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Research paper

Imiquimod-loaded nanocapsules improve cytotoxicity in cervical cancer cell line

L.A. Frank[a,*], R.P. Gazzi[b], P. de Andrade Mello[c,d], A. Buffon[b], A.R. Pohlmann[a,e], S.S. Guterres[a,*]

[a] Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
[b] Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
[c] Laboratorio de Biologia Celular, Universidade Federal de Ciências da Saude de Porto Alegre (UFCSPA), Porto Alegre 90050-170, Rio Grande do Sul, Brazil
[d] Laboratorio de Análises Bioquímicas e Citológicas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre 90610-000, Rio Grande do Sul, Brazil
[e] Departamento de Química Orgânica, Instituto de Química, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil

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ABSTRACT

This paper proposes the development of imiquimod-loaded polymeric nanocapsules formulation for the treatment of cervical cancer. The mechanism of death involved in the reduction of the cell viability as well as the production of an inflammation marker (IL-6) after the treatment in cell line SiHa have been evaluated. The formulation has significantly decreased the viability of the cells in a time-dependent manner, after 24, 48 and 72 h. Additionally, results showed a cellular decrease of almost 80% of the cells after 72 h of treatment. The formulation induced death by apoptosis, necrosis, autophagy, and increased the percentage of SubG1subpopulation of SiHa cells after 72 h. After the same time-interval, the formulation significantly prevented the appearance of colonies, showing effectiveness against SiHa. Finally, the formulation stimulated SiHa cells to release IL-6. These findings open new possibilities for the development of aqueous nanosuspension containing imiquimod as a novel strategy for the treatment of cervical cancer.

1. Introduction

Cervical cancer is the fourth most common cancer among women [1]. Epidemiological studies have pointed out a strong correlation between cervical cancer incidence and some specific types of human papillomavirus (HPV) infection, which represents the most common sexually transmitted viral disease [2]. HPV is subdivided in low and high-risk according to their malignant potential and cell-transforming capacity *in vitro*. The subtypes of HPV low-risk, for example HPV 6 and HPV 11, are causative agents of genital warts, while the high-risk subtypes HPV 16 and HPV 18 are those directly associated to the development of cervical cancer [3]. Conventional treatments against this cancer such as radical hysterectomy, exenterations, radiotherapy or chemotherapy are considered painful and expensive, affecting significantly women's quality of life [1].

An alternative way proposed to deal with this kind of cancer is by the use of topical application of imiquimod to improve the immune response against the HPV virus. Results of the use of imiquimod have shown a beneficial reduction of HPV load in patients with external genital warts [4,5]. While most of the immunomodulatory agents available or in development act as inhibitors of the pathways involved in immune activation, imiquimod is the only one that activates immune function [6]. Imiquimod acts as an agonist of toll-like receptor (TLR) 7, activating both innate (monocytes, macrophages and dendritic cells) and adaptive cellular immunity (Th1) through the induction of pro-inflammatory cytokines, such as interferon alpha (IFN-α), tumor necrosis factor (TNF) and interleukins (IL) 1, 6, 8 and 12. In parallel, this drug induces cell apoptosis and activates B lymphocytes, potentializing the immune response [7–9] for which high doses of the drug are required.

Recently, the nanoencapsulation of drugs, including imiquimod [10], has been proposed to increase drug selectivity and efficacy in the treatment of HPV related diseases such as cancer [11–13]. Nanoencapsulated antitumor drugs have been able to significantly decrease the tumor cells viability in comparison with their respective control (free drug) [14,10]. This effect is attributed to the polymeric nanocapsules ability to modulate the interaction between drug and cells, consequently delivering a greater amount of drug in the tumor environment [15]. The use of imiquimod as an antitumor drug has already been demonstrated in different cancer cell lines such as prostate

* Corresponding authors at: Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga, 2752/405, CEP 90610-000 Porto Alegre, RS, Brazil.
E-mail addresses: luiza.frank@ufrgs.br (L.A. Frank), silvia.guterres@ufrgs.br (S.S. Guterres).

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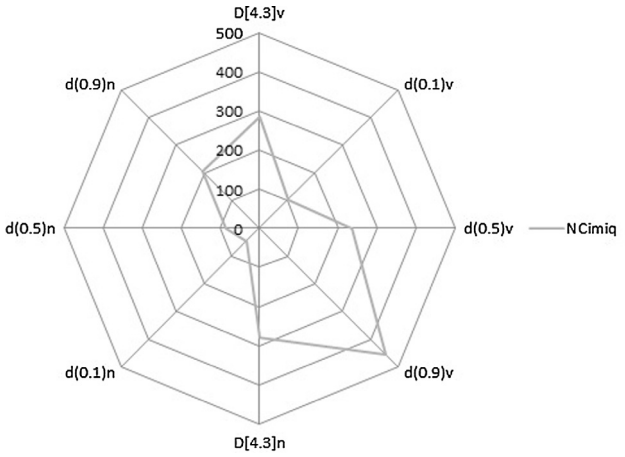

Fig. 1. Radar chart presenting the volume-weighted mean diameters (D[4,3]) and the diameters at percentiles 10, 50 and 90 under the size distribution curves by volume and by number of particle.

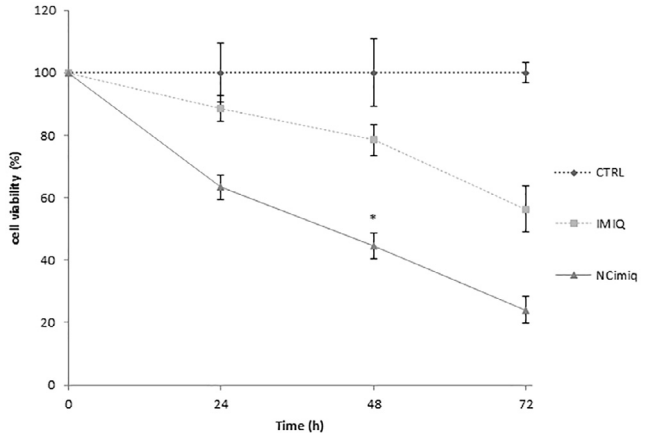

Fig. 2. Number of viable SiHa cells after 3 μM NCimiq and IMIQ after treatment for 24, 48, and 72 h n = 3 and $^{*}p < 0.05$ compared with control (one-way ANOVA, followed by Tukey's test.

cancer (TRAMP-C2 and PC-3) [16], basal cell carcinoma (BCC/KMC-1 and A375) [17–19] and squamous cell carcinoma (SCC12) [20]. However, the mechanism of action involved on this imiquimod-loaded polymeric nanocapsules effect is still unknown. According to literature, imiquimod per se is able to decrease the viability of cells through induction of apoptosis [16–18,20], cell cycle arrest [16] and autophagy [17,18]. In this study, we aim to further elucidate the intracellular mechanism by which the imiquimod-loaded nanocapsule formulation induced cervical cancer cells (SiHa) death, as well as to evaluate its capability to stimulate cancer cells production of proinflammatory interleukin (IL-6) in order to clarify its utility as a new formulation in HPV-related cancer therapy.

