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Neuartiger Oxaliplatin Analoga

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Mag. Sergey Abramkin

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Ph.D.–Thesis

Synthesis and Characterization

of Novel Oxaliplatin Analogs

Written by

Sergey Abramkin

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This PhD thesis is based on the following papers, which are presented in the original format.

Abramkin, S.A.; Jungwirth, U.; Valiahdi, S. M.; Dworak, C.; Habala, L.; Meelich, K.; Berger, W.; Jakupec, M. A.; Hartinger, C. G.; Nazarov, A. A.; Galanski, M. and Keppler, B.K. {(1*R*,2*R*,4*R*)-4-methyl-1,2-cyclohexanediamine}oxalatoplatinum(II): a novel enantiomerically pure oxaliplatin derivative showing improved anticancer activity in vivo. *J Med Chem.* **2010**, 53, 7356

Abramkin, S; Valiahdi, S. M.; Jakupec, M. A.; Galanski, M.; Metzler-Nolte, N. and Keppler, B. K. Solid-phase synthesis of oxaliplatin-TAT peptide bioconjugates. *Dalton T.* **2012**, 41, 3001

Further development of conjugates synthesis is described in chapter „Optimization of the Coupling Strategy of Pt(IV) Complexes to Peptides“

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Abstract

Platinum-based drugs are the established class of cancer therapeutics. Antiproliferative properties of the compounds were discovered in the 1960-s. Cisplatin, diamminedichloridoplatinum(II), was the first complex to show potency against cancer. Successful application encouraged chemists to search for better molecules in order to reduce severe side effects of cisplatin therapy and to broaden the application spectrum. The extensive search gave only several structures of cisplatin analogs, although thousands of complexes were synthesized.

Oxaliplatin was the last one to be approved by the FDA (2002) and used worldwide for colorectal cancer treatment. It has lower oto- and nephrotoxicity compared to cisplatin. The easiest and most straightforward way to develop a new drug is to modify already existing ones, to optimize their structures. In some extent all the platinum-based drugs are cisplatin's posterity.

In this work, different modifications of oxaliplatin are described. In the first project the methyl-substituted cyclohexanediamine ligands were synthesized and corresponding oxaliplatin derivatives were obtained. This small substituent may take different positions related to the cyclohexane ring: both axial and equatorial isomers were prepared, characterized with NMR, elemental analysis and mass spectrometry. Their biological activity was tested *in vitro* and *in vivo*. In cytotoxicity tests, the one with axial orientation of the methyl group showed better results, the IC_{50} values were generally lower compared to oxaliplatin. The isomer with an equatorial orientation of the methyl group showed comparable or slightly higher values. The results of *in vivo* investigations were the opposite, in tests on leukemia mouse models better activity was shown by the equatorially substituted oxaliplatin. It's also noteworthy to mention, that general toxicity was lower compared to oxaliplatin, what allowed increasing dosage of the drug.

In the second project, conjugates of oxaliplatin featuring the TAT peptide (Trans-Acting Activator of Transcription) were prepared making the cellular transport more efficient. Inefficient delivery of complexes into the cell is one of the shortcomings of platinum-based therapy, and using delivery vectors could increase cellular accumulation of the drug. Oxaliplatin was oxidized to its Pt(IV) analog and equipped with a linker, capable of conjugation with a peptide via standard amide coupling. The peptide was synthesized on solid

phase, the Pt complex was appended after the last amino acid. The obtained mono- and bisconjugates were characterized by NMR, ESI and MALDI MS and liquid chromatography. Both substances showed better cytotoxicity than the Pt precursor or the analogous amide without biologically relevant group.

Zusammenfassung

Platinbasierte Arzneistoffe sind etablierte Krebstherapeutika; die antiproliferative Eigenschaft der Komplexe wurde 1965 erstmals beschrieben. Diammindichloridoplatin(II) (Cisplatin) war der erste Komplex, der eine Wirksamkeit gegen Krebs zeigte. Die erfolgreiche Anwendung führte zur Entwicklung analoger Verbindungen, zur Minderung der schweren Nebenwirkungen und zur Erweiterung des Anwendungsspektums. Trotz der Synthese tausender Cisplatin Analoga, konnte nur wenige mit einem interessanten Wirkspektrum identifiziert werden.

Oxaliplatin wurde als letzter Platinkomplex 2002 von der FDA zugelassen; es ist derzeit nahezu weltweit zur Behandlung von kolorektalen Karzinomen im Einsatz. Im Vergleich zu Cisplatin hat es eine niedrigere Oto- und Nephrotoxizität. Die einfachste Art, neue Wirkstoffe zu entwickeln, ist bereits bekannte Wirkstoffe zu verändern, immer im Hinblick auf eine Optimierung der Struktur-Aktivitäts Beziehungen. Gewissermaßen sind alle platinbasierten Therapeutika ein direktes Derivat des Cisplatins.

In der vorliegenden Arbeit wurden unterschiedliche Derivate des Oxaliplatins entwickelt. Im ersten Projekt wurden methylsubstituierte Cyclohexandiaminliganden synthetisiert, und die entsprechenden Oxaliplatinanaloga hergestellt. Der kleine Substituent (Methylgruppe) kann unterschiedliche Positionen im Bezug auf den Cyclohexandiaminring einnehmen, sowohl das axiale, als auch das equatoriale Isomere wurden hergestellt und mittels NMR, Elementaranalyse und Massenspektrometrie eindeutig charakterisiert. Die biologische Wirksamkeit wurde *in vitro* und *in vivo* getestet. Das Isomer mit der axial orientierten Methylgruppe zeigte bessere Ergebnisse bezüglich der IC_{50} Werte, diese waren im Allgemeinen niedriger im Vergleich zu Oxaliplatin. Das Isomere mit der equatorial orientierten Methylgruppe zeigte vergleichbare oder etwas höhere IC_{50} Werte. Der Effekt in der *in vivo* Untersuchung (Mausmodell, L1210) war jedoch entgegengesetzt, das equatorial substituierte Oxaliplatin zeigte eine bessere Aktivität. Bemerkenswert ist in diesem Zusammenhang auch, dass die systemische Toxizität niedriger war im Vergleich zu Oxaliplatin, was die Erhöhung der Dosierung ermöglichte.

Im zweiten Projekt wurden zur Effizienzsteigerung des zellulären Transportes, neuartige Konjugate bestehend aus Oxaliplatin und dem TAT Peptid (Trans-Acting Activator of Transcription) synthetisiert. Die ungenügende Akkumulation der Komplexe in der Zelle ist

einer der wichtigsten Probleme der platinbasierten Therapie; eine Steigerung der zellulären Anreicherung kann mittels Verwendung geeigneter Trägermoleküle gemäß des Konzeptes des Drug targeting erreicht werden. Hierbei wurde Oxaliplatin zu dem entsprechenden Pt(IV) Analogon oxidiert und mit einem Linker, der mit einem Peptid mittels Amindkopplung verknüpft werden konnte, ausgestattet. Das Peptid wurde durch Festphasensynthese hergestellt, wobei der Platinkomplex nach der letzten Aminosäure angefügt wurde. Die isolierten Mono- und Biskonjugate wurden mit NMR, ESI und MALDI MS bzw. Flüssigkeitschromatografie charakterisiert. Die beiden Substanzen zeigten eine bessere Zytotoxizität im Vergleich zum Ausgangskomplex bzw. zu einem Amidanalogon, das keine Gruppe im Sinne des Drug Targetings aufwies.

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I. Introduction

1. Drug Design

1.1 Rational Drug Design in a Historical Prospect

Therapy as a phenomenon cannot have any clear beginning in human history. The wish to be cured and get rid of pain is as basic as hunger. Without any doubt, people got sick, and consequently they tried to find cures, exactly as we do now. One can speculate that instincts were much more important at the early age of mankind, and treating wounds, finding medical plants can be described as their extrapolation. The development of healing into modern drug design was by no means straightforward, the way often led into a dead end, it was a child of its time.

The beginning of European medicine is often counted from Hippocrates, he used a scientific approach based on observation. Previously, it was a mixture of superstition, magic and religion,[1] the general explanation was provided exceptionally by belief. In ancient Egypt benign and malicious tumours were already known, and natural substances like castor oil and pig's ears were used for treatment.[2] Hippocratic medicine had a different base – diseases had natural causes, medicine was thus transferred from irrational field into a (more) rational philosophy. The title of his book "On the Sacred Disease" about epilepsy already contains the starting point for his polemic: he described it not as a divine, but as a hereditary disease of the brain where the soul and feelings were located. According to his concept of 4 humours:[3]

> *"The body of man has in itself blood, phlegm, yellow bile, and black bile; these make up the nature of the body, and through these he feels pain or enjoys health. Now, he enjoys the most perfect health when these elements are duly proportioned to one another in respect to compounding, power and bulk, and when they are perfectly mingled. Pain is felt when one of these elements is in defect or excess, or is isolated in the body without being compounded with all the others."*

Imbalance of liquids would cause disease, which could be treated by balancing the humours of a patient.

A similar scheme existed in Chinese medicine. Organs were classified according to five basic elements of nature. Together with successful application of vegetable remedies, other substances bearing a symbolic and metaphorical meaning were used like rhinoceros horn or tiger penis. Ascription of healing potency, based on some other properties of the object, –

could it be called in modern language a "rational drug design"? Compensating excess of yin with yang-food – isn't it rational decision? The only difference is the rationale itself.

One can consider rationality to be an accumulation of empirical facts and their metamorphosis into a new concept of cause-effect relationship. Depending on depth of the insight into the matter the concept of "therapy" changed. If Hippocrates is called a father of medicine, Paracelsus is the father of pharmacy. Like his predecessors, he considered bodies to consist of several basic elements. He distinguished between three of these: Salt, Mercury, and Sulphur. These are the names of elements we still use nowadays, but the meaning changed considerably:[4]

> *A different Sulfur is found in gold, another in silver, another in iron, lead, zinc, and so forth-the same is true of Salt and Mercury. They are still more divided, so that there is not only one kind of gold, hut many kinds of gold, just as there is not only one kind of pear or apple but many kinds. Therefore, there are just as many different kinds of Sulfurs of gold, Salts of gold, Mercuries of gold.*

Paracelsus used both organic and mineral compounds to treat different diseases, some of them may seem to be absurd, but still there is rationality behind it. In the end of the 15th century, a violent epidemic of syphilis occurred in Europe. It was either imported from the New World or existed previously, but had mutated considerably. Paracelsus had his own explanation: it was caused by astral constellation of Venus combined with sexual libertinism.[5] His treatment was in accordance with the proverb "Two minutes with Venus, two years with Mercury": the patients were prescribed mercury to balance the elements in the body and to excrete the disease with saliva, three pints needed to be produced in order for the treatment to be considered effective.

Mercury was used conventionally for syphilis treatment till the beginning of the 20th century. By that time, the first exact analyses and syntheses of pure substances became possible. With the boom of organic chemistry, the new methods of experimental tests laid the foundation for pharmacy, and by the end of the century it became science.[6]

1.2 Base of Modern Drug Development

In 1878 the medicine student Paul Ehrlich wrote his dissertation titled "Contributions to the Theory and Practice of Histological Staining". Injecting dyes into living organisms, he observed that cells of different organs were stained in different ways. The blood cells and microorganisms were of special interest to him. Ehrlich wanted to stain them without affecting other tissues. The successful experiments encouraged him to set a new task: to make

these selective substances toxic for microorganisms. He spent years treating samples with chemically modified substances to achieve this goal. The idea was revolutionary at that time, and nowadays it's the common method of drug development.[7]

"In a first place we want to affect the parasites solely, that means we need to learn to target, to target chemically. For this purpose the methods offer possibly versatile variation of considered substances on a way of chemical syntheses."

In 1863 the French pharmacist Antoine Béchamp synthesised an arsenorganic compound, which was named "Atoxyl" (Figure 1) because of the low toxicity observed. The observation turned out to be wrong, after using the substance in Africa for sleeping dropsy treatment a serious side effect was found – the drug was affecting the optic nerve.

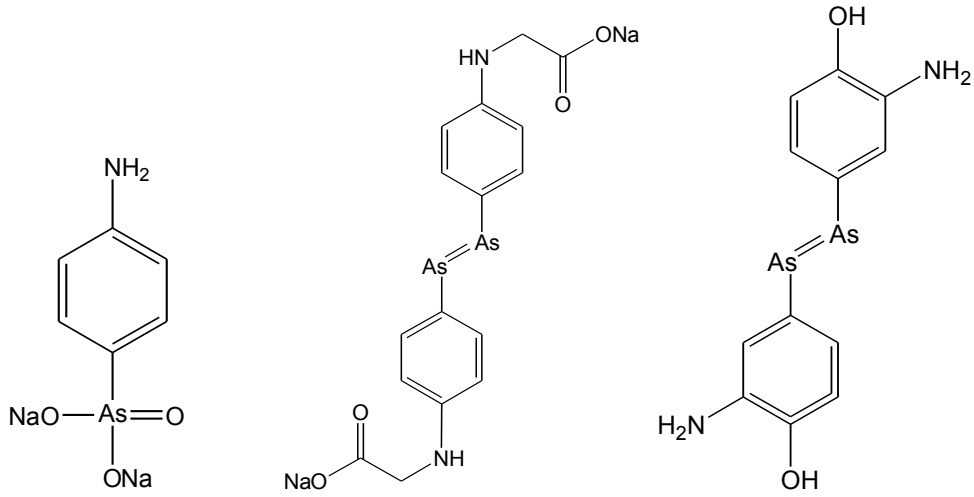


Figure 1. Atoxyl, arsonophenylglycin and salvarsan (depicted in monomer forms)

Following his concept, Ehrlich started to vary the structure, and after some hundreds of trials a first success was achieved. It was "arsenophenylglycin", compound number 418. The substance was not really effective for the treatment of sleeping dropsy, but showed potency against spirochetes and, at the same time, low toxicity. Research was carried out further, tests were performed on the first feasible animal model, syphilis infected rabbits brought from Japan. The investigation of analogous compounds revealed the most interesting one, number 606. Toxicity was lower as compared to the derivatives of As(V) due to the intentionally changed oxidation state of arsenic in the molecule following the idea of bioreduction of arsenic in the human body. One year later, the substance was marketed under the name salvarsan.

The proposed structure with As=As (by analogy with azo compounds) was under debate almost until present. In 2005 ESI-MS investigation showed that the predominant species in

aqueous solution are rings with 3 and 5 As atoms in the molecule.[8]

The discovery and strategy of structure optimization of salvarsan were important but maybe of even higher importance were the concepts proposed by Ehrlich. The investigations on "toxin – antitoxin" interactions of the natural toxins abrin and ricin, the experiments on "natural and acquired immunity" led to in his general "side-chain" theory. By the "side chains" he meant macromolecules on surface of a cell that could interact stoichiometrically with their counterparts (e.g. toxins – antitoxins) on the cell surface. In 1900, he introduced the term "receptor" instead of "side-chain". The principle that toxins, nutrients and drugs bind selectively and the observation that tissues could be stained with different dyes, allowed him to establish connection between a chemical structure of the applied compounds and the nature of a biological object. Extrapolating these molecular interactions he proposed the idea of a "magic bullet" – an absolutely selective drug. His statement "wir müssen chemisch zielen lernen" ("we have to learn how to aim chemically") as well as the strategy of optimizing chemical structure became the new paradigm for chemical treatment of the 20th century (Figure 2). Much later, his idea was realized literally in antibody therapy of, for example, non-Hodkin lymphoma,[9] and was conceptually implemented in targeted therapy. But the general principle was applied much sooner. During tests on nitrogen mustard as a potential chemical weapon, an unexpected property was discovered: it was more toxic to lymphoma and myeloma cells as compared to healthy ones and in 1942 clinical trials for lymphoma treatment were started. The ongoing development of molecular biology allowed deeper insights into molecular mechanisms of the mustard gas's action: DNA was found to be the target. A search for agents, that were potentially reactive towards DNA, started after establishing this fact. One of the first successes were the synthetic nucleobases, fluorinated pyrimidines, which were prepared to hinder the replication process in 1957.[10]

Afterwards, a plenitude of DNA-attacking molecules was discovered. They were modified and brought to clinical practice, still making use of the same principle – to stop DNA polymerisation and thus cell division.

