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Drug Delivery

Evaluation of New Palladium Cages as Potential Delivery Systems for the Anticancer Drug Cisplatin

Andrea Schmidt,^[a] Viviana Molano,^[b] Manuela Hollering,^[a] Alexander Pöthig,^[a] Angela Casini,^{*,[b, c]} and Fritz E. Kühn^{*,[a]}

Abstract: Self-assembled metallocages are very promising drug-delivery systems among supramolecular complexes. Thus, *exo*-functionalized Pd₂L₄ (L = ligand) cages were synthesized and characterized, and the encapsulation of the anticancer drug cisplatin in their cavity has been documented. The antiproliferative effects of the metallocages and their combination with cisplatin were examined in vitro in cancer cell lines, while fluorescence microscopy was used to monitor their uptake. Notably, the hydroxymethyl-functionalized Pd^{II} cage encapsulating cisplatin showed improved cytotoxic effect against human ovarian cancer cells compared to free cisplatin. The toxicity of Pd₂L₄ cages was evaluated for the first time *ex vivo* in healthy rat-liver tissues using the precision cut-tissue slices technology, demonstrating in some cases scarce effects on liver viability. These results further highlight the potential of self-assembled Pd₂L₄ cages for biological applications.

Metal-mediated self-assembly of supramolecular coordination complexes (SCCs) has been explored—among other areas of potential usage—for biological applications.^[1] Indeed, metallo-supramolecular systems have been shown to possess a plethora of interesting chemical–physical properties, as well as host–guest interactions, which make them suitable for molecular recognition and encapsulation of a variety of organic and inorganic guest molecules.^[2] Concerning biological studies, SCCs have been found to interact with DNA,^[3] possess anticancer activity,^[4,5] and function as drug-delivery vectors.^[6–8] The advantages of SCCs over other inorganic and hybrid porous materi-

als, for example, molecular organic frameworks (MOFs), include being well-defined and discrete molecular entities with increased solubility in biological media.

Thus, an arene ruthenium metallocage was the first coordination cage used as drug-delivery system showing an increased cytotoxic effect against human A2780 ovarian cancer cells upon encapsulation of palladium and platinum acetylacetonato complexes.^[6] Ru-based trigonal prisms have also been designed to encapsulate organic anticancer drugs for delivery.^[8] Recently, a new strategy was developed using a self-assembled hexanuclear Pt^{II} cage to deliver Pt^{IV} prodrugs to cancer cells.^[9] Moreover, the first *in vivo* studies of SCCs as anticancer agents were conducted with rhomboidal Pt^{II} metallocycles.^[5] However, in this case, the cytotoxic effects were exerted by the cages alone, and no drug was encapsulated in their cavity.

In spite of the various reports on the synthesis of metallocages as drug-delivery systems, this research area is still in its infancy, and further studies are necessary to explore their uses in medicinal chemistry. Within this frame, a particularly attractive feature of SCCs is the self-assembly of M₂L₄ (M = metal, L = ligand) metallocages,^[10] in which optimization/fine tuning of the cage's properties is achieved by functionalization of the ligand structure.^[11–13] For example, the fluorescent properties, the solubility, and the guest-encapsulation properties of M₂L₄ cage compounds are influenced by variation of the ligand structure, also in terms of their *exo*- and *endo*-functionalization. For instance, an *exo*-functionalized glucose cage was found to be water soluble,^[12] whereas Yoshizawa et al. reported a fluorescent cage having anthracene-based ligands.^[11a] Interestingly, the M₂L₄ metallocages are capable to encapsulate anions,^[14] cations,^[15] and neutral molecules.^[16]

The combination of pyridyl ligands and square-planar Pd^{II} or Pt^{II} cations has proven to be a very reliable recipe for the realization of supramolecular self-assemblies of this type.^[17] As an example, Pd^{II} and Pt^{II} cages have been studied for their drug-encapsulation properties,^[18,19] as well as for their intrinsic cytotoxic effects.^[20] Based on these promising results, herein, a series of *exo*-functionalized Pd₂L₄ metallocages using bis(pyridyl) ligands and square-planar coordinated Pd^{II} ions has been synthesized by metal-mediated self-assembly and tested for its biological properties. *Exo*-functionalization with amino and hydroxyl moieties was selected, because it may be exploited for further conjugation of the cages to targeting moieties (e.g., peptides and antibodies) or to fluorescence tags. Thus, the encapsulation of cisplatin within the reported cages has been

