Cymantrene conjugation modulates the intracellular distribution and induces high cytotoxicity of a cell-penetrating peptide†‡

Ines Neundorf,*[a] Jan Hoyer,[a] Katrin Splith,[a] Robert Rennert,[a] Harmel W. Peindy N'Dongo[b] and Ulrich Schatzschneider*[b]

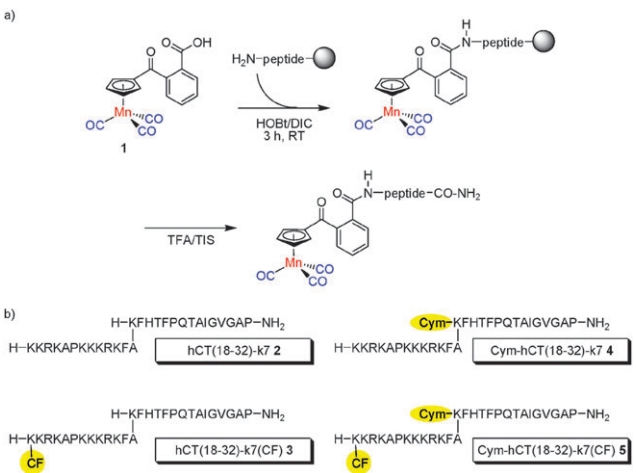
Received (in Cambridge, UK) 25th July 2008, Accepted 12th September 2008
First published as an Advance Article on the web 26th September 2008
DOI: 10.1039/b812799a

The conjugation of cymantrene $CpMn(CO)_3$ to cell-penetrating peptide hCT(18-32)-k7 alters the intracellular distribution in MCF-7 cells compared to the unmodified peptide, as visualized by fluorescence microscopy, and leads to an increased nuclear accumulation; the peptide and cymantrene compound themselves are not toxic, but the bioconjugate shows a significant cytotoxicity with an IC_{50} value of 36 μmol l^{-1}.

The last years have seen a steadily growing interest in the development of potent metal complexes for biological applications. Several of these compounds show promising activity as antibiotics and anti-cancer therapeutics. In addition, a number of metal-based building blocks have been coupled to bioactive molecules for modulating and increasing their biological effect. In particular, metallocenes have been in the focus of intense research efforts and recently they have been conjugated to steroids, saccharides, and antibiotics to generate new derivatives with improved activity.[1] However, problems in the use of such compounds frequently arise from their poor bioavailability, which is due to a limited solubility in water and insufficient uptake by living cells. Also, selective transport to specific cellular target structures is necessary in order to exert the desired biological activity.

During the last decade, the discovery of cell-penetrating peptides (CPPs) has helped to overcome some of these limitations.[2–5] These peptides are able to translocate into several cell lines without the need for a special receptor and can thereby transport different cargos across the cell membrane. Among them, CPPs derived from the native hormone human calcitonin (hCT) have been identified as highly potent delivery systems.[6] In particular, branched hCT-based carrier peptides like hCT(18-32)-k7 turned out to be excellent in cell internalization as well as very effective as transport vectors. Recently, we reported the efficient transfection of several cell lines, including primary cells, with plasmid DNA encoding for the green fluorescent protein.[7] Furthermore, with this peptide, we could demonstrate the successful transport of covalently coupled nanoparticles, *i.e.* quantum dots, into HEK 293 and HeLa cells.[8] The functionalization of such peptides with organometallic compounds has also found considerable interest in the field of bioorganometallic chemistry.[9–11] This includes, for example, coupling of metal complexes to nuclear localization sequences[12] or peptide-based receptor ligands.[13] In this work, we have utilized hCT(18-32)-k7 as a carrier for a cymantrene ($CpMn(CO)_3$) derivative, which was chosen as a robust and easy-to-functionalize organometallic marker.[14–17] The keto carboxylic acid **1** was prepared by Friedel–Crafts acylation of cymantrene as recently reported.[18–20]§ Carrier peptide **2** was synthesized by automated solid phase peptide synthesis (SPPS) on a Rink amide resin. Manual coupling of **1** to the *N*-terminus of the hCT(18-32) chain was achieved by activation with HOBt–DIC (Scheme 1). Subsequent cleavage from the resin occurred by reaction with trifluoroacetic acid (TFA) under addition of triisopropylsilane (TIS). MALDI-TOF/TOF-MS showed a signal for **4** of $[M + H]^+$. However, the most intensive signal was found for $[M + H - Mn(CO)_3]^+$, indicating the loss of the $Mn(CO)_3$ moiety under MALDI conditions. On the other hand, ESI-MS showed the $[M + 5H]^{5+}$ and $[M + 6H]^{6+}$ signals as the major peaks.

