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Solid lipid nanoparticles loaded with fluorescent labelled cyclosporine A: anti-inflammatory activity *in vitro*.

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Abstract

FMOC-isocyclosporine A, a fluorescent labeled cyclosporine A, was encapsulated in solid lipid nanoparticles (SLN) prepared by the coacervation technique, and its anti-inflammatory activity was evaluated. The anti-inflammatory activity of the fluorescent labelled molecule, measured as inhibition of TNF-α secretion, is similar to the native one. SLN were compared to commercial formulations, through measurement of cytokine release and drug uptake in rat peripheral blood mononuclear cells. Drug-loaded SLN inhibit TNF-α secretion in a lower extent than commercial formulations, probably due to a lower uptake by the cells, but the increase of IL-10 secretion caused by the lipid matrix itself makes this formulation interesting for its anti-inflammatory activity.

Keywords

Solid lipid nanoparticles, coacervation, cyclosporine A, FMOC, cytokines, inflammation

Introduction

Cyclosporine A (CyA), a water insoluble molecule, has been used to prevent immune rejection in solid tissue transplantation and to treat systemic inflammatory diseases, especially focusing on topical [1] and ophthalmic diseases [2]. Anti-inflammatory activity of CyA is due to the inhibition of calcium/calmodulin-dependent protein phosphatase calcineurin. This step prevents the dephosphorylation of the nuclear factor of activated T-lymphocytes (NFAT) and consequently its translocation to the nucleus leading to NFAT activity inhibition. This inhibition thus prevents the transcriptional induction of several cytokine and the activation of the immune response. Furthermore, CyA might exert side effects such as acute and chronic nephrotoxicity, that are thought in part due to calcineurin inhibition in non lymphatic tissues [3].

A labelled CyA can be useful to investigate the interaction of this molecule with cells and organs *in vitro* and *in vivo*. Although H^3-cyclosporine (H^3-CyA) was already used for *in vitro* and *in vivo* studies [4], fluorescent labelled CyA allows larger scale production and easier handling compared to H^3-CyA. Recently [5] our research group developed a procedure for quantitative production of fluorescent labelled CyA (FMOC-isocyclosporine A), basing on a method described in literature [6]. The chemico-physical properties of the native molecule should not be extensively altered, because of its molecular weight higher than that of the chromophore, but more investigation is needed for the pharmacological properties of the labelled molecule, with especial regard to the anti-inflammatory properties.

Due to its low water solubility, CyA needs a vehicle to be delivered. Currently, two formulations are marketed as Sandimmun® (a micellar solution) and Neoral® (a self emulsifying drug delivery system).

Nowadays nanotechnology-based drug delivery systems, like nanosuspensions, SLN and liposomes are widely used for the delivery of poorly soluble drugs and can be considered a suitable vehicle for CyA. In literature a better oral uptake has been demonstrated for nanoparticulate CyA, compared to commercial formulations [7].

Solid lipid nanoparticles (SLN) are disperse systems with mean particle size comprised between 50 and 1000 nm, whose main advantage over polymeric nanoparticles in drug delivery is the use of biocompatible lipids with a safe history of use in therapy [8]. Recently, a new SLN preparation technique has been worked out, without the use of solvents. Briefly, a fatty acid alkaline salt micellar solution is prepared, then pH is lowered by acidification causing fatty acid precipitation, owing to proton exchange between the added acid solution and the soap: this process was named as "coacervation" [9].

In this experimental work the anti-inflammatory activity of FMOC-isocyclosporine A (FMOC-isoCyA) has been compared to that of native CyA. FMOC-isoCyA has then been encapsulated in behenic acid SLN, prepared according to the coacervation method. This formulation has been compared to commercial formulations labelled with the fluorescent conjugate, regarding the anti-inflammatory activity and the uptake in rat lymphocytes. In particular, we used a whole-blood model in which cytokine release in rat peripheral blood mononuclear cells (PBMC) was measured after stimulation by lipopolysaccharide (LPS) and the influence of the different CyA formulations was evaluated on cytokines with pro-inflammatory actions (TNF-α) and with implied functions in the Th1/Th2 balance (IL-10).

