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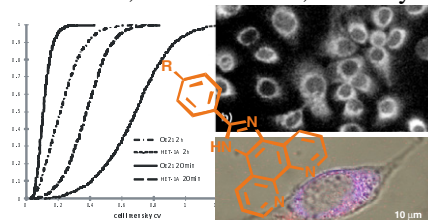


Graphical Abstract

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Simple but powerful: Phenanthroline-based small molecules for cellular imaging and cancer screening

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ABSTRACT

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A two-step synthetic procedure gives highly fluorescent phenanthroline molecular probes. The compounds localize in the endoplasmic reticulum and their potential as bioactive probes was evaluated. The materials are quickly taken up by living cells within 5 min. Preliminary *in vitro* studies have shown that these compounds are selective to esophageal cancer cells and can be used as selective markers in intracellular cancer diagnostics. The materials show a remarkable cytotoxicity towards cancer cells vs. normal as 7 to 1.

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Fluorescence cell imaging has garnered significant attention in the scientific community over the past years.¹ Due to the clinical significance of the topic, a vast body of data has been generated for compounds ranging from simple organic molecules to highly sophisticated fluorescent proteins and bionanotags.² High-content screening³ is a high-throughput technical approach based on fluorescent materials targeting cellular functions and processes, and is widely used in biomedicine, cancer research and drug development. These fully automated systems provide quick analyses and generate a massive amount of information in a relatively short period of time. For example, they can deliver 10⁸ individual physiological measurements at the cellular level in less than a day.⁴ This integrated approach also allows screening under more relevant physiological and biological conditions in comparison to classical biochemical scenarios.

Cancer is a plague of the 21st century, increasing the number of its victims every year due to overall population aging, environmental conditions and life style choices. Esophageal cancer is the eighth of most common type and sixth most common cause of death from cancer worldwide.^{5,6} Due to late detection, the survival rates in Europe are about 10%.^{6,7} It has shown a dramatic increase in Europe⁸ and North America in the past few years.^{9,10} According to the Esophageal Cancer Research Fund (OCRF), the rates of the disease in Ireland and UK are amongst the highest in Europe and increase faster than in other European countries.¹¹ This necessitates the development of better screening techniques for this and other types of cancer.

Small fluorescent molecules offer significant potential in this

area above.¹² We have found that the small molecules such as phenanthroline derivatives can be used as versatile materials for cellular imaging and cancer screening studies. They show strong absorption in the 280-400 nm region, exhibit high fluorescence upon excitation and large Stokes shifts. These optical properties do not alter drastically with changes of the solvent systems and the compounds are stable in aqueous media with no decay in fluorescence over time.^{13a} The compounds **2** and **3** are soluble in aquatic phosphate buffered salen solution (PBS) and exhibit a strong fluorescence in PBS at pH 7.4 upon excitation at 405 nm for our studies. (Fig. 1)

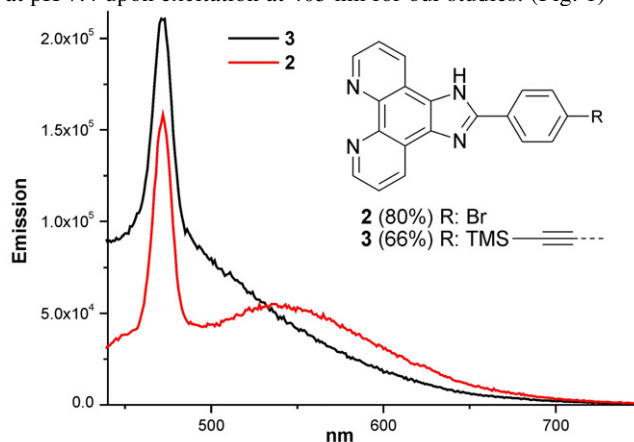
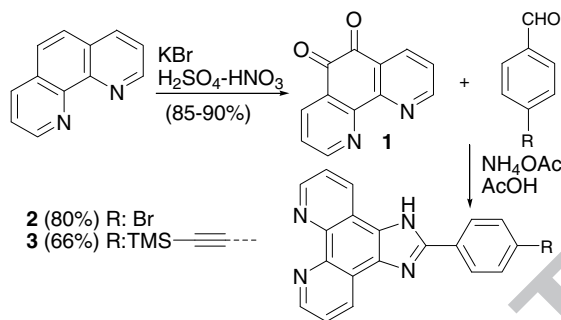
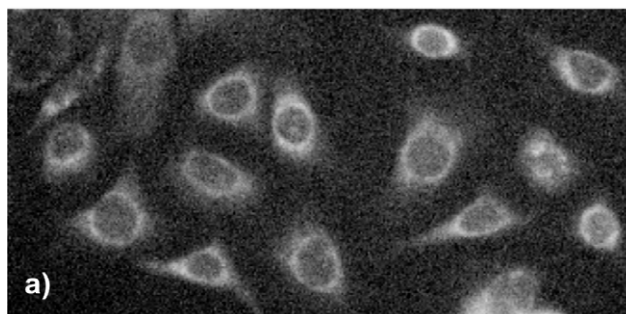


Figure 1. Emission spectra of **2** and **3** at excitation of 405 nm in PBS at pH 7.4 (10 μ M).

