Supporting Information

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

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CONTENT

EXPERIMENTAL SECTION

PROTOCOL FOR LIVE FLUORESCENT STAINING

Supernatants were collected from each well and 50 µl of pre-warmed 1X MPCT1 Fluor Solution in cell media were added to each well. After incubation for 30 minutes at 37°C, supernatants were aspirated and 100 µl of pre-warmed PFA 3% were added to each well (fixation procedure). Pre-warming the fixative solution was critical to maintain cell integrity. The 96-wells plates were incubated in a sterile environment at room temperature for 15 minutes. PFA was removed and plates were washed trice with 150 µl/well of 1X Wash Buffer. After washings, 200 µl/well of 1X Wash Buffer were added, and each plate was then sealed for multiple automated analyses by HCSA.

Major details on the staining Kit used can be found on the ThermoScientific website, at the following link:
http://www.thermoscientific.com/wps/portal/ts/products/detail?navigationId=L10763&categoryId=82098&productId=12811558.

GENERAL ELISA ASSAYS PROTOCOL

Plate Preparation Capture antibody was diluted to the working concentration in PBS without carrier protein. 96-wells plates were filled with 100 µl/well of the diluted capture antibody. The plates were then sealed with an adhesive strip and incubated overnight at room temperature. Each well was aspirated and washed with wash buffer, repeating the process two times for a total of three washes. Complete removal of liquid at each step was performed by inverting the plate and blotting it against clean paper towels. 300 µl of reagent diluent were added to each well and plates were incubated at room temperature for an hour. The aspiration/wash step was repeated, as described before.

Assay Procedure 100 µl/well of sample or standard in reagent diluent were added and incubated 2

hours at room temperature. After repeating the aspiration/wash step, 100 µl of the detection antibody, diluted in reagent diluent, were added to each well. The plates were covered with a new adhesive strip and incubate 2 hours at room temperature. 100 µl of the working dilution of streptavidin-HRP were added to each well after aspiration/wash and incubated for 20 minutes at room temperature in the dark. The aspiration/wash step was repeated and 100 µl/well of substrate solution were added and incubated for 20 minutes at room temperature in the dark. Finally, 50 µl of stop solution were added to each well and the optical density of each well was determined using a VERSAmax microplate reader (Molecular Devices, USA) set to 450 nm and 540 nm. Readings at 540 nm were subtracted from the readings at 450 nm in order to correct the optical imperfections in the plate.

ADDITIONAL FIGURES AND RESULTS

HIGH CONTENT SCREENING AND ANALYSIS (HCSA)