Materials

CyA, fluorenylmethyloxicarbonyl-chloride (FMOC-Cl), sodium myristate (SM), sodium palmitate (SP), sodium stearate (SS), arachidic (AA) and behenic acid (BA) were from Fluka (Buchs, Switzerland). 80% hydrolysed PVA 9000–10000 Mw (PVA 9000), and sodium myristate (SM) were from Sigma (Dorset, UK). Polyoxyethylated castor oil was from BASF (Ludwigshafen, Germany). Corn oil was from ACEF (Concorezzo, Italy). Sodium behenate (SB) and sodium arachidate (SA) were obtained by adding a stoichiometric amount of NaOH ethanolic solution to the ethanolic solution of BA and AA respectively: the soap was purified by recrystallisation and stored in an essicator at room temperature. Deionised water was obtained by a MilliQ system (Millipore,

Bedford, MO). Cell culture medium and reagents were purchased from Sigma-Aldrich (Milano, Italy). All other chemicals were analytical grade and used without any further purification.

Methods

Synthesis of FMOC-isoCyA

A fluorescent conjugate of CyA was synthesised according to a slightly modified literature method (Fois and Ashley 1991): CyA was converted to isocyclosporine A (isoCyA) by heating (50°C) a 10% CyA solution in acid anhydrous methanol (1.2% methanesulfonic acid) for 18 hours [10], thus exposing a secondary amine group on the molecule backbone. Unreacted CyA was precipitated by water addition and the resulting suspension was centrifuged (40000 g, Allegra^{TM} R64 centrifuge, Beckman Coulter, USA) and sodium carbonate was added to the supernatant in order to precipitate isoCyA, owing to amino group deprotonation. The obtained suspension was centrifuged (40000 g), and the precipitated pure isoCyA dried under vacuum. Every reaction step was checked by silica TLC. The mobile phase consisted of $CH_3Cl/CH_3OH/CH_3COOH$ (90/4/6).

The fluorescent probe was conjugated to isoCyA adding a molar excess of FMOC-Cl (1:2 w/w ratio) in hot acetonitrile solution (0.7% isoCyA, 70° C), in the presence of sodium carbonate, in order to remove HCl forming during the reaction. After 3 hour-reaction, the mixture was filtered to remove sodium carbonate, acetonitrile was evaporated, and the residue dissolved in chloroform. Each reaction step was checked by silica TLC. The mobile phase consisted of $CH_3Cl/CH_3OH/CH_3COOH$ (92/2/6). FMOC-isoCyA was separated from the reaction mixture by gradient column liquid chromatography (Kieselgel 60 – Merck, Darmstadt). Mobile phase A was CH_3Cl, mobile phase B was CH_3OH. The gradient was: eluent A for 30 minutes, then shift from 100% A to 100% B in 10 minutes, finally eluent B for 30 minutes. Fractions were assayed by silica TLC - mobile phase $CH_3Cl/CH_3OH/CH_3COOH$ (92/2/6). The conjugate was dried under vacuum, re-dissolved in CH_3OH, from which it was re-crystallised by water addition, and finally dried under

vacuum. FMOC-isoCyA was identified by mass spectrometry in chemical ionisation (TSQ 700, Finnigan-Mat).

SLN preparation

Blank SLN were prepared according to the coacervation method [9], as reported in Table 1. Briefly, fatty acid sodium salts were dispersed in water with PVA 9000 and the mixture was then heated under stirring (300 rpm), to obtain a clear solution. The acidifying solutions (coacervating solutions) were added drop-wise to the mixture until complete fatty acid precipitation. The obtained suspension was then cooled under stirring at 300 rpm until 15° C temperature was reached.

Drug loaded SLN (SLN Fluo) were prepared with the same procedure, but adding FMOC-isoCyA ethanol solution (25 mg/ml) to the fatty acid sodium salt solution before the acidification step and keeping under stirring until complete dissolution. Drug final concentration in suspension was 0.05% w/v.

SLN characterisation

SLN particle size distribution was determined by the laser light scattering technique (LLS - Brookhaven, New York, USA). The dispersions were diluted with water (1:1000) and measurements were done at an angle of 90° with a laser beam of 675 nm.

Drug entrapment efficiency (EE %) was calculated as the ratio between the amount of drug entrapped within the lipid matrix and the amount used to prepare SLN. The entrapped drug was determined by centrifuging the SLN suspension at 55000 g and extracting the precipitate with CH_3OH. The lipid fraction was selectively precipitated by adding a small volume of water and the supernatant was centrifuged and analysed by HPLC.

