Redox-active N4Py-metal complexes in human cell cultures

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Chapter 6

Conclusions and Perspectives

A small overview of the most important achievements and conclusions from each chapter will be given, followed by a general overview and proposed mechanism in which this information is combined. This discussion will be followed by some suggestions for future research on this topic.

6.1 Introduction

The research described in this thesis has its origins in a collaboration between the groups of Feringa and Que, which resulted in the synthesis and characterization of the pentadentate ligand N4Py, which proved to be a good synthetic mimic of the metal binding domain of the antitumor drug BLM (Figure 1a).[1] The low-spin Fe(II) complex $[(N4Py)Fe(CH_3CN)](ClO_4)_2$ (**1**, Fe(II)-N4Py) (Figure 1b) was shown to be able to react with H_2O_2 in methanol or acetone and form a low-spin Fe(III)-OOH species (Figure 1c) with spectroscopic characteristics similar to 'activated BLM'.[2–6] Initially, complex **1** was used as oxidizing agent for various organic reactants.[7] Later, efficient single-strand cleavage of plasmid DNA was observed with **1**, even without the presence of a reductant and subsequent mechanistic investigations revealed an important role for superoxide radicals.[8,9]

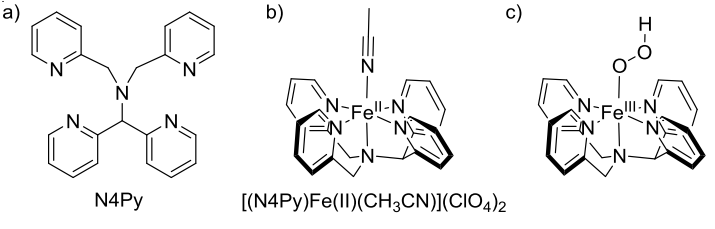

Figure 1. (a) ligand *N*,*N*-bis(2-pyridylmethyl)-*N*-bis(2-pyridyl)-methylamine (N4Py) (b) $[(N4Py)Fe(II)(CH_3CN)](ClO_4)_2$ (**1**) (c) low-spin Fe(III)-OOH species generated from **1**.

More recently, our group presented the first data on the use of Fe(II)-N4Py in cultured cells.[10] Fe(II)-N4Py was shown to induce cell death *via* a different mechanistic pathway than BLM. A sudden drop in metabolic activity was observed over a small concentration range. In addition, Fe(II)-N4Py appears to be a cytotoxic agent with roughly half of its induced cell death caused by caspase-dependent apoptosis. In contrast, BLM is a cytostatic agent, causing G_2/M-phase cell cycle arrest. Moreover, about half the amount of dsDNA damage observed by treatment of cells with Fe(II)-N4Py was ascribed to indirect damage *via* apoptosis, while BLM is believed to mainly cause dsDNA damage by direct cleavage of the DNA backbone.

As mentioned in chapter 1, the aim of this research project was to determine the chemical nature and localization of N4Py in living cells, as well as to further manipulate its structure in order to enhance its favorable properties in a cellular environment, since this could ultimately lead to a successful BLM mimic with good anticancer activity. This last chapter will use the knowledge obtained from the

previous chapters together with previous work on this topic to present new insights with regard to the function of N4Py in a cellular environment. A small overview of the most important achievements and conclusions from each chapter will be given, followed by a general overview and proposed mechanism in which this information is combined. This discussion will be followed by some suggestions for future research on this topic.

6.2 Summary of Previous Chapters Regarding the Behavior of N4Py in Living Cells

6.2.1 Metal Coordination of N4Py in a Cellular Environment

The chemical experiments in combination with biological studies of chapter 2 show that it is clearly not one N4Py metal species that can be present in a cell. Instead, a mixture of Fe(II)-, Cu(II)- and Zn(II)-N4Py complexes is proposed, of which the iron complex seems to be responsible for the observed activity of N4Py in the cell. The active complex can therefore differ from the complex that was initially added to the cell culture medium, regardless of the initial metal ion coordinated.

The mechanism of action is mostly oxidative, since hROS formation was demonstrated with the APF probe and the effects were largely diminished by addition of an antioxidant.[11] These findings are in agreement with previous reports by our group regarding the inherent DNA cleavage ability of Fe(II)-N4Py on plasmid DNA.[9] In addition to oxidation, metal scavenging from the active site of proteins or from co-factors is also believed to be involved in its mechanism of action, especially when cells are treated with the uncoordinated ligand.