2. Materialsandmethods

2.1. Materials

Poly (ε-caprolactone) (PCL) (Mn 80 kg mol^{-1}) and sorbitan monostearate (Span 60®) were purchased from Sigma-Aldrich (Steinheim, Germany). Polysorbate 80 (Tween 80®) was purchased from Henrifarma (São Paulo, Brazil) and copaiba oil was kindly donated by Inovam-Da Lamarta&cia Ltda. Imiquimod (IMIQ) was purchased from Chemical Goods (Guangdong, China). Annexin V, and propidium iodide were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Acridine orange (AO) were purchased from Sigma-Aldrich (St. Louis, MO). The cervical carcinoma cell line SiHa was purchased from American Type Culture Collection, Rockville, MD. All solvents and reagents were of analytical or pharmaceutical grade.

2.2. Methods

2.2.1. Production of imiquimod-loaded nanocapsules

The nanocapsules were produced by the interfacial deposition of preformed polymer method [21]. Briefly, an organic phase consisting of imiquimod (5 mg), PCL (100 mg), copaiba oil (334 μl) and Span 60® (38.4 mg) was dissolved in acetone (27 mL). The solution was maintained under magnetic stirring at 37 °C during 15 min. Then, the organic phase was injected into an aqueous phase (53 mL) containing polysorbate 80 (76 mg). After 10 min, the turbid solution was evaporated under reduced pressure in a rotative evaporator at 37 °C (Büchi, Switzerland) to approximately 10 mL. The formulation produced was named NC_{imiq}.

2.2.2. Characterization of nanocapsules

The nanocapsules were characterized in terms of pH, size and zeta potential, immediately after production. The pH analysis was performed by direct measurement using potentiometry (B474 Micronal). The nanocapsules size was measured by different techniques (Laser diffraction: Mastersizer 2000, Nano ZS, Malvern; and Dynamic light scattering: Zetasizer Nano ZS, Malvern) by dilution of nanocapsules in bidistilled water. For determination of the zeta potential, the nanocapsules were diluted in NaCl solution (10 mM) and analyzed by electrophoretic mobility (Zetasizer, Nano ZS, Malvern). Analyses were performed in triplicate.

The drug content (n = 3) was determined after the imiquimod extraction from the nanocapsule aqueous dispersions by High Performance Liquid Chromatography with detection in the ultraviolet (HPLC-UV, Series 200, PerkinElmer, Waltham, MA, USA). The quantification method was adapted [22] and validated according to our purposes. A C18 reversed phase column (Merck & Co, Inc, Whitehouse Station, NJ, USA) was used as stationary phase and acetonitrile:acetate buffer (pH4.0; 100 mM):diethylamine (30:69:85:0.15 v/v), as mobile phase. An injection volume of 20 μl was used and the drug was detected at 242 nm. Calibration curves (n = 3) were made to determine the drug concentration showing linearity ($r = 0.998$) in the range of 1–25 μg mL^{-1}.

The encapsulation efficiency (EE%) was calculated using Eq. (1).

$$EE\% = \frac{C_t - C_{free}}{C_t} \times 100 \quad (1)$$

were C_t is the drug content and C_{free} is the imiquimod concentration determined by HPLC-UV in the ultrafiltrate, which was obtained by using the ultrafiltration/centrifugation technique (Ultrafree-MC 10,000 MW, Millipore, Billerica, USA) at 4120*g* for 10 min.

2.2.3. Cell culture

Cervical carcinoma cell line SiHa containing integrated HPV 16 (American Type Culture Collection, Rockville, MD) was used to verify the antitumor capability of the formulations tested. Cells were maintained in low glucose DMEM supplemented with 10% FBS and penicillin/streptomycin antibiotics (0.5 U mL/95% air at 37 °C).

2.2.4. Cell treatment

Cells were seeded and treated after 24 h with the formulation NC_{imiq}. The free drug (drug solution in DMSO at the same concentration of the nanoformulation) was also used as control. The cultures were exposed to the formulations for 24, 48 and 72 h with concentrations of 3.0 μmol L^{-1} of nanocapsule aqueous dispersions in culture media. All materials were previously sterilized (autoclaving process), and the nanocapsule suspensions were prepared under aseptic conditions.

