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Medicinal Applications of Metal–Peptide Bioconjugates

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Abstract: Organometal–peptide bioconjugates have a variety of applications in biomedical studies, such as anti-proliferative and anti-bacterial activity, as well as to aid the elucidation of the mode of action of the conjugated metal complexes. Our group has developed the solid-phase synthesis of organometal–peptides by different synthesis schemes, their characterization and use in biomedical studies. Selected examples are presented in this review.

Keywords: Bioconjugates · Bioorganometallic chemistry · Ferrocene · Peptides · Solid-phase synthesis

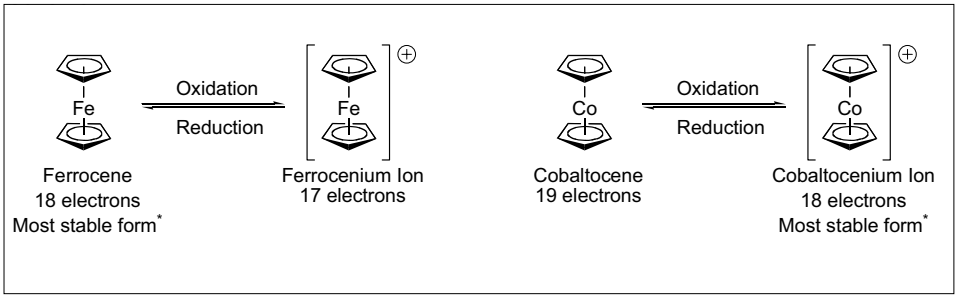
1. Introduction

The discovery of the organo-arsenic compound Salvarsan (Paul Ehrlich's compound no. 606) marks the advent of modern medicinal chemistry. For the first time, a disease – syphilis – became curable by chemotherapy. In the course of the research leading up to this compound, a structure–activity relationship was, also for the first time, established in a systematic way. It is interesting to note that such a relationship was thus originally established for an inorganic compound.[1] Moreover, Salvarsan also meets the definition of an organometallic compound in that it has direct covalent metal–carbon bonds. In recent years, there has been a renewed interest in medicinal applications of organometallic compounds.[2] Two prime examples, for which a structure–activity relationship was also successfully established, are the ferrocene-containing molecules ferrocifen and ferroquine.[3] Both compounds are in (ferroquine), or very close to clinical trials (ferrocifen), and they are discussed in other articles of this issue of CHIMIA. For the background of this article, it is interesting to note that ferrocene itself has a biological activity (Scheme 1). In 1984, Köpf and Köpf-Maier reported that simple ferrocenium salts have significant anti-proliferative activity.[4] Soon after, it was established that aqueous solubility was an important factor for activity. In a series of papers, Neuse and coworkers found that binding of ferrocene to high-molecular weight organic polymers (such as poly-arginine) greatly enhances the biological activity. This was, at least in part, attributed to a better aqueous solubility.[5]

Given the very simple structure of ferrocene, one might wonder what exactly is the mechanism of action of ferrocene? Suggestions from the literature include DNA interaction and DNA damage, possibly by generation of oxygen radicals *via* a Fenton-type mechanism, enzyme inhibition, or cell-wall interaction and possibly damage.[6] While all of these suggestions seem to be founded on experimental data, they are not readily combined in an overall picture, and are indeed partly contradictory. At present, it is not even clear whether the neutral ferrocene (Fe(II)) or its one-electron oxidized ferrocenium form (Fe(III)) are the active species (Scheme 1). For a Fenton-type mechanism of radical generation, redox cycling between the two states would indeed be required. Therefore, the question of the mechanism of action of ferrocene or ferrocenium salts remains unanswered.

As indicated by the above list of possible targets, neither the molecular target nor the place of action are well understood. We have therefore embarked on a different strategy to reduce the complexity of the problem. Rather than searching for the molecular target, our aim is to identify the location inside the cell where a particular metal complex – ferrocene in this example – will be active. For example, a metal complex that will damage the DNA should be more active if it were selectively transported into the cell nucleus where the DNA is found. Conversely, it should be inactive if located in the cytoplasm.

This new approach, however, raises a different question: How can one achieve directed delivery of metal complexes to a single, well-defined cellular compartment? For this purpose, nature uses small peptides,



Scheme 1. Ferrocene, the ferrocenium cation, cobaltocene, the cobaltocenium cation, and their interconversion by one-electron redox reactions. The designation 'most stable form' refers to physiological conditions.

