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A Cytotoxic Ruthenium Tris(Bipyridyl) Complex that Accumulates at Plasma Membranes

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Ruthenium tris(bipyridyl) complexes in which the bipyridyl ligand is derivatised with groups exhibiting different lipophilicities have been evaluated in vitro. The most lipophilic complex is cytotoxic, but the less lipophilic compounds are not. Confocal microscopy was used to gain insights into the uptake and localisation of the luminescent complexes in cells. The most lipophilic complex adhered to the plasma membrane whereas the other complexes penetrated the membrane and accumulated in small organelles in the cytoplasm. None of the compounds appears to accumulate in the cell nucleus, and this calls into question the relevance of DNA as a target for this class of compound.

Ruthenium polypyridyl complexes have been widely used in a variety of applications due to their chemical stability and the ease by which their spectral and redox properties can be tuned by changing the substituent groups on the polypyridyl ligand. Notably, ruthenium(II) polypyridyl complexes have been employed as charge-transfer sensitizers in dye-sensitized solar cells due to their favourable ground and exited state redox properties.[1] The electrochemical behaviour of these metal complexes comprises a one-Ru^{II}-based oxidation and several ligand-based reductions. Depending on the electron density of the ligands the redox potential of metal complexes can be tuned to a specific potential. Indeed, ruthenium polypyridyl complexes have proven to be particularly versatile in high-energy lithium batteries involving both molecular wiring and targeting approaches[2,3] and in electrochemical biosensors.[4]

Ruthenium complexes have considerable potential in a number of biomedical applications such as diagnostics and therapeutics and a wide range of ruthenium compounds have been evaluated as putative anticancer agents.[5] Two ruthenium compounds, albeit not containing polypyridyl ligands, have even completed phase I clinical trials.[6,7] The biological properties of ruthenium complexes with bipyridyl ligands not too dissimilar from the ruthenium dyes employed in the above-mentioned applications have been extensively studied with a focus on how such compounds interact with DNA.[8,9]

In this communication, we describe a new feature of ruthenium polypyridyl dyes. We harnesseed their luminescent properties to show that they can target the cell membrane or small organelles and induce cell death via a non-DNA pathway. (Figure 1)

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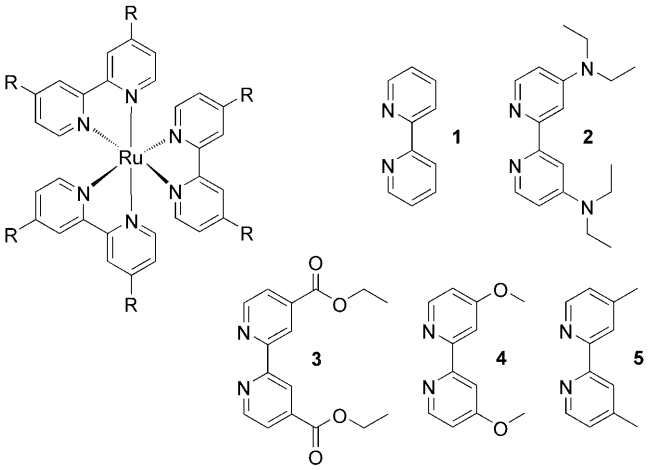

Figure 1. Ruthenium(II) tris(bipyridyl) complexes used in this study.

To assess the effect on cell growth, ovarian cancer cells were grown for three days at 37 °C in the presence of various concentrations of the ruthenium tris(bipyridyl) complexes, and following incubation, cell survival was monitored by using the MTT assay. As shown in the table, the compounds exhibit similar cytotoxicities towards both the A2780 cell line and the cisplatin-resistant A2780cisR variant. Compounds **1**, **3**, **4** and **5** are not particularly cytotoxic, but **2** is highly cytotoxic (<1 μM; Table 1). The high toxicity of **2** could be explained either by a high lipophilicity or by positive charges forming as its diethylamine groups are protonated.[10] We therefore determined its pK_a (5.8) and measured the $\log P_{oct/w}$ of the different compounds. At the pH of the culture medium (pH 7), **2** is in its unprotonated (and most lipophilic) form, making it more prone to bind to the plasma membrane, and possibly explaining its high cytotoxicity.