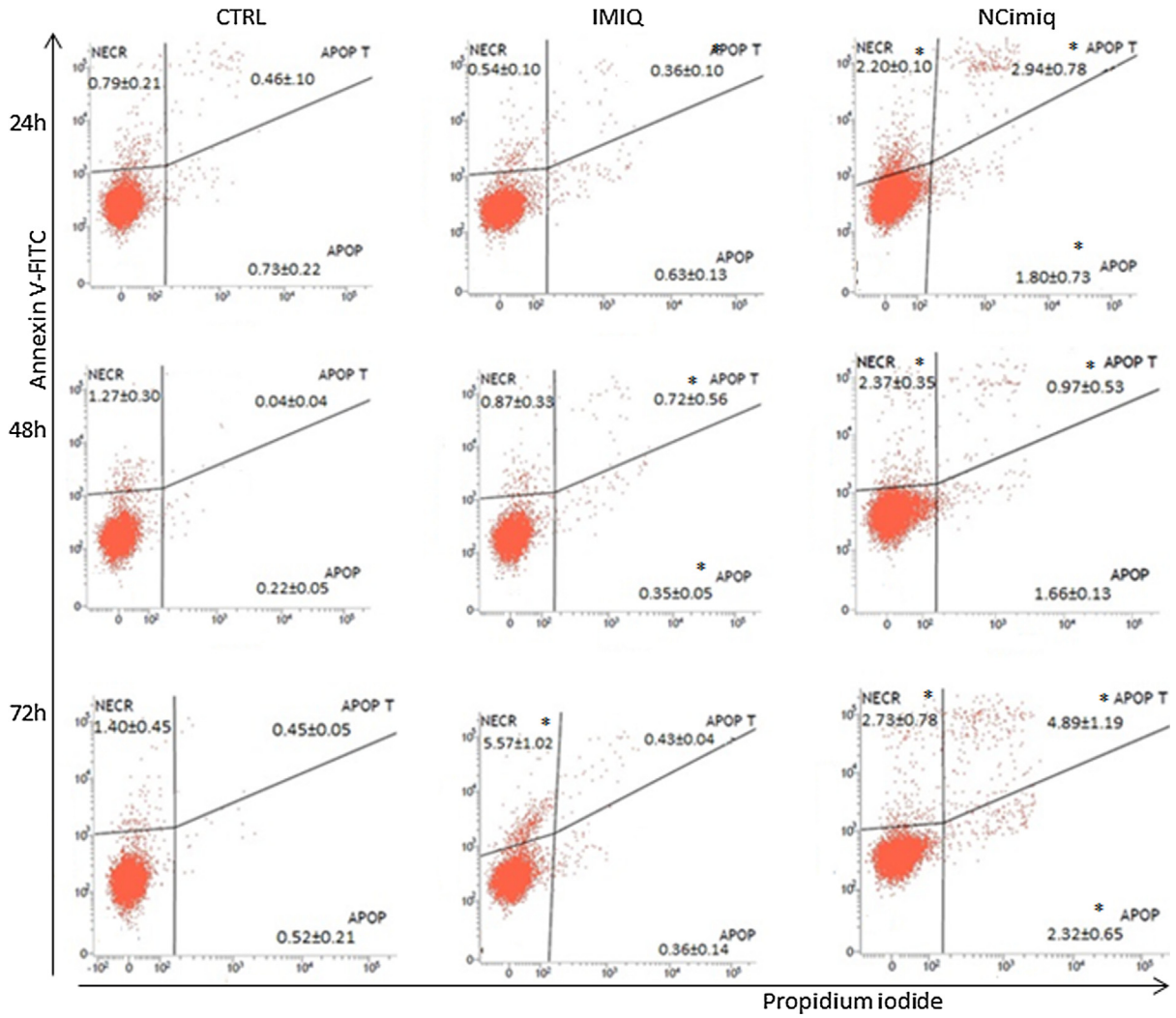

Fig. 3. SiHa was exposed or not for 24, 48 and 72 h with 3 μm NCimiq and IMIQ. Apoptosis and necrosis were measured according to annexin V/PI binding. n = 3 and $^{*}p < 0.05$ compared with control (one-way ANOVA, followed by Tukey's test). Note: PerCP = annexin V-FITC, FITC-A = propidium iodide.

2.2.5. Cell viability

Cell lines (40,000 cells/well) were seeded on 24-well plates and 24 h later they were treated with formulations according to described above. At the end of treatment, medium was removed, cells were washed with 1× PBS, 200 μl of 0.25% trypsin/EDTA was added to detach the cells and 400 μl of DMEM + 10% FBS was added to inactivate trypsin. The viable number of cells was then counted by flow cytometry using FACSVerse flow cytometer (BD Biosciences, San Jose, CA, USA). Negative controls were used by treating cells with DMEM supplemented with 10% FBS. Results were expressed in percentage values regarding the control.

2.2.6. Annexin v and propidium iodide staining

Phosphatidylserine externalization was determined by the annexin fluorescence signal of an annexin V–fluorescein isothiocyanate conjugate (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) according to the manufacturer's protocol. Cell cultures were treated, trypsinized, and centrifuged for 6 min at 1600 rpm, and the supernatant was discarded. The pellet was suspended with 150 μl of annexin binding buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), incubated with annexin V at 0.75 μl/sample and PI at 15 μl/sample for 15 min at room temperature in the dark, and analyzed in a FACSVerse flow cytometer, using FACSVerse software for analysis (BD Biosciences, San Jose, CA, USA). Cisplatin, 40 μM, was used as positive control for apoptosis, and 0.1% Triton X-100 was used as a positive control for necrosis.

2.2.7. Labeling the nucleus of cells with hoechst dye

Cell nuclei were stained with Hoescht35565665 (1500 μg/mL) according to the manufacturer's instruction. The dye labeling was done after treatment at the established time intervals (24, 48 and 72 h) by fluorescence microscopy.

2.2.8. Detection of autophagy using acridine orange (AO)

The development of acidic vesicular organelles (AVO) was quantified.AVO formation is a typical feature of autophagy, and its development indicates autophagosomes maturation and an efficient autophagic process, since only mature/late autophagosomes are acidic [23,24]. For this experiment, cells (20,000 cells/well) were seeded on 24-well multiwell plates, waited to growth for 24 h and exposed to 3 μM NC_{imiq} and IMIQ for 24, 48 and 72 h. The development of acidic vesicular organelles (AVO) was quantified. AVO formation is a typical feature of autophagy, and its development indicates autophagosomes maturation and an efficient autophagic process, since only mature/late autophagosomes are acidic [23,24]. Rapmicyn (200 nM) was used in parallel as a positive control of autophagy inducer.

2.2.9. Cell cycle analyses

After treatment with NC_{imiq} and IMIQ according to described above, cells were detached and centrifuged at 1500 rpm for 5 min.

