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Authors: Helma Wennemers, Michal S Shoshan, Thomas Vonderach, and Bodo Hattendorf

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Peptide-coated Platinum Nanoparticles with Selective Toxicity against Liver Cancer Cells

Michal S. Shoshan,[a] Thomas Vonderach,[b] Bodo Hattendorf,[b] and Helma Wennemers[a]*

Abstract: *Peptide-stabilized platinum nanoparticles (PtNPs) were developed that have significantly greater toxicity against hepatic cancer cells (HepG2) compared to other cancer cells and noncancerous liver cells. The peptide H-Lys-Pro-Gly-DLys-NH_2 was identified by a combinatorial screening and further optimized to allow for the formation of water-soluble, monodisperse PtNPs with average diameters of 2.5 nm that are stable for years. In comparison to cisplatin, the peptide-coated PtNPs are not only more toxic against hepatic cancer cells but have a significantly higher tumor cell selectivity. Cell viability and uptake studies revealed that high cellular uptake and an oxidative environment are key for the selective cytotoxicity of the peptide-coated PtNPs.*

Hepatocellular carcinoma (HCC) is the sixth most frequent cancer and the second leading cause of death from cancer worldwide.[1] Sorafenib is the most commonly used FDA-approved systemic drug for treating advanced HCC, but suffers from low efficacy and severe side effects.[2,3] Thus, there is a need for specific chemotherapeutics against HCC. Cisplatin is an effective chemotherapeutic against many cancer cells but is also toxic to healthy organs.[4,5] Platinum nanoparticles (PtNPs) are promising alternatives to cisplatin and other Pt-based complexes.[6-10] However, previous studies with various cancer cell-lines neither showed improved cytotoxicity nor tumor selectivity of PtNPs over cisplatin.[6-9]

Within PtNPs, cytotoxic Pt(II) ions[5] are masked as inert Pt(0) atoms and oxidation of Pt(0) to Pt(II) is required to unleash cytotoxicity. PtNPs should therefore have a particularly high toxicity in cellular environments with a high oxidation potential. Liver cancer cells have a higher oxidative state than other cells due to high concentrations of reactive oxygen species.[11,12] PtNPs should therefore be ideally suited to target HCC. A recent report on pH-responsive polymer particles that were functionalized with a targeting ligand for HCC and contained PtNPs supported this assumption.[10] Upon entry of the multicomponent system into HCC cells, the polymer matrix disassembled and the released PtNPs exhibited cytotoxicity. We envisioned that suitably functionalized PtNPs should allow for selective toxicity against HCC cells without the need for immobilization in a polymeric matrix. Such PtNPs have to be water soluble, small,[13] and bear a coat of molecules that a) prevent aggregation of the PtNPs, b) enable cellular uptake, and c) allow for oxidation of Pt(0) to Pt(II) inside HCC cells.

Herein, we present peptide-coated PtNPs that are toxic against hepatic cancer cells but not, or only barely, against other cancer and noncancerous cells. The peptide, which was identified in a combinatorial screening, endows the PtNPs with high stability and monodispersity and can be functionalized with additional moieties, *e.g.*, glucose to enhance the uptake into cancer cells.

Peptides are valuable for the formation of biocompatible noble metal nanoparticles.[14-17] We therefore envisioned that peptidic ligands would also enable the preparation of stable PtNPs in aqueous solution and, moreover, could be tailored to allow for their uptake into liver cancer cells and subsequent oxidation of Pt(0) to Pt(II). Split-and-mix libraries have proven useful for the identification of short-chain peptides that control the formation of PdNPs and AgNPs and endow them with stability against aggregation in water.[17] We therefore started our studies by a combinatorial screening for peptides that enable PtNP formation with a library of maximally 15^3 = 3375 different tri- and tetrapeptides (Figure 1a).[18] Upon incubating the library with K_2PtCl_4 and addition of $NaBH_4$ several dark beads were observed (Figure 1a). The brown/black color indicates PtNPs and suggests that the peptides on these beads had complexed Pt(II) and enabled PtNP formation upon reduction. Analysis of the peptides on several of the colored beads revealed H-Lys-Pro-Gly-DLys-NH_2 (**1**) as a consensus sequence.[19,20]

