

PPARs are mediators of anti-cancer properties of superparamagnetic iron oxide nanoparticles (SPIONs) functionalized with conjugated linoleic acid

Marina Ricci<sup>a</sup>, Marta Miola<sup>b</sup>, Cristina Multari<sup>c</sup>, Ester Borroni<sup>d</sup>, Rosa Angela Canuto<sup>e</sup>, Noemi Congiusta<sup>a</sup>, Enrica Vernè<sup>\*f</sup>, Antonia Follenzi<sup>\*g</sup>, Giuliana Muzio<sup>h</sup>

<sup>a</sup> Department of Clinical and Biological Sciences, University of Torino, Corso Raffaello 30, 10125, Turin, Italy. [marina.ricci@unito.it](mailto:marina.ricci@unito.it)

<sup>b</sup> Department of Applied Science and Technology, Politecnico di Torino, Corso Duca degli Abruzzi 24, 10129 Turin, Italy. [marta.miola@polito.it](mailto:marta.miola@polito.it)

<sup>c</sup> Department of Applied Science and Technology, Politecnico di Torino, Corso Duca degli Abruzzi 24, 10129 Turin, Italy. [crisrina.multari@polito.it](mailto:crisrina.multari@polito.it)

<sup>d</sup> Department of Health Sciences, University "Amedeo Avogadro" of East Piedmont, Via Solaroli 17, 28100, Novara, Italy. [ester.borroni@med.uniupo.it](mailto:ester.borroni@med.uniupo.it)

<sup>e</sup> Department of Clinical and Biological Sciences, University of Torino, Corso Raffaello 30, 10125, Turin, Italy. [rosangela.canuto@unito.it](mailto:rosangela.canuto@unito.it)

<sup>f</sup> Department of Applied Science and Technology, Politecnico di Torino, Corso Duca degli Abruzzi 24, 10129 Turin, Italy. [enrica.verne@polito.it](mailto:enrica.verne@polito.it)

<sup>g</sup> Department of Health Sciences, University "Amedeo Avogadro" of East Piedmont, Via Solaroli 17, 28100, Novara, Italy. [antonia.follenzi@med.uniupo.it](mailto:antonia.follenzi@med.uniupo.it)

<sup>h</sup> Department of Clinical and Biological Sciences, University of Torino, Corso Raffaello 30, 10125, Turin, Italy. [giuliana.muzio@unito.it](mailto:giuliana.muzio@unito.it)

*\*co-shared authorship*

Address of corresponding authors:

Dr. Giuliana Muzio

Dipartimento di Scienze Cliniche e Biologiche, Università di Torino

Corso Raffaello 30, 10125 TORINO (ITALY),

Phone n. +39 011 6707750, fax n. +39 011 6707753, e-mail: [giuliana.muzio@unito.it](mailto:giuliana.muzio@unito.it)

## **Abstract**

Breast cancer chemotherapy can cause side effects due to nonspecific drug delivery, low solubility and fast metabolism of drugs used in conventional therapy. Moreover, the therapeutic effect of the drugs is often reduced by the strengthening of chemoresistance, which occurs via a variety of mechanisms. Different strategies have been developed to reduce multidrug resistance (MDR)-associated gene expressions including the use of surfactants and polymers. In this study superparamagnetic iron oxide nanoparticles (SPIONs) functionalized with conjugated linoleic acid (CLA) reduced the number and viability of cells in comparison with both untreated cells or cells treated with SPIONs alone. This cytostatic effect correlated with the increase of peroxisome proliferator-activated receptors  $\gamma$  (PPAR $\gamma$ ). The necrotic cell death induced, as a consequence, an inflammatory process, as evidenced by the decrease of the anti-inflammatory PPAR $\alpha$  and increase of pro-inflammatory TNF $\alpha$  and IL-1 $\beta$ . PPARs were examined because CLA is one of their natural ligands. The antitumor effect observed was accompanied by a down-regulation of p-glycoprotein (P-gp), which was the first important discovered efflux transporter belonging to MDR, and of ALDH3A1, an enzyme able to metabolize some drugs, reducing their effects. The down-regulation of P-gp correlated with the increase of cytokines. The ALDH3A1 decrease correlated with the increase of PPAR $\gamma$ . Based on these results, PPARs are molecular mediators of anti-cancer effect of SPIONs functionalized with CLA, being changes in these nuclear receptors correlated with induction of cytotoxicity and inflammation, and decreased ability of cancer cells in blocking anti-cancer drug effect.

