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. Photochromic molecules, which can be switched between isomorphic 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, in vivo imaging, biomolecule function, sensors/probes, and even to induce reversible paralysis in C. Elegans nematodes.

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 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. 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. No attempts were made to investigate the effect of photoisomerization.

The structures and the isomerization scheme of SP derivative used in this study are shown in Scheme 1. The photochromic unit is the 6-nitro BIPS moiety, the alkyl linked ammonium group was attached to increase water solubility. The closed spiropyran isomer is isomerized to the open merocyanine form with a time constant of 4.7 min at 23°C, yielding in a 50/50 ratio (determined by NMR experiments). Hydrolysis of the merocyanine form is also observed, although on a much longer time scale (time constant 50 h). The closed form absorbs mainly in the UV region with absorption maxima at 270 nm and 351 nm, whereas the open form absorbs also in the visible region with bands centered around 356 nm and 510 nm (Fig. S5, ESI†). The open form 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, whereas polar zwitterions like 1o typically display poor membrane penetrating properties. 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 spiropyran can be pass through the cell membrane and that can be photoswitched to 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. 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 \rightarrow 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. 

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 \rightarrow 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 affected cells ceased to bleb 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 present to induce cell blebbing, and that the 10 s UV dose alone is not sufficient to initiate the effect.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Exposed cells</th>
<th>Blebbing cells</th>
<th>Blebbing cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV</td>
<td>145</td>
<td>4</td>
<td>3 ± 2 %</td>
</tr>
<tr>
<td>UV+SP</td>
<td>127</td>
<td>70</td>
<td>52 ± 9 %</td>
</tr>
</tbody>
</table>

(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
it with necrosis. 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†). 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 $1c \rightarrow 1o$. Judging by the relative fluorescence intensities, however, it seems that the concentration of $1o$ in the cell nuclei 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 $\rightarrow$ merocyanine conversion of SP derivatives like 1, even inside live cells. In this approach appears very attractive, since 720 nm light is well inside the tissue optical window, where the penetration depth is maximized.

In summary, we have presented a photoactivatable 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.

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### Notes and references

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† 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/b000000z/

‡‡ 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. The corresponding SP derivative equipped with a quaternary ammonium group instead of the tertiary ammonium group is 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.

### References


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