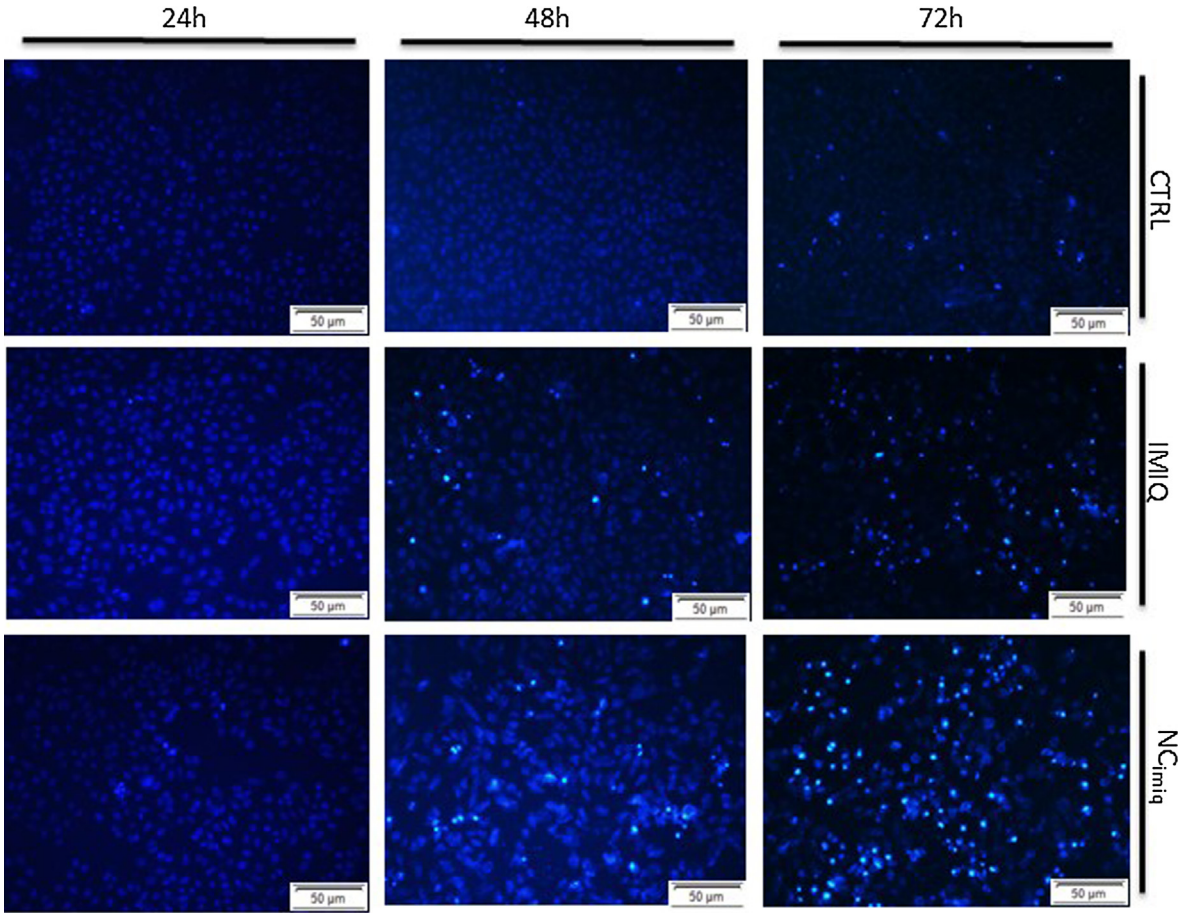

Fig. 4. Images of SiHa cell treatment with 3.0 µM NCimiq and IMIQ for 24, 48, and 72 h. Cell nuclei was stained with Hoescht 35565665 according to manufacturer's instruction. Note apoptotic features and fragmented nuclei when cells were treated with imiquimod. Scale bars, 20 µm; magnification, 20×.

Subsequently, cells were washed 1× with 200 µl of PBS and centrifuged at 1500 rpm for 5 min. Then cells were fixed in 70% ethanol for 2 h at 4 °C, followed bya new wash with PBS and centrifugation. Finally, propidium iodide (12 µg/ml), 0.1% triton X-100 and RNAase (50 µg/ml) were added and incubated with the cell suspension for 30 min at room temperature, protected from light [25]. Cells were subsequently analyzed by FACSVerseflow cytometry. Mitomycin C (5 µg/mL) was used in parallel as positive control of cell cycle arrest.

2.2.10. Clonogenic survival assay

Cells were assayed for the cytotoxic effect of imiquimod after the cell survival according to established methods for clonogenic assay [24,26]. Subconfluent cultures were exposed to 3 mM of the formulations (NC_{imiq} and IMIQ) for 24, 48, and 72 h. Then the surviving adherent cells were washed with PBS preheated to 37 °C, trypsinized, counted, and replated in six-well plates (100 cells/well). After 10 days of incubation in complete culture medium, the colonies, formed from each cell plated, were stained with crystal violet after fixation with methanol and counted manually. In each case results are expressed as survival fraction, which was obtained by dividing the number of colonies that arise after treatment of cells by the number of cells seeded and plate efficiency (PE: number of colonies formed by untreated cells/number of cells seeded), multiplied by 100.

2.2.11. Measurement of IL-6 released by tumor cells

After treating the cells with the formulations (NC_{imiq} and IMIQ), the culture medium was withdrawn, centrifuged and the resultant supernatant was collected and frozen at −20 °C until analysis. The amount of the IL-6 inflammatory mediator was determined by ELISA kit (boster biological technology, Valley Ave, Pleasanton, CA), according to the manufacturer's protocol. Calibration curves were made to determine the IL-6 concentration in culture medium in the range of 4.69–300 pg/ml.

2.2.12. Data analysis

Statistical analyses were performed by means of one-way Analysis of Variance (ANOVA) followed by the post-hoc Tukey's test for multiple comparison of means ($\alpha = 0.05$). The software SPSS statistics 17.0® was used for the statistical analyses.

3. Results and discussion

3.1. Production of polymeric nanocapsules

Imiquimod-loaded nanocapsule formulation (NC_{imiq}) was produced following our previous report [10]. Laser diffraction analysis showed unimodal size distributions for formulation. The volume-weighted mean diameters (D[4,3]) and the diameters at percentiles 10, 50 and 90 under the size distribution curves by volume and by number of particles were plotted in a radar chart (Fig. 1). The shape of the curves in the radar chart are the fingerprint characteristic of unimodal particle size distributions, as previously determined for different polymeric nanocapsules [27]. NC_{imiq} showed d(0.9)v values lower than 500 nm and d(0.5)n values lower than 200 nm, which is in accordance with the findings from Frank and co-workers [10].

