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Modification with Organometallic Compounds Improves Crossing of the Blood–Brain Barrier of [Leu^5]-Enkephalin Derivatives in an In Vitro Model System

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Enkephalin peptides are thought to be suitable vectors for the passage of the blood–brain barrier (BBB). Modifications that do not alter the amino acid sequence are often used to improve the permeation through living membrane systems. As a new type of modification we introduce organometallic compounds, in particular ferrocene carboxylic acid. Derivatives of [Leu^5]-enkephalin were synthesised and labelled with organometallic compounds by using solid-phase synthesis techniques. All new metal–peptide bioconjugates were comprehensively characterised by HPLC, NMR spectroscopy and mass spectrometry and found to be at least 95% pure. For the first time, permeation coefficients in a BBB model for organometal–peptide derivatives were determined in this work. The uptake and localisation of fluorescein-labelled enkephalins was monitored by fluorescence microscopy on three cancer cell lines. Octanol/H_2O partition coefficients of the compounds were measured by HPLC. The introduction of the organometallic moiety enhances the uptake into cells and the permeation coefficient of [Leu^5]-enkephalin. This could be due to an increase in lipophilicity caused by the organometallic label. The metal–peptide conjugates were found to be nontoxic up to mM concentrations. The low cytotoxicity encourages further experiments that could take advantage of the selectivity of enkephalin derivatives for opioid receptors.

Introduction

The isolation of the peptide neurotransmitters [Leu^5]-enkephalin and [Met^5]-enkephalin from the brain of mammalians in 1975[1] and the discovery of their analgesic effect[2] led to intense pharmacological research of the enkephalins.

The five amino acid-containing enkephalins and most of the other small neuropeptides are cleaved from large precursor proteins or peptides, which are produced by protein biosynthesis directly inside neuronal cells and are rapidly cleared out of the liquor and bloodstream due to their instability towards peptidases.[3–5] It has been shown that the relatively small enkephalins and other small peptides are primarily taken up by the brain through a saturable peptide transport mechanism[6–8] or by adsorptive-mediated endocytosis.[9,10] However, the amount of enkephalin that penetrates through the blood–brain barrier (BBB) is very limited and insufficient for biomedical studies. The BBB is the interface between blood and brain and is primarily formed of brain capillary endothelial cells (BCEC), which line all cerebral blood vessels.[11] BCEC have special neuroprotective characteristics, for instance, tight junctions and low vesicular transport, which impede the transport of compounds in a range from a few hundred Daltons to several kD; this makes the BBB the most important physical, metabolic and immunological barrier for most solutes.[12] In the last two decades lipidation and glycosylation of peptides was under investigation because such modifications alter the interaction between peptides and membranes as well as their metabolic stability.[13–15]

Nowadays the development of solid-phase synthesis (SPS) lipidation and glycosylation methods make it possible to synthetically produce peptides labelled with lipids, glycosides and neoglycosides.[16–18] Additionally, lipidation can be used to enhance drug delivery by increasing the lipophilicity of the transfecting cationic peptides,[19] whereas glycosylation greatly influences serum stability and biodistribution of peptides, including penetration of the BBB.[15,20]

In the case of enkephalins both modifications were investigated to improve uptake into the brain. Adamantyl-labelled derivatives were synthesised that show higher in vivo activities than the nonlipidated compound.[21] Further modifications include the conjugation of enkephalins to lipidated amino acids; similar modifications were also successfully applied to other therapeutic peptides.[22] Glycoenkephalin derivatives were synthesised early on, but the interest at the time focussed on receptor binding and structural conformation.[23–25]

Increased BBB passage was demonstrated in the following years indirectly by an increased analgesic effect of in vivo administered glycosylated enkephalin derivatives, which were subsequently improved.[26–30] Lipidation of peptides improves BBB penetration, similar to the fact that an increase in lipophi-

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licity usually increases the efficacy of small-molecule, CNS-related drugs. This approach, however, does not work for glycosylated peptides. Investigation of interactions with membranes led to the recognition that these peptides follow the concept of Biousian theory,[31] which does not rely on lipophilicity but rather on the amphiphilic nature of glycopeptides.

This work was inspired by our observation that covalent labelling of bioactive peptides with organometallic compounds leads to significant cellular uptake of such conjugates even for peptides that are not usually readily incorporated into cells, such as the simian virus nuclear localization sequences (NLSs).[32,33] We thus hypothesised that the functionalization of neuropeptides with organometallic groups might be a way to increase their permeation through the BBB. We show here that ferrocene–enkephalin conjugates in particular are readily prepared, stable and nontoxic organometallic peptide derivatives with increased BBB penetration. These results are supported by the fact that such conjugates are readily incorporated even into cell lines that do not express the enkephalin receptor; hence, this rules out receptor-mediated uptake.

Ferrocene (dicyclopentadienyliron, Cp_2Fe, FcH) is a well-known and thoroughly characterised organometallic compound that is formed by a central iron atom and two cyclopentadienyl rings to give a stable so-called "sandwich" complex that fulfils the 18-electron rule. The isoelectronic cobaltocinium cation possesses very similar properties except for an additional positive charge, and in this way achieves the stable 18-electron configuration.

Administration of ferrocene to dogs and mice in high doses over a long period of time led to moderate, reversible side effects and intact ferrocene molecules were extracted from the faeces of the dogs, which suggests high metabolic stability and a low intrinsic toxicity for ferrocene compounds.[34,35]

Furthermore ferrocene-containing compounds have recently been proposed as promising anticancer (Ferrocifen)[36] and antimalarial drug candidates (Ferroquine, successfully finished phase II clinical trials in France).[37]

Several approaches to introduce ferrocene into the side-chain of enkephalin were investigated in the past, with limited success mostly due to experimental problems. The replacement of Phe4 in the enkephalin sequence by the artificial amino acid ferrocenylalanine (Fer) led to reduced receptor affinity. Furthermore it was difficult to obtain enantiomerically pure compounds.[38,39] Several groups have explored ways to couple ferrocene derivatives to amino acids.[40,41] In our group, mild conjugation methods were successfully employed to link ferrocene to amino acid side-chains and peptide nucleic acids (PNA), such as Sonogashira coupling[42] and "click chemistry".[43,44] The most promising metallocene derivatives for standard solid-phase peptide chemistry (SPPS), however, are still carboxylic acid derivatives as shown in Figure 1 for ferrocene and cobaltocenium. This is due to their facile synthesis, insensitivity to air and moisture, and high stability under the conditions of SPPS.[45–49]

We present the synthesis and biological characterisation of metallocenoyl [Leu^5]-enkephalin derivatives. BBB permeation experiments were conducted in a porcine BBB model and correlated to experimentally determined log *P* values of the conjugates. To the best of our knowledge, permeation of the BBB has never been investigated for metal–peptide conjugates before. Furthermore, live-cell uptake experiments were carried out on fluorescein-labelled ferrocenoyl enkephalin derivatives.