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which are attached to larger biomolecules such as proteins. A cellular machinery then exists that will transport the conjugate to the location for which the attached peptide encodes. The aim of our work was to use such peptides and 'highjack' the cellular machinery for the directed delivery of metal complexes inside a cell. Individual steps of this project thus were:

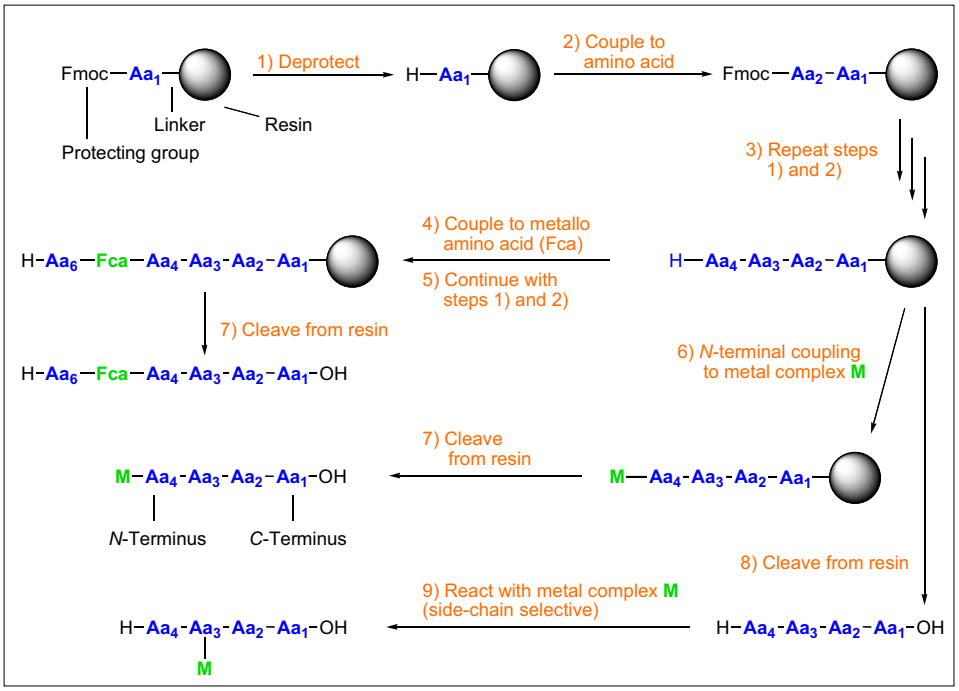
i) synthesis of organometal-peptide conjugates;
ii) purification and characterization of organometal-peptide conjugates;
iii) study of cellular uptake and intra-cellular localization, and
iv) study of their biological activity.

This account will roughly follow and present our results along these four steps.

2. Synthesis of Organometal–Peptide Conjugates by SPPS

Solid-phase peptide synthesis (SPPS, also called Merrifield synthesis after its inventor, the 1984 Nobel Laureate Bruce Merrifield) is the method of choice for the chemical synthesis of small to medium-sized peptides. The challenge in this project was to modify or adapt existing SPPS methods in such a way that the overall scheme becomes compatible with the chemical properties (in particular, the chemical stability) of the metal complex that is to be incorporated. A first indication that this task is all but trivial originates from the fact that there are only a small handful of reports in the literature on the use of SPPS for the synthesis of organometal–peptide bioconjugates, mostly using ferrocene and derivatives. Almost all of these were published by a French group in the 1980s.[7] The same group honestly reports problems in isolating some of the compounds, and by today's standards, the characterization of the conjugates is rather thin. More work has been published recently on ferrocene–DNA conjugates for applications in electrochemical biosensors,[8] and for the radiolabelling of peptides with metal complexes.[9,10] However, the labelling reaction in the latter case is usually carried out in solution just prior to biological testing of the conjugate and is thus not directly comparable to the work we will discuss herein, where the metal complex will be introduced as part of the SPPS scheme.

