Determination of spiropyran cytotoxicity by High Content Screening and Analysis for safe application in bio-nanosensing

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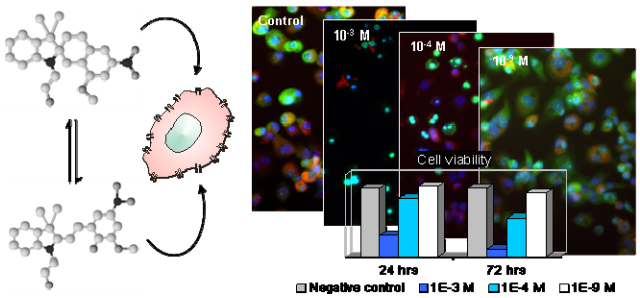

ABSTRACT The *in vitro* toxic response of spiropyrans in cellular models has not been previously addressed, despite the fact that such photoswitchable molecules have shown great potential as versatile and tuneable components for bio-nanodevices and imaging agents. In this study, we examine the cytotoxic effects of a spiropyran, namely 8-methoxy-6-nitro-BIPS or **(**1',3'-dihydro-1'-ethanol-3',3'-dimethyl-8-methoxy-6-nitro-spiro(2H-1-benzopyran-2,2'-(2H)-indole) **[1]**, in three cultured cellular models (THP-1, AGS and A549 cell lines) by High Content Screening and Analysis (HCSA) and by enzyme-linked immunosorbent (ELISA) assays (Interleukin-6 and Tumour Necrosis Factor-alpha). The HCSA results show that low concentrations of 8-methoxy-6-nitro-BIPS (10^{-6}, 10^{-8} and 10^{-9} M) do not induce any cytotoxic response after 24 and 72 h exposure, while at the highest concentrations ($10^{-3}$and 10^{-4} M) the exposure time becomes a critical parameter of the toxic response. The cell viability is reduced by 60% for THP-1 cells, 50% for AGS cells, and 40% for A549 cells at spiropyran concentration of 10^{-3} M after 24 h incubation, whereas at 72 h the cell loss increases above 90%. Interestingly, at 10^{-4} M no significant cytotoxic response is registered after 24 h exposure; where contrarily cytotoxicity is verified after 72 h. Our ELISA results shown that, consistently with the HCSA analysis, a robust inflammatory response is present at 10^{-3} M after 24 h exposure, and at 10^{-3} M and 10^{-4} M after 72 hr, in all the three cell lines investigated.

[1]FOOTNOTES Abbreviations: AGS, human gastric cancer cell line; A549, human adenocarcinomic

epithelial cell line; ELISA, enzyme-linked immunosorbent assay; HCSA, High Content Screening and Analysis; IL-6, Interleukin-6; ME, merocyanine; MEH, protonated merocyanine; MSDS, Materials Safety Data Sheet; OECD, Organization for the Economic Co-operation and Development; REACH, Registration, Evaluation, Authorisation and Restriction of Chemicals; SP, spiropyran; THP-1, human monocytic leukaemia cell line; TNF-α; Tumour Necrosis Factor-alpha.

INTRODUCTION Since the discovery of spiropyrans in 1952 (*1*), researchers have investigated these compounds for various applications. Spiropyrans are molecular switches that can be reversibly converted between two or more stable states in response to stimuli (*1*). They may exist in three different forms: the colourless closed or spiro form (SP^1), the highly conjugated coloured open form referred to as merocyanine (ME) and the protonated open form (MEH). Switching among these three states is possible upon irradiation with UV and visible light and by addition of an acid or a base (*2-4*).

As a result of their ability to alter their structural conformation in response to various photochemical stimuli, growing interest in the application of spiropyrans has been driven towards the selective chelation or release of certain metal-ions (*5*), and imaging in living cells by optical lock-in detection (OLID) approach (*6, 7*) and stimulated emission depletion (STED) microscopy (*8*). More recently these molecules have been integrated in nanometre-sized molecular machines (*9, 10*), showing that such compounds have also considerable potential for biological applications as light-controlled nanosensors, drug delivery nanosystems (*11*) and nanofluidic systems (*12*). It has been reported, for example, that the opening/closing process of a nanovalve derived from a channel protein (*10, 12*), which was covalently modified with a spiropyran, can be controlled by light and might be used to drive molecules across compartments of a nanodevice.

With the rapid growth of research towards the development of nanosystems, there has been a strong drive of both the International and European regulatory organizations to identify the methodologies for the assessment of the "Health, Safety and Environmental impact" of the new nanotechnologies (*13*). The

Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulates and assesses the toxicity of all chemicals sold in Europe in large quantities (*14*). However, in 2008, the Organization for the Economic Co-operation and Development (OECD) expressed its concern on the relative inadequacy of toxicological data available on the new bio-nanotechnologies and all their fundamental constituents (*15*). To date, the main uncertainties regarding the potential integration of spiropyrans in nanoactuators, as well as their application as imaging agents, are related to their chemical instability in aqueous environment (*16*) and to the lack of data on the toxicity or cytotoxicity attributed to these molecules. In fact, to date, the only toxicity information available comes from the Materials Safety Data Sheet (MSDS) for the 6-nitro-BIPS **[2]** (Scheme 2), a compound commercially available from Sigma-Aldrich (CAS No.: 1498-88-0), which demonstrates a 50% Lethal Dose (LD_{50}) of 56 mg/kg by intravenous administration in mouse.

In compliance with the aforementioned OECD directives and the scarcity of toxicological data on spiropyrans, this study aims to investigate whether the functional condition of macrophages (THP-1 cell line), gastric cells (AGS cell line) and epithelial cells (A549 cell line) could be significantly altered by the exposure to 8-methoxy-6-nitro-BIPS **[1]** (Scheme 1) (*17*). The objective of this work is twofold: (i) the investigation of the chemical stability of the spiropyran **[1]** in aqueous environment by optical spectroscopy; and (ii) the evaluation of the dose- and time-dependent cytotoxic response to this photochromic compound in various cultured cell models. Since access to the human body can occur through skin absorption (and consequent perfusion into the systemic blood circulation), ingestion and/or inhalation (*18*), THP-1, AGS and A549 cell lines were chosen as the most suitable models to investigate the potential toxicity associated with the use of 8-methoxy-6-nitro-BIPS **[1]** as chemical nanosensor and/or component of functioning nanomachines (*18*). Five different concentrations of spiropyrans ranging from 10^{-3} M to 10^{-9} M were prepared and tested at two endpoints, set at 24 and 72 h.