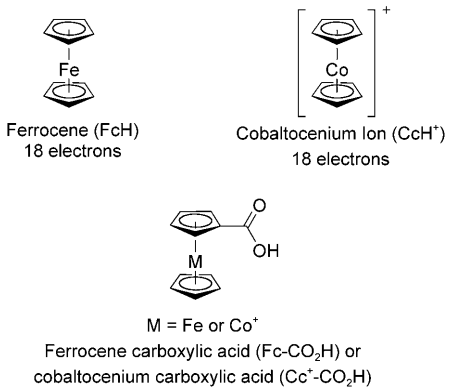

Figure 1. Metallocenes that were used in this study.

Results

All compounds in this study were synthesised by following standard solid-phase peptide synthesis protocols with slight modifications to incorporate the metallocenes.[50] After completion of the syntheses, peptides were cleaved from the resin, precipitated with cold ether, lyophilized and purified by preparative HPLC when necessary. In this way, $>95\%$ purity was ensured for all compounds. In addition to metallocene derivatisation, we have also introduced fluorescein to enable fluorescence microscopy studies. To this end, an additional 4-methyltrityl-N^6-protected lysine was introduced after completion of the enkephalin sequence. This was selectively deprotected by 1% trifluoroacetic acid (TFA) on the resin. Reaction with fluorescein isothiocyanate (FITC) leads to fluorescein–enkephalin derivatives, which were subsequently N-terminally derivatised with ferrocene. The identity of the peptides was confirmed by electrospray ionisation mass spectrometry (ESI-MS) and NMR spectroscopy. All signals of the peptide could be assigned by standard homo- and heteronuclear 1D and 2D NMR spectroscopic techniques. Characteristic signals for the metallocenes around 4–5 ppm provide an easy means of identification of metallocene–peptide conjugates.

Determination of log *P* values by RP-HPLC

The octanol/water partition coefficient (log *P* value) describes a compound's lipophilicity. Because ferrocene is a lipophilic compound, conjugation to a hydrophilic peptide should lead to a shift towards higher log *P* values for the modified peptide, which would also improve permeation through the BBB. To prove this assumption, $\log k_w$ values of all compounds were determined by reverse-phase high-performance liquid chromatography (RP-HPLC) and converted to log *P* values by comparison to reference compounds.[51] The log *P* values are listed in

Table 1. The parent [Leu^5]-enkephalin **1** is hydrophilic and thus has a negative log *P* value (Figure 2).

N-terminal modification by acetylation (**2**) or iodophenylation (**7**) of [Leu^5]-enkephalin shifts the distribution of the compound towards the octanol phase, thus increasing log k_w and consequently log *P*. Conjugation of **1** to metallocenes in **3** and **4** also increased the log *P* value significantly. The ferrocenoyl–enkephalin **3** actually has a higher log *P* value than ferrocene itself.[52] Addition of fluorescein as in **9** leads to a further increase in log *P*. For comparison, we have also prepared N_ε-fluorescein-lysine with (**6**) and without (**5**) ferrocene.

Table 1. Octanol/water partition coefficients for the compounds that were used in this study.[a]

Compound	log k_w	log *P*	Compound	log k_w	log *P*
1	−1.74	−1.20	**2**	4.35	4.90
3	5.23	5.79	**4**	1.63	2.18
5	7.99	8.45	**6**	9.53	10.00
7	6.55	7.11	**9**	7.51	7.97
ferrocene	–	2.66/3.54[b]			

[a] log *P* and log k_w determined by the method of Minick et al.[51] [b] Data from ref. [52] (experimental/calculated).

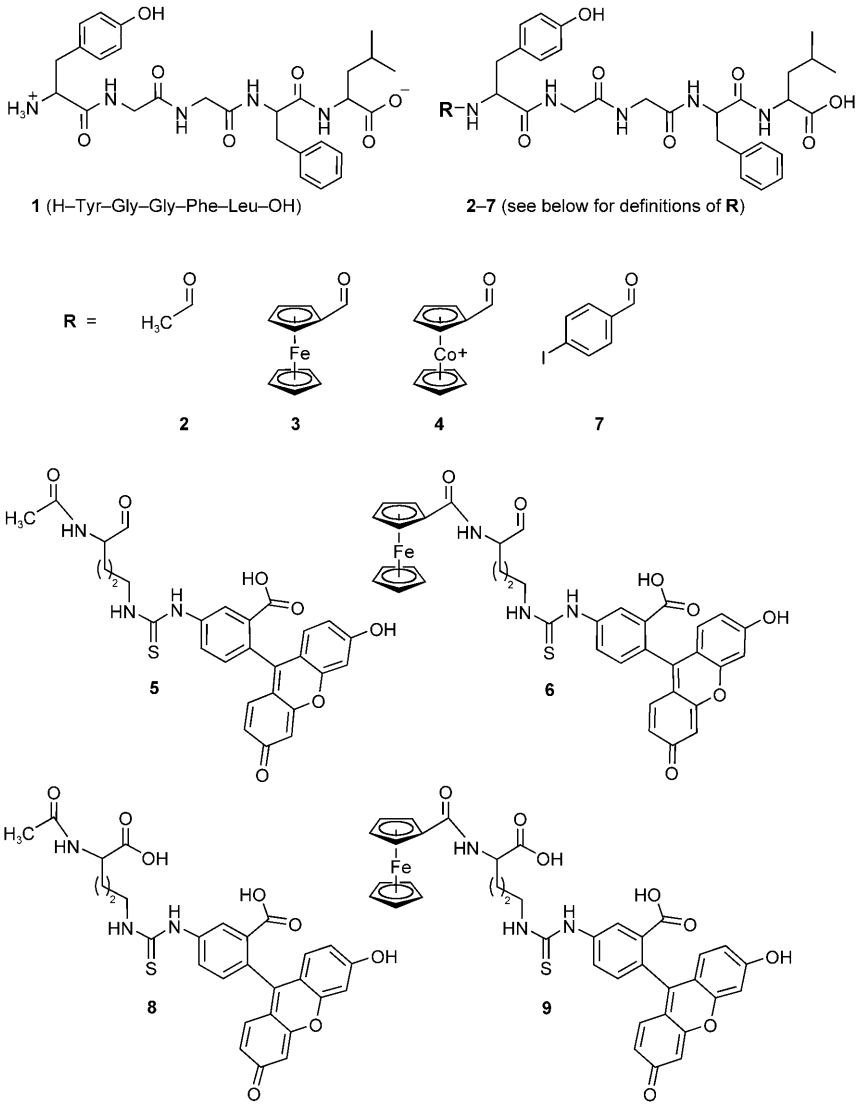

Figure 2. Metallocene [Leu^5]-enkephalins and other compounds that were used in this study.