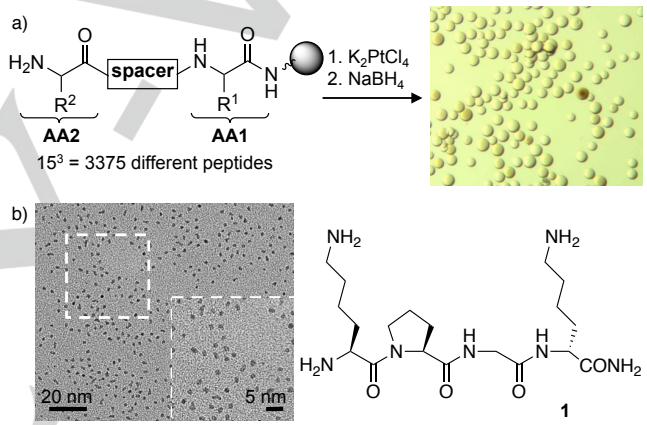

Figure 1. a) Combinatorial library and assay for PtNP formation; bead size ~100 μm. b) TEM micrographs of PtNP-**1**.

We then resynthesized **1** and evaluated its ability to form and stabilize PtNPs in solution. Testing of different parameters (*e.g.*, stoichiometry, concentration, reducing agent) showed that addition of $NaBH_4$ (1.2 equiv) to a solution of the peptide (1 equiv) and Pt(II) ions (1 equiv) in a mixture of deionized water and CH_3CN (3:1) is optimal for the formation of small PtNPs with a narrow size distribution and an average diameter of 1.9±0.8 nm (Figure 1b). The peptide-coated PtNPs proved stable for months in the reaction mixture but aggregated upon dialysis, which was necessary prior to the biological tests to remove residual reagents. We therefore performed structure–activity relationship (SAR) studies with analogues of **1** to elucidate which functional groups are important for the formation of the PtNPs and further improve the structure of the peptide to obtain even more robust PtNPs.[19] These SAR experiments revealed the following key features of **1** for the formation of stable PtNPs: a) the amino groups of the lysine (Lys) residues and at the N-terminus are essential, b) the C-terminal CO_2H group must be capped, and c) the two Lys residues must be in the i, i+3 positions. The absolute configuration of the backbone α-carbon atoms plays a minor role. Replacement of the proline (Pro) and glycine (Gly) residues by alanine led to stable NPs with lower monodispersity. These findings suggest that the amino groups

complex Pt(II) ions and stabilize the resulting PtNP by coordination to Pt(0). The results also suggest that the Pro and Gly residues can be modified, *e.g.*, for functionalization with additional moieties. We therefore elongated the all-L-configured diastereoisomer of **1** with an additional unit of Lys-Pro-Gly to the heptamer **2** H-Lys-[Pro-Gly-Lys]$_2$-NH$_2$.[21] Reassuringly, small PtNPs formed also in the presence of **2** with a narrow size distribution (2.5±0.7 nm, Figure 2a). These nanoparticles did not aggregate upon dialysis and the purified PtNPs proved to be stable for >1 year even upon repeated lyophilization and resuspension as judged by inspections by TEM. Such a high level of stability is remarkable and typically only achieved with ligands (*e.g.*, thiols) that form covalent bonds with the noble metal NP.[22]

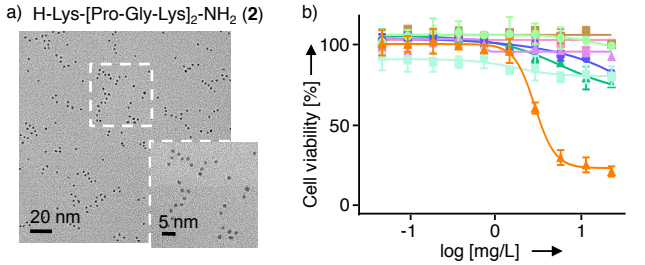

Figure 2. a) TEM micrographs of PtNP-**2**. b) Viability of human cancer cells treated with PtNP-**2**: HepG2 (▲), HT-29 (■), MCF-7 (■), HeLa (▲), PC3 (■), A431 (●), A549 (■), A2780 (▲).