The dynamic light scattering showed hydrodynamic mean diameters of 242.1 ± 17 nm (NC_{imiq}) with polydispersity indexes of 0.17 ± 0.1. The results demonstrated that nanocapsule aqueous

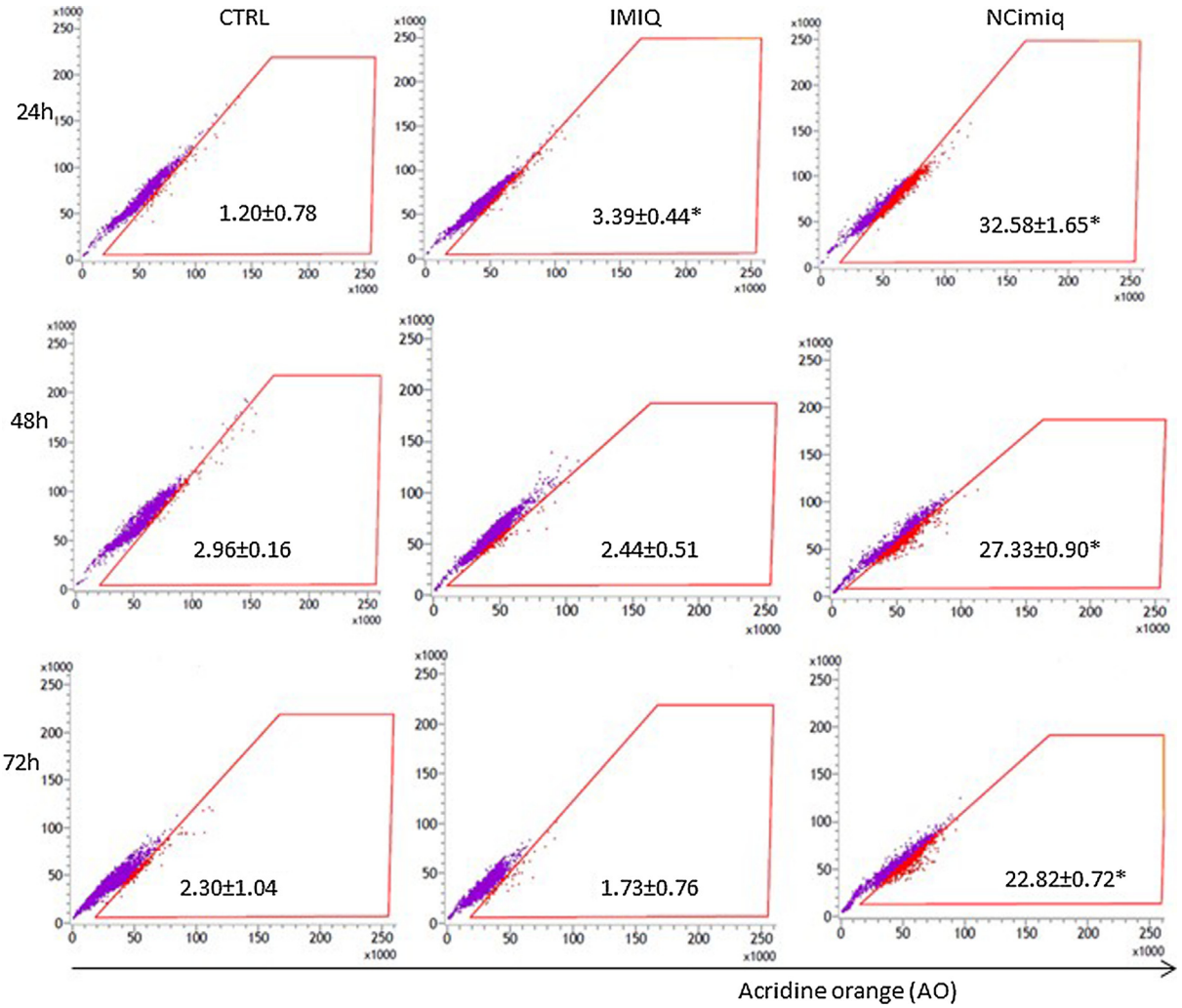

Fig. 5. SiHa cells were left untreated or treated with 3 μM of NC_{imiq} and IMIQ for 24, 48 and 72 h and autophagy was measured according to the acridine orange (AO) staining. $^{*}p < 0.05$ compared with control (one-way ANOVA, followed by Tukey's test). Note: PE-A: positive AO staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dispersions have narrow unimodal size distributions (data not shown) with high homogeneity of sizes. The zeta potential of NC_{imiq} was -8.9 ± 0.3 mV, which value is in accordance with other formulations prepared usingpoly(ε-caprolactone), as shell, and polysorbate 80, as coating material [28]. The pH value for the nanocapsule aqueous dispersions was6.1 ± 0.07. The drug content showed $0.49 \pm 0.05\ mg\ mL^{-1}$ of imiquimod in NC_{imiq}. Regarding the encapsulation efficiency, NC_{imiq} presented 98 ± 0.2% of imiquimod retained in the dispersed phase.

3.1.1. Nc_{imiq} significantly reduces cervical cancer cell viability in a time-dependent manner

According to Fig. 2, NC_{imiq} formulation was more effective against the cervical cancer cell line (SiHa), significantly decreasing the cell viability in a time-dependent manner as compared to the free drug in the same concentration. This result is in agreement with our previously published data, which demonstrated that this formulation decreased cervical cancer cell viability at the concentrations of 1.5 and $3.0\ \mu mol\ L^{-1}$ after 24 h of treatment [10]. Other works have also shown that polymeric nanocapsules loaded with antitumor drugs (i.e. doxorubicin, bromelain) are capable to significantly decrease the viability of the cells (MCF-7) compared to the drug in a DMSO/water solution, reinforcing the potential use of nanocapsules to boost traditional antitumor drugs [29,14]. Similarly, Chen and co-workers [30] demonstrated that polymeric nanocapsules containing doxorubicin decrease the viability of MCF-7 at concentrations ranging from 0.2 to 40 μM. They also showed that the uptake by cells was greater than the free drug at 1, 8 and 24 h. Different results were found by Zanotto-Filho and co-workers [31] regarding the delivery of curcumin into glioma cells (C6). In this case, early evaluated points of time showed higher uptake for the non-encapsulated drug, whereas for late points of time (48 h or more), the uptake was increased by nanoencapsulation. However, in the *in vivo* experiments they observed better results for the nanoencapsulated formulation.

