Chalmers Publication Library

This document is the accepted manuscript version of a published work that appeared in final form in *Chemical Communications*, © Royal Society of Chemistry, after peer review and technical editing by the publisher. To access the final edited and published work, see http://dx.doi.org/10.1039/C1CC13561A

(Article begins on next page)

Chem Comm

Dynamic Article Links ►

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

Communication

Light-Induced Cytotoxicity of a Photochromic Spiropyran†

Jesper R. Nilsson[a], Shiming Li[a], Björn Önfelt*[b] and Joakim Andréasson*[a]

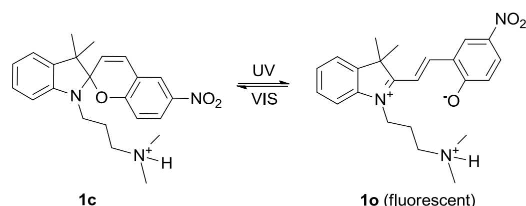
Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

In this work we present a novel water soluble spiropyran photoswitch that can be photonically activated inside live cells from a form that has no significant effect on the cellular survival to a form that induces a dramatic toxic response.

The main problem with today's chemotherapeutical methods is that many of the existing drugs display very low specificity to cancer cells, resulting in severe side effects. If the chemical properties are not sufficient to target drugs to a specific tissue or tumour it is attractive to introduce another level of control, allowing for a locally induced cytotoxic effect. One way to achieve that is to use photo-activatable molecules that can be selectively activated from a non-toxic form to a toxic form only in certain areas.[1-4] Photochromic molecules, which can be switched between isomeric forms of different chemical (and possibly toxic) properties are potential candidates for this purpose. Several examples have been reported of their use in biological contexts such as live cell imaging,[5-11] *in vivo* imaging,[12] biomolecule function,[13-18] sensors/probes,[19-20] and even to induce reversible paralysis in *C. Elegans* nematodes[21]. Here, we report for the first time a novel photochromic molecule from the spiropyran (SP) family that can be photo-activated inside live cells from a form that does not affect the cellular survival to a form that induces a dramatic toxic response. SP photoswitches[22] (typically attached to nano-particles or proteins) have previously been introduced to live cells. The main focus of these studies, however, has been on bioimaging applications.[6-10] The only study addressing the potential cytotoxicity so far was reported by Giordani and co-workers, and it was found that the thermally stable spiro form is unlikely to display strong toxic effects.[23] No attempts were made to investigate the effect of photoisomerization.

The structures and the isomerization scheme of SP derivative **1** used in this study are shown in Scheme 1.[‡a] The photochromic unit is the 6-nitro BIPS moiety, the alkyl linked ammonium group was attached to increase water solubility. The closed spiro form **1c** is isomerized to the open merocyanine form **1o** with a time constant of 4.7 min in aqueous solution using 254 nm UV-light (700 μW/cm^2). The photostationary distribution reached is ca. 40/60 **1c**/**1o**. The reverse reaction is induced by visible light ($\lambda > 465$ nm, 35 mW/cm^2) and converts the sample to virtually 100% **1c** ($t_{1/2} = 10$ s). The quantum yield of the opening (**1c** → **1o**) and closing (**1o** → **1c**) reactions were determined to ca. 0.1 for both processes. Left in the dark, a thermal equilibrium is established with a time constant of 6 h at 23°C, yielding **1c**/**1o** in a 50/50 ratio (determined by NMR experiments). Hydrolysis of **1o** is also observed[24], although on a much longer time scale (time constant 50 h). The closed form **1c** absorbs mainly in the UV region with absorption maxima at 270 nm and 351 nm, whereas **1o** absorbs also in the visible region with bands centered around 356 nm and 510 nm (Fig. S5, ESI†). The open form **1o** is the only fluorescent isomer (quantum yield = 0.01 ± 20%), and the emission maximum is found at 641 nm.



Scheme 1 Structures of the closed spiro form (**1c**), and the open merocyanine form (**1o**) of SP photoswitch **1**.