We chose the HCSA system as the ideal tool in multiparametric evaluation of cellular responses, such as viability, membrane permeability, lysosomal mass/pH changes, and nuclear staining intensity/size,

using established fluorescent biomarkers (*19*). HCSA system is becoming increasingly important as powerful tool for the screening of large sets of drug candidates (*20-22*), nanoparticles (*23-25*) and nanostructures (*26*), for both efficacy and toxicity. Since responses to external agents are commonly not limited to a single cellular target or isolated mechanism of interaction (*27*), HCSA provided a reliable and reproducible system for toxicity screening of the selected molecular switch.

To further corroborate the HCSA multiparameter results presented, parallel ELISA assays were carried out on the relevant (glycol-) protein signalling molecules (cytokines). ELISA assay allowed quantifying the extent of protein secretion related to the cell stress-induced signalling cascade and transduction activity induced by the exposure of the three cellular models to the 8-methoxy-6-nitro-BIPS **[1]**.

EXPERIMENTAL PROCEDURES

OPTICAL STUDIES Synthesis, purification and the X-ray crystal structure of 1',3'-dihydro-1'-ethanol-3',3'-dimethyl-8-methoxy-6-nitro-spiro(2H-1-benzopyran-2,2'-(2H)-indole) (8-methoxy-6-nitro-BIPS) **[1]** (Scheme 1) have been reported previously (*17*). 8-methoxy-6-nitro-BIPS **[1]** was dispersed in Milli-Q water by sonication at concentration of 10^{-3} M. Solution at 10^{-4} M was obtained by progressive dilution with fresh Milli-Q water. The solution was incubated at 37 °C in the dark up to 72 h; it showed a neutral pH before and after spectroscopic experiments. UV/vis absorption measurements were carried out in accordance with the *in vitro* incubation conditions (37 °C, 3 time points), with a PerkinElmer UV/vis Lambda 35 Absorption Spectrophotometer. Further absorption spectra were collected following cycles of vis (878 nm) and UV (254 nm) irradiation. The vis emission spectra were recorded on a PerkinElmer LS 55 Emission Spectrophotometer at excitation wavelength (λ_{exc}) of 530 nm.

SPIROPYRAN SOLUTIONS IN CELL MEDIA 8-methoxy-6-nitro-BIPS **[1]** was added to RPMI 1640 cell media (GIBCO®, Invitrogen, USA) or Hams F12 media (GIBCO®, Invitrogen, USA) in sterile

environment at concentration of 10^{-3} M. The dispersion was sonicated for 7 hours in sonic bath in order to obtain a homogeneous solution. All the other concentrations (10^{-4}, 10^{-6}, 10^{-8} and 10^{-9} M) were obtained by subsequent dilutions with fresh cell media. For all the concentrations, neutral pH was measured.

CELL CULTURE Three established commercial available cell lines (all from ATCC™, VA, USA) were used: human monocytic leukaemia cell line (THP-1), human gastric cancer cell line (AGS), and human alveolar epithelial cell line (A549). Full cell line characterization (e.g., cell line profile, morphological images, DNA profile, and cytogeneticity), subculturing, propagation, doubling time, and preservation were also supplied by ATCC™ at the delivery. The passage number was restricted between five and eight.

THP-1 cells Human monocytic leukaemia cell line (THP-1 cell line) were cultured in modified RPMI 1640 media (supplemented with streptomycin, penicillin and L-glutamine) in T75 tissue culture flasks and incubated at 37 °C and 5% CO_2 until a 60% confluence. THP-1 cells were seeded in a 96-well plate at concentration of 20,000 cells/well (final volume: 200 µl/well) using a Matrix WellMate (ThermoFisher Scientific, USA), and activated with phorbol-12-myristate-13-acetate (PMA) (Sigma-Aldrich, USA) for 72 h to induce their differentiation into adherent macrophages and stop their natural proliferation.

AGS cells Human gastric cancer cell line (AGS cell line) were cultured in modified Hams F12 media (supplemented with 1% penicillin/streptomycin and 10% foetal bovine serum) in T75 tissue culture flasks and incubated at 37 °C and 5% CO_2 until a 70-80% confluence. AGS cells were seeded in a 96-well plate at concentration of 4,000 cells/well (final volume: 200 µl/well) using a Matrix WellMate (ThermoFisher Scientific, USA), and incubated at 37 °C and 5% CO_2 for 24 h for complete adhesion. Differently than THP-1 cells, this cell line was distinguished by cellular proliferation during the entire experiment.

A549 cells Human alveolar epithelial adenocarcinomic cells (A549 cell line) were cultured in

modified Hams F12 media (supplemented with 1% penicillin/streptomycin and 10% foetal bovine serum) in T75 tissue culture flasks and incubated at 37 °C and 5% CO_2 until a 70-80% confluence. A549 cells were seeded in a 96-well plate at concentration of 4,000 cells/well (final volume: 200 µl/well) using a Matrix WellMate (ThermoFisher Scientific, USA), and incubated at 37 °C and 5% CO_2 for 24 h for complete adhesion. Similarly to AGS cells, this cell line was distinguished by cellular proliferation during the entire experiment.

CYTOTOXICITY METHODOLOGY After removing the cell media, adherent THP-1, AGS and A549 cells were exposed to spiropyran **[1]** solutions prepared with fresh cell media. The final volume of solution added was 300 µl/well. To assess the dose-dependent cytotoxicity, the experiment design consisted of a total of five different doses (10^{-3}, 10^{-4}, 10^{-6}, 10^{-8} and 10^{-9} M), one negative control (cells plated with cell media), and one positive control, in triplicate wells. Detergent damage (Triton-X 0.05%) was used as positive control in THP-1 cell line, while an anticancer drug (Pacliataxel 4 µM) was used as positive control in AGS and A549 cells. The exposure times chosen for each experiment were 24 h and 72 h, to assess the time-dependent cytotoxicity. Positive and negative controls, temperature, and pH were controlled and kept constant across the experiments.