These highly fluorescent compounds can be synthesized easily and on a large scale making them commercially affordable and cheap. The compounds **2** and **3** have been prepared via two-step procedure in the yields of 80 % and 66 %, respectively.^{13a} Commercially available 1,10-phenanthroline can be easily oxidized with KBr in H₂SO₄-HNO₃ to 1,10-phenanthroline-5,6-dione **1** in 95 % followed by reaction with an appropriate aldehyde and NH₄OAc in glacial acetic acid (Scheme 1).

Studies on localization, incubation times, concentration ranges and toxicity were carried out in two cell lines OE21 (human esophageal squamous carcinoma) and HET-1A (human normal esophageal epithelium).^{13a} An analysis and evaluation of the HET-1A and OE21 live-cells stained with the novel compounds at different time points (5 min to 24 h) and concentration ranges (2-27 μM) was performed with high-content screening techniques using the IN Cell 1000 and IN Cell Investigator Image Analysis Software.



Scheme 1. Synthesis of compounds **2** and **3**.

Remarkably, cellular images could be collected after 5 min of incubation for both compounds in contrast to some metal complexes¹⁴ for which longer times are required (Fig. 2).

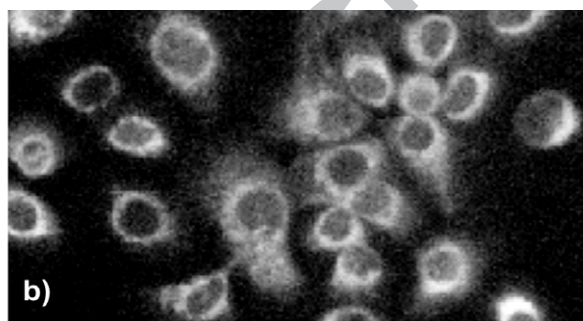


Figure 2. IN Cell images of the OE21 live-cells at 405nm irradiation and 535nm emission: a) **2**, 5 min, 9 μM; b) **3**, 20 min, 27 μM.

The dyes **2** and **3** accumulate in THE endoplasmic reticulum (ER). The ER is vital cellular organelle¹⁵ which is involved in the regulation of crucial cell events such as intracellular signaling (ER performs a calcium storage function involving release and uptake of Ca²⁺) and participates in the initiation of apoptotic cell death pathways.^{16,17}

The intracellular localization of the dyes **2** and **3** was studied by confocal microscopy with a LSM 700 confocal laser scanning microscope. Living HET-1A cells were incubated with the commercially available ER red-tracker for 20 min at 37 °C and stained with **3** (18 μM). The ER tracker was illuminated at 560 nm without any interference from the dye **3**, and images were collected at 575 nm. The fluorescence of **3** was detected at 420-480 nm upon excitation at 405 nm. Superimposition of the intracellular images of the red ER stain and **3** confirmed that the latter accumulates in the endoplasmic reticulum (Fig. 3).

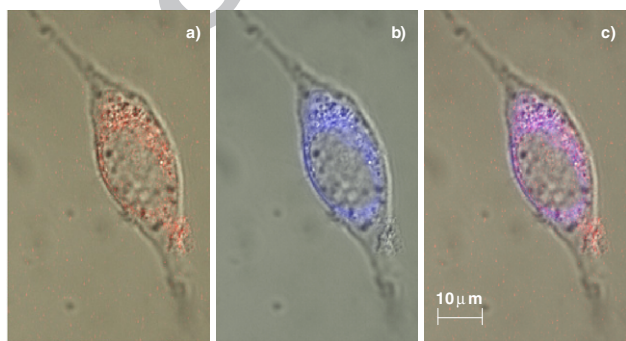


Figure 3. Confocal fluorescence images of HET-1A live-cells incubated with: (a) **3**; (b) red ER tracker; (c) superimposition of **3** and red ER tracker.

Similar results were obtained for **2** as well.^{12a} The experiments were carried out in OE21 live-cells and showed the same

localization of the dyes in both cell lines. Moreover, we carried out the same *in vitro* experiments using fixed cells. Noteworthy, ER-tracker has to be administered to living, incubated, and if an experiment requires cells can be fixed later. In our case the compounds **2** and **3** can be used to stain living as well as fixed cells, both exhibit the same intracellular localization.

Additionally, both dyes show remarkable cytotoxicity in cancer cells and can be used as potential therapeutics for cancer treatment. Cytotoxicity tests were performed on both cell lines and carried out using an MTT assay.¹³ The cells were incubated with **2** and **3** for 24 h at a concentration range of 0.5-15 μM in DMSO¹³ (Table 1).