Permeation of the blood-brain barrier in vitro

To assess the potential of blood–brain barrier permeation of **1**–**4**, the permeability of these compounds was tested in an in vitro brain endothelial cell (porcine brain capillary endothelial cell, PBCEC) system. PBCEC were isolated from pig brains as recently described.[53,54] Briefly, cells were isolated from brains obtained from the local slaughterhouse by a combined mechanical/enzymatic procedure and were cultured as cell monolayers on permeable filter supports. Confluency was reached after seven days of culture. The integrity of the monolayers was checked by determination of the permeability of carboxyfluorescein, a nonpermeable paracellular marker. Then, the substances were applied at 50 μM concentration on the apical side ("blood-oriented" cell surface). Samples were taken from the basal side ("brain-oriented" cell surface) at 30, 60 and 90 min after the start of the experiment. The concentrations of the compounds at the basal side were determined by HPLC. From the slopes of the concentration–incubation time curves the apparent permeation coefficients (P_{app}) were calculated as described in the Experimental Section.

The permeated amount of substances **1**–**4** is plotted against the permeation time in Figure 3, calculated P_{app} values are summarized in Table 2. Compounds **2**–**4** all reach higher P_{app} values than unmodified [Leu^5]-enkephalin **1**. The ferrocenoyl–enkephalin **3** has the highest P_{app} value, whereas the permeation coefficient for the acetylated and the cobaltacenium derivatives are almost identical.

Uptake of fluorescence-labelled enkephalins in cancer cell lines

As shown by the BBB experiments, metallocene labels improve the membrane-crossing properties of [Leu^5]-enkephalin. To further investigate this property, the cellular uptake of met-

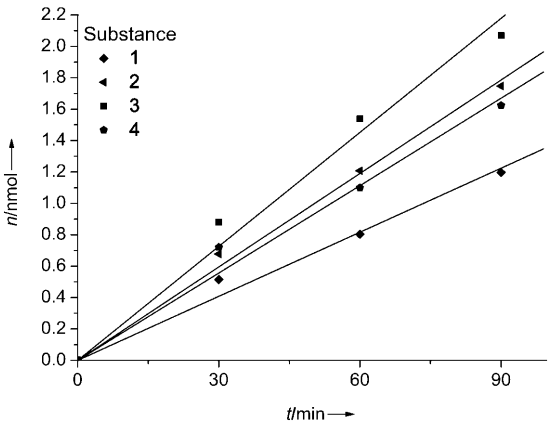

Figure 3. Plot of permeated amount of substances **1**–**4** in nmol against permeation time. Lines show the linear fit of the obtained data points

Table 2. Apparent permeation coefficients (P_{app} values). All values were determined in triplicate (mean value and standard error).

Compound	P_{app} [cm s^{-1}]	Error [cm s^{-1}]	Compound	P_{app} [cm s^{-1}]	Error [cm s^{-1}]
1	0.38×10^{-5}	0.04×10^{-5}	**2**	0.57×10^{-5}	0.17×10^{-5}
3	0.68×10^{-5}	0.13×10^{-5}	**4**	0.52×10^{-5}	0.12×10^{-5}

allocene enkephalins was determined by fluorescence spectroscopy. HeLa, HepG2 and HT-29 cell lines were incubated for 6 and 24 h with the fluorescein-labelled enkephalins. The cancer cells were incubated with a 20 μM solution of compounds **5**, **6**, **8** and **9**. Compound **5** only was also evaluated at 85 μM. At the end of the incubation period, cellular integrity was confirmed with propidium iodide. After several washing cycles, phase contrast and fluorescence microscopy pictures were recorded with a wide-field microscope. Fluorescence was quantified in ten representative, manually chosen regions of interest (ROIs).

The determined values are shown in Figure 4. For control compounds **8** and **9**, fluorescence intensity was low after 6 h and 24 h. Incubation with a 20 μM solution of compound **5** led to low fluorescence intensity after 6 h and to a slight increase after 24 h. The higher 85 μM concentration of **5** gave rise to a high intensity after 6 h which strongly decreased after 24 h. Compound **6** clearly shows better accumulation in HeLa and HepG2 cells than **5** after 24 h. The fluorescence intensity for **6** in HT29 cells was low after 6 h and did not increase significantly after 24 h; this is different from the Hela and HepG2 cells. One reason could be the pronounced extracellular matrix of HT-29 cells.[55] Observation with a 100× oil-immersion objective showed green fluorescent vesicular structures, which were more abundant with compound **6**, in all cell lines.

Cytotoxicity of compounds 3 and 4 on cancer cell lines

Compounds **3** and **4** were tested for cytotoxicity on the three cancer cell lines (Hela, HT-29 and HepG2). The viability of the cells was determined by the colorimetric resazurin assay, which relies on mitochondrial activity. Crystal violet assays were performed simultaneously to monitor the cell biomass. Both assays gave similar results. The substance was applied to the cells regardless of the concentration in RPMI medium with 0.5% DMSO for 48 h. Data for compound **3** are presented in Figure 5. At low-micromolar concentrations no detrimental effect could be seen on any of the cell lines. Increasing the concentration to 750 μM and 1500 μM decreased cell viability. Similar results were obtained for compound **4** (see the Supporting Information).

Discussion

The metallocene peptides could be easily synthesised in high yield and purity by following standard peptide synthesis procedures. To prevent the decomposition of ferrocene during cleavage, phenol was used in place of water. A purity of at least 95% was achieved and confirmed by HPLC. Spectroscopic data confirm the constitution of the peptides and characteristic signals for the metallocenes can be readily used for identification. An examination of compound **9** after three weeks of storage at 4 °C in culture medium by HPLC did not show any signs of