Scheme 2 gives an overview of the steps of SPPS. Somewhere along this scheme, a metal complex needs to be inserted. Naturally, all subsequent steps must be compatible with the metal complex as well as all other groups in the peptide.[11] For example, traditional Merrifield synthesis as used by Tartar, Sergheraert and coworkers uses neat HF to cleave the bioconjugate from the resin. Very few organometallic complexes,



Scheme 2. Solid-phase synthesis scheme

however, will withstand prolonged treatment with HF without decomposition. Ferrocene, and most of its derivatives, generally are not stable in the presence of strong acids without special precautions. In early work, we have therefore used the HMBA linker for the synthesis of ferrocene–peptide conjugates. The peptide is cleaved from the resin with NH_3 in MeOH, thus avoiding strong acids all together. By this method, the C-terminal carboxamide is obtained. Upon a suggestion from Prof. H.-B. Kraatz, we established that ferrocene also survives treatment with concentrated TFA, provided that *ca.* 10 % phenol is added as an anti-oxidant. It then depends on the linker, whether the C-terminal carboxamide (Rink amide linker) or the free acid (Wang resin) is obtained. In most cases, acid cleavage offers greater flexibility, especially with respect to commercially available amino acids with particular side-chain protecting groups.

One of the first peptide derivatives that we have prepared is ferrocenoyl–enkephalin (entry 1 in the Table). Enkephalin (Enk) is a five-amino acid neuropeptide with the primary sequence Tyr-Gly-Gly-Phe-Leu. It is the natural ligand for the opiate receptor and exists in two forms which only differ in the C-terminal amino acid (Leu or Met). We have concentrated on [Leu^5]-enkephalin. Chemically, its synthesis is fairly straightforward as it only requires one side-chain protecting group on the tyrosine phenol group. Later on, chemically more complex peptides were synthesized by our group, such as the nuclear localization signal (NLS) or HIV-derived TAT peptide. Both sequences require additional protecting groups for lysine and/or arginine side chains. More recently, we have also prepared a number of peptides with anti-bacterial activity, which are composed of Arg and Trp amino acids (see below). An overview of some ferrocene peptides that were prepared in our group is given in the Table.

As described above, our initial efforts concentrated on ferrocene bioconjugates with a view to the study of their biological activity. However, as the field of organometal bioconjugates was largely unexplored at the onset of our investigations, we were also curious to extend the range of available metal complexes for SPPS. On going from ferrocene to the isoelectronic cobaltocenium cation, even more stable metal conjugates were obtained. The cobaltocenium cation is also an 18-electron complex with almost identical geometry to ferrocene. It is, however, chemically even more robust than ferrocene. Unlike ferrocene, which is reversibly oxidized to yield the 17-electron ferrocenium cation at moderate potentials, the 18-electron cobaltocenium cation is one-electron reduced at far more negative potential (Scheme 1). By careful comparison of the two complexes, we can thus study the influence of (positive) charge and different redox chemistry in biological systems.

In addition to simple metallocenes, we have also investigated the use of other metal complexes in SPPS. Fig. 1 lists some of these compounds with the neuropeptide enkephalin. The electrochemistry, structure, and dynamics of the complex Mo(allyl)$(CO)_2$(His) (His = N_δ, N, O-histidinate) were studied in detail by a variety of spectroscopic means as well as computationally.[18]

Table. Selected ferrocene–peptide conjugates prepared in our group

Entry no.	Peptide name/ group (Primary sequence)	Abbreviation	Amino acid sequence	Refs
1	[Leu^5]-Enkephalin (Tyr-Gly-Gly-Phe-Leu)	FcCO–Enk-OH	**Fc**CO-Tyr-Gly-Gly-Phe-Leu-OH and **Fc**CO-Tyr-Gly-Gly-Phe-Leu-NH_2 [b]	[12]
2		[*p*-CC-Fc-Phe^4]–Enk	H-Tyr-Gly-Gly-Phe(*p*-CC-**Fc**)-Leu-OH	[13]
3		[*p*-CC-CR_2-NHCOFc-Phe^4]–Enk	H-Tyr-Gly-Gly-Phe(*p*-CC-CR_2-NHCO**Fc**)-Leu-OH	[13]
4			Boc-**Fca**-Ala-Gly-Val-Leu-NH_2	
5			Ac-Val-Gly-Ala-**Fca**-Ala-Gly-Val-Leu-NH_2	
6	Nuclear Localization Sequence, NLS (Pro-Lys-Lys-Lys-Arg-Lys-Val)	FcCO–NLS	**Fc**CO-Lys-Pro-Lys-Lys-Lys-Arg-Lys-Val-NH_2 [a, b]	[14,15]
7	HIV-TAT (Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg)	FcCO–TAT	**Fc**CO-Lys-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-OH [a, b]	[14]
8	Artificial anti-microbial peptides		**Fc**CO-Arg-Trp-Arg-Trp-Arg-NH_2 [b, c]	[16]
9			**Fc**CO-Trp-Arg-Trp-Arg-Trp- NH_2 [b, c]	[16,17]

[a] also fluorescein thiourea derivative on Lys^1; [b] Also cobaltocenium derivative prepared; [c] Several shorter peptide derivatives were also prepared but found to be less active.