In this study, the primary objectives were to investigate both the cellular uptake and the cytotoxicity of **1c** and **1o**. Merely judging from the structures, **1c** could pass through the cell membrane due to the non-polar nature[20], whereas polar zwitterions like **1o** typically display poor membrane penetrating properties[‡b]. To assess the cellular uptake, a 50/50 **1c**/**1o** solution (6 μM total concentration of **1**) was prepared and incubated with HEK 293 cells at 37 °C for 15 min. This sample was imaged by confocal fluorescence microscopy with excitation at 488 nm for observation of the open merocyanine form **1o**. As shown in Fig. 2a, no fluorescence is observed from inside the cells immediately after incubation (the weak fluorescence seen originates mainly from the extracellular areas), indicating that the intracellular concentration of **1o** is very low. Next, the sample was exposed to 10 s UV-light (300 nm < λ < 400 nm), and the fluorescence was again monitored by 488 nm excitation. Intense fluorescence was now observed from inside the cells (Fig. 2b), clearly demonstrating the presence of **1o**. This shows that only the closed spiro form **1c** is able to pass the cell membrane and that **1c** can be photoswitched to **1o** inside the cells. Interestingly, this intracellular fluorescence remained (even if somewhat declined) although the sample was illuminated by 488 nm for an extended time, which normally would lead to isomerization **1o** → **1c**. This observation can only be explained by some process/processes inhibiting isomerization, *e.g.* binding to

intracellular molecules. No significant difference in cellular uptake upon incubation at 4 °C (on ice) vs. 37 °C is noted (Figs. S6 and S7, ESI†), suggesting uptake mainly by passive diffusion. Diffusion across the cell membrane followed by a thermal intracellular conversion **1c** → **1o** would be another potential route from **1c** (extracellular) to **1o** (intracellular). The thermal intracellular isomerization, however, is extremely slow and yields a negligible intracellular **1o** concentration compared to the photoinduced isomerization (Video 1, ESI†). A possible explanation could be that **1** experiences a lower polarity in the cytosol compared to aqueous solution, which would favour **1c** at thermal equilibrium. Furthermore, cells incubated with 50/50 **1c**/**1o** for 9 h show no signs of decreased viability, *i.e.*, in the absence of UV-light there is no or very little effect on the cellular survival at a concentration of 6 μM (Video 1, ESI†). This observation is consistent with the study by Giordani and co-workers, where the closed spiro form of an SP derivative similar to **1c** was found to induce no cytotoxic response in the μM concentration regime.[23]

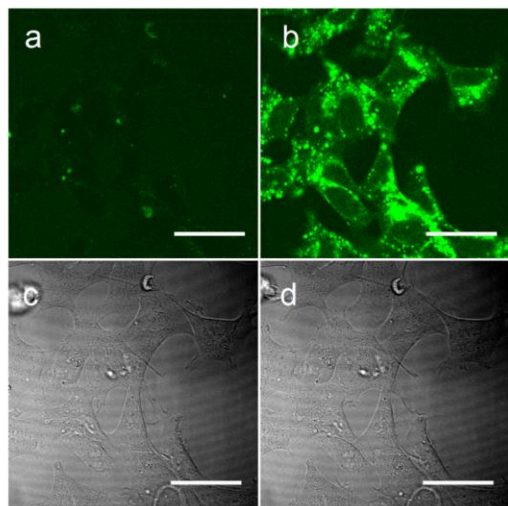


Fig. 1 Confocal microscopy images of HEK 293 cells incubated for 15 minutes with 50/50 **1c**/**1o** before (a,c) and immediately after (b,d) 10 s UV exposure. The wavelength of the probing light is 488 nm. Scale bar = 30 μm.

In order to follow the effects of intracellular photoisomerization **1c** → **1o**, cells incubated with SP for 15 min were exposed to a 10 s UV dose. To highlight the effect of the UV-light, the exposed region was limited to the circles marked in Fig. 2. Shortly after the UV stimulus, the exposed SP-incubated cells marked by white arrows detached from the glass surface, "rounded up", and extensive dynamic membrane blebbing was initiated (Fig. 2b). This was followed by a stage where the effected cells ceased to blebb and re-assumed a spherical shape. Finally the cell membrane of the rounded cells burst – the cells were dead (red arrows Fig. 2c, the entire sequence is vividly illustrated in Video 2, ESI†). The cells not exposed to UV-light, however, do not display any of the abovementioned morphological changes, again showing that the presence of intracellular **1c** / extracellular **1o** has no or very little effect on the cellular survival. UV-light alone is known to kill cells if the damage to their DNA is extensive enough. To exclude the 10 s UV dose being the reason for the observed cell death, rather than the cytotoxic effect of intracellular **1o**, the corresponding control experiment was performed on cells not incubated with **1**. The results are shown in Fig. 2d-f, and the exposed cells appeared to be unaffected by the 10 s UV dose (Video 3, ESI†). The toxic effect from the combination of **1** and UV-light was also studied on larger cell populations using an objective with lower magnification.