Table 1. Cytotoxicity and lipophilicity of **1**–**5**.

	A2780 IC_{50} [μM]	A2780cisR IC_{50} [μM]	log *P* (pH 7)
1	158	163	−1.21
2	<1	<1	0.55
3	100	121	−0.28
4	85	88	−0.37
5	>200	>200	−0.99

In order to gain insights into the uptake of the compounds, we took advantage of their intrinsic fluorescence (Figure 2). Compound **1** did not accumulate in the cell; this is consistent with results found in the literature.[11] Compounds **3, 4** and **5** were observed to accumulate in dot-like structures in the cyto-

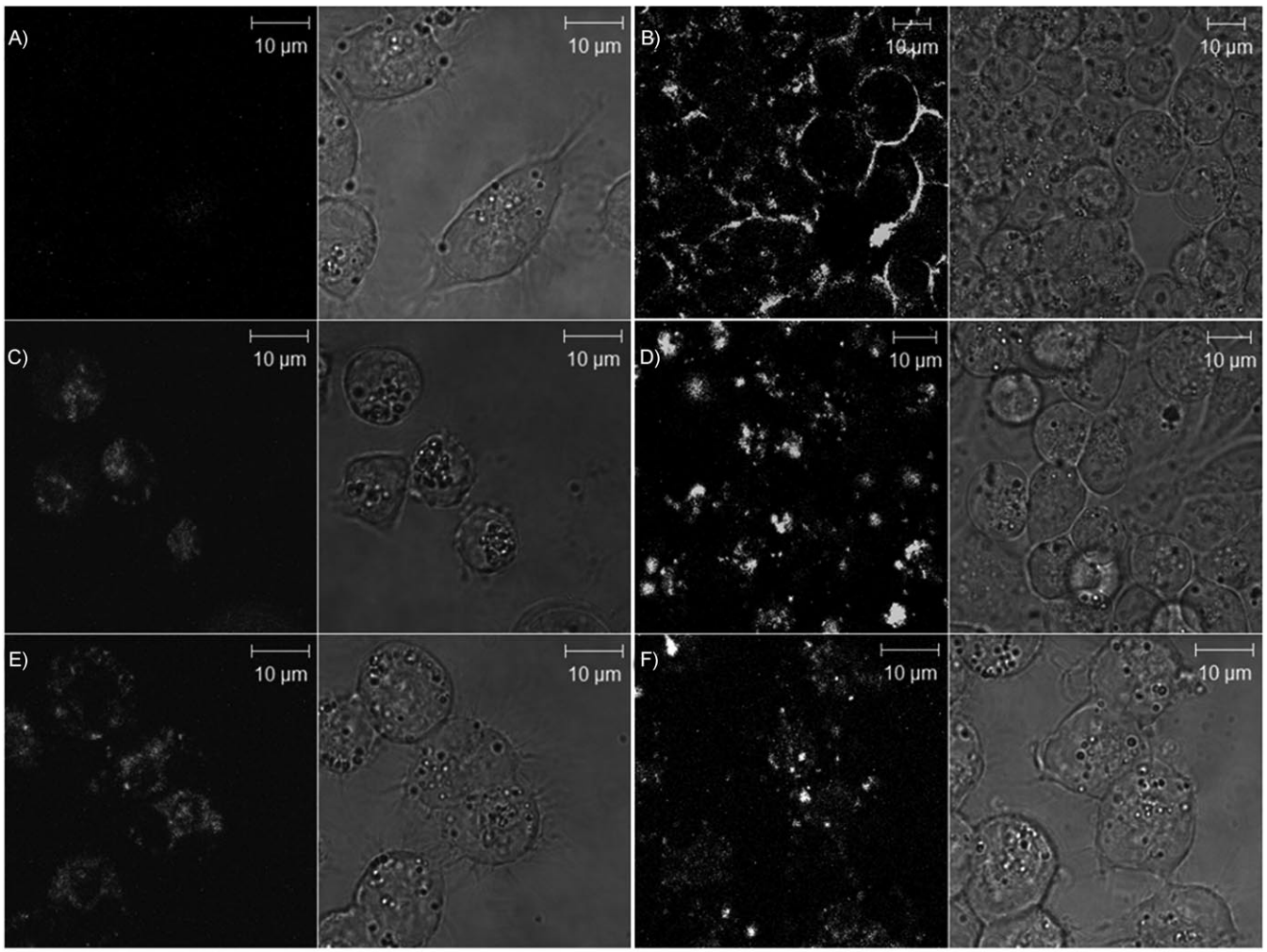

Figure 2. Confocal fluorescence microscopy images showing that **2** labels the plasma membrane whereas **3**, **4** and **5** accumulate in cytoplasmic organelles. A) Control and cells incubated for 24 h at 37 °C with B) **2** (10 μM), C) **2** (1 μM), D) **3** (50 μM), E) **4** (50 μM), and F) **5** (200 μM). Pictures show the fluorescence of the compounds (left) and transmitted light (right).

plasm, a feature previously reported for other ruthenium compounds[12,13] and probably corresponding to uptake via endocytosis.[14] Remarkably, the cells exposed to 10 μM of **2** showed a strong membrane-associated fluorescence pattern, whereas at lower concentration (1 μM) **2** was observed in cytoplasmic structures. The concentration dependency of the localisation of **2** is not in agreement with a passive diffusion of the complex across the membrane,[15] but fits with a model where the internalisation of the compound depends on cellular machinery that is either saturated or inhibited by high concentrations of the compound.