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studied by different methods. Moreover, the Pd_2L_4 cages were evaluated in vitro in cancer cells, and their uptake was monitored by fluorescence microscopy. To exclude possible toxic effects of the metallogages and of their components in healthy cells, toxicity of three selected cages were performed by using rat precision-cut liver slices (PCLS) ex vivo.^[21] This technology allows cells to remain in their natural environment with maintenance of the original cell–cell and cell–matrix contacts, which is absent in classical 2D cell cultures in vitro. Notably, the technique is a FDA-approved model for drug toxicity and metabolism studies, and it has been previously used for the assessment of toxicity of metal-based compounds, such as cisplatin,^[22] experimental organometallic compounds,^[23] aminoferrocene-containing pro-drugs,^[24] and ruthenium-based kinase inhibitors.^[25]

The rigid bidentate pyridyl ligands **1a–d** were synthesized according to procedures reported in the Experimental Section (see the Supporting Information). Afterwards, reaction of **1a–d** and palladium precursor $[\text{Pd}(\text{NCCH}_3)_4](\text{BF}_4)_2$ in a 2:1 ligand/metal ratio in DMSO at room temperature gave the cage compounds $[\text{Pd}_2(\textbf{1})_4](\text{BF}_4)_4$ **2a–d** within one hour (Scheme 1). ^1H NMR spectra of the product showed that the pyridyl proton signals ($\text{H}_a\text{--H}_d$) are significantly downfield shifted upon cage formation, especially the signals of H_a and H_b are shifted by approximately 1 ppm (see Figure S13 in the Supporting Information).

Scheme 1. Synthesis of Pd^{II} cages **2** using bidentate ligands **1** and a Pd precursor via self-assembly.

Diffusion-ordered NMR spectroscopy (DOSY) provides additional evidence for the successful cage formation in solution (see Tables S1 and S2 in the Supporting Information). ^1H DOSY measurements of the ligands **1** and of the cages **2** gave diffusion coefficients (D) in the range of 1.86 to $2.18 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ and 1.02 to $1.12 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$, respectively, resulting in $D_{\text{ligand}}/D_{\text{complex}}$ ratio of about 2:1, which are in line with literature values.^[13] In addition, DOSY NMR was used to estimate the size of the cage compounds by using the Stokes–Einstein equation. The hydrodynamic radii r_s of the cage compounds were determined to be approximately 1 nm.

Complex formation was further confirmed by ESI MS showing correct isotope pattern for $[\text{Pd}_2(\textbf{1})_4]^{4+}$ and $[\text{Pd}_2(\textbf{1})_4\text{BF}_4]^{3+}$. Exemplarily, the molecular structure of **2b** has been determined by single-crystal X-ray diffraction (see Figure S14 in the

Supporting Information). Based on spectroscopic evidence, the structures of **2a**, **c**, and **d** are, as expected, alike.

The spectroscopic properties of metallogages (see Table S3 and Figures S15–S22 in the Supporting Information), particularly the emissive ones, are of interest to follow the uptake and distribution of the cages in cells by fluorescence microscopy. The quantum yields differ significantly between the ligands, from high values of 52% for **1a** over 21% for **1b** to low yields of about 1% for **1c** and **d**. Upon cage formation, a significant quenching of the fluorescence occurs due to the presence of heavy metal ions resulting in quantum yields of 17 and 6% for **2a** and **b**, respectively.