Qualitative data at 72 h exposure

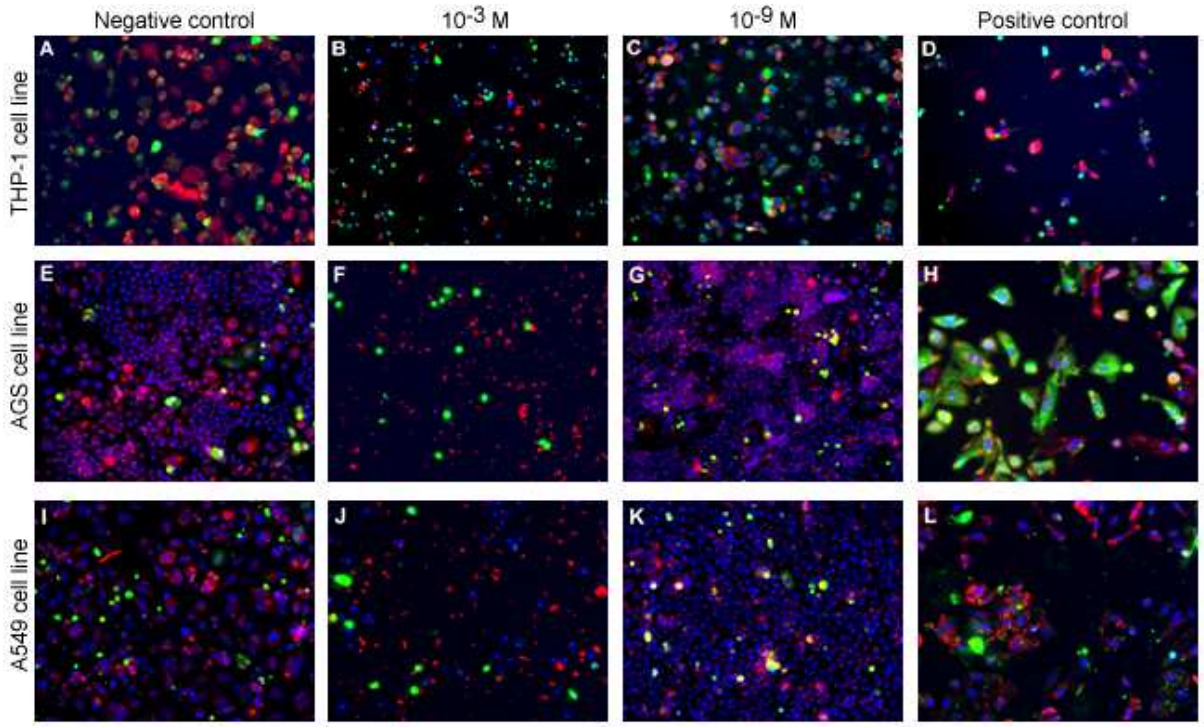

Figure S1. HCSA qualitative results for THP-1 cells (Figure A, B, C and D), AGS cells (Figure E, F, G and H) and A549 cells (Figure I, J, K and L) at 72 h exposure to the spiropyran derivative **[1]**. The composite images show the cells stained for: (i) nuclei (in blue); (ii) cell membrane permeability (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).

Nuclear area and nuclear staining intensity at 24 and 72 h exposure Following a toxic insult, cells may respond with changes in nuclear size and/or staining intensity depending on the cell type, the compound and the intersection between the two (*1*). A decreased value of nuclear area and an increased value of nuclear staining intensity are generally associated with cell stress and subsequential cell nuclei collapse. Additionally, in our study this process may also be induced by the intra-nuclear uptake of the small molecules of the photochromic compound tested. Changes in nuclear area and nuclear staining intensity were therefore investigated after incubation of 8-methoxy-6-nitro-BIPS **[1]** in THP-1, AGS, and A549 cells **(**Figure S2).

In THP-1 cells, the spiropyran effect on the nuclear area and intensity were found to be strongly correlated with the results reported in the manuscript for cell viability, cell membrane permeability, and lysosomal mass/pH changes. Decreasing nuclear area and increasing nuclear staining intensity were shown, in fact, at 10^{-3} M at 24 and 72 h. Decreasing nuclear area was also seen in AGS and A549 cell at 10^{-3} M, while the nuclear intensity did not show highly significant changes in these two cell lines. Interestingly, decreased nuclear area was observed in A549 cells at 72 h at concentrations ranging from 10^{-6} to 10^{-9} M. This phenomenon was correlated to the high cell proliferation of the A549 cell line at these concentrations, which caused cell shrinkage due to the space limits of the wells.

Statistical analysis of the changes of nuclear area and nuclear staining intensity confirmed the dose- and time-dependent cytotoxicity of 8-methoxy-6-nitro-BIPS **[1]** in THP-1, AGS and A549 cell lines.