## **Highlights**

1. SPIONs+CLA reduce cell number and induce necrotic cell death in mouse breast 4T1 cells.
2. The decreased cell proliferation correlates with PPAR $\gamma$  increase.
3. SPIONs+CLA increase pro-inflammatory cytokines correlated with PPAR $\alpha$  and P-gp decrease.
4. The increase of PPAR $\gamma$  also correlates with the decrease of ALDH3A1.
5. CLA functionalization of SPIONs may ameliorate the anti-cancer potential of nanoparticles through PPARs.

**Keywords:** SPIONs; CLA; Breast cancer 4T1 cells; PPARs; P-gp; ALDH3A1

## 1. Introduction

Breast cancer is the most common neoplasm, among all cancer types affecting females. It represents 25% of all cancer cases and the leading cause of cancer mortality (15%). The number of those who die of this neoplasm remains high, even if in the last years screening, early diagnosis and improved therapeutic protocols contributed to a fall in total cancer mortality, with a favourable trend also for breast cancer [1].

Conventional chemotherapy for breast cancer can cause severe side effects due to distribution to off-target tissues [2,3]. Moreover, the therapeutic potential of these drugs is often reduced by the onset of chemoresistance that occurs in a variety of distinct mechanisms and pathways. These include a reduction of intracellular pro-drug activation and an increased drug inactivation by Phase I and/or II enzymes [4].

In order to overcome the critical aspects of conventional chemotherapy, new nanotechnology-based approaches are currently under investigation. In particular, different types of nanocarriers, including polymeric nanoparticles, liposomes, micelles and magnetic nanoparticles were experimentally tested for cancer diagnosis, imaging and treatment [5]. In particular, superparamagnetic iron oxide nanoparticles (SPIONs) have been proposed for diagnostic assays, for local hyperthermia-based cancer therapy and intra-tumour delivery of conventional drugs or monoclonal antibodies, viral vectors engineered with genes or shRNAs [6–8]. Due to the chance of targeting tumor with SPIONs, their side effects on non-cancerous tissues can be reduced [9].

Physico-chemical and biological properties of SPIONs can be improved through surface modifications, increasing also the nanoparticle dispersion [10,11]. Our previous study highlighted that SPIONs functionalized with CLA, known for its anticancer properties, have an increased dispersion and a stronger cytotoxic effect on mouse breast cancer cells 4T1 in comparison with SPIONs alone [12].

In the present study, the mechanisms underlying the effect of CLA-functionalized SPIONs (SPIONs+CLA) were investigated. Particular attention was paid to the type of cell death induced, to the effect on inflammation process, to the possible involvement of PPARs and to the effect on P-gp and ALDH3A1 which are involved in cancer chemoresistance. PPARs were investigated because CLA is a natural ligand of these nuclear hormone receptors that regulate the expression of genes involved in several intracellular signal transduction pathways, including proliferation and survival.

## 2. Materials and Methods

### 2.1 Materials

Unless otherwise specified reagents were purchased from Sigma-Aldrich, Germany

### 2.2 SPION preparation

Synthesis and characterization of SPIONs were carried out as previously described [12]. In the experiments reported here SPIONs alone and SPIONs coated with a single layer of CLA, at the highest of the two amounts that were initially synthesized (4.5  $\mu\text{l}$  CLA/ml of SPION suspension, SPIONs+CLA), were used for cell treatment.

