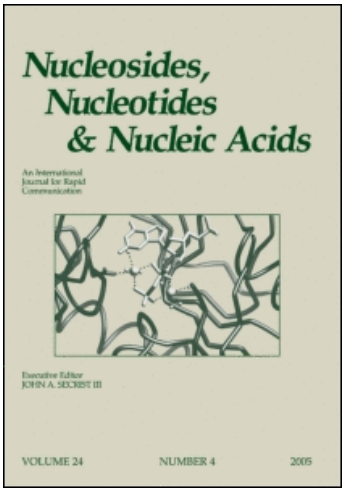

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DNA STAINING IN AGAROSE GELS WITH ZN^{2+}-CYCLEN-PYRENE

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□ *A pyrene-labeled Zn^{2+}-cyclen complex for the staining of DNA in agarose gels is reported. The metal chelate coordinates reversibly to the DNA phosphate backbone, which induces the formation of pyrene excimers. The typical pyrene excimer emission is used for the detection of the DNA. Staining is limited to agarose gels and is less sensitive than ethidium bromide, but DNA amounts as low as 10 ng and short DNA strands (∼300 b.p.) are detectable. Gel extraction as a standard technique in molecular biology was successfully performed after staining with Zn^{2+}-cyclen-pyrene. Cytotoxicity tests on HeLa and V-79 cells reveal that the zinc-cyclen pyrene probe is significant less toxic compared to ethidium bromide.*

Keywords DNA staining; Zn^{2+}-cyclen; pyrene; excimer emission

INTRODUCTION

DNA separation on agarose gels is a standard technique in molecular biology. Ethidium bromide is the most commonly used reagent for visualization of the separated fragments.[1] However, its genotoxic potential is controversially discussed. At physiologically relevant conditions, intercalation is the predominantly occurring binding mode of ethidium bromide to DNA.[2,3] Intercalation is often associated with mutagenic potential, as insertion of such intercalators between π-stacked base pairs results in local structural changes in DNA (e.g., unwinding and lengthening of the DNA double helix). Consequently, interaction with DNA polymerases and other DNA-related proteins is disrupted, and thus, DNA transcription is affected. Further, frameshift mutations are conceivable, since the widening of π-stacked base pairs especially in repetitive DNA sequences can lead to

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either leaving a single unpaired base or bulging several bases out of the helix. This propensity was indeed observed, however, only in bacteriophage and bacterial assays, but not in mammalian cells.[4–6] In contrast, the Ames test reveals that only metabolites of ethidium bromide are mutagenic in *Salmonella typhimurium*[7] and a mutagenic potential of ethidium bromide in *E. coli* was reported to depend on ultraviolet light exposure.[8]

Ethidium bromide should, therefore, be handled with care, generating additional costs in gel staining and imaging and disposal of waste.[9–11] To overcome drawbacks in DNA staining, a variety of alternative stains are available: SYBR Green I/II, SYBR Gold, SYBR Safe, PicoGreen, GelGreen, GelRed, Midori Green, RedSafe, OliGreen, BlueView are stated to be not genotoxic (as derived from substance data sheets provided by the manufacturers) but the structure of the dyes, conditions of the mutagenicity tests, and composition of the DMSO stock solutions is not always available.[8] Furthermore, these reagents are often less stable than ethidium bromide and, therefore, are suggested not to be stored at room temperature. Nile Blue A and Methylene Blue are water-soluble DNA stains and suggested to be non mutagenic. However, the sensitivity of Methylene Blue for DNA staining is significantly lower compared to ethidium bromide.

Herein, we report a water-soluble fluorescent DNA staining reagent Zn^{2+}-cyclen-pyrene **1**. Coordination of the pyrene metal-chelate to DNA induces pyrene-pyrene excimer formation, which is easily detected. By this, staining down to 10 ng of DNA in agarose gels was achieved. Preliminary cytotoxicity studies on two different cell lines indicate that Zn^{2+}-cyclen-pyrene **1** may have lower cytotoxicity compared to ethidium bromide. Moreover, storage at special conditions is not necessary.