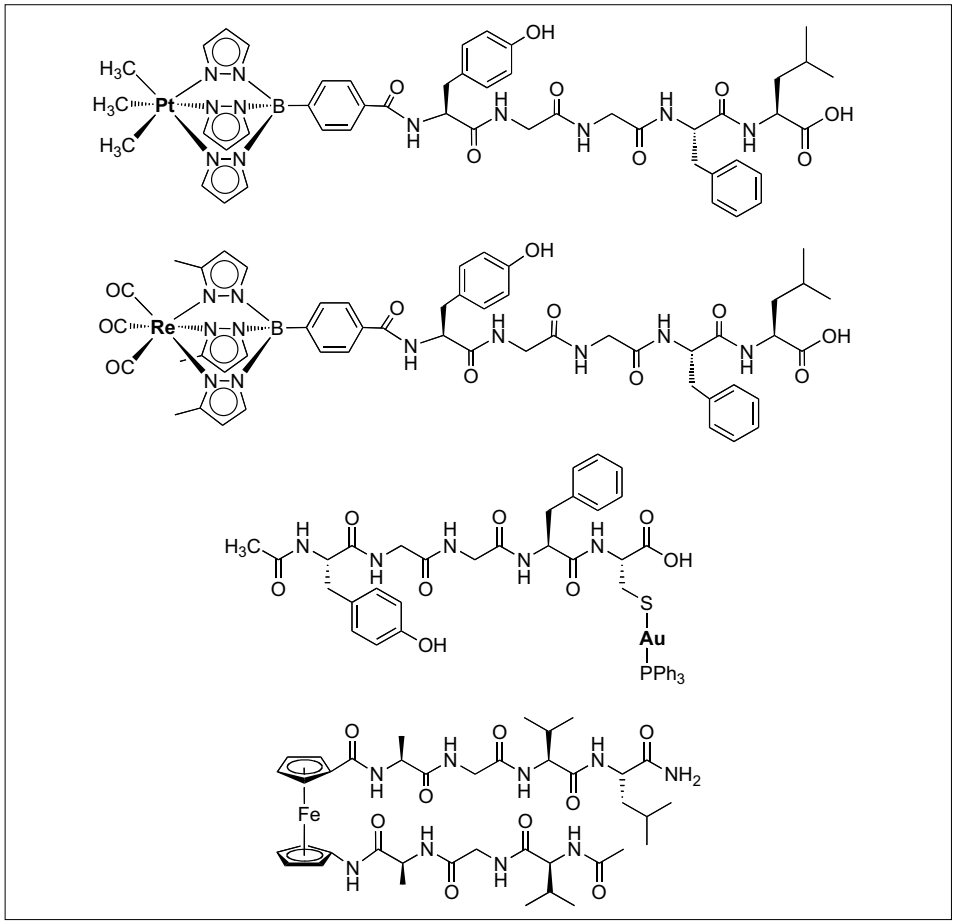


Fig. 1. Organometallic enkephalin and Fca bioconjugates prepared in our group by SPPS

This complex is also stable enough to be used in SPPS with an appropriate linker on the histidinate ligand.[19] More recently, we have explored the use of the tris(pyrazolyl) borate (Tp) ligand family. Boron-functionalized Tp ligands can be prepared which have a benzoic acid functionality. These ligands can be converted into a variety of metal complexes, which can then be used to label peptides. Two examples are shown in Fig. 1. The X-ray single crystal structure of a (Tp)$PtMe_3$ derivative with phenylalanine was also reported.[20] This compound, as well as a related (Tp')$Re(CO)_3$ complex were successfully linked to enkephalin.[21] In both cases, however, the standard methods for SPPS had to be modified. We have also used another $Re(CO)_3$ complex with the 3,3-bis(2-imidazolyl) propionic acid ligand in bioconjugates with peptide nucleic acid oligomers (PNA).[22]