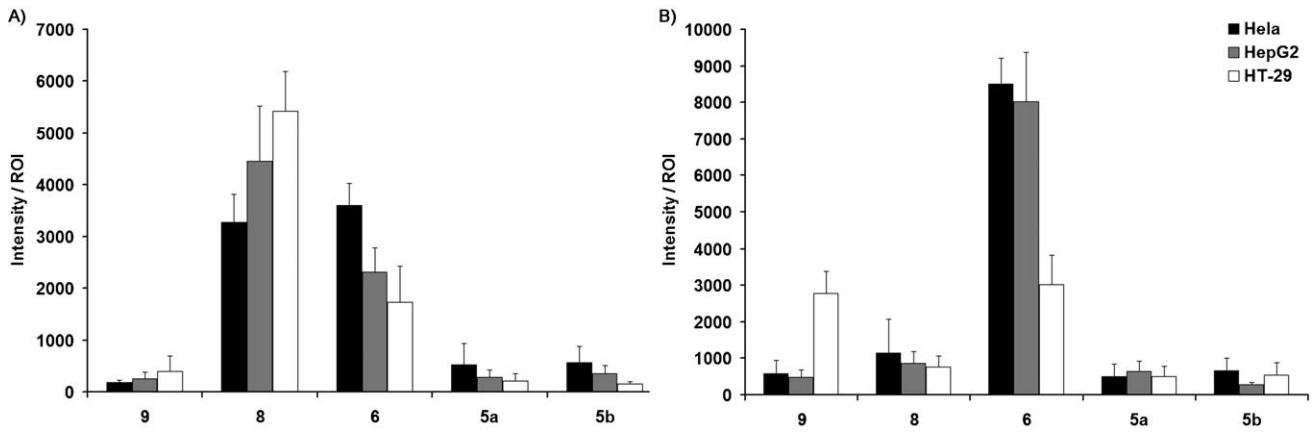

Figure 4. Fluorescence intensity determination on cancer cell lines. A) After 6 h and B) 24 h of incubation of HepG2, HT-29 and HeLa cells with the compounds **5**, **6**, **8** and **9**. 20 μM Concentration was used for compounds **6**, **8** and **9**. For compound **5** two concentrations were tested: 20 μM (**5a**) and 85 μM (**5b**). Ten ROIs per picture were determined. Data shown are average values ± standard error of the mean (SEM).

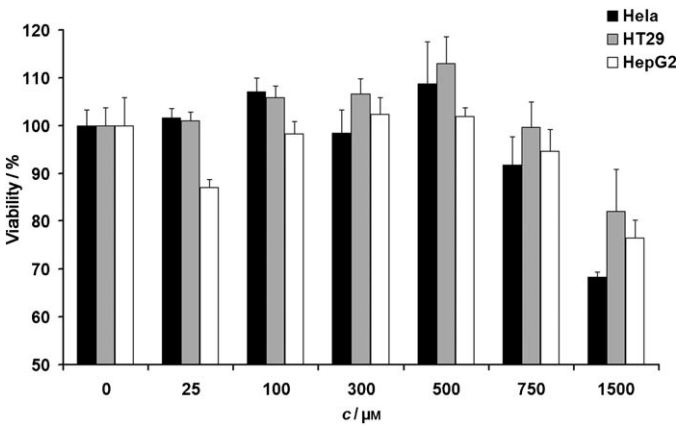

Figure 5. Cytotoxicity of **3** on HeLa, HT29 and HepG2 cells. Viability is shown in percent ± SEM (standard error mean). Substances were applied in the given concentrations in culture medium 0.5% DMSO. Viability at 0 μM was set to 100%.

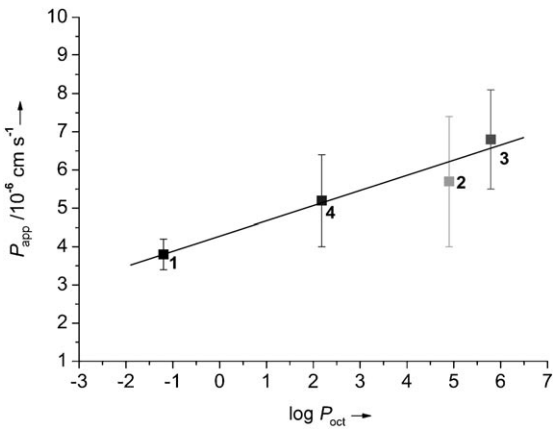

Figure 6. Correlation of log *P* versus apparent permeation coefficient P_{app} of compounds **1**–**4**. Data shown are ± standard error. The black line represents a linear correlation.

degradation (data not shown); this indicates good chemical stability and resistance to proteases, which could positively influence the accumulation in the cell culture experiments.

The log *P* values of peptides and metallocene peptides in this study span a wide range. A log *P* value of around −1.2 for [Leu^5]-enkephalin **1** is consistent with the literature.[56] All of the studied modifications of enkephalin lead to an increase in log *P*. N-terminal acylation thus generally increases lipophilicity by suppressing protonation or hydrogen-bond formation at the N-terminal nitrogen atom. Iodophenylation, which is known to strongly increase lipophilicity, leads to a log *P* of 7.11 in compound **7**. The metallocenes in **3** and **4** cause the same effect, but to a lesser extent. In case of the cobaltocenium peptide **4**, the effect is obviously diminished due to the positive charge of cobaltocenium group. Ferrocenoylation in **3**, however, raises the log *P* value above the calculated and experimental value for unmodified ferrocene;[52] this reveals a synergistic effect between peptide and metal organic moiety, which was also reported for other ferrocene-modified molecules.[57] Additionally the log *P* of 5.79 for **3** suggests suitability for brain targeting as suggested by structure–activity relationship studies (SARS) of other neuropharmaceuticals.[58] Overall, these results confirm our working hypothesis: the addition of a metallocene moiety can increase the lipophilicity of peptides, with or without a fluorescein label.

Ferrocene conjugates reach log *P* values that could be favourable for membrane permeation, in particular permeation of the BBB. This assumption was confirmed in a BBB model system consisting of a confluently grown PBCEC monolayer. In the PBCEC monolayer experiments, the highest apparent permeation coefficient was determined for **3**, the ferrocene-modified enkephalin. The P_{app} values from BBB experiments in Table 2 correlate well with the log *P* values of **1**–**4** and thus with lipophilicity, as can be seen from Figure 6.

A correlation between lipophilicity and permeation, as observed in our experiments, concurs most obviously with passive diffusion, however, it does not strictly rule out a receptor-mediated uptake. The good correlation between permeation and lipophilicity suggests no or only a very low interaction of all compounds with the export proteins that are expressed to a large extent in the BBB.[59] Although the permeation coefficient values are relatively low, modification with ferrocene significantly enhanced the permeation. Similar results could also be found in cellular uptake experiments on three tumour cell lines, in which significantly increased fluorescence of the ferrocene-modified enkephalins could be detected in two out of three cell lines. For cobaltocenium peptides a higher uptake could also be detected in previous work.[32] The cell lines were chosen because under cell culture conditions they do not express receptors with a high affinity for [Leu^5]-enkephalin.[60–62] Most probably the localised cellular fluorescence results from a slow endocytotic process like adsorptive endocytosis, because an increase in the fluorescence intensity for **6** is clearly visible after 24 h hours, whereas in the case of diffusion or active transport a higher uptake after 6 h would be expected. If adsorptive endocytosis were the mechanism of membrane crossing, this could explain why no bigger increase in BBB permeation could be achieved, because PBCEC show reduced endocytosis as one of their neuroprotective properties.[12]

The amount of substance used in the fluorescence experiments was noncytotoxic for the cell lines, as can be seen from the cytotoxicity results. Only very high concentrations (mM) of compound **3** seem to damage cells. The mechanism of cytotoxicity of ferrocene compounds has been debated controversially.[63] Most likely, cytotoxicity is derived from Fenton chemistry of the ferrocene core, and this leads to damage of the cell membranes. Along those lines, an increased uptake into endosomes and intracellular localisation would actually diminish toxicity in the sense that less free ferrocene would be available for interaction with the cell membranes.