6.2.2 Cellular Localization and Mechanism of Uptake of N4Py-fluorophore Conjugates

Chapter 3 described the effect of fluorophore conjugation to N4Py on its mode of action, localization and function in the cell. Even though conjugation had little effect on the intrinsic DNA cleavage activity of N4Py, the cellular behavior was greatly affected. N4Py-Fluorescein was found to be unable to pass through the cellular membrane and the compound did not show any change in cellular activity compared to solvent controls. It can therefore be concluded that, in order for N4Py to inflict oxidative damage to the cell, it has to be able to pass through the cellular membrane and damage the cell from inside.

The N4Py conjugates of Rhodamine B and Cy5, N4Py-RhodamineB and N4Py-Cy5,

were clearly able to enter the cell, but localized in different compartments. Whereas N4Py-Cy5 could passively enter the cell and predominantly localize in the mitochondria, N4Py-RhodamineB was actively transported into the cell and remained largely trapped in the lysosomes. This also explains why N4Py-RhodamineB remained largely inactive in the cell, while mitochondrial localization of N4Py-Cy5 seemed to even improve the cytotoxicity of N4Py. Contrary to N4Py itself, N4Py-Cy5 clearly damages the mitochondrial DNA and causes mitochondrial production of $O_2^{\cdot-}$. In addition, Fe(II)-N4Py induces caspase-dependent apoptosis, while this is not the case for N4Py-Cy5. From these results it can be concluded that conjugation of fluorophores can change both its cellular localization as well as the mode of cell death. In addition, due to the obtained data for the fluorophore conjugates, localization of Fe(II)-N4Py in the lysosomes or mitochondria seems highly unlikely. This data also seems to suggest that N4Py passively enters the cell.

6.2.3 Receptor-mediated Transport and High Selectivity for Folate-expressing Cancer Cells

Studies with N4Py on various cancer cell lines and (conditionally) immortalized cell lines have shown little to no cell selectivity. A particularly promising target for selective drug delivery known in literature is the folate receptor (FR), which has been used in numerous clinical and preclinical studies.[12–17] Indeed, conjugation of N4Py to a molecule of folate resulted in selective delivery of N4Py to FR(+) KB cancer cells *via* receptor-mediated endocytosis (chapter 4). In addition, the introduction of a redox sensitive disulfide linker between N4Py and folate resulted in a significant enhancement of its biological activity, with a decrease in metabolic activity approaching that of Fe(II)-N4Py after a period of 72 h, while a minimum effect was observed in the same period of time for FR(-) MCF-7 breast cancer cells. Furthermore, the disulfide conjugate appeared to be also functionally active and selective for IGROV1 cells which have a 30 to 50 times lower folate receptor density on the cell membrane compared to KB cells and therefore give a better representation of physiologically relevant expression of the folate receptor.[18] These findings show the potential for obtaining an N4Py derivative with good potency and reduced toxicity compared to the generic working of N4Py itself.

6.2.4 Exploring the Potential of Fe(II)-N4Py as Photosensitizer in Cultured Cells

Previous work from our group showed the potential of Fe(II)-N4Py as photosensitizer

in cleavage studies with 'naked' plasmid DNA.[19] The work in Chapter 5 however showed that no elevated hROS- and cell death levels were observed by treatment of irradiated samples of MCF7 breast cancer cells treated with Fe(II)-N4Py. It was concluded that the Fe(II)-N4Py complex does not fulfill the most optimal requirements for use as a photosensitizer in cultured cells, since a 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}), long triplet state lifetimes ($\tau > 100$ µs) and, preferably, maximum light absorbance at long wavelengths (typically 700-1200 nm).[20–23] Even though an actual effect of irradiation of the complex cannot be excluded, the effect might be too small to be observed in cell cultures under the experimental conditions used or simply be non-existing.

6.3 Plausible Mechanism of Action for N4Py in Cells

Based on the cumulative data obtained from the previous chapters, a plausible mechanism for the behavior of N4Py in- and outside of cultured cells can be compiled, which is by no means fully complete but tries to give an overview of the most likely scenarios. The studies with N4Py-Fluorescein (chapter 3) reveal that N4Py does not inflict significant damage to the outside of cells to the extent that it can lead to cell death. Therefore, cellular uptake seems vital for its mechanism of action.

The uptake mechanism appears to be passive for both the Fe(II)-N4Py complex as well as the unbound ligand. This finding is based on a comparison of experiments performed at 37 °C and 4 °C. Even at 4 °C, when active membrane transport mechanisms are effectively shut down,[24,25] A2780 cells treated with N4Py and Fe(II)-N4Py clearly showed a change in morphology occurring for both compounds at both temperatures. This is a clear indication of passive transport of both the ligand as well as the complex (data not shown). At 4 °C, a significant amount of swelling of the cells was observed at 10 µM concentrations for both compounds, although swelling seems to occur faster for the uncomplexed ligand than for the iron complex. A similar observation was made for experiments run at 37 °C: an extensive amount of cell death was observed at 10 µM concentrations, but the cells treated with N4Py seemed to deteriorate faster than the ones treated with the Fe(II) complex. It is clear from these observations that both the ligand as well as the metal complex seem to be able to passively diffuse through the cell membrane. In addition, treatment with high concentrations (~ > 20µM) of Fe(II)-N4Py and N4Py at 37 °C resulted in clear morphological changes within 2h, which is also observed for the passively diffusing

N4Py-Cy5 conjugate, but not for the actively transported N4Py-RhodamineB, for which no uptake was observed within this time period.