LIVE FLUORESCENT STAINING. Supernatants were collected from each well for post-exposure assays and Multiparameter Cytotoxicity 1 HitKit HCSA Reagent Kit (Cat. No.: 8400101, ThermoFisher, USA) was used for staining the three cell lines following the optimized Kit procedure provided for live cell staining (the detailed protocol is reported in Supporting Information). The Cytotoxicity 1 HitKit is formed by a cocktail of three fluorescent dyes (MPCT1 Fluor Cocktail), which contains the following components: (i) Hoechst Dye (blue dye) for labelling nuclei; (ii) cell permeability indicator (green dye) for labelling permeabilized cells; (iii) fluorescent weak base (red dye) for labelling acidic organelles and measuring pH and mass. This Kit offers therefore the ability to detect changes to many cellular properties, such as (i) cell viability (determined as cell loss compared to the negative controls); (ii) cell membrane permeability; (iii) lysosomal mass/pH changes; (iv) nuclear DNA staining intensity; (v)

nuclear size; and (vi) cellular morphology. Complete list of the parameters acquired in this study, along with explanations, is given in Table 1.

IMAGING AND MULTIPARAMETRIC ANALYSIS Measurements were carried out using the IN-Cell 1000 automated fluorescent microscope system (GE Healthcare, USA) and its associated analysis software (In Cell Analyzer System, GE Healthcare, USA). 96-well plates prepared as described above were read on the IN-Cell Analyzer 1000 using three detection channels with three different excitation filters. These included a $\lambda<503$ nm filter, which detected the blue fluorescence at 461 nm; a $\lambda>509$ nm filter, which detected the green fluorescence at 509 nm; and a $\lambda>599$ nm filter, which detected the red fluorescence at 599 nm. Ten random microscopic fields were sequentially acquired by the IN-Cell 1000 automated fluorescent microscope system at magnification 10X, after carrying out image calibration and maintaining constant the acquisition time, contrast, and brightness throughout all the experiment. Acquired images for each exposure time and dose were then analyzed by the IN-Cell Analyzer software, which automatically identifies objects based on a fluorescent stain following calibration on the control samples. To obtain the cell viability data, an algorithm-based software analysis was applied to detect and count the stained nuclei (blue channel) per field. From the microscopic fields, the fluorescent staining intensities reflecting cell permeability (green channel) and lysosomal mass/pH changes (red channel) were also quantified for each individual cell present in the fields, by means of the analysis software. Further algorithms were applied to measure the average intensity (nuclear intensity) and the intensity distribution (nuclear area) of blue pixels confined within the nuclear outline.

CYTOKINE ASSAYS To correlate the cytotoxicity with the downstream functional responses at the level of protein secretion, the concentrations of natural human Interleukin 6 (IL-6) and human Tumour Necrosis Factor-alpha (TNF-α) secreted by the exposed THP-1, AGS and A549 cells were measured (human TNF-α/TNFSF1A, cat. No: DY210; human IL-6, cat. No: DY206; DuoSet ELISA Development kit, R&D Systems). Cell cytokines expression due to the exposure to the spiropyran **[1]** was evaluated by ELISA assays and compared to the relevant controls, following the standard procedure (Supporting

Information). The assays were repeated in duplicate (n_{test} = 2). Optical density of each well at 450 nm was determined by means of a VERSAMax Plus microplate reader (Molecular Devices, USA), calibrated against standards and corrected by subtracting the optical aberration of the 96-wells plastic plate. Cell count for each well was carried out by HCSA system in order to quantify the cytokine production as picogram per cell (pg/cell) for the different concentrations and time points.

STATISTICAL ANALYSIS A two-way analysis of variance (ANOVA) followed by a Bonferroni post-test analysis were carried out for all HCSA and cytokine parameters to measure the significance of dose- and time-interdependencies between the negative controls and the five spiropyran concentrations chosen in this study (Prism; GraphPad Software Inc., USA). A $p<0.05$ was considered statistically significant. A comprehensive list of the p values at each concentration and time point has been summarized in Table S1-S5, in the Supporting Information. The HCSA data are presented in the graphs as mean values (n_{test} = 3 in THP-1 cells; n_{test} = 2 in AGS and A549 cells; $n_{replicates}$ = 3 in all cell lines) ± standard error mean, and normalized to the negative control cells, unless differently specified in the graph caption. The histograms showing the ELISA results are plotted as mean values (n_{test} = 2) ± standard error mean.

RESULTS

OPTICAL STUDIES

We monitored the stability of 8-methoxy-6-nitro-BIPS **[1]** in water, after incubation of the aqueous solution at 37 °C and at various time points (0, 24 and 72 h) by optical spectroscopy.

The absorption spectrum of the spiropyran derivative **[1]** in aqueous solution **(**black solid line in Figure 1A) showed two bands at 390 nm and 526 nm, revealing the presence of the open merocyanine form, confirmed by an emission band at 688 nm **(**black dashed line in Figure 1A). The absorption and emission spectra did not change much after storing the solutions for 24 h at 37 ºC. (grey solid and dashed lines in Figure 1A), while significant spectroscopic changes were recorded after 72 h. The

intensity of the absorption band at 526 nm and the emission band at 688 nm dramatically decreased, and four new absorptions bands appeared at 263 nm, 305 nm, 374 nm, and 413 nm, indicating decomposition of the spiropyran derivative after 72 h storage of the aqueous solution at 37 ºC. In particular, the small absorption band at 305 nm was assigned to the 5-nitrosalicylaldehyde generated by decomposition of the 8-methoxy-6-nitro-BIPS **[1]** (*16*).

The absorbance at 526 nm can be modulated by alternating visible and UV radiations. The data points in Figure 1B illustrate this effect for three consecutive switching cycles.