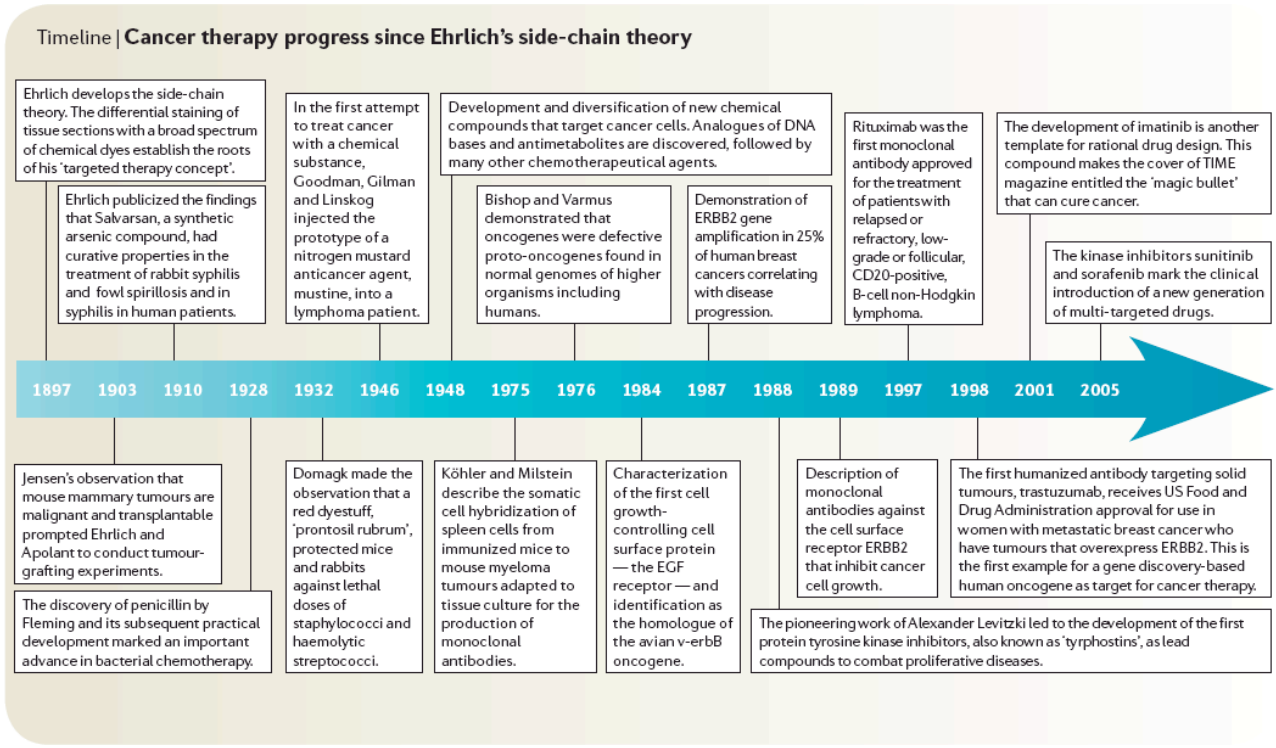


Figure 2. Progress in drug developments starting from Ehrlich's "Side-Chain Theory" (taken from ref. 11)

Further investigation of carcinogenesis revealed other cell targets, potentially useful for developing therapies: the cell proliferation could be interfered at any stage of the cell cycle. Cyclin-dependent kinases were found to be an attractive object of investigation due to their abundance and necessity in all cells. The close structural similarity of many kinases and thus possible interactions of a drug molecule with more then one of them changed the Ehrlich's postulate of the "magic bullet" (one drug – one target) into a "multitargeted drug", a shotgun, to spread Ehrich's metaphor.[12]

The principle of drug design, laid by Ehrlich and successfully used in development of salvarsan, may be considered to be rational, but it was not *the optimization,* it was variation of structure to achieve better therapeutic impact. This kind of half-blind search was extensively used in the second half of the 20th century. Plenty of compounds, usually, organic ones, were screened for their biological activity, and this work resulted in an enormous amount of information on physical, chemical and biological properties. With the development of synthetic techniques, combinatorial synthesis became possible but the results were rather disappointing. The number of usable hits and consequently the value of the method, was very low, although the quantity of synthesised substances was enormous.[13]

1.3 Langmuir's Approach

The connections between composition and properties of substance were the core idea of chemistry since Mendeleev's systematization of the elements. Hammet demonstrated the influence of structure on the reactivity of organic compounds in the 1930-ies. His theory became useful in predicting biochemical properties of the substances. Although empirical, it could quantify the influence of substituents on sterical and electronic properties, molar refractivity and lipophilicity.[14]

Hammet's outlook was a consecutive, but definitely more exact development of the concept proposed by Langmuir in 1919. His article "Isomorphism, Isosterism and Covalence"[15] begins with the previously formulated ideas on structure of matter: "In recent papers I have described a theory of valence which I have called the octet theory." Further the scale is enlarged, comparing physical properties of numerous gases and inorganic salts (both cations and anions), he called the compounds with similar properties *isosteres*. In some cases their resemblance is rather concealed: N_2 and CO, F^- and O^{2-}, He and Li^+, the conclusions on the base are even more surprising: *"The relation between argon and nitrogen enables us to trace an equally close relationship between the cyanogen and the chlorine ions",* but the concept was largely applied in biochemical research.

The term "bioisosterism" was introduced by Friedman in 1951, then was redefined as "compounds or groups that possess near-equal molecular shapes and volumes, approximately the same distribution of electrons, and which exhibit similar physical properties."

Similarity of atomic radii of hydrogen and fluorine allow this isosteric replacement, the strategy was successfully pursued in the development of 5-fluorouracil, a synthetic inhibitor of thymidylate synthase. The enzyme is necessary for DNA replication, and its strong covalent binding to 5-fluoro-2'-deoxyuridylic acid (metabolite of 5-fluorouracil) results in deactivation and thus ceases cancer cell proliferation. The small spatial difference between uracil and its fluorinated analogue allows successful mimicking, while a strong difference in electronegativity of H and F change their interaction with the enzyme significantly.[16]

Not only atoms, but whole groups can be substituted according to their isosterical properties. The first attempt to classify them scientifically was performed by Grimm in 1929.[17] He arranged isosters ("pseudoatoms") in columns:

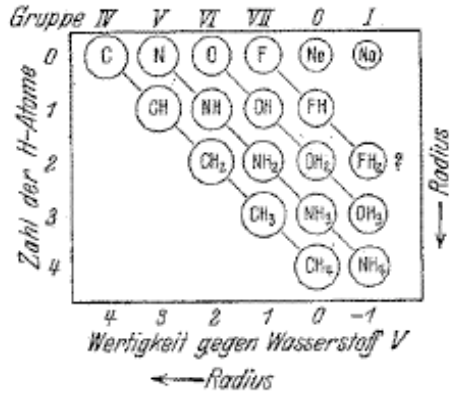


Figure 3. Isosteric replacements, proposed by Grimm

This approach can be efficient in the case that the synthesis of multiple drug candidates was not troublesome and economically unrealistic, but it covers only known molecules, either discovered drugs or extracted from natural material. Later the strategy was called “Ligand-Based Drug Design”, as opposed to the “Structure-Based” one.

Improved preparative methods afforded straightforward synthesis of desired structures, but not all of them could be subjected to biological testing. In order to increase the efficiency of biological screening, filters were required to guide the chemist to the optimal result. The most famous of these filters is Lipinski’s rule of 5, based on the statistical analysis of properties of thousands of drugs. Similarities among them allowed to formulate the following rules for a drug to have bad absorption and permeation:[18]

There are more than 5 H-bond donors (the sum of OH's and NH's)

The molecular weight is over 500

The Log P is over 5 (or MLogP is over 4.15)

There are more than 10 H-bond acceptors (expressed as the sum of Ns and Os);

Compound classes that are substrates for biological transporters are exceptions to the rule.

The sum of criteria gave a new tongue-twisting adjective to describe a compound as a potential medicine – “drugelikeness”. The established paradigm was abundantly quoted and motivated many researchers to expand it, to find further characteristics of molecules that could be useful for their biochemical application. In 2001, Oprea noticed that 70% of drug-like compounds had between zero and two hydrogen bond donors, between two and nine hydrogen bond acceptors, between two and eight rotatable bonds and between one and four rings. Verber found other parameters to be important for oral bioavailability of drugs in rat:

the number of rotable bonds has to be smaller than 10 and a polar surface not larger than 140Å.[19]

As Ehrlich proposed and was established experimentally later, the drug molecule reaches its target in the body. The complimentary (to structural variation) approach is to investigate the target itself and to find out how to facilitate its interaction with a drug molecule. Potentially interesting proteins (targets) are isolated and cloned. Numerous NMR methods, X-ray crystallography and mass-spectrometry give information on the 3D structure in the solid state and in solution. When the geometry is known the active site can be found. The pocket is packed either with a molecule from a database, or the filling is constructed from atoms and functional groups. Further operations are performed *in silico*, so called *de novo* drug design (Figure 4). After the shape of a drug is determined, other properties, necessary for effective binding, are computed. Complex binding of a small molecule and a target consists of hydrogen bonding and hydrophobic and electrostatic interactions. The first being the strongest among them, this determines the key interaction site of the pair and narrows down the range of possible structures.[20]

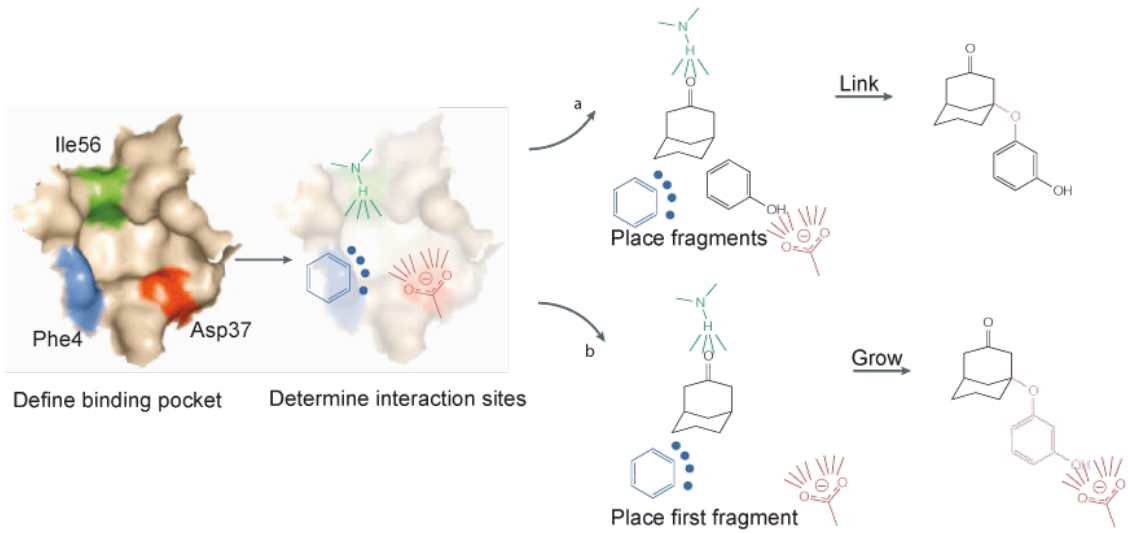


Figure 4. *De novo* drug design (Taken from reference 20)

2. Platinum-Based Therapy

2.1 Platinum Drug Development

Cisplatin, the first platinum-based drug, was discovered accidentally but not by chance. In 1961 young physist Barnett Rosenberg investigated the influence of an electric field on the growth of *Escherichia Coli* bacteria. The idea, or better to say, the intuition behind consisted in the similar appearance of a mitotic spindle and the lines of a magnetic field (Figure 5), seen on iron filings around a magnet bar. "It looks like a dipole field," – he said.[21]

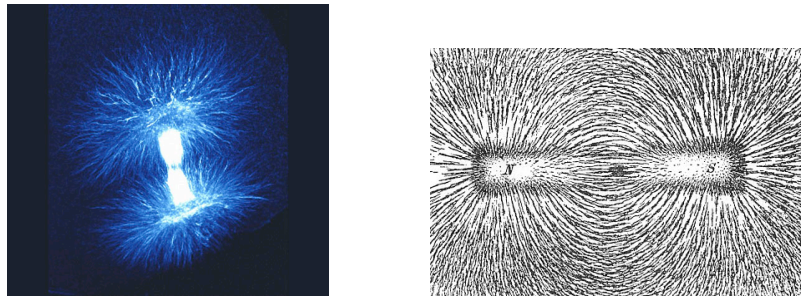


Figure 5 Spindle apparatus (left) and iron spinings in magnetic field (right) [22]

Electrical current passed through a medium with *E. Coli* inhibited bacteria division. Thorough examinations of the experiment parameters (frequency of voltage, pH, presence of oxygen, temperature, UV irradiation) were carried out. The exploration reminded more of a detective story, than a cold scientific report. Finally, a substance was proposed to be responsible for the cease of bacteria division (and not the electric current itself). It was a platinum salt $(NH_4)_2[PtCl_6]$ which was formed upon attack of chloride ions from the electrolyzed solution on platinum electrodes.[23] Other metal compounds were tested as well, and rhodium salts also caused bacteria elongation. The nature of the effect was unclear. The role of oxidation state of metal, ligands around them and the geometry of complexes were stressed as undefined. As was established in a pioneering work on biological effects of inorganic compounds in 1931, not the metal alone, but also its companions should be considered.[24] The authors tested metals and their salts dissolved in physiological solution and suspended in olive oil if the solubility was too low. These were injected subcutaneously to trace the patterns of biological effects, and found metals in zero oxidation state to be ineffective. The most fundamentally important statement was that "similar compounds of the same metal often show absolutely different impact."

This was proved in further investigations of Rosenberg, when *cis*- and *trans*-isomers (Figure 6) of $[PtCl_4(NH_3)_2]$ were synthesized separately and tested on *E. coli*. The *trans*

complex was inefficient as an inhibitor of bacteria division, but the *cis* species showed the same activity as was observed in the experiment with platinum electrodes. Simultaneously, the corresponding Pt(II) complexes were tested. Again, the *cis* isomer was effective and *trans* did not show any impact.[25]

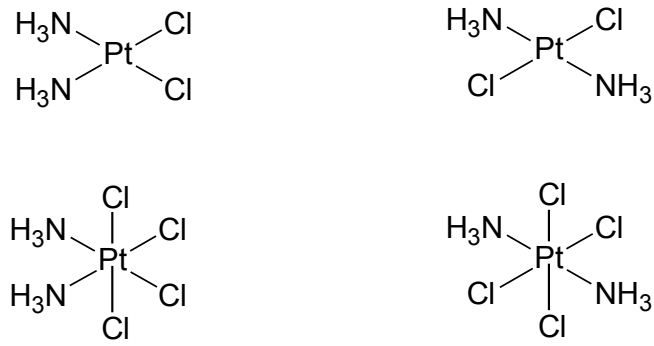


Figure 6. *cis*- (left) and *trans*- (right) diamminedichloridoplatinum (above) and their corresponding Pt(IV) analogs (below)

This was the first structure-activity relation that afterwards had been proved for the analogues of initial cisplatin. Two possible explanations for the configuration were proposed: 1) *trans*-complexes are hydrolysed significantly faster and thus react faster and less selectively, 2) *cis*-complexes can form chelates which could be the reason for antitumor activity. Numerous analogues of the parental structure (focusing on the *cis*-configuration) were synthesized and tested for biological activity.

Very soon after Rosenberg's discovery, in 1970, DNA was suggested to be a target for the complexes.[26] Some similarities to alkylating agents like mustard gas were found. The anticancer activity could be explained by intrastrand DNA cross-linking based on the possibility of *cis*-isomers to form chelates. Because of this parallel, cisplatin was classified as "alkylating" type of anticancer drugs (or "alkylating-like" sometimes), although the pure inorganic molecule has nothing to do with organic alkylation reactions. After intravenous injection, cisplatin diffuses into the cells (passively or supported by copper transporters) and undergoes aquation. In the bloodstream the reaction is supressed by a higher chloride concentration, but the intracellular concentration is much lower and the dichlorido complex is activated for DNA-attack. The most favourable coordination site of DNA is the electron-rich N7 atom of guanines (Figure 7) and adenines. After formation of the monoadduct, another chloride is substituted by neighbouring nucleobase. Intrastrand (i.e. located on the same strand of DNA) cross-links with two neighbouring guanines are the most common adduct for cisplatin. As a result, the DNA replication is hindered, which leads to apoptosis.

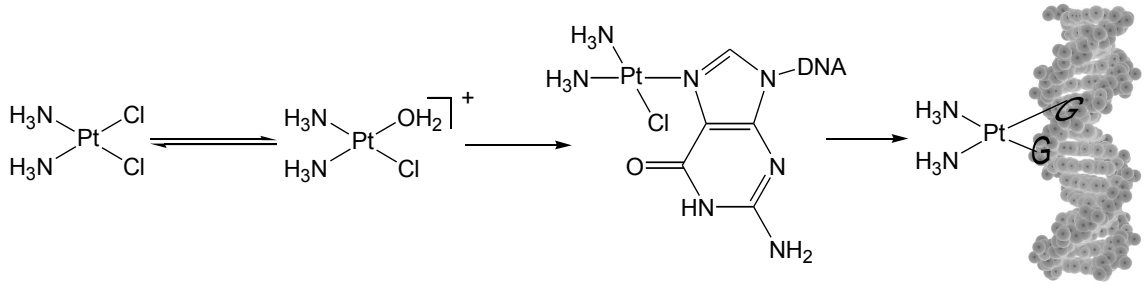


Figure 7. Hydrolysis of cisplatin, formation of N7-guanine monoadduct, and its conversion into intrastrand G-G crosslink

The successful treatment and rapid approval by the American Food and Drug Administration in 1978 (first trials began in 1971) was a strong impulse for the search for complexes with improved properties as some tumours turned out to be resistant to cisplatin and additionally severe side effects of the therapy were observed. Thousands of complexes were synthesized and tested for anticancer activity, and soon further regularities were established: chlorido ligands react, and ammoniac molecules remain coordinated. Structure variations and studies on their biological impact were performed already in 1973 within less than 10 years after discovery of the Pt-induced cellular effects.[27] Higher lability of dissociating ligands such as H_2O, NO_3^- resulted in immediate and unselective toxicity, and inert leaving groups like rhodanido and nitrito decreased the potency of complexes as they hinder hydrolytic activation and the following intracellular reactions. The range of labile ligands is limited to halogens and O-donors, because some softer atoms like P, S form too strong bonds with the soft Pt central atom. The (pseudo)halogenido ligands were exchanged against chelating oxalate and malonate and these complexes showed promising results in some cancers. The strategy of increasing drug stability using chelates brought a second generation drug with lower nephro- and neurotoxicity – carboplatin (Figure 8). The adducts formed with DNA are the same due to the same ammine ligands. A reason for decreased side toxicity is the hydrolysis rate.

