the vast number of applications in different fields, including biomedicine.[5] An appropriate functionalization of the calixarene skeleton has provided suitable derivatives to be used as carriers for drug delivery.[6]

We initially tested the potential of an amphiphilic calixarene derivative (**1**) self-assembling in well-defined nanoaggregates in PBS medium (pH 7.4). Then we focused on the ability of the calixarene nanoassembly to solubilize and preserve curcumin from degradation in PBS medium, and we further characterized the calixarene-curcumin supramolecular assembly (Calix-Cur) with respect to size, polydispersity index, surface potential, morphology, structure, drug loading. Moreover, feasibility studies, using *in vitro* and *in vivo* models of ocular inflammation, were carried out to demonstrate the potential of the calixarene nanoaggregate as a delivery system of curcumin and to evaluate its anti-inflammatory activity.

METHODS



Design and chemical synthesis: An amphiphilic self-assembling calix[4]arene derivative bearing 4 choline groups was loaded with curcumin to give a clear colloidal solution in PBS. Size, polydispersity index, and surface charge of the nanostructured system were derived from **DLS analysis** and **zeta potential** measurements. **UV-vis spectroscopy** and **HPLC analyses** were performed to study solubility and chemical stability of curcumin alone and curcumin-loaded calixarene system.

***In vitro* studies**: Curcumin, calixarene and curcumin-loaded calixarene were used to test biocompatibility on **corneal cells and J774 murine macrophage cells** (**MTT assay**) and to evaluate *in vitro/in vivo* anti-inflammatory effects. On **J774 cells** pre-treated (2h) and post-treated (24h) after **LPS-stimulation**, western blot analysis were performed to test the expression of inflammation markers. The anti-inflammatory activity was shown through the modulation of **NF-κB activation**, reduction of **IκBα degradation**, **COX-2**, **iNOS expression and nitrite levels**.

***In vivo* studies**: Male Lewis rats (160–180 g) were obtained from Harlan Nossan, Italy. Endotoxin Induced Uveitis (EIU) was obtained by injection in the footpad of 200 mg of LPS from Salmonella typhimurium (Sigma-Aldrich, St Louis, MO, USA) that had been diluted in 0.2 mL of PBS, pH 7.4. Following treatment 3 days before and 7 days after induction of **uveitis**, rats were sacrificed (16 and 72 hrs) and eyes taken to perform histology and immunohistochemical staining. In particular immunohistochemical staining for the expression of adhesion molecule **ICAM-1**, of **nitrotyrosine** and of **VEGF** were performed.

RESULTS

Design and chemical synthesis

To develop a novel cationic nanocarrier for ocular delivery of curcumin, we designed calix[4]arene derivative **1**. The introduction of four long alkyl chains at the calix[4]arene lower rim (Fig.1) was planned to (i) promote the formation of well-defined nanoaggregates, (ii) provide a greater viscosity and stability to the nanoaggregates. The cationic choline groups introduced at the calixarene upper rim, were selected for: i) confering amphiphilicity to the hydrophobic scaffold, ii) establishing charge interactions with charged curcumin, iii) taking advantage of the choline homing properties: binding of choline to cognate transporters present in cornea, [7] iris and retina can favour the ocular penetration.

A phase solubility method was used to nanoencapsulate curcumin in the nanoaggregates of derivate **1**. The amount of calixarene and curcumin in the Calix-Cur colloidal solution corresponding to a 2:1 calixarene:curcumin molar ratio suggested an assembly model in which one curcumin molecule, compatible to its symmetric structure (Fig.2), establishes interactions with two calixarene molecules (Fig.3). Calixarene nanoassembly solubilizes and preserves curcumin from degradation in PBS medium (Fig.4). In table 1 results of physico-chemical characterization of nanocarrier are reported.

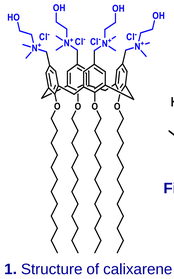


Fig. 1. Structure of calixarene derivative **1**

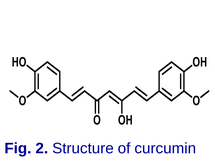


Fig. 2. Structure of curcumin

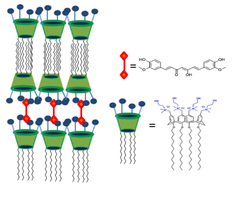


Fig. 3. Schematic representation of the hypothesized structural organization of Calix-Cur.