The dynamic switching between ME (fluorescent form) and SP (non-fluorescent form) in water was seen under an epifluorescent microscope[1] by live imaging of a freshly prepared aqueous solution of 8-methoxy-6-nitro-BIPS **[1]** (the video is available free of charge *via* the Internet at http://pubs.acs.org as Web-Enhanced Object).

HIGH CONTENT SCREENING AND ANALYSIS The cytotoxic outcomes of the cell-spiropyran **[1]** interaction were evaluated by HCSA in three cellular models (THP-1, AGS and A549 cells) after 24 and 72 h exposure. The evaluation of the HCSA images acquired (Figure 2 and Figure S1), showed that the cytotoxic response to 8-methoxy-6-nitro-BIPS **[1]** was both dose- and time-dependent. The multiparametric analysis provided quantitative information on the changes in five commonly used cellular parameters (*20, 22-26*), including cell viability, membrane permeability, lysosomal mass/pH changes, nuclear area and nuclear staining intensity, as explained in detail in Table 1. The quantitative results are shown in Figure 3 and in Figure S2 (in Supporting Information).

Cell viability By assuming that changes in cell viability are directly correlated to the toxic effects of the compound tested, we found that the 8-methoxy-6-nitro-BIPS **[1]** did not affect the cellular survival of THP-1, AGS and A549 cells at 24 h, for concentrations ranging from 10^{-4} M to 10^{-9} M (Figure 3A, 3D and 3G). On the other hand, at 10^{-3} M a significant decrease in cell viability down to 40%, 50% and

[1] λ_{exc} = 450 nm; λ_{em} = 596 nm; CCD camera: Olympus BX501M, Japan.

60% was shown for THP-1, AGS, and A549 cells respectively, when compared to negative controls. At 72 h we found a further decrease in cell viability down to 20%at 10^{-3} M in all cell lines. At 10^{-4} M the cytotoxic responses to spiropyran **[1]** were cell type-dependent at 72 h. THP-1 cells showed an overall cell loss of 40%, while AGS and A549 cells had a reduction of approximately 50% in their proliferative activity. For all the other concentrations, the cell-spiropyran interactions at 72 h were found to be negligible across the three cell lines investigated. This was reflected by the 100% viability of the THP-1 cells, and the threefold increase in cell number of both AGS and A549, as a clear indication of their normal proliferation.

The dose-dependent cell loss was found to be significant at 10^{-3} M in THP-1 cells, at 10^{-3} M and 10^{-4} M in AGS cells. Significant time-dependent cell viability changes were shown at 10^{-3} M and 10^{-4} M in the AGS cell line, and at 10^{-3} M, 10^{-6} M, 10^{-8} M and 10^{-9} M in A549 cells, as summarised in table S1, S2 and S3 (Supporting Information).

Cell membrane permeability It has been reported that changes in the cell membrane permeability are often associated with an ongoing toxic or apoptotic responses, and the loss of cell membrane integrity is a common phenotypic feature of marked cytotoxicity (*28*). We used this as a key parameter for the evaluation of the cell-spiropyran interaction in addition to the cell viability.

A significant increase in the cell membrane permeability (evaluated by green-fluorescence emission) was registered at 10^{-3} M at 24 h, and at 10^{-3} M and 10^{-4} M at 72 h, in all cellular models tested (as shown in figure 3B, 3E, and 3H for THP-1, AGS and A549 cells, respectively). Interestingly for the AGS cell model, an onset of toxicity, shown by increased cell membrane permeability, was measured at 10^{-4} M after only 24 h exposure; this response was not registered for any other cell lines tested in this study.

The dose-dependent increment in cell membrane permeability was significant at 10^{-3} M and 10^{-4} M in THP-1, AGS and A549 cells. The time-dependent changes in cell membrane permeability were prominent at 10^{-3} M in AGS cells, and at 10^{-3} M and 10^{-4} M in THP-1 and A549 cells.

Lysosomal mass/pH changes Changes in the mass/pH of lysosomes were also investigated at 24 and

72 h (Figure 3C, 3F and 3I), since it has been reported that external agents (such as drugs and nanoparticles) can interfere with the normal cell physiology, by affecting the mass, the number and/or the function of lysosomes and endosomes (*29*). In our study high values in the lysosomal mass/pH parameter (evaluated by the red-fluorescence emission within the cellular cytoplasm) may also be associated with the endocytosis of the small spiropyran molecules (mass wt = 382 g/mol), and subsequent hydrolysis and storage in the lysosomes.

For THP-1, AGS and A549 cells, increasing values of lysosomal mass/pH were found at 10^{-3} M after 24 h exposure. Conversely, while increasing values were observed in A549 cells at concentrations ranging between 10^{-4} M to 10^{-9} M, at 24 h, the red fluorescence intensities were comparable to the untreated controls in THP-1 and AGS cell models. Interestingly, THP-1 cells showed no significant changes in their lysosomal mass/pH response after 72 h exposure, whereas rising values of red-fluorescence intensity were still detected at 72 h, when 8-methoxy-6-nitro-BIPS **[1]** was incubated at 10^{-3} M in AGS and A549 cell lines. Furthermore, lysosomal mass/pH changes were also observed at 72 h in AGS and A549 cell lines across all the remaining concentrations (10^{-4} M – 10^{-9} M).

Dose-dependent lysosomal mass/pH changes were verified at 10^{-3} M in all cellular models tested. Time-dependent lysosomal mass/pH responses were significant at 10^{-3} M in all cell lines, at 10^{-6} M and 10^{-8} M in AGS cells, and from 10^{-4} M to 10^{-6} M in A549 cells.

Nuclear area and intensity The changes in nuclear area and nuclear staining intensity confirmed and strengthened the dose- and time-dependent cytotoxic trend showed by cell viability, cell membrane permeability and lysosomal mass/pH changes (Figure S2, Supporting Information).

