


Pro-Inflammatory Cytokines at Ultra-Low Dose Exert Anti-Inflammatory Effect In Vitro: A Possible Mode of Action Involving Sub-Micron Particles?

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Abstract

Tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) are pro-inflammatory cytokines involved in acute and chronic inflammatory diseases. Indeed, immunotherapy blocking these 2 cytokines has been developed. Micro-immunotherapy (MI) also uses ultra-low doses (ULD) of pro-inflammatory cytokines, impregnated on lactose-sucrose pillules, to counteract their over-expression. The study has been conducted with 2 objectives: examine the anti-inflammatory effect in vitro and the capacity of 2 unitary medicines, TNF- α (27 CH) and IL-1 β (27 CH), to reduce the secretion of TNF- α in human primary monocytes and THP-1 cells differentiated with phorbol-12-myristate-13-acetate, after lipopolysaccharide (LPS) exposure; then, investigate the presence of particles possibly containing starting materials using tunable resistive pulse sensing technique. The results show that the unitary medicines, tested at 3 pillules concentrations (5.5, 11 and 22 mM), have reduced the secretion of TNF- α in both models by about 10–20% vs. vehicle control, depending on concentration. In this exploratory study, particles (150–1000 nm) have been detected in MI ULD-impregnated pillules and a hypothesis for MI medicines mode of action has been proposed. Conscious that more evaluations are necessary, authors are cautious in the conclusions because the findings described in the study are still limited, and future investigations may lead to different hypothesis.

Keywords

micro-immunotherapy, ultra-low doses, anti-inflammatory unitary medicines, sub-micron particles

Abbreviations

TNF- α , tumor necrosis factor-alpha; IMIDs, immune-mediated inflammatory diseases; IL-1 β , interleukin-1beta; MI, micro-immunotherapy; ULD, ultra-low doses; LD, low doses; CH, Centesimal Hahnemannian dilutions; LPS, lipopolysaccharide; PMA, phorbol-12-myristate-13-acetate; NPs, nanoparticles; TRPS, tunable resistive pulse sensing; NTA, nanoparticle tracking analysis

Introduction

Tumor necrosis factor-alpha (TNF- α) is a pro-inflammatory cytokine involved in a wide range of acute and chronic inflammatory diseases. Indeed, TNF- α inhibitors have been developed to manage immune-mediated inflammatory diseases (IMIDs), such as rheumatoid arthritis (RA), ankylosing spondylitis, inflammatory bowel diseases, and psoriasis with or without complicating arthritis.¹ TNF- α was the first cytokine to be fully validated as a therapeutic target for the treatment of RA.¹ Interleukin-1 β (IL-1 β) is a highly active pro-inflammatory cytokine also involved in IMIDs, in pain,² and in tissue damage after

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chronic inflammation.³ Monotherapies blocking IL-1 activity, as well as TNF- α inhibitors, have been developed to reduce IMIDs symptoms severity.³ Beside the benefits of approved pro-inflammatory cytokines inhibitors, many side effects have been reported.⁴ In addition, individual variability in drug response has been described, sometimes a progressive loss of response, or even a complete lack of response, which confirms the complexity of the immune responses and the existence of an individual drug compensatory adaptation.⁵

On the other side, micro-immunotherapy (MI) aims to help the organism using its resources and strategies taking advantage of immune system adaptation.

MI medicines are lactose-sucrose pillules, also called globules, for oromucosal administration, which are impregnated with ethanolic preparations of immune mediators and nucleic acids at ultra-low doses (ULD), or at low doses (LD). The composition of MI medicines is expressed as number of Centesimal Hahnemannian dilutions (CH), as in traditional homeopathic medicines.⁶⁻¹³ LD range from 3 to 7 CH and aim at stimulating the expression of specific proteins and molecular pathways, for example to reduce oxidative stress,⁷ while ULD aim at regulating (from 8 to 10 CH) and/or inhibiting (higher than 10 CH) the expression of certain key proteins involved in IMIDs, to reestablish the homeostasis. TNF- α and IL-1 β are therapeutic targets used in MI at inhibiting ULD to counteract their overexpression.⁶

It has been shown that 2LARTH[®], which contains the cytokines TNF- α and IL-1 β at 17 CH, as well as other immune regulators, exerts an anti-inflammatory effect *in vitro*⁶ and *in vivo*⁹ respectively, by reducing the TNF- α and IL-1 β secretion on human primary monocytes exposed to lipopolysaccharide (LPS), and by reducing the systemic levels of TNF- α in a murine model of RA.

In order to perform fundamental research, it has been examined the anti-inflammatory effect of 1 single pro-inflammatory cytokine at a time, TNF- α or IL-1 β , both at 27 CH, using 2 different cellular models of inflammation exposed to LPS: human primary enriched monocytes from healthy donors, and THP-1 cell line differentiated with phorbol-12-myristate-13-acetate (PMA).

In the last couple of years, *in vitro*,⁶⁻⁸ and *in vivo*^{9,10} studies reported evidence about the safety, the biological activity, and the efficacy of MI medicines. Furthermore, clinical data also described the potential benefit of the therapy in several acute¹¹ and chronic diseases.^{12,13} Nevertheless, further investigations are needed to comprehend, at the molecular level, the mechanism of MI. The hypothesized mode of action of LD and ULD, on which MI medicines are based, mainly involves 2 conserved core biological features: i) all organisms, cells and tissues, particularly the immune system, can respond to a multitude of stimuli, in a wide range of concentrations, according to a non-linear dose-response curve, also called biphasic or hormetic response¹⁴; ii) all organisms, tissues and cells have the capacity to adapt to environmental signals and stressors with a high plasticity and with the aim to reestablish the homeostasis.¹⁵

Recent discoveries conducted by the Jayesh Bellare's team on homeopathic remedies revealed that ultra-high potencies do not reach the point where no molecule of the active substance is left, but always retain starting material from the original preparation in form of nanoparticles (NPs), reaching an asymptote of few pg/mL.¹⁶ The studies of Chikramane et al. describe "how" and "why" the intense turbulence caused by the succussion/dynamization process, induces the formation of air-nanobubbles and the further NPs levitation to the surface.^{16,17} According to the so-called "surface monolayer hypothesis," the NPs of starting material form a monolayer on the liquid surface, that is preserved in spite of the serial dilutions, and that it is responsible of the asymptote formation.¹⁷ It is important to highlight that Bellare's team used metal powders as starting raw materials, and 90% v/v ethanol as solvent, to prepare the high potencies. The research group has gone further in their investigation with the homeopathic potencies, evaluating their biological effects and demonstrating that metal NPs are able to induce hormetic responses *in vitro*.¹⁸ Other research groups, including Van Wassenhoven *et al*, investigated the presence of metal NPs in homeopathic dilutions that use water as solvent, but did not confirm the same findings.¹⁹

Another line of evidence proposes the "silica hypothesis," which is based on the presence of silica nanostructures in homeopathic potencies, generated during the succussion/dynamization process, by detachment from the walls of the glass containers used.²⁰

Since MI medicines are manufactured according to the fundamental processes of homeopathic remedies, it has been performed an exploratory study aimed at investigating the presence of small particles possibly containing starting materials directly in the final pharmaceutical product, lactose-sucrose pillules impregnated with TNF- α at 27 CH, or with IL-1 β at 27 CH. A proper vehicle control was included in the tests, lactose-sucrose pillules impregnated with the only solvent without active substances.