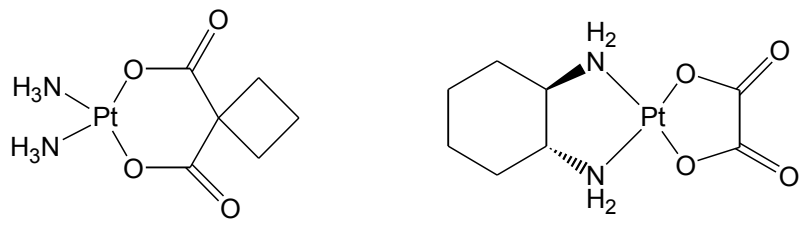


Figure 8. Approved Pt-based drugs with dicarboxylates as leaving groups, carboplatin (left) and oxaliplatin (right)

2.2 Rules of Activity

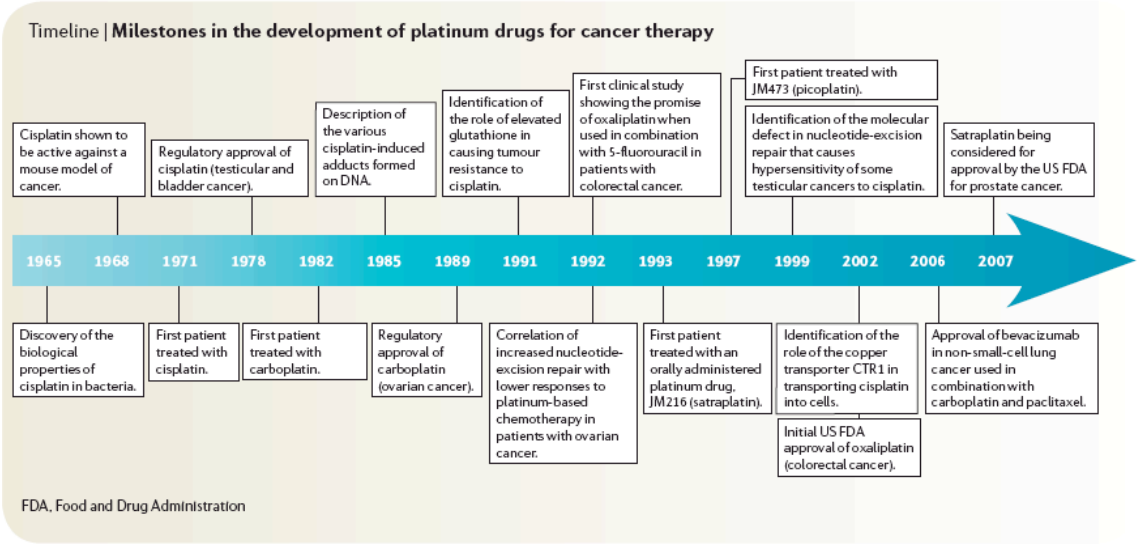


Figure 9. Development of Pt-based drugs (taken from reference 28)

The leaving group determines the reactivity of complexes, the am(m)ine fragment is responsible for steric properties and lipophilicity. The highest activity was observed for ammonia and primary amines. Any further substitution at the nitrogen atom decreased activity and solubility of the complexes, exceptions are rare. Also, interactions of hydrogens at the coordinated nitrogen atom with biomolecules were expected to play an important role in the activity of the drug.

The empirical facts were summarized in four rules, necessary for anticancer active Pt(II) complexes:[28]

1) The complex should be uncharged.

2) *cis*-Configuration of the leaving groups is essential.

3) Lability of leaving groups should be comparable with to the one of chlorides in cisplatin.

4) The other ligands should be inert and neutral.

The 4 rules left not much space for structure variation. They were expanded to another oxidation state of Pt. Investigation of Pt(IV) complexes revealed the necessity of *cis*-configuration of amine ligands as well (1975),[29] and it was demonstrated in 1985, that antitumor activity of Pt(IV) complexes is in fact the activity of Pt(II) analogues, that are generated upon reduction of the initial species.[30]

All Pt-based drugs, approved for marketing or being candidates for approval follow the rules, which can clearly be seen on a superposition of structures of these complexes.

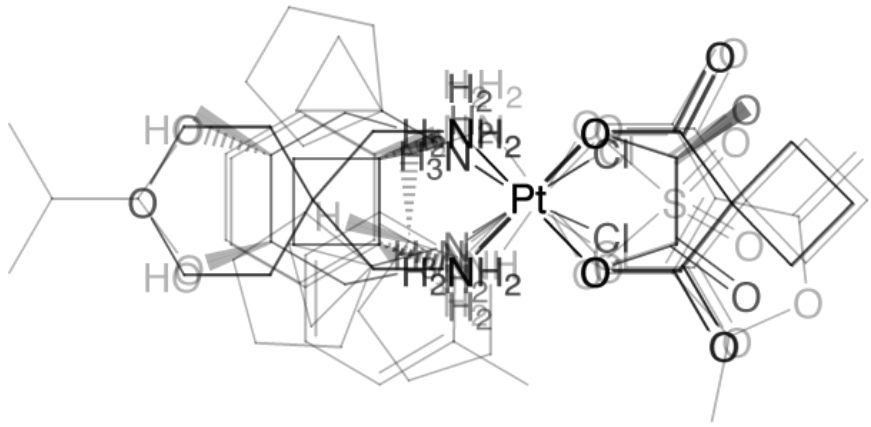


Figure 10. Superposition of approved and promising Pt-based drugs.

The rules facilitated the development of numerous active compounds, but together with guiding the researchers they narrowed their sight field and "the rule has sometimes been invoked more prescriptively".[31]

2.3 Rulebrakers

As any framework, when pressure of contradicting facts is getting too high, it turns useless. These four rules lost their validity upon subsequent investigation of Pt complexes and as insight into biological action became deeper. The majority of complexes that possess the described properties were significantly less active as compared to cisplatin. *cis*-Complexes with linear alkylamines lost their activity with the elongation of the hydrocarbon chain, branched alkyl radicals did not show good results as well.

The first *trans* complexes to demonstrate higher cytotoxicity as compared to the corresponding *cis* isomers were discovered in 1989.[32]

A fast hydrolysis rate was found to be one of the reasons for decreased activity of *trans* isomers. The active hydrolyzed species were undergoing faster side reactions and were unable to reach the DNA. But the 5-fold difference in ID_{50} values of *trans* and *cis* dipyridine complexes could not simply be explained with the hydrolysis rate as they were found not to differ dramatically and the obtained facts entailed more questions than answers.

If rapid hydrolysis is undesired, it should be hindered. Knowing that the ligand-exchange reaction proceeds via a 5-coordinated intermediate state, it could have been slowed down by bulkier substituents hindering the axial positions of the complex. The concept was used for the preparation of *trans*-complexes with quinoline ligands.[33] Significant activity of the

compounds with *trans*-configuration was also observed in Pt(IV) analogues as well as complexes with a bulky cyclohexylamine substituent.

Approximately at the same time, in 1989, the rule of neutrality was violated.[34] Activity of cisplatin analogues with one chloride substituted for heterocyclic amines in murine tumour models was higher than that of cisplatin itself. In 1991 both rules of *cis*-configuration and zero charge were broken by Gibson *et al*, when the covalent binding of Pt complexes to DNA was reinforced with the intercalating properties of an appended antraquinone ligand. The isomers (*cis* and *trans*, Figure 11) showed similar activity.[35]

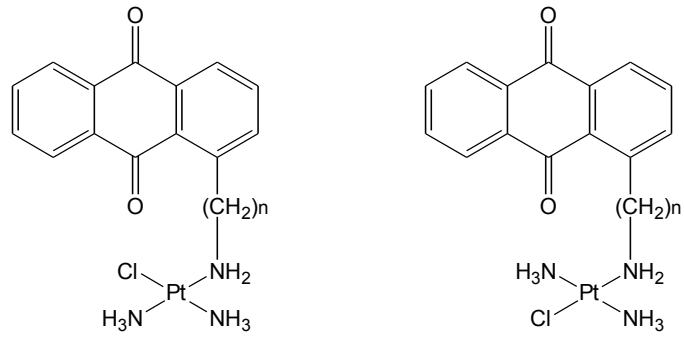


Figure 11. *cis*- (left) and *trans*- (right) isomers of Pt(II) complexes with an antraquinone ligand

Multinuclear platinum-based complexes first described by Farrell,[36] can also be considered as *trans*-configured ones. The *trans* position to chlorine is occupied by a diamine linker, that transfers the second DNA binding site to another metal centre.

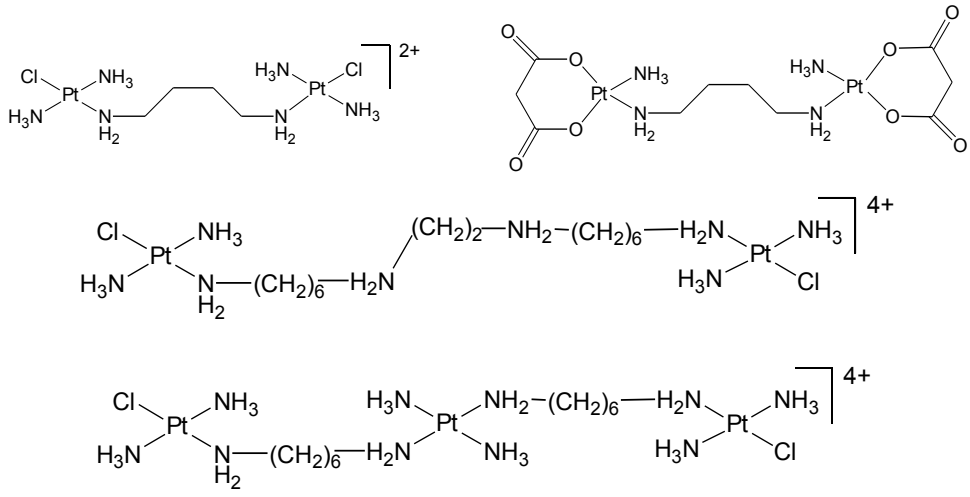
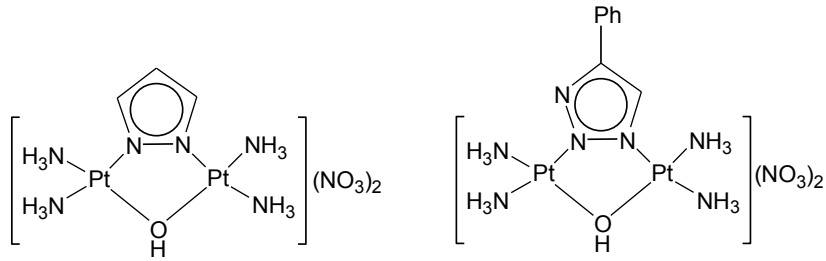


Figure 12. Di- and trinuclear Pt(II) complexes with linear linkers

The idea behind designing these compounds was to make different DNA adducts possible, that would be recognized and repaired in a different way in order to overcome cisplatin

resistance. Numerous linkers of different nature were introduced between two platinum moieties.[37] Complexes with bridging azoles showed activity in a cisplatin resistant cell line, and investigation of their binding showed isomerization of nucleobase adducts, which could lead to different DNA distortions and thus recognition.[38]



Picture 13. Dinuclear Pt(II) complexes with bridging azoles

The platinum fragments were linked via linear polyamines with prospect of hydrogen bonding between the central amino group and H-donors of the DNA, but the best results were obtained by a trinuclear complex with one additional platinum moiety as linker. This is the only non-classical compound (i.e. rule-braking) to pass Phase II clinical studies.[39] A noteworthy fact is, that the total charge of the trinuclear complex cation is 4+.

2.4 Pt(IV) Complexes as Anti-Cancer Drugs

The only thing that remained of the initial rules is the classification: complexes that obey the rules are called "classic" ones, all others "non-classic" complexes. But there is a vast class of platinum complexes that were already considered by Rosenberg, but left aside in further development, namely Pt(IV) complexes.

The first promising Pt(IV) compound was *cis,trans,cis*-dichloridodihydroxido-bis(isopropylamine)platinum(IV), initially named CHIP and then "iproplatin" (Figure 14). Comparison with cisplatin (called in that paper "neoplatin") showed an equal antitumor effect and lower toxicity.[40] The importance of the development of analogues with smaller side effects like hearing loss and nephrotoxicity brought the compound into phase I clinical trials.[41] In further examinations, the cross-resistance with cisplatin was stressed as an advantage of the compound,[42] but the activity in phase II was just modest.[43]

The second Pt(IV) compound brought into investigation was tetrachlorido(*dl-trans*)-1,2-cyclohexanediamineplatinum(IV) called tetraplatin (Figure 14). The stated rationale was again the cisplatin resistance and the limited range of responsive cancers.[44] Nevertheless, its toxicity (neuropathy) was higher than the therapeutic effect and it was abandoned.

The structure of tetraplatin was synthetically completed, and no further modifications

were possible, besides a non-leaving cyclohexanediamine moiety. Iproplatin with its two axial hydroxido groups had more space for modifications. In 1992, Khokhar used common reaction of organic chemistry to modify dihydroxido Pt(IV) complexes – they reacted as alcohols, forming inorganic ester analogues. A series of different carboxylato complexes was synthesized, and trifluoracetato derivatives were found to be more effective when compared to acetato ones. This could be explained by a higher lipophilicity of the trifluormethyl groups, but an influence of the stronger inductive acceptor on the reduction of the Pt(IV) complex to its Pt(II) analogue was also not excluded.[45] Recently, it was established that not the potential alone is responsible for reduction, the thermodynamically driven reaction also faces a kinetic obstacle. Electron transfer is facilitated by the formation of a bridge between complex and reducing agent, thus the reaction rate is very slow, when the coordination sphere consists only of amines and carboxylates.[46]

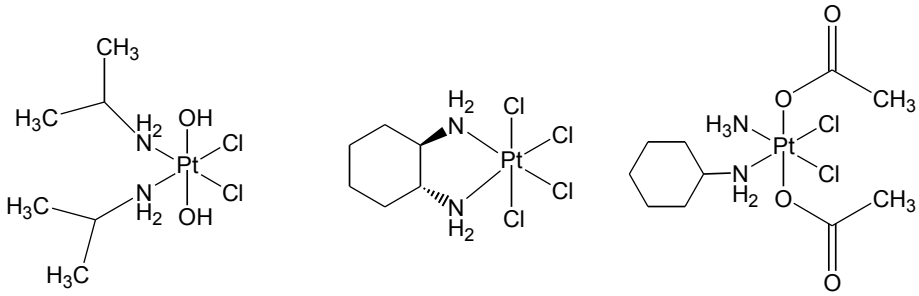


Figure 14. Pt(IV) candidates: iproplatin, tetraplatin and satraplatin

In 1993 another Pt(IV) compound was prepared as a potentially orally available drug by Kelland *et al.* and Brystol-Myers: JM216, later on named satraplatin. Four years before an attempt to introduce carboplatin orally caused nausea and vomiting even when offered in lemonade syrup and adsorption was poor compared to intravenous injection.[47] A Pt(IV) formulation was proposed due to higher kinetic stability of these complexes, two additional ligands increased lipophilicity, however the different mode of administration may have changed the pharmacokinetics of the drug.

The easiest and most logical modification of satraplatin would be a variation of axial acetates. The ligands are cleaved upon reduction to the Pt(II) species, they do not affect binding to intracellular targets, but change the lipophilicity and the reduction potential of the complex.

Acylated complexes could be afforded either via anhydrides or acid chlorides. The reaction of hydroxide complexes with cyclic anhydrides yielded molecules with a free carboxylic group,[48] and the functionality was extensively used for derivatization. Activation

and coupling reactions of carboxylic acids are well developed in organic chemistry and numerous esters and amides can be produced for the fine-tuning of lipophilicity of drugs.[49] As delivery vectors also more complex molecules were appended like hormones,[50] carbon nanotubes[51] and peptides.

The empirically determined and calculated properties of "rationally designed" Pt(IV) complexes were subjected to QSAR systematization with the declared goal to develop a reliable predictive model for cytotoxicity of complexes in given cell lines.[52] Models could be built using experimentally determined parameters like lipophilicity and reduction potential or even calculated numbers of C, N, O atoms in a molecule:

$\log 1/IC_{50}(\text{ovarian}) = -0.015 + 0.25(\pm 0.03)nCs - 0.27(\pm 0.07)nO,$

where nCs is the number of sp^3 carbon atoms, and nO the number of oxygen atoms.

One can also see from the equation that the more carbons the molecule contains, the higher is its cytotoxicity. Lower water solubility of compounds with long alkyl residues is thus not considered. The importance of reduction kinetics and not only the potentials was mentioned above,[46] and the contribution of reduction potential in vivo may significantly differ from cytotoxicity tests.

As one can see, this regression model is applicable only to closely related compounds and may be considered a compact form to describe the IC_{50} values of a class of substances for a given cell line, but is not the optimal tool for finding new drugs.

2.5 Other Strategies for Improvement

Besides the variation of a drug structure itself, combinations of already established drugs with supportive pharmaceuticals were extensively investigated. One of the reasons for a lower activity of Pt compounds in many cancers is the binding of complexes to glutathione (cysteine-containing tripeptide), and buthionine sulfoxime was used to suppress glutathione synthesis.[53] Another reason for cisplatin resistance is mismatch repair, and addition of DNA demethylating agents helped to sensitize tumours resistant to cisplatin.[54] The addition of bevacizumab, an antibody targeting the vascular endothelial growth factor receptors, to the carboplatin – paclitaxel regimen improved overall survival by "increase of drug delivery" to the tumors.[55] In reality, the medicine was not target-oriented (i.e. brought to the tumour), but could reach it more easily.

