Drug Targeting

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Organometallic Rhenium Complexes Divert Doxorubicin to the Mitochondria

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Abstract: *Doxorubicin, a well-established chemotherapeutic agent, is known to accumulate in the cell nucleus. By using ICP-MS, we show that the conjugation of two small organometallic rhenium complexes to this structural motif results in a significant redirection of the conjugates from the nucleus to the mitochondria. Despite this relocation, the two bioconjugates display excellent toxicity toward HeLa cells. In addition, we carried out a preliminarily investigation of aspects of cytotoxicity and present evidence that the conjugates disrupt the mitochondrial membrane potential, are strong inhibitors of human Topoisomerase II, and induce apoptosis. Such derivatives may enhance the therapeutic index of the aggressive parent drug and overcome drug resistance by influencing nuclear and mitochondrial homeostasis.*

The mode of action and efficacy of chemotherapeutic agents is fundamentally influenced by localization and uptake in the target cells. While much effort has been put into the rational design and synthesis of such agents, the results are sometimes counterintuitive. It has been shown that small alterations to established lead structures can unexpectedly alter their cellular localization.[1] In this work, we investigated a significant change in the cellular distribution of a clinically relevant chemotherapeutic upon conjugation to small rhenium complexes.

Doxorubicin (Dox) is a broad-spectrum antineoplastic agent, known for its accumulation in the nuclear compartment and inhibition of topoisomerase II upon intercalation into DNA.[2] Although it shows excellent activity against a broad range of cancers, therapeutic protocols are aggressive and rarely well-tolerated owing to severe off-target toxicity.[3] By far most worrisome is the much-dreaded cardiotoxicity that limits therapy to a predefined dose.[4] Moreover, Dox and related anthracyclines are subject to multidrug resistance (MDR), an intrinsic or acquired form of resistance after multiple therapy cycles, resulting in poor remission.[5] New strategies are being exploited to improve its efficacy and reduce its side effects, for example, delivery with liposomes[6] or in hydrogels,[7] conjugation to nanoparticles,[8] and derivatization of Dox itself.[9]

Mitochondria have been proposed as an alternative target for Dox to force new mechanisms of action by which the therapeutic profile could be extended and MDR could be overcome. Since Dox accumulates in the nucleus, efforts have been undertaken to redirect the drug into these organelles by derivatization with a mitochondria-targeting peptide[10] or delivery in mitochondria-targeted nanocarriers.[11] Recently, two reports have shown mitochondrial localization by linking Dox to rationally designed targeting functions for mitochondria.[12] In these approaches, Han and co-workers conjugated Dox at its primary amine to the known MitoTracker QCy7HA or to the triphenylphosphonium cation. While all of the mentioned approaches indicated an improved activity in Dox resistant cells, none of the studies presented quantified uptake values in mitochondria.

In this work, we present two organometallic rhenium cyclopentadienyl (Cp) complexes that shift the cellular distribution of Dox from a pure nuclear localization to significant mitochondrial uptake. We quantified the uptake with inductively-coupled plasma mass spectrometry (ICP-MS) and conducted a preliminary investigation of the observed cytotoxicity.

The metalloconjugates Cp-Dox and Cp-*N*-Dox (Figure 1) were prepared by coupling two previously reported rhenium–Cp complexes[14] to Dox through HBTU-mediated amide bond formation. After HPLC purification, the identity of the compounds was confirmed by NMR spectroscopy and high-resolution mass spectrometry (Charts S1–S8 in the Supporting Information). The complexes were then incubated with HeLa cells, the well-defined mitochondria of which can readily be imaged.[15] Figure 1 shows fluorescence images of cells incubated with native Dox and the two novel conjugates. Both conjugates were taken up rapidly, since fluorescence was visible already 10 min post incubation. While native Dox accumulates solely in the nuclear compartment, Cp-Dox and Cp-N-Dox showed cytoplasmic staining only. However, the cytoplasmic fluorescence of Cp-Dox was clearly confined to defined structures in the cytosol. This effect was less pronounced for Cp-*N*-Dox, for which a fluorescence signal was observed mainly in the perinuclear region. Co-staining of both compounds with a mitochondria-selective probe exhibited an excellent overlay, thus indicating mitochondrial uptake of the Dox conjugates (see Chart S9 for a full set of microscopy images).