CYTOKINES SECRETION In the presence of an inflammogenic compound or a foreign body, THP-1, AGS and A549 cells release inflammatory cytokines (*30*), the levels of which are clearly associated with cell-based inflammatory reactions (*31*). To refine the investigation on the dose- and time-response of THP-1, AGS and A549 cells to spiropyran exposure, the secretion levels of IL-6 and TNF-α were measured on the supernatants of the exposed cells by ELISA assays. IL-6 and TNF-α are, in fact,

multifunctional cytokines known to be secreted by cells to regulate their acute phase reactivity (*32*). The results on the concentrations of IL-6 and TNF-α secreted by THP-1, AGS and A549 cells after 24 and 72 h exposure to 8-methoxy-6-nitro-BIPS **[1]** are shown in Figure 4. In THP-1 cells, the cytokines secretion levels were significantly high at 10^{-3} M at 24 h, and at 10^{-3} M and 10^{-4} M at 72 h. This suggested that high concentrations and longer exposure times to 8-methoxy-6-nitro-BIPS **[1]** induced a pleiotropic inflammatory response, triggering the release of both cytokines by exposed THP-1 cells. At 72 h, the IL-6 and TNF-α secretion levels increased twofold. This phenomenon was due to the extended exposure exercised on the THP-1 cells, which triggered the onset of acute phase reaction. In AGS and A549 cells, high TNF-α concentrations were found at 10^{-3} M and 10^{-4} M at 24 h, and 10^{-3} M at 72 exposure. Furthermore, the secretion of IL-6 was prominent at 10^{-3} M at 24 h, and outstanding at both 10^{-3} M and 10^{-4} M at 72 h.

IL-6 and TNF-α secretion levels were dose-dependent at 10^{-3} M in AGS cells, and at 10^{-3} M and 10^{-4} M in A549 cell line, as summarized in Table S4 and S5 (Supporting Information). Interestingly, in THP-1 cells the secretion of IL-6 and TNF- α proved to be dose-dependent for all concentrations tested. The time-dependent IL-6 secretion was shown to be significant at 10^{-3} M in all three cell lines, and at 10^{-4} M in A549 cells. TNF-α secretion was significantly time-dependent at 10^{-3} M in all three cell lines, and at 10^{-4} M only in AGS cell lines.

DISCUSSION

Several cellular parameters (such as cell viability, cell membrane permeability, lysosomal mass/pH changes, and nuclear DNA binding) were measured *via* HCSA system. From the combined qualitative and quantitative HCSA analysis at 24 and 72 h exposure, it was possible to conclude that 8-methoxy-6-nitro-BIPS **[1]** was not cytotoxic for the three cellular models tested below a certain threshold. Thus, after 24 h THP-1, AGS and A549 cells were not significantly damaged at concentrations below or equal to 10^{-4} M. Clear cytotoxic effect was observed only at 10^{-3} M. At this concentration, the cell viability

and the nuclear area consistently decreased in conjunction with a marked increase in cell membrane permeability. This phenomenon is generally associated with an ongoing dose-dependent cell insult. Similarly, time-dependent cytotoxicity was verified, as increased cytotoxic response was registered at 10^{-4} M at 72 h exposure in all three cell lines. At this concentration and time point, the cell viability and nuclear area decreased when compared to the results at 24 h, while the cell membrane permeability and nuclear intensity increased. This suggested that the cytotoxic event was predominantly determined by the exposure time rather than the immediate toxic effect of the products generated by the hydrolysis of 8-methoxy-6-nitro-BIPS **[1]**. It is of interest that at 10^{-4} M the cell viability remained above 50% for all cellular models investigated even after 72 h exposure. This clearly showed that THP-1, AGS and A549 cells were relevantly resistant to the presence of high concentrations of 8-methoxy-6-nitro-BIPS **[1]**.

HCSA also allowed for the detection of subtle differences in the cytotoxic response among the three cell lines tested. THP-1 cells showed clear changes in the lysosomal mass/pH at 10^{-3} M after 24 h exposure. This result, combined with the increase in nuclear staining and decreased nuclear area, lead to speculate that THP-1 cells were undergoing endocytosis of the photochromic compound after initial exposure. Since changes in the lysosomal mass/pH were not shown after 72 h, it is possible that such uptake reached saturation levels after the first 24 h exposure and was exhausted after 72 h. Similar pattern could be seen in A549 cells, whereas AGS cells showed an increasing cell membrane permeability at 10^{-4} M after 24 h incubation. This result could define a relative sensitivity of AGS cells towards exposure to 8-methoxy-6-nitro-BIPS **[1]**, but it was not supported by analogous changes in the other parameters analyzed.

The data obtained from the cytokines assays corroborated the HCSA results and clearly showed that the pro-inflammatory cytokine production was both dose- and time-dependent. An overall remarkable inflammatory response of the three cellular models tested was seen at 10^{-3} M after 24 h exposure, and at $10^{-3}$and 10^{-4} M after 72 h. From the results it emerged that AGS and A549 cells secreted lower concentrations of TNF-α, as compared to THP-1 cells. These results also revealed that the inflammatory

response of AGS and A549 cells to 8-methoxy-6-nitro-BIPS **[1]** was exhausted after 24 h exposure. This is in agreement with the physiological differences between the cellular models tested, since, for instance, cytokines from the TNF family are mainly produced by macrophages. The secretion of both cytokines (TNF-α and IL-6) was found to be consistently dose-dependent.

In conclusion, we have demonstrated that strong toxic effects on macrophage, gastric and epithelial cells are unlikely as a result of continuous exposure to the spiropyran at micromolar concentrations over a 72 h exposure period. . Significant cytotoxic effects were found in fact only at 10^{-3} M and for longer exposures at 10^{-4} M. By HCSA we have shown that the dose- and time-dependent cytotoxic responses to 8-methoxy-6-nitro-BIPS **[1]** in THP-1, AGS and A549 cells are statistically significant, while by ELISA assays we have validated the HCSA system as a highly precise tool for further testing and screening the effects of spiropyrans in a larger range of cell types and exposure conditions. While our cytotoxic results clearly open the potential for the future integration of spiropyrans in chemical nanosensors to future exploitation and experimental validation, the optical studies indicate that the degradation process of 8-methoxy-6-nitro-BIPS **[1]** in water started after 24 h at 37 °C. Therefore, limitations to the incorporation of spiropyran derivatives in living systems could still derivate from the instability of such molecules in the aqueous environment and possible toxic effects of light-induced switching inside the cells..Our results, validating the HCSA system as powerful system for the evaluation of spiropyran cytotoxicity, open the way towards the screening of the *in vitro* switching cytotoxic response.