Furthermore, the fluorophore (5,6)-carboxyfluorescein (CF) was introduced at the *N*-terminus of the side sequence k7 as reported recently,[8] to allow study of the cellular uptake with



Scheme 1 (a) Synthesis of the cymantrene peptide conjugate was achieved by activation of 5 eq. cymantrene keto carboxylic acid **1** with 5 eq. HOBt–DIC in DMF. (HOBt: *N*-hydroxybenzotriazole; DIC: *N*,*N*′-diisopropylcarbodiimide; TFA: trifluoroacetic acid; TIS: triisopropylsilane; DMF: *N*,*N*′-dimethylformamide); (b) sequence and position of modification of the four peptides **2** to **5** studied in this work.

[a] *Institut für Biochemie, Universität Leipzig, Brüderstr. 34, D-04103 Leipzig, Germany. E-mail: ines.neundorf@uni-leipzig.de; Fax: (+)49 341 97-36909*
[b] *Lehrstuhl für Anorganische Chemie I-Bioanorganische Chemie, Ruhr-Univeristät Bochum NC 3/74, Universitätsstr. 150, D-44801 Bochum, Germany. E-mail: ulrich.schatzschneider@rub.de; Fax: (+)49 234 32-14378*
† In memoriam Prof. Dr. Peter Welzel.
‡ Electronic supplementary information (ESI) available: Synthesis of **1**. See DOI: 10.1039/b812799a

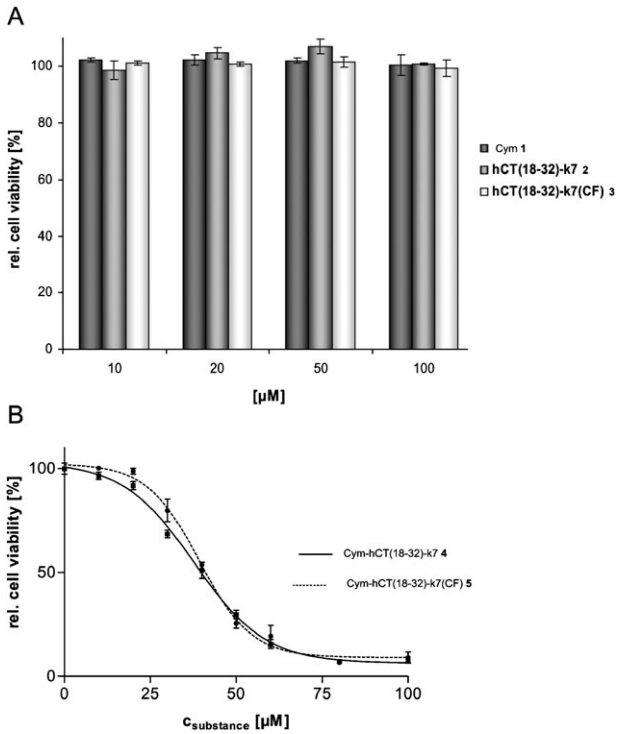


Fig. 1 Relative cell viability of human breast adenocarcinoma MCF-7 cells. A: after treatment for 24 h with compounds **1**, **2**, and **3**. B: representative IC_{50} curves for compounds **4** (solid line) and **5** (dashed line), respectively. Data are normalised to untreated cells (100% viability). All experiments were done with *n* (number of experiments) ≥ 2 in triplicate.

fluorescence microscopy. Coupling of the cymantrene acid **1** and cleavage from the resin was achieved as described above. The product **5** was identified by MALDI-TOF/TOF and ESI mass spectrometry, showing again the $[M + H]^+$ and $[M + H - Mn(CO)_3]^+$ signals in the MALDI and the $[M + 5H]^{5+}$ and $[M + 6H]^{6+}$ signals in the ESI-MS. All peptides **2** to **5** were purified by semi-preparative HPLC and are stable over a period of six months in frozen aqueous solution when stored at −20 °C.