A derivative of the naturally occurring [Leu^5]-Enk is [Cys^5]-Enk, which has a cysteine instead of leucine as the C-terminal amino acid. This peptide was prepared on Wang resin, the cysteine was selectively deprotected, and a Au(PPh_3) fragment complexed to the thiol group. Following a modified cleavage procedure, the gold-Enk peptide was isolated in >90% purity as the first synthetic Au(I) peptide prepared by SPPS.[23]

More drastic modifications were required to incorporate the unnatural amino acid 1'-amino-ferrocene-1-carboxylic acid (Fca).[8] This compound is one of the simplest organometallic amino acids one can imagine. Ferrocene amino acid and peptide conjugates were studied in great detail, in particular with a view on their propensity to act as peptide turn-mimetics. Details of their structures and methods for investigation were published in another review.[24] At the time of our study, Fmoc-protected Fca was not readily available. It was, however, later used by Heinze and coworkers.[25] In our case, a tetrapeptide was assembled on the resin by standard Fmoc chemistry. It was then reacted with Boc-Fca-OH, the Boc group was removed by HCl/CH_2Cl_2, and the Fmoc synthesis was continued after neutralization. Cleavage from the resin was achieved by NH_3/MeOH (HMBA linker, entries 4 and 5 in the Table).[26] As in all other cases, the product peptide (Fig. 1) was purified by preparative HPLC and analyzed by the usual methods, in particular HPLC, MS, NMR and other appropriate spectroscopic methods.

3. Labelling of Peptides with Organometallics in Solution

All of the above-mentioned metal bioconjugates were prepared on the resin, *i.e.* the metal complex was introduced as one

step of the SPPS scheme. This provides the usual advantages of SPPS, *i.e.* good yield of the labelling reaction and high purity of the conjugates. The challenge in all cases was to make the SPPS compatible with the chemical stability of the metal complex, as described above. The conjugates generally undergo one more purification step by preparative HPLC. If that is not possible or feasible, *e.g.* in the case of very unstable complexes or labelling with radioactive compounds, then a different synthetic strategy may be applied. A ligand or functional organic group is introduced into the peptide during HPLC. After cleavage and purification, the peptide, which is purely organic up to this moment, can be stored as long as required. A suitable metal precursor will then react specifically with this functional group or ligand just prior to the biological application of the conjugate. This scheme is particularly useful for labelling with radioactive complexes and a variety of ligands have been proposed. For organometallic compounds, the $Tc(CO)_3$ fragment deserves particular mention, which is readily available and has been used for the labelling of a variety of peptides.[10,27] In collaboration with Alberto's group in Zurich, we have introduced a histidine-based ligand into enkephalin and used this approach for the C-terminal labelling with $Tc(CO)_3$.[28]

Alternatively, we have introduced a functionalized bis(2-picolylamine) ligand at the N-terminus of enkephalin by SPPS. After cleavage and purification, the peptide was readily reacted with $Mo(CO)_3(EtCN)_3$, to yield the $Mo(CO)_3$(Enk-bpa) conjugate in quantitative yield in solution (Fig. 2).[19] A more elegant, and maybe more specific way was used for the formation of Co-carbonyl peptide conjugates. During SPPS, an alkyne group was introduced into the peptide, *e.g.* attached to the N-terminus as propargylic acid. After cleavage and purification, the alkyne reacts cleanly and quantitatively with $Co_2(CO)_8$ to yield the $Co_2(CO)_6$(alkyne) complex, as shown in Fig. 2.[29] The conjugate shows the expected metal carbonyl bands around 2000 cm^{-1}. In the ESI-MS, a characteristic fragmentation pattern from consecutive loss of all six CO ligands is observed, which is very indicative for this type of complex. The alkyne coordination of $Co_2(CO)_8$ is highly specific and occurs in the presence of other potentially coordinating groups that are likely present in a peptide.

Sonogashira coupling offers another means of selective peptide derivatization. We have reported the use of this Pd-catalyzed C–C coupling reaction for the labelling of amino acids and peptides with ferrocene derivatives.[30] The methodology was extended to label Enk on the phenylalanine side chain.[13] During the SPPS of Enk, *p*-iodo-phenylalanine was used in place of normal Phe. The peptide was cleaved and purified as usual. Sonogashira coupling with ferrocenyl-alkynes was then performed in solution using the $Pd(PPh_3)_2Cl_2$/CuI system. Again, the reaction worked selectively and produced the compounds shown in Fig. 2 (entries 2 and 3 in the Table).