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Supporting Information Available: Experimental methods and statistical analysis tables; nuclear area and nuclear intensity changes at 24 h and 72 h exposure; HCSA qualitative results at 72 h exposure; live imaging of a freshly prepared spiropyran aqueous solution by means of epifluorescent microscope. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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TABLES AND TABLE CAPTIONS

Table 1. Summary of the assay strategy for measuring the cellular parameters of interest by HCSA analysis. This description is in agreement with previously published work (*24*).

Cellular parameter	Description	Target	Model of action	Fluorescence emission
Cell count	Number of nuclei identified per field	Nucleus	DNA binding	Blue (λ_{em}: 461 nm)
Cell membrane permeability	Average fluorescence intensity of pixels within the cytoplasm	Cell membranes	Cell membrane is permeable when compromised	Green (λ_{em}: 509 nm)
Lysosomal mass/pH changes	Average fluorescence intensity of pixels within the cytoplasm	Acid organelles	Increase fluorescence corresponds to decreased pH or increased lysosomes number	Red (λ_{em}: 599 nm)
Nuclear intensity	Average fluorescence intensity of pixels within nucleus	Nucleus	DNA binding	Blue (λ_{em}: 461 nm)
Nuclear area	Number of pixels within the nucleus	Nucleus	DNA binding	Blue (λ_{em}: 461 nm)

FIGURES AND FIGURE LEGENDS

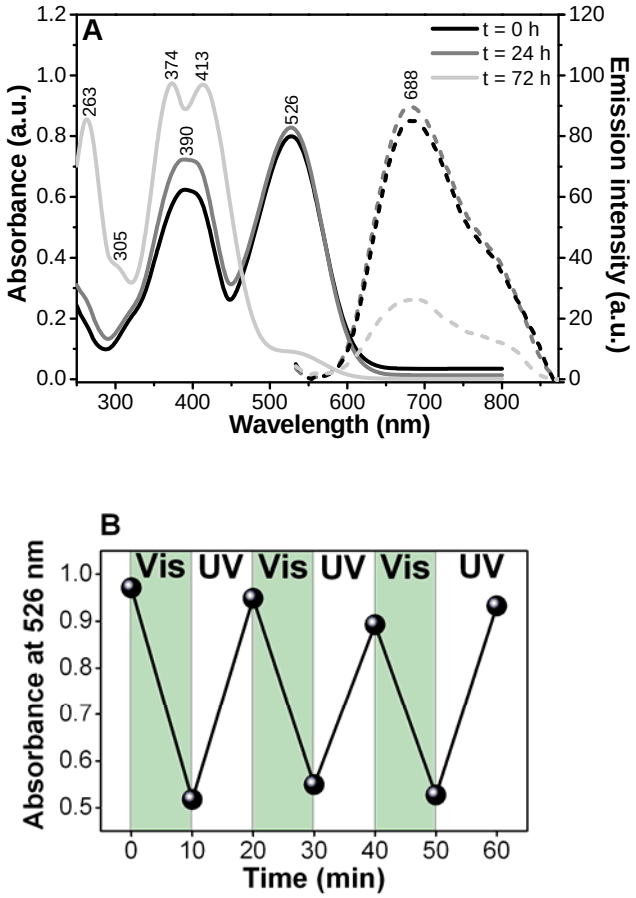


Figure 1 (A) Absorption (solid lines) and emission spectra (dashed lines) of **[1]** (1.0 x 10^{-4} M, water, 37 °C) after 0, 24 and 72 h incubation. (B) Changes in absorbance at 526 nm under the influence of visible and UV light for three consecutive cycles.

