Synthesis and cytotoxicity of a cobaltcarbonyl–alkyne enkephalin bioconjugate†

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A solid-phase synthesized propargyl derivative of the neuropeptide leucine-enkephalin (Enk) reacts rapidly and quantitatively with $Co_2(CO)_8$ to give the $Co_2(CO)_6$–alkyne labeled peptide 2, which is the first organometallic peptide bioconjugate to show significant toxicity against two different tumor cell lines.

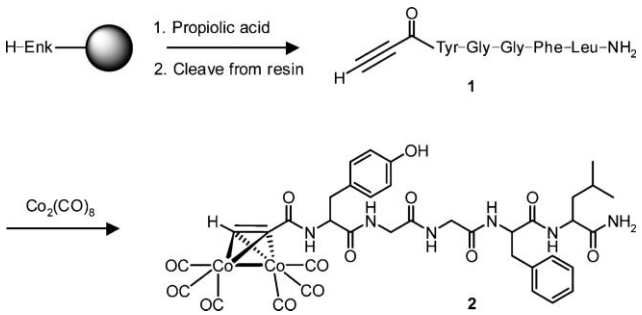
The quest for novel metal-based anti-cancer drugs is an active and important area in medicinal inorganic chemistry. Organometallic compounds are among the most promising candidates in this field.[1,2] A few years ago, cobaltcarbonyl–alkyne derivatives of common analgetics (non-steroidal anti-inflammatory drugs, NSAIDs) were identified as promising lead structures.[3] In particular, a derivative of acetylsalicylic acid (ASS, Aspirin®) shows an anti-proliferative potency similar to that of the well-known anti-cancer drug cisplatin.[4,5] Its mode of action has been the subject of investigation, and interaction with cyclooxygenase enzymes, which are also a target for NSAIDs, has been suggested as the primary mode of activity.[6,7] If verified, this would be a very significant finding, because only drugs with a completely new mechanism of action have the potential to overcome resistance to existing anti-cancer drugs.[2]

In parallel, the development of targeted anti-cancer drugs is of increasing importance, *i.e.* such compounds which will affect exclusively, or at least preferably, cancer cells. One promising approach is to conjugate established anti-cancer drugs to biomolecules such as peptides, which will be selectively internalized only by cancer cells. While this approach has been successfully applied to tumor imaging and diagnosis,[8] applications to tumor therapy are in their infancy still.[9] For example, conjugation of platinum moieties to peptides has been achieved in a combinatorial manner.[10] Disappointingly however, the resulting conjugates lack the anti-proliferative activity of the platinum fragment.[11] For most other metal fragments, not even the necessary chemistry to prepare metal conjugates by solid-phase peptide synthesis (SPPS) methods has been established. Although some synthetic progress has recently been made by our group[12–14] and others,[15] no organometallic peptide conjugates were tested for their anti-proliferative activity so far.

In this work, we set out to prepare a model peptide conjugate with a cobaltcarbonyl–alkyne group and to evaluate its anti-cancer activity. We thereby aim to contribute to the understanding of the mode of action of this class of metal-based drugs and secondly establish the necessary chemistry for the development of tumor-targeting organometallic peptide conjugates.

The neuropeptide leucine-enkephalin ([Leu^5]-Enk, primary amino acid sequence Tyr-Gly-Gly-Phe-Leu), which is a natural ligand to the opiate receptor, was chosen as the target for labeling. Enk was synthesised in good yield and purity by standard Fmoc SPPS on a Rink amide resin as described previously.[13,14,16] After Fmoc deprotection of Tyr, the amino terminus of the resin-bound Enk is readily accessible for functionalization with propiolic acid to give **1** (Scheme 1). The standard SPPS coupling protocol[16] however had to be modified because propiolic acid precipitates upon addition of DIPEA to the coupling mixture. Initially, HATU-activated propiolic acid was added for 5 min to the resin without adding additional base, which resulted in incomplete coupling. Secondly, the resin was treated for 1 min with 10% DIPEA in DMF. These two steps are repeated up to eight times, until a negative Kaiser test indicated coupling to all available *N*-terminal amino groups. For comparison, acetylated enkephalin (Ac-Enk-NH_2, **3**) was obtained by treatment of Enk on the resin with a mixture of acetic anhydride and DIPEA.