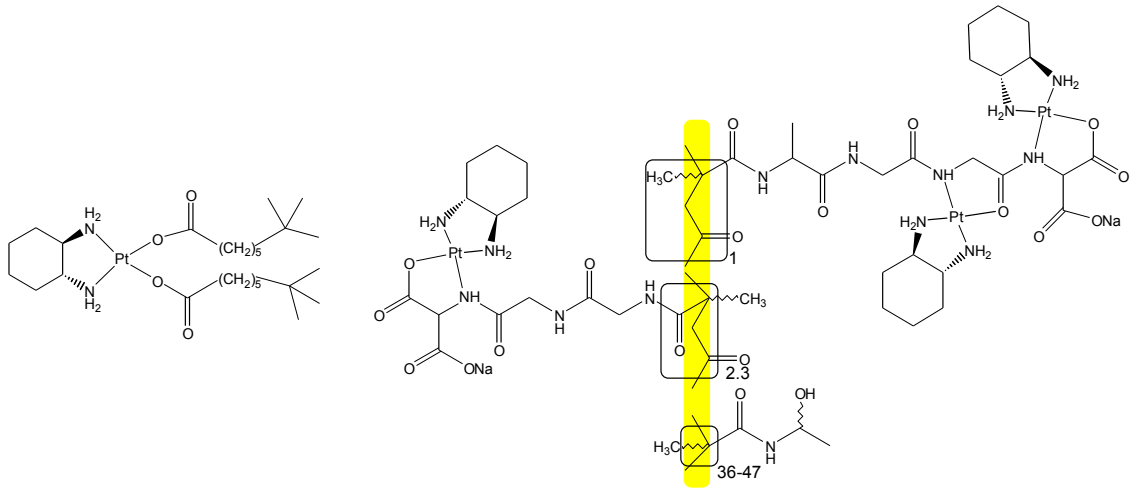


Figure 15. Aroplatin and prolindac, carrier-supported drugs

Drug delivery in its literal sense is yet another booming area in pharmacy, and platinum-based drugs are not an exception. One strategy exploited was to trap cisplatin into PEG-ylated micelles in order to increase its stability in blood. The formulation got the name “Lipoplatin”, it was clinically investigated parallel to free cisplatin and carboplatin. The Phase III trials showed lower toxicity together with retained activity in non-small cell lung cancer.[56] Slow release of cisplatin from liposomes is the main problem of the application of the drug, and ultrasound was applied to increase its availability. The method, though, is limited to superficial tumours, because ultrasound cannot reach deeper tissues.[57] The type of cancer seems to play an important role in Lipoplatin: early attempts to treat osteosarcoma in dogs were not successful, probably because of the low availability of cisplatin. The osteosarcoma is less relevant as a cancer model, because it misses leaky vasculature important for drug accumulation in the tumour region.[58]

Another goal was set to encapsulate cyclohexanediamine complexes of platinum with two lipophilic carboxylates. Liposomic formulations made administration of water-insoluble compounds possible and the efficiency of encapsulation was also increased as compared to cisplatin.[59] Development of this strategy resulted in aroplatin (Figure 15), an encapsulated (*R,R*-1,2-cyclohexanediamine)-bis(neodecanoato)platinum (II), that was tested in phase II as a drug candidate for colorectal carcinoma. It was well-tolerated and similarly active to oxaliplatin.[60]

A similar logic stands behind the polymer-bound (*R,R*-1,2-cyclohexanediamine)platinum, prolindac (Figure 15), evaluated in phase II clinical studies.[61] The long hydroxy-propylmethacrylamide polymer chains should be retained in the leaky blood vasculature of tumours (enhanced permeability and retention effect, EPR) and upon hydrolysis of the

amidomalonato group the DACH-platinum cation is released. The hydrolysis of complexes is faster at a lower pH, thus the selectivity for cancer sites is increased.[62]

Liposomes, polymers, micelles and nanoparticles are used as carriers for drugs with selectivity based on macrocharacteristics of cancer like a lower pH in tumour area or leaky blood vessels. Later, platinum complexes were conjugated to short peptides with the RGD-motive that are recognized by integrins upregulated on certain cancer cells. The peptide-conjugated complexes showed higher efficacy compared to the single aminoacid derived ones and showed potential as more selective drugs.[63] Besides the presented results, another aspect is important in the article. The tethered peptides are called "tumour-targeting-device". Appending receptor-binding peptides is also integrated into the term "targeting", but the term is not quite clear even on the webpage of he National Cancer Institute (NCI). "Targeted therapy" is defined as "a type of treatment that uses drugs or other substances, such as monoclonal antibodies, to identify and attack specific cancer cells," [64] but even in the factsheet of the same institute a more detailed description says: "Targeted cancer therapies are drugs or other substances that block the growth and spread of cancer by interfering with specific molecules involved in tumor growth and progression. Because scientists often call these molecules "molecular targets," targeted cancer therapies are sometimes called "molecularly targeted drugs," "molecularly targeted therapies," or other similar names." [65]

"Targeting" and all its paronyms thus became a symbol of new, better – rational drugs. Market analyses already describe them separate from classical anticancer therapeutics, because the growth of the integer oncology market is mostly attributed to rapid development of molecularly targeted drugs.[66] The ratio of US sales of chemotherapies versus targeted cancer therapies changed from 0.84 to 1.28 within 4 years (Figure 16), which makes the concept of targeted therapy desirable in drug marketing.

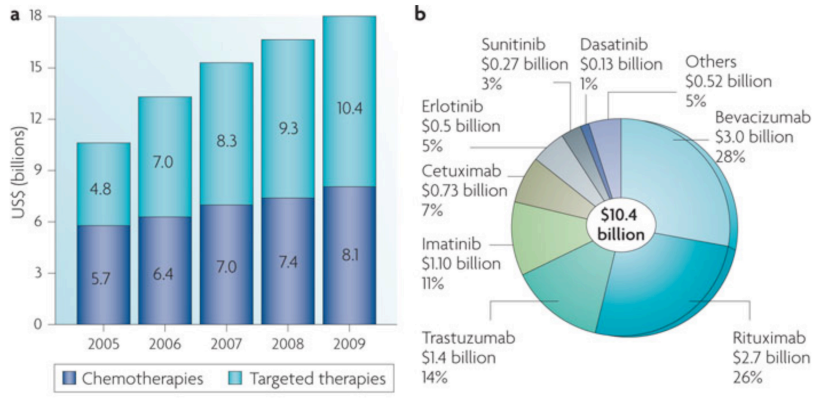
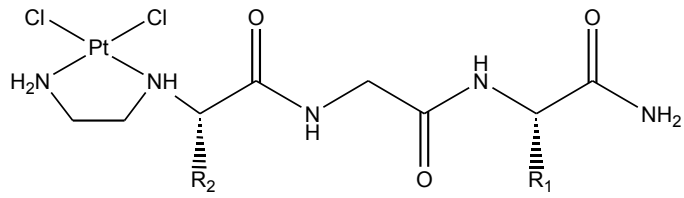


Figure 16. Market growth of targeted and classical chemotherapeuticals
(fig. taken from the ref. 66)

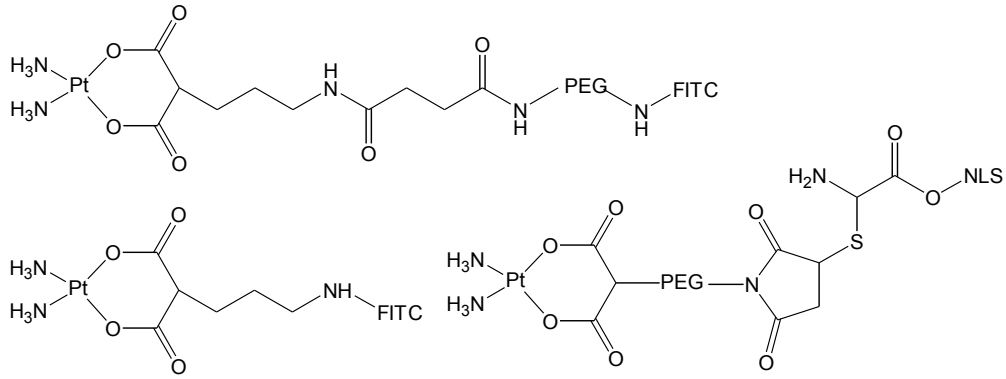
The adjective “rational” experienced the same fate, as “targeted”. On the same NCI webpage they are mentioned together: “targeted therapies are often referred to as the product of “rational drug design.” Osella *et al.* stressed, that the series of synthesized complexes was well thought-out, but in general the term is used for molecules adapted to their targets.[67]

It is interesting to mention, that in the first article about Pt conjugation the peptide was not appended as a delivering moiety (Picture 17), guanidine residues (R_1=$(CH_2)_3NHC(NH)NH_2$, R_2=H) of arginine should have changed the binding properties of the complex to DNA.[68] Soon, the synthesis was performed in an automated mode[69] and a dinuclear complex was prepared,[70] but none of the compounds showed any significant cytotoxic activity. In the investigated series of N-substituted ethylene diamine ligands with dipeptide fragment the best results were observed with least sterically hindering glycine derivatives (R_1=R_2=H), the lowest effect was observed with R_2=Benzyl and R_1=H, sending back to the old postulate of minimal substitution of the coordinated nitrogen atom.



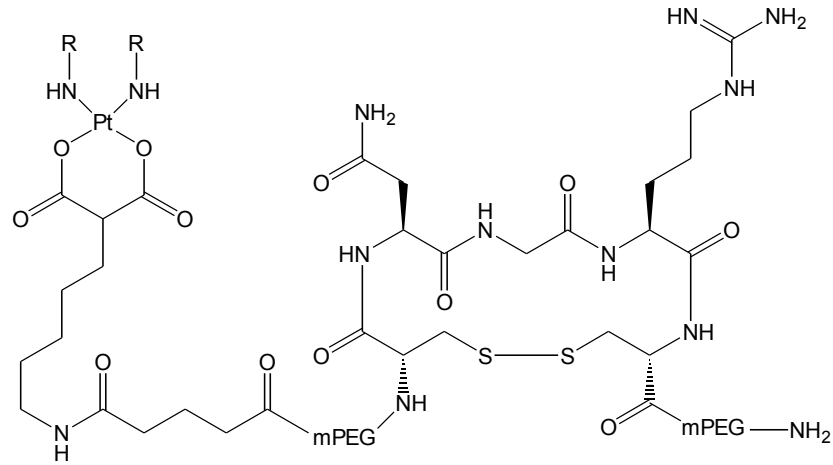
Picture 17. Pt(II) peptide conjugate

Peptide tethering of the leaving malonato group did not bring much success as well. In a thorough article of Gibson *et al.,* the diammineplatinum fragment was appended to a nuclear localization signal (NLS), a short cationic peptide facilitating transport into the cell nucleus. The following molecules were synthesized and investigated:[71]



Picture 18. Conjugates of the diammine Pt(II) fragment with NLS (FITC = fluorescein isothiocyanate, PEG = polyethylene glycol)

NLS was necessary for delivery, fluorescein allowed microscopic monitoring of label distribution within cells and PEG-ylation was performed analogically to other investigated non-platinum anticancer drugs. Confocal microscopy showed accumulation of NLS-vectored folic acid (a model cargo) in the nucleus. The molecules without peptide remained distributed in the cytoplasm. But the results of expriments with Pt-conjugates were unexpected: the unconjugated complex showed better cytotoxicity and DNA platination. The phenomenon was explained by insufficient activation of the initial molecule: before reacting with DNA, the complex needs to be hydrolyzed to form the active species *cis*-$[Pt(NH_3)_2]^{2+}$. Probably it was not efficiently released in the nucleus and the hydrolysis in cytosol was necessary.



Picture 19. NGR-tethered Pt(II) conjugates, R=H or R=C_2H_5.

Conjugation to a different peptide indirectly proved Gibson's assumption: when malonate was appended to a peptide with the NGR motif,[72] able to bind to endothelium of angiogenetic blood vessels and tumour cells in murine breast carcinoma models, the uptake was significantly increased in parallel with cytotoxicity. In this case, the leaving group could dissociate in the cytoplasm, before the activated diam(m)ineplatinum cation reached DNA.

3. Concluding remarks

The complexes of Gibson should have also been a poor drug candidate according to the rules of Lipinski and Cleare: a high charge of the molecule due to 5 basic amino acids (and large polar surface area), probably, low lipophilicity because of the same reason and high molecular weight. But they were not potent because of absolutely different properties of the substance. Oxaliplatin conjugates with the TAT peptide, described in the second article enclosed, would also be bad candidates according to the listed requirements, but the cytotoxic activity was significantly better, compared to the unconjugated analogues.

Insufficient cell uptake of cisplatin and its analogues remains one of the main problems of therapy, and increased influx of the drug can make them much more efficient. Pt(IV) complexes with axial ligands, which are cleaved upon reduction to Pt(II) derivatives, offer the best opportunity for drug delivery, because the reduction products are the investigated Pt(II) analogues. Amine ligands are responsible for the form of DNA adducts, chemical modification of their periphery has its optimum – in case of the cyclohexanediamines, the best results were observed with 4-methyl substituted rings, larger substituents decreased the activity of complexes.[73] The leaving groups determine the hydrolysis (and thus activation) rate. Inert complex are vain, too reactive ones suffer side reactions before they reach DNA. If both groups can be optimized to their limit, the only remaining site is the axial position. Conjugation with carriers, delivery vectors, or any other molecules via cleavable linker offers the best strategy to modify properties of drugs, optimized for intracellular action in future development of platinum-based drugs.

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{(1*R*,2*R*,4*R*)-4-Methyl-1,2-cyclohexanediamine}oxalatoplatinum(II): A Novel Enantiomerically Pure Oxaliplatin Derivative Showing Improved Anticancer Activity in Vivo

Sergey A. Abramkin,[†] Ute Jungwirth,[‡,⊥] Seied M. Valiahdi,[†,⊥] Claudia Dworak,[†] Ladislav Habala,[†] Kristof Meelich,[†] Walter Berger,[‡,§] Michael A. Jakupec,[†] Christian G. Hartinger,[†] Alexey A. Nazarov,*[,∥] Markus Galanski,*[,†] and Bernhard K. Keppler*[,†,§]

[†]*University of Vienna, Institute of Inorganic Chemistry, Währinger Strasse 42, A-1090 Vienna, Austria,* [‡]*Department of Medicine I, Institute of Cancer Research, Medical University of Vienna, Borschkegasse 8a, A-1090 Vienna, Austria,* [§]*Research Platform "Translational Cancer Therapy Research" University of Vienna, Währinger Strasse 42, A-1090 Vienna, Austria, and* [∥]*Institut des Sciences et Ingénierie Chimiques, Ecole Polytechnique Fédérale de Lausanne (EPFL), CH-1015 Lausanne, Switzerland.* [⊥] *U.J. and S.M.V. contributed equally to this work*

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Novel derivatives of the clinically established anticancer drug oxaliplatin were synthesized. Cytotoxicity of the compounds was studied in six human cancer cell lines by means of the MTT assay. Additionally, most promising complexes were also investigated in cisplatin- and oxaliplatin-resistant human cancer cell models. The therapeutic efficacy in vivo was studied in the murine L1210 leukemia model. Most remarkably, {(1*R*,2*R*,4*R*)-4-methyl-1,2-cyclohexanediamine}oxalatoplatinum(II), comprising an equatorial methyl substituent at position 4 of the cyclohexane ring, was as potent as oxaliplatin in vitro but distinctly more effective in the L1210 model in vivo at the optimal dose. The advantage observed in the in vivo situation was mainly based on a more favorable therapeutic index. The maximum tolerated dose of the novel analogue was higher than that of oxaliplatin and caused a greater increase in life span (>200% versus 152%), with more animals experiencing long-term survival (5/6 versus 2/6). These data support further (pre)clinical development of the methyl-substituted oxaliplatin analogue with improved anticancer activity.

Introduction

The cytotoxic properties of cisplatin, *cis*-diamminedichloridoplatinum(II),[1] were found by serendipity in Barnett Rosenberg's laboratory in 1965.[2,3] Cisplatin was finally approved by the Food and Drug Administration (FDA[a]) in 1978. Exchange of the two chloride leaving groups of cisplatin with the more kinetically inert 1,1-cyclobutanedicarboxylato ligand resulted in carboplatin, *cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II), which received approval in 1989 as second-generation platinum-based agent. In 2004, the FDA approved the third generation platinum drug, oxaliplatin (Figure 1), {(1*R*,2*R*)-cyclohexanediamine}oxalatoplatinum(II),[4,5] for the first line treatment of colorectal carcinoma in combination with 5-fluorouracil and leucovorin (5-FU/LV); neurotoxicity was found to be dose limiting.[6] Clinical trials based on different tumors of the gastrointestinal tract and breast and lung carcinomas are ongoing. Remarkably, oxaliplatin shows a different spectrum of cytotoxicity compared to cis- and carboplatin as deduced from the 60 cell line panel of the NCI, being in accord with its activity in the inherently cis- and carboplatin resistant colorectal carcinoma.[7]

Compared to cisplatin, the volume of distribution (serves as a measure, how effectively a drug is cleared from the bloodstream and going into tissue) of oxaliplatin is considerably high. Moreover, the terminal elimination phase is longer than that for cisplatin.[8] The mechanism of cellular oxaliplatin accumulation is still unclear. In contrast to cisplatin, accumulation of oxaliplatin in cancer cells is less dependent on the copper transporter CTR1.[9] Like cisplatin, the ultimate target of oxaliplatin is the genomic DNA and it forms the same types of inter- and intrastrand DNA cross-links as cisplatin,[10,11] however, the reactivity toward isolated and cellular DNA is lower.[12] Because the cytotoxic potency of oxaliplatin is generally well comparable or even higher than that of cisplatin in some cell lines, oxaliplatin-induced DNA adducts have to be more cytotoxic than those arising from cisplatin.[13] Cisplatin−DNA adducts are effectively recognized by the MMR complex and by HMG1 but not those adducts deriving from oxaliplatin.[14,15] Postreplicative bypass is also different and more efficient past oxaliplatin−GG adducts.[16] On a molecular level, the adducts of oxaliplatin[17] and cisplatin[18] with the same dodecamer duplex were investigated by crystal structure

*To whom correspondence should be addressed. For A.A.N.: phone, +41 (0)21 693 98 60; fax, +41 (0)21 693 98 85; E-mail, alexey.nazarov@epfl.ch, For M.G.: phone, +43-1-4277-52603; fax, +43-1-4277-52680; E-mail, markus.galanski@univie.ac.at. For B.K.K.: phone, +43-1-4277-52602; fax, +43-1-4277-52680; E-mail, bernhard.keppler@univie.ac.at.

[a] Abbreviations: 5-FU, 5-fluorouracil; Cbz, carbobenzoxy; COSY, correlated spectroscopy; CTR, copper transporter; DACH, diaminocyclohexane; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; d-OHP, *S*,*S*-enantiomer of oxaliplatin; ESI, electrospray ionization; FDA, Food and Drug Administration; G, guanine; HMG, high-mobility group protein; ILS, increase in life span; LV, leucovorin; MEM, minimal essential medium; MMR, mismatch repair; MsCl, methanesulfonyl chloride; MTD, maximum tolerated dose; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; NCI, National Cancer Institute; Py, pyridine.

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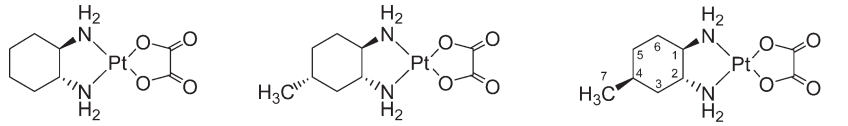


Figure 1. Structures of oxaliplatin and the novel enantiomerically pure oxaliplatin derivatives with either equatorial (**3a**) or axial (**3c**) methyl substituent with NMR numbering scheme.

diffraction, revealing a relatively similar geometry. From that point of view, it is difficult to understand the differences in their cytotoxic potential. However, it has to be taken into account that the situation in solution may vary from that in the solid state as could be demonstrated recently.[19]

The cyclohexanediamine carrier ligand in oxaliplatin comprises a *trans*-1*R*,2*R* configuration with both amino substituents in equatorial position; the cyclohexane ring is situated nearly perfectly within the square planar coordination plane.[20] The same holds true for the *trans*-1*S*,2*S* enantiomer. In the case of the *cis*-1*R*,2*S* counterpart, one amino substituent is in an equatorial and the other in an axial position, leading to a nearly perpendicular arrangement of the cyclohexane ring with respect to the coordination plane.[21] The antiproliferative properties of all three (cyclohexanediamine)-oxalatoplatinum(II) complexes have been investigated with the following structure−activity relationship (DACH = diaminocyclohexane): *trans*-1*R*,2*R*-DACH enantiomer > *trans*-1*R*,2*R*/1*S*,2*S*-DACH racemate > *trans*-1*S*,2*S*-DACH enantiomer > *cis*-1*R*,2*S*-DACH diastereomer.[22,23] The difference between *trans*- and *cis*-configured complexes is easily explainable by the shape of the *cis* analogue (perpendicular arrangement of the cyclohexane ring). Indeed, due to steric hindrance, adduct formation with DNA was slow and also the conversion of the monoadduct to the bisadduct.[24] However, in the case of both *trans*-isomers (same shape), the DNA seems to act as chiral discriminator as could be shown by Lippard and colleagues.[17] Formation of a hydrogen bond between the pseudoequatorial NH hydrogen of the (1*R*,2*R*)-cyclohexanediamineplatinum(II) fragment and O^6 of a neighboring guanine was found, which is not possible in the case of *trans*-1*S*,2*S*-configuration.