### 2.3 Cell Cultures and Treatment of cells with SPIONs

Mouse breast cancer cells 4T1 were seeded (12,500 cells/cm<sup>2</sup>) in DMEM/F-12 medium supplemented with 2 mM glutamine, 1% (v/v) antibiotic/antimycotic solution, 10% (v/v) fetal bovine serum (FBS) and maintained at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> in air.

24 hours after cell seeding, culture medium was removed and replaced with the same amount of medium not supplemented or supplemented with SPIONs or SPIONs+CLA, both at the concentrations of 8  $\mu\text{g}$ , 16  $\mu\text{g}$  or 32  $\mu\text{g}$  SPION/10<sup>5</sup> cells. Untreated 4T1 cells were used as control.

Human lung A427 cells (ATCC, MD, USA) were cultured (20,000 cells/cm<sup>2</sup>) in DMEM/F12 medium supplemented with 2 mM glutamine, 1 % antibiotic/antimycotic solution and 10 % FBS

A549 human lung-adenocarcinoma cells were from ATCC (Rockville, MD, USA) and cultured (25,000 cm<sup>-2</sup>) in HAM F-12K medium supplemented with 2 mM glutamine, 1% antibiotic/antimycotic solution, 1% non-essential amino acids and 10% (v/v) FBS.

24 hours after cell seeding, culture medium was removed and replaced with the same amount of medium not supplemented or supplemented with SPIONs or SPIONs+CLA 16  $\mu\text{g}$  SPION/10<sup>5</sup> cells.

### 2.4 Cell growth and viability

At the different experimental times (12, 24, 48 or 72 h) 4T1 adherent cells were detached by trypsin/EDTA (0.5%/0.03 mM), and culture media were collected. Aliquots of both adherent cells and culture medium were used for counting cells by flow cytometry analysis.

Remaining cells and culture medium were combined, centrifuged at 400 g at 4°C for 5 minutes, washed with PBS, and resuspended in Annexin-binding buffer (0.5x10<sup>6</sup> cells/ml) in order to evaluate the percentage of viable, necrotic or apoptotic cells by flow cytometry (Annexin V-FITC Apoptosis Detection Kit, eBioscience, CA). Briefly, 195  $\mu\text{l}$  of cell suspension were added with 5  $\mu\text{l}$

of Annexin V-FITC and incubated at room temperature for 10 min. Then, cells were washed with 200  $\mu$ l of binding buffer (1X), resuspended in 190  $\mu$ l of the same buffer, and added with 10  $\mu$ l (20 $\mu$ g/ml) of propidium iodide. Samples were analysed (20,000 events) by flow cytometry (BD Accuri C6, BD Biosciences, CA).

A549 and A427 cells were detached by trypsin/EDTA (0.5%/0.03 mM) at 72 h and used for growth evaluation only.

### *2.5 Inhibition of PPAR $\gamma$ by antagonist GW9662*

Mouse breast cancer cells 4T1 treated as reported in section 2.3 were also supplemented with 10  $\mu$ M GW9662, a well known PPAR $\gamma$  antagonist. In these experiments the following groups were analysed: C, untreated cells; C + Gw9662, untreated cells exposed to 10  $\mu$ M GW9662; M, cells treated with SPIONs (16  $\mu$ g/10<sup>5</sup> cells); M + GW9662, cells treated with SPIONs (16  $\mu$ g/10<sup>5</sup> cells) + 10  $\mu$ M GW9662; M + CLA, cells treated with SPIONs + CLA (16  $\mu$ g/10<sup>5</sup> cells); M + CLA + GW9662, cells treated with SPIONs + CLA (16  $\mu$ g/10<sup>5</sup> cells) + 10  $\mu$ M GW9662. GW9862 was added to cell cultures simultaneously with SPIONs. The evaluation of cell growth was carried out at 24 and 48 h treatment.

