Redox-active N4Py-metal complexes in human cell cultures

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Chapter 5

Photosensitizing Properties of N4Py in Cultured Cells

Previous work from our group revealed the potential of Fe(II)-N4Py as photosensitizer in cleavage studies with 'naked' plasmid DNA. This chapter investigates whether it would be possible to use this iron complex as a photosensitizer in cultured cells as a form of photodynamic therapy (PDT).

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5.1 Introduction

Most conventional anti-tumor drugs are administered intravenously and have a cytotoxic mechanism of action. They target rapidly dividing cells with little discrimination between malignant tissues of tumors and the surrounding healthy tissues. As a result, many patients experience a range of adverse side effects such as bone marrow suppression, gastrointestinal symptoms, alopecia, permanent infertility and heart, kidney, liver and lung damage.[1–3] In order to increase the treatment selectivity towards malignant tissues and potentially reduce the dose-limiting side effects, new therapeutic strategies are being developed, which includes the emerging field of photodynamic therapy (PDT). PDT is already applied in ophthalmology and dermatology for well over a decade[4,5] and is rapidly gaining ground in cancer research.

In PDT treatment, cellular and tissue damage can be induced by a synergistic effect that is obtained by a photosensitizer (PS), light and molecular oxygen.[6] The PS is ideally non-toxic to the cells, but can be activated upon exposure to light of a specific wavelength to occupy an excited singlet state (^{1}PS*), which subsequently undergoes intersystem crossing (ISC) to the long lived triplet state (^{3}PS*). At this point, two different mechanisms can occur that either involve electron transfer or energy transfer. They are called type I and type II, respectively (Figure 1).[2,7,8]

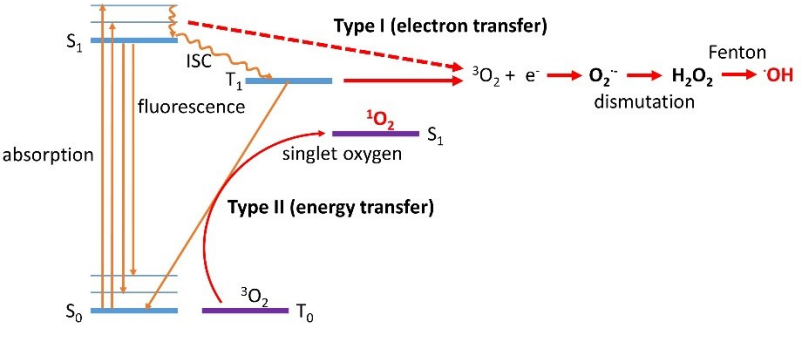

Figure 1. A simplified Jablonski diagram illustrating generation of excited states and ROS by type I and II photosensitization reactions. Energy ground states are represented by a thick line and electronic transitions are represented by orange arrows.

In a type I reaction, electron transfer of ^{3}PS* to ground state triplet oxygen ($^3O_2$) can form superoxide anion radicals ($O_2^{\bullet-}$) that can undergo spontaneous dismutation to generate hydrogen peroxide (H_2O_2). Highly reactive oxygen species (hROS) such as

hydroxyl radicals (•OH) can potentially form from H_2O_2 by Fenton-type reactions.[7,8] Alternatively, a molecule in the ^{3}PS* state can transfer an electron to a surrounding biological substrate, which leads to formation of radicals that can further interact with $^3O_2$ to form ROS. A type II reaction involves energy transfer from ^{3}PS* to $^3O_2$ which generates singlet oxygen ($^1O_2$), a notorious ROS species. $^1O_2$ can react with unsaturated lipids (including cholesterol), guanine nucleobases and the amino acids tryptophan, histidine and methionine.[9]

The ratio between type I and II reactions depends largely on the type of sensitizer used.[6] The diffusion distances of ROS are generally small (50-100 nm for $^1O_2$, 0.8-6.0 nm for •OH) enabling them to cause high localized damage to cellular components.[10–13] Malignant tumor tissue can be killed by PDT by means of three mechanisms: (i) generated ROS can kill the cells directly; (ii) tumor-associated vascular damage that leads to local hypoxia and infarction; (iii) activation of an immune response against the tumor cells.[6,8,14]

The vast majority of currently clinically approved photosensitizers are based on tetrapyrrole structures.[8,15–17] Over the last decade, an extensive amount of potential metal polypyridyl based photosensitizers have been reported, which were based especially on copper,[18] oxovanadium,[2,19,20] lanthanides,[21] rhodium and ruthenium.[2,22–25] Interestingly however, only two groups have explored iron-based polypyridyl complexes, even though iron is a bio-essential metal and the presence of a charge transfer band with high molar absorptivity could provide iron complexes with potentially interesting properties as photosensitizers.