The enkephalins were cleaved from the resin with 95% TFA to give the peptide amide. After precipitation with cold ether, dissolving in H_2O–CH_3CN and lyophilization, the peptides **1** and **3** were obtained in almost quantitative yields. Analytical HPLC showed 95% purity and therefore, both peptides were used without further purification. To obtain the cobaltcarbonyl–alkyne enkephalin **2**, **1** was reacted under Schlenk conditions stoichiometrically with $Co_2(CO)_8$ in THF.[17] After complete addition, no further CO evolution was observed, the solution was filtered, and



Scheme 1 Synthesis of the cobalt–alkyne Enkephalin conjugate **2**, see text for details. Standard SPPS conditions for the preparation of resin-bound Enk: coupling with 5 eq. amino acid, 4.9 eq. TBTU, 5 eq. HOBt, 10 eq. DIPEA in DMF for 20 min, deprotection with 20% piperidine in DMF for 10 min.

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the solvent was carefully evaporated. Compound **2** was obtained almost quantitatively and in over 90% purity as shown by analytical HPLC. The reaction is quantitative within minutes, tolerates functional groups present in peptides, and is not sensitive to temperature and light. The organometallic peptide conjugate is stable under the conditions for biological testing, *i. e.* it is not sensitive to air or water (see Fig. S4–S6, ESI, for additional data on stability of **2**).†

All peptides were fully characterized using HPLC, MS, NMR and IR spectroscopy.‡† The cobaltcarbonyl–alkyne enkephalin **2** exhibits three strong absorption bands from the cobalt-bound carbonyls at 2103, 2064 and 2034 cm^{-1}. Peptides do not exhibit any infrared bands between 1800 and 2300 cm^{-1}, which is therefore an ideal spectroscopic window (Fig. 1).[18]

In electrospray ionization mass spectrometry (ESI-MS neg.), all peptides show the $[M - H]^-$ peak as the base peak. In positive ion detection mode (ESI-MS pos.) for **2**, the $[M + H]^+$ peak is also clearly detectable. Upon fragmentation of this peak (MS^2), a characteristic pattern of sequential loss of all six CO ligands is observed, leading to the fragment $[M + H - 6\ CO]^+$ as the base peak. ^{1}H- and ^{13}C-NMR signals of the peptides could be assigned completely using standard 2D NMR-spectroscopy based on previously reported data for metal–enkephalin derivatives.[13] A broad signal for Co–CO at 200 ppm in the ^{13}C-NMR is indicative of metal-bound CO.

In vitro cytotoxicity of the peptides **1**, **2** and **3** was studied on HeLa epithelial cervix carcinoma cells and HepG2 hepatocytoma cells. Cell viability, which correlates with the metabolic activity of a cell, was determined by the resazurin assay.[19] In addition to the cell viability, absolute cell numbers were determined by the crystal violet assay,[20] which can be applied after elution of resazurin.†

The assays have been carried out on 96 well plates. As a positive control, the established cytostatic vinblastine was used. The negative control consisted of untreated cells. Due to the poor water solubility of the peptide conjugates, DMSO stock solutions had to be used. Because DMSO itself is cytotoxic in higher concentrations, final DMSO concentrations were adjusted to 1% in all samples.

Solutions of the conjugates **1**, **2**, **3** and the controls were applied after 12 h of preincubation. The cells were treated with concentrations of 1 mM, 0.5 mM and 0.1 mM in quadruplicate. After incubation with the compounds for 48 h the resazurin assay was carried out, followed by the crystal violet assay. The relative cell viability or cell numbers, respectively, were calculated as the percentage absorption of treated cells compared to untreated cells. The results for both assays on the two different cell lines are comparable.‡ Data for the crystal violet assay on HeLa cells are shown in Fig. 2 as a representative example.