### *2.6 Western Blot and Real-time PCR analyses*

Proteins and total RNA were isolated from 4T1 cells using the Tri-Reagent Kit according to the manufacturer's protocol.

35  $\mu$ g of proteins were separated by SDS–polyacrylamide gel electrophoresis and electro-transferred to a polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific, MA), which was then blocked for 1 hour with TBS containing 5% non-fat dry milk. The membrane was then rinsed and incubated overnight with polyclonal anti-PPAR $\alpha$  or anti-PPAR $\gamma$  antibodies (Santa-Cruz Biotechnology Inc., CA) and monoclonal anti- $\beta$ -actin antibodies. Protein bands were visualized using a chemiluminescent detection system (Pierce® ECL Plus Western Blotting Substrate, Thermo Scientific, IL)

1  $\mu$ g of total RNA was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA). 16  $\mu$ l of a PCR mixture containing 2  $\mu$ l of cDNA template, 5 pmoles each of forward and reverse primers (Table 1), bidistilled water and the *Power SYBR® Green PCR Master Mix* (Applied Biosystems, CA) were amplified using an iCycler PCR instrument (Bio-Rad, CA) with an initial denaturation at 95 °C for 10 min, followed by 35–40 cycles at 95 °C for 30 s, annealing at 52 °C for 40 s, extension at 72°C for 40 s. Each sample was tested in duplicate and threshold cycle (Ct) values were averaged out from each reaction. The

change in expression was defined as that detected in the treated 4T1 cells versus that detected in the untreated cells, calculated as  $2^{-\Delta\Delta C_t}$ , where  $\Delta C_t = C_{t_{\text{sample}}} - C_{t_{\text{GAPDH}}}$  and  $\Delta\Delta C_t = \Delta C_{t_{\text{sample}}} - \Delta C_{t_{\text{control}}}$ .

### 2.7 Protein content determination

Protein content was measured using the DC<sup>TM</sup> Protein Assay Kit, following the manufacturer's instructions (Bio-Rad Laboratories Inc., CA).

### 2.8 Pro-inflammatory cytokine protein content

TNF $\alpha$  and IL-1 $\beta$  content was evaluated in the culture medium by Enzyme Linked Immuno Sorbent Assay (Invitrogen Corporation, Frederick, MD).

### 2.9 Statistical Analysis

All data were expressed as means  $\pm$  SD. Differences between group means were assessed by analysis of variance followed by the *post hoc* Newman–Keuls test.

## 3. Results

Mouse breast cancer 4T1 cells were exposed to 8, 16 or 32  $\mu\text{g}/10^5$  cells of SPIONs or SPIONs+CLA and the variations in cell number were recorded for up to 72 hours as reported in Figure 1. All the concentrations of both types of nanoparticles caused a reduction of cell number in comparison with control cells. Regarding the two treatments, the biggest effect was observed in presence of SPIONs+CLA. The reduction of cell number with this SPIONs was dose-dependent until 48 hours after the treatment, reaching the major reduction at 48 h with 32  $\mu\text{g}/10^5$  cells (55.2 %). Results at 72 hours evidenced that the numbers of cells were similar to those observed at 48 hours for 8 and 16  $\mu\text{g}/10^5$  cells, whereas for 32  $\mu\text{g}/10^5$  cells a growth recovery was observed.

The flow cytometry analysis of viability and of type of cell death is reported in Figure 2. The results evidenced that both treatments induced necrosis and a few apoptosis, but the cytotoxic effect induced by SPIONs+CLA was bigger. The cytotoxic effect is dose-dependent until 48 h of treatment, whereas at 72 h the cells were more viable than after 48 h, being the increase of viable cells about 11% in presence of SPIONs+CLA 8 and 16  $\mu\text{g}/10^5$  cells and 18% in case of SPIONs+CLA 32  $\mu\text{g}/10^5$  cells. It is important to underline that functionalization with CLA increased the cytotoxic effect of SPIONs alone of about 100%.