Definitely, the study has been carried out with 2 major objectives: first of all, to examine the anti-inflammatory effect and the capacity of 2 unitary medicines, TNF- α (27 CH) and IL-1 β (27 CH), to reduce the secretion of TNF- α induced by LPS in human primary enriched monocytes and in PMA-differentiated THP-1 cell line; then, to investigate the presence of small particles possibly containing starting materials in the same 2 unitary medicines using the qNano Gold, an instrument produced and commercialized by Izon Science, that utilizes the technique of tunable resistive pulse sensing (TRPS). TRPS is a method for sensing and analyzing small particles dispersed in aqueous electrolytes. It uses an electric field which is applied across a pore, also called nanopore. Ions move between the electrodes through the nanopore, creating a baseline current and, when a particle passes through the pore, the ionic current is momentarily blocked and a "resistive pulse" is observed. Finally, these resistive pulses reveal information about particles size, counting, and concentration.²¹

Materials and Methods

Tested Items

The 2 unitary medicines consist of lactose-sucrose pillules impregnated with ethanolic preparation (96% water/ethanol) of human recombinant (hr)-TNF- α or hr-IL-1 β at 27 CH. The 2 cytokines were produced by recombinant technology, under good manufacturing practice (GMP) conditions, in the clean room of Labo'Life Spain's laboratory. ULD are obtained by diluting 1 μ g of the cytokines in 1 ml of endotoxin free water for injection (Ph. Eur.) and up to 1 CH, then in ethanol 96 per cent V/V (Ph. Eur. BDH Chemicals) up to 27 CH. Serial Kinetic Process (SKP) was achieved using STELLA scro 88 amber 20 ml glass bottle (Ph. Eur. STELLA Kunststofftechnik GmbH.), closed with STELLA scro 88 II polypropylene screw cap, with sealing disk (Ph. Eur. STELLA Kunststofftechnik GmbH.). SKP consists of a kinetic vertical processing of 100 succussions generated by a dynamizator (Dynamat 50CS, Labotics bvba). Both unitary medicines were prepared according to the European Pharmacopeia monographs 1038 and 2371 (current edition). The lactose-sucrose pillules impregnated with the only solvent, ethanol 96 per cent V/V (Ph. Eur. BDH Chemicals), without any active substance, were used as vehicle control in all experiments.

Cytotoxicity Assay

Blood samples from 3 healthy human donors (male or female, aged between 18 and 68 years, blood tested as for transfusion, full information and medical questionnaire available in German on www.uniklinik-freiburg.de), were collected at the University Medical Center of Freiburg (Germany). Written informed consent was obtained from all donors. The study has been approved by the Ethics Committee of Albert Ludwigs University of Freiburg.

Human primary enriched monocytes were prepared from buffy coats following a standardized procedure,²² recently described.⁶ Cells were seeded in 96-well plates at a density of 220,000 cells/well.⁶ Both unitary medicines and vehicle pillules were dissolved in cell culture media and cells were exposed to 6 different lactose/sucrose concentrations (1.37, 2.75, 5.5, 11, 22 and 44 mM). After 24 hours of incubation, 10 μ l of AlamarBlue[®] (Thermo Fisher Scientific, Waltham, MA, USA) was added to each well. Then, fluorescence was measured with a fluorescence spectrophotometer using 544EX nm /590EM nm filter settings. The experiment was performed in quadruplicates.

TNF- α Secretion in Human Primary Enriched Monocytes Exposed to LPS and Treated With Unitary Medicines

Cells were seeded in 24-well plates (2.2 million cells/well) and incubated at 37°C with 5% CO₂. As previously described, 30 minutes prior to LPS stimulation, cells were exposed to different concentrations (5.5, 11 and 44 mM) of unitary medicines (TNF- α at 27 CH or IL-1 β at 27 CH) or vehicle and

incubated for 24 hours at 37°C and 5% CO₂.⁶ Then, supernatants were removed, centrifuged, and investigated for TNF- α secretion using commercially available single analyte ELISA kits (Immunotools, Friesoythe, Germany). The protocol used followed the manufacturer's instruction. The experiment was performed in triplicates.

TNF- α Secretion in Differentiated THP-1 Cells Exposed to LPS or IL-1 β to Induce Inflammation

THP-1 cells were obtained from ATCC (Manassas, VA, USA) and cultured in RPMI 1440 supplemented with 10% FCS and antibiotics. 500,000 cells/well were seeded in 24-well plates and treated with PMA (150 nM) for 24 hours. After differentiation, cells were exposed to LPS (1 μ g/mL), IL-1 β (10 ng/mL) or IL-1 β (50 ng/mL) to induce inflammation. Then, supernatants were removed, centrifuged, and investigated for TNF- α secretion using commercially available single analyte ELISA kits (Immunotools, Friesoythe, Germany). The protocol used followed the manufacturer's instruction. The experiment was performed in triplicates.

TNF- α Secretion in Differentiated THP-1 Cells Exposed to LPS and Treated With Unitary Medicines

PMA-differentiated THP-1 cells were pre-treated with different concentrations (5.5, 11 and 44 mM) of unitary medicines (TNF- α at 27 CH or IL-1 β at 27 CH) or vehicle for 30 min and stimulated with LPS (1 μ g/mL) for 24 h at 37°C and 5% CO₂. Then supernatants were removed, centrifuged, and investigated for TNF- α concentrations (pg/mL) using commercially available single analyte ELISA kits (Immunotools, Friesoythe, Germany). The protocol used followed the manufacturer's instruction. Three independent experiments were performed in triplicates.