MATERIALS AND METHODS

Synthesis

Detailed information on the synthetic procedures and compound characterization data are provided in the Supporting Information.

Electrophoresis of DNA on Agarose Gels

Plasmid DNA (circular and linearized) was electrophoretically resolved on 0.8% agarose in TAE buffer (20 mM Tris/acetate, 1 mM EDTA) at a voltage of 100 V. The loading buffer consisted of 0.25% (w/v) bromophenol-blue, 0.25% (w/v) xylenecyanol, and 40% (w/w) sucrose. For short *ds*DNA fragments (~300 b.p.), agarose gels of 2% were prepared.

Zn^{2+}-Cyclen-Pyrene Staining

Staining with Zn^{2+}-cyclen-pyrene **1** was done by soaking the gels in a solution of the fluorescent metal complex in deionized water at a probe

concentration of 10^{-4}–10^{-3} M. In general, incubating for 5–10 minutes was sufficient to fully resolve the DNA bands on the gels. Destaining in pure deionized water was not strictly necessary, but helpful when staining was performed in highly concentrated solutions of compound **1** to improve the contrast. To test the staining ability of the pyrene moiety itself, a highly concentrated solution of the metal-free ligand of compound **1** in MeOH was prepared and diluted with deionized water ($\sim 10^{-3}$ M). After soaking the gel in this staining bath for up to 1 hour, destaining was performed in water.

Staining with Compounds 2–4

Staining with fluorescent metal-chelates **2–4** was done according to the procedure for staining with compound **1** as described above.

Ethidium Bromide Staining

Staining with ethidium bromide was performed according to standard procedures by treating the gels with an aqueous solution of ethidium bromide (~1 mM when freshly prepared). Depending on the concentration of the staining bath, gels were soaked about 5–30 minutes. If necessary, gels were destained by washing with pure deionized water or an aqueous $MgCl_2$ (~mM) solution for up to 30 minutes.

Imaging

Zn^{2+}-cyclen-pyrene gels were placed on a PeqLab Superbright UV table ($\lambda_{max} = 316$ nm) and images were taken either by a Pentax K10D or a Canon EOS 450D. Ethidium bromide gels were imaged on either a standard UV table and photographed or as described for Zn^{2+}-cyclen-pyrene gels.

Plasmid Digestion

phMGFP DNA (Monster Green Fluorescent Protein phMGFP vector Promega GmbH, Mannheim, Germany) was digested using *Nde*I restriction enzyme (CA/TATG) incubated in NEBuffer 4 (both New England Biolabs Inc., Frankfurt/Main, Germany) for 2 hours at 37°C. By this, phMGFP (4707 b.p.) is cut at position 387 and 2628.

Gel Extraction

For gel extraction, a QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany) was used. Gel extraction was performed according to manufacturer instructions.

Cytotoxicity Investigations

Cell Lines and Cell Culture Conditions

The human epithelial cervical cancer cell line HeLa [CCL-17, American Type Culture Collection (ATCC)] was cultured in MEM Earle's medium. An amount of 500 mL MEM were supplemented with 0.8 mL amphotericin B (250 μg/mL), 5 mL penicillin/streptomycin (10,000 U/mL/ 10,000 μg/mL), 5 mL L-glutamine (200 mM), 5 mL NEA (100×), and 50 mL fetal bovine serum (all from Biochrom AG, Berlin, Germany). Cells were split in 1:6 ratio twice a week. Chinese hamster lung fibroblast cells V-79 (HGPRT+) (ATCC, Manassas VA, USA) were maintained in DMEM (PAN Biotech, Aidenbach, Germany) supplemented with 10 μg/mL thioguanine (Sigma Aldrich, Taufkirchen, Germany) and 10% fetal calf serum (PAN Biotech, Aidenbach, Germany) and split in 1:5 ratio every 7 days. All cells were cultured in a humidified incubator at 37°C in a 5% CO_2 atmosphere.