To test if the difference of localisation of the various compounds at high concentrations is due to different mechanisms of cell uptake, their internalisation was studied by microscopy at shorter exposure times and at different temperatures. A2780 cells were examined after incubation with **2**, **3**, **4** and **5** for 15 and 35 min at 37 °C and following incubation at 4 °C for 35 min (Figure 3). Incubation at 37 °C for such short time exposures, corresponding to tens of membrane cycling,[16] allows the early events of internalisation of the different compounds to be explored, and incubation at 4 °C, a temperature at which active cellular mechanisms such as endocytosis are inhibited, allow the uptake mechanism to be established. Compounds **1** and **2** were not included in this experiment because they were not sufficiently internalised at the concentration of 10 μM.

Compound **2** (1 μM) rapidly stained the plasma membrane after 10 min of incubation at 37 °C, but none of the other compounds was visible on the cell membranes or within the cells. After 35 min of incubation at 37 °C, all the compounds weakly stained cytoplasmic structures, and compound **2** showed additionally a persistent membrane binding. It therefore appears that the staining of the plasma membrane is unique to compound **2**. After 35 min of incubation with **2**–**5** at 4 °C, no accumulation of any of these compounds in the cell was observed (data not shown), although **2** showed a faint plasma membrane staining; therefore the binding of **2** to the membrane could be a passive phenomenon, even though its internalisation is inhibited at 4 °C (data not shown).

To test if the differences in cytotoxicity and cell internalisation are associated with diverging modes of actions of the compounds, we compared their effects on cell apoptosis. Apoptosis is a well-established mechanism of cell death involv-