The new cymantrene–CPP conjugates **1** to **5** were then investigated for their cytotoxicity on human breast adenocarcinoma MCF-7 cells using a fluorescent resazurin-based cell proliferation assay. Cells were incubated for 24 h at 37 °C with cymantrene acid **1**, the unmodified peptide **2**, fluorescently-labelled **3** and the two cymantrene-carrier peptide derivatives, **4** and **5**, respectively.

The parent cymantrene keto carboxylic acid **1** as well as the two hCT-peptides **2** and **3** have no effect on cell viability in the concentration range tested (Fig. 1A). However, upon cymantrene conjugation to the carrier peptide, a highly toxic effect on MCF-7 cells was observed. We determined very similar IC_{50} values of (36.0 ± 1.8) μM for **4** and (36.4 ± 2.8) μM for **5**. This also indicates that the introduction of the CF fluorescence label does not alter the cytotoxicity of the peptide or the organometallic bioconjugate. Furthermore, only the combination of the CPP and the cymantrene keto acid leads to the cytotoxic effect observed since both parts alone have no influence on the cell viability. To gain more insight into the fate of the organometallic bioconjugate, we investigated the intracellular distribution by fluorescence microscopy. Living unfixed MCF-7 cells were incubated with different concentrations of cymantrene-modified peptide **5** (1, 10, and 20 μM) for 30 to 90 min at 4 and 37 °C. After removal of the peptide solution, remaining extracellular fluorescence was quenched by the addition of Trypan blue solution to the cells. After washing the cells twice, they were inspected in HBSS buffer (Hanks' balanced salt solution). As a reference, we also included fluorescence microscopy studies with peptide **3**. We found that uptake of both compounds is concentration- and temperature-dependent. At 4 °C, no fluorescence was observed inside the cells (data not shown), pointing to an energy-dependent uptake pathway. Incubating the cells with 1 μM of the bioconjugate **5** as well as unmodified peptide **3** at 37 °C only led to a minor uptake (data not shown), whereas at higher concentrations the uptake significantly increased.

As seen in Fig. 2 left, after an incubation time of 90 min, a dot-like distribution pattern with vesicles near the nucleus is observed for the carrier peptide **3**. Vesicular uptake by endocytosis was recently described to be the main entry pathway for hCT-derived carrier peptides.[21] Especially for the peptide **3**, it was demonstrated that internalization occurs mainly by lipid raft-dependent endocytosis depending on the cell line investigated.[7] In contrast, a significantly different