These findings were additionally corroborated by ICP-MS, since the presence of rhenium in the derivatives allows sensitive quantification of the metal content in the cellular

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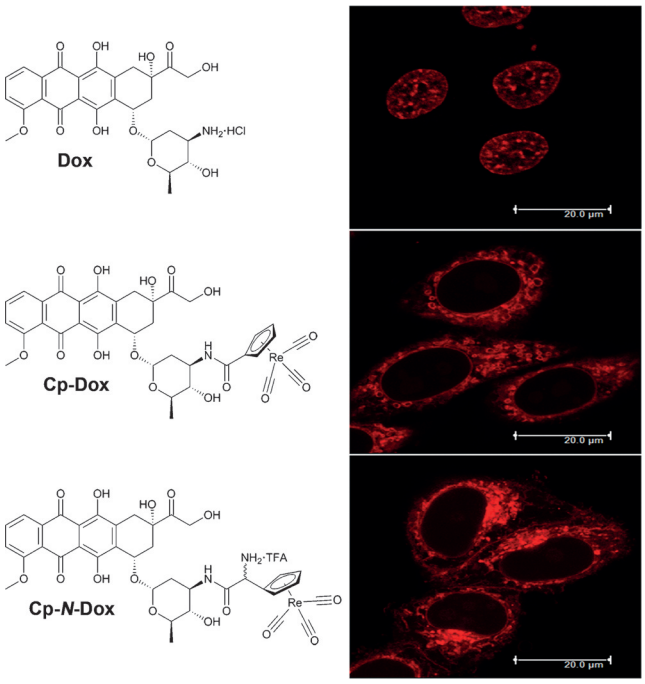

Figure 1. Confocal fluorescence microscopy images of Dox (1 µM), Cp-Dox (5 µM), and Cp-*N*-Dox (5 µM) in HeLa cells after 2 h of incubation. Excitation at 488 nm, emission above 600 nm. Scale bars: 20 µm.

compartments. The results are summarized in Table 1. Remarkably, the Re quantification evidenced a "sponge-like" behavior of HeLa cells towards Cp-Dox and Cp-*N*-Dox, since after only 2 h, 68 and 83 % of the absolute Re amount in the medium, was taken up, respectively (Chart S10). Moreover, ICP-MS confirmed a significant accumulation of both derivatives in the mitochondria. This observation was in good agreement with the microscopic colocalization studies. Interestingly, the derivatization of Dox with these small Re complexes changes its cellular distribution, since under identical incubation conditions, an accumulation of Dox in mitochondria was excluded. Of note, the majority of both conjugates was still recovered from the nuclear fractions. At first sight, this seems counterintuitive, since virtually no fluorescence was observed in this compartment. However, we attribute this effect to nearly quantitative quenching of DNA-intercalated conjugates. We have recently made similar observations for more bulky rhenium conjugates of Dox, where fluorescence microscopy suggested perinuclear and cytosolic uptake, but quantification by ICP-MS clearly evidenced a major uptake into the nucleus and only to a very limited extent into mitochondria (1–2 %).[16] With Cp-Dox and Cp-*N*-Dox, we observed for the first time, noteworthy uptake of a Dox derivative into mitochondria. Even though earlier reports have presented mitochondrial accumulation of Dox derivatives, they all rely on fluorescence microscopy or flow cytometry. Both methods are at least questionable to make a firm statement about the intracellular distribution of Dox derivatives, since the apparent uptake into mitochondria may be over-interpreted by the unknown amount of Dox in the nucleus, which is invisible owing to quenching upon intercalation into DNA.

It is known that lipophilic, cationic compounds are taken up into mitochondria owing to the electrochemical gradient between the inner and outer membranes.[17] Cp-Dox and Cp-*N*-Dox are indeed very lipophilic: their partition coefficients ($\log D_{octanol/PBS}$) have been determined to be 1.86 ± 0.10 and 1.75 ± 0.02, respectively. Additionally, Cp-*N*-Dox is protonated at physiological pH, which may explain the mitochondrial uptake. However, this explanation does not hold true for Cp-Dox, since this complex is uncharged but shows a higher mitochondrial uptake than its protonated congener. Under closer scrutiny of the fluorescence images, Cp-Dox appears to selectively stain the mitochondrial membrane rather than the matrix, thus suggesting a lipophilic interaction with the membrane rather than translocation into the organelle (Figure 2).

Overall, the partitioning of these lipophilic Dox derivatives between the mitochondria and the nucleus could indicate an additional activity on mitochondria that is unknown for the parent drug. To investigate influences of Cp-Dox and Cp-*N*-Dox on the nucleus and mitochondria, we performed a series of additional experiments to make a preliminary assessment of compartment-specific mechanisms of toxicity.

Mitochondria play a central role in cellular homeostasis. The integrity of the mitochondrial membrane and especially an intact electrochemical gradient are responsible for energy production, while dysfunction of the latter is linked to the release of pro-apoptotic factors.[18] On the basis that the Re

Table 1: In vitro and in situ biological data for the Dox conjugates.