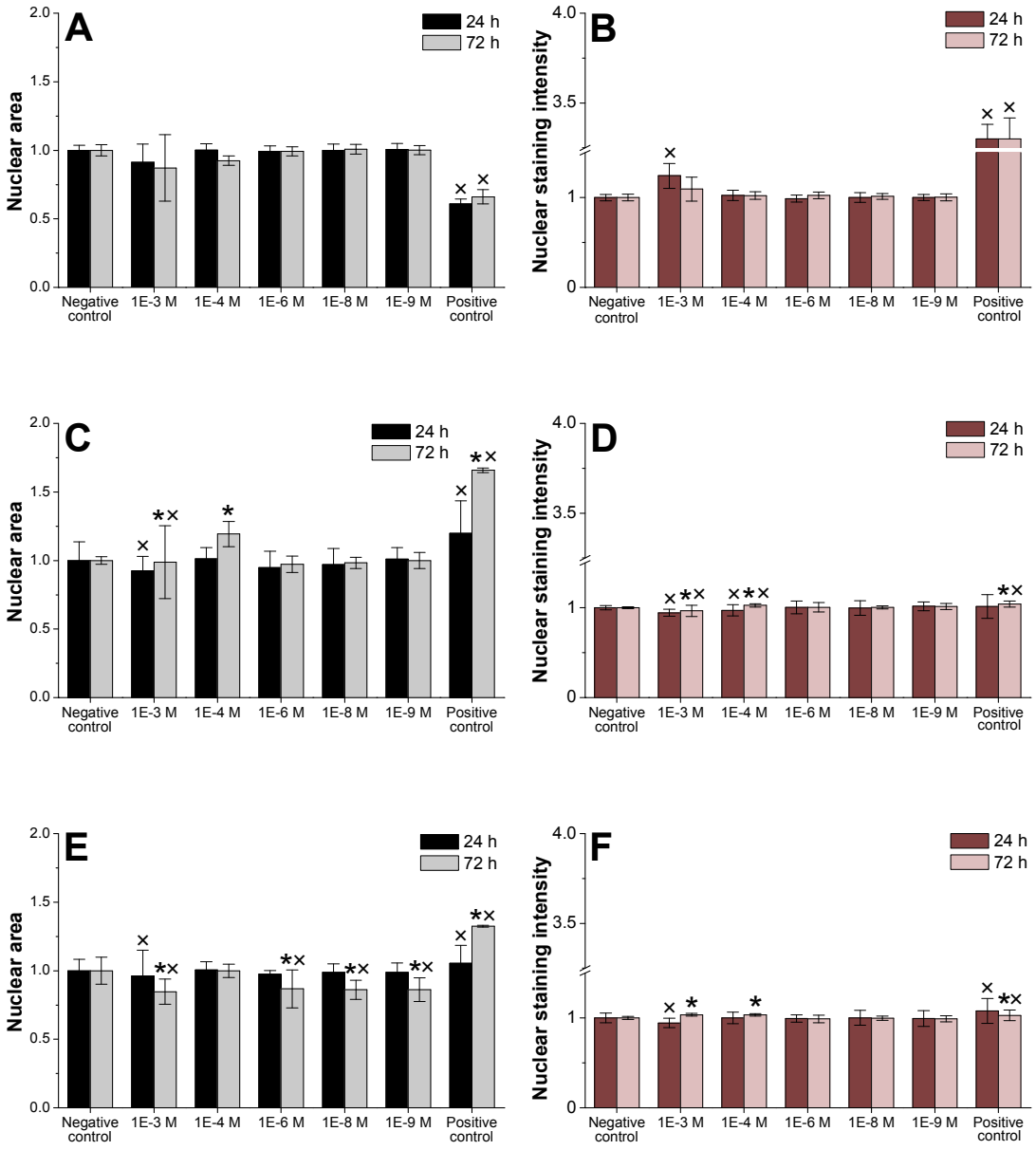

Figure S2. HCSA quantitative results for the dose- and time-exposure of THP-1, AGS and A549 cells to the spiropyran derivative **[1]** at five different concentrations (10^{-3}, 10^{-4}, 10^{-6}, 10^{-8} and 10^{-9} M) and at two endpoints (24 and 72 h). In black, nuclear area changes associated with the exposure of (A) THP-1, (C) AGS and (E) A549 cells to spiropyran **[1]**. In brawn, the nuclear intensity changes of (B) THP-1, (D) AGS and (F) A549 cells when exposed to the spiropyran **[1]**. 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, $p<0.05$).

ADDITIONAL TABLES

Table S1. Statistical analysis of (A) dose-, and (B) time--interdependent changes of cellular responses between the negative controls and THP-1 cells exposed to five concentrations of 8-methoxy-6-nitro-BIPS **[1]**. *p* values are reported for each dose and exposure time; *ns* indicates not significant data ($p>0.05$).

		THP-1 cell line				
		A – *DOSE-DEPENDENCY*				
		Cell viability	Cell membrane permeability	Lysosomal mass/pH	Nuclear area	Nuclear staining intensity
10^{-3} M	24 h	$p<0.01$	$p<0.001$	$p<0.01$	*ns*	$p<0.05$
	72 h	$p<0.001$	$p<0.001$	*ns*	*ns*	*ns*
10^{-4} M	24 h	*ns*	*ns*	*ns*	*ns*	*ns*
	72 h	*ns*	$p<0.05$	*ns*	*ns*	*ns*
10^{-6} M	24 h	*ns*	*ns*	*ns*	*ns*	*ns*
	72 h	*ns*	*ns*	*ns*	*ns*	*ns*
10^{-8} M	24 h	*ns*	*ns*	*ns*	*ns*	*ns*
	72 h	*ns*	*ns*	*ns*	*ns*	*ns*
10^{-9} M	24 h	*ns*	*ns*	*ns*	*ns*	*ns*
	72 h	*ns*	*ns*	*ns*	*ns*	*ns*
Positive control	24 h	*ns*	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$
	72 h	*ns*	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$
		B – *TIME-DEPENDENCY*				
		Cell viability	Cell membrane permeability	Lysosomal mass/pH	Nuclear area	Nuclear staining intensity
10^{-3} M		*ns*	$p<0.01$	$p<0.001$	*ns*	*ns*
10^{-4} M		*ns*	*ns*	*ns*	*ns*	*ns*
10^{-6} M		*ns*	*ns*	*ns*	*ns*	*ns*
10^{-8} M		*ns*	*ns*	*ns*	*ns*	*ns*
10^{-9} M		*ns*	*ns*	*ns*	*ns*	*ns*
Positive control		*ns*	*ns*	*ns*	*ns*	*ns*