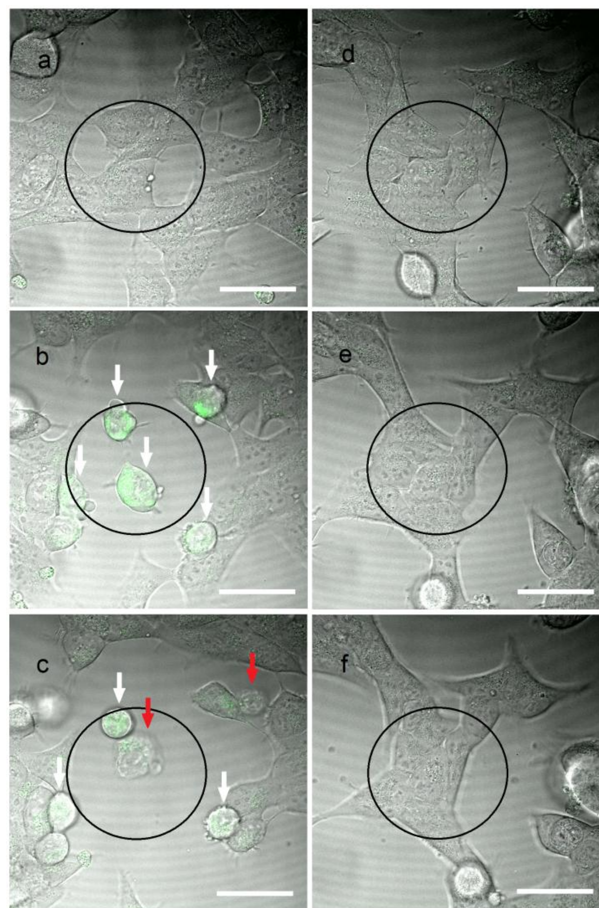


Fig. 2 Effect of UV-light exposure on cells incubated with **1** (a-c) and untreated cells (d-f). The circles indicate the areas where UV-light was applied. The arrows in b and c show the cells initially exposed to UV-light. Red arrows indicate ruptured cells. Images were acquired with a 60 s interval. Displayed time points are t=0 (a,d), t=29 min (b,e) and t=118 min (c,f). Scale bar = 30 μm.

UV-light was directed to the entire field of view and the membrane blebbing was assessed for cells with or without the presence of **1**. The results, summarized in Table 1, clearly illustrate the importance of having the SP derivative **1** present to induce cell blebbing, and that the 10 s UV dose alone is not sufficient to initiate the effect.

Table 1 Cell blebbing effect after UV-light exposure

Conditions[(a)]	Exposed cells[(b)]	Blebbing cells[(b)]	Blebbing cells[(c)]
UV	145	4	3 ± 2 %
UV+SP	127	70	52 ± 9%

(a) UV-light exposure time was 10 s and the total concentration of **1** was 6 μM. (b) Mean of duplicate experiments. (c) Mean ± SD of duplicate experiments.

Light microscopy is considered as one of the best experimental methods to define apoptosis (programmed cell death) and contrast

This journal is © The Royal Society of Chemistry [year]

it with necrosis.[25] Although the nature of the involved mechanism is not established, the similarities of the dramatic photoinduced effect observed and the morphological changes associated with apoptosis are noticeable. The apoptotic morphological changes, which involve cell contraction, dynamic membrane blebbing, and cell fragmentation are all identified in the time lapse series shown in Fig. 2a-c (Video 2, ESI†).[26-27] Previous studies in our laboratory have shown that also the DNA-binding properties of SP derivatives similar to **1** are activated by the UV-induced isomerization **1c** → **1o**.[28-29] Judging by the relative fluorescence intensities, however, it seems that the concentration of **1o** in the cell nulei is low compared to the corresponding concentration in the cytoplasm, suggesting that **1** cannot efficiently penetrate through the nuclear envelope to reach the DNA (Fig. 1b). Hence, it is more likely that the cytotoxic effect of **1o** is due to interference with processes in the cytoplasm (*e.g.* disruption of vital enzyme functions by intermolecular interactions, or generation of reactive oxygen species) rather than being associated with binding to nuclear DNA. The mechanism of action is presently being investigated.