The cytotoxicity profile for N4Py and Fe(II)-N4Py appears to be different from that of N4Py-RhodamineB, which is localized mainly in the lysosomes, and N4Py-Cy5, localized in the mitochondria. Based on these findings and the proposed passive transport through the cell membrane, localization in the cytoplasm appears to be most likely. Localization in an organelle other than the mitochondria or lysosomes can however not be completely excluded.

Based on the chemical experiments performed in chapter 2 alone, it cannot be concluded which coordination complexes of N4Py exist inside the cell. These experiments did however show that, under the cell-free test conditions, the ligand can exchange metal ions and a mixture of Fe(II)-, Cu(II)- and Zn(II)-N4Py complexes can be formed. The equilibrium between these complexes will also depend on the relative concentrations of the metal ions in the cell. An oxidative mechanism of action seems to be the major contributor to cellular damage, with clear formation of hROS and a largely diminished effect upon addition of an antioxidant.[11] Experiments with Cu(II)-N4Py in the presence of NAC revealed that Cu(II) interaction and complexation with GSH can result in reduction of Cu(II) to form a Cu(I)-$(GSH)_2$ type complex with the additional formation of $O_2^{\cdot-}$ and eventual formation of hROS.[26–29] A large fraction of the observed oxidative damage is however ascribed to direct oxidation by Fe(II)-N4Py.

N4Py appears to be a cytotoxic agent and based on experiments with the broad range caspase-inhibitor ZVAD-FMK, about half of the sustained damage results in caspase-dependent apoptosis. Metal chelation induced apoptosis might play a large role, since N4Py is known to inhibit the X-linked inhibitor of apoptosis by Zn(II) chelation,[30] and is able to mobilize iron from ferritin.[31] Deprivation of cancer cells from iron is an often used approach in cancer treatment and can often lead to apoptosis.[32–34] In addition to the mentioned modes of cell death, many other forms are known that could, to a greater or lesser extent, play a role in the cell death mechanism induced by N4Py.[35] The roles of ferroptosis[36] and programmed necrosis such as parthanatos,[37–39] can for example not be excluded.

6.4 Prospects

In this final paragraph, the prospects for the use of N4Py in biology are discussed. Some future experiments are recommended and new strategies are suggested. It is clear from the work described in the previous chapters and before that N4Py does not always behave the way it was expected based on our knowledge of its mechanism in cell free systems. To date, treatment of cultured cells with N4Py still raises many questions. Continued studies of this ligand and its complexes will undoubtedly lead to a better understanding of its mechanism and naturally also lead to new questions.

Even though passive transport of the Fe(II)-N4Py complex was postulated, it is not yet fully proven. Unfortunately, ICP-MS studies proofed to be unreliable because of the high natural abundance of iron in living cells. A recent paper does however show that it is possible to use ICP-MS with ^{58}Fe-labelled material in combination with biological samples.[40] Alternatively, the group of Chakravarty showed that it is possible to get good estimates of cellular iron content with inductively coupled plasma optical emission spectrometry (ICP-OES).[41] Another possibility would be to use the ruthenium complex of N4Py. Since ruthenium is in the same group in the periodic table as iron, its physical and chemical properties are quite similar. In addition, ruthenium polypyridyl complexes are generally very stable to the extent that chemical reactions can be performed on the complexes.[42,43] Since ruthenium does not have high background levels in the cell, it should be possible to determine the ruthenium content reliably by ICP-MS. Extrapolation of this data to Fe(II)-N4Py could indicate the likelihood of N4Py entering a cell coordinated to iron.

A perhaps more challenging project could involve tests performed in human tissues and eventual animal testing with derivatives of N4Py. Since the selectivity towards tumor cells was demonstrated for folate receptor (FR) expressing tumors by conjugation of folate to N4Py, the currently developed folate-N4Py conjugate with a disulfide moiety could be a good starting point. Integration of an organelle specific localization signal, e.g. a nuclear localization signal, might subsequently target the nucleus after cleavage of the disulfide bond and result in enhanced nuclear DNA cleavage. The exact biological mode of action of such molecules would have to be further studied. Effectors and signaling pathways in the cell would have to be mapped in order to better comprehend its cellular behavior.