Table 1. Cytotoxicity of **2** and **3**. IC₅₀ values for OE21 and HET-1A cell lines.

| Cell line | Compound | IC ₅₀ value, μM (μg/ml) ± SE |
|-----------|----------|---|
| OE21 | 2 | 0.77 (0.29) ± 0.13 |
| Het-1a | 2 | 3.15 (1.18) ± 0.08 |
| OE21 | 3 | 0.44 (0.16) ± 0.005 |
| Het-1a | 3 | 3.02 (1.11) ± 0.32 |

IC₅₀ - inhibitory concentration at 50 % survival; SE - standard error.¹³

Overall, the cytotoxicity shown is very high and comparable to established anti-cancer drugs, for example, cisplatin, carboplatin and 5-fluorouracil, which are used in combination with radiotherapy.¹⁸ Notably, compounds **2** and **3** are more cytotoxic to cancer cells than for normal HET-1A cells. Compound **2** is in four times and **3** is seven times more effective towards the cancer cells compared to the normal cell line.

Another remarkable feature of compounds **2** and **3**, especially compound **2**, is their potential to be used as a probe for cancer screening *in vitro*. A few methods have been established to diagnose cancer *in vitro*, for example antibody-based

immunodiagnostic assays involving monoclonal antibodies. However they are expensive and difficult to perform.¹⁹ We evaluated the different parameters based on localization and intensity of **2** and **3** in both OE21 and HET-1A cell lines using cell fluorescence analysis. The experiments were carried out on ca. 1500 cells for each cell line at different concentrations and incubation times (20 min to 2 h). Total area and intensity of the inclusions (distribution of the materials within cytoplasm), cell intensity and cell intensity CV²⁰ (excluding nuclei) were chosen for use in high-content screening as the key elements. The analysis can already be performed after 20 min of incubation in

living cells. The intensity of the inclusions for both cell lines remains the same and it can be used as a quality control to check an even probe distribution. The analysis shows a significant difference between the two cell lines using cell intensity CV, which is a normalized parameter, describing the coefficient of cell intensity variation (Fig. 4).

Thus, the assay described here can be used as a primary evaluation analysis of abnormal vs. normal cells *in vitro*.

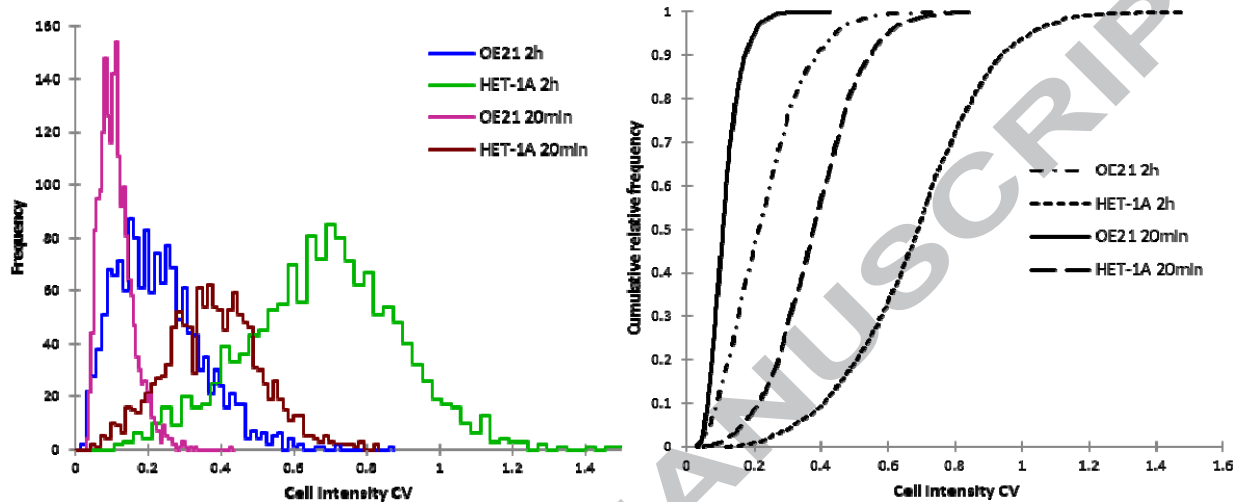


Figure 4. IN Cell analysis of the HET-1A and OE21 live-cells incubated for 20 min and 2h with **2**.

In conclusion, compounds **2** and **3** can be used in bioassays as fluorescent markers in living cells. The incubation times are very short and initial images can be acquired after 5-20 min. Localization studies showed that the dyes accumulate in the endoplasmic reticulum and can be used as facile ER-markers. The materials proved to be cytotoxic with a remarkable preference for cancer cells and therefore can be considered as potential candidates for cancer treatment. Additionally, intracellular multiparametric analysis using high-content screening technique indicates a high prospect to employ **2** and **3** as cancer diagnostics. Currently we are investigating the relative cytotoxicity of these compounds in relation to established cancer drugs, are expanding the tests to other cell lines and focus on the intracellular uptake mechanism.

Acknowledgments

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20. Coefficient of variation of the fluorescence intensity of pixels within the cytoplasm.

Supplementary Material

Supplementary material contains details on chemical synthesis and characterization; methods and materials, MTT results, confocal studies and statistical data.

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