The group of Chakravarty reported their first ternary Fe(III) complex with photosensitizing properties in 2007,[26] after which cell studies on the complexes followed two years later.[27] Their initial strategy was to use well known photosensitizer-cum-DNA binder ligands such as dipyridoquinoxaline (dpq), dipyridophenazine (dppz) or 1,10-phenanthroline (phen), together with a tetradentate ligand to stabilize the ferric state (Figure 2a). The results indicated a clearly enhanced toxicity upon irradiation of the cells, with good phototoxic indices (ratio of its IC_{50} in the dark to its IC_{50} upon light irradiation). Later work showed an improved phototoxic index and higher wavelength ligand-to-metal charge-transfer (LMCT) band (620-850 nm) due to its dipicolylamine ligand with pendant anthracenyl moiety and additional catecholate ligand, respectively (Figure 2b).[28] The group of Hussain also reported Fe(III) complexes which use biologically important molecules, such as vitamin B_6[29]

and curcumin[30], as ligands. Their results, however, indicate high energy charge transfer bands and rather low phototoxic indices.

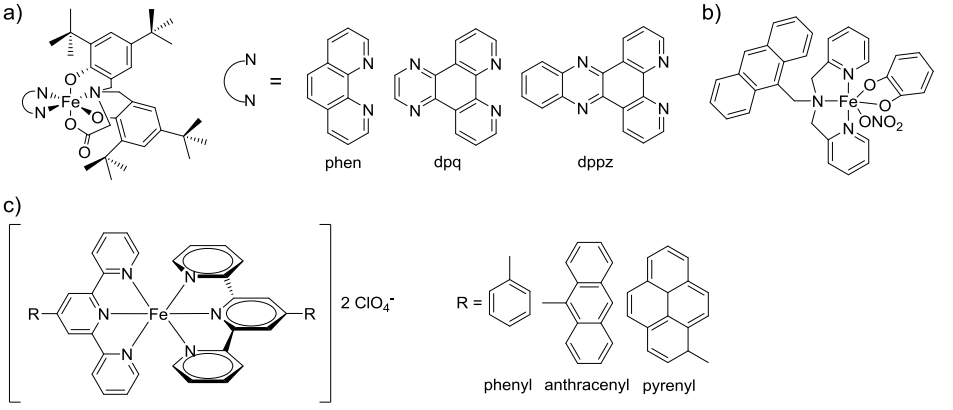

Figure 2. Structures of iron complexes with photosensitizing properties.

Chakravarty *et al.* further studied the phototoxic properties of Fe(II) complexes, since Fe(III) complexes are often prone to reduction in cells by cellular reducing agents such as glutathione and ascorbate.[31] This can affect the stability and cytotoxicity of the complex in the dark, hence lowering the phototoxic index of the compound. Even though it was demonstrated that Fe(II) complexes based on dpq ligands were ineffective as photosensitizers,[32] terpyridine (tpy) ligands with a photoactive anthracenyl or pyrenyl moiety could serve as photosensitizer-cum-DNA binder and stabilized the 2+ oxidation state of iron (Figure 2c).[33] Good phototoxic indices were obtained, albeit with relatively high energy irradiation.

The DNA cleavage ability of the Fe(II) complex of the pentadentate ligand *N,N*-bis(2-pyridylmethyl)-*N*-bis(2-pyridyl)methylamine (N4Py) was reported previously on 'naked' plasmid DNA by our group.[34] It was found that $[(N4Py)Fe(CH_3CN)](ClO_4)_2$ (Fe(II)-N4Py) displays significantly enhanced DNA cleavage activity under photoirradiation that depends on the wavelength and power of irradiation. In addition, the dominant contributor to the DNA cleavage ability under photoirradiation as well as ambient lighting is $O_2^{\bullet-}$. This indicates that, while the DNA cleavage process is more efficient as a result of photoirradiation, it does not fundamentally seem to alter the DNA cleavage chemistry. A more thorough study into the photochemistry of Fe(II)-N4Py revealed that irradiation of the complex in solution causes a perturbation of its intrinsic spin equilibrium.[35] Interconversion between the $^1A_1$ and $^5T_2$ spin states is expected to involve an intermediate ^3T spin state, which would facilitate electron

transfer to $^3O_2$ that leads to formation of $O_2^{\bullet-}$. Disproportionation of $O_2^{\bullet-}$ forms H_2O_2 that can react with Fe(II)-N4Py to form highly reactive Fe-OOH species. The photoexcitation is believed to increase the steady state population of the intermediate ^3T states, which seems to sufficiently affect the rate of oxidation. Inspired by the photosensitizing results with iron complexes reported by others (*vide supra*), the Fe(II)-N4Py complex was tested in cancer cells in order to discover its potential as a photosensitizer. The results of these studies are described in this chapter.