Table 2 reports data relative to the effect of SPIONs functionalized or not with CLA on human lung cancer cells A549 and A427., showing that CLA presence induced a higher inhibition of cell growth in more malignant A427 cells than in less malignant A549.

Since CLA is a ligand of PPARs, as other polyunsaturated fatty acids, PPAR $\gamma$  protein content was evaluated. Figure 3 shows an increase of PPAR $\gamma$  at 24 h in cells treated with both SPIONs in comparison with the control cells, the increase being bigger in cells treated with SPIONs+CLA and consistent with the reduction of cell number and cell toxicity.

The involvement of PPAR $\gamma$  in reducing 4T1 cell growth has been investigated using GW9662, a well known antagonist of this nuclear receptor. Results reported in Table 3 evidenced that the inhibition of PPAR $\gamma$  by GW9662 always increased the cell numbers, being the major recovery present in case of SPIONs + CLA.

The presence of cell necrosis suggested to evaluate some inflammation-related parameters: PPAR $\alpha$ , TNF $\alpha$  and IL-1 $\beta$  at the experimental time of 72 h. Figure 4 shows that PPAR $\alpha$  protein content decreased with both types of SPIONs and, again, the highest decrease was observed in cells treated with SPIONs+CLA in comparison with SPIONs alone and control cells. The decrease of PPAR $\alpha$  was accompanied by the increase of cytokine protein content, like other parameters examined, i.e. there was a bigger increase in cells treated with SPIONs+CLA in comparison with SPIONs alone and control cells.

P-gp and ALDH3A1 were evaluated in order to investigate the effect of these types of SPIONs on the mechanism involved in cancer chemoresistance. Figure 5 shows a decrease of P-gp and ALDH3A1 in 4T1 cells treated with both types of SPIONs, being SPIONs+CLA the most efficient in the decrease.

#### **4. Discussion**

In a previous study we evidenced that SPIONs functionalized with CLA affected viability of 4T1 breast cancer cells, being the effect observed bigger if compared to SPIONs alone. This effect is due to the increased colloidal stability and the anti-cancer properties of the CLA [13].

In this study we investigated the molecular pathways triggered by SPIONs+CLA, paying particular attention to the effect on cell proliferation and cell death. Moreover, to confirm the ability of SPIONs in reducing cancer cell growth, their effect has been investigated in a preliminary way in human lung cancer cells. The flow cytometry analysis on 4T1 breast cancer cells evidenced that the

decreased cell viability was mainly due to the induction of necrotic cell death, while the percentage of the apoptotic cells was close to 0 %. The decreased efficacy of SPIONs + CLA in reducing cell growth observed at 72 h could be the consequence of CLA intracellular metabolism, leading to a decrease of intracellular free CLA able to trigger signal transduction pathways.

Interestingly, results on human lung cancer cells seemed to indicate that SPIONs + CLA show a major effect on more malignant cell line.

The decreased cell number seems negatively correlated with the increased level of PPAR $\gamma$  after 24 h from treatment. This result agrees with what observed in different tumour cell lines treated with CLA, where the decreased cell proliferation associated with the increase of PPAR $\gamma$  level [14]. With regard to the present study, the pivotal role played by this nuclear receptor in modulating SPIONs cytostatic effect has been evidenced by the observation that the antagonist GW9662 completely reversed the decrease of cell number. Moreover, the increased cell number occurring in control cells treated with GW9662 alone further confirm the inverse correlation between PPAR $\gamma$  and proliferation.

Concerning the absence of the apoptotic process, this may partially be the consequence of the non-increasing PPAR $\alpha$  levels. In fact, a previous study with SK-HEP-1 cells, a human hepatocarcinoma cell line, showed that CLA-induced apoptosis was associated with an increase in PPAR $\alpha$  protein. Meanwhile in MDA-MB-231, a human breast cancer cell line, the CLA-induced inhibition of cell proliferation was not associated with apoptosis and was not accompanied by an increased content of PPAR $\alpha$  protein [14,15].