	ICP-MS quantification in HeLa[a] (ng Re/mg protein)			MMP disruption after 48 h[b]	Topoisomerase II Inhibition[c] [µM]		Cytotoxicity[d] [µM]		
	Mitochondria	Nucleus	Total	HeLa	hTopoII α	hTopoII β	HeLa	RPE1-hTERT	Ratio[e]
Dox	n/a	n/a	n/a	14 %	1.6 ± 0.2[13]	1.1 ± 0.2[13]	0.11 ± 0.02	0.08 ± 0.02	0.73
Cp-Dox	93.8 ± 25.8 (28 %)	196.3 ± 25.8 (59 %)	328.9 ± 13.3	52 %	8.3 ± 0.9	3.3 ± 0.4	0.34 ± 0.03	1.82 ± 0.54	5.4
Cp-*N*-Dox	47.9 ± 7.6 (20 %)	153.1 ± 47.5 (64 %)	235.9 ± 4.1	30 %	2.0 ± 0.2	2.0 ± 0.3	1.65 ± 0.26	1.27 ± 0.53	0.77

[a] Values indicate the mean ± standard deviation of three experiments. Numbers in brackets represent relative uptake in the fraction compared to the total Re content in the cells. Residual fraction not shown (see the Supporting Information). [b] See the Supporting Information for values at earlier time points. [c] Values indicate the mean ± standard deviation of triplicate measurements made for the enzymes side by side. [d] Values indicate the mean ± standard deviation of triplicate experiments. [e] IC_{50} value in healthy cells divided by the IC_{50} value in cancer cells.

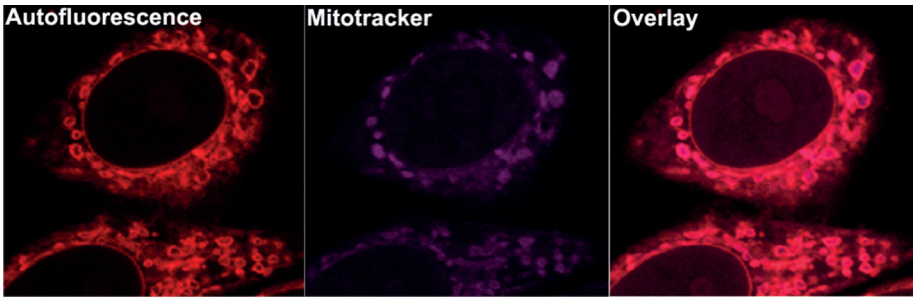

Figure 2. Magnified images showing an overlay of Cp-Dox autofluoresnce and MitoTracker fluorescence in HeLa cells, suggesting an accumulation of the Dox conjugate in the mitochondrial membrane.

complexes demonstrate partial accumulation in mitochondria, it was of high interest to investigate the influence of the Re complexes on the mitochondrial membrane potential (MMP). Remarkably, we indeed found a correlation between the amount of compound taken up into mitochondria and depolarization of the mitochondrial membrane. Significant uncoupling of the MMP was detected in a time-dependent manner. After 48 h, Cp-Dox and Cp-*N*-Dox exhibited disrupted MMPs of 52 % and 30 %, respectively (Chart S11). We would like to point out that native Dox showed merely 14 % MMP disruption under the same conditions, thus reinforcing the hypothesis that treating HeLa cells with the presented metalloconjugates has an influence on mitochondrial homeostasis. To corroborate the idea that Cp-Dox and Cp-*N*-Dox are capable of exerting part of their action in the cell nucleus, we studied their interaction with DNA (binding affinity, K_b) and potential to inhibit human topoisomerase II (hTopoII). The parent Dox is known to intercalate strongly into DNA and is an effective poison for the hTopoII enzyme. The binding affinity for DNA was studied by titration with calf-thymus (ct) DNA and monitoring of the autofluorescence intensity. Since the luminescence of the anthraquinone chromophore is quenched upon intercalation into DNA, thermodynamic parameters can be extracted by fitting the spectroscopy data to the non-cooperative model for DNA binding (Charts S12,13).[19] Cp-*N*-Dox exhibited strong binding affinity towards DNA ($K_b = 1.14 \pm 0.50 \times 10^6\ mol^{-1}$), which was comparable to that of the parent Dox ($K_b = 4.98 \pm 0.50 \times 10^6\ mol^{-1}$), thus evidencing a durable interaction with double-stranded DNA. Of note, the binding affinity of Cp-Dox could not be determined because the initially quenched fluorescence signal quickly recovered after some minutes of equilibration. We tentatively attribute this effect to a very weak, possibly reversible interaction with DNA.