Determination of Cytotoxicity

The cytotoxic effect of Zn^{2+}-cyclen-pyrene **1** and ethidium bromide, respectively, was determined using two different cell lines, non-metabolizing HeLa (ATCC CCL-17) and metabolizing V-79 (HGPRT+).

For evaluation of cytotoxicity on HeLa cells, the colorimetric MTT (tetrazolium) assay was conducted as previously described by Mosmann[12] in a modified manner according to Heilmann et al.[13] In brief, following trypsinization, HeLa cells were counted and 11,250 cells/well were seeded into a 96 well plate (Techno Plastic Products AG, Trasadingen, Switzerland). Afterward, cells were exposed to compound **1** or ethidium bromide dissolved in 30% of EtOH (final concentrations: 0.1, 1, 10, 25, 50, 100 μM) for 72 hours at 37°C and 5% CO_2. For quantification of viability, cells were incubated with 15 μL/well MTT (tetrazolium) solution (4 mg/mL) for 4 hours, which was then converted into insoluble, violet formazan. After removal of the supernatant, 150 μL SDS solution (10%) was added to dissolve the formazan crystals. The next day, absorption was measured at 560 nm using a SpectraFluorPlus microplate reader (Tecan GmbH, Crailsheim/Germany).

In a second cell-culture-based experiment according to Lindl[14] V-79 cells were also tested on the cytotoxic potential of RedSafe (Intron Biotechnology Inc., Seongnam, South Korea) beyond Zn^{2+}-cyclen-pyrene **1** and ethidium bromide. In this assay format, approximately 200 V-79 cells were seeded into Petri dishes of 6 cm in diameter (Corning Incorporated, Corning NY, USA) and exposed to compound **1** and ethidium bromide dissolved in H_2O at the same final concentrations described above; in the case of RedSafe, defined amounts of a commercially available 20,000× stock solution in DMSO were added to the cell medium to obtain the final concentrations (1:10 000, 1:20 000, 1:40 000). After 72 hours of incubation at 37°C in

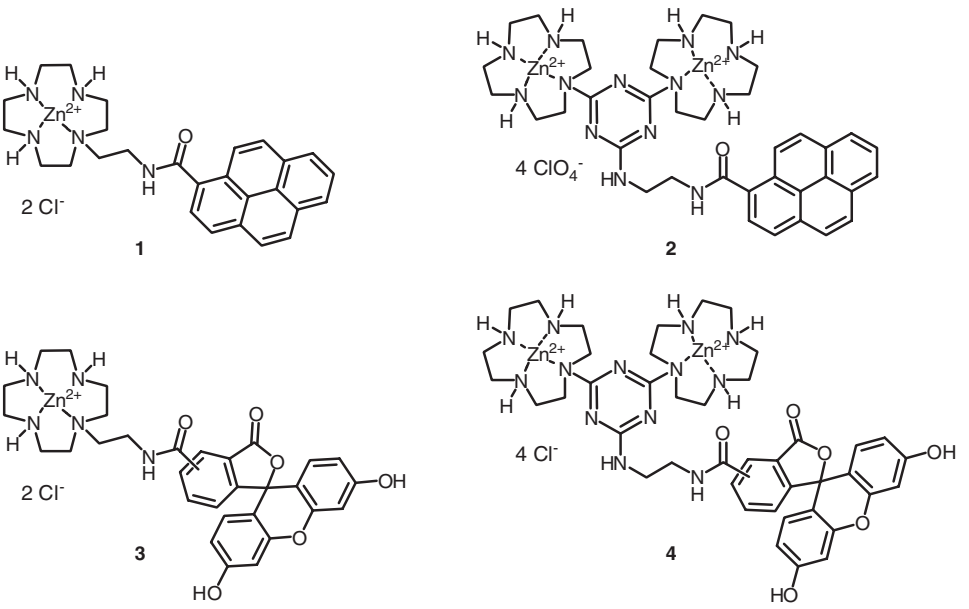

FIGURE 1 Dye-labelled Zn^{2+}-cyclen complexes used in gel staining. Compounds **1** and **2** were previously found to discriminate uridine-phosphates from other nucleotides by analyte induced excimer formation);[25] compound **4** was reported to be a phospho-protein staining reagent by emission increase upon binding to phosphate groups in proteins.[23]

5% CO_2 atmosphere, cells were fixed (methanol:glacial acetic acid = 3:1), stained (Giemsa solution) and colonies were counted.