Conclusions

To the best of our knowledge, the present work presents the first experiments on the permeation of the BBB for metal–peptide conjugates. Such investigations are highly relevant, given

the rising interest in medicinal organometallic chemistry, which is progressing from a mainly synthesis-driven discipline to the study of relevant biological properties of the new compounds and compound classes.[64–68]

The results presented herein suggest that metallocenes can be used to modify endogenous peptides and alter their physicochemical properties, which in turn leads to improved pharmacokinetics in vitro and probably in vivo. The versatility of the system and molecular uptake mechanisms have been studied for NLS derivatives in detail.[33,33] The increase in lipophilicity makes metallocenes suitable to modify peptide drugs that target the brain. Recently, such an approach was successfully adapted for ferroquine, a ferrocene-containing structural mimic of the antimalarial compound cloroquine, which also shows improved pharmacokinetics.[37] The good stability and ready chemical modification, along with low toxicity, makes the metallocenes investigated herein well suited for applications in solid-phase synthesis techniques and biological applications at the same time. Moreover, the specific properties of the metal make such conjugates amenable to metal-specific detection techniques, such as atomic absorption spectroscopy (AAS), which has been recently used by our group to quantify the uptake of peptides and peptide-PNA conjugates in HepG2 cells.[69] The present work indicates that organometal–peptide conjugates might also be advantageously used for imaging and targeting of brain tissues in vivo.

Experimental Section

All materials and reagents that were used were of peptide grade or analytical grade purity. Reagents for peptide chemistry were purchased from Novabiochem (Darmstadt, Germany) or Iris Biotech (Marktredwitz, Germany). All amino acids were enantiomerically pure L amino acids. Mass spectrometry: peptides were dissolved in MeOH and analysed by a Bruker Esquire 6000 Ion Trap with an electrospray ionisation source (ESI-MS). NMR spectroscopy: ^{1}H and ^{13}C NMR spectra were recorded on Bruker NMR spectrometers operating at ^{1}H frequencies of 300, 360 or 400 MHz as detailed below. NMR spectra were referenced to TMS by using the ^{13}C or residual protio signals of the deuterated solvents as internal standards.

Metallocene and solid-phase peptide synthesis: Ferrocene and cobaltocenium carboxylic acid synthesis was carried out according to the literature.[48,49] If not stated otherwise, all solid-phase syntheses have been carried out according to standard procedures.[50] Fmoc-Leu-Wang resin was swelled for 1 h in dimethylformamide (DMF). The N-terminal Fmoc protecting group was removed by treatment with a solution of piperidine (20%) in DMF.[70] After each deprotection and coupling step, the resin was excessively washed with DMF to remove any residual activation or deprotection reagents. Amino acids (fourfold excess) were mixed with equimolar amounts of *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) and 1-hydroxybenzotriazole (HOBt) until a clear solution was obtained. The coupling was performed for 45 min for all amino acids and 1–2 h for metallocene compounds. N-terminal acetylation was carried out by treatment with a mixture of Ac_2O and iPr_2EtN (5%). For selective deprotection Fmoc-Lys-(Mtt)-OH was introduced N-terminally into the peptide chain, which was treated with up to 7×2 min with TFA (trifluoroacetic acid, 1%) in CH_2Cl_2.[71] For fluorescence labeling a fourfold excess of FITC and ten equivalents of iPr_2EtN were used to couple to the unprotected side-chain of lysine. After the synthesis was finished, the peptidyl-Wang resin was treated with a mixture of TFA (95%), H_2O (2.5%) and triisopropylsilane (TIS) (2.5%, 6 mL per g resin) for 3 h. For ferrocene conjugates, the cleavage mixture consisted of 85% TFA, 10% phenol and 5% TIS.[50]

H-Tyr-Gly-Gly-Phe-Leu-OH (1): $C_{28}H_{37}N_5O_7$ (555.27 g mol^{-1}): ^{1}H NMR ([D$_6$]DMSO, 300.14 MHz): δ = 12.61 (br s, 1H; COOH), 9.35 (br s, 1H; OH_{Tyr}), 8.74 (t, J = 5.0 Hz, 1H; NH_{Gly}), 8.35 (d, J = 7.8 Hz, 1H; NH_{Leu}), 8.11 (t, J = 5.4 Hz, 1H; NH_{Gly}), 8.04 (d, J = 7.3 Hz, 1H; NH_{Phe}), 7.26 (m, 5H; $H_{Ar,Phe}$), 7.06 (d, J = 8.2 Hz, 2H; $H_{Ar,Tyr}$), 6.71 (d, J = 8.2 Hz, 2H; $H_{Ar,Tyr}$), 4.58 (dt, J = 3.6 Hz, J = 9.2 Hz, 1H; $C_\alpha H_{Phe}$), 4.22 (m, 1H; $C_\alpha H_{Tyr}$), 3.98 (m, 1H; $C_\alpha H_{Leu}$), 3.74 (m, 4H; $C_\alpha H_{Gly}$), 2.90 (m, 4H; $C_\beta H_{Phe,Tyr}$), 1.64 (m, 1H; $CH(CH_3)_2$), 1.55 (m, 2H; $C_\beta H_{Leu}$), 0.88 (dd, J = 6.2, 17.4 Hz, 6H; $CH(CH_3)_2$); ^{13}C NMR ([D$_6$]DMSO, 75.47 MHz): δ = 173.7 (COO), 171.0, 168.4, 168.0 (CON), 155.6 ($C_{Ar}OH_{Tyr}$), 137.6 ($C_{Ar,q,Phe}$), 130.3 ($C_{Ar,q,Tyr}$), 129.1, 127.9, 126.1 (C_{Ar}), 124.7, 115.2 (C_{Ar}), 53.6 ($C_{\alpha,Phe}$), 53.4 ($C_{\alpha,Tyr}$), 50.2 ($C_{\alpha,Leu}$), 41.8, 41.5 ($C_{\alpha,Gly}$), 37.6 ($C_{\beta,\ Phe}$), 36.1 ($C_{\beta,\ Tyr}$), 24.2 ($C_{\beta,\ Leu}$), 22.7 ($CH_{3,\ Leu}$), 21.2 ($CH(CH_3)_2$); MS (ESI, −ve): m/z 1109.18 [2M−H] 554.27 [M−H].