The cisplatin encapsulation properties of the cage compounds were investigated by solution NMR and by single-crystal X-ray diffraction. In ^1H NMR spectrum of cage **2c** (Figure 1), the inward directed proton signals H_a and H_e undergo significant shifts upon encapsulation of the Pt complex. For the pyridyl proton H_a , a downfield shift of $\Delta\delta = 0.06$ ppm and for the benzyl proton H_e an upfield shift of $\Delta\delta = -0.04$ ppm and a broadening of both signals was observed. These significant shifts indicate the successful cisplatin encapsulation and are

Figure 1. ^1H NMR spectra (400 MHz, $[\text{D}_7]\text{DMF}$) of ligand **1c** (a), cage **2c** (b), $[(\text{cisplatin})_2 \cdot 2\textbf{c}]$ (c), $[(\text{cisplatin})_2 \cdot 2\textbf{c}]$ after 72 h (d).

consistent with results previously reported for a tri(pyridyl) cage.^[12] It is important to note that cisplatin remains encapsulated in **2c** in the presence of water after 72 h. Because the cavity of cage **2c** is structurally equal to complexes **2a** and **b**, the encapsulation properties can be assumed to be very similar. Encapsulation of cisplatin in the tri(pyridyl) cage **2d** could be also verified (see Figure S23 in the Supporting Information).

The formation of a host–guest complex $[(\text{cisplatin})_2 \cdot 2\textbf{d}]$ was additionally proven by single-crystal X-ray diffraction. As shown in Figure 2, two cisplatin molecules are encapsulated in the cavity with a size of approximately $11.2 \times 11.1 \times 11.3 \text{ \AA}^3$. The guest molecules in the cavity are stabilized by hydrogen bonds (see Figure S24 in the Supporting Information) between the amine ligands of cisplatin and the central pyridine moiety of the cage ($\text{N} \cdots \text{H} \cdots \text{N}$), as well as between the chlorido ligands of cisplatin and the inward directed protons ($\text{Cl} \cdots \text{H} \cdots \text{C}$). In addition, both cisplatin molecules interact with each other by hydrogen bonds ($\text{N} \cdots \text{H} \cdots \text{Cl}$). The Pt $\cdots$ Pt distance between the two cisplatin molecules is $3.3326(1) \text{ \AA}$. This is in accordance with

Figure 2. Molecular structure of cage **2d** in the solid state encapsulating two cisplatin molecules. Ellipsoids are shown at 50% probability. Hydrogen atoms, counterions, and solvent molecules are omitted for clarity. Selected hydrogen bond lengths [Å]: N–H...N 2.057/2.9314(1), Cl...H–C 2.550/3.3705(1), N–H...Cl 2.666/3.3605(1). Due to an inversion center in the center of the cavity, only half of the cage and one cisplatin molecule are present in the asymmetric unit. Symmetry code: 1/2–x, 1/2–y, 1–z.

previously reported studies by Crowley and co-workers on similar systems.^[18]

The antiproliferative effects of the coordination cages **2a–d**, and the respective ligands **1a–d**, as well as of the palladium precursor [Pd(NCCH₃)₄][BF₄]₂, were evaluated against a series of human cancer cell lines - A549, SKOV-3, and HepG2 - in comparison to cisplatin (see the Experimental Section). The half maximal inhibitory concentration (IC₅₀) values of the compounds are summarized in Table 1. Overall, the metallocages are less effective in all tested cancer cells than cisplatin, with the exception of **2c**, which exhibits a higher toxicity in SKOV-3 cells. In general, metallocages **2a** and **c** are more toxic to cancer cells (IC₅₀ in the range 8–17 μM) than **2b** and **d** (30–66 μM). Notably, the palladium precursor [Pd(NCCH₃)₄][BF₄]₂ and the bidentate ligands **1a–d** are inactive (IC₅₀ > 100 μM) in the selected cell lines with the exception of ligand **1c**, which is moderately cytotoxic (IC₅₀ between 36–62 μM).

Taking advantage of the fluorescent properties of the metallocages, their accumulation was investigated in A549 cells using fluorescence microscopy. Representative uptake studies of complex **2a** are shown in Figure 3 and Figure S25 in the Supporting Information. Although diffuse cytoplasm localization could be observed, co-localization of the cage fluorescence with PI staining indicate accumulation also in the cell nuclei. Notably, the corresponding ligand **1a** could not be visualized in cells, suggesting very limited uptake of the compound in line with its scarce cytotoxic effects.