The cobaltcarbonyl–alkyne enkephalin **2** shows a cytotoxicity comparable to that of vinblastine on both HeLa and HepG2 cells in every concentration tested. The non-metallated peptides **1** and **3** show no toxic influence on the cells under the same conditions. Also, $Co_2(CO)_8$ itself is far less cytotoxic than **2** (Fig. S7, ESI).† As further reference points, the cytotoxicity of cobaltcarbonyl complexes of different alkynes has been studied by Gust and co-workers and generally found to be lower than that of the cobalt–ASS lead structure.[7]

There is considerable interest in new cytostatics with a different mechanism of action. Metal-based drugs in particular offer great potential.[2] Cobaltcarbonyl–alkyne derivatives of estradiol and other hormones were proposed for the study of receptor interactions and as analytical tools.[21] Jung, Gust, and co-workers discovered cobaltcarbonyl–alkyne derivatives of NSAIDs as promising organometallic lead structures.[3,4] It was suggested that this class of compound acts *via* inhibition of cyclooxygenase enzymes (COX),[2,6,7] which are also the molecular targets of most NSAIDs.[22] Although there is a regulatory dependence between enkephalins and COX inhibitors, Enks do not act as direct COX inhibitors. On the other hand, the cobaltcarbonyl–alkyne peptide conjugate **2** still exhibits strong anti-proliferative activity on two cancer cell lines. Control experiments show that this activity is due to the organometallic fragment. This finding questions COX inhibition as the mode of action for anti-proliferative activity at least for this cobaltcarbonyl–alkyne derivative.

Moreover, conjugation of a cytotoxic metal fragment to a peptide paves the way to targeted cytostatics, in which the peptide delivers the active moiety selectively to cancer cells. Suitable peptides can be readily prepared by SPPS. As shown in this work, cobaltcarbonyl–alkyne derivatives are easily and quantitatively available and are sufficiently stable under physiological conditions. We propose that in the present work the relatively lipophilic enkephalin peptide serves as an uptake vector to deliver the metal

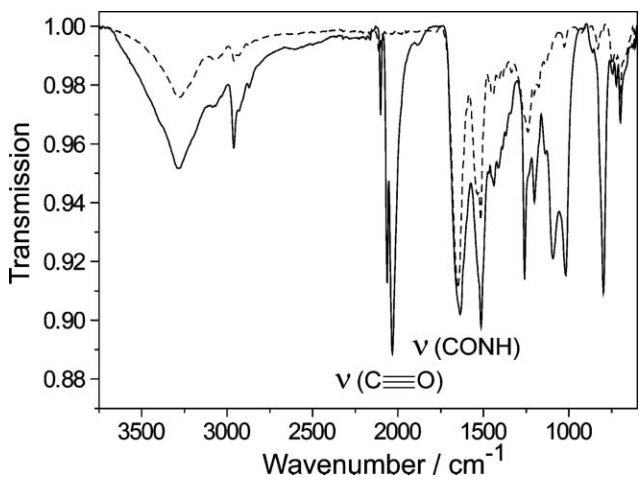

Fig. 1 IR-spectra (ATR) of **1** (dotted line) and the $Co_2(CO)_6$ complex **2** (solid line).

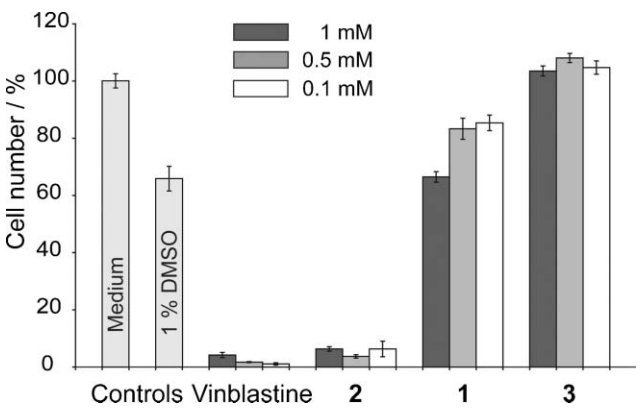

Fig. 2 Cytotoxicity studies of **1**–**3** on HeLa cells using crystal violet staining.

carbonyl alkyne moiety into the cells, where the organometallic fragment exerts its cytotoxic effect. Results from this work also suggest that continued effort to search for the mechanism of action of this interesting and promising class of compound is needed. Further research along those lines, as well as to identify suitable tumor-specific peptide conjugates with the $Co_2(CO)_6$–alkyne group, is already under way in our laboratory.