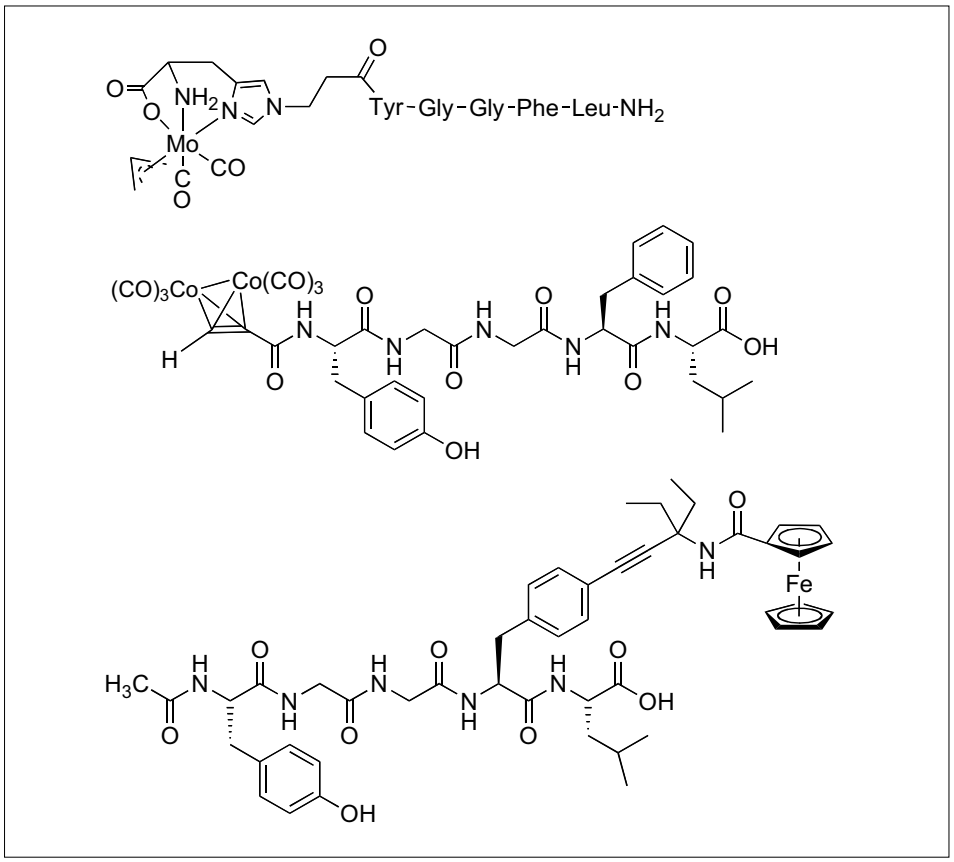


Fig. 2. Organometal–enekphalin derivatives prepared by metal complexation in solution after SPPS

4. Applications of Organometal–Peptide Conjugates for Biomedical Studies

The project of ferrocene–peptide conjugates was initiated to discover the cellular target compartment for the biological activity of ferrocene. Thus, it was important to study cellular uptake and intra-cellular localization of ferrocene–peptide conjugates. In order to detect these conjugates, an additional Mtt-protected lysine residue was incorporated at the N-terminus of the peptides. The Mtt group can be selectively removed by mild acid treatment, and the free amino group was allowed to react with fluorescein-isothiocyanate (FITC). SPPS was then finalized as described before. Cellular uptake studies were carried out in our laboratory and in collaborations.[14,15] A typical result is shown in Fig. 3. On the left, a live cell fluorescence microscopy image of HepG2 cells is shown after incubation with a ferrocene–NLS conjugate (entry 6 in the Table). Clearly, the green fluorescence is taken up into the cell and mostly localized in the nucleus. Similar results were obtained for a NLS–cobaltocenium conjugate, which is also readily taken up and mainly localized in the nucleus.[15] The middle picture shows the uptake of a ferrocene–TAT conjugate (entry 7 in the Table). In this case, the conjugate is taken up readily and evenly distributed in the cytoplasm. It is not, however, transported into the nucleus. The picture on the right shows a TAT conjugate with cobaltocenium in place of the ferrocene. Clearly, this conjugate is hardly incorporated into the cell at all. The reason for this drastically different behavior is not clear at present. It is, however, clear, that subtle changes in the metallocene (in this case, addition of a positive charge in an otherwise almost identical molecule) does make a significant difference. Propidium iodide staining showed that all cells were intact and the compounds did not induce necrosis. Further cytotoxicity testing did not show a significant activity of any of these conjugates up to mM concentrations.