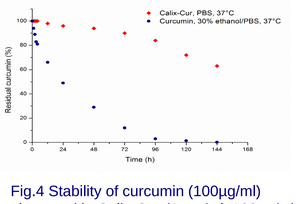


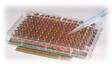
Fig.4 Stability of curcumin (100μg/ml) alone and in Calix-Cur (1 mg/ml ,100μg/m)

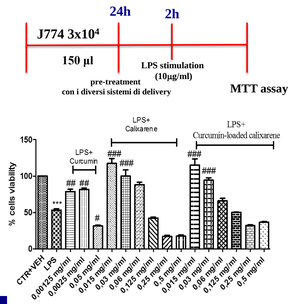
Table1

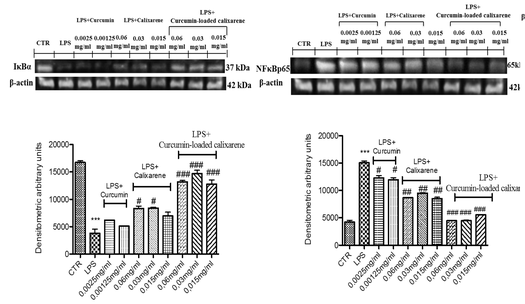
Physico-chemical characterization	Carrier-curcumin
Size	Z average 82 nm
Polydispersity index	0.2
Zeta potential	23 mV
Loading capacity	10% [carrier] 606 μM [drug] 271 μM

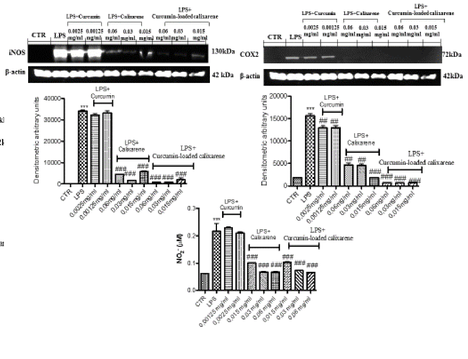
RESULTS

In vitro studies

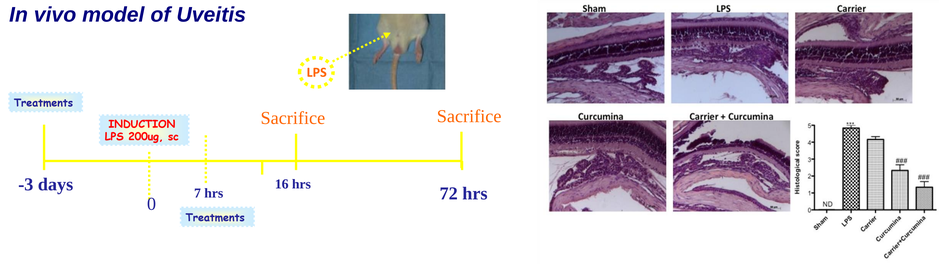


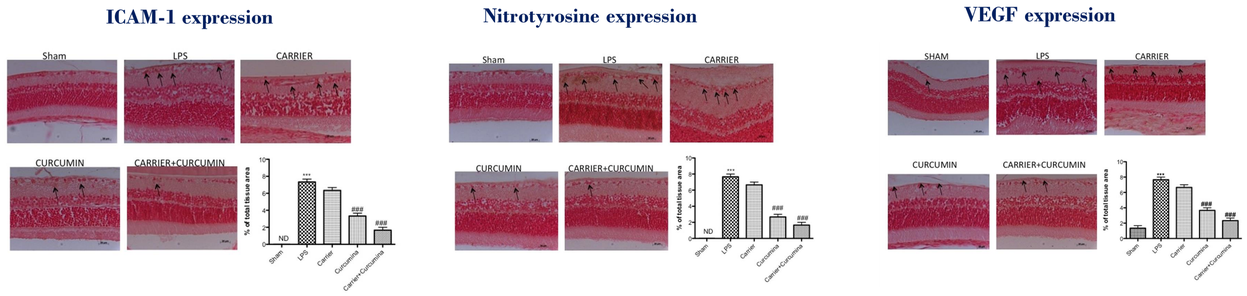






In vivo model of Uveitis





CONCLUSIONS

For the first time a calix[4]arene-based nanoassembly was investigated as a carrier for ophtalmic drug delivery. Curcumin solubilized in calix[4]arene nanoaggregate maintained its anti-inflammatory effects *in vitro* and *in vivo* experiments. Pre-treatment with Calix-Cur, in J774 macrophages exposed to LPS oxidative stress, was shown to inhibit IκBα degradation, to modulate NF-κB activation, to reduce COX-2, iNOS expression and nitrite levels ($p<0.0001$ vs LPS control). Moreover, Calix-Cur reduced signs of LPS-induced uveitis in rats as shown in histological and immunohistochemical analyses. Reduction of nucleophilis infiltration, ICAM-1 and nitrotyrosine levels in iris ciliary-body, protein level in aqueous humor and VEGF expression in retina were also observed. These results strongly support the role of the calixarene nanoassembly as a very promising ocular drug delivery system.[8]

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