Here, we show that low concentration of the formulation NC_{imiq} was able to significant decrease SiHa cell line viability in a time-dependent manner, indicating that NC_{imiq} formulation increases the cervical cancer cell death.

3.2. NC_{imiq}-induced cell death through apoptosis and necrosis

In order to elucidate if NC_{imiq} is able to promote cancer cell death through apoptosis and/or necrosis we evaluated the percentage of annexin V^+/PI^+ cells after exposure to NC_{imiq} or IMIQ for 24, 48 and 72 h. According to Fig. 4, NC_{imiq} enhanced the number of both PI^+ (necrosis) and annexin V^+/IP^+ (apoptosis) subpopulation in all times tested in relation to the control. Particularly at 72 h, IMIQ induced an increase in the number of PI^+(necrosis) subpopulation only. Those

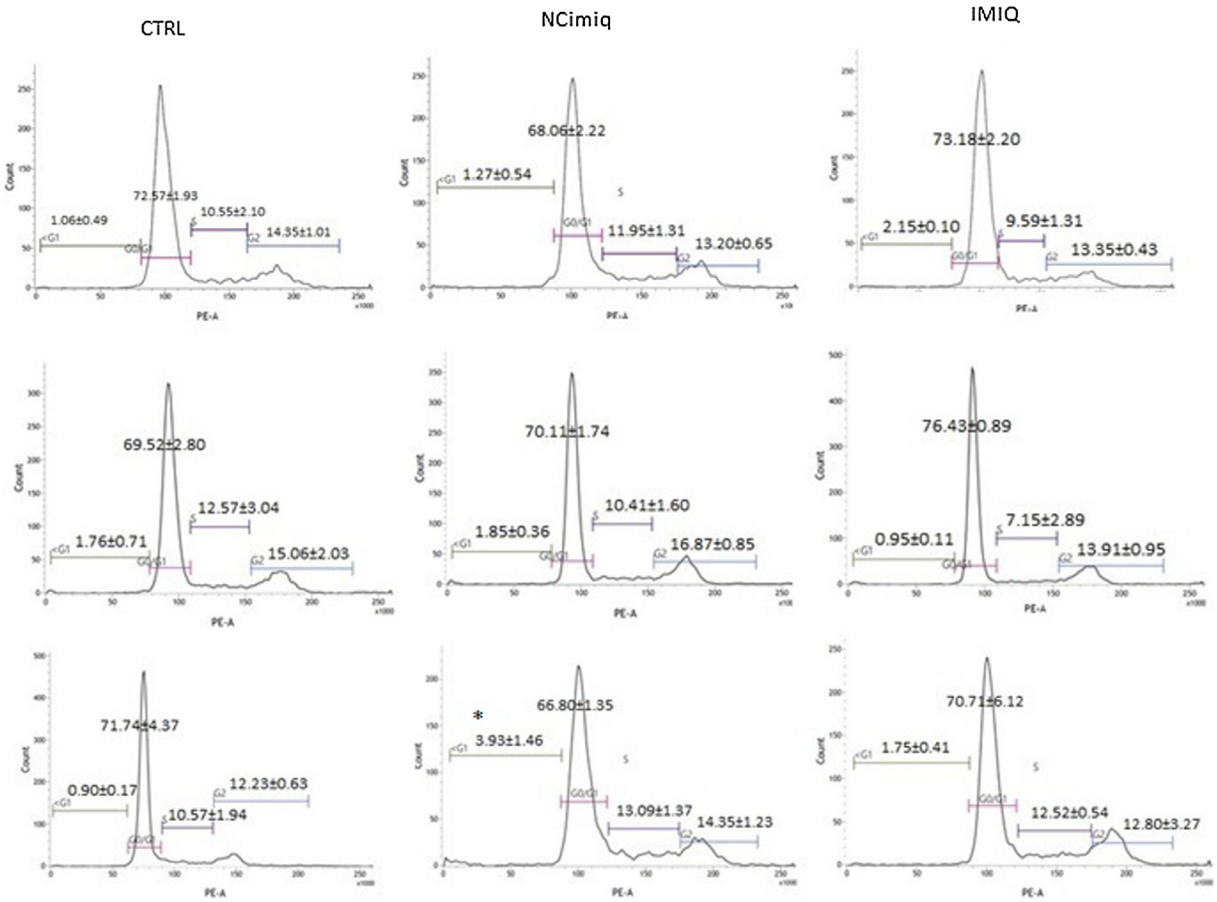

Fig. 6. The effect of imiquimod on the cell cycle of SiHa cells determined by DNA content assay. $^{*}p < 0.05$ compared with control (one-way ANOVA, followed by Tukey's test).

differences may justify the enhanced effect on tumor cell death provoked by the NC_{imiq} formulation.

Imiquimod induced cell death through apoptosis is a long standing observation and similar mechanism was identified for many types of cell lines such as FL, A375, TRAMP-C2, SCC12, BCC, BLM [16,17,19,20,32,33]. However, in such works, imiquimod induced cell death at higher concentrations (20–150 μg/ml) than those used in this present work (3.75 μg/ml). As an example, Han and co-workers [16] showed that 20 μg/ml of imiquimod was able to induce 8.32% of apoptotic cell death in prostate cancer cells (TRAMPC-2) after 48 h of treatment. In the same sense, Sohn and co-workers [20] demonstrated that 150 μg/ml of imiquimod caused 30% of skin carcinoma cells (SCC12) death through apoptosis after 16 h of treatment. Similarly, Huang and co-workers [17] observed that 50 μg/ml of imiquimod elicited 45% of the skin cancer cells (BCC) death after 48 h of treatment. The higher percentage of apoptotic cells induced by imiquimod in those settings could be attributed to the higher amount of drug used in those works. Here we demonstrated that NC_{imiq} was able to reproduce the imiquimod-related mechanism of cell death, but differently from others the amount of drug used in this formulation was, at least, six times smaller. Moreover, at this lower concentration the free drug was not able to trigger cell apoptosis.

3.3. Nc_{imiq} increases cervical cancer cell shrinkage, membrane blebbing and chromatin condensation

Fig. 3 shows that both NC_{imiq} and IMIQ are able to produce cell alterations that resembles apoptotic features such as membrane blebbing, cell shrinkage (upper part) and chromatin condensation (bottom part) [24]. In turn, NC_{imiq} formulation showed more pronounced effects, mainly at 72 h after treatment, when compared to IMIQ, suggesting the higher efficacy of the former in inducing cancer cell apoptosis and these results are in agreement with the findings represented in Fig. 4.