Finally, what we really do know about the mode of action of oxaliplatin and what may serve as basis for the development of novel oxaliplatin derivatives with improved cytotoxic and anticancer properties are: (i) distribution in the body and cellular accumulation of cisplatin and oxaliplatin are different and (ii) recognition and processing of DNA adducts are not the same. These features are, at least in part, the result of the *trans*-(1*R*,2*R*)-cyclohexanediamine carrier ligand, providing lipophilicity along with steric bulk to oxaliplatin. Consequently, we have focused on further increasing the lipophilicity/steric bulk of the cyclohexanediamine moiety. Here, we report for the first time on the synthesis, characterization, in vitro cytotoxicity, and in vivo anticancer activity of a novel enantiomerically pure oxaliplatin derivative, {(1*R*,2*R*,4*R*)-4-methyl-1,2-cyclohexanediamine}oxalatoplatinum(II), exhibiting an equatorial methyl group at position 4 of the cyclohexane ring. We also demonstrate that bigger substituents (ethyl or *t*-butyl) do not lead to an improvement of the cytotoxic and anticancer properties.

Results and Discussion

The aim of the present work was to synthesize close analogues of oxaliplatin, which are equipped with an increased lipophilicity, additionally having an influence on the structure of adducts formed with the final target DNA. Consequently, the intention was to improve the biological properties of oxaliplatin and not to develop completely different types of complexes ("The most fruitful basis for the discovery of a new drug is to start with an old drug" Sir James Black, Nobel Prize in Physiology or Medicine in 1988).[25] The oxalato leaving group was left unchanged because it affects the solubility, the biodistribution, and the systemic toxicity but of course not the structure of DNA adducts. The {*trans*-(1*R*,2*R*)-cyclohexanediamine}platinum(II) fragment binds to DNA in the major groove (Supporting Information, Figure S1), generating there an unpolar region/steric bulk. From that point of view, it seems to be reasonable to derivatize the fragment at position 4 of the cyclohexane ring (preferably in equatorial position), offering a maximal distance from the coordination site. Substitution at positions close to the metal center, or even at the coordinated nitrogen atoms, would significantly influence the structural changes of DNA adducts and were therefore not favored.

Synthesis and Characterization. Racemic (1*R*,2*R*,4*R*/1*S*,2*S*,4*S*)-4-methyl-1,2-cyclohexane-diamine was synthesized as reported recently.[26] Shortly, 4-methylcyclohex-1-ene was converted into the vicinal *trans*-diol (Scheme 1) by treatment with hydrogen peroxide in the presence of formic acid. In a next step, the corresponding mesylate was formed via reaction with methanesulfonyl chloride/pyridine, which was then transferred with sodium azide into 4-methyl-*trans*-1,2-cyclohexanediazide. The diamine was subsequently obtained by reduction with hydrogen at Pd on $CaCO_3$ (Lindlar's catalyst).

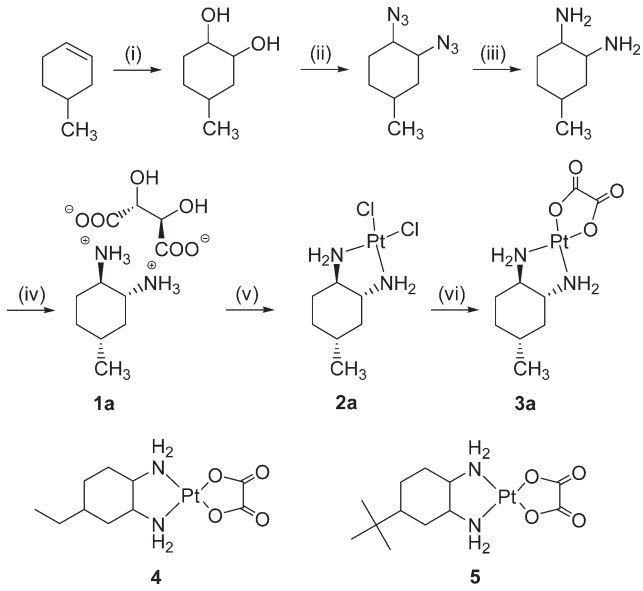
Enantiomer separation of racemic (1*R*,2*R*,4*R*/1*S*,2*S*,4*S*)-4-methyl-1,2-cyclohexanediamine was performed via recrystallization of the respective L- or D-tartrates in an ethanol−water mixture (Supporting Information, Scheme S1). Enantiomerical purity was proven by chiral HPLC. Prior to HPLC analysis, the diamines were derivatized with benzyloxycarbonyl chloride in order to obtain UV-active compounds (**6** + **7**, Supporting Information, Scheme S1 and Figure S2).

Enantiomerically pure (1*R*,2*R*,4*R*)-4-methyl-1,2-cyclohexanediaminium L-tartrate, **1a**, was then reacted with K_2PtCl_4 in the presence of NaOH, resulting in the yellow dichloridoplatinum(II) complex **2a**. The chlorido ligands were released with $AgNO_3$ and addition of potassium oxalate to the formed diaquaplatinum(II) species finally resulted in the white target complex **3a**, {(1*R*,2*R*,4*R*)-4-methyl-1,2-cyclohexanediamine}oxalatoplatinum(II). The corresponding 1*S*,2*S*,4*S*-enantiomer **3b** was obtained by following the same procedure and starting from (1*S*,2*S*,4*S*)-4-methyl-1,2-cyclohexanediaminium D-tartrate **1b**.

Oxalatoplatinum(II) complexes **3a** and **3b** were fully characterized by elemental analysis and multinuclear (^1H, ^{13}C, and ^{195}Pt) one- and two-dimensional NMR spectroscopy. The coordination sphere around the platinum atom can best

be judged by measuring a ^{195}Pt NMR spectrum. The resonance at −365 ppm for **3a** (Figure 2) is indicative for a PtN_2O_2 coordination and is in accordance with the chemical shift found for oxaliplatin at −361 ppm.[27] The corresponding ^{195}Pt signal for the dichlorido precursor complex **2a** with PtN_2Cl_2 coordination was detected significantly upfield at −644 ppm.

Scheme 1. Synthesis of {(1*R*,2*R*,4*R*)-4-Methyl-1,2-cyclohexanediamine}oxalatoplatinum(II), **3a**[a]



[a] (i) H_2O_2/HCOOH, (ii) MsCl/Py, NaN_3, (iii) Pd/$CaCO_3$, H_2 (3 bar), (iv) L-tartaric acid, (v) K_2PtCl_4/NaOH (0.25 M), and (vi) $AgNO_3$, potassium oxalate. Structures of 4-ethyl- and 4-*t*-butyl oxaliplatin derivatives **4** and **5**, synthesized as enantiomeric mixtures (all substituents at the cyclohexane ring in equatorial positions).

In **3a**, all substituents (two amino and one methyl group) of the cyclohexane ring are in an equatorial position; consequently, protons at carbon atoms 1, 2, and 4 are axially oriented, which can be proven by the Karplus relationship for NMR spin−spin coupling constants (vicinal H−C−C−H coupling constants are large at angles of 180° with $^3J_{H,H} \approx 11-12$ Hz, and small when the torsion angle is 60° with a coupling of around 5 Hz).

Indeed, large couplings of the axial proton H-1ax at 2.31 ppm with the neighboring protons H-2ax and H-6ax with $^3J_{H,H} = 11.5$ Hz, and a small $^3J_{H,H}$ coupling to H-6eq of 4 Hz were detected (Figure 2), proving the equatorial position of the amino substituent. The same holds true for proton H-2ax, demonstrating the trans-configuration at C1−C2. In the case of proton H-3ax, resonating at 0.89 ppm, a quartet with a coupling constant of 12 Hz was found as a result of two neighboring axial protons (H-2ax, and H-4ax) and additionally the large geminal $^3J_{H,H}$ coupling to proton H-3eq. Axial position of H-4ax finally proves that the methyl group at C-4 is equatorially oriented. Signal assignment of all remaining protons is based on two-dimensional ^{1}H,^{1}H- and ^{1}H,^{13}C correlated NMR spectra. Of note is the diastereotopic splitting of the methylene protons at C-3, C-4, and C-6, giving rise to six single shift correlation signals in the ^{1}H,^{13}C NMR spectrum. Synthesis and characterization of racemic 4-ethyl and 4-t-butyl-1,2-cyclohexanediamine}oxalatoplatinum(II) complexes **4** and **5** (all substituents at the cyclohexane ring equatorial position, Scheme 1) were described recently.[28]

With the intention of further optimizing structure−activity relationships, the platinum complex **3c**, {(1*R*,2*R*,4*S*)-4-methyl-1,2-cyclohexanediamine}oxalatoplatinum(II), exhibiting

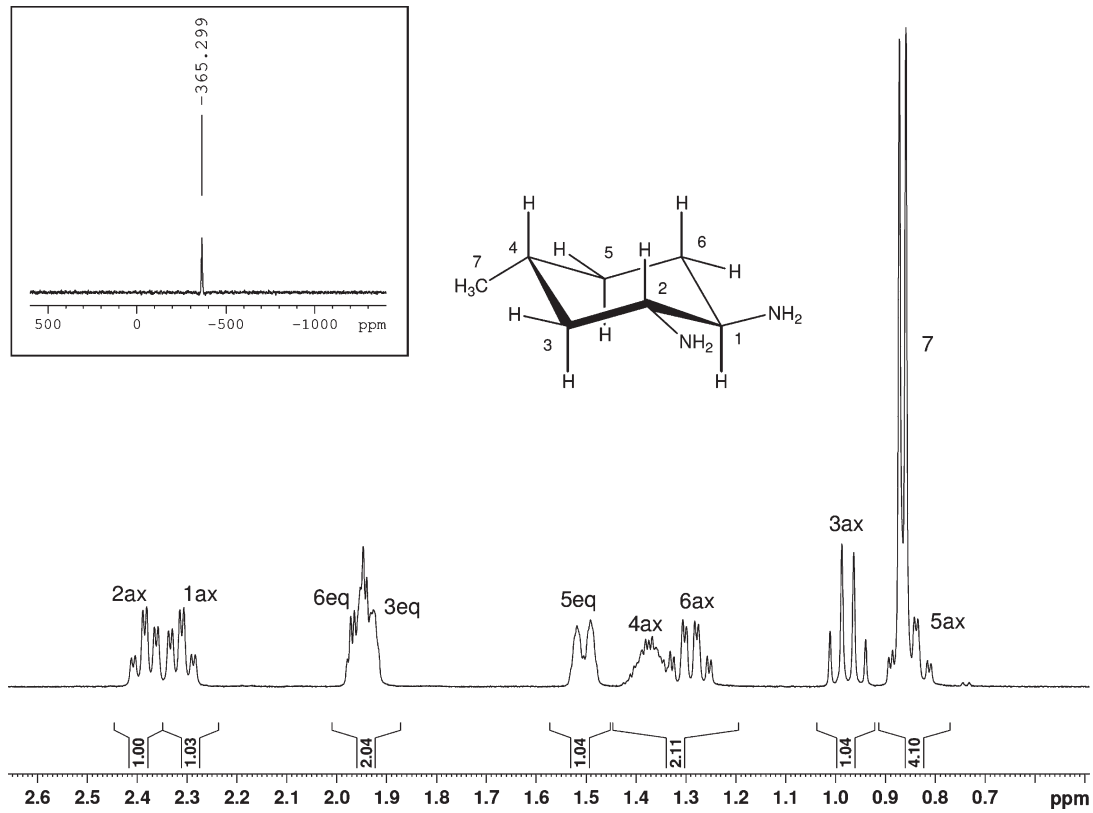


Figure 2. ^{1}H and ^{195}Pt (small insert) NMR spectrum of {(1*R*,2*R*,4*R*)-4-methyl-1,2-cyclohexanediamine}oxalatoplatinum(II) **3a** with NMR numbering scheme and signal assignment. The oxalatoplatinum(II) fragment has been omitted for clarity.

Table 1. Cytotoxicity of Oxaliplatin Derivatives Compared to Oxaliplatin in Six Human Cancer Cell Lines

	IC_{50} (μM)[a]					
compd	CH1	HeLa	HCT-15	HCT-116	SW480	U-2 OS
oxaliplatin	0.33 ± 0.09	0.35 ± 0.07	1.5 ± 0.6	0.38 ± 0.03	0.30 ± 0.08	0.72 ± 0.24
d-OHP[b]	1.0 ± 0.04	1.1 ± 0.4	4.4 ± 1.6	1.7 ± 0.1	1.3 ± 0.3	2.5 ± 0.7
3a	0.28 ± 0.09	0.21 ± 0.06	1.6 ± 0.1	0.81 ± 0.31	0.67 ± 0.31	0.71 ± 0.41
3b	0.69 ± 0.10	0.88 ± 0.38	4.6 ± 0.7	2.9 ± 0.7	2.3 ± 1.2	1.8 ± 0.6
3c	0.10 ± 0.01	0.24 ± 0.02	0.33 ± 0.07	0.32 ± 0.05	0.33 ± 0.14	0.52 ± 0.03
4	0.38 ± 0.08	0.66 ± 0.13	3.4 ± 1.0	1.6 ± 0.1	0.92 ± 0.30	4.0 ± 0.3
5	4.1 ± 0.8	11 ± 2	39 ± 3	31 ± 2	24 ± 3	24 ± 5

[a] 50% inhibitory concentrations in the MTT assay (96 h exposure). Values are means ± standard deviations obtained from at least two (mostly three) independent experiments. [b] d-OHP is the (1*S*,2*S*)-enantiomer of oxaliplatin.

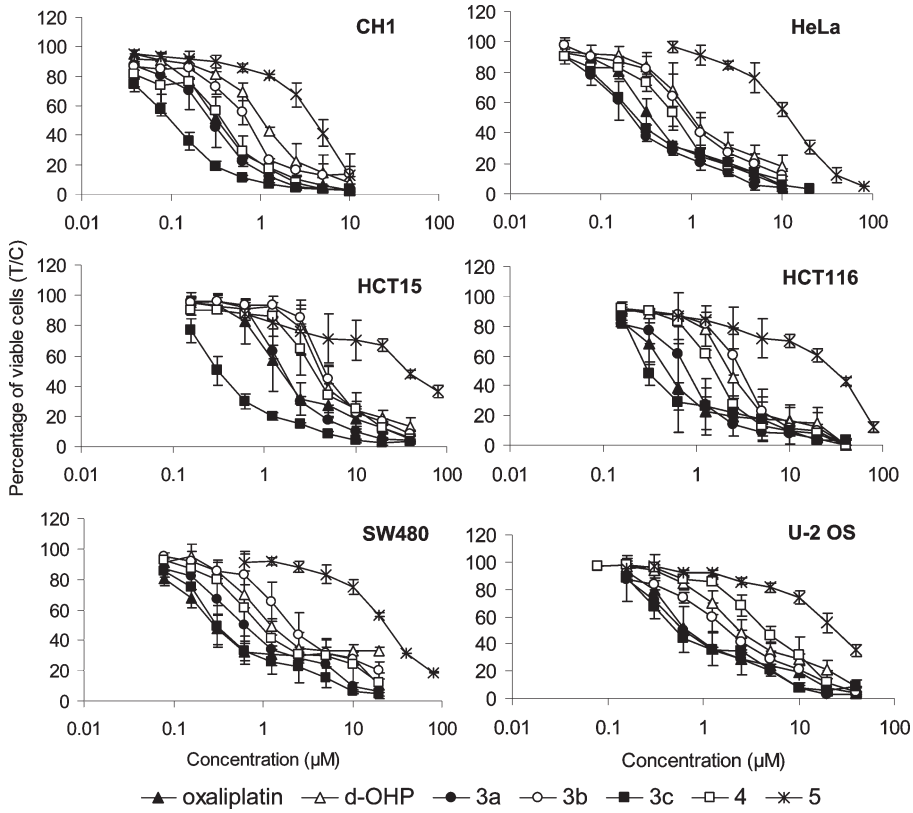


Figure 3. Concentration−effect curves of ring-substituted oxaliplatin derivatives in comparison to oxaliplatin and its *S*,*S* analogue (d-OHP) in CH1, HeLa, U-2 OS, HCT-15, HCT-116, and SW480 cells, obtained by the MTT assay (96 h exposure).

an axial methyl substituent at position 4 of the cyclohexane ring, was synthesized (Supporting Information, Scheme S2). Starting from enantiomerically pure (*S*)-citronellal, (*S*)-4-methylcyclohexene was prepared, which was converted to the corresponding diazide exhibiting trans-configuration at C1/C2. After reduction, (1*R*,2*R*,4*S*)-4-methyl-1,2-cyclohexanediaminium L-tartrate, **1c**, was obtained (diastereomeric purity was proven by chiral HPLC in the form of the Cbz derivative, Supporting Information, Figure S3). In analogy to **3a** and **3b**, the oxalato complex **3c** was synthesized and characterized. Besides a distinctly different ^{195}Pt chemical shift (**3c**, −381 ppm; **3a**, −365 ppm), the axial position of the methyl group could be judged by comparison of ^{1}H (Supporting Information, Figure S4) and ^{13}C NMR spectra of **3a** and **3c**, respectively. As expected, the chemical shift difference of protons H-1ax and H-2ax ($\Delta\delta$ = 0.27 ppm) in **3c** is significantly bigger compared to **3a** ($\Delta\delta$ = 0.08 ppm). The same holds true for chemical shift differences of carbon atoms C1 and C2 in **3c** ($\Delta\delta$ = 4.9 ppm) and **3a** ($\Delta\delta$ = 0.2 ppm). However, most indicative are the differences (shapes and chemical shifts, compare Figures 2 and Supporting Information, Figure S4) in the proton NMR spectra for ^{1}H resonances at C3, C4 and C5.