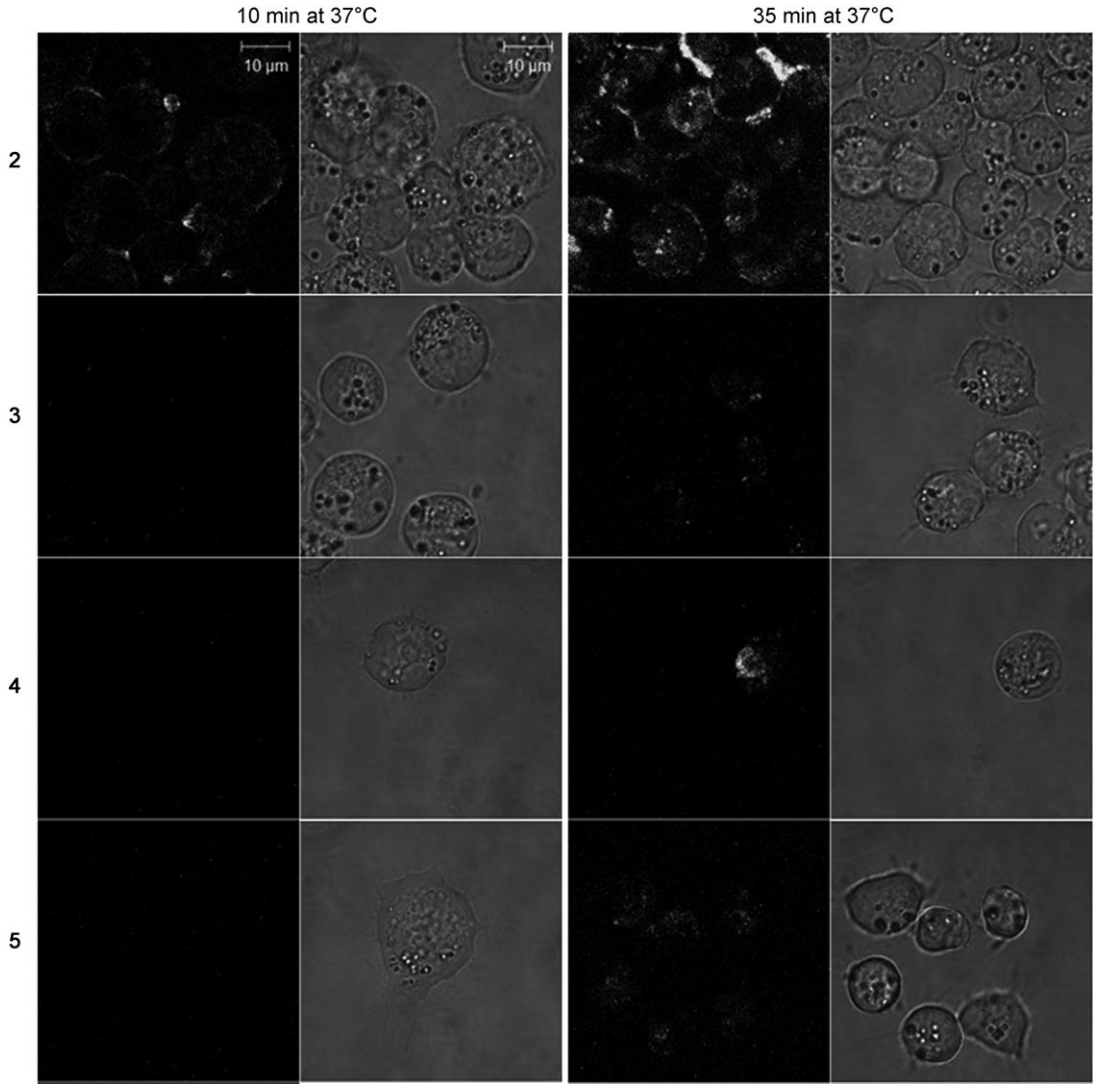

Figure 3. Confocal fluorescence microscopy images showing the cell internalisation of the various compounds after incubation at 37 °C, for 10 or 35 min. Pictures show the fluorescence of the compounds (left) and transmitted light (right).

ing active and precisely planed cellular functions and signalling pathways, as opposed to necrosis, in which cell death is initiated by exogenous wounding and is thought to be passive. One of the manifestations of apoptosis is a chromatin condensation, which can be detected by the observation of the cell nucleus morphology.[17] 4′,6-Diamidino-2-phenylindole (DAPI), a fluorescent stain for DNA, was therefore used to compare the nuclei of A2780 cells treated with **2** or cisplatin (cisPt), a platinum complex widely used in chemotherapy known to induce apoptosis.[18] Hence the percentage of cells presenting a condensation of the chromatin was determined. Figure 4 shows that 5 h after incubation with the compounds, there is no significant increase of the proportion of condensed nuclei upon treatment with **2** versus untreated cells, whereas cisplatin clearly causes a chromatin condensation. These results indicate that **2** triggers cell death via necrosis.

The intracellular trafficking of three different fluorescent ruthenium tris(bipyridyl) complexes was studied in order to gain insights in their uptake mechanisms. Related ruthenium compounds with bipyridyl ligands have been extensively studied as DNA-damaging agents,[19,20] although in vitro cellular studies are rare.[21,22] Growth inhibition tests performed on A2780 ovarian cancer cells showed that the most lipophilic compound, **2**, is extremely cytotoxic whereas the more hydrophilic compounds **1**, **3** and **4** are essentially not toxic. Microscopy studies indicate that the subcellular localisation of the compounds vary greatly, with **3** and **4** showing an intracellular localisation comparable to other ruthenium complexes,[21,23] and **2** targeting the plasma membrane.