3.4. Nc_{imiq} induces increased acridine orange staining in SiHa cells

Increased acridine orange (AO) staining is a suggestive feature of autophagy induction [23]. Depending on its levels of stimulation, autophagy can contribute to tumor cell death. Therefore, we next evaluated if autophagy is involved with the mechanism of NC_{imiq} induced cervical cancer cell death. The results demonstrated that NC_{imiq} was capable to promote increased amount of AO^+ cells (Fig. 6), suggesting the induction of autophagy process. Moreover, this effect was exclusively related to the NC_{imiq} formulation and it occurred in all time point tested. According to the percentage of AO^+ cells we can notice that autophagy is strongly triggered in the first 24 h of treatment, despite the number of cells AO^+ are still high after 48 h and 72 h of treatment. Interestingly, IMIQ was not able to increase the amount of AO^+ cells and presented similar values to those from the control group ($p > 0.05$) for the times 48 and 72 h. This lack of effect could be explained by the fact that IMIQ concentration is very low to trigger such process in to the cells, which is, on the other hand, potentiated by the NC_{imiq} formulation.

Autophagy is a usual process triggered by normal cells exposed to stress condition such as under privation of nutrients, hormones or energy and infection by pathogens [34]. In this context, autophagy prevents the transformation of normal cells to malignant cells by reducing oxygen reactive species, damage to the DNA, protein aggregation and mitochondrial abnormality [35]. It is now understood that, in cancer,

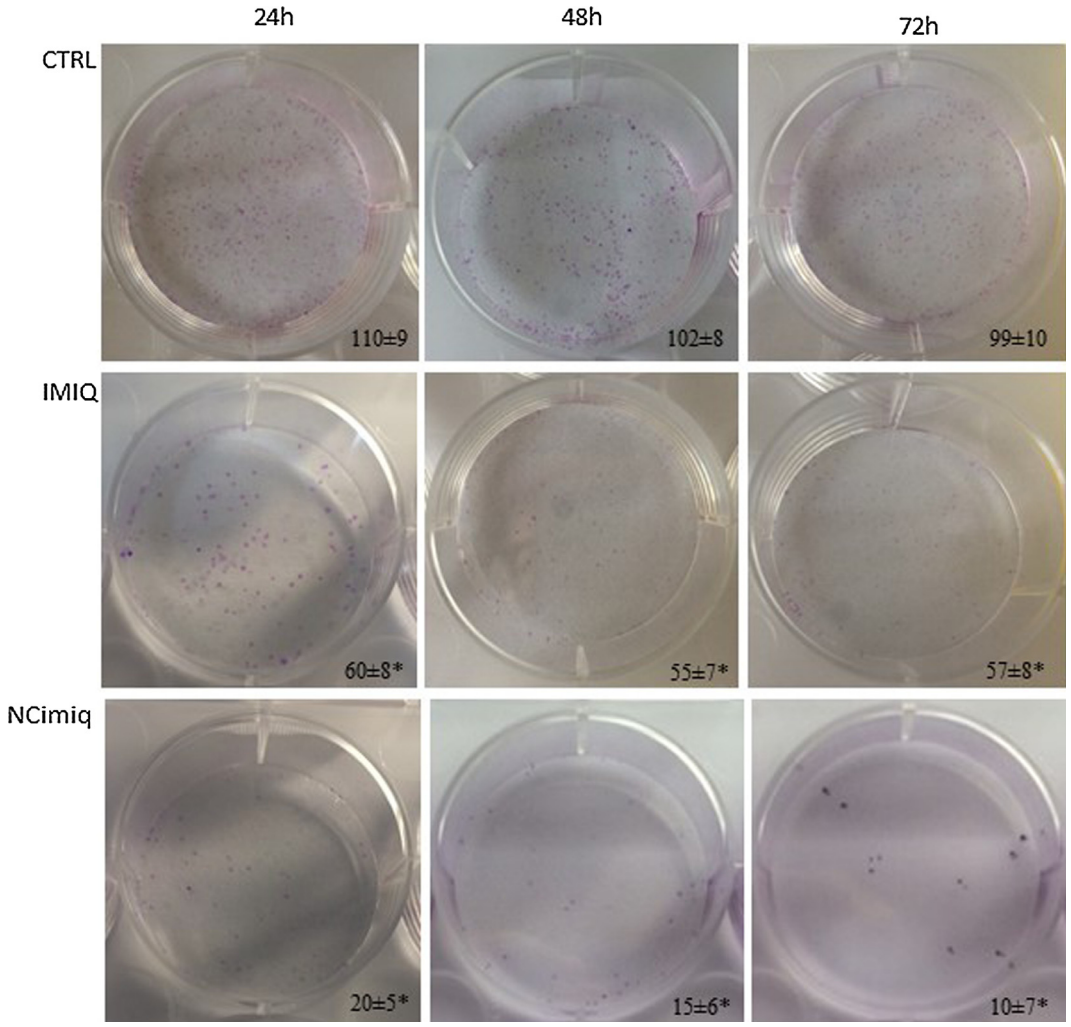

Fig. 7. Clonogenic assay: 100 viable cells were seeded in clonogenic assay, and colony formation was evaluated. $^{*}p < 0.05$ compared with control (one-way ANOVA, followed by Tukey's test).