Particles Measurements and Data Collection

TRPS measurements were performed using the qNano Gold (Izon Science). In order to be analyzed, samples must be diluted in an electrolyte solution. Therefore, all samples were prepared by diluting the content of each capsule (380 mg of sucrose-lactose pillules) of TNF- α (27 CH), IL-1 β (27 CH), and vehicle, in phosphate-buffered saline (PBS). PBS was sterilized by filtration through 0.22 μ m membrane filter before being used to dilute the sugar pillules.

The first test was performed using the membrane pore (or nanopore) NP200 rated for 80–500 nm particles. The samples were diluted in 1 mL of PBS before being analyzed. The pulse signal was calibrated with a 200 nm polystyrene particle standard supplied by Izon Science with a 1 x 10¹² particles/mL concentration.

Because particles bigger than 500 nm were present in samples, obtruding sometimes the nanopore, 2 more tests were performed on 2 different days.

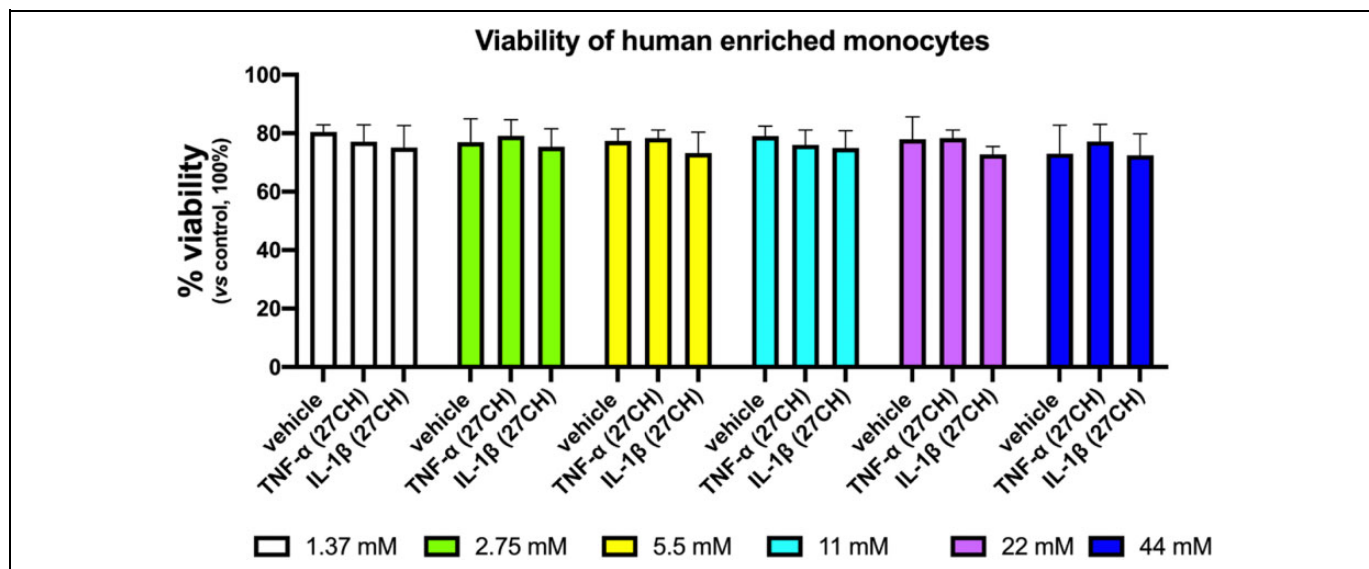


Figure 1. Human enriched monocytes viability examination after exposure to unitary MI medicines or vehicle diluted pillules. Human enriched monocytes were treated for 24 hours with: complete medium (control), or 6 different concentrations, ranging from 1.37 mM to 44 mM, of unitary medicines or vehicle lactose-sucrose pillules. The graph shows the percentage of cell viability evaluated by AlamarBlue[®]. Data were expressed as mean \pm SD percentage (vs. control, 100%) of 3 donors measured in quadruplicates. Minimum and maximum values are indicated in the graph.

For the second and third tests, samples were analyzed by using the NP1000, rated for 490–2900 nm particles. Each sample was diluted in 1 mL of PBS, then was further diluted in PBS 1:2 before being analyzed. The pulse signal was calibrated with a 950 nm polystyrene particle standard supplied by Izon Science, with a 5.2×10^{10} particles/mL concentration. Measurements were made with 46.95 mm of stretch being applied to the elastic membrane and a potential 0.10 V being applied across the pore. 35 μ L of diluted sample was loaded into the top fluid cell and 75 μ L of measurement electrolyte PBS was loaded into the bottom fluid cell. 10 Pa of pressure was applied to the top fluid cell.

Izon Control Suite Software has collected all data about particles size and concentration, and it has generated histograms and graphs to represent the particles distribution.

Statistical Analysis

Authors have followed the recent recommendations encouraging data transparency²³ by plotting independent data points in graphs so that readers may “interpret the data for themselves, rather than showing possibly misleading *P* values or error bars.” Indeed, descriptive statistics was preferred to statistical inferences.

Results

Cell Viability Test

Human enriched monocytes from 3 donors were treated with 6 different lactose-sucrose pillules concentrations, ranging from

1.37 mM to 44 mM, of vehicle or unitary medicines: TNF- α (27 CH), or IL 1 β (27 CH). The experiment included the condition in which cells were cultured only with complete medium, (control). After 24 hours, cell viability was evaluated by AlamarBlue[®]. Samples were run in quadruplicates. Viability in control was set 100%. As shown in Figure 1, all tested concentrations of unitary MI medicines, and of vehicle, have not affected the viability of human primary enriched monocytes, that ranged between 64% (minimum) and 86% (maximum).