Topoisomerase II inhibition was studied with a recently published assay, which exploits the preferred binding of a fluorescently labeled oligonucleotide $(TTC)_3$ to double-stranded plasmid DNA containing a triplex forming sequence after relaxation by hTopoII α or β versus the supercoiled plasmid (Chart S14).[13,20] The results are summarized in Table 1. The assay clearly demonstrated that the studied Dox conjugates are able to inhibit both isoforms of hTopoII, since Cp-Dox as well as Cp-*N*-Dox exhibited half maximal inhibitory concentration (IC_{50}) values in the same range as Dox itself. Cp-*N*-Dox, in particular, approaches the inhibitory potency of the parent drug, and the slightly reduced IC_{50} values correlate with the reduced binding affinity for DNA of this conjugate compared to Dox. Moreover, Cp-Dox showed the lowest potency in inhibiting hTopoII, thus supporting the afore-mentioned assumption that its interaction with DNA may indeed be very weak. A firm and lasting interaction with DNA is a prerequisite for TopoII inhibitors to induce subsequent DNA double-strand breaks, since they stabilize the so called TopoII–DNA cleavable complex only upon binding to DNA.[21]

Both strong disruption of the MMP and inhibition of topoisomerase II can induce apoptosis, which is the programmed cell death pathway triggered by the parent Dox.[2,22] To confirm this pathway also for the Dox conjugates, we examined Caspase 3/7 activation, which is a typical marker for the induction of apoptosis (Chart S15).[23] Indeed, the activity of this enzyme was found to increase in a time-dependent manner upon treatment of HeLa cells with the conjugates. While Cp-Dox displayed similar Caspase 3/7 activation to that of native Dox, we would like to highlight that Cp-*N*-Dox exhibited an outstanding profile, reaching nearly a three-fold higher activity.

As a direct confirmation of induced apoptosis, antiproliferative studies with Cp-Dox and Cp-*N*-Dox in HeLa cells evidenced formidable toxicity values, with IC_{50} values in the nanomolar and low micromolar ranges, respectively (see Table 1 and Chart S16). Under identical experimental conditions, native Dox exhibited an IC_{50} of 0.11 ± 0.02 μM. Cp-Dox closely approximates this value and both conjugates clearly outperform cisplatin (IC_{50}: 9.6 ± 1.1 μM), a cytostatic standard, thus demonstrating that these Re derivatives can reach comparably useful inhibitory potencies in cancer cells. This is not self-evident, since alterations of the amino sugar can markedly reduce the activity of the derivative, as has been shown for a good number of Dox congeners.[24] This phenomenon is even exploited in novel orthogonal prodrug strategies.[25] While a large portion of the toxicity of our conjugates can likely be attributed to the Dox pharmacophore, we speculate that the organometallic Cp complex may have an advantageous influence on its activity; over recent years it has become apparent that *fac*-$\{Re(CO)_3\}^+$ complexes possess interesting anticancer properties.[26] It is noteworthy that incubating the derivatives with non-cancerous RPE1-hTERT cells showed Cp-Dox to be five times less toxic compared to the results in HeLa cells, possibly indicating a superior therapeutic index, since native Dox (and cisplatin; Chart S16) exhibited nearly equal IC_{50} values in both cell lines.

In summary, we have demonstrated that appending small organometallic rhenium–Cp complexes to an established anticancer drug markedly changes its in vitro distribution. While the parent Dox is known to target the nucleus, the presented derivatives partially accumulate in mitochondria. Upon treatment of HeLa cells with the metalloconjugates, we

observed disruption of the MMP in vitro as well as an efficient inhibition of topoisomerase II in situ, thus suggesting a possible influence on the homeostasis of both target cellular compartments. Both conjugates showed excellent toxicity in the range of established antineoplastic benchmarks and induce the apoptotic cell death pathway.

Although the two compounds share similarities in their structure, there may be inherently different mechanisms of action at work. Maintaining the primary -NH_2 group in Cp-*N*-Dox yields a derivative that functionally resembles the parent Dox compound, as evident from the quantitative data in Table 1. However, its mitochondrial uptake is already clearly accentuated in comparison to the native drug. This is in contrast to Cp-Dox. Apparently, the missing -NH_2 group results in a strongly reduced DNA binding capacity but significant uptake into mitochondria of nearly 30%. Considering these differences in structure and biological profile, it is safe to state that Cp-*N*-Dox is "half way" between Dox and Cp-Dox. The observed toxicity and subcellular distribution between the nucleus and mitochondria therefore warrant a more thorough understanding of their in vitro profile to corroborate a true dual mechanism of action. We are currently investigating the effect of the conjugates on mitochondria and their inhibitory potential towards Dox-resistant cancer cells.

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