The evident necrotic process focused our attention on the resulting inflammation induction. The analysis of some of the parameters involved in the inflammatory process evidenced an increase in pro-inflammatory cytokines TNF $\alpha$  and IL-1 $\beta$ . This increase inversely correlates with the decreased level of PPAR $\alpha$ . These results confirm what was previously seen in human lung adenocarcinoma A427 cells treated with CLA [16].

An important characteristic of tumor cells is their ability to block the effect of drugs, developing resistance by different mechanisms. One possibility is represented by the alterations of the efflux transporters such as P-gp [17], while, another one, is to metabolize the drug, making it inactive.

The acquired resistance to drugs (MDR) could be due to copy number alterations or increased expression of different genes [16] and is an important impediment for successful chemotherapy [18,19]. The most important and well-studied MDR-genes are: *ABCB 1* that encodes the P-glycoprotein (P-gp), the multidrug resistance protein 1 (*MDR1*), *ABCC 1–3* that encodes the multi-drug resistance associated protein (*MRP*) 1–3 and *ABCG 2* encoding the breast cancer



resistance protein (*BCRP*). Different strategies were developed to reduce MDR-associated gene expressions including the use of surfactants that inhibit the drug-efflux and polymers for their ability to improve the intracellular transport of chemotherapeutics [16]. Recently, different studies have evaluated the capability of various nanoparticle formulation in overcoming MDR in breast cancer, showing that drugs encapsulated by nanomaterials enter the cancer cells through different mechanisms overcoming the drug transporters and reversing MDR [17].

P-gp was one of the first efflux transporter discovered [20–22]; it provides, in physiological conditions, protection from xenobiotics during brain development and it was highlighted to be up-regulated by glucocorticoids and inhibited by pro-inflammatory cytokines [22]. In different “in vivo” and “in vitro” liver and colon tumor models TNF $\alpha$  downregulated *ABCB 1* expression, causing a reduction of drug efflux and an increase of cytotoxicity [23,24]. This correlation was confirmed also in this study where the functionalization with CLA increased the production of TNF $\alpha$  and IL-1 $\beta$  and decreased the expression of P-gp in comparison with control cells and cells treated with SPIONs alone.

The ability of cancer cells to block the effect of drugs is also due to the increased expression of *ALDH3A1*. SPIONs + CLA reduced the expression of *ALDH3A1* through the increase of PPAR $\gamma$ . This inverse correlation between *ALDH3A1* and PPAR $\gamma$  was shown in our previous study on human lung tumor A549 cells where the *ALDH3A1* expression was decreased by arachidonic acid, another ligand of PPARs, via the activation of PPAR $\gamma$  and the consequent inhibition of NF- $\kappa$ B transcriptional activity. In fact, *ALDH3A1* gene possesses several NF- $\kappa$ B binding sites [25,26] which are involved in the positive and negative regulation of its expression [27].

## 5. Conclusions

This research demonstrated the SPIONs functionalized with CLA are able to reduce cell number, induce necrotic cell death and inflammation in mouse breast 4T1 cells. These effects are mediated by PPARs: the increase of PPAR $\gamma$  correlated with the decreased cell proliferation and the decrease of PPAR $\alpha$  with the increase of pro-inflammatory cytokines. In turn, this increase is responsible for the decrease of P-gp mRNA level. The increase of PPAR $\gamma$  also correlates with the decrease of *ALDH3A1*.

The study results indicate that the anti-cancer properties of SPIONs functionalized with CLA are mediated by PPARs, being changes in these nuclear receptors correlated with induction of

cytotoxicity and inflammation, and decreased ability of cancer cells in blocking anticancer drug effect.

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## **7. Declaration of interest statement**

All the authors declare that there is no conflict of interest.