The Effect of 2 Unitary MI Medicines on TNF- α Secretion in Human Primary Enriched Monocytes

Human monocytes respond to LPS by expressing many inflammatory cytokines.^{6,24} In particular, the authors investigated the secretion of TNF- α in LPS-exposed enriched monocytes from 3 donors, exposed for 24 hours to different pillules concentrations (5.5, 11 and 22 mM) of unitary MI medicines TNF- α (27 CH), IL-1 β (27 CH) or vehicle. Figure 2 shows the secreted levels of TNF- α , expressed as mean \pm SD percentage vs. vehicle, set 100%. At the pillules concentration of 5.5 mM, the 2 tested unitary MI medicines have not exerted an effect on TNF- α secretion levels compared to vehicle (Figure 2A); at the pillules concentration of 11 mM, the 3 donors responded in a similar manner to both unitary medicines with a reduction of TNF- α levels of about 20% compared to vehicle (Figure 2B). The responses of the 3 donors to both unitary medicines are comparable, as shown in Supplementary Figure 1B. Doubling

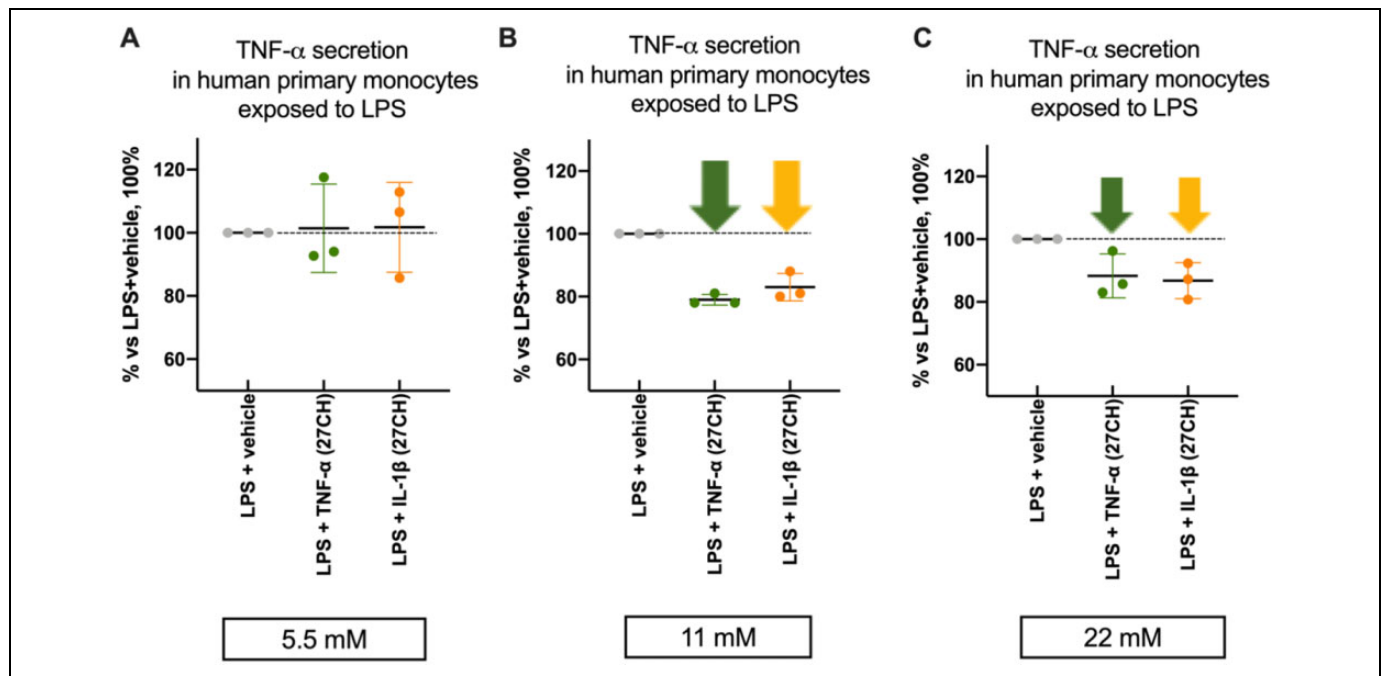


Figure 2. Effect of unitary medicines on TNF- α secretion in human primary monocytes exposed to LPS to induce inflammation. Cultured human enriched monocytes from 3 donors, exposed to 10 ng/mL of LPS for 24 hours, were treated with 3 concentrations: (A) 5.5 mM, (B) 11 mM, or (C) 22 mM of unitary MI medicines (TNF- α at 27 CH represented with green dots; or IL-1 β at 27 CH represented with orange dots) or vehicle (gray dots). Cell medium was collected and TNF- α secretion was measured by ELISA. In graphs are represented individual values, mean \pm SD percentage vs. LPS + vehicle, which was set at 100%.

the pillules concentration from 11 to 22 mM, the effect did not increase; on the contrary, TNF- α secretion appears to have diminished as it can be observed a reduction of about 10% compared to vehicle (Figure 2C).

The Effect of 2 Unitary MI Medicines on TNF- α Secretion in PMA-Exposed THP-1 Cells

In order to evaluate the effect on a cell line, authors established another *in vitro* model of inflammation to test the 2 unitary MI medicines. THP-1 cells were differentiated with PMA (150 nM) for 24 hours, then cells were exposed to LPS (1 μ g/mL), or IL-1 β (10 ng/mL), or IL-1 β (50 ng/mL) for 24 hours, to induce the secretion of cytokines, in particular of TNF- α . As the highest levels of TNF- α were obtained using LPS as inflammatory stimulus (Figure 3), PMA-differentiated and LPS-exposed THP-1 cells have been selected and used as a model of inflammation to test the specific anti-inflammatory effect of the 2 medicines.

At the pillules concentration of 5.5 mM, the 2 tested unitary medicines have reduced the TNF- α secretion compared to vehicle by about 20% (Figure 4A). At the pillules concentration of 11 mM, the effect of both tested items on TNF- α secretion is similar to the one observed in cells treated with 5.5 mM (Figure 4B). When doubling the pillules concentration from 11 to 22 mM, the effect did not increase, but on the contrary, it diminished (Figure 4C).

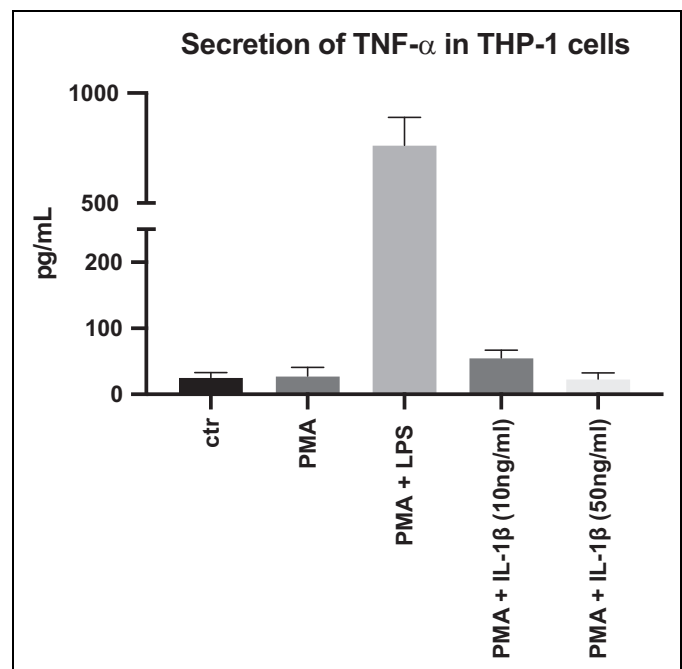


Figure 3. TNF- α secretion in differentiated THP-1 cells exposed to LPS or IL-1 β to induce inflammation. THP-1 cells were differentiated with the use of PMA (150 nM) for 24 h. After differentiation, cells were exposed to LPS (1 μ g/mL), IL-1 β (10 ng/mL) or IL-1 β (50 ng/mL) to induce inflammation. Untreated cells were included in the experiment (ctr), too. After 24 hours, cell medium was collected and TNF- α secretion was evaluated by ELISA. In graphs are represented mean values (pg/mL) \pm SD.