We did, however, observe strong anti-proliferative effects in the Co-carbonyl alkyne conjugates described in section 3.[29] The cytotoxicity of Co-carbonyl alkyne complexes was first discovered by Jung and Gust on derivatives of acetyl salicylic acid (ASS, also known as Aspirin®).[31] In

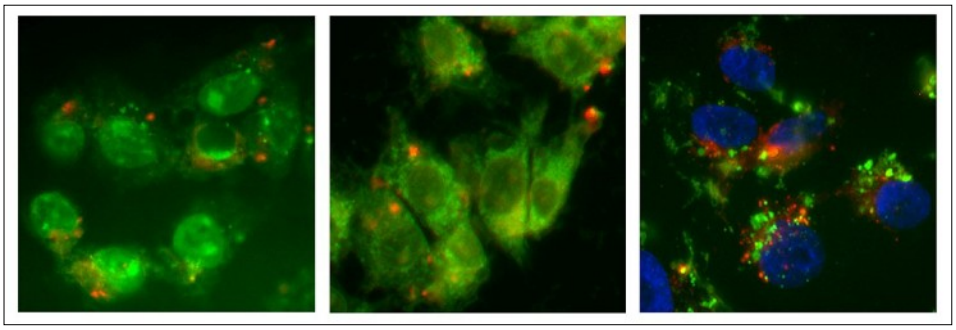


Fig. 3. Cell uptake (HepG2) of FITC-labelled metallocene–peptide conjugates (green color). Left: Ferrocene–NLS (entry 6 in the Table), middle: Ferrocene–TAT (entry 7 in the Table), right: Cobaltocenium–TAT.

the following, Co-carbonyl derivatives of many other anti-inflammatory drugs (non-steroidal anti-inflammatory drugs, NSAID) were studied, mostly with far less activity. Efforts are ongoing to disclose their mechanism of action.[32] The peptide derivatives reported from our group are the first non-NSAID derivatives with high anti-proliferative potency.

As described above, the anti-proliferative activity of our metallocene–peptide conjugates was low. However, we discovered some ferrocene–peptides that were highly active as anti-bacterials.[16,17] A group of anti-microbial peptides (AMPs) has long been known. These peptides are mostly well over ten amino acids long and serve to protect plants and other organisms against bacterial infections. Their mode of action is thought to involve membrane interactions. Their structures were studied in detail, and artificial AMPs were designed. We have prepared a number of metallocene peptide conjugates from Trp and Arg amino acids (entries 8 and 9 in the Table). The amino acids were systematically varied, and the metallocene was usually attached to the N-terminus *via* a carboxylic acid group. The most active derivative, FcCO-Trp-Arg-Trp-Arg-Trp-NH2 (entry 9 in the Table) showed a minimum inhibitory concentration (MIC) of 28 μM against *P. aeruginosa*, and only 7 μM against *S. aureus*.[16] This number compares favorably with the twenty amino acid naturally occurring Pilosulin 2, which has MIC values of 4 and 12 μM against *P. aeruginosa* and *S. aureus*, respectively, under the same experimental conditions. It is of note that unlike Pilosulin 2 and all other derivatives tested (including the related cobaltocenium derivatives), the ferrocene conjugate shows a *higher* activity against the Gram-positive *S. aureus*. In the light of the growing resistance of many bacteria against common drugs like penicillins, and given the health concerns associated with multi-drug resistant strains of *S. aureus* in particular, these results are of high interest.

Far more work is certainly needed to establish the mechanism of these conjugates. Already, the results demonstrate the enormous potential of metal derivatives of biomolecules in general. Not only may we alter their physiological properties, but the incorporation of metals may bring about new and unprecedented activity or selectivity, or it may offer additional, metal-specific means of detetion, such as Atomic Absorption Spectroscopy (AAS).[33]

Acknowledgements

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