Table S2. Statistical analysis (two-way ANOVA) of the (A) dose-, and (B) time-interdependent changes of cellular responses between the negative controls and AGS cells exposed to five concentrations of 8-methoxy-6-nitro-BIPS **[1]**. *p* values are reported for each dose and exposure time; *ns* indicates not significant data ($p>0.05$).

		AGS cell line				
		A – *DOSE-DEPENDENCY*				
		Cell viability	Cell membrane permeability	Lysosomal mass/pH	Nuclear area	Nuclear staining intensity
10^{-3} M	24 h	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.05$	$p<0.001$
	72 h	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$
10^{-4} M	24 h	$p<0.001$	$p<0.05$	*ns*	*ns*	$p<0.01$
	72 h	$p<0.001$	$p<0.001$	*ns*	*ns*	$p<0.01$
10^{-6} M	24 h	*ns*	*ns*	*ns*	*ns*	*ns*
	72 h	*ns*	*ns*	*ns*	$p<0.001$	*ns*
10^{-8} M	24 h	*ns*	*ns*	*ns*	*ns*	*ns*
	72 h	*ns*	*ns*	*ns*	$p<0.001$	*ns*
10^{-9} M	24 h	*ns*	*ns*	*ns*	*ns*	*ns*
	72 h	*ns*	*ns*	*ns*	$p<0.001$	*ns*
Positive control	24 h	$p<0.001$	$p<0.001$	*ns*	$p<0.001$	*ns*
	72 h	$p<0.001$	$p<0.001$	*ns*	$p<0.001$	$p<0.001$
		B – *TIME-DEPENDENCY*				
		Cell viability	Cell membrane permeability	Lysosomal mass/pH	Nuclear area	Nuclear staining intensity
10^{-3} M		$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$
10^{-4} M		$p<0.001$	*ns*	*ns*	$p<0.001$	$p<0.001$
10^{-6} M		*ns*	*ns*	$p<0.01$	*ns*	*ns*
10^{-8} M		*ns*	*ns*	$p<0.01$	*ns*	*ns*
10^{-9} M		*ns*	*ns*	*ns*	*ns*	*ns*
Positive control		$p<0.001$	$p<0.001$	*ns*	$p<0.001$	$p<0.001$

Table S3. Statistical analysis (two-way ANOVA) of the (A) dose-, and (B) time-interdependent changes of cellular responses between the negative controls and A549 cells exposed to five concentrations of 8-methoxy-6-nitro-BIPS **[1]**. *p* values are reported for each dose and exposure time; *ns* indicates not significant data ($p>0.05$).

		A549 cell line				
		A – *DOSE-DEPENDENCY*				
		Cell viability	Cell membrane permeability	Lysosomal mass/pH	Nuclear area	Nuclear staining intensity
10^{-3} M	24 h	*ns*	*ns*	$p<0.001$	$p<0.05$	$p<0.001$
	72 h	*ns*	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$
10^{-4} M	24 h	*ns*	*ns*	$p<0.001$	*ns*	*ns*
	72 h	*ns*	$p<0.001$	*ns*	*ns*	$p<0.01$
10^{-6} M	24 h	*ns*	*ns*	*ns*	*ns*	*ns*
	72 h	*ns*	*ns*	*ns*	$p<0.001$	*ns*
10^{-8} M	24 h	*ns*	*ns*	$p<0.05$	*ns*	*ns*
	72 h	*ns*	*ns*	*ns*	$p<0.001$	*ns*
10^{-9} M	24 h	*ns*	*ns*	*ns*	*ns*	*ns*
	72 h	*ns*	*ns*	*ns*	$p<0.001$	*ns*
Positive control	24 h	*ns*	$p<0.01$	*ns*	$p<0.001$	$p<0.001$
	72 h	*ns*	$p<0.001$	$p<0.01$	$p<0.001$	$p<0.01$
		B – *TIME-DEPENDENCY*				
		Cell viability	Cell membrane permeability	Lysosomal mass/pH	Nuclear area	Nuclear staining intensity
10^{-3} M		$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$
10^{-4} M		*ns*	$p<0.001$	$p<0.001$	*ns*	$p<0.001$
10^{-6} M		$p<0.001$	*ns*	$p<0.05$	$p<0.001$	*ns*
10^{-8} M		$p<0.001$	*ns*	$p<0.001$	$p<0.001$	*ns*
10^{-9} M		$p<0.001$	$p<0.01$	$p<0.05$	$p<0.001$	*ns*
Positive control		$p<0.001$	$p<0.001$	*ns*	$p<0.001$	$p<0.001$