Afterwards, the anticancer activity of the host–guest complex [(cisplatin)₂C**2c**] (cisplatin/**2c** stoichiometric ratio 1:0.5) was studied in human ovarian carcinoma SKOV-3 (Table 1). Interestingly, this complex showed an approximately tenfold en-

Table 1. Effects of cages, ligands, Pd²⁺ precursor, and complex [(cisplatin)₂C**2c**] on cell viability in human lung carcinoma A549, in human ovarian cancer SKOV-3, and in human hepatocellular carcinoma HepG2 cells after 72 h incubation.

Compound	A549	IC ₅₀ [μM] ^[a] SKOV-3	HepG2
1a	> 100	> 100	> 100
2a	16.5 ± 4.3	16.7 ± 2.0	8.2 ± 1.6
1b	> 100	> 100	–
2b	47.3 ± 1.8	66.0 ± 10.8	–
1c	59.4 ± 11.0	62.0 ± 4.4	35.9 ± 4.2
2c	13.2 ± 2.4	11.6 ± 1.7	10.7 ± 0.6
1d	> 100	> 100	> 100
2d	32.0 ± 9.7	44.0 ± 6.2	29.7 ± 2.6
[Pd(NCCH ₃) ₄][BF ₄] ₂	> 100	> 100	> 100
cisplatin	8.9 ± 4.2	15.4 ± 2.2	6.4 ± 1.5
[(cisplatin) ₂ C 2c]	–	1.9 ± 0.5	–
cisplatin + 1c + Pd ²⁺	–	22.1 ± 4.4	–

[a] Mean ± SD of at least three independent experiments.

Figure 3. Fluorescence microscopy visualization of metallocage **2a**. A549 cells were incubated for 30 min with 70 μM of **2a** at 37 °C. Scale bars: 50 μm.

hanced toxic effect in cancer cells compared to cisplatin and cage **2c** alone. It is worth mentioning that co-administration of cisplatin, ligand **1c** and Pd²⁺ precursor to the cell-culture medium did not lead to the same increase of antiproliferative activity (see Table 1).

Finally, the toxicity of the cages and their ligands were tested ex vivo in healthy rat-liver tissues using the above-mentioned PCLS technology. All experiments were approved by the committee for care and use of laboratory animals of the University of Groningen and were performed according to strict governmental and international guidelines. Thus, liver slices were incubated with different concentrations of compounds, and after 24 h, incubation the viability of the tissues was evaluated measuring the ATP content. Results for cages **2a**, **c**, and **d** are reported in Figure 4. Although cages **2a** and **d** showed some toxic effects at 30 μM concentration, cage **2c** reduced significantly the viability only at approximately 100 μM or higher. In accordance with the cell studies, the ligand and Pd precursors did not affect tissue viability even at the highest tested concentration of 150 μM (see Table S4 in the Supporting Information).

In conclusion, we have designed a new series of exo-functionalized self-assembled Pd₄L₄ cages able to encapsulate the anticancer drug cisplatin, as was shown by NMR spectroscopy and single-crystal X-ray diffraction. Some of them are cytotoxic in human cancer cell lines, which make them suitable as anti-cancer agents by itself. Notably, an increased cytotoxic effect

Figure 4. Viability of rat liver PCTs after 24 h treatment with cages **2a**, **c**, and **d** at different concentrations. The viability of control slices was set to 100%. Values represent means $\pm$ SD of at least two independent experiments performed in triplicate.

was observed for the host–guest system [(cisplatin)₂C**2c**] compared to cisplatin and to the cage alone. Moreover, **2c** displays very limited toxicity in healthy liver tissues *ex vivo*. Overall, the encapsulation properties, as well as reported biological effects make these cage compounds attractive candidates for further exploration as drug-delivery system. The evaluation of drug release from metallocages within cells and tissues is currently under investigation in our laboratories, as well as their coupling to targeting moieties through the *exo*-functionalized groups.

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Keywords: cisplatin • drug delivery • metallocages • palladium • self-assembly

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