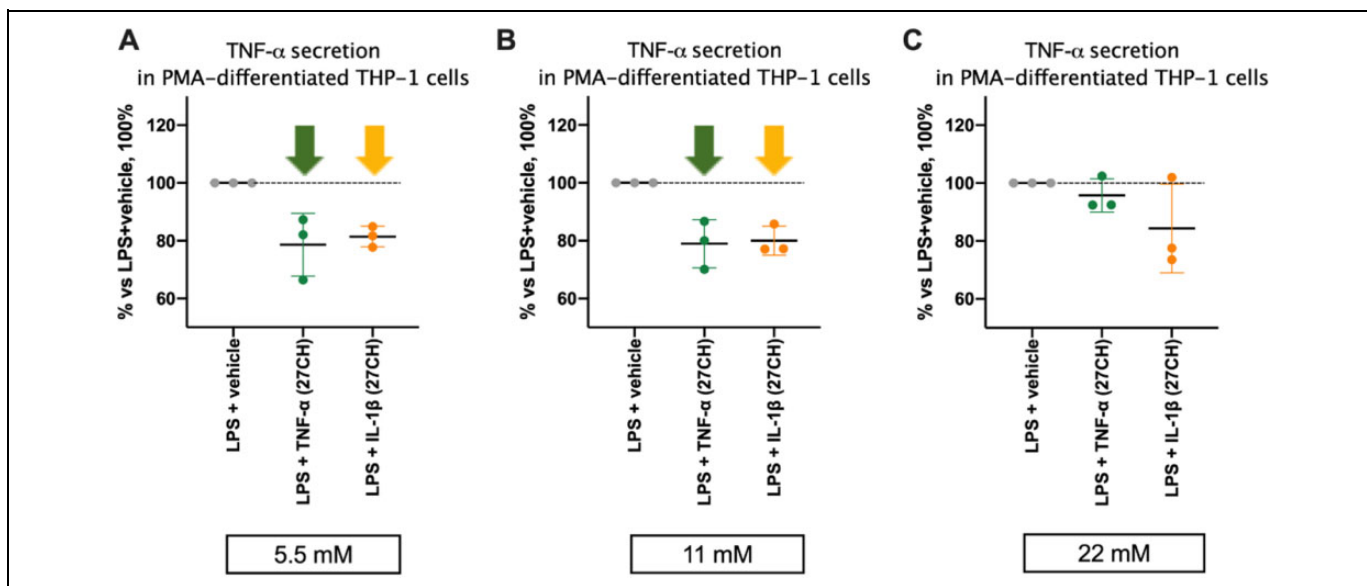


Figure 4. Effect of unitary MI medicines on TNF- α secretion in treated PMA-differentiated THP-1 cells exposed to LPS to induce inflammation. THP-1 cells were treated with PMA (150 nM) for 24 h. Then, cells were pre-treated with 3 concentrations: (A) 5.5 mM, (B) 11 mM, or (C) 22 mM of unitary MI medicines (TNF- α at 27 CH represented with green dots; IL-1 β at 27 CH represented with orange dots; vehicle represented with gray dots) for 30 min and stimulated with LPS (1 μ g/mL) for 24 h. Cell medium was collected and TNF- α secretion was evaluated by ELISA. In graphs are represented individual values, mean (of 3 independent experiments made in triplicates) \pm SD percentage vs. LPS + vehicle, which was set at 100%.

Raw Particles Concentration and Distribution in Impregnated Pillules

Pillules (vehicle and unitary medicines) have been diluted in PBS in order to allow the passage of the ionic current throughout the nanopore and to be analyzed by the qNano, as described in material and methods.

The first measurement has been performed using the nanopore NP200, which is rated for particles with a diameter ranging from 80 to 500 nm. Figure 5A shows the raw concentration values (or number of particles per mL) of particles found in diluted vehicle-impregnated pillules (6.7×10^7 particles/mL) and TNF- α (27 CH)- or IL-1 β (27 CH)-impregnated pillules (respectively 2.2×10^8 particles/mL, and 8.4×10^8 particles/mL).

In the light of the results, even if those pillules impregnated with the only vehicle solution do contain small particles, TNF- α (27 CH)-impregnated pillules have been found to be 3.3 more concentrated than vehicle, and IL-1 β (27 CH)-impregnated pillules appeared to be 12 times more concentrated than vehicle.

qNano can provide more information, like the arithmetic mean of particles diameter. These are the values obtained in the first test for vehicle, TNF- α (27 CH) and IL-1 β (27 CH): 185 nm, 157 nm and 209 nm, respectively. In addition, the software can analyze the data generating graphs that combine data from multiple samples. In order to highlight eventual differences in particle distribution between vehicle and unitary medicines, 2 graphs have been generated, shown in Figure 6 A and B, representing the particles distribution of TNF- α (27

CH) together with the vehicle, or IL-1 β (27 CH) together with the vehicle.

The second and the third measurements, performed on 2 different days, employed the use of a larger nanopore, NP1000, rated for particles ranging from 490 to 2900 nm. Figure 5B shows the raw concentration values present in vehicle-impregnated pillules ($1.5\text{--}3.9 \times 10^7$ particles/mL) and ULD-impregnated pillules ($5.2\text{--}6.5 \times 10^7$ particles/mL and $8.9\text{--}9.1 \times 10^7$ particles/mL) respectively in TNF- α (27 CH)- and IL-1 β (27 CH)-impregnated pillules. ULD-impregnated pillules were found to be more concentrated than vehicle for bigger particles too. Particles diameter means for vehicle, TNF- α (27 CH) and IL-1 β (27 CH) samples were: 638 nm, 781 nm, and 729 nm (found in the second measurement), and 684 nm, 723 nm, and 675 nm (detected in the third measurement). Differences between vehicle and unitary medicines in bigger particles distribution could be visualized, as well as for small particles, in the 2 graphs shown in Figure 6 C and D.