Table S4. Statistical analysis of the (A) dose-, and (B) time- interdependent changes of IL-6 secretion between the negative controls and THP-1, AGS, and A549 cells exposed to five concentrations of 8-methoxy-6-nitro-BIPS **[1]**. *p* values are reported for each cell line, dose, and exposure time; *ns* indicates not significant data ($p>0.05$).

		A – *DOSE-DEPENDENCY*		
		THP-1 cell line	*AGS cell line*	*A549 cell line*
10^{-3} M	24 h	$p<0.01$	*ns*	$p<0.01$
	72 h	$p<0.001$	$p<0.001$	$p<0.001$
10^{-4} M	24 h	*ns*	*ns*	*ns*
	72 h	$p<0.001$	*ns*	$p<0.001$
10^{-6} M	24 h	*ns*	*ns*	*ns*
	72 h	$p<0.001$	*ns*	*ns*
10^{-8} M	24 h	*ns*	*ns*	*ns*
	72 h	$p<0.05$	*ns*	*ns*
10^{-9} M	24 h	*ns*	*ns*	*ns*
	72 h	$p<0.001$	*ns*	*ns*
Positive control	24 h	$p<0.001$	*ns*	*ns*
	72 h	$p<0.001$	$p<0.001$	$p<0.001$
		B – *TIME-DEPENDENCY*		
		THP-1 cell line	*AGS cell line*	*A549 cell line*
10^{-3} M		$p<0.001$	$p<0.001$	$p<0.001$
10^{-4} M		$p<0.001$	*ns*	$p<0.001$
10^{-6} M		$p<0.001$	*ns*	*ns*
10^{-8} M		$p<0.001$	*ns*	*ns*
10^{-9} M		$p<0.001$	*ns*	*ns*
Positive control		$p<0.001$	$p<0.001$	$p<0.001$

Table S5. Statistical analysis of the (A) dose-, and (B) time-interdependent changes of TNF-α secretion between the negative controls and THP-1, AGS, and A549 cells exposed to five concentrations of 8-methoxy-6-nitro-BIPS. *p* values are reported for each cell line, dose, and exposure time; *ns* indicates not significant data ($p>0.05$).

		A – *DOSE-DEPENDENCY*		
		THP-1 cell line	*AGS cell line*	*A549 cell line*
10^{-3} M	24 h	$p<0.001$	$p<0.05$	$p<0.05$
	72 h	$p<0.001$	$p<0.001$	$p<0.001$
10^{-4} M	24 h	$p<0.001$	$p<0.05$	$p<0.001$
	72 h	$p<0.001$	*ns*	$p<0.05$
10^{-6} M	24 h	*ns*	*ns*	*ns*
	72 h	$p<0.01$	*ns*	*ns*
10^{-8} M	24 h	*ns*	*ns*	*ns*
	72 h	$p<0.001$	*ns*	*ns*
10^{-9} M	24 h	*ns*	*ns*	*ns*
	72 h	$p<0.001$	*ns*	*ns*
Positive control	24 h	$p<0.001$	$p<0.05$	$p<0.05$
	72 h	$p<0.001$	$p<0.001$	$p<0.001$
		B – *TIME-DEPENDENCY*		
		THP-1 cell line	*AGS cell line*	*A549 cell line*
10^{-3} M		$p<0.001$	$p<0.001$	$p<0.001$
10^{-4} M		$p<0.001$	$p<0.05$	*ns*
10^{-6} M		$p<0.001$	*ns*	$p<0.001$
10^{-8} M		$p<0.001$	*ns*	*ns*
10^{-9} M		$p<0.001$	*ns*	*ns*
Positive control		$p<0.001$	$p<0.001$	$p<0.001$

REFERENCES

(1) Majno, G. and Joris, I. (1995) Apoptosis, Oncosis and Necrosis - An overview of cell-death. *Am. J. Pathol. 146*, 3-15.