Ac-Tyr-Gly-Gly-Phe-Leu-OH (2): $C_{30}H_{39}N_5O_8$ (597.28 g mol^{-1}): ^{1}H NMR ([D$_6$]DMSO, 400.13 MHz): δ = 12.55 (br s, 1H; COOH), 9.12 (br s, 1H; OH_{Tyr}),8.22 (d, J = 8.0 Hz, 1H; NH_{Leu}), 8.19 (t, J = 5.2 Hz, 1H; NH_{Gly}), 8.03 (d, J = 8.1 Hz, 1H; NH_{Tyr}), 7.97 (d, J = 8.0 Hz, 1H; NH_{Phe}), 7.89 (t, J = 5.3 Hz, 1H; NH_{Gly}), 7.23 (m, 5H; $H_{Ar,Phe}$), 7.00 (d, 2H; J = 8.2 Hz, $H_{Ar,Tyr}$), 6.61 (d, J = 8.2 Hz, 2H; $H_{Ar,Tyr}$), 4.54 (dt, J = 3.4 Hz, J = 8.6 Hz, 1H; $C_\alpha H_{Phe}$), 4.38 (m, 1H; $C_\alpha H_{Tyr}$), 4.21 (m, 1H; $C_\alpha H_{Leu}$), 3.63 (m, 4H; $C_\alpha H_{Gly}$), 2.89 (m, 4H; $C_\beta H_{Phe,Tyr}$), 1.75 (s, 3H; CH_3, acetyl), 1.62 (m, 1H; $CH(CH_3)_2$), 1.53 (m, 2H; $C_\beta H_{Leu}$), 0.86 (dd, 6H; J = 6.4, 16.4 Hz, $CH(CH_3)_2$). ^{13}C NMR ([D$_6$]DMSO, 100.61 MHz): δ = 173.8 (COO), 171.9, 171.0, 169.3, 168.9, 168.2 (CON), 155.7 ($C_{Ar}OH_{Tyr}$), 137.7 ($C_{Ar,q,Phe}$), 139.9 ($C_{Ar,q,Tyr}$), 129.2, 128.0, 127.9, 126.2, 114.6 (C_{Ar}), 54.4 ($C_{\alpha,Phe}$), 53.5 ($C_{\alpha,Tyr}$), 50.3 ($C_{\alpha,Leu}$), 42.0, 41.7 ($C_{\alpha,Gly}$), 37.6 ($C_{\beta,Phe}$), 36.6 ($C_{\beta,Tyr}$), 24.2 ($C_{\beta,Leu}$), 22.7 ($CH_{3,Leu}$), 22.4 (CH_3, acetyl), 21.3 ($CH(CH_3)_2$); MS (ESI, neg): m/z 1193.73 [2M−H] 596.30 [M−H].

Fc-CO-Tyr-Gly-Gly-Phe-Leu-OH (3): $C_{39}H_{45}FeN_5O_8$ (767.26 g mol^{-1}): ^{1}H NMR ([D$_6$]DMSO, 300.14 MHz): δ = 12.62 (br s, 1H; COOH), 9.12 (br s, 1H; OH_{Tyr}), 8.28 (d, J = 7.8 Hz, 1H; NH_{Leu}), 8.19 (t, J = 5.2 Hz, 1H; NH_{Gly}), 8.07 (d, J = 8.4 Hz, 1H; NH_{Phe}), 7.79 (d, J = 8.2 Hz, 1H; NH_{Tyr}), 7.25 (m, 5H; $H_{Ar,Phe}$), 7.17 (d, J = 8.1 Hz, 2H; $H_{Ar,Tyr}$), 6.67 (d, J = 8.3 Hz, 2H; $H_{Ar,Tyr}$), 4.83/4.72 (2s, 1H; $CpH_{2',5}$), 4.59 (m, 2H; $C_\alpha H_{Phe,Tyr}$), 4.30 (m, 2H; CpH3,4), 4.22 (m, 1H; $C_\alpha H_{Leu}$), 3.95 (s, 5H; CpH), 3.67 (m, 4H; $C_\alpha H_{Gly}$), 2.92 (m, 4H; $C_\beta H_{Phe,Tyr}$), 1.62 (m, 1H; $CH(CH_3)_2$), 1.54 (m, 2H; $C_\beta H_{Leu}$), 0.87 (dd, J = 6.3, 16.4 Hz, 6H; $CH(CH_3)_2$); ^{13}C NMR ([D$_6$]DMSO, 75.47 MHz): δ = 173.5 (COO), 171.8, 170.7, 168.8, 168.5, 167.8 (CON), 155.4 ($C_{Ar}OH_{Tyr}$), 137.4 ($C_{Ar,q,Phe}$), 129.6, 128.8 (C_{Ar}), 128.2 ($C_{Ar,q,Tyr}$), 127.6, 125.8, 114.5 (C_{Ar}), 75.5 ($CCp_{,1}$), 69.5 ($CCp_{,\ 3',4}$), 68.9 (CCp'), 68.2/67.4 ($CCp_{,\ 2',5}$), 54.3 ($C_{\alpha,Phe}$), 53.1 ($C_{\alpha,Tyr}$), 49.9 ($C_{\alpha,Leu}$), 41.7, 41.3 ($C_{\alpha,Gly}$), 37.2 ($C_{\beta,\ Phe}$), 35.7 ($C_{\beta,\ Tyr}$), 23.9 ($C_{\beta,\ Leu}$22.4 ($CH_{3,\ Leu}$), 21.0 ($CH(CH_3)_2$; MS (ESI, +ve): m/z 768.4 [M+H]$^+$, 790.5 [M+Na]$^+$, 806.4 [M+K]$^+$, MS (ESI, −ve): m/z 766.4 [M−H]$^-$.

Cc-CO-Tyr-Gly-Gly-Phe-Leu-OH (4): $C_{39}H_{44}CoN_5O_8$ (771.7 g mol^{-1}): ^{1}H NMR ([D$_6$]DMSO, 500.13 MHz): δ = 12.50 (br s, 1H; COOH), 9.24 (br s, 1H; OH_{Tyr}), 8.87 (d, J = 7.8 Hz, 1H; NHLeu), 8.19 (t, J = 5.2 Hz, 1H; NH_{Gly}), 8.07 (d, J = 8.4 Hz, 1H; NHPhe), 7.79 (d, J = 8.2 Hz, 1H; NH_{Tyr}), 7.25 (m, 5H; $H_{Ar,Phe}$), 7.17 (d, J = 8.1 Hz, 2H; $H_{Ar,Tyr}$), 6.67 (d, J = 8.3 Hz, 2H; $H_{Ar,Tyr}$), 4.83, 4.72 (2s, 1H; $CpH_{2,5}$), 4.59 (m, 2H; $C_\alpha H_{Phe,Tyr}$), 4.30 (m, 2H; $CpH_{3,4}$), 4.22 (dd, J = 8.4, 13.9 Hz, 1H; $C_\alpha H_{Leu}$), 3.95 (s, 5H; CpH), 3.67 (m, 4H; $C_\alpha H_{Gly}$), 2.92 (m, 4H; C_βHPhe,Tyr), 1.62 (m, 1H; $CH(CH_3)_2$), 1.54 (m, 2H; $C_\beta H_{Leu}$), 0.87 (dd,