5.2 Results

In order to determine the minimum irradiation time needed to affect cells, MCF7 breast cancer cells were treated with a 0.03 % dimethylsulfoxide (DMSO) solution and irradiated for varying amounts of time with a collimated LED light irradiating at 455 nm. This wavelength was chosen since it corresponds to the absorbance region of the two ^{1}MLCT bands of Fe(II)-N4Py[36] and lies close to the wavelength used in the photoenhanced DNA cleavage studies described earlier (*vide supra*).[34] The cytotoxicity by irradiation was measured with a propidium iodide (PI)/FACS assay and indicate a slow and steady increase in PI positive cells upon increased irradiation (Figure 3). Observations under a light microscope revealed that cells irradiated for 30 min or less still had a normal morphology and looked healthy directly after irradiation. Contrary, 1 h irradiation resulted in about 50 % of the cells having a changed, more round shaped and unhealthy morphology. This amount of cells increased to roughly 75 % after 2 h irradiation. It was important to make sure that the cells received enough irradiation to be able to photoactivate the Fe(II)-N4Py complex. Therefore, it was deemed necessary to see an irradiation effect with 0.03 % DMSO treated control cells, which was clearly visible, albeit still not very lethal, after 1h irradiation. An irradiation time of 1 h was therefore used in all experiments.

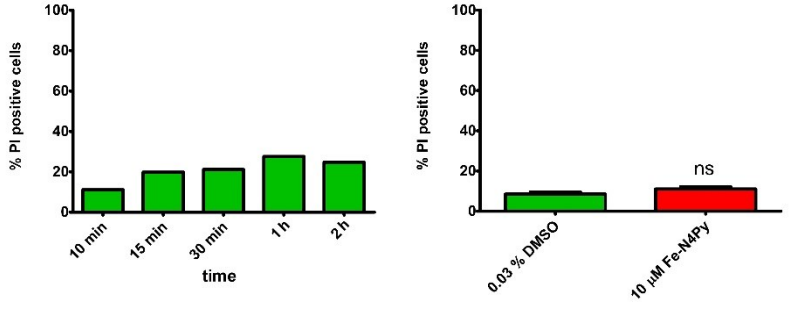

Figure 3. (previous page) (a) PI/FACS assay with MCF7 cells treated with 0.03 % DMSO and irradiated at 455 nm for t = 10, 15, 30, 60 and 120 min with subsequent incubation with fresh medium in an incubator for 24h at 37 °C in a humidified 5 % CO_2 incubator; N=1 (b) PI/FACS assay with non-irradiated MCF7 cells treated with 10 µM Fe(II)-N4Py and 0.03 % DMSO for 2 h and subsequent incubation with fresh medium for 24 h at 37 °C in a humidified 5 % CO2 incubator; n=3. Data are presented as the mean ± SEM, ns = non-significant.

Previous studies with MCF7 cells treated with Fe(II)-N4Py revealed an IC_{50} value of around 25 µM with almost no observed cell death at 10 µM concentrations (chapter 4). Since PDT is based upon a photosensitizer that would ideally be non-toxic in the dark and become activated upon exposure to light of a specific wavelength, a 10 µM concentration of Fe(II)-N4Py seemed a good starting point for this study. In order to confirm this under the current conditions, a short experiment was performed in which MCF7 cells were treated with 10 µM Fe(II)-N4Py. No significant cytotoxicity was caused by the complex in comparison to the 0.03 % DMSO control over a period of 2 h (Figure 3b).

Cells were subsequently treated with 10 µM Fe(II)-N4Py upon continuous irradiation for 1 h and cytotoxicity levels were compared with cells treated with 0.03 % DMSO with 1 h irradiation and with non-irradiated cells treated with 10 µM Fe(II)-N4Py or 0.03 % DMSO. Observations under a light microscope after 24 h revealed no significant changes in morphology for the non-irradiated samples, about 50 % of round unhealthy looking cells for the irradiated cells treated with 0.03 % DMSO and a slightly higher amount of cells (~60 %) with unhealthy looking morphology for the 10 µM Fe(II)-N4Py treated sample. The results of the PI/FACS assay 24 h after irradiation do however not show any significant changes in cytotoxicity (Figure 4a).