All tests were performed in quadruplicates and all experiments have been repeated two times. Solvent control was set to 100%. Results were plotted as bar graphs and are expressed as means ± s.d. (range).

RESULTS AND DISCUSSION

The ability of Zn^{2+}-cyclen complexes to bind phosphate anions and imide functional groups in aqueous solution is well known.[15–21] Dye-labeled Zn^{2+}-cyclen complexes should, therefore, allow the staining of oligonucleotides in gels, as previously reported for phosphorylated proteins.[22–24] Figure 1 shows the Zn^{2+}-cyclen-dye conjugates used in this study. The pyrene-labeled compounds **1** and **2** are intended to signal the presence of an oligonucleotide by analyte induced excimer formation.

Plasmid DNA phMGFP was enzymatically cleaved by *Nde*I giving two linear strands of *ds*DNA (2241 b.p., 2466 b.p.). This linear *ds*DNA and the circular plasmid were electrophoretically resolved on an agarose gel and stained by Zn^{2+}-cyclen-pyrene **1** or ethidium bromide. Both procedures yield identical staining patterns as shown in Figure 2. Compound **1** indicates the presence of the DNA by its enhanced excimer emission. Compounds **2–4**

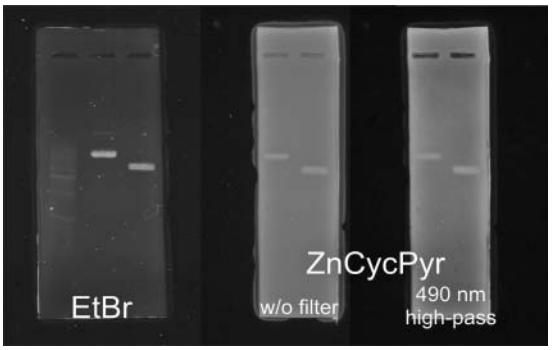

FIGURE 2 Staining of a linearized and a circular plasmid phMGFP with ethidium bromide or complex **1**. Gel run of linearized (left lanes) and circular (right lanes) plasmid DNA on an agarose gel (0.8%) stained with either Zn^{2+}-cyclen-pyrene **1** or ethidium bromide; images were taken on a PeqLab Superbright UV table ($\lambda_{max} = 316$ nm) with a Canon EOS 450D. (Using a high-pass filter (490 nm) bluish gel background emission would be observed in greenish color.)

were investigated likewise on their staining ability, but failed to identify DNA in the gel by a significant emission signal. All metal complexes **1–4** are expected to interact with the oligophosphate backbone of the DNA and, to a smaller extent, with thymine imide moieties. The experiments show that the affinity of the complexes to the DNA analyte under the experimental conditions is not sufficient to achieve a noncovalent labeling. Only in the case of **1**, presumably by stabilizing the DNA-complex aggregate by pyrene-pyrene interactions, a sufficient affinity is reached (Figure 3).

Uridinemonophosphate (UMP), which is able to induce enhanced excimer emission of **1**[25] was used to investigate the response of the probes' emission properties to gel matrices. (Induction of excimer formation in homogeneous solution is restricted to UMP. *ss*DNA pentamer *p*TACCG does not lead to pyrene excimers if added to HEPES buffered solutions of **1**.) Agarose and polyacrylamide gels containing UMP behave different when soaked in a solution of **1**: While the agarose gel quickly develops the typical turquoise excimer emission upon irradiation at 366 nm, no excimer emission is observed for polyacrylamide.