FMOC-isoCyA HPLC analysis

HPLC was performed using a LC9 pump (Shimadzu, Kyoto, Japan), coupled with a RF 551 (Shimadzu, Kyoto, Japan) fluorimeter (λ_{exc} = 265, λ_{em} = 315).

Analytical conditions were as follows: column: Chromosystem 5 mm×15 cm; mobile phase: CH_3CN/H_2O 75:25; flux: 1 ml/min; rt: 12 min

Preparation of fluorescent labelled formulations with the same composition of Sandimmun® and Neoral®.

Drug free formulations were as follows: a micellar solution with the same excipients of Sandimmun®, without CyA (Formulation A); a SEDDS vehicle with the same excipients of Neoral®, without CyA (Formulation B). FMOC-isoCyA was then dissolved in both formulations at the same molar concentration of CyA in commercial Sandimmun® and Neoral®. In this way Formulation A Fluo and Formulation B Fluo were obtained.

Prior to use, Formulation A Fluo and Formulation B Fluo were diluted with water, to reach the same drug concentration of SLN Fluo.

PBMC isolation and culture

According to the method of Liu et al. [11], PBMC were isolated from heparinised peripheral rat blood samples by density-gradient centrifugation over Ficoll-Paque Premium 1.084 (Pharmacia Biotech, Sweden). Then 5×10^5/ml viable cells were cultured in 24-well culture plates in a RPMI 1640 medium containing 10% fetal calf serum (v/v), 2 mM L-glutamine, 100 U streptomycin/ml, 200U penicillin/ml and 1 μg/ml LPS (Sigma–Aldrich, Italy) at 37 °C and 5% CO_2 for 24 h. PBMC were incubated with 1 and 10 μM CyA (diluted form commercial Sandimmun®) or FMOC-isoCyA (diluted from Formulation A Fluo and Formulation B Fluo and SLN Fluo) at the start of the culture period. A trypan blue dye exclusion assay was performed for each condition after 24 h incubation to

evaluate cell viability. Three independent experiments were performed and the study was approved by the local Ethics Committee.

Immunoassays for cytokines

An enzyme-like immunosorbent assay for the quantitative detection of rat TNF-α and IL-10 (eBioscience, USA) in PBMC culture supernatants after 24 h incubation was used according to the manufacturer's instructions.

Isocyclosporine A-FMOC uptake by rat lymphocytes

5×10^5/ml rat PBMC were incubated with 10 μM FMOC-isoCyA as SLN Fluo, Formulation A Fluo and Formulation B Fluo. After 24 h cells were detached with 0.025% trypsin-EDTA, harvested by centrifugation and washed twice with PBS. FMOC-isoCyA was extracted from harvested cells with 0,2 ml CH_3OH overnight. The samples were then centrifuged and injected to HPLC.

Results and discussion

The aim of this experimental work was the formulation of SLN loaded with the fluorescent conjugate FMOC-isoCyA and the evaluation of their effect at cellular level, including the secretion of cytokines, in comparison with commercial formulations. Cytokine production, one of the major regulatory mechanisms of the immune system, is a self-restricted phenomenon. Mediators produced by mononuclear phagocytes in response to infective agents such as IFN-β, TNF-α and IL-1β, and regulators of immuno-mediated inflammation elicit non-specific inflammatory cells through the antigenic specific recognition by T lymphocytes. Moreover, IL-10 produced by monocytes acquires anti-inflammatory properties such as inhibiting nuclear transcription, macrophage and monocyte pro-inflammatory cytokine production and promoting phenotypic changes in the lymphocyte Th2 phenotype [12].

In this experimental work, an increase of TNF-α secretion was considered as direct marker for inflammatory activity, while an increase of IL-10 secretion was considered as functional to an anti-inflammatory activity.

In Table 1 blank SLN composition and mean diameter of myristic (MA), palmitic (PA), stearic (SA), arachidic (AA) and behenic (BA) acid are reported.