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Notes and references

‡ Selected spectroscopic data. **1**: R_t = 12.2 min, MS (ESI$^-$, MeOH): *m/z* 605 [M − H]$^-$, ^{1}H NMR (MeOH-d_4, 600.13 MHz): 7.31 (m, $\mathbf{H}_{o,m\text{-Phe}}$), 7.25 (m, $\mathbf{H}_{p\text{-Phe}}$), 7.08 (d, $\mathbf{H}_{Tyr}$), 6.74 (d, $\mathbf{H}_{Tyr}$), 4.63 (m, $C_{\alpha,Tyr}\mathbf{H}$), 4.57 (t, $C_{\alpha,Phe}\mathbf{H}$), 4.38 (m, $C_{\alpha,Leu}\mathbf{H}$), 3.82 (m, $C_{\alpha,Gly}\mathbf{H_2}$), 3.63 (s, C≡C**H**), 3.22 (dd, $C_{\beta,Tyr}\mathbf{H}$), 3.08 (dd, $C_{\beta,Phe}\mathbf{H}$), 3.03 (dd, $C_{\beta,Tyr}\mathbf{H}$), 2.91 (dd, $C_{\beta,Phe}\mathbf{H}$), 1.65 (m, $C_{\gamma,Leu}\mathbf{H}$, $C_{\beta,Leu}\mathbf{H_2}$), 0.94 (dd, CH($C_{\delta,Leu}\mathbf{H_3}$)$_2$). **2**: R_t = 16.4 min, MS (ESI$^+$, MeOH): *m/z* 725 [M − 6 × CO + H]$^+$, ^{1}H NMR (DMSO-d_6, 400.13 MHz): 9.08 (s, $O_{Tyr}\mathbf{H}$), 8.27 (t, $N_{Gly}\mathbf{H}$), 8.10 (m, 4H, $N_{Gly,Phe,Tyr,Leu}\mathbf{H}$), 7.24 (m, $\mathbf{H}_{Phe}$), 7.01 (d, $\mathbf{H}_{Tyr}$), 7.00 (d, CON$\mathbf{H_2}$), 6.61 (s, $Co_2C\mathbf{H}$), 6.58 (d, $\mathbf{H}_{Tyr}$), 4.55 (m, $C_{\alpha,Tyr,Phe}\mathbf{H}$), 4.20 (m, $C_{\alpha,Leu}\mathbf{H}$), 3.74 (m, $C_{\alpha,Gly}\mathbf{H_2}$), 3.01–2.79 (m, $C_{\beta,Tyr,Phe}\mathbf{H}$), 1.56 (m, $C_{\gamma,Leu}\mathbf{H}$), 1.47 (t, $C_{\beta,Leu}\mathbf{H_2}$), 0.85 (dd, CH($C_{\delta,Leu}\mathbf{H_3}$)$_2$). **3**: R_t = 11.9 min, MS (ESI$^-$, MeOH): *m/z* 595 [M − H]$^-$.

Cell viability [% of control]: HeLa/CV: (1% DMSO: 66) Vin: 4/2/1 (1 mM/0.5 mM/0.1 mM), **1**: 67/83/85, **2**: 6/3/6, **3**: 103/108/105; HeLa/Res: (62) Vin: 11/3/3, **1**: 87/91/92, **2**: 4/6/18, **3**: 103/108/100; HepG2/CV: (71) Vin: 5/3/4, **1**: 26/36/62, **2**: 10/3/3, **3**: 83/85/82; HepG2/Res: (78) Vin: 4/5/15, **1**: 54/50/37, **2**: 6/6/25, **3**: 86/84/100.

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