To exclude the intercalation of the pyrene moiety into the *ds*DNA as binding motif, the staining ability of Zn^{2+}-cyclen-pyrene **1** was compared to its noncomplexed ligand. No staining was observed with the ligand (Figure 4) showing that the zinc(II) complex is essential for the staining ability of compound **1**.

To estimate the sensitivity of the staining reagent, dilution series ($m_{DNA/lane}$ [ng] $\sim$ 700, 350, 175, 88, 44, 22, 11, 5) of plasmid phMGFP were transferred on two independent agarose gels and stained after the gel run with Zn^{2+}-cyclen-pyrene **1** and ethidium bromide, respectively, at comparable concentrations ($\sim$1 mM). As deduced from the obtained images shown

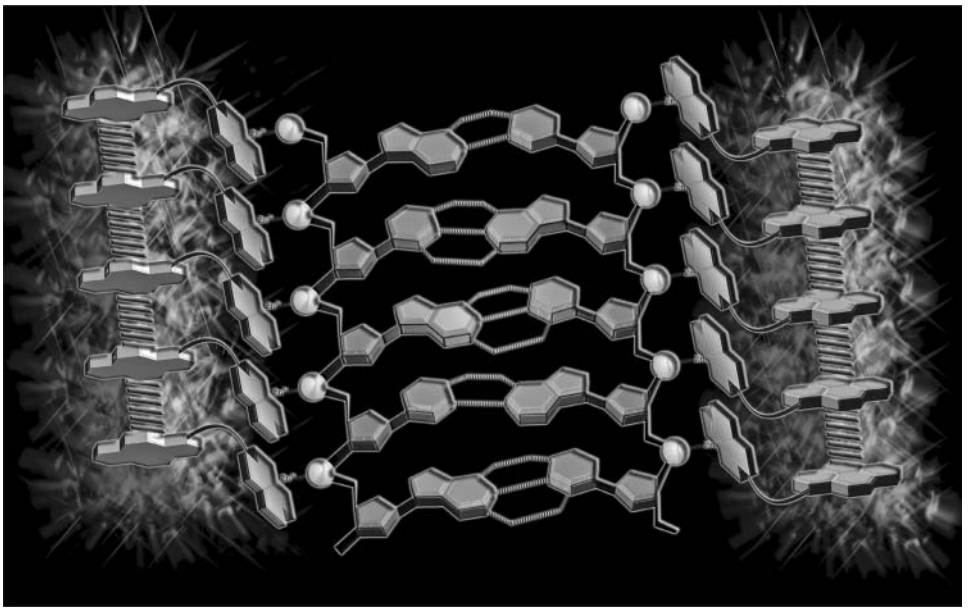

FIGURE 3 Proposed interaction of Zn^{2+}-cyclen-pyrene with DNA and staining mechanism due to analyte induced pyrene excimer formation. Metal-chelates are suggested to bind to phosphodiesters of the backbone while pyrene-pyrene interactions additionally stabilize the binding to DNA by π-stacking; due to formed pyrene-excimers upon irradiation, pyrene-emission is changed that finally allows the detection of lightened up DNA bands on the gel.

in Figure 5, compound **1** is not as sensitive as ethidium bromide. However, DNA amounts of ~10 ng could be visualized with Zn^{2+}-cyclen-pyrene.

Gel extraction experiments of the Zn^{2+}-cyclen pyrene stained plasmid were performed. Gel extraction is a standard technique to isolate DNA from gels for further amplification or cloning. In addition, the experiment shows if compound **1** initiates major chemical modifications in the DNA (e.g., DNA cleavage) when bound to it. Plasmid phMGFP was transferred on a gel and treated with compound **1** staining solution. The fluorescent DNA band was cut out and subsequently, extracted using a QIAquick Gel Extraction Kit. Afterward, the recycled DNA was again electrophorized and then stained with ethidium bromide. Comparison of re-extracted plasmid and a control sample of the plasmid confirmed that Zn^{2+}-cyclen-pyrene **1** has not affected the plasmid phMGFP since only one DNA band with comparable migration characteristics could be identified.