Table 1: blank SLN composition and mean diameter

	MA SLN	PA SLN	SA SLN	AA SLN	BA SLN
SM	109 mg*				
SP		108 mg*			
SS			107 mg*		
SA				107 mg*	
SB					106 mg*
PVA 9000	100 mg	100 mg	100 mg	200 mg	200 mg
1M Citric acid	0.5 ml	0.2 ml			
1M Na_2HPO_4	0.1 ml				
1M lactic acid			0.5 ml		
1M NaH_2PO_4				0.2 ml	0.2 ml
1M H_3PO_4				0.3 ml	
1M HCl					0.3 ml
Water	9.4 ml	9.8 ml	9.5 ml	9.5 ml	9.5 ml
Mean diameter	483±54	270±15	295±10	320±14	356±25
Polydispersity	0.200	0.058	0.025	0.046	0.101

* corresponding to 100 mg fatty acid

Prior to test, drug-loaded formulations and SLN prepared by coacervation with different fatty acids were tested on rat lymphocytes for TNF-α secretion, to evaluate the eventual pro-inflammatory activity of the vehicle itself. Fatty acid concentration used in the experiment was 70 μM.

As it can be noticed form figure 1, only BA SLN did not cause any increase in TNF-α level when compared to the control; all other fatty acid SLN determined an increase in TNF-α secretion, with a maximum extent in the case of PA SLN. This is in agreement with literature data, which state that

fatty acids can increase cytokines secretion if administered systemically [13, 14], with pro-inflammatory effect. According to these experimental data, BA is an exception among other fatty acids.

Since in this preliminary experiment BA showed no rat PBMC enhanced secretion of TNF-α, drug loaded BA SLN were used in the next steps of this study.

The pharmacological anti-inflammatory activity of FMOC-isoCyA was preliminarily compared to the one of native CyA, by testing the secretion of different cytokines from rat lymphocytes: TNF-α inhibition and IL-10 induction tests were performed. Sandimmun® and Formulation A Fluo were diluted at 1 and 10 μM drug concentration. As it can be noted from figure 2, FMOC-isoCyA and CyA have comparable efficacy in inhibiting the secretion of TNF-α. Therefore it can be assessed that the anti-inflammatory activity of CyA is substantially preserved when it is conjugated with the fluorescent probe. As a consequence, FMOC-isoCyA was used instead of CyA throughout this experimental study, due to the possibility of fluorimetric detection in cell lines experiments. It should be noted also that FMOC-isoCyA has no effect on IL-10 induction, whereas CyA is effective.

FMOC-isoCyA-loaded BA SLN (SLN Fluo) were then prepared: they show a mean size of 382±35 nm (polydispersity 0.125) and a drug encapsulation efficiency of 82%. The antinflammatory activity of SLN Fluo was then compared with Formulation A Fluo and Formulation B Fluo by testing the secretion of the same cytokines at 1 μM and 10 μM drug concentrations; blank BA SLN were tested as control at a concentration of 70 μM fatty acid (70 μM fatty acid corresponds to 10 μM FMOC-isoCyA for SLN Fluo). As shown in figure 3, Formulation A Fluo has similar effect to Formulation B Fluo on the two cytokines; SLN Fluo inhibit TNF-α to a lesser extent compared to commercial formulation. This agrees with the experimental data concerning the intracellular accumulation of FMOC-isoCyA, that is shown in Table 2: FMOC-isoCyA is internalised two folds

less when delivered in SLN than in commercial formulations, probably because the solid state of nanoparticles hampers the free diffusion of the drug towards the cells.

Table 2: cell internalisation of isocyclosporine A-FMOC

	pg/cell
Formulation A Fluo	1,29
Formulation B Fluo	1,40
SLN Fluo	0,60

It should also be noted that both SLN Fluo and blank BA SLN have a promoting effect on IL-10 secretion: this effect can be ascribed to the lipid matrix itself, since neither Formulation B Fluo nor Formulation A Fluo have any important induction effect on the secretion of IL-10.

Conclusions

In this experimental study SLN loaded with fluorescent labelled CyA, the so called FMOC-isoCyA, were prepared. According to the experiments performed with SLN on PBMC cell lines it can be concluded that:

- BA, used as lipid matrix for SLN preparation, is not pro-inflammatory, contrarily to other fatty acids
- the anti-inflammatory activity of CyA is substantially preserved when it is conjugated with the fluorescent probe (FMOC-isoCyA)
- the inhibition of the FMOC-isoCyA on TNF-α secretion is lower when it's delivered in SLN compared to commercial formulation, probably due to a lower uptake by the cells.
- the secretion of IL-10 is higher in the presence of BA SLN than that observed in the presence of commercial formulations. This effect can be ascribed to the lipid matrix itself.