Next, we examined the effect of PtNP-**2** on the viability of hepatocellular carcinoma cells (HepG2) and other human carcinoma cells that included colon (HT-29), breast (MCF-7), cervical (HeLa), prostate (PC3), epidermoid (A431), lung (A549), and ovarian (A2780) cells. Different amounts of the PtNPs were incubated with the cells for three days and their viability was then determined by MTT assays.[23] For the liver cancer cells (HepG2) an IC_{50} value of 2.9±0.3 mg/L and a maximal inhibition value of 79% were observed (Figure 2b, orange). None of the other carcinoma cells were affected to a comparable extent by the PtNP-**2** under the same conditions. This result demonstrates the selective toxicity of the peptide-coated PtNPs against liver carcinoma cells.

Cancer cells have a higher glucose metabolism and more glucose transporters on their surface than noncancerous cells.[24] We envisioned that functionalization of the peptide with glucose could enhance the cellular uptake and effective cytotoxicity of the peptide-coated PtNPs. We therefore prepared peptide **2a** that bears a glucose moiety and used it to form PtNPs (Figure 3a).[25] The PtNPs have similar stability (>1 year), size and monodispersity (2.7±0.8 nm), and coverage of the peptide on the NP surface (~4.8 peptides/nm^2) as those formed in the presence of peptide **2**.[19,22] This result shows the tolerance of the PtNP formation process toward a functional moiety on peptide **2**. Cell viability tests with PtNP-**2a** showed that they inhibit the growth of hepatic cancer cells HepG2 almost completely (maximal inhibition 97%; IC_{50} = 2.4±0.6 mg/L; Figure 3b). In contrast, the other cancer cell-lines were not affected to nearly the same extent, which further highlights the specific toxicity of the PtNPs against liver cancer cells.[26]

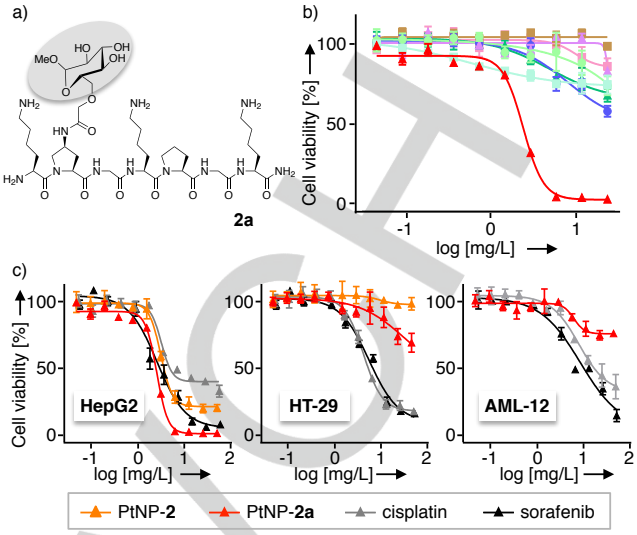

Figure 3. a) Structure of peptide **2a**. b) Viability of human cancer cells treated with PtNPs-**2a**: HepG2 (▲), HT-29 (■), MCF-7 (■), HeLa (▲), PC3 (■), A431 (●), A549 (■), A2780 (▲). c) Cell viability of HepG2 (left), HT-29 (middle), and AML-12 (right) cells against sorafenib, cisplatin, PtNP-**2**, and PtNP-**2a**. Values are mean±SD of ≥3 repeats performed each in triplicate.