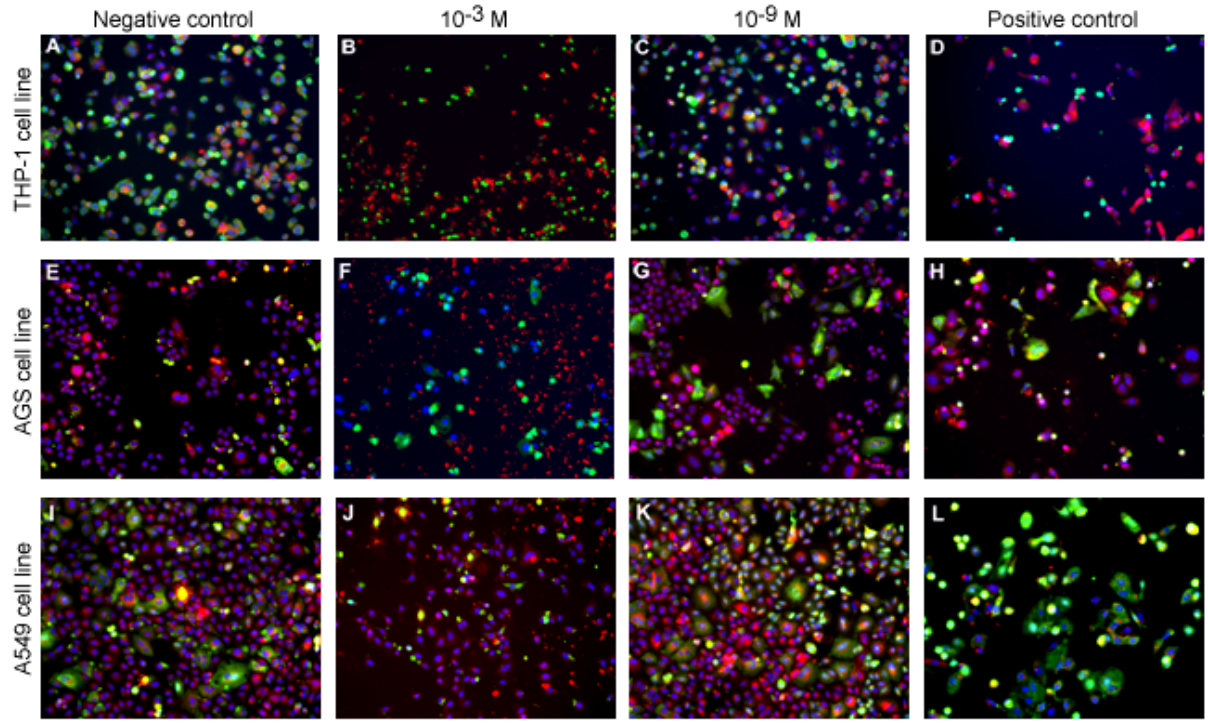


Figure 2. HCSA qualitative results for THP-1 cells (Figure A, B, C and D), AGS cells (Figure E, F, G and H) and A549 cells (Figure I, J, K and L) at 24 h exposure to the spiropyran derivative **[1]**. The composite images show the cells stained for: (i) nuclei (in blue); (ii) cell membrane permeability (in green); and (iii) lysosomal mass/pH changes (in red). The results for two representative concentrations (10^{-3} M and 10^{-9} M) are reported. Figure B, F and J are significant image fields showing decreased cell viability and increased cell membrane permeability at 10^{-3} M, as compared to the negative controls (Figure A, E and I). The cell viability results comparable to the negative controls at 10^{-9} M (Figure C, G and K). Figures D, H and L are significant fields of the positive controls. Image size: 0.897mm × 0.671 mm (10X objective).

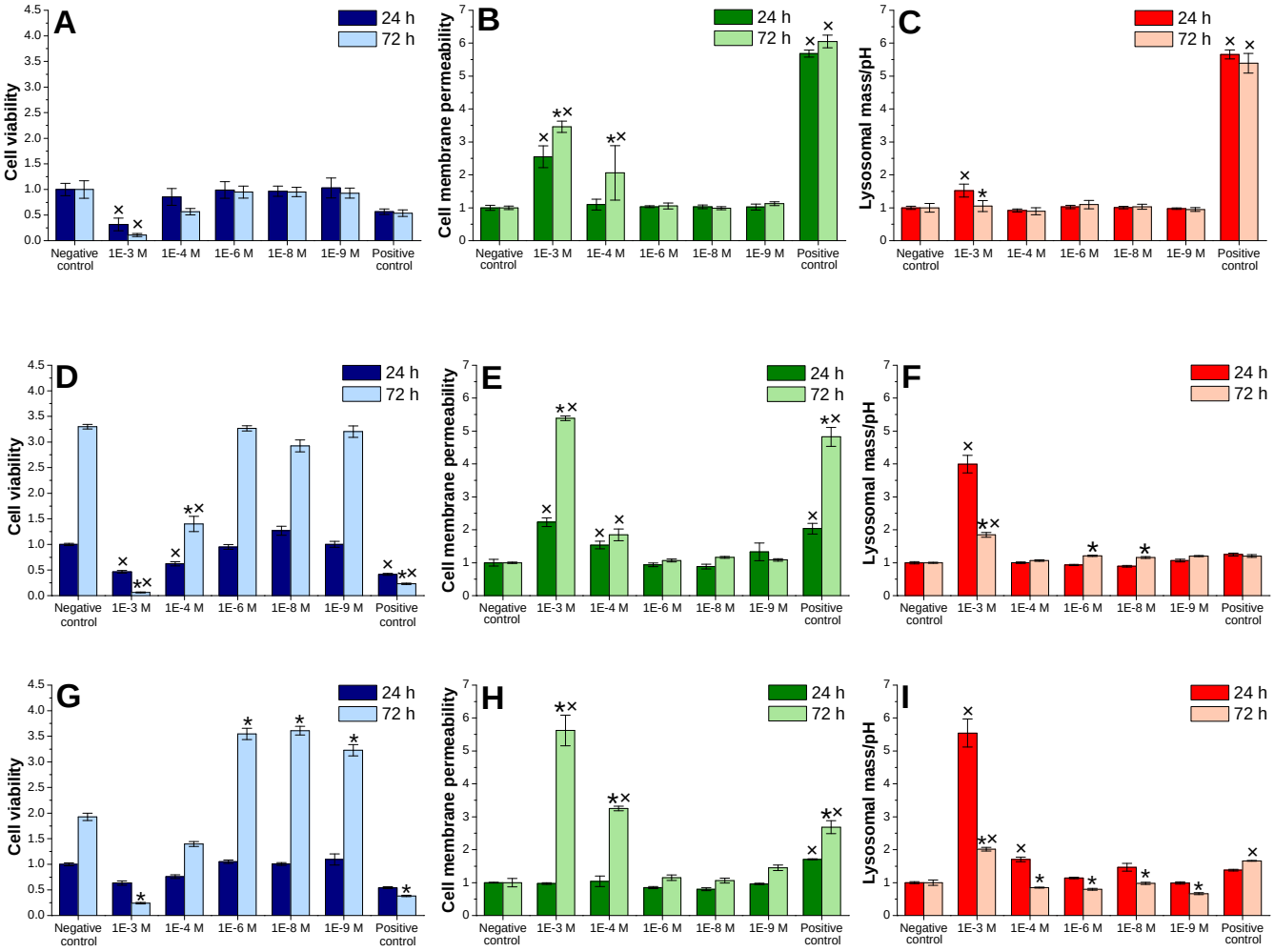


Figure 3. HCSA quantitative results for THP-1, AGS and A549 cells to the spiropyran derivative **[1]** at five different concentrations (10^{-3}, 10^{-4}, 10^{-6}, 10^{-8} and 10^{-9} M) for two endpoints (24 and 72 h). In blue, dose- and time- response of (A) THP-1, (D) AGS and (G) A549 cell population viability to spiropyran exposure; the data are normalized to the negative control at 24 h. In green, cell membrane permeability changes of (B) THP-1, (E) AGS and (H) A549 cells associated with the exposure to the spiropyran; the data for each time point are normalized to the matching negative control. In red, lysosomal mass/pH changes of (C) THP-1, (F) AGS and (I) A549 cells associated with the exposure to the spiropyran are shown; the data for each endpoint are normalized to the matching negative control. The symbol (×) above the bars indicates dose-dependent cytotoxicity as compared to the negative controls (two-way ANOVA, $p<0.05$), while the symbol (*) indicates statistically significant time-dependent cytotoxicity (two-way ANOVA, $p<0.05$).