Discussion

The results of the first part of the present study show that MI pillules impregnated with ULD of 1 single pro-inflammatory cytokine at a time are not cytotoxic, and exert anti-inflammatory properties *in vitro*.

To evaluate potential cytotoxic effects, viability of human monocytes from 3 donors exposed to vehicle, TNF- α (27 CH), or IL-1 β (27 CH), in a wide range of pillules concentrations (from 1.37 to 44 mM), have been assessed. On the basis of the results, the 2 tested ULD unitary medicines are non-cytotoxic

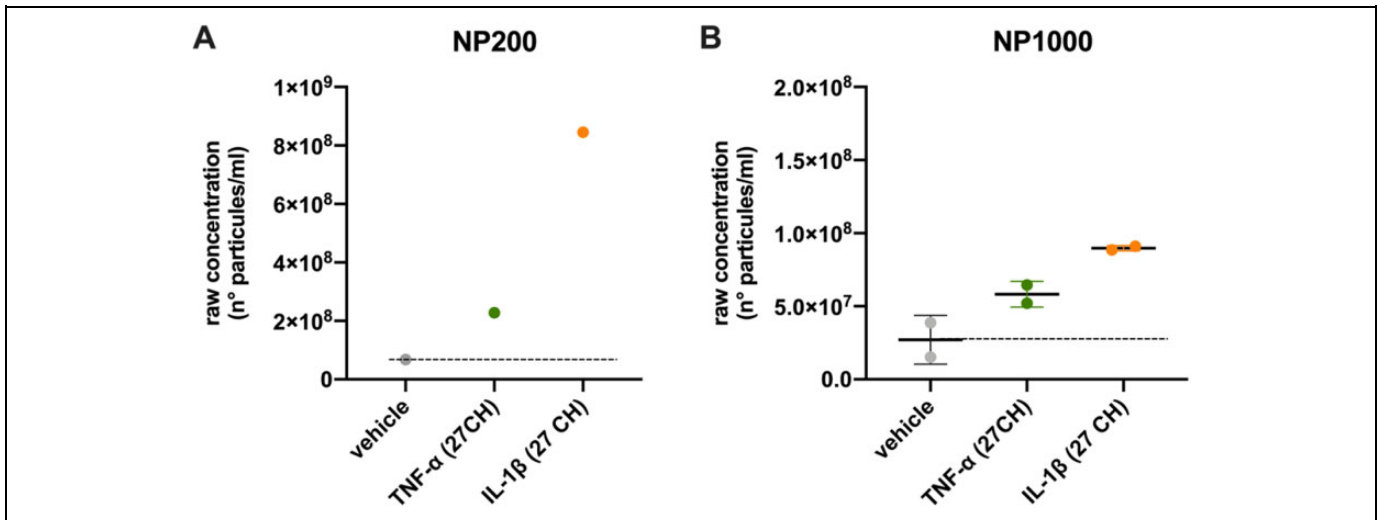


Figure 5. Raw concentrations of particles present in impregnated pillsules, obtained by using 2 different nanopores, NP200 and NP1000. (A) The 2 unitary MI medicines TNF- α (27 CH), IL-1 β (27 CH), as well as vehicle impregnated pillsules, were diluted in PBS (1 capsule of 380 mg in 1 mL), then analyzed by qNano using a NP200 nanopore, rated for 80–500 nm particles. The values of the raw concentrations, expressing the number of particles per mL, are reported in the graph. (B) Because the first analysis revealed the presence of particles bigger than 500 nm, 2 more tests were performed using a NP1000 nanopore, rated for 490–2900 nm particles. TNF- α (27 CH), IL-1 β (27 CH), or vehicle impregnated pillsules were diluted in PBS, (1 capsule of 380 mg in 1 mL, then further diluted 1:2) before being analyzed. The values of the raw concentrations, expressing the number of particles per mL, measured in 2 different days, are shown in the graph.

under the experimental conditions and pillsules concentrations tested (Figure 1).

It is important to remind that pillsules are composed of lactose and sucrose, sugars that mammalian cells are not able to hydrolyze. Already in 1969, Cohn and his collaborators observed how macrophages exposed to indigestible disaccharides are uptaken by pinocytosis and stored in secondary lysosomes.²⁵ In the 90 s, more studies confirmed the findings,²⁶ and discovered that sucrose can activate autophagy.^{27–29} The absence of cytotoxic effect has been proven at high sucrose concentration (100 mM), as well as any effect in apoptosis, while the effects on gene expressions for those proteins involved in autophagy via AKT/mTOR pathways, increase with time of treatment, and in a dose-dependent manner, starting with 25 mM.²⁹

Because the disaccharides that compose the vehicle of MI medicines can produce biological effects, activating autophagy or possible other pathways, *in vitro* efficacy is often tested at the “safe” concentration of 11 mM^{7,8} or into a “safe” range of concentrations, below that or equal to 22 mM.⁶

In the light of the results, among the 3 pillsules concentrations (5.5, 11 and 22 mM) tested, 11 mM represents the good compromise and the suitable concentration to evaluate the biological effects of MI medicines *in vitro*.

TNF- α (27 CH) has exerted an anti-inflammatory effect reducing the secretion of TNF- α in LPS-stimulated monocytes from 3 donors compared to vehicle by about 20% when pillsules were diluted at 11 mM (Figure 2B), and by about 10% at the concentration of 22 mM (Figure 2C). Doubling the pillsules concentration from 11 to 22 mM, the anti-inflammatory effect of ULD of TNF- α slightly

decreased, probably because the effect of sucrose and lactose molecules at 22 mM have influenced and masked somehow the effect of the unitary MI medicine. Reducing the concentration by half, from 11 mM to 5.5 mM, the anti-inflammatory effect on TNF- α secretion disappeared, probably because the ULD of TNF- α is too diluted in cell media (Figure 2A).

In a very similar manner, in primary enriched monocytes exposed to LPS and treated with ULD of IL-1 β , levels of TNF- α secretion reduced compared to vehicle-treated cells (Figure 2A–C). It might be explained with the fact that the 2 cytokines are strongly linked by an autocrine positive loop that keep upregulated the levels of pro-inflammatory cytokines in monocytes, after LPS exposure,³⁰ and IL-1 β (27 CH) probably acts via the inhibition of IL-1 β .