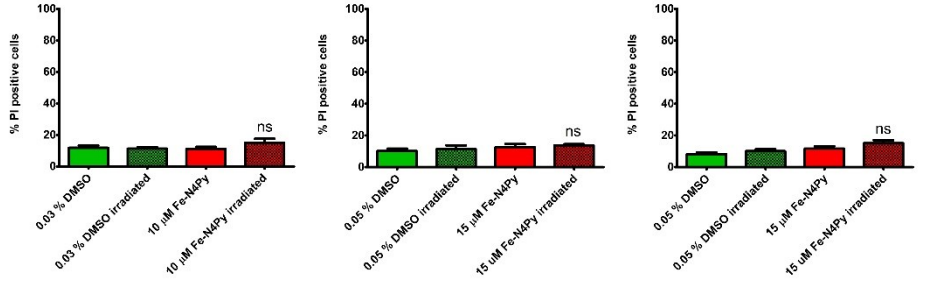

Figure 4. PI/FACS assay with MCF7 cells treated with Fe(II)-N4Py (in red) or DMSO control (in green) either with irradiation at 455 nm for 1 h (blocked boxes) or without irradiation (clear boxes) with subsequent washing and 24 h incubation at 37 °C in a humidified 5 % CO_2 incubator.(a) Treatment with 10 µM Fe(II)-N4Py in a well with approximate growth area of 3.8 cm^2. (b) Treatment with 15 µM Fe(II)-N4Py in a well with approximate growth area of 3.8 cm^2.

(c) Treatment with 10 μM Fe(II)-N4Py in a well with approximate growth area of 1.9 cm^2. ns = non-significant.

Since insignificant changes in cell death were observed by treatment with 10 μM Fe(II)-N4Py compared to all controls, the experiments were repeated with a slightly higher concentration of 15 μM Fe(II)-N4Py. Observations under the light microscope 24 h after irradiation revealed again no significant changes in morphology for the non-irradiated samples, with about 50 % of round unhealthy looking cells for the irradiated cells treated with 0.05 % DMSO and 70-80 % of the cells with unhealthy looking morphology for the 10 μM Fe(II)-N4Py treated sample. The results of the PI/FACS assay 24 h after irradiation however, again do not show any significant changes in cytotoxicity at this concentration (Figure 4b). Higher concentrations of Fe(II)-N4Py (20 μM and 30 μM) were tested, but resulted in heavy damage after just 1 h of incubation without irradiation. Therefore, the concentration limit for Fe(II)-N4Py as possible photosensitizer was reached at 15 μM and improvements had to be made by changing other experimental factors.

It was observed that the irradiation of the collimated LED did not have enough power to harm the cells on the outside of the well as much as in the middle of the well. Cells were therefore seeded into a 24-well plate instead of the previously used 12-well plate, reducing the surface area from 3.8 cm^2 to 1.9 cm^2. Unfortunately, the irradiation still seemed to be unevenly divided over the surface area of the well and the results of the PI/FACS assay seemed to be largely in correlation with those of a larger surface area (Figure 4c).

The above results clearly indicated that irradiation of the cells with the LED light source, did not cause sufficient damage to the cells over a period of 24 h after irradiation in order to result in significant amounts of cell death. It was however envisioned that, even though the sustained damage did not lead to cell death, irradiation by light in combination with the oxidative mechanism of Fe(II)-N4Py could yield significant ROS production in the cells. The hROS probe APF was therefore used to quantify the amount of hROS produced in a period of 1 h irradiation and simultaneous incubation with 15 μM Fe(II)-N4Py (Figure 5a). No significant increase in fluorescence from the cleavage of APF was observed for irradiated cells treated with Fe(II)-N4Py in comparison to non-irradiated cells treated with Fe(II)-N4Py.