Accumulation in the cell nucleus was not observed for any of the compounds; this indicates that DNA damage as a mechanism of cell death for this class of compounds is unlikely. An alternative mode of action for **2** involving interference with the dynamics of the plasma membrane presumably leads to cell death. Indeed, ruthenium complexes have been shown to interact with purified lipids in vitro.[24,25] Compounds based on ruthenium have been developed specifically for tracking applications in cell biology, mostly to gain insights into the uptake

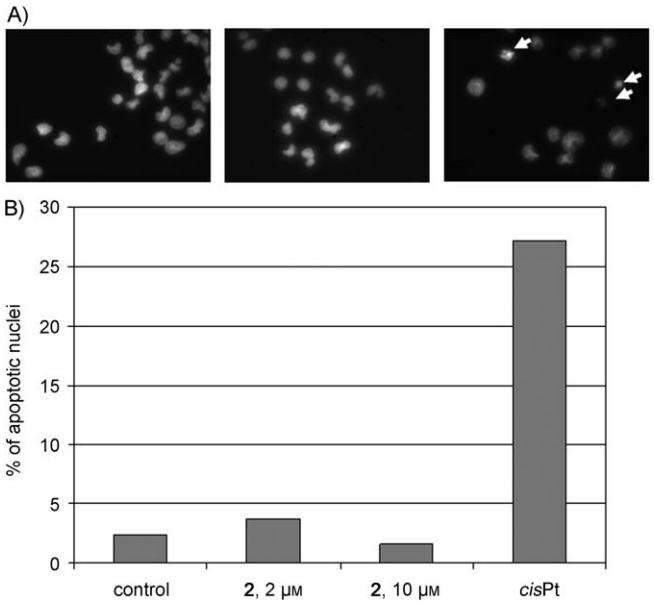

Figure 4. A) Fluorescence microscopy images showing the nuclei morphology of A2780 cells treated with **2** or cisplatin. Left: Control, untreated cells; Middle: cells incubated at 37 °C with **2** (10 μM); Right: cells incubated at 37 °C with cisplatin (10 μM). Arrows point to condensed nuclei characteristic of apoptotic cells. B) Histogram showing the proportion of condensed nuclei in the populations of cells treated with **2** (at concentrations of 2 and 10 μM) and cisplatin (cisPt, at a concentration of 10 μM).

of drugs, but some are also in routine use for staining.[26] Until recently ruthenium complexes were generally thought to be internalised through endocytosis, with some emphasis on the involvement of the transferrin receptor in the active uptake of such molecules.[27] Neugebauer et al. recently described the behaviour of a polypyridyl compound that associated to the plasma membrane and rapidly diffused in the cytoplasm.[28] It was hypothesised that the compound passively diffused through the plasma membrane because its cellular uptake was shown to be insensitive to both chemical metabolism inhibitors and low temperature. Our results suggest an alternative mechanism for **2**, showing both energy-independent plasma membrane binding and saturable and energy-dependent internalisation. This conclusion is based on the two following observations; first, at a high concentration, **2** tends to accumulate at the plasma membrane, whereas at lower concentrations it is efficiently internalised, and second, when the cells are incubated with **2** at 4 °C, internalisation of the complex is blocked, but not its binding to the plasma membrane. A passive adsorption of **2** on the plasma membrane, probably favoured by its lipophilicity, would then be followed by an active internalisation involving membrane receptors or transporters. Hence, membrane staining would not be inhibited by either receptor saturation or reduced temperatures, whereas the staining of cytoplasmic organelles would be impaired at low temperature and reduced in the presence of high concentrations of **2**.

Ruthenium red is a prominent example of a ruthenium complex known to interact with the plasma membrane. Due to this property it has been widely used in optical or electron microscopy to label the cell membrane and study membrane recycling dynamics. It is thought to bind to proteoglycans present in the cell coat.[29–31] Therefore such glycosylated proteins could be a target for **2** and could mediate its uptake depending on the ratio between metal complex and proteoglycan concentrations.