It is well-known that primary cells are more sensitive than a cell line, and maintain much better the functions seen *in vivo* than a cell line. On the other side, the inter-individual variability among donors could lead to donor-dependent responses. For that reason, while the number of donors is low, and no conclusions can be drawn, authors have represented singly each donor response for the concentration of 11 mM in Supplementary Figure 1. It can be visualized that the responses on the % of TNF- α reduction is very similar among the 3 donors, while concentrations (expressed as pg/ml) of vehicle-treated cells are not equivalent among donors.

In order to evaluate the anti-inflammatory effects of the 2 unitary medicines also in cell line, authors opted for PMA-differentiated THP-1 cell line, broadly used to study monocytes/macrophages functions because of the biological similarity with human peripheral blood mononuclear cell

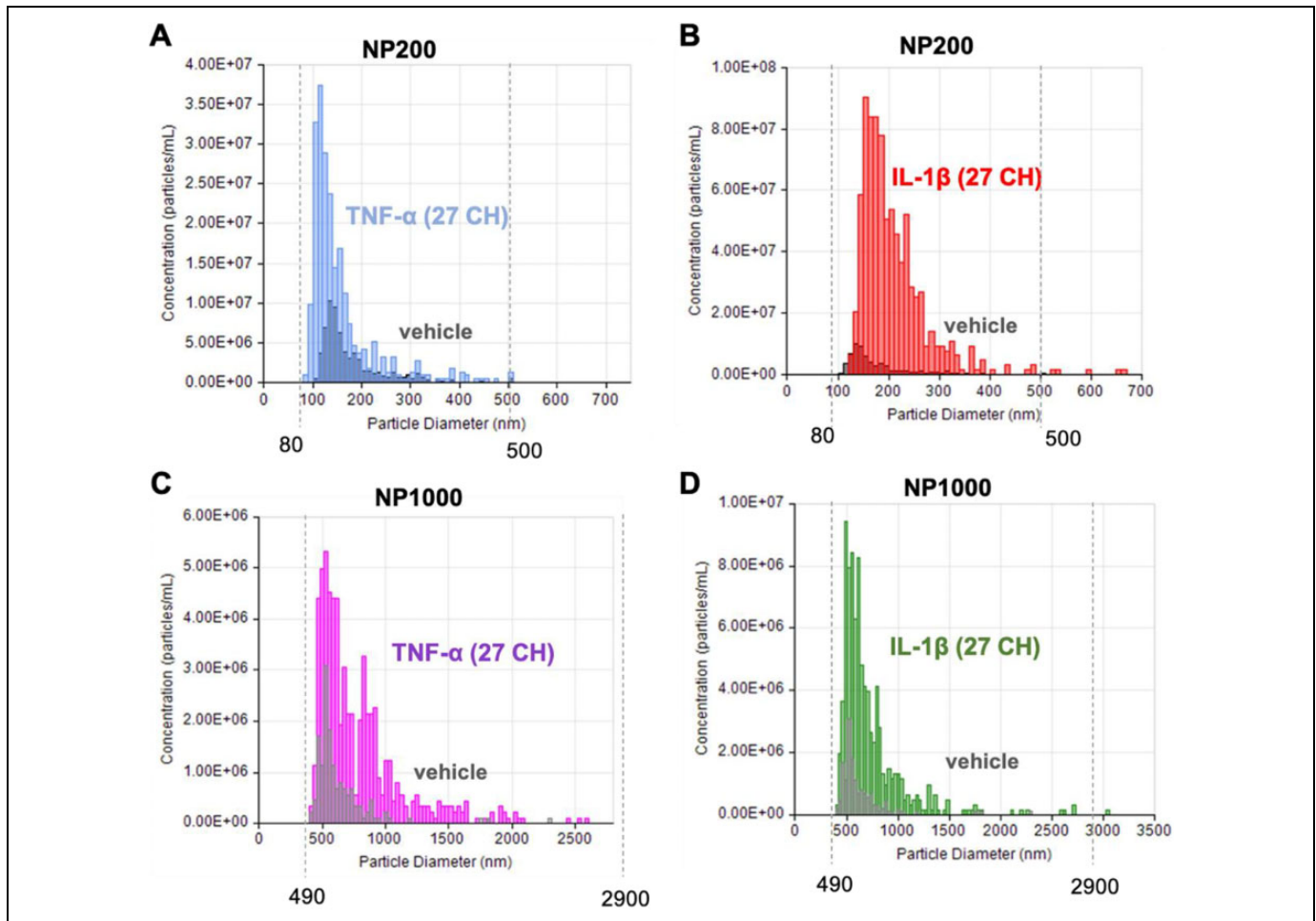


Figure 6. Distribution of particles present in impregnated pillules and acquired by using 2 different nanopores, NP200 and NP1000. The particles distribution measured in diluted pillules, TNF- α (27 CH) together with vehicle-impregnated pillules (A), as well as the distribution of particles in diluted pillules impregnated with IL-1 β (27 CH) or with vehicle, obtained in the first analysis with NP200, is represented in graphs. Samples were also run using the NP1000 nanopore, rated for 490–2900 nm particles. The distribution of bigger particles measured in diluted pillules, impregnated with TNF- α (27 CH) and vehicle (C), as well as with IL-1 β (27 CH) and vehicle (D), is represented in graphs.

derived-monocytes and macrophages.^{31,32} To establish this *in vitro* model, different cell culture conditions were investigated: PMA-differentiated THP-1 were exposed to LPS (1 $\mu\text{g}/\text{mL}$), or IL-1 β (10 ng/mL), or IL-1 β (50 ng/mL) to induce inflammation. After 24 hours, cell medium was collected and TNF- α secretion was evaluated. The highest levels of TNF- α secretion have been observed in LPS-stimulated cells, as shown in Figure 3. Therefore, the anti-inflammatory effect of the 2 unitary medicines has been evaluated in PMA-differentiated THP-1 cell line after LPS exposure. Figure 4 shows the results of 3 independent experiments and 3 pillules concentrations (5.5, 11 and 22 mM). At the lowest concentration (5.5 mM), both tested ULD medicines have reduced the secretion of TNF- α by about 20% compared to vehicle (Figure 4A). Doubling the concentration, from 5.5 to 11 mM , the response of ULD-treated cells compared to vehicle is approximately the same (Figure 4B). Doubling again, from 11 mM to 22 mM , the anti-inflammatory effect was reduced and almost disappeared (Figure 4C), probably because the pillules concentration was

too high and the disaccharides of vehicle mask the effect of the ULD. The anti-inflammatory effect of IL-1 β (27 CH) on TNF- α secretion has been observed also in LPS-stimulated PMA-differentiated THP-1 cells, as already mentioned, probably via the inhibition of IL-1 β . That hypothesis is sustained by data of 2 independent experiments made on LPS-stimulated PMA-differentiated THP-1 cells, showing that the exposure to IL-1 β (27 CH) has reduced the secretion of IL-1 β by about 10% vs. vehicle at 2 pillules concentrations, 11 and 22 mM (Supplementary Figure 2). While the data are encouraging additional experiments, kinetic and molecular studies are necessary to validate that hypothesis.