It is particularly attractive that the active merocyanine form cannot penetrate the cell membrane, as diffusion of extracellular **1o** from the UV exposed target region will not lead to the buildup of intracellular **1o** in other regions. This will increase the spatial control even further. Finally, the poor penetration depth of UV-light into biological tissue is acknowledged. It has been shown, however, that two-photon excitation at 720 nm can effectively trigger the spiro → merocyanine conversion of SP derivatives like **1**, even inside live cells.[6-7] This approach appears very attractive, since 720 nm light is well inside the tissue optical window, where the penetration depth is maximized.[30]

In summary, we have presented a photoactivateable spiropyran derivative, showing light-induced cytotoxicity to human cancer cells. From these *in vitro* studies on live cells, it was seen that the thermally stable spiro form entered the cells, displaying no cytotoxic effects until a dose of UV-light was applied to trigger the isomerization to the active merocyanine form inside the cells. The toxic response observed showed many similarities with the morphological changes associated with programmed cell death. In the negative control (no spiropyran introduced) the cells appeared to be unaffected by the applied UV dose, showing that the cytotoxic effect is owing to the intracellular isomerization to the active merocyanine form rather than to the UV-light itself. We envision that this approach may stimulate the identification of novel photoinitiated methods for cancer treatment in addition to conventional photodynamic therapy.

The authors gratefully acknowledge the support from the Swedish Research Council (VR, grants 2007-5520 and 622-2010-280), the European Research Council (ERC FP7/2007-2013 No. 203952), the Swedish Foundation for Strategic Research, and Carl Trygger Foundation for Scientific Research.

Notes and references

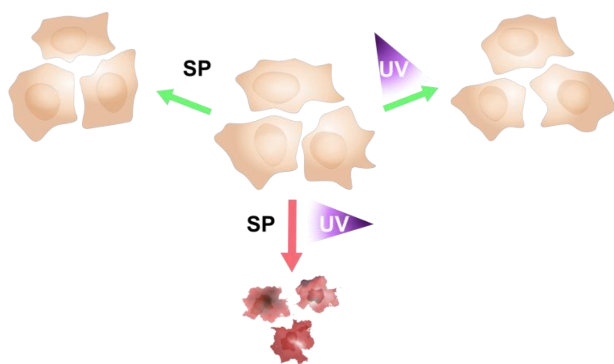
[a] *Department of Chemical and Biological Engineering, Physical Chemistry, Chalmers University of Technology, Göteborg, 412 96, Sweden. Fax: (+46) 31-772 38 58; Tel: (+46) 31-772 28 38; E-mail: a-son@chalmers.se*

[b] *Department of Applied Physics, Division of Cell Physics, The Royal Institute of Technology, Stockholm, 106 91, Sweden and Department of Microbiology, Tumour and Cell Biology, Karolinska Institutet, Stockholm, 171 77, Sweden. Fax: (+46) 8-31 11 01; E-mail: bjorn.onfelt@ki.se*

† Electronic Supplementary Information (ESI) available: Details for synthesis and spectroscopic characterization of SP **1**, experimental procedure and time-lapse confocal microscopy image compilations. See DOI: 10.1039/b000000x/

‡[a] At lower pH, the phenolate oxygen of **1o** is protonated to form **1oH**$^+$. Protonation does not inhibit photoisomerization to **1c**, although the rate of the corresponding thermal isomerization is virtually zero. ‡[b] The corresponding SP derivative equipped with a quaternary ammonium group instead of the tertiary ammonium group was also investigated, but displayed poor membrane penetrating properties. Deprotonation of the ammonium group of **1c** gives a charge-neutral species. This is likely to facilitate transport through the cell membrane.