We then compared the cytotoxicity of PtNP-**2** and PtNP-**2a** with that of the drugs cisplatin and sorafenib (Figure 3c). Aside from the liver cancer cells (HepG2) we used colon cancer cells (HT-29) for comparison with a cell-line that was not significantly affected by the PtNPs. These experiments revealed that the glucose-functionalized PtNP-**2a** have a comparable toxicity against HepG2 cells (maximal inhibition 97%; IC_{50} = 2.4±0.6 mg/L) as sorafenib (maximal inhibition 93%; IC_{50} = 2.6±0.2 mg/L) and are significantly more toxic than cisplatin (maximal inhibition 66%; IC_{50} = 3.1±0.4 mg/L, Figure 3c, left). Sorafenib and cisplatin are also toxic against HT-29 cells (maximal inhibition: 84% and 82%; IC_{50} = 5.3±0.4 mg/L and 4.2±0.5 mg/L). In contrast, both PtNPs affected this cell-line to a much lesser extent (maximal inhibition <5% (PtNP-**2**), 31% (PtNP-**2a**); IC_{50} >100 mg/L; Figure 3c, middle). Thus, whereas cisplatin and sorafenib have comparable toxicities against different types of cancer cells,[2-5,27] the peptide-coated PtNPs are significantly more toxic against liver cancer cells than other cancer cells.

We also evaluated the toxicity of PtNP-**2a**, cisplatin and sorafenib towards noncancerous mouse liver cells (AML-12).[28] Cisplatin (IC_{50} = 8.3±0.5 mg/L, maximal inhibition ≥ 64%) and sorafenib (IC_{50} = 9.1±0.6 mg/L, maximal inhibition ≥ 86%) are toxic to these noncancerous cells,[29] but the peptide-coated PtNPs have only a small effect on their viability (IC_{50} >100 mg/L; maximal inhibition 25%, Figure 3c, right). Thus, the PtNPs are not only more toxic against hepatic compared to other cancer cells, but also differentiate between noncancerous and carcinogenic liver cells.

The observed toxicity profile could be due to higher uptake of the PtNPs, particularly those functionalized with glucose, or increased oxidation of Pt(0) to Pt(II) inside the hepatic cancer cells compared to other cells; or a combination of both effects. Accordingly, we quantified the cellular uptake of PtNP-**2**, PtNP-**2a**, and cisplatin into HepG2 and HT-29 cells with inductively

coupled plasma mass spectrometry (ICP-MS, Table 1).[30] These studies revealed higher uptake of both types of PtNPs (up to 20-fold) than of cisplatin into HepG2 and HT-29 cells (Table 1, entries 1 and 2). PtNP-**2a** have an even higher cellular uptake (2–3-fold) than PtNP-**2** as expected based on the increased glucose metabolism in cancer cells.[24] The same amount of cisplatin (5.5 ng Pt/mg cells) was internalized in HepG2 and HT-29 cells, which corroborates the lack of cell-type selectivity of cisplatin. In contrast, the uptake of the peptide-coated PtNPs into HepG2 cells is twice as high as their uptake into HT-29 cells, presumably due to the higher metabolism in liver compared to colon cells.[31] The highest amount of Pt (107±14 ng Pt/mg cells) was detected in the HepG2 cells after incubation with the glucose-functionalized PtNP-**2a** (Table 1, entry 1).

Table 1. Quantification of platinum inside cells

		[Pt] ng/ cells mg[a]		
Entry	Cell-line	Cisplatin	PtNP-**2**	PtNP-**2a**
Whole cells[b]				
1	HepG2	5.3 ± 0.2	40 ± 1	107 ± 14
2	HT-29	5.5 ± 1.4	19 ± 5	61 ± 7
Nuclei[c]				
3	HepG2	n.d.[d]	29 ± 5 (72%)	71 ± 10 (66%)
4	HT-29	n.d.[d]	5 ± 3 (26%)	16 ± 7 (27%)

[a] Values are mean±SD of ≥3 repeats performed each in triplicate. [b] Pt in whole cells. [c] Pt in the nuclei; in brackets: percentage from whole cells. [d] not determined.