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## Figure Captions

Figure 1. Number of mouse breast cancer 4T1 cells untreated or treated with SPIONs functionalized or not with CLA.

The number of cells is expressed as percentage of untreated cells set equal to 100 %. The values are means  $\pm$  S.D. of 4 experiments. For each group (SPIONs or SPIONs+CLA) means with different letters are significantly different from one another ( $p < 0.05$ ) as determined by analysis of variance followed by a post-hoc Newman-Keuls test.

SPIONs: cells treated with superparamagnetic iron oxide nanoparticles; SPIONs+CLA: cells treated with superparamagnetic iron oxide nanoparticles functionalized with conjugated linoleic acid; CLA: conjugated linoleic acid; C: untreated cells (black line); 8  $\mu$ g, 16  $\mu$ g, 32  $\mu$ g quantity of various SPIONs added to 100,000 cells.

Figure 2. Viability of mouse breast cancer 4T1 cells untreated or treated with SPIONs functionalized or not with CLA.

Data are means  $\pm$  S.D. of 4 experiments and indicate the percentage of cells that are viable, in apoptosis or in necrosis vs. the total number of cells analyzed by flow cytometry, taken as 100%. SD, not reported, is less than 10% in all cases. For each experimental time analyzed (12, 24, 48 or 72 h), means with different letters are significantly different from one another ( $p < 0.05$ ) as determined by analysis of variance followed by a post-hoc Newman-Keuls test.

SPIONs: cells treated with superparamagnetic iron oxide nanoparticles; SPIONs+CLA: cells treated with superparamagnetic iron oxide nanoparticles functionalized with conjugated linoleic acid; CLA: conjugated linoleic acid; C: untreated cells; 8  $\mu$ g, 16  $\mu$ g, 32  $\mu$ g: quantity of various SPIONs added to 100,000 cells.

Figure 3. PPAR $\gamma$  protein content in mouse breast cancer 4T1 cells untreated or treated with SPIONs functionalized or not with CLA.

The protein content was determined by Western blot analysis. Band intensities were normalized to the corresponding  $\beta$ -actin value and expressed as a percentage, setting the value of untreated cells (C) arbitrarily at 100%. Data are means  $\pm$  S.D. of 4 experiments. For each experimental time considered (24 or 72 h), means with different letters are significantly different from one another ( $p < 0.05$ ) as determined by analysis of variance followed by a post-hoc Newman-Keuls test.

See legend in Fig. 2 for acronyms.

Figure 4. PPAR $\alpha$ , TNF $\alpha$  and IL-1 $\beta$  protein content evaluated after 72 h treatment in mouse breast cancer 4T1 cells untreated or treated with SPIONs functionalized or not with CLA.

The PPAR $\gamma$  protein content was determined by Western blot analysis. Band intensities were normalized to the corresponding  $\beta$ -actin value and expressed as a percentage, setting the value of untreated cells (C) arbitrarily at 100%.

TNF $\alpha$  and IL-1 $\beta$  release, determined by ELISA assay, was expressed as % of untreated cells set equal to 100 %.

Data are means  $\pm$  S.D. of 4 experiments. For each protein (PPAR $\alpha$ , TNF $\alpha$  or IL-1 $\beta$ ) means with different letters are significantly different from one another ( $p < 0.05$ ) as determined by analysis of variance followed by a post-hoc Newman-Keuls test.

See legend in Fig. 2 for acronyms.

Figure 5. P-glycoprotein and ALDH3A1. mRNA content evaluated after 72 h in mouse breast cancer 4T1 cells untreated or treated with SPIONs functionalized or not with CLA.

The mRNA, determined by real time PCR, was expressed as % of untreated cells set equal to 100 %. Data are means  $\pm$  S.D. of 4 experiments. Means with different letters are significantly different from one another ( $p < 0.05$ ) as determined by analysis of variance followed by a post-hoc Newman-Keuls test.

See legend in Fig. 1 for acronyms.