Cytotoxicity in Cancer Cell Lines and Structure−Activity Relationships. Cytotoxicity of oxaliplatin and the here presented derivatives with equatorial alkyl substituents in position 4 of the cyclohexane ring was compared by means of a colorimetric microculture assay (MTT assay) in six human cancer cell lines representing four tumor entities: ovarian carcinoma (CH1), cervical carcinoma (HeLa), osteosarcoma (U-2 OS), and colon carcinoma (HCT-15, HCT-116, SW480), yielding IC_{50} values mostly in the low micromolar or even submicromolar range (Table 1, Figure 3).

Oxaliplatin and the 4-methyl derivative **3a** show quite similar cytotoxic potencies in all six cell lines. Notably, **3a** is at least as active as oxaliplatin in four of them, except for the two colon cancer cell lines HCT-116 and SW480. Generally, the (1*R*,2*R*,4*R*)-enantiomer **3a** is by factors of 2−4 more cytotoxic than the (1*S*,2*S*,4*S*)-enantiomer **3b**, indicating that the well-known structure−activity relationship observed

Table 2. Cytotoxicity of Oxaliplatin Derivatives Compared to Oxaliplatin in Cisplatin- And Oxaliplatin-Resistant Human Cancer Cell Models

	IC_{50} (μM)[a]								
compd	GLC4	GLC4/CDDP	-fold resistance	A2780	A2780/cis	-fold resistance	HCT116	HCT116 oxR	-fold resistance
cisplatin	1.7 ± 0.5	18.0	10.6	1.8 ± 0.2	> 10	> 5	4.7 ± 0.1	7.3 ± 0.1	1.6
oxaliplatin	0.6 ± 0.2	1.2 ± 0.3	2.0	0.4 ± 0.2	0.7 ± 0.4	1.8	0.6 ± 0.1	22.0 ± 0.1	36.7
3a	0.8 ± 0.1	2.1 ± 0.1	2.6	0.5 ± 0.1	1.6 ± 0.8	3.2	1.5 ± 0.1	11.7 ± 0.1	7.8
3c	0.3 ± 0.2	0.6 ± 0.1	2.0	0.2 ± 0.1	0.5 ± 0.3	2.5	0.5 ± 0.1	9.3 ± 0.1	18.6

[a] 50% inhibitory concentrations in the MTT assay (72 h exposure). Values are means ± standard deviations obtained from at least two (mostly three) independent experiments.

with oxaliplatin and its (1*S*,2*S*)-enantiomer (d-OHP) also applies to the alkyl-substituted derivatives. The other 4-methyl derivative **3c** (methyl substituent in axial position) is even more cytotoxic than oxaliplatin in the majority of cell lines and thus the most potent of the studied compounds, whereas the racemic 4-ethyl derivative **4** yields IC_{50} values that are mostly intermediate between those of **3a** and **3b** (with the exception of U-2 OS cells).

In contrast, the IC_{50} values of the racemic 4-*t*-butyl derivative **5** are on average an order of magnitude higher than those of **4**. These data support a previous study, which showed that large alkyl substituents will result in a decrease of cytotoxicity.[29] Generally, the cytotoxicity profiles of these compounds resemble each other, with CH1 and HeLa being the most sensitive cell lines and HCT-15 being the least sensitive cell line. Therefore, fundamental differences in the underlying molecular mechanisms of action are unlikely.

The cytotoxic properties of compounds **3a** and **3c** were also analyzed in cisplatin- and oxaliplatin-resistant cell models derived from small cell lung (GLC4), ovarian cancer (A2780), and colon cancer (HCT-116) (Table 2). Both highly cisplatin-resistant cell models (GLC4/CDDP and A2780/cis) were (comparable to oxaliplatin) significantly but very moderately (up to 3.2-fold) cross-resistant to the novel derivatives. The resistance of oxaliplatin-insensitive HCT-116 oxR against the new derivatives was, as expected, more distinct (up to 18.6-fold) as compared to that of cisplatin. Nevertheless, it was significantly weaker for both tested derivatives (**3a** and **3c**) as compared to oxaliplatin (36.7-fold).

In Vivo Anticancer Activity. Anticancer activity in vivo was investigated in the murine L1210 leukemia model. Compounds were administered intraperitoneally on three consecutive days. A transient decrease of body weight to not less than 85% was considered tolerable, and for each compound the tolerable dose resulting in the highest increase in life span (ILS) was considered optimal. Except for **5**, which did not allow dose escalation beyond 12 mg/kg/day because of much lower solubility, the maximum tolerated dose (MTD) was reached for all compounds. The optimal dose in terms of therapeutic efficacy is mostly identical with the MTD, except for d-OHP, the (*S*,*S*)-analogue of oxaliplatin (Table 3). Remarkably, a mere 50% dose increase over the MTD (6 mg/kg/day) turned oxaliplatin in a 100% lethal compound, indicating a steep lethality curve, whereas in the case of the new derivatives, a 50% increase over the respective MTD resulted only in 0−2 toxic deaths (in groups of six animals each). With the exception of **3b**, all compounds were tolerated in at least 50% higher doses (9 mg/kg/day) than oxaliplatin.

All compounds tested prolonged the survival of leukemic mice with high statistical significance ($p < 0.001$) but to different extents. The optimal therapeutic effect of the parent compound oxaliplatin caused an increase in life-span (ILS) by 152% and long-term survival of two animals (Table 3, Figure 4A). Although in equal doses the efficacy of the 4-methyl derivative **3a** is not higher than that of oxaliplatin, the lower toxicity of this compound enables the application of a higher dose, resulting in a clearly superior therapeutic effect with as much as five long-term survivors and an ILS of more than 200% (Figure 4B). Surprisingly, the analogous complex **3c** (with the 4-methyl substituent in axial instead of equatorial position) causes an ILS of only 122%. Thus, this compound is much less active than **3a** and even somewhat less active than oxaliplatin despite a cytotoxic potency superior to all other compounds studied in vitro. The 4-ethyl derivative **4** is also slightly less effective than oxaliplatin and causes an ILS of 129%, with one long-term surviving animal at the optimal dose. However, this compound is a racemic mixture, and improved therapeutic efficacy might still be expected from the pure (1*R*,2*R*,4*R*)-enantiomer. The lower efficacy of (1*S*,2*S*)-enantiomers is confirmed by data obtained with d-OHP and **3b**, which are no more than half as effective as oxaliplatin and **3a**, respectively. Finally, further increasing the size of the substituent to *t*-butyl (compound **5**) results in a tremendous loss of activity in applicable doses, which parallels the structure−activity relationships observed in vitro.

A synopsis of in vitro and in vivo data reveals that small alkyl substituents (methyl, perhaps also ethyl) on the cyclohexane ring result in an optimum with respect to the therapeutic index mainly due to a higher tolerability than unsubstituted oxaliplatin and a higher activity than derivatives with larger substituents. However, the geometric orientation (axial vs equatorial) of the substituent obviously plays a decisive role, having opposite effects on cytotoxicity in vitro and efficacy in vivo. Apart from that, structure−activity relationships in vitro and in vivo nicely parallel each other within the 4-substituted oxaliplatin derivatives, but this aberrant example demonstrates that caution should be exercised when selecting candidate compounds for drug development on the basis of in vitro data. The L1210 leukemia model employed may seem remote from the actual clinical indication of oxaliplatin, i.e. colorectal cancer, but it should be called in mind that it was one of the few tumor models in which the promising activity of oxaliplatin was originally recognized and therefore proved particularly valuable in this context.[30,31] Nevertheless, further evaluation of the compounds will require additional models, in particular, solid tumors.

As the cytotoxicity profiles of the studied compounds argue against fundamental differences in the mode of action, the reasons for the differences in therapeutic index and the favorable cross-resistance patterns may be found in the combination of characteristics such as lipophilicity and bulkiness and their subtle consequences for drug transport, biotransformation, and interactions with DNA as well as DNA repair systems.

Comparing both methyl derivatives **3a** (equatorial methyl group) and **3c** (axial methyl group), it is remarkable that **3c** is

Table 3. Tolerability and Efficacy of Intraperitoneal Treatment with Oxaliplatin Derivatives Compared to Oxaliplatin in L1210 Leukemia-Bearing Mice (Therapeutic Effects at the Respective Optimum Dose Are Marked in Bold Type)

compd	dose (mg/kg/day)	min body weight (%)	toxic deaths	ILS (%)[a]	long-term survivors[b]
oxaliplatin	9		6/6		0/6
	6 (MTD)[c]	93.2	0/6	**152**	**2/6**
	4.5	91.3	0/6	89	1/6
	3	94.1	0/6	94	1/6
d-OHP	12		1/6		0/6
	9 (MTD)[c]	85.9	0/6	63	0/6
	6	93.1	0/6	**73**	**1/6**
	4.5	93.6	0/6	26	0/6
3a	12		2/6		4/6
	9 (MTD)[c]	88.6	0/6	**> 200**	**5/6**
	6	97.5	0/6	119	0/6
	4.5	94.6	0/6	84	1/6
	3	97.3	0/6	90	0/6
3b	9		1/6		0/6
	6 (MTD)[c]	92.0	0/6	**103**	1/6
	4.5	93.4	0/6	68	**2/6**
	3	97.9	0/6	68	1/6
3c	12		2/6		1/6
	9 (MTD)[c]	86.4	0/6	**122**	**2/6**
	6	87.3	0/6	78	1/6
	4.5	87.9	0/6	111	2/6
4	12	83.2	0/6	144	2/6
	9 (MTD)[c]	89.9	0/6	**129**	**1/6**
	6	93.6	0/6	89	0/6
	3	98.4	0/6	61	0/6
5	12	100.0	0/6	33	0/6
	6	100.0	0/6	25	0/6
	3	98.5	0/6	17	0/6

[a] Increase in life span compared to untreated controls, based on median survival; all values indicate life prolongation with high statistical significance ($p < 0.001$). [b] Animals with ILS > 200% without any signs of leukemia. [c] Maximum tolerated dose.

equipped with a higher cytotoxicity in vitro, but in parallel showing a lower anticancer activity in vivo. Differences between **3a** and **3c** in the cellular accumulation and DNA adduct formation are expected to be very similar in vitro and in vivo. Nevertheless, one might assume the existence of interacting factors like altered transport by drug uptake and/or efflux pumps which might impact on the pharmacological characteristics of these stereoisomeric complexes in vivo but not in vitro. The identification of these interacting factors is a matter of ongoing investigations.

Conclusions

In this study, we successfully developed an oxaliplatin derivative with enhanced antitumor activity compared to the parental drug by introducing a small alkyl substituent on the cyclohexane ring in equatorial position. Using the L1210 murine leukemia model, the highest anticancer activity was proven for {(1*R*,2*R*,4*R*)-4-methyl-1,2-cyclohexanediamine}oxalatoplatinum(II), resulting in both increased life span and enhanced long-term survival. Although in vitro this compound is not consistently more cytotoxic than oxaliplatin, the better tolerability enables application of higher doses, resulting in enhanced efficacy in vivo. Considering that adverse effects are a central problem of clinical oxaliplatin application, the evaluation of the new derivative in (pre)clinical studies is highly desirable. These studies should primarily focus on colorectal cancer, where oxaliplatin is used as therapeutic standard, but also on other tumors recently suggested to be sensitive against this third-generation platinum compound like certain leukemias[32] or esophago-gastric cancer.[33]

Experimental Section

If necessary, the reactions were carried out in dry solvents and under argon atmosphere. Potassium tetrachloridoplatinate(II) was obtained from Johnson Matthey (Switzerland). All other chemicals obtained from commercial suppliers were used as received and were of analytical grade. Water was prior to use doubly distilled. The synthetic procedures were carried out in light protected environment when platinum complexes were involved. ^{1}H, ^{13}C, ^{15}N, ^{195}Pt, and two-dimensional COSY NMR spectra were recorded with a Bruker Avance DPX 400 instrument (Ultrashield Magnet) at 400.13 MHz (^{1}H) and 100.63 MHz (^{13}C) or with a Bruker Avance III 500 MHz NMR spectrometer at 500.32 (^{1}H), 125.81 (^{13}C), 107.55 (^{195}Pt), and 50.70 MHz (^{15}N) in DMF-d_7, D_2O, or $CDCl_3$ at 298 K. ^{15}N chemical shifts were referenced relative to external NH_4Cl, whereas ^{195}Pt chemical shifts were referenced relative to external $K_2[PtCl_4]$. HPLC analysis was carried out using a Dionex Summit system. The sample concentration was 1.0 mg/mL, with an injection volume of 10 μL introduced onto a Chiralcel OD column (250 mm × 4.6 mm) protected by a guard column (50 mm × 4.6 mm) at 5 °C. The flow rate of the mobile phase (hexane:*i*-propyl alcohol 9:1) was 1 mL/min. Melting points were determined with a Büchi B-540 apparatus and are

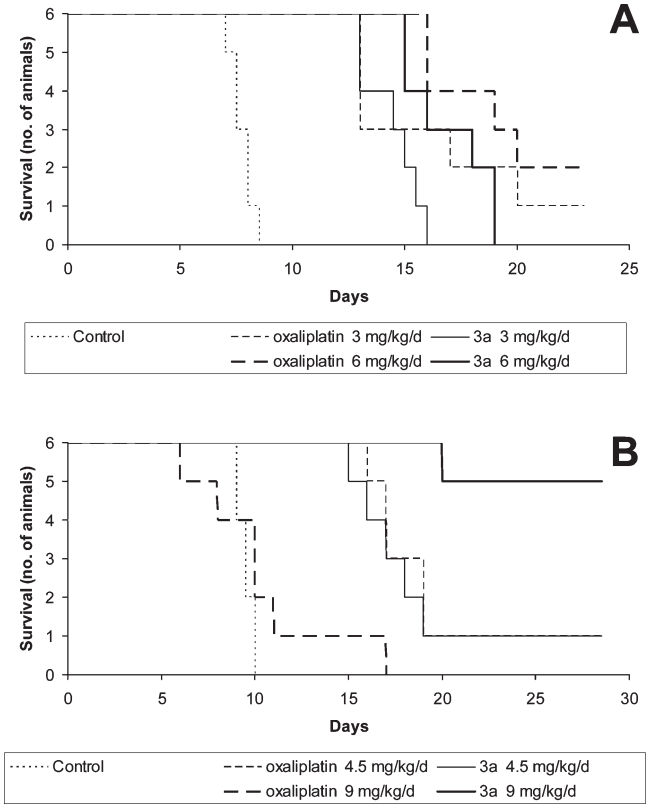


Figure 4. Kaplan−Meier plots showing the survival (days after tumor implantation) of L1210 leukemia-bearing mice treated intraperitoneally with different doses of oxaliplatin or **3a** in comparison to untreated controls, in groups of six animals each. Note that mortality in animals treated with 9 mg/kg/day oxaliplatin is drug-related in all cases (A), whereas the same dose of **3a** results in long-term survival of five animals (B).

uncorrected. Electrospray ionization mass spectra were recorded on a Bruker esquire$_{3000}$ in positive ion mode. Elemental analyses of prepared compounds were carried out on a Perkin-Elmer 2400 CHN elemental analyzer or a Carlo Erba microanalyzer at the Microanalytical Laboratory of the University of Vienna and are within ±0.4% of the calculated values, confirming their ≥95% purity. Silica gel (Polygram SIL G/UV$_{254}$) was used for thin layer chromatography.

(*SP*-4-3)-[(1*R*,2*R*,4*R*)-4-Methyl-1,2-cyclohexanediamine- κ^2N,N'][ethanedioato(2-)-$\kappa^2O1,O2$]platinum(II) 3a. $AgNO_3$ (167 mg, 0.98 mmol) was added to a suspension of (*SP*-4-3)-dichlorido-[(1*R*,2*R*,4*R*)-4-methyl-1,2-cyclohexanediamine-κ^2N,N']platinum-(II) **2a** (200 mg, 0.5 mmol) in water (3 mL). The reaction mixture was stirred for 24 h at room temperature, the precipitated silver chloride was filtered off, and an in situ prepared solution of potassium oxalate from oxalic acid dihydrate (61 mg, 0.48 mmol) and KOH (0.96 mL, 0.5 M, 0.48 mmol) in water (2 mL) was added to the diaqua complex. The reaction mixture was stirred for 24 h at room temperature, the solvent was removed in vacuum, and the pure white complex was obtained after recrystallization in water (1 mL). The product was filtered off and dried under reduced pressure over P_4O_{10}. Yield: 0.82 mg (39%), mp 282−290 °C (decomp). Elemental analysis, Found: C, 26.05; H, 3.65; N, 6.55. Calcd for $C_9H_{16}N_2O_4Pt$: C, 26.28; H, 3.92; N, 6.81. MS (ESI$^+$) *m*/*z* 434 [M + Na]$^+$. ^{1}H NMR in D_2O: δ = 0.85 (dq, $^3J_{H,H}$ = $^2J_{H,H}$ = 13 Hz, $^3J_{H,H}$ = 3.5 Hz, 1H, H-5ax), 0.86 (d, $^3J_{H,H}$ = 6.5 Hz, 3H, H-7), 0.98 (q, $^3J_{H,H}$ = $^2J_{H,H}$ = 12 Hz, 1H, H-3ax), 1.29 (dddd, $J_{H,H}$ = 12.5 Hz, $J_{H,H}$ = 12.5 Hz, $J_{H,H}$ = 12.0 Hz, $J_{H,H}$ = 3.5 Hz, 1H, H-6ax), 1.37 (m, 1H, H-4ax), 1.51 (m, 1H, H-5eq), 1.88−2.00 (m + m, 2H, H-3eq, H-6eq), 2.31 (dt, $^3J_{H,H}$ = 11.5 Hz, $^3J_{H,H}$ = 4.0 Hz, 1H, H-1ax), 2.39 (dt, $^3J_{H,H}$ = 11.5 Hz, $^3J_{H,H}$ = 4.0 Hz, 1H, H-2ax). ^{13}C NMR in D_2O: δ = 20.0 (C-7), 30.6 (C-6), 30.9 (C-4), 32.2 (C-5), 39.5 (C-3), 62.0 (C-2), 62.2 (C-1), 168.3 (COO). ^{195}Pt NMR in D_2O: δ = −365.