The higher cellular uptake of the PtNPs correlates well with their higher toxicity compared to that of cisplatin in the HepG2 cells (Figure 3c, left). This is not the case in HT-29 cells. Despite their higher internalization, the PtNPs are significantly less toxic against HT-29 cells than cisplatin (Figure 3c, middle). These results show that the amount of Pt alone does not determine the cytotoxicity of PtNPs but that the environment inside the cells plays a key role. In HT-29 cells, the PtNPs with neutral Pt(0) atoms are barely toxic, consistent with toxic Pt(II) ions being formed more readily in the oxidative environment of the hepatic cancer cells.[32]

Pt(II) ions can damage DNA.[5] We therefore reasoned that the amount of Pt in the nuclei should be higher in HepG2 than in HT-29 cells. Thus, we isolated the nuclei of HT-29 and HepG2 cells after incubation with PtNP-**2** and PtNP-**2a** and quantified the Pt in the nuclear fraction versus that in the whole cells (Table 1, entries 3 and 4).[19] In the HepG2 cells, ~70 % of the Pt accumulated in the nuclei. In contrast, only ~25 % of the Pt inside the HT-29 cells localized in the nuclei. The high proportion of Pt in the nuclei of HepG2 cells arises presumably from better penetration of Pt(II) ions compared to PtNPs through the nuclear membrane.

In conclusion, the screening of a combinatorial library enabled the development of the peptide H-Lys-[Pro-Gly-Lys]$_2$-NH_2 as an additive for the formation of small, monodisperse PtNPs. The peptide-coated PtNPs are highly toxic against hepatic cancer cells but do not affect the viability of other cancer or noncancerous cells to nearly the same extent. To the best of our knowledge, these PtNPs have the highest cytotoxicity combined with selectivity for hepatic cancer cells achieved so far. Cellular uptake combined with cell viability studies revealed that the combination of high cellular uptake and an oxidative environment is key for the cytotoxicity of PtNPs. These results open exciting prospects for the development of PtNP-based therapeutics.

Acknowledgements

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No 705970. We also thank the Swiss National Science Foundation for support by Sinergia Grant CRSII2_160805. We are grateful to Leyla Hernandez for assistance in cell culture. We thank the Scientific Center for Optical and Electron Microscopy (ScopeM) and Prof. Detlef Günther (ETH Zurich) for access to high resolution TEM and ICP-MS instruments, respectively.

Keywords: peptides • Platinum nanoparticles • Cytotoxicity • Selectivity • Hepatocellular carcinoma

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[21] Further elongation to the decamer H-Lys-[Pro-Gly-Lys]$_3$-NH$_2$ (**3**) led to PtNPs with comparable size, monodispersity, and stability as PtNP-**2**. PtNPs formed in the presence of tredecamer H-Lys-[Pro-Gly-Lys]$_4$-NH$_2$ (**4**) were polydisperse and bigger. See the supporting information for details.

[22] EDX and STEM analysis confirmed the formation of ~2.5 nm sized PtNP-**2**. TGA, ICP-MS and ζ potential analysis revealed a coverage of ~4.5 peptides per nm^2 on the PtNPs and a ζ potential of +3.6 ± 0.8 mV. See the supporting information for details.

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[32] The necessity of an oxidative environment for unleashing the toxicity of PtNPs was further supported by incubating PtNP-**2** with HT-29 cells in the absence and presence of H_2O_2. Whereas PtNPs-**2** are barely toxic, toxicity was observed upon addition of H_2O_2 (at a concentration which is non-toxic) against HT-29 cells, see the supporting information for details.

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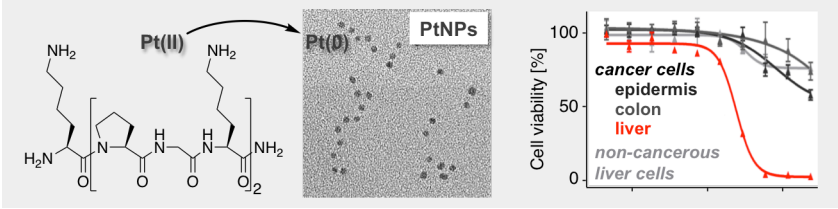

Selective Toxicity. Peptide-stabilized, monodisperse platinum nanoparticles were developed that selectively kill liver cancer cells over other cancer and non-cancerous cells. The peptide was identified in a combinatorial screening and enables high stability of the PtNPs, their cellular uptake, and release of toxic Pt(II) ions in an oxidative environment.

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