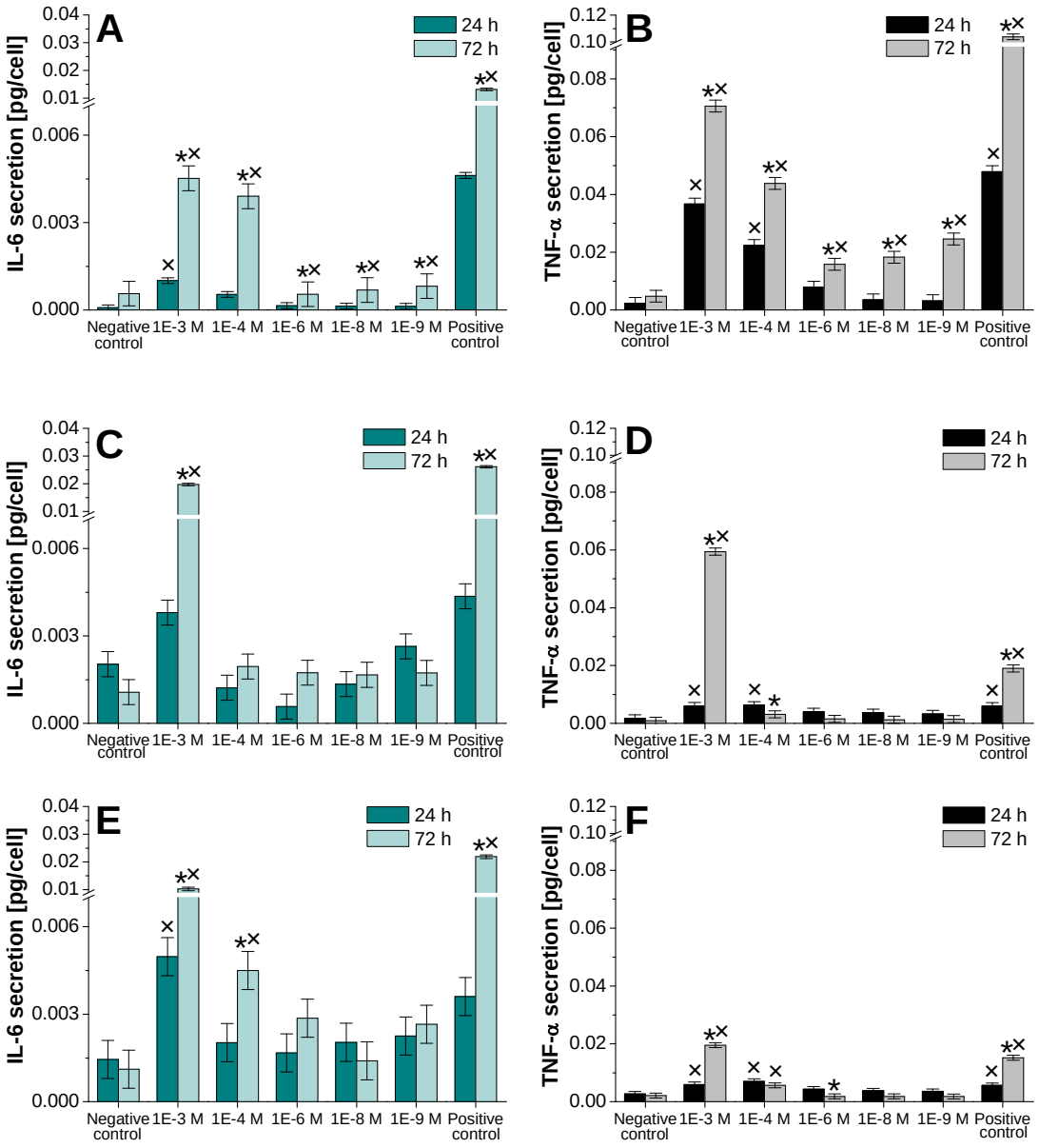
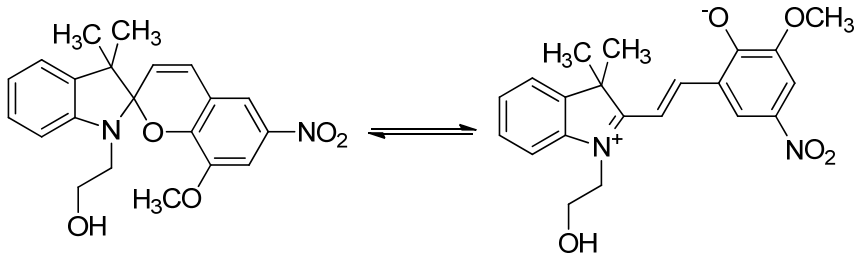


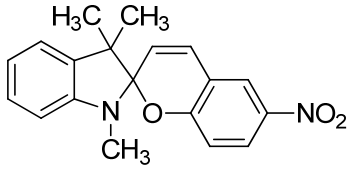
Figure 4. Concentration of pro-inflammatory cytokines released from: (A, B) THP-1 cells, (C, D) AGS cells, and (E, F) A549 cells, after 24 and 72 h incubation of five different concentrations of 8-methoxy-6-nitro-BIPS **[1]** (10^{-3}, 10^{-4}, 10^{-6}, 10^{-8} and 10^{-9} M). In cyan, concentration of IL-6; in black, concentration of TNF-α. The data are represented as average (n_{test} =2) ± s.e.m. picograms (pg) per cell. The symbol (×) above the bars indicates dose-dependent cytotoxicity as compared to the negative controls (two-way ANOVA, p<0.05), while the symbol (*) indicates statistically significant time-dependent cytotoxicity (two-way ANOVA, p<0.05).

Scheme 1. Isomeric forms of the 8-methoxy-6-nitro-BIPS **[1]**. On the right, molecular structure of the coloured merocyanine form (ME). In water, the molecule can be switched to the colourless closed form (SP) upon vis irradiation.



SP **ME**

Scheme 2. Molecular structures of 6-nitro-BIPS **[2]**.



[2]