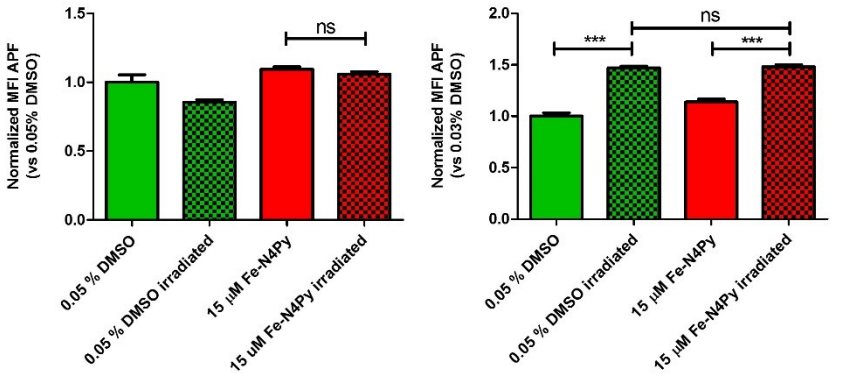

Figure 5. Detection of hROS formation in MCF7 cells upon treatment with 15 µM Fe(II)-N4Py (in red) or 0.05 % DMSO control (green), either with irradiation for 1 h (blocked boxes) or without irradiation (clear boxes). (a) Irradiation and treatment time of 1 h, with subsequent washing and 24 h incubation at 37 °C in a humidified 5 % CO_2 incubator. (b) Irradiation time of 1 h and 24 h treatment time with 15 µM Fe(II)-N4Py or 0.05 % DMSO at 37 °C in a humidified 5 % CO_2 incubator. *** = p<0.001, ns = non-significant.

Even though no significant hROS formation was observed after 1 h irradiation of MCF7 cells, in a final attempt, hROS formation was studied after 1 h irradiation and treatment of the cells with 15 µM Fe(II)-N4Py or 0.05 % DMSO for 24 h. No washing step was performed after the irradiation (Figure 5b). Whilst the irradiated samples showed a small but significant increase of fluorescence compared to the non-irradiated samples, the difference between irradiated samples of 0.05 % DMSO and 15 µM Fe(II)-N4Py turned out to be insignificant.

5.3 Discussion

The results indicate that, under the investigated treatment conditions, no significant photosensitizing effect could be obtained by irradiation of Fe(II)-N4Py treated MCF7 cancer cells. Two factors are considered to be most important for the possible success of photosensitizers in PDT: the light source and the metal complex.[6,37]

The power of the collimated LED light source cannot be increased much further, since cell damage was already observed by light microscopy with DMSO treated control cell cultures after 1h irradiation. In addition, although the irradiation power at the sample was not determined exactly, the power of the lamp and irradiation times seem to be at least similar if not higher and longer than used in most reported cases of PDT.[38–42] Even though it was noted that the irradiation of the LED was not evenly distributed over the entire well, reducing the surface area from 3.8 cm^2 to 1.9 cm^2 did

not resolve this issue. A further reduction in surface area is believed to result in having too few cells for accurate read-out by FACS analysis, which would increase the error margins. Even if a different read-out would be chosen, it remains to be seen if any significant effect would be observed.

The greatest bottleneck in this project is considered to be the metal complex. Ideally, a PDT photosensitizer should have high quantum yields of the triplet state ($\varphi_T > 0.4$), high molar absorptivities ($\varepsilon > 50{,}000$ L mol^{-1} cm^{-1}) and long triplet state lifetimes ($\tau >$ 100 µs), in order to ensure efficient ROS production to the malignant tissues.[9,43,44] In addition, maximum light transmittance into tissue can take place at long wavelengths (typically 700-1200 nm), which also avoids cytotoxicity from high energy photons.[7] Even though Fe(II)-N4Py does have ^{1}MLCT bands with intensities that have been reported for other photosensitizers,[44] their intensities are lower than ideally required for PDT and the other photophysical requirements are also not met.[35,45] A possible, albeit likely small, photosensitizing effect might be obtained by adapting the structure of the iron complex such to include a photosensitizer-cum-DNA binding moiety such as acridine. In a previous study, the Fe(II)-N4Py complex showed a DNA cleavage rate for pUC18 plasmid DNA that is about 2-fold increased by irradiation at 473 nm or 400.8 nm, while the acridine derivative shows a 2.4-fold and 5-fold increase at by irradiation at 473 nm or 400.8 nm respectively.[34] Since the experimental error in a biological experiment might be quite large,[46] it is plausible that the enhanced effect of Fe(II)-N4Py caused by irradiation is too small to actually observe in cell cultures. It therefore remains to be seen whether optimization of the light source would be enough to observe this effect, since the photophysical properties of Fe(II)-N4Py are far from optimal for usage in PDT.