J=6.3, 16.4 Hz, 6H; $CH(CH_3)_2$); ^{13}C NMR ([D$_6$]DMSO, 75.47 MHz): δ= 173.5 (COO), 171.8, 170.7, 168.8, 168.5, 167.8 (CON), 155.4 ($C_{Ar}OH_{Tyr}$), 137.4 ($C_{Ar,q,Phe}$), 129.6, 128.8 (C_{Ar}), 128.2 ($C_{Ar,q,Tyr}$), 127.6, 125.8, 114.5 (C_{Ar}), 75.5 (CCp$_{r1}$), 69.5 (CCp$_{3r4}$), 68.9 (CCp'), 68.2, 67.4 (CCp$_{2r5}$), 54.3 ($C_{\alpha,Phe}$), 53.1 ($C_{\alpha,Tyr}$), 49.9 ($C_{\alpha,Leu}$), 41.7, 41.3 ($C_{\alpha,Gly}$), 37.2 ($C_{\beta,Phe}$), 35.7 ($C_{\beta,Tyr}$), 23.9 ($C_{\beta,Leu}$), 22.4 ($CH_{3,Leu}$), 21.0 ($CH(CH_3)_2$); MS (ESI+): m/z 770.5 $[M-H]^+$, 404.9 $[M+K]^{2+}$.

Ac-Lys(FITC)-Tyr-Gly-Gly-Phe-Leu-OH (5): $C_{57}H_{62}N_8O_{14}S$ (1114.41 g mol^{-1}): MS (ESI−): m/z 724.3 $[M-\text{FITC-H}^+]^-$, 1113.3 $[M-H^+]^-$.

Fc-CO-Lys(FITC)-Tyr-Gly-Gly-Phe-Leu-OH (6): $C_{66}H_{68}FeN_8O_{14}S$ (12.84,39 g mol^{-1}): MS (ESI−): m/z 554.2 $[M-\text{Fc-Lys-FITC-H}^+]^-$, 1283.2 $[M-H^+]^-$.

***p*-I-C$_6$H$_4$-CO-Tyr-Gly-Gly-Phe-Leu (7)**: $C_{35}H_{40}IN_5O_8$ (785.19 g mol^{-1}): ^1H NMR ([D$_6$]DMSO, 360.14 MHz): δ=12.54 (br s, 1H; COOH), 9.12 (br s, 1H; OH_{Tyr}), 8.61 (d, J=8.2 Hz, 1H; NH_{Tyr}), 8.31 (t, J=5.5 Hz, 1H; NH_{Gly}), 8.26 (d, J=7.9 Hz, 1H; NH_{Leu}), 8.04 (d, J=8.4 Hz, 1H; NH_{Phe}), 7.96 (t, J=5.5 Hz, 1H; NH_{Gly}), 7.82 (d, J=8.4 Hz, 2H; $H_{Ar,I\text{-}Ph}$), 7.57 (d, J=8.4 Hz, 2H; $H_{Ar,I\text{-}Ph}$), 7.25 (m, 5H; $H_{Ar,Phe}$), 7.09 (d, J= 8.3 Hz, 2H; $H_{Ar,Tyr}$), 6.61 (d, J=8.4 Hz, 2H; $H_{Ar,Tyr}$), 4.59 (m, 2H; $C_\alpha H_{Phe,Tyr}$), 4.22 (dd, J=8.4, 14.0 Hz, 1H; $C_\alpha H_{Leu}$), 3.63 (m, 4H; $C_\alpha H_{Gly}$), 2.85 (m, 4H; $C_\beta H_{Phe,Tyr}$), 1.64 (m, 1H; $CH(CH_3)_2$), 1.54 (m, 2H; $C_\beta H_{Leu}$), 0.87 (dd, J=6.3, 16.8 Hz, 6H; $CH(CH_3)_2$); ^{13}C NMR ([D$_6$]DMSO, 90.56 MHz): δ=173.7 (COO), 171.6, 170.9, 168.8, 168.1 (CON), 165.6 ($CON_{I\text{-}Ph}$), 155.6 ($C_{Ar}OH_{Tyr}$), 137.6 ($C_{Ar,q,Phe}$), 136.9 ($C_{Ar,I\text{-}Ph}$), 133.4 ($C_{Ar,q,I\text{-}Ph}$), 129.9, 129.3, 129.1 (C_{Ar}), 128.3 ($C_{Ar,q,Tyr}$), 127.9, 126.1, 114.8 (C_{Ar}), 98.7 ($C_{Ar,q,I\text{-}Ph}$), 55.2 ($C_{\alpha,Phe}$), 52.7 ($C_{\alpha,Phe}$), 53.4 ($C_{\alpha,Tyr}$), 50.2 ($C_{\alpha,Leu}$), 42.0, 41.6 ($C_{\alpha,Gly}$), 37.5 ($C_{\beta,Phe}$), 36.1 ($C_{\beta,Tyr}$), 24.2 ($C_{\beta,Leu}$), 22.7 ($CH_{3,Leu}$), 21.2 ($CH(CH_3)_2$); MS (FAB): m/z 808 $[M+Na]+$, 786 $[M+H]^+$, 655 $[M-\text{H-Leu-OH}]^+$, 508 $[M-\text{H-Phe-Leu-OH}]^+$, 451 $[M-\text{H-Gly-Phe-Leu-OH}]^+$

Ac-Lys(FITC)-Lys-OH (8): $C_{29}H_{27}N_3O_8S$ (577.17 g mol^{-1}): MS (ESI−): m/z 576.3 $[M-H^+]^-$

Fc-Lys(FITC)-Lys-OH (9): $C_{38}H_{33}FeN_3O_8S$ (747.13 g mol^{-1}): MS (ESI−): m/z 357.0 $[M-\text{FITC-H}^+]^-$, 746.1 $[M-H^+]^-$

Standard HPLC conditions: HPLC-grade solvents were bought from Mallinkrodt–Baker (Deventer, Netherlands). High-performance liquid chromatography was performed by using a customised Varian instrument with Varian Dynastar C-18 reversed-phase column for analytical (250×4.6 mm) and semi-preparative (250× 10 mm). H_2O and CH_3CN were used as eluents, each containing TFA (0.1%).