(*SP*-4-3)-[(1*S*,2*S*,4*S*)-4-Methyl-1,2-cyclohexanediamine- κ^2N,N'][ethanedioato(2-)-$\kappa^2O1,O2$]platinum(II) 3b. Following the same procedure as described for **3a**, complex **3b** was obtained from (*SP*-4-3)-dichlorido[(*1S*,*2S*,*4S*)-4-methyl-1,2-cyclohexanediamine-κ^2N,N']platinum(II) **2b** (200 mg, 0.5 mmol). Yield: 0.82 mg (38%), mp 275−280 °C (decomp). Elemental analysis, Found: C, 26.07; H, 3.66; N, 6.83. Calcd for $C_9H_{16}N_2O_4Pt$: C, 26.28; H, 3.92; N, 6.81. MS (ESI$^+$) *m*/*z* 434 [M + Na]$^+$. ^{1}H NMR in D_2O: δ = 0.85 (dq, $^3J_{H,H}$=$^2J_{H,H}$ = 13 Hz, $^3J_{H,H}$ = 3.5 Hz, 1H, H-5ax), 0.86 (d, $^3J_{H,H}$ = 6.5 Hz, 3H, H-7), 0.97 (q, $^3J_{H,H}$ = $^2J_{H,H}$=12 Hz, 1H, H-3ax), 1.28 (dddd, $J_{H,H}$ = 12.5 Hz, $J_{H,H}$ = 12.5 Hz, $J_{H,H}$ = 12.0 Hz, $J_{H,H}$ = 3.5 Hz, 1H, H-6ax), 1.36 (m, 1H, H-4ax), 1.50 (m, 1H, H-5eq), 1.88−2.00 (m + m, 2H, H-3eq, H-6eq), 2.30 (dt, $^3J_{H,H}$ = 11.5 Hz, $^3J_{H,H}$ = 4.0 Hz, 1H, H-1ax), 2.38 (dt, $^3J_{H,H}$ = 11.5 Hz, $^3J_{H,H}$ = 4.0 Hz, 1H, H-2ax). ^{13}C NMR in D_2O: δ =20.0 (C-7), 30.6 (C-6), 30.9 (C-4), 32.2 (C-5), 39.5 (C-3), 62.0 (C-2), 62.2 (C-1), 168.3 (COO). ^{195}Pt NMR in D_2O: δ = −365.

(*SP*-4-3)-[(1*R*,2*R*,4*S*)-4-Methyl-1,2-cyclohexanediamine- κ^2N,N']-[ethanedioato(2-)-$\kappa^2O1,O2$]platinum(II) 3c. (*SP*-4-3)-{Dichlorido-[(1*R*,2*R*,4*S*)-4-methyl-1,2-cyclohexanediamine-κ^2N,N']platinum(II)} (1.767 g, 4.48 mmol) was suspended in 80 mL of water and a solution of $AgNO_3$ (1.462 g; 8.60 mmol) in 10 mL of water was added. The suspension was stirred for 24 h at room temperature. The AgCl formed was filtered off with a G4 glass sinter filter with an additional MN GF3 filter. The clear, slightly yellowish solution was treated with conditioned basic ion-exchange resin IRA402 for 24 h at room temperature (shaking). [Conditioning of IRA402: 150 g of IRA402 chloride form was stirred with 2 M NaOH (400 mL) for 30 min. Then the resin was washed with deionized water until achieving neutral pH followed by washing with distilled water twice.] The ion-exchange resin was separated from the solution by decantation and washed with water three times. The combined aqueous solutions were filtered through a G4 glass sinter filter, and oxalic acid dihydrate (0.565 g, 4.48 mmol) was added. The mixture was put into a round flask, and the volume was reduced to ca. 80 mL within 3 h, whereupon the liquid became cloudy. The latter was kept at room temperature for 12 h and was further reduced to ca. 40 mL. A white solid formed which was finally filtered with a G4 glass sinter filter after 1 h, washed with water twice, and dried in vacuum over P_4O_{10}. Yield: 0.534 g (29%). Elemental analysis, Found: C, 26.07; H, 3.88; N, 6.66. Calcd for $C_9H_{16}N_2O_4Pt$: C, 26.28; H, 3.92; N, 6.81. ^{1}H NMR in D_2O: δ = 0.84 (d, $^3J_{H,H}$ = 7.5 Hz, 3H, H-7), 1.22−1.55 (m + m + m + m, 4H, H-5eq, H-5ax, H-3ax, H-6ax) 1.71−1.90 (m + m + m, 3H, H-3eq, H-6eq, H-4eq), 2.24 (dt, $^3J_{H,H}$ = 12.0 Hz, $^3J_{H,H}$ = 4.5 Hz, 1H, H-1ax), 2.51 (dt, $^3J_{H,H}$ = 12.0 Hz, $^3J_{H,H}$ = 4.0 Hz, 1H, H-2ax). ^{13}C NMR in D_2O: δ = 16.7 (C-7), 26.4 (C-6), 27.0 (C-4), 29.3 (C-5), 37.2 (C-3), 57.8 (C-2), 62.7 (C-1), 168.3 (COO). ^{195}Pt NMR in D_2O: δ = −381.

Cell Lines and Culture Conditions. CH1 (ovarian carcinoma, human) cells were kindly provided by Lloyd R. Kelland (CRC Centre for Cancer Therapeutics, Institute of Cancer Research, Sutton, UK). HeLa (cervical carcinoma, human) and U-2 OS (osteosarcoma, human) cells were donated by Thomas Czerny (Institute of Genetics, University of Veterinary Medicine Vienna, Austria). SW480, HCT116, and HCT15 (all colon carcinoma, human) cells were kindly provided by Brigitte Marian (Institute of Cancer Research, Medical University of Vienna, Austria). In addition, cell models comprising a parental drug-sensitive and respective cisplatin- or oxaliplatin-resistant subline were used. GLC4 small cell lung cancer cells together with cisplatin-resistant GLC4/CDDP cells were kindly supplied by Elisabeth de Vries, University of Groningen. The ovarian carcinoma cell line A2780 together with the cisplatin-resistant subline A2780/cis were obtained from Sigma. The oxaliplatin-resistant subline HCT-116 oxR was established by stepwise drug selection of HCT-116 colon cancer cells.

GLC4 and A2780 cell lines and derivatives were grown in RPMI1680 medium with 10% fetal bovine serum. All other cells

were cultured in complete culture medium, i.e., Minimal Essential Medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 4 mM L-glutamine, and 1% nonessential amino acids (100×) (all purchased from Sigma-Aldrich). Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air.

Cytotoxicity Tests in Cancer Cell Lines. Antiproliferative activity was determined by a colorimetric microculture assay (MTT assay, MTT = 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide). For this purpose, cells were harvested from culture flasks by trypsinization and seeded in 100-μL aliquots into 96-well microculture plates (Iwaki/Asahi Technoglass) in the following densities: 1.5×10^3 (CH1, HeLa), 2.0×10^3 (GLC4 cell model), 2.5×10^3 (SW480, HCT-116 cell model, HCT-15), and 3.2×10^3 (U2OS, A2780 cell model) viable cells/well. Cells were allowed to settle and resume adherent growth in drug-free complete culture medium for 24 h, followed by the addition of dilutions of the test compounds in 100 μL/well complete culture medium and incubation for 96 h. In experiments involving drug-resistance models, the incubation time was 72 h. At the end of exposure, medium was replaced by 100 μL/well RPMI 1640 medium (supplemented with 10% heat-inactivated fetal bovine serum and 4 mM L-glutamine) plus 20 μL/well MTT solution in phosphate-buffered saline (5 mg/mL). After incubation for 4 h, medium was removed and the formazan product formed by viable cells was dissolved in DMSO (150 μL/well). Optical densities at 550 nm were measured with a microplate reader (Tecan Spectra Classic). The quantity of viable cells was expressed in terms of T/C values by comparison to untreated controls, and 50% inhibitory concentrations (IC_{50}) were calculated from concentration−effect curves by interpolation. Evaluation is based on means from at least two (mostly three) independent experiments, each comprising six replicates per concentration level.

Antileukemic Activity in Vivo. Animal experiments were performed in accordance with the European Community Guidelines for the use of experimental animals in the animal facility at the Cancer Research Institute, Slovak Academy of Sciences, Bratislava, Slovak Republic. L1210 murine leukemia cells (1×10^5) were injected in a volume of 0.5 mL into DBA/2J mice (weighing 18−22 g) intraperitoneally on day 0. The test compounds were administered intraperitoneally in a volume of 0.5 mL aqua ad injectionem per mouse per day in a split-dose regimen (days 1, 2, and 3 after implantation of cells) to groups of six animals per dose. Toxicity of the test compounds was monitored by daily observation of animals and registration of their body weight. Therapeutic efficacy of the test compounds was monitored by recording the lengths of survival of experimental mice compared to untreated control animals. The experiment was terminated after the 3-fold of the survival time of untreated controls, and animals experiencing an increase in life span of more than 200% without any signs of leukemia were considered long-term survivors. The absence of malignant disease in the abdominal cavity was confirmed by dissection. Statistical significance of survival data was calculated using the unpaired t test.

Acknowledgment. We are indebted to the FFG−Austrian Research Promotion Agency (811591), the Austrian Council for Research and Technology Development (IS526001), the FWF (Austrian Science Fund), and COST D39.

Supporting Information Available: Figure with the crystal structure of an oxaliplatin 1,2-d(GpG) intrastrand cross-link in a DNA dodecamer duplex; scheme with the separation of racemic (1*R*,2*R*,4*R*/1*S*,2*S*,4*S*)-4-methyl-1,2-cyclohexanediamine and with structures of CBz analogues used for chiral HPLC analysis, figure with the confirmation of enantiomeric purity of (1*R*,2*R*,4*R*)- and (1*S*,2*S*,4*S*)-4-methyl-1,2-cyclohexanediamine by chiral HPLC, scheme with the synthesis of {(1*R*,2*R*,4*S*)-4-methyl-1,2-cyclohexanediamine}oxalatoplatinum(II), figure with the confirmation of diastereomeric purity of (1*R*,2*R*,4*S*)-4-methyl-1,2-cyclohexanediamine after transformation into the CBz-derivative, figure with ^{1}H- and ^{195}Pt NMR spectra of {(1*R*,2*R*,4*S*)-4-methyl-1,2-cyclohexanediamine}oxalatoplatinum(II) **3c**, synthesis and characterization of (*S*)-4-methylcyclohexene, 4-methyl-1,2-cyclohexanediaminium tartrates **1a**−**1c**, (*SP*-4-3)-dichlorido[4-methyl-1,2-cyclohexanediamine-κ^2N,N']platinum(II) complexes **2a**−**2c**, and N,N'-bis(benzyloxycarbonyl)-4-methyl-1,2-cyclohexanedicarbamates **6** and **7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Supporting Information

{(1*R*,2*R*,4*R*)-4-Methyl-1,2-cyclohexanediamine}oxalatoplatinum(II) – a novel enantiomerically pure oxaliplatin derivative showing improved anticancer activity in vivo

Sergey A. Abramkin,[†] Ute Jungwirth,[‡,**] Seied M. Valiahdi,[†,**] Claudia Dworak,[†] Ladislav Habala,[†] Kristof Meelich,[†] Walter Berger,[‡,§] Michael A. Jakupec,[†] Christian G. Hartinger,[†] Alexey A. Nazarov,[#,*] Markus Galanski,[†,*] Bernhard K. Keppler[†,§,*]

[†] University of Vienna, Institute of Inorganic Chemistry, Währinger Strasse 42, A-1090 Vienna, Austria

[‡] Department of Medicine I, Institute of Cancer Research, Medical University of Vienna, Borschkegasse 8a, A-1090 Vienna, Austria

[§] Research Platform "Translational Cancer Therapy Research" University of Vienna, Währinger Strasse 42, A-1090 Vienna, Austria

[#] Institut des Sciences et Ingénierie Chimiques, Ecole Polytechnique Fédérale de Lausanne (EPFL), CH-1015 Lausanne, Switzerland

[**] U. J. and S. M. V. contributed equally to this work

Contents

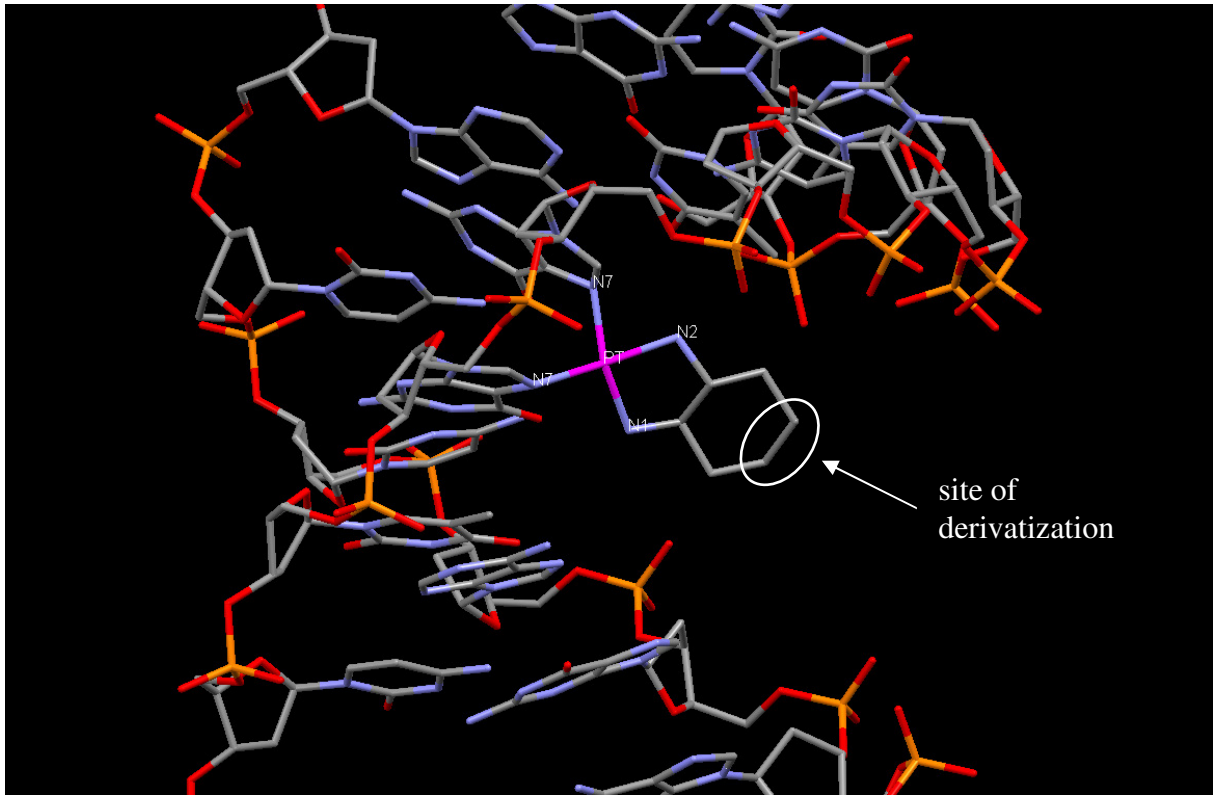
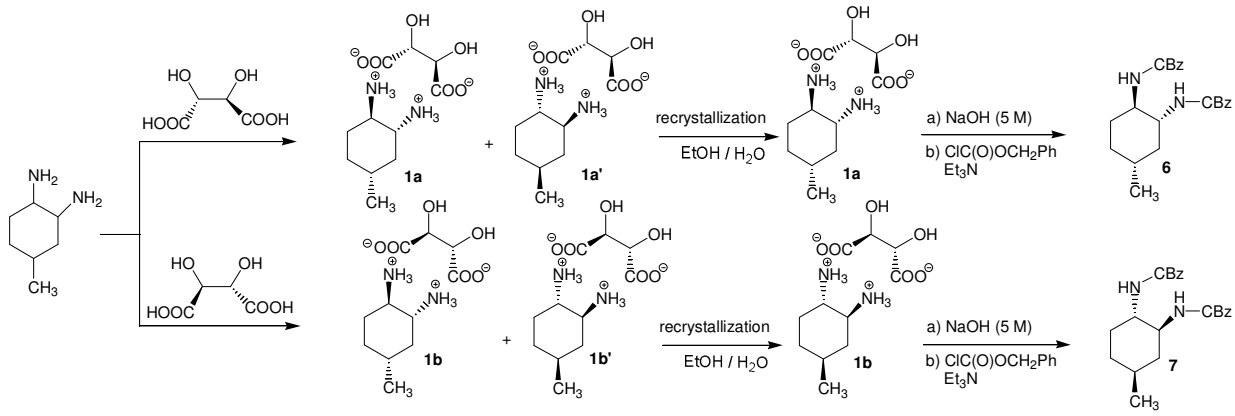


Figure S1. Crystal structure of an oxaliplatin 1,2-d(GpG) intrastrand cross-link in a DNA dodecamer duplex.
Protein Data Bank, PDB ID: 1IHH, Spingler, B., Whittington, D.A., Lippard, S.J. 2001, 2.4 Angstrom Crystal Structure of an Oxaliplatin 1,2-d(GpG) Intrastrand Cross-Link in a DNA Dodecamer Duplex, Inorg. Chem., 40, 5596.



Scheme S1. Separation of the racemic mixture of (1*R*,2*R*,4*R*)- and (1*S*,2*S*,4*S*)-4-methyl-1,2-cyclohexanediamine via recrystallization with the L- or D-tartrate and conversion of the diaminium tartrates into the UV-active CBz analogs used for chiral HPLC analysis.