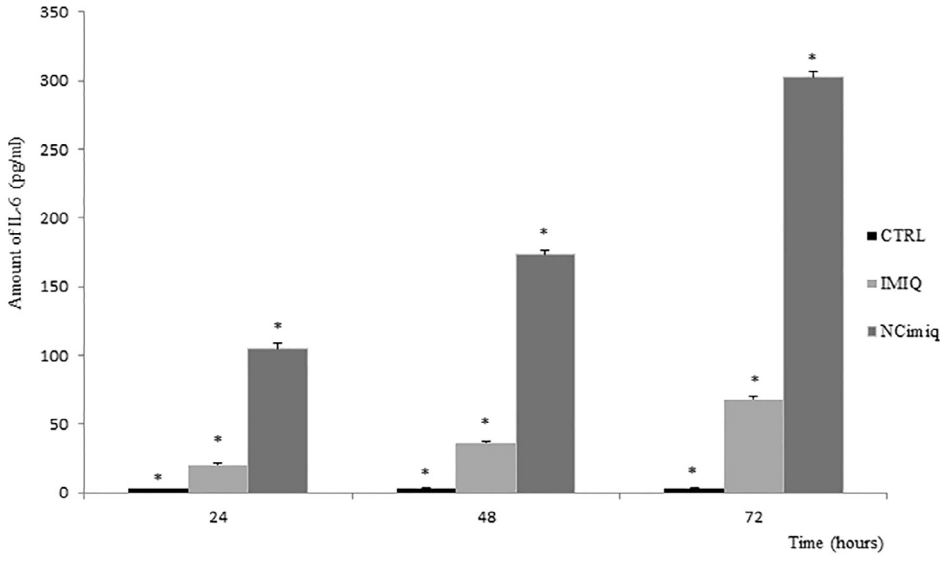

Fig. 8. Amount of interleukin 6 after treatment of SiHa cells with the formulations.n = 3 and $^{*}p < 0.05$ compared with control (one-way ANOVA, followed by Tukey's test).

autophagy can exert a double role: it can either inhibit tumors formation by avoiding the accumulation of organic substrates and protein aggregates or stimulates tumor growth by serving as a mechanism of cellular survivor [36]. Imiquimod-induced autophagy has already been demonstrated by Wang and co-workers [18] and Huang and co-workers [17]. According to these studies, high concentration of free imiquimod (50 μg/mL) was responsible to trigger cell (BBC) autophagy and contribute to cell death. Here, we demonstrated that low level of imiquimod was also able to promote autophagy, but only after being encapsulated in polymeric nanocapsules, reinforcing the promising effect of nanocapsules to increase drug effect.

3.5. NC_{imiq} increases the percentage of SubG1subpopulation of SiHa cells after 72 h

In order to reinforce the evidences that NC_{imiq} promotes cervical cancer cell death through apoptosis, the content of DNA fragmentation by cell cycle analyses was also evaluated (Fig. 5). The eukaryotic cellular cycle is traditionally divided in two main periods: the interphase and the mitosis (M). An entire mammalian cell cycle in culture usually takes 16 h and is divided in the 3 periods: G1 (growing and preparation for the chromosomes replication), S (DNA synthesis), G2 (preparation for the mitotic division). However, when cells are undergoing apoptosis and the DNA fragmentation occurs, a subpopulation of cells accumulates in a sub G1 phase. This sub G1 phase is seeing to the left of the G1 peak on the cell cycle graphic. According to Fig. 5, after 72 h of treatment NC_{imiq} showed a significant increase in the percentage of cells at phase < G1 (Sub G1) in relation to the control and IMIQ group, suggesting DNA fragmentation and cell apoptosis.

Differently from the present work, data from literature showed that 50 μg/ml of imiquimod induced an increment of subG1 subpopulation in the basocellular carcinoma cells (CCB and A375) [18] and 20 μg/ml of imiquimod increased the amount of TRAMP-C2 cells in G2/M, while it decreases the percentage of cells at the G1 phase Han and co-workers [16]. Despite these differences, our result supports the idea that imiquimod induce cancer cell death through apoptosis and the nanoformulation have a better efficiency in producing this effect.

3.6. Clonogenic survival assays

Besides acting as a cytotoxic agent, imiquimod also inhibit survival SiHa cells to proliferate and form new colonies. According to the clonogenic assay (Fig. 7) single SiHa cell were not capable to grow up in a colony after being exposed to both, formulations NC_{imiq} and IMIQ. Again, this effect was more pronounced when cells were exposed for long period of time (72 h) with the NC_{imiq} formulation. To the best of our knowledge, we are the first group to demonstrate this long lasting effect caused by imiquimod per se as well as the high activity caused by its nanoencapsulation.

3.7. NC_{imiq} stimulates SiHa cells to release IL-6

Fig. 8 depicts the property of both NC_{imiq} and IMIQ formulations in stimulating SiHa cells to release IL-6 in the culture medium. This effect was already expected, since imiquimod is broadly known an immune system activator [6]. Once more, NC_{imiq} formulation was able to boost IL-6 release, triggering the release of higher levels of this cytokine in comparison to IMIQ formulation ($p < 0.01$) for all analyzed times. IL-6 is a potent proinflammatory cytokine, which stimulates the maturation and activation of neutrophils and macrophages as well as the differentiation and maintenance of T lymphocytes, modulating both innate and adaptive immune response [29,37]. Increased circulating levels of IL-6 have already been observed in diverse inflammatory related diseases, including gynecologic cancer [1] and this explains the IL-6 values found for the CTRL. In this context, Tjiong and co-workers [38] showed that IL-6 levels were highly elevated in the blood of patients with cervical cancer (HPV positive) in comparison to the control healthy group (HPV negative), pointing a role for this cytokine in the cervical cancer development. Therefore, NC_{imiq} formulation might be an attractive strategy to treat HPV-related disease such as cervical cancer videlicet its superior capacity to potentiate tumor cell death at the same time that stimulates the immune response by releasing IL-6 in the tumor microenvironment.

In previous studies have been shown that IL-6 concentrations in cancer patients were higher than healthy controls [39], however it is still not completely clear how this protein is involved with cancer. In our findings, we identified that the NC_{imiq} formulation increases the concentration of IL-6 *in vitro*, but although some authors relate this cytokine to carcinogenesis, we believe the cytotoxic effect of our formulation may overcome the pro-tumoral cytokine effect. However, this assumption can only be confirmed after *in vivo* studies.

4. Conclusions

In the present study, we successfully demonstrated the efficacy of NC_{imiq} in inducing cervical cancer cell death through multiple of downstream mechanisms, being apoptosis and autophagy the mainstream of those processes. In addition, the drug-loaded nanocapsule formulation significantly prevented the appearance of colonies. Moreover, our results demonstrated that NC_{imiq} stimulates tumor cells to release the proinflammatory cytokine IL-6, reinforcing a role for imiquimod in boosting the immune response. These findings open new possibilities for these polymeric nanocapsules aqueous suspensions containing imiquimod as a novel strategy for the treatment of cervical cancer.

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Disclosure of interest

The authors report no conflicts of interest.

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