The ability to stain short DNA strands (~300 b.p.) was investigated comparing Zn^{2+}-cyclen-pyrene **1** and ethidium bromide. Staining of short strands (<150 b.p.) using ethidium bromide generally results in cloudy, not well-defined bands. In this case, staining with compound **1** should be an advantage. In fact, direct comparison shows that for Zn^{2+}-cyclen-pyrene **1** a better resolution of the band was observed, but with lower intensity (Figure 6).

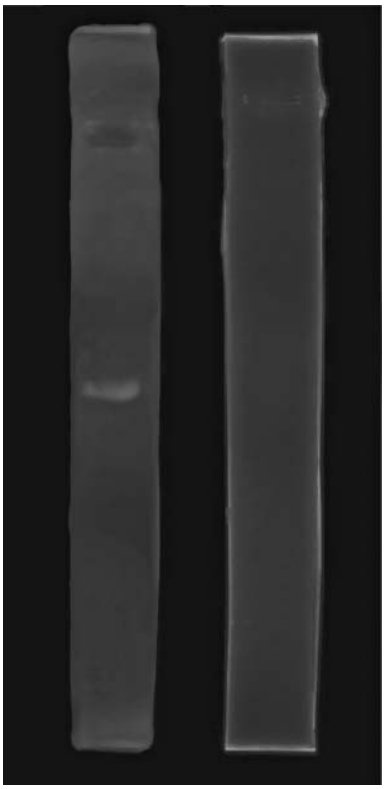

FIGURE 4 Comparison of gel staining with complex **1** and the corresponding zinc-ion free ligand. Gel runs of plasmid DNA phMGFP stained with either Zn^{2+}-cyclen-pyrene **1** (left) or the non complexed ligand (right) at comparable conditions; (left) distinct visualization of DNA band as pronounced fluorescent signal; (right) no staining of DNA could be observed when using the noncomplexed, pyrene-labeled aza-macrocycle suggesting that 1) coordinative interactions are crucial for this staining method and 2) pyrene intercalation does not occur. This image was taken without using any filter and, thus, shows the total fluorescence of the stain upon irradiation.

Preliminary studies were performed to compare the cytotoxic potential of complex **1** to ethidium bromide. In a first series, HeLa cells were cultivated, transferred in a well-plate and treated in separate trials with ethidium bromide and Zn^{2+}-cyclen-pyrene **1**, respectively (100, 50, 25, 10, 1, 0.1 μM). Upon incubation for 3 days, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added, whose conversion into purple-colored formazan is proportional to the cell viability rate.[12] Results obtained from fluorescence read out of the well-plates suggest that Zn^{2+}-cyclen-pyrene **1** is less toxic compared to ethidium bromide: While more than 60% of HeLa cells survived the treatment with 100 μM of compound **1**, the same concentration of ethidium bromide caused complete cell mortality (Figure 7). In a second series, V-79 cells were tested under comparable

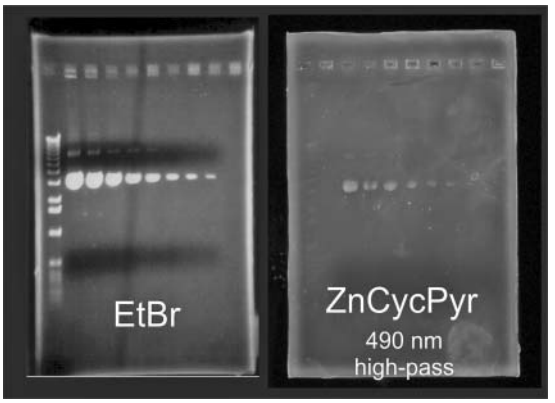

FIGURE 5 Sensitivity comparison of Zn^{2+}-cyclen-pyrene and ethidium bromide as staining reagents. Identical dilution rows (from left to right: $m_{DNA/lane}$ [ng] ~ 700, 350, 175, 88, 44, 22, 11, 5) were transferred in two separate gels followed by electrophoresis; staining with (left) ethidium bromide (~1 mM) and (right) compound **1** (~1 mM) demonstrate the staining performance of both. In comparison, ethidium bromide shows better sensitivity; however, compound **1** is able to visualize concentrations of DNA of ~10 ng.