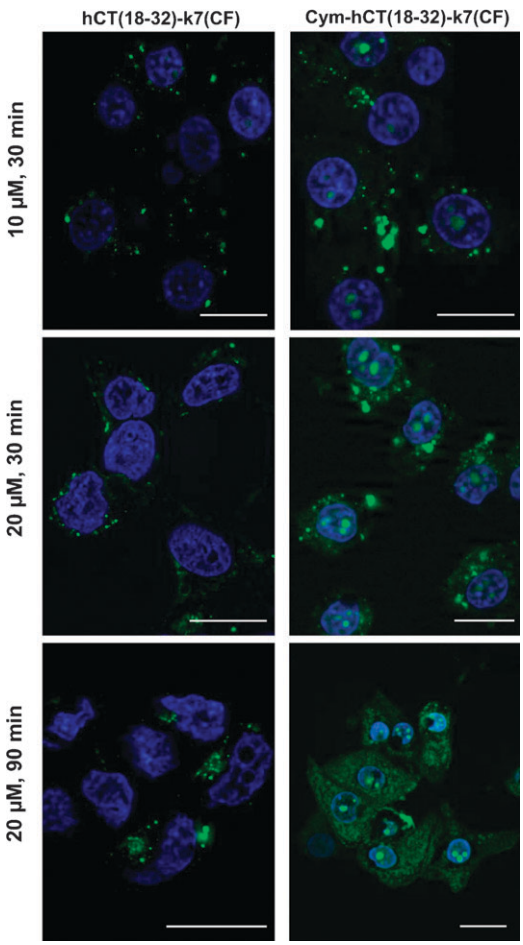


Fig. 2 Cellular uptake of CF-labelled carrier peptides **3** (left) and **5** (right) by human breast adenocarcinoma MCF-7 cells. Scale bars are 20 μm.

picture was obtained after incubating the cells with the organometallic-modified carrier peptide **5**. In this case, after 90 min, the bioconjugate is distributed throughout the whole cytosol. In addition, a large amount of fluorescence is found inside the cell nucleus. Since the peptide alone was not able to cross the nuclear membrane, we assume that this is mediated by the conjugation of the cymantrene unit to the CPP and that the intact bioconjugate reaches the nucleus.

In this work, we have thus shown that the coupling of the organometallic cymantrene moiety to a cell-penetrating peptide generates bioconjugates with a biological activity very distinct from that of the individual building blocks. Significant nuclear uptake is achieved for Cym-hCT(18-32)-k7 and might be responsible for the observed cytotoxicity, although the precise mechanism of biological action is still under investigation. These observations show that very interesting new organometallic compounds for biological studies can be obtained by a proper combination of metal moiety, linker, and peptide. In our opinion, these results have strong implications on the development of non-radioactive organometallic bioconjugates useful for future application in chemotherapy.

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) within FOR 630 ''Biological function of organometallic compounds''. The authors thank Prof. Dr Annette Beck-Sickinger (Leipzig) and Prof. Dr Nils Metzler-Nolte (Bochum) for generous access to all resources of the institutes.

Notes and references

§ Cymantrene keto carboxylic acid **1** was prepared and purified by a reported procedure.[18–20] Peptides **2** and **3** were synthesized according to standard Fmoc/*t*Bu-strategy using automated solid phase peptide synthesis.[8] *N*-Terminal coupling of cymantrene keto carboxylic acid **1** to peptides **2** or **3** was performed manually using 5 eq. of **1** and 5 eq. HOBt–DIC in DMF. The reaction mixture was shaken for 3 h at room temperature. Kaiser test demonstrated the successful coupling. Cleavage from the resin was done using a mixture of 95% TFA, 2.5% TIS and 2.5% water. The bioconjugate was precipitated in cold diethyl ether, dried, and lyophilised to obtain the *C*-terminal amide. After semi-preparative HPLC, we isolated the Cym–peptide conjugates **4** and **5** in high purity (>95%). **4**: MALDI-TOF/TOF-MS: ($MW_{cal.}$ 3499 Da) *m*/*z* 3499.8 $[M + H]^+$, 3362.3 $[M + H - Mn(CO)_3]^+$. ESI-MS: *m*/*z* 701.0 $[M + 5H]^{5+}$, 584.3 $[M + 6H]^{6+}$. **5**: MALDI-TOF/TOF-MS: ($MW_{cal.}$ 3857 Da) *m*/*z* 3720.2 $[M + H - Mn(CO)_3]^+$. ESI-MS: *m*/*z* 772.6 $[M + 5H]^{5+}$, 644.1 $[M + 6H]^{6+}$. Cell viability was determined using a fluorimetric resazurin-based cytotoxicity assay. Human breast adenocarcinoma MCF-7 cells were grown to subconfluency in 96-well plates and then incubated for 24 h under standard growth conditions with up to 100 μM peptide solution. As a negative control, cells were treated for 15 min with pure buffer. Following incubation, cells were washed twice with DMEM (Dulbecco's modified eagle medium) and subsequently incubated at 37 °C for 2 h with a 10% solution of resazurin in DMEM. Finally, conversion of resazurin to the reduced resorufin was measured fluorimetrically at 595 nm (λ_{EX} = 550 nm) with a Spectrafluor plus multiwell reader (Tecan, Crailsheim, Germany). To investigate the cellular uptake of peptide **3** and cymantrene conjugate **5** by fluorescence microscopy, unfixed MCF-7 cells were used. After growing to subconfluency, cells were incubated with 1 to 25 μM of the CF-labelled conjugate in OptiMEM at 37 °C for 30 to 90 min. The cell nuclei were stained with benzimide H33342 for 10 min prior to the end of peptide incubation. After incubation, the conjugate solution was removed, cells were treated for 1 min with Trypan blue (6.5 mM in sodium acetate buffer, pH 4.5) to quench external CF fluorescence and washed twice with HBSS (Hanks' balanced salt solution). Visualization was done with a Zeiss Axiovert 200 inverted fluorescence microscope with ApoTome.