A general recurrent issue in the cell studies was the lack of influx of N4Py conjugates.

Various N4Py conjugates, including those containing biotin and lucifer yellow moieties as well as ditopic N4Py ligands did not seem to enter the cell in significant quantities. Encasing drugs in nanoparticles is a currently a popular and efficient way of trafficking drugs through cell membranes. However, uptake of nanoparticles often involves endocytosis, which traps the cargo in the endosomes.[44,45]

Recently, the group of Stachowiak described a very elegant approach to possibly overcome this issue by using so called connectosomes to deliver cargo directly into the cytoplasm.[46] Cells are able to exchange molecular cargo such as peptides, siRNA, signaling molecules and metabolites with neighboring cells using gap junctions. These gap junctions are formed by proteins that are called connexins. Self-assembly of connexins results in formation of connexons, hexameric pores present on the plasma membrane. Connexons from two neighboring cells can fuse together and form a gap junction channel that enables molecules to move directly from the cytoplasm of one cell to that of the other. Connectosomes are lipid vesicles that contain connexons, which can fuse with cells to form gap junctions and release their cargo into the cytoplasm. The authors showed that the channels are able to open and close in the absence or presence of Ca(II). Connectosomes can be harvested from special donor cells and the cargo can be loaded into the connectosomes either by incubation in the donor cells or by incubation of connectosomes in a solution of the desired cargo. As a proof of concept, the authors showed that the therapeutically effective dose (LD_{50}) of doxorubicin was reduced by more than one order of magnitude compared to free doxorubicin treatment and even several orders of magnitude lower compared to doxorubicin constrained in liposomes. The use of connectosomes for the treatment of cells with N4Py derivatives that, due to their molecular characteristics have difficulties entering the cells, could open up various new possibilities for future research.

The interesting structural features and biological functions of G-quadruplexes (GQs), guanine-rich sequences that can form non-canonical four-stranded nucleic acid structures, have become increasingly popular for the discovery of new anti-cancer drugs.[47] GQs are especially abundant in telomeric DNA repeats and some oncogenic promoter regions and it is suggested that GQs play important roles in chromosome maintenance and transcriptional- and translational control of proliferation-associated genes.[48–51] Ligands for GQs can interfere with telomere maintenance.[52] In short, ligands that stabilize GQs can interfere with telomere replication by blocking the elongation of DNA by telomerase.[53,54] This can induce telomere shortening and

senescence of the cell. Several of these GC ligands have been reported.[52,55–59] The group of Cowan showed an interesting example, in which an amino-terminal copper binding motif (GGHK) was coupled to an acridine-based GQ ligand (Figure 2).[60] Strong binding (K_d = ~ 0.5 μM) was observed for the complex to the tested GQ oligonucleotide in addition to significant telomeric DNA shortening, inhibition of cell proliferation and induction of apoptosis of MCF7 cells. Since the used acridine ligand resembles the structure of the previously reported amino-acridine derivative of N4Py,[8] this good DNA cleaving agent[9] seems to be a good starting point for some preliminary studies in this emerging field.

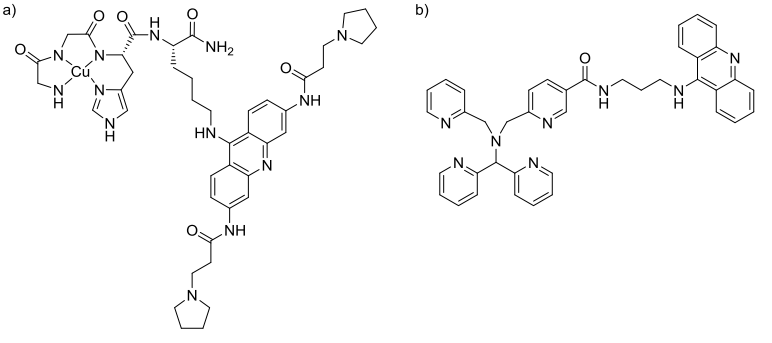

Figure 2. Chemical structures of: (a) CuGGHK-Acr (b) N4Py-acridine.

The suggestions represented above illustrate the endless possibilities in this area of research. Clearly, quite some work should still be performed in order to get a deeper knowledge of the cellular working of N4Py. It is however the author's opinion that valuable lessons can still be learned from future cellular research on N4Py. Since the chemistry of N4Py is well known, it is well suited as a model compound to explore new possibilities in chemical biology. It is clear to the author that advances in various fields of science and human curiosity can offer great potential for future research projects regarding N4Py for years to come.

6.5 References

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