To summarize, the findings of that first part of the study provide *in vitro* evidence about the specific anti-inflammatory effect on TNF- α secretion levels of TNF- α (27 CH), or IL-1 β (27 CH), in comparison to the vehicle control.

To explore the possible involvement of small particles possibly containing starting materials in the mode of action of ULD, the second objective of the study was to investigate the

presence of particles by using TRPS technique in diluted pillules impregnated with TNF- α (27 CH) or IL-1 β (27 CH). A proper control was included, too.

According to the EU Commission, the term nanomaterials, and nanoparticles, is described as “a manufactured or natural material that possesses unbound, aggregated or agglomerated particles where external dimensions are between 1–100 nm size range,”³³ while sub-micron particles have a diameter bigger than 100 nm and lower than, or very closed, to 1 micron.

TRPS, a very sensitive technique of nanotechnology, has been preferred to other techniques, like nanoparticle tracking analysis (NTA), because TRPS is more suitable for polydisperse system, as it can measure particle size distributions of a complex polydisperse samples with high resolution and accuracy.³⁴ TRPS utilizes a size tunable pore, or nanopore, that is formed by mechanically puncturing a micron sized hole into an elastic membrane. An inconvenience of this technique is the issue of pore blockages due to particles that are too large to fit through the pore, or particles that, due to their chemical properties, are attracted to the pore surface and form aggregates within or around the pore, causing an increase in time devoting to cleaning and washing the machine. On the other side, the positive point of using a nanopore is that the concentration is calculated into a defined size range, allowing the users to make sure there is no over or under expression of particles.

All items—vehicle, TNF- α (27 CH) and IL-1 β (27 CH)—were equally managed and, just before being analyzed, the solutions were prepared by diluting pillules in sterile filtered (0.22 μ m filter) PBS. The solutions were highly concentrated in those 2 disaccharides, sucrose and lactose, of which MI pillules are composed (380 mg/mL in the first measure, or 190 mg/mL in the second and the third); however, this did not influence the measurement. It could not be excluded that the sugar molecules have formed aggregates of different size, making the interpretation of the results complicated. For that reason, in order to highlight eventual differences in size and particles concentrations between vehicle control and active samples, the authors made the graphs shown in Figure 6. The use of ULD preparation could have solved that issue, nevertheless, it would have been necessary to dilute the ethanol at least 1,000 times to avoid the oxidation of the metallic silver-silver (Ag/AgCl) electrode.

The first test performed with the NP200, which is rated for particles with a diameter ranging from 80 to 500 nm, have revealed the presence of particles having a diameter average of about 150–200 nm in all tested samples. While particles were also detected in the vehicle control, the raw concentration was lower than the 2 concentrations observed in ULD-impregnated pillules (Figure 5A).

During the detection, the nanopore was obstructed by particles bigger than 500 nm. Consequently, the same items were analyzed by using the NP1000, rated for particles having a diameter ranging from 490 to 2900 nm. In that way, bigger particles were also detected in all tested samples, including vehicle. The particles diameter average ranged from about 630 to 780 nm. As shown in Figure 5B, the lowest

concentration was again observed in diluted vehicle-impregnated pillules.

In the light of the results of the second part, for the first time, the possibility that MI based on ULD, may retain starting materials on impregnated pillules has been discussed. Because glass bottles are used to prepare ULD, it can be speculated that silica particles are formed during the SKP process, establishing stable tridimensional structures with proteins.³⁵ Silica particles present in high potencies might be the carrier of the active substances used in MI medicines. Then, those particles can be transferred to the sugar pillules during the pillules impregnation, the last manufacturing pharmaceutical process.

Depending on the size, the mode of action could change: while very small particles, characterized by a high bioavailability, can cross cellular membranes and induce specific cell responses,³⁶ bigger particles could activate the immune system³⁷ inducing specific biological responses.

Hormetic responses, already applied as a plausible mode of action for homeopathic medicines, can be involved also in the mode of action of LD- and ULD-based MI medicines.

All the research carried out in the last years on high potencies and homeopathic remedies, including the results discussed in the present study, raise still more questions than answers. With the implementation of nanotechnology, novel knowledge, and, on the other side, new difficulties have been added in that area of research. In parallel, the research aimed at evaluating the biological effects *in vitro* and *in vivo* must continue, to legitimate the importance of that field of research, and to develop the potential of that therapy.

Conclusions

Sub-micron particles possibly containing starting materials in a very low dose, transferred on impregnated MI pillules, may exert their biologic effects in cells and/or in organisms via hormesis. Aware about the limitation of the study, it is important to highlight that, for the first time, the presence of particles, in a wide range of size, has been disclosed in unitary-ULD-(27 CH)-impregnated pillules, at concentrations that appeared higher than vehicle. That discovery has been associated to a specific anti-inflammatory effect of the 2 unitary MI medicines in 2 different *in vitro* models. With this association, a materialistic hypothesis based on the “surface monolayer hypothesis” and the “silica hypothesis” has been proposed for MI. Nevertheless, authors are cautious in the conclusions because the findings described in the study are still insufficient, and future investigation may unveil unexpected results and different hypothesis.

Other studies have previously detected the presence of NPs in commercial homeopathic control solutions.^{38,39} The principal interrogation about the possible contamination cannot be ignored, and authors prefer to be cautious and do not jump to conclusions. More evaluations of LD and ULD-impregnated pillules are compulsory and should use different methods, like transmission electron microscopy, scanning electron microscopy, and atomic force microscopy, together with the recent

nanotechnology techniques, in order to also characterize the nature and the chemical composition of those particles present in MI pillules.

Authors' Note

IF and BL designed the study. TR, JACR and CR performed the experiments. IF, BL, and KA coordinated the overall undertaking of the study. IF wrote the manuscript and prepared the figures. BL, KA and CR revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The data of the current study are available from the corresponding author on reasonable request.


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Supplemental Material

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