conditions for cytotoxic reaction in presence of compound **1**, ethidium bromide and the commercially available DNA staining reagent RedSafe, which is offered as stock solution in DMSO (20,000×) and is described as very safe. Cell staining with Giemsa solution and counting of stained cell colonies surprisingly showed a significantly enhanced cytotoxic effect for RedSafe and metabolites on this metabolizing cell line. Even the highest dilution (1:40 000

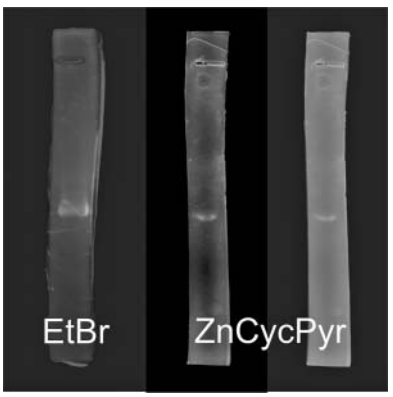

FIGURE 6 Short *ds*DNA (~300 b.p.)—comparison of staining ability of ethidium bromide and Zn^{2+}-cyclen-pyrene **1**. Two identical gels with *ds*DNA with a length of about 300 b.p. were stained in a solution of either ethidium bromide or compound **1**; as generally known for ethidium bromide, short DNA fragments (<150 b.p.) lead to cloudy and not well-defined bands as also demonstrated on the left side for this length; staining with Zn^{2+}-cyclen-pyrene **1** leads obviously to DNA bands with improved resolution, however, intensity is lower compared to ethidium bromide. In this case, ethidium bromide stained gel was photographed in parallel with compound **1** stained gel on a PeqLab Superbright UV table ($\lambda_{max} = 316$ nm) with a Canon EOS 450D and using a high-pass filter (490 nm).

of a 20,000× stock solution) added to the cells lead to complete cell mortality in the culture solution. While metabolites of ethidium bromide only lead to noticeable cell mortality at concentrations >25 μM, Zn^{2+}-cyclen-pyrene **1** and its metabolites seem not to affect the cells at all (Figure 7).