1 G. Mayer and A. Heckel, *Angew. Chem. Int. Ed.*, 2006, **45**, 4900.
2 Q. Shao and B. G. Xing, *Chem. Soc. Rev.*, 2010, **39**, 2835.
3 I. Willner, *Acc. Chem. Res.*, 1997, **30**, 347.
4 D. D. Young and A. Deiters, *Org. Biomol. Chem.*, 2007, **5**, 999.
5 M. W. Berns, T. Krasieva, C. H. Sun, A. Dvornikov and P. M. Rentzepis, *J. Photochem. Photobiol. B*, 2004, **75**, 51.
6 S. Mao, R. K. P. Benninger, Y. L. Yan, C. Petchprayoon, D. Jackson, C. J. Easley, D. W. Piston and G. Marriott, *Biophys. J.*, 2008, **94**, 4515.
7 G. Marriott, S. Mao, T. Sakata, J. Ran, D. K. Jackson, C. Petchprayoon, T. J. Gomez, E. Warp, O. Tulyathan, H. L. Aaron, E. Y. Isacoff and Y. L. Yan, *Proc. Natl. Acad. of Sci. USA*, 2008, **105**, 17789.
8 Z. Y. Tian, W. W. Wu, W. Wan and A. D. Q. Li, *J. Am. Chem. Soc.*, 2009, **131**, 4245.
9 L. Y. Zhu, W. W. Wu, M. Q. Zhu, J. J. Han, J. K. Hurst and A. D. Q. Li, *J. Am. Chem. Soc.*, 2007, **129**, 3524.
10 M. Q. Zhu, G. F. Zhang, C. Li, M. P. Aldred, E. Chang, R. A. Drezek and A. D. Q. Li, *J. Am. Chem. Soc.*, 2011, **133**, 365.
11 Y. Zou, T. Yi, S. Z. Xiao, F. Y. Li, C. Y. Li, X. Gao, J. C. Wu, M. X. Yu and C. H. Huang, *J. Am. Chem. Soc.*, 2008, **130**, 15750.
12 A. A. Beharry, L. Wong, V. Tropepe and G. A. Woolley, *Angew. Chem. Int. Ed.*, 2011, **50**, 1325.
13 H. Asanuma, X. G. Liang, T. Yoshida and M. Komiyama, *Chembiochem*, 2001, **2**, 39.
14 T. Sakata, Y. L. Yan and G. Marriott, *Proc. Natl. Acad. Sci. USA*, 2005, **102**, 4759.
15 T. Stafforst and D. Hilvert, *Angew. Chem. Int. Ed.*, 2010, **49**, 9998.
16 D. Vomasta, C. Hogner, N. R. Branda and B. Konig, *Angew. Chem. Int. Ed.*, 2008, **47**, 7644.
17 F. Z. Zhang, K. A. Timm, K. M. Arndt and G. A. Woolley, *Angew. Chem. Int. Ed.*, 2010, **49**, 3943.
18 D. D. Young and A. Deiters, *Chembiochem*, 2008, **9**, 1225.
19 M. Inouye, K. Kim and T. Kitao, *J. Am. Chem. Soc.*, 1992, **114**, 778.
20 N. Shao, J. Y. Jin, H. Wang, J. Zheng, R. H. Yang, W. H. Chan and Z. Abliz, *J. Am. Chem. Soc.*, 2010, **132**, 725.
21 U. Al-Atar, R. Fernandes, B. Johnsen, D. Baillie and N. R. Branda, *J. Am. Chem. Soc.*, 2009, **131**, 15966.
22 R. C. Bertelson, in *Organic Photochromic and Thermochromic Compounds*, Vol. 1 (Eds: J. C. Crano, R. J. Guglielmetti), Plenum Press, New York, pp. 11.
23 D. Movia, A. Prina-Mello, Y. Volkov and S. Giordani, *Chem. Res. Toxicol.*, 2010, **23**, 1459.
24 T. Stafforst and D. Hilvert, *Chem. Commun.*, 2009, 287.
25 F. Doonan and T. G. Cotter, *Methods*, 2008, **44**, 200.
26 J. C. Mills, N. L. Stone and R. N. Pittman, *J. Cell Biol.*, 1999, **146**, 703.
27 O. Ndozangue-Touriguine, J. Hamelin and J. Breard, *Biochem. Pharmacol.*, 2008, **76**, 11.
28 J. Andersson, S. M. Li, P. Lincoln and J. Andréasson, *J. Am. Chem. Soc.*, 2008, **130**, 11836.
29 M. Hammarson, J. Andersson, S. M. Li, P. Lincoln and J. Andréasson, *Chem. Comm.*, 2010, **46**, 7130.
30 B. J. Tromberg, N. Shah, R. Lanning, A. Cerussi, J. Espinoza, T. Pham, L. Svaasand and J. Butler, *Neoplasia*, 2000, **2**, 26.



5 In this work we present a novel water soluble spiropyran photoswitch that can be photonically activated inside live cells from a form that has no significant effect on the cellular survival to a form that induces a dramatic toxic response.

This journal is © The Royal Society of Chemistry [year]