1 D. R. van Staveren and N. Metzler-Nolte, *Chem. Rev.*, 2004, **104**, 5931.
2 Ü. Langel, *Handbook of Cell-Penetrating Peptides*, 2nd edn, CRC Press, Boca Raton, 2006.
3 A. Joliot and A. Prochiantz, *Nat. Cell Biol.*, 2004, **6**, 189.
4 S. Pujals, J. Fernandez-Carneado, C. Lopez-Iglesias, M. J. Kogan and E. Giralt, *Biochim. Biophys. Acta, Biomembr.*, 2006, **1758**, 264.
5 M. Mäe and Ü. Langel, *Curr. Opin. Pharmacol.*, 2006, **6**, 509.
6 R. Rennert, I. Neundorf and A. Beck-Sickinger, *Adv. Drug Delivery Rev.*, 2008, **60**, 485.
7 R. Rennert, I. Neundorf, H. G. Jahnke, P. Suchowerskyj, P. Dournaud, A. Robitzki and A. Beck-Sickinger, *ChemMedChem*, 2008, **3**, 241.
8 C. Walther, K. Meyer, R. Rennert and I. Neundorf, *Bioconjugate Chem.*, 2008, accepted.
9 G. Jaouen, *Bioorganometallics*, Wiley-VCH, Weinheim, 2006.
10 N. Metzler-Nolte, *Chimia*, 2007, **61**, 736.
11 G. Dirscher and B. König, *Eur. J. Org. Chem.*, 2008, 597.
12 F. Noor, A. Wüstholz, R. Kinscherf and N. Metzler-Nolte, *Angew. Chem.*, 2005, **117**, 2481.
13 S. Mukhopadhyay, C. M. Barnes, A. Haskel, S. M. Short, K. R. Barnes and S. J. Lippard, *Bioconjugate Chem.*, 2008, **19**, 39.
14 A. G. Ginzburg, *Russ. Chem. Rev.*, 1993, **62**, 1025.
15 I. Lavastre, J. Besancon, P. Brossier and C. Moise, *Appl. Organomet. Chem.*, 1990, **4**, 9.
16 M. Salmain, N. Fischer-Durand, L. Cavalier, R. Bogna, J. Zakrzewski and G. Jaouen, *Bioconjugate Chem.*, 2002, **13**, 693.
17 M. Hromadova, M. Salmain, N. Fischer-Durand, L. Pospisil and G. Jaouen, *Langmuir*, 2006, **22**, 506.
18 R. Dabard and M. Le Plouzennec, *Bull. Soc. Chim. Fr.*, 1972, 3594.
19 M. Le Plouzennec and R. Dabard, *Bull. Soc. Chim. Fr.*, 1972, 3600.
20 H. W. Peindy N'Dongo, I. Neundorf, K. Merz and U. Schatzschneider, *J. Inorg. Biochem.*, 2008, DOI: 10.1016/j.jinorgbio.2008.07.01.
21 C. Foerg, U. Ziegler, J. Fernandez-Carneado, E. Giralt, R. Rennert, A. Beck-Sickinger and H. P. Merkle, *Biochemistry*, 2005, **44**, 72.