The encapsulation in SLN of FMOC-isoCyA (a fluorescent conjugate of CyA with anti-inflammatory activity) maintains the anti-inflammatory activity of the drug, even if reduced due to a lower uptake by the cells; moreover a enhanced secretion of IL-10, caused by the vehicle itself, can be functional to the anti-inflammatory activity. Further *in vivo* studies have to be performed to investigate if the delivery of FMOC-isoCyA in SLN can enhance the oral uptake of the drug compared to commercial formulations.

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Figure captions

Figure 1 TNF-α secretion in PBMC culture supernatants analysed by ELISA at 24 h. * $p < 0.05$ and ** $p < 0.01$ (PBMC exposed to SLN made of different fatty acids *versus* control PBMC)

Figure 2 a) TNF-α and b) IL−10 secretion in PBMC culture supernatants analysed by ELISA at 24 h. * $p < 0.05$ and ** $p < 0.01$ (Sandimmun® Fluo *versus* Sandimmun®)

Figure 3 a) TNF-α and b) IL−10 secretion in PBMC culture supernatants analysed by ELISA at 24 h. * $p < 0.05$ and ** $p < 0.01$ (Formulation A Fluo or Formulation B Fluo *versus* SLN Fluo)

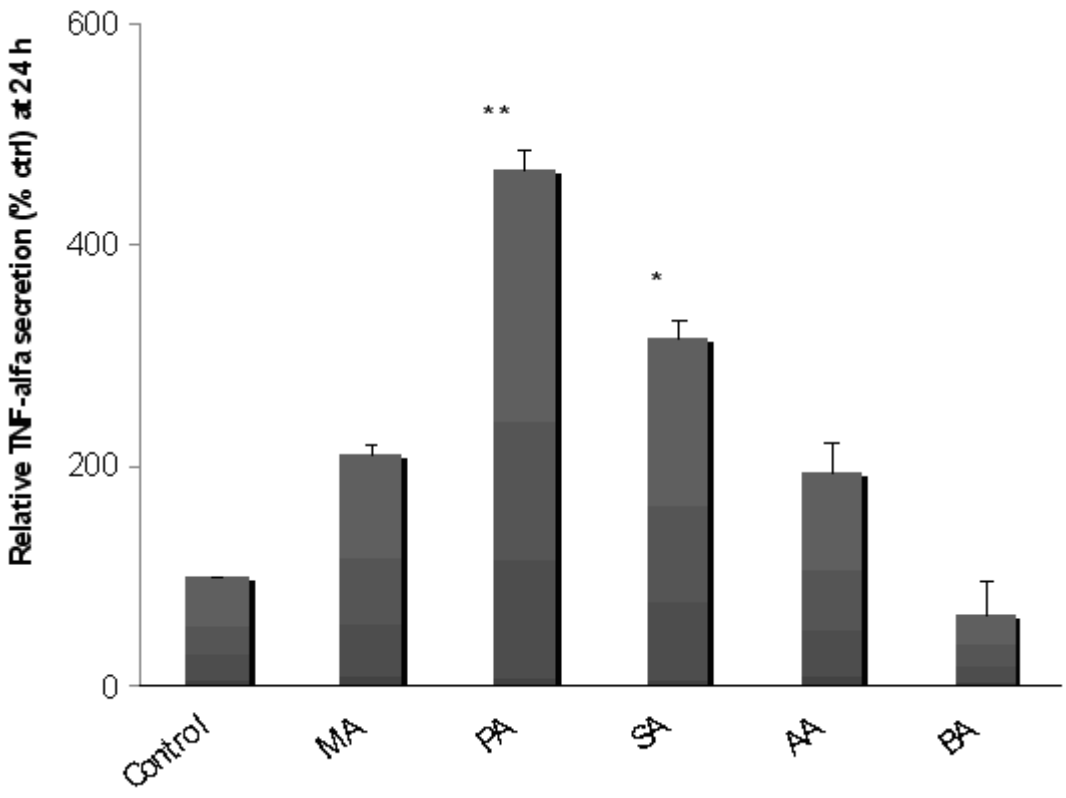

Figure 1

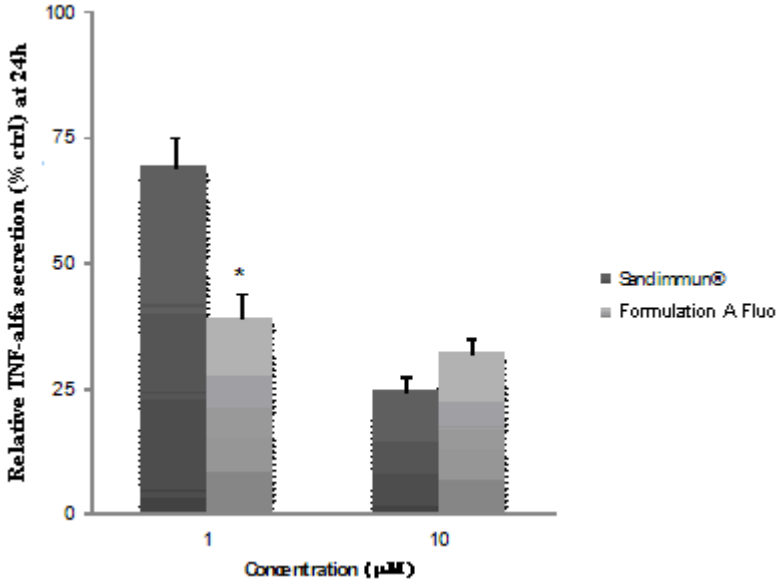

Figure 2a

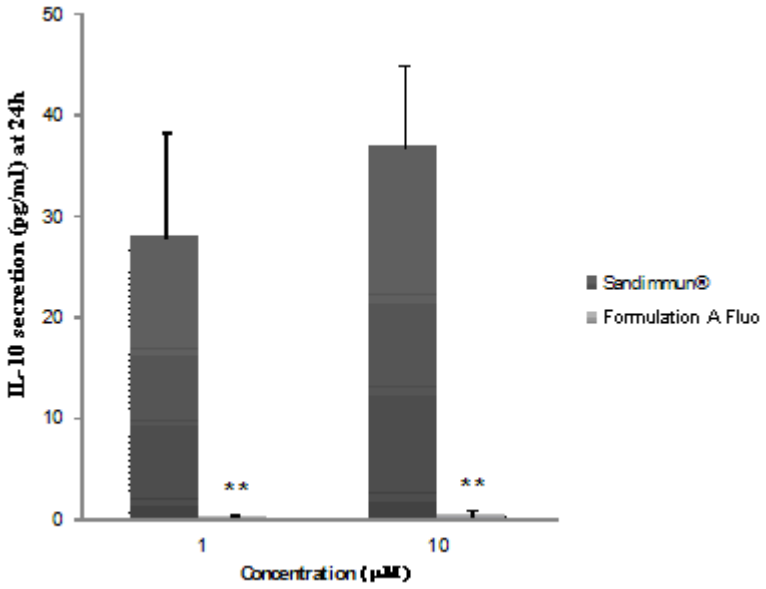

Figure 2b

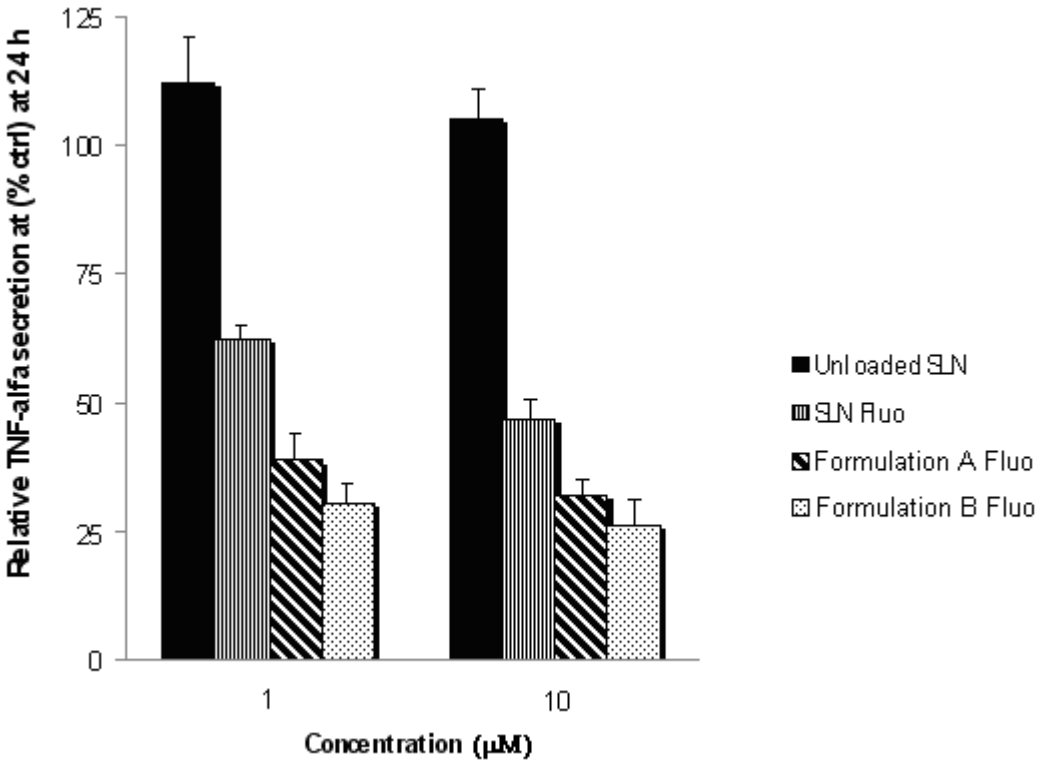

Figure 3a

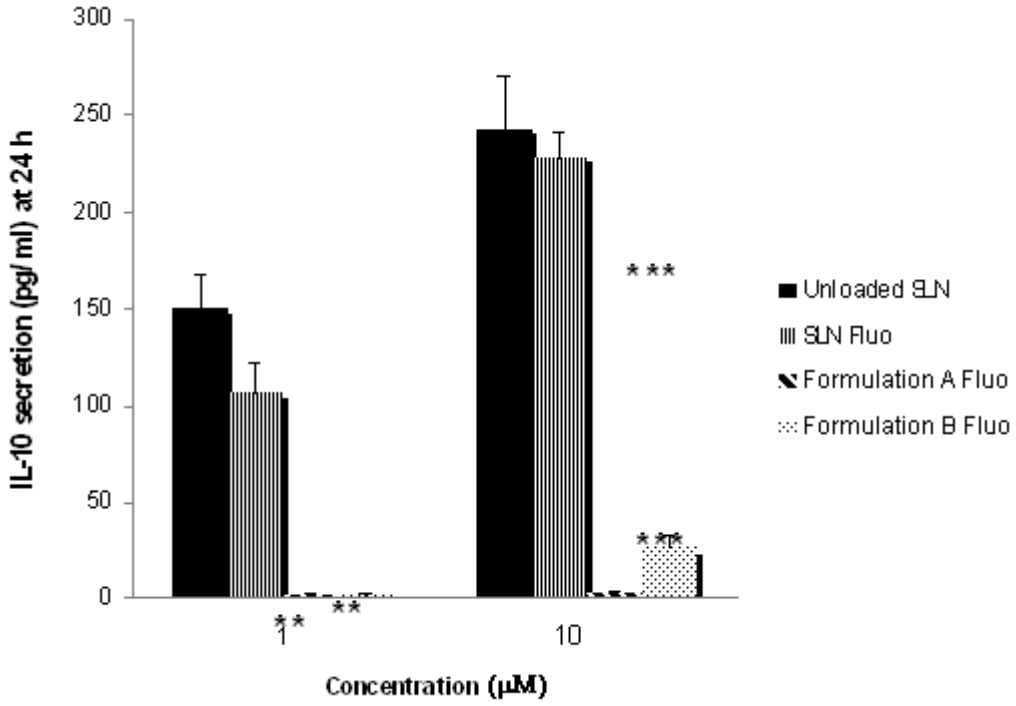
300
250
200
150
100
50
0
IL-10 secretion (pg/ml) at 24 h
* **
* **
* **
* **
1
10
Concentration (µM)
Unloaded SLN
SLN Fluo
Formulation A Fluo
Formulation B Fluo

Figure 3b