In conclusion, our studies show that, by changing the substituent groups attached to the bipyridine ligand, a variety of metal complexes can be obtained that exhibit significantly different cytotoxicities and uptake mechanisms. DNA does not appear to be a target for any of the studied compounds, as they are not seen to accumulate in the cell nucleus, and future studies will focus on delineating the actual biomolecular target or targets that are responsible for cell death.

Experimental Section

Synthesis of ruthenium tris(substituted-bipyridine) complexes $[RuL_3](Cl)_2$: Compounds **2** and **4** were prepared as reported earlier and **1** and **3** were prepared by following the same procedure with the appropriate ligands.[4]

Cells and cell treatment: Human A2780 and A2780cisR cells were obtained from the European Centre of Cell Cultures (ECACC, Porton Down, UK). All cell culture reagents were obtained from Gibco-BRL (Basel, Switzerland). The cells were grown in RPMI 1640 medium containing 10% foetal calf serum (FCS) and antibiotics. The organometallic complexes were dissolved in DMSO as 40 mM for stock solution and then diluted in complete medium to the required concentration. DMSO at comparable concentrations did not show any effects on cell cytotoxicity (results not shown).

Determination of cytotoxicity: Cells were grown in 96-well cell culture plates (Corning, NY) at a density of 2.5×10^4 cells per well. The culture medium was replaced with fresh medium containing complexes **1**–**4** at concentrations varying from 0 to 200 μM, with an exposure time of 72 h. Thereafter the medium was replaced by fresh medium and cell survival was measured by using the MTT test as previously described.[32] Briefly, 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyltetrazolium bromide (MTT, Merck) was added at 250 μg mL^{-1} and incubation was continued for 2 h. Then the cell culture supernatants were removed, the cell layer was dissolved in DMSO, and absorbance at 540 nm was measured in a 96-well multiwell-plate reader (iEMS Reader MF, Labsystems, Bioconcept, Switzerland) and compared to the values of control cells incubated without complexes. Experiments were conducted in quadruplicate wells and repeated at least twice.

Determination of pK_a: The pK_a of **2** was determined by titration of **2** (100 μM), supplemented with HCl (0.6 mM), against NaOH (2.5 mM). The titration was followed by potentiometry.

Determination of log $P_{oct/w}$: The lipophilicity of the compounds **1**–**5** was determined by using the shake-flask method of octanol/water phase partition. The compounds were dissolved in PBS (pH 7) at concentrations corresponding to absorbance of 0.4 at 488 nm. Octanol (500 μL) was added to a solution of the ruthenium compounds in PBS (500 μL) in 1.5 mL microcentrifuge tubes. The tubes were vigourously shaken for 10 min and the phases were separated by centrifugation at 2000 *g* for 5 min. The concentration of ruthenium compounds were determined by their absorbance at 488 nm and used to calculate log $P_{oct/w}$.

Microscopy experiments: Cells were grown for 24 h on chambered coverglass (Lab-Tek, NUNC) slides in complete medium at a

density of 1×10^4 and later exposed to **2**, **3** and **4** at 37 °C in the dark. Excess complex was washed away with PBS before observation. The measurement chamber was placed on the stage of an inverted confocal fluorescence microscope (LSM510, Zeiss) equipped with a 63× water immersion objective of 1.2 NA (Zeiss). The laser line for excitation and filter for detection of Ru compounds were 488 nm and >650 nm, respectively. Fluorescence signal intensities were evaluated by using Zeiss LSM software.

DAPI staining of cell nucleus: Cells were grown for 24 h on chambered coverglass (Lab-Tek, NUNC) slides in complete medium at a density of 1×10^4 and later exposed to **2** and cisPt at 37 °C in the dark. The medium was then discarded, cells were rinsed twice with PBS and fixed by incubation in Carnoy solution for 25 min at −20 °C. Cells were rinsed with MeOH and dried overnight. Cell nuclei were stained with DAPI (10 μg mL^{-1}) for 15 min at room temperature in the dark and rinsed with PBS. Cells were mounted in PBS before being observed by fluorescence microscopy by using a Zeiss Axiovert 200 M microscope equipped with a 40× air immersion objective. The filters used for excitation and detection of DAPI were 345 and 548 nm respectively. Fluorescence signal intensities were evaluated by using MetaMorph software and cell nuclei were manually counted.

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