5.4 Conclusion

In this study, the complex $[(N4Py)Fe(CH_3CN)](ClO_4)_2$ (Fe(II)-N4Py) was studied for its potential use as a photosensitizer in cultured cells. Even though previous work by our group revealed the potential of Fe(II)-N4Py as photosensitizer in cleavage studies with ‘naked’ plasmid DNA, no elevated hROS levels and cell death were observed by treatment of irradiated samples of MCF7 breast cancer cells treated with Fe(II)-N4Py. The uneven distribution of light by the LED light source might have had a slight influence on the results. However, the Fe(II)-N4Py complex does not fulfill the most optimal requirements for use as a photosensitizer in PDT, which is expected to be the major reason for not observing any significant effect. It cannot be fully

excluded that there is an actual effect by irradiation of the complex which is actually too small to be observed in cell cultures under the experimental conditions used.

5.5 Experimental Section

5.5.1 Materials and Instrumentation

Solvents and chemicals were of reagent grade or higher and were used without any further purification. The complex $[(N4Py)Fe(CH_3CN)](ClO_4)_2$ (Fe(II)-N4Py) was synthesized as previously reported.[47,48] Irradiation experiments were performed with an M455L4-C5 collimated LED (ThorLabs, Newton, NJ, USA), which consists of an M455L3 mounted LED with a nominal wavelength of 455 nm and a lamphouse-port-compatible housing that contains an aspheric optic. It was supplied with a constant current of 700 mA and has an approximate beam power of 500 mW at the maximum current of 1000 mA.

5.5.2 Biological Experiments

5.5.2.1 Cell Culture

MCF7 cells (human mammary gland adenocarcinoma) were purchased from flow laboratories (Rockville, MD, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Verviers, Belgium) supplemented with 10 % FCS (Perbio Hyclone, Etten-Leur, The Netherlands), 50 µg/mL gentamycine sulfate (Invitrogen, Breda, The Netherlands), 2 mM *L*-glutamine (Lonza) and incubated at 37 °C, in a humidified 5 % CO_2 incubator.

5.5.2.2 Irradiation Experiments and Cell Death (PI/FACS)

Cells were seeded at a density of $8 \cdot 10^4$ cells in a 12-well plate and treated for 1h with Fe(II)-N4Py or DMSO control with or without irradiation. Samples were irradiated by placing the 12-well plate on a solid optical breadboard to which a standard post holder was mounted with a lens tube clamp to hold the M455L4-C5 collimated LED in place. The light beam faced downwards to the 12-well plate, with the position of the 12-well plate marked to ensure the same positioning between experiments. Cells were washed once with PBS buffer and treated with the indicated concentration of Fe(II)-N4Py or DMSO control in PBS buffer. Samples were irradiated for 1h at room temperature under atmospheric conditions, after which the cells were washed once with PBS buffer and incubated for 24h in DMEM medium at 37 °C, in a humidified 5 % CO_2 incubator. After treatment, cells were harvested and stained with 5 µg/mL PI (Sigma-Aldrich)/PBS. After a 10 min incubation at 4 °C in the dark, fluorescence was

measured using the FL-2 channel of a FACScalibur flow cytometer (Beckton Dickenson Biosciences, San Jose, CA). Each experiment was performed at least three times. The percentage PI positive cells was determined with Kaluza 1.2 (Beckman Coulter) software.

In the case of a 24 h treatment time with Fe(II)-N4Py or DMSO control (Figure 5b) the procedure was adapted as follows: before treatment, cells were washed once with PBS buffer and treated with the indicated concentration of Fe(II)-N4Py or DMSO control in DMEM no phenol red medium. After sample irradiation for 1 h, the cells were incubated without washing or changing the medium.

5.5.2.3 ROS Detection

The PI/FACS procedure was adapted as follows: after sample irradiation, the cells were washed once with PBS buffer and treated with 5 µM APF (Molecular Probes) and 100 µM H_2O_2 for 30 min at 37 °C. Cells were washed with PBS (3x) and collected. APF fluorescence was detected in the FL1 channel of a flow cytometer (BD FACSCalibur, BD Biosciences). Data was analyzed with Kaluza 1.2 (Beckman Coulter) software.

5.5.2.4 Statistics

Statistical analysis was performed using Graphpad Prism 5 software. Single group and multiple group comparisons were performed with the student's t-test or one-way ANOVA followed by Dunnett's post hoc test, respectively. A p-value of 0.05 or less was considered statistically significant.

5.6 References

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