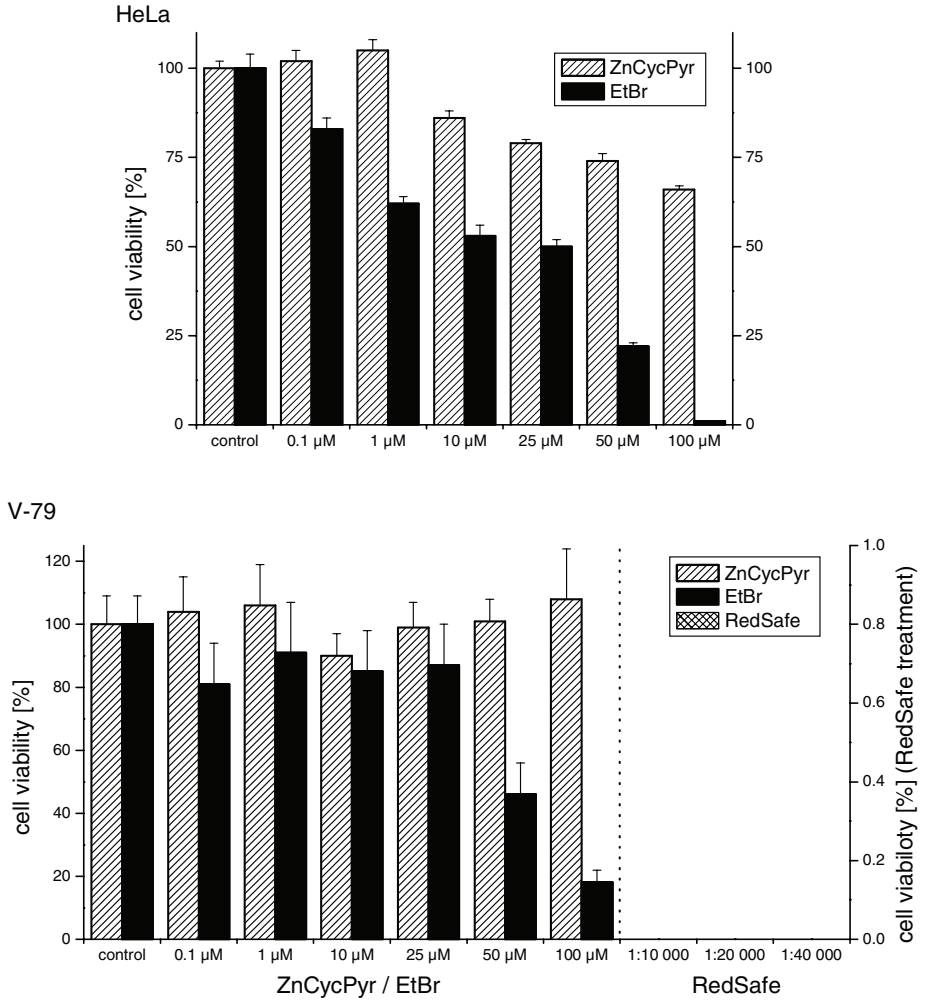

FIGURE 7 Results of preliminary cytotoxicity studies obtained for HeLa and V-79 cell lines. HeLa cells were incubated in a well-plate in independent trials for 3 days with Zn^{2+}-cyclen-pyrene **1** and ethidium bromide, respectively (100, 50, 25, 10, 1, 0.1 μM); V-79 cells were investigated under comparable conditions in Petri culture dishes in the presence of Zn^{2+}-cyclen-pyrene **1**, ethidium bromide (both at 100, 50, 25, 10, 1, 0.1 μM) and RedSafe (1:10 000, 1:20 000, 1:40 000 of a 20,000× stock solution in DMSO); fluorescence read out due to conversion of MTT into formazan by surviving cells (HeLa) or counting intact cell colonies after Giemsa staining (V-79) represent cell viability that was finally plotted as columns (means ± s.d.) against concentration of substances investigated; solvent control was set to 100%; Zn^{2+}-cyclen-pyrene **1** was found to hardly influence non-metabolizing HeLa and to be affectless on metabolizing V-79 cells. In comparison, treatment of both cell lines with ethidium bromide resulted in higher cell mortality, while RedSafe was found to show obviously dramatically increased cytotoxicity as shown on V-79 cells.

A likely reason for the increased cytotoxicity of RedSafe may be the presence of DMSO, which permeates cell membranes. Hence, the uptake of RedSafe in cells may be dramatically increased compared to the trials performed with aqueous solutions of either compound **1** or ethidium bromide.

CONCLUDING REMARKS

Herein, the application of Zn^{2+}-cyclen-pyrene **1** as DNA staining reagent on agarose gels was demonstrated. Based on coordinative interactions between the metal-chelate and the phosphate backbone in DNA being additionally stabilized by pyrene-pyrene π-stacking interactions, irradiation results in formation of pyrene excimers. These excimers allowed the detection of DNA bands as brightened up, well-resolved signals on gels. In contrast, intercalation of the pyrene moiety into DNA could be excluded. In comparison to ethidium bromide as a standard staining reagent, compound **1** was found to be marginally less sensitive, but showed better resolution of short *ds*DNA fragments (~300 b.p.). However, this staining method is limited to agarose gels. Nevertheless, important features of **1** are excellent water-solubility and a presumably low cytotoxic potential according to preliminary cell-based studies presented inhere. Although, to further confirm its comparably low risks, a more comprehensive cytotoxicity profile has to be drawn and also mutagenicity studies would be of high interest.

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