Determination of log*P* values by RP-HPLC: The HPLC method of Minick et al. was used,[51] which proved is a simple and reproducible procedure for the log*P* value determination. As eluents, a 3-morpholinopropane-1-sulfonic acid buffer (MOPS; pH 7.4) with decylamine (0.15% *v/v*) and MeOH with octan-1-ol (0.25% *v/v*) were used. The stationary phase was the same as for analysis of peptides. Four different standards with known log*P* (4-methoxyanilin, 4-bromoanilin, naphthalene, *tert*-butylbenzene) were chosen for calibrating the system and to establish a correlation between $\log k_W$ and log*P*. These substances were dissolved in MeOH, and uracil was added to determine the system's dead time. The standard mixture was run with isocratic gradients containing from 55% to 85% of MeOH. The retention times, together with the dead times obtained from uracil, were used to calculate the corresponding capacity factors k' H_2O/MeOH ratio. The extrapolation to 100% H_2O (0% MeOH) gave the desired $\log k_W$ value for each standard. All four $\log k_W$ values of the reference substances were plotted against the literature log*P* values of the reference compounds to obtain a linear function that correlates both parameters. This function was then used to determine all log*P* from their $\log k_W$ values, which were measured in the same way as those of the references. (For graphs see the Supporting Information).

Blood–brain barrier permeation of selected [Leu^5]-enkephalins: PBCECs were extracted from the brain of 4- to 6-month-old pigs. PBCECs were cultured on 12 mm polyester filter inserts of a 12-well Transwell Clear© plate (0.4 μm pore size), that had been previously treated with rat-tail collagen (5 μg cm^{-2}). Each well was seeded with 250000 cells and cultivated for seven days before the experiments were carried out. Cell monolayer growth and confluence were monitored by light microscopy. In addition, the integrity of cell monolayers was confirmed by carboxyfluorescein as a paracellular marker. Cells were grown in "medium 199" containing Pen-Strep, HEPES and glutamine, as well as defined equine serum (9%). The medium was replaced on days 1, 2 and 4. On the sixth day it was exchanged by DMEM/Ham's F12 (1:1) medium, which was free of serum. After one week of cell cultivation the medium was removed and the filters containing the cell monolayers were washed twice with Krebs–Ringer buffer (KRB) and subsequently inserted into a freshly prepared 12-well plate, containing 1500 μL of KRB in the acceptor wells. Each of the selected enkephalin derivatives, was prepared as a 50 μM solution in KRB of which 500 μL were used for incubation of the PBCEC layer in the donor vial. Samples (200 μL) were taken from the acceptor vial after 30, 60 and 90 min and immediately replaced by the same volume of KRB. The apparent permeation coefficient (P_{app}) was calculated from the permeation rate by following Equation (1).

$$P_{app} = \frac{dA}{dt} \times \frac{V_{acc}}{A} \times \frac{1}{C_0} \times \frac{1}{x} \quad (1)$$

P_{app} = apparent permeation coefficient [cm s^{-1}], dA/dt = permeation rate [% s^{-1}], V_{acc} = acceptor volume [mL], A = area of the filter insert [cm^2], C_0 = initial concentration of donor [100%], x = volume correction factor (ratio acceptor volume/donor volume).

The amount of permeated substance was determined by RP-HPLC by integration of the substance peaks. All samples, taken in triplicate, were directly frozen at −70 °C and only defrosted prior to HPLC injection. For test substances, stock solutions (50 μM) were used to generate calibration curves by injection of 1, 2, 5, 10, 25, 50, 75 and 100 μL of each solution onto the HPLC. An aliquot (100 μL) of each 200 μL sample was injected into HPLC running a gradient from CH_3CN (5%) in H_2O (t=0 min.) to CH_3CN (100%, t= 20 min.). The gradient then returned to initial conditions at 25 min and equilibrated for another 5 min. Both eluents contained TFA (0.1%) for improved solubility. The signals, recorded at 220 nm, were manually integrated, and the amount of substance was calculated from the linear regression function of the corresponding calibration curve. For the calculation of transported amounts of substance all three measurements have been used to create mean values.

Detection of fluorescently labelled enkephalins in cancer cell lines: Hela, HepG2 and HT-29 cells were kept in culture with RPMI 1640 medium supplemented with FCS (10%), L-glutamine (2 mM) and the antibiotic penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}; all PAA, Cölbe, Germany) under CO_2 (5%) atmosphere.

Substances **5**, **6**, **8** and **9** were dissolved in culture medium shortly before use. Cells were seeded in 6-well plates and incubated 24 h before substance application. After addition of the substances in culture medium the cells were incubated for another 6 or 24 h.

Before microscopy, dead cells were stained with propidium iodide (4 μM) for 15 min and the wells were washed four times with colourless RPMI 1640 medium. Pictures were taken with an Olympus IX-51 with a colorview CCD camera with a long-working-distance objective (20×, NA 0.4). The pictures were background corrected and analysed with ImageJ.[72] Fluorescence intensity was determined from 10×10 pixel-sized ROI, which were placed on cells. At least 10 ROI per picture were evaluated.

For microscopy with the 100× oil-immersion objective (NA 1.4) a similar procedure was used. Cells were grown on gelatine-coated coverslips and additionally stained with H 33342. The coverslips were sealed with a mixture of vaseline, lanolin and paraffin on glass slides with culture medium.

Cytotoxicity assays: The same cell culture conditions as for fluorescence microscopy were used. In vitro cytotoxicity of the peptides **3** and **4** was studied on Hela, HT-29 and HepG2 cells. Cell viability, which correlates with the metabolic activity of a cell, was determined by the resazurin assay.[73] In addition to cell viability, absolute cell numbers were determined by the crystal violet assay,[74,75] which can be applied after elution of resazurin. The cells were seeded in 96-well microtiter plates (MTP) coated with gelatine (0.2%). After seeding, the cells were grown for 24 h under standard conditions. Then compound **3** was dissolved in culture medium with DMSO (0.5%) and applied to the cells in various concentrations for 48 h. Compound **4** could be dissolved without the aid of DMSO. Every concentration was tested sixfold. Before resazurin (Sigma–Aldrich) was added to the cells, they were washed three times with phenol-red-free RPMI-1640 medium (Sigma–Aldrich). Then, phenol-red-free RPMI medium with resazurin (10%) was added. Absorbance at 600 nm was directly measured with a Tecan Sapphire^2 microplate reader (Tecan, Germany) at 37 °C. After 2 h of incubation at 37 °C and CO_2 (5%) the measurement was repeated. The decrease in absorbance gave the viability. Resazurin was removed and the cells were fixed with paraformaldehyde (PFA, 4%; Riedel de Haen) in PBS for 15 min at room temperature. PFA was eluted with PBS (3×), and membranes were permeabilised by Triton X100 (0.1%, Sigma) in PBS for 10 min. Afterwards an crystal violet solution (0.04%) was added to the cells, and the microtitre plate was mechanically shaken for 1 h. Next, the cells were washed seven times with H_2O, and crystal violet was eluted with EtOH (96%) for 4 h. Then absorbance was determined at 570 nm, after subtraction of 24 h pre-substance-incubation absorbance values, cell biomass could be calculated.

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Keywords: bioorganometallic chemistry · blood–brain barrier · neuropeptides · peptides · solid-phase synthesis

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