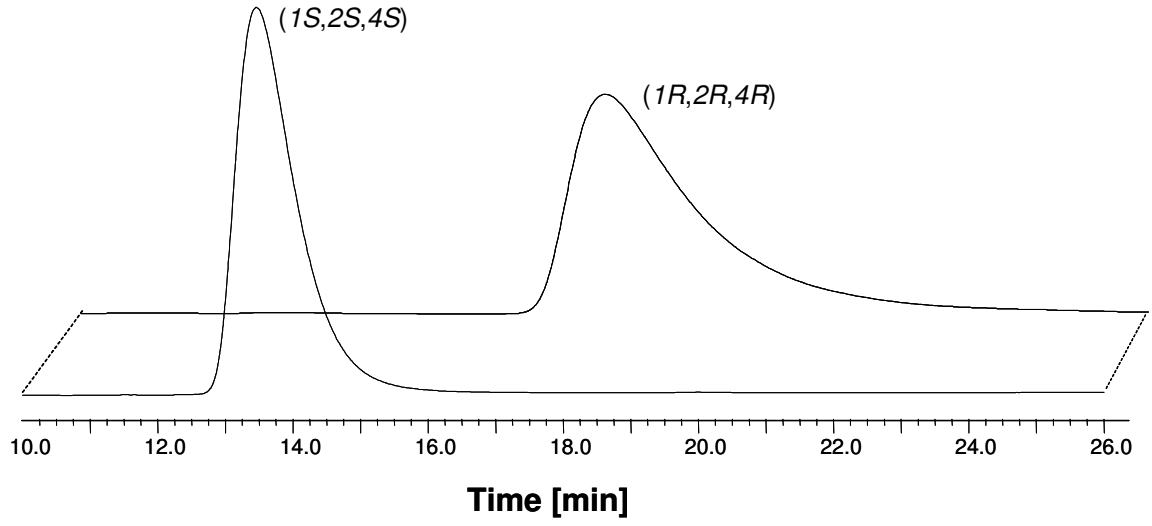
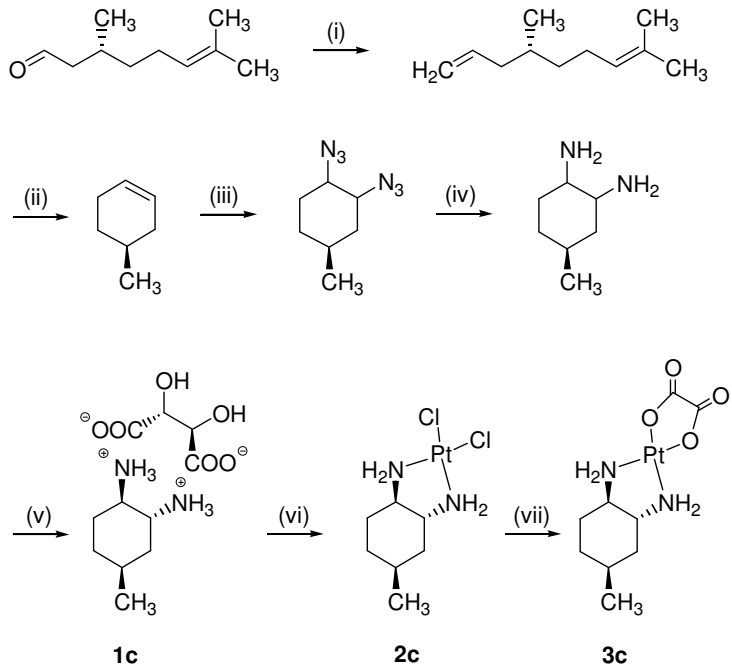


Figure S2. Confirmation of enantiomeric purity of (1*R*,2*R*,4*R*)- and (1*S*,2*S*,4*S*)-4-methyl-1,2-cyclohexanediamine after transformation into the CBz-derivatives by chiral HPLC.



Scheme S2. Synthesis of {(1*R*,2*R*,4*S*)-4-methyl-1,2-cyclohexanediamine}oxalato-platinum(II), **3c**. (i) $Me(PPh_3)_3Br/BuLi$, (ii) trans-$WOCl_2(OAr)_2$ (Ar = 2,6-dibromophenyl)/ tetraethyllead, (iii) $NaN_3/Mn(OAc)_3$, (iv) $Pd/CaCO_3$, H_2 (10 bar), (v) L-tartaric acid, (vi) K_2PtCl_4/NaOH and (vii) $AgNO_3$, basic ion exchanger, oxalic acid.

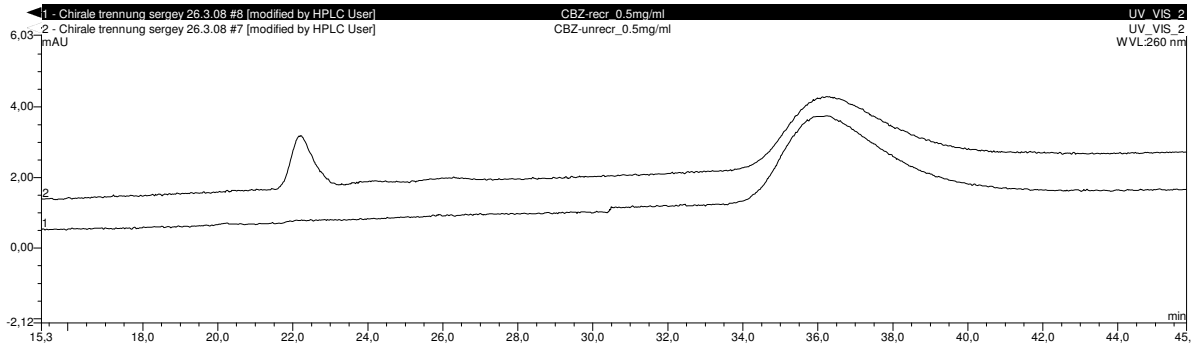


Figure S3. Confirmation of diastereomeric purity of (1*R*,2*R*,4*S*)-4-methyl-1,2-cyclohexanediamine after transformation into the CBz-derivatives by chiral HPLC (upper curve, mixture of (1*R*,2*R*,4*S*)- and (1*S*,2*S*,4*S*)-isomers; lower curve, pure (1*R*,2*R*,4*S*)-isomer.

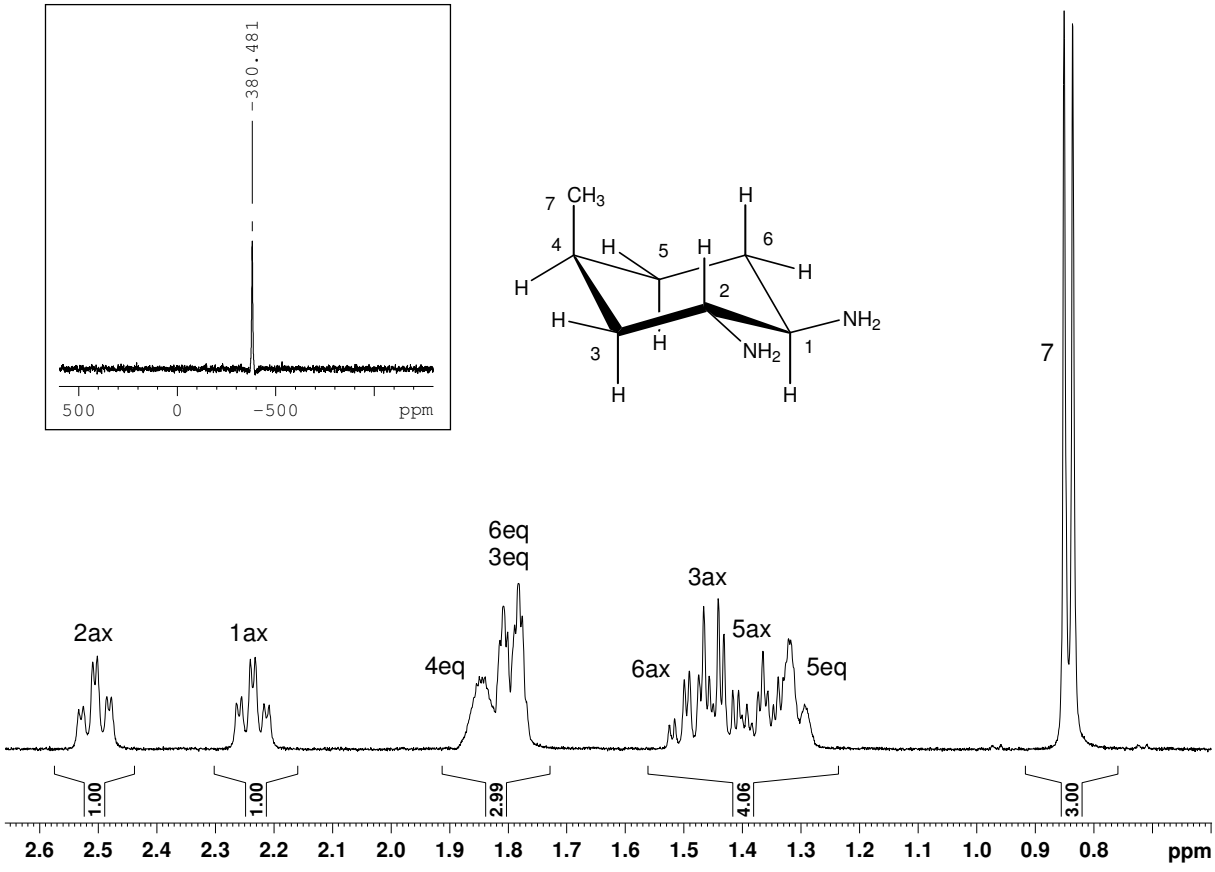


Figure S4. ^{1}H- and ^{195}Pt (small insert) NMR spectrum of {(1*R*,2*R*,4*S*)-4-methyl-1,2-cyclohexanediamine}oxalatoplatinum(II) **3c** with NMR numbering scheme and signal assignment, the oxalatoplatinum(II) fragment has been omitted for clarity.

Synthesis of (*S*)-4-Methylcyclohexene[1]

(*S*)-4,8-Dimethyl-l,7-nonadiene

A suspension of methyltriphenylphosphonium bromide (22.9 g, 64.1 mmol) in absolute tetrahydrofuran (400 mL) was filled into a three-necked flask under inert conditions and cooled to -20°C. Butyllithium (1.60 M in hexane; 38.0 mL, 60.8 mmol) was added via syringe. The orange mixture was stirred for 30 min. at room temperature and then cooled to -78°C. Thereafter, (*S*)-3,7-dimethyl-6-octenal ((*S*)-citronellal; 11.0 mL, 60.7 mmol) was added slowly, keeping the temperature low. The mixture was stirred for 1 h at -78°C and was then allowed to warm up slowly to room temperature. Acetone (10 mL) was added and the reaction mixture was stirred for 30 min to quench the excess of reagent. Eventually, the mixture was added to hexane (800 mL) and filtered. From the filtrate the solvent was removed with a rotary evaporator at reduced pressure, the solid formed was filtered off and washed with hexane. Again, hexane was removed using a rotary evaporator and the raw product was distilled under vacuum to afford the diene as a colorless liquid. Yield: 6.56 g (71%). ^{1}H NMR in $CDCl_3$: δ = 0.90 (d, ^{3}J=6.5 Hz, 3H), 1.12-1.21 (m, 1H), 1.33-1.41 (m, 1H), 1.48-1.57 (m, 1H), 1.62 (s, 3H), 1.70 (s, 3H), 1.87-2.13 (m, 4H), 4.97-5.04 (m, 2H), 5.12 (t, 1H), 5.74-5.86 (m, 1H).

***trans*-Dichlorobis(2,6-dibromphenoxy)oxotungsten(VI)**

A mixture of tungsten(VI) oxychloride (1.71 g, 5.00 mmol) and 2,6-dibromphenol (2.52 g, 10.0 mmol) in absolute toluene (25 mL) was heated at reflux for 1 h under argon. After the mixture was cooled down to room temperature, the solvent was removed under vacuum. The residue was stirred with absolute dichloromethane (100 mL) under argon for 10 min. The

[1] Nugent, W. A.; Feldman, J.; Calabrese, J. C. Practical Catalyst for Cyclic Metathesis. Synthesis of Functional and/or Enantiopure Cycloalkenes. *J. Am. Chem. Soc.* **1995**, *117*, 8992–8998.

solution was filtered inertly and filled into a sidearm round flask. Thereafter the solvent was removed in vacuum and the resulting amorphous, dark-red solid was stored under argon. The flask was directly used for the next reaction step.

(*S*)-4-Methylcyclohexene

trans-Dichlorobis(2,6-dibromphenoxy)oxotungsten(VI) was dissolved in dry trichlorobenzene (180 mL) under argon in a Schlenk flask, and (*S*)-4,8-dimethyl-l,7-nonadiene (31.5 g, 228 mmol), as well as tetraethyllead (50% solution in xylenes, 10g, 15 mmol) were added. The dark red mixture was heated up to 90°C, whereby a color change from deep-red to dark-blue, as well as a gas evolvement were observed. The mixture was kept at 90° for 2 h. After cooling down to room temperature, the solution was stirred shortly with silica (10 g) and filtered; a colorless liquid was obtained. Distillation at 97-120 °C produced a crude product containing about 10% of dissolved xylenes, which did not with subsequent transformations of the product. Yield: 19,5 g with about 10% xylenes (about 17,5 g of the product, 89 %). ^{1}H NMR in $CDCl_3$: δ = 0.97 (d, ^{3}J=6.5 Hz, 3H), 1.17-1.30 (m, 1H), 1.59-1.74 (m, 3H), 2.00-2.14 (m, 3H), 5.64-5.71 (m, 2H).

(*1R*,*2R*,*4R*)-4-Methyl-1,2-cyclohexanediaminium L-tartrate 1a

A mixture of 4-methyl-*trans*-(±)-1,2-cyclohexanediazide (2.1 g, 14.0 mmol) and palladium on $CaCO_3$ (Lindlar's catalyst, 5% Pd, 420 mg) in dry ethanol (30 mL) was stirred for 24 h under hydrogen atmosphere (3 bar). The catalyst was filtered off, the volume of the solvent was reduced to 2/3 and 4-methyl-*trans*-1,2-cyclohexanediaminium L-tartrate **1a/1a'** was precipitated by addition of L-tartaric acid until a pH of 7 was reached. The diaminium salt was filtered off, washed with diethyl ether (2 × 50 mL), and dried in vacuum. Yield 1.23 g (35%). A suspension of 4-methyl-1,2-*trans*-cyclohexanediaminium L-tartrate **1a/1a'** (3.0 g) in absolute ethanol (50 mL) was refluxed for 15 min, then water was added dropwise to obtain a clear solution. The solution was allowed to cool down to room temperature and was kept in the refrigerator for 12 h at 4 °C. The enantiomer **1a** was filtered off and dried in vacuum. Yield: 0.54 g (36%), m.p. 258–260 °C, $[\alpha]_{20}^{D}$ = +12 (c 0.5, acetone). Elemental analysis, found: C, 47.28; H, 8.04; N, 10.34. Calcd for $C_{11}H_{22}N_2O_6$: C, 47.47; H, 7.97; N, 10.07. MS (ESI^+) *m/z* 129 $[M+H]^+$. ^1H NMR in D_2O: δ = 0.85 (d, $^3J_{H,H}$ = 6.5 Hz, 3H, H-7), 0.99 (m, 1H, H-5), 1.15 (m, 1H, H-3), 1.41–1.58 (m, 2H, H-4, H-6), 1.71 (m, 1H, H-5`), 1.98–2.10 (m, 2H, H-6`, H-3`), 3.20–3.38 (m, 2H, H-1, H-2) 4.24 (s, 2H, CHOH). ^{13}C NMR in D_2O: δ = 20.7 (C-7), 29.5 (C-6), 30.2 (C-4), 31.5 (C-5), 37.6(C-3), 52.4 (C-1), 52.5 (C-2), 74.2 (CHOH), 178.9 (COO).

(*1S*,*2S*,*4S*)-4-Methyl-1,2-cyclohexanediaminium D-tartrate 1b

Following the same procedure as described for **1a**, compound **1b** was synthesized. Yield: 0.86 g (57%), m.p. 257–259 °C, $[\alpha]_{20}^{D}$ = -12 (c 0.5, acetone). Elemental analysis, found: C, 47.51; H, 7.79; N, 9.84. Calcd for $C_{11}H_{22}N_2O_6$: C, 47.47; H, 7.97; N, 10.07. MS (ESI^+) *m/z* 129 $[M+H]^+$. ^1H NMR in D_2O: δ = 0.85 (d, $^3J_{H,H}$ = 6.5 Hz, 3H, H-7), 0.98 (m, 1H, H-5), 1.14 (m, 1H, H-3), 1.43–1.53 (m, 2H, H-4, H-6), 1.71 (m, 1H, H-5`), 2.00–2.07 (m, 2H, H-6`, H-3`), 3.20–3.35 (m, 2H, H-1, H-2) 4.23 (s, 2H, CHOH). ^{13}C NMR in D_2O: δ = 20.7 (C-7),

29.6 (C-6), 30.3 (C-4), 31.5 (C-5), 37.7 (C-3), 52.4 (C-1), 52.5 (C-2), 74.2 (CHOH), 178.9 (COO).

(*1R,2R,4S*)-4-Methyl-1,2-cyclohexanediaminium L-tartrate 1c

(*S*)-4-Methylcyclohexene (10 g, 0.105 mol) and trifluoroacetic acid (120 mL) were added to a suspension of manganese acetate dihydrate (83.5 g, 0.31 mol) and sodium azide (34 g, 0.52 mol) in acetonitrile (1200 mL) under argon at -20°C. The reaction mixture was stirred for 3 h at -20°C and for 12 h at room temperature. Thereafter, $Na_2S_2O_5$ (10%, 300 mL) was added till the solution became clear. The intermediate diazide was extracted with petroleum ether (4 × 150 mL), washed with saturated Na_2CO_3 solution (2 × 100 mL) and brine (2 × 100 mL) and dried over Na_2SO_4. The solvent was removed in vacuum and the resulting oil was sissolved in 90 mL of ethanol. One third of the intermediate (30 mL of solution) was mixed with Lindlar catalyst (Pd on $CaCO_3$, 5% Pd, 1.6 g) and filled into an autoclave, which was then evacuated and pressurized with 10 bar of hydrogen. The suspension was stirred for 24 h at room temperature. The catalyst was filtered off and the product was precipitated by addition of a 20% stoichiometric excess of L-tartaric acid (6.3 g, 0.042 mol) in ethanol. The salt was filtered off, washed with diethyl ether (2 × 30 mL) and dried in vacuum. The product was recrystallyzed twice from a ethanol/water (1/1) mixture. The other two parts of the diazide intermediate were treated in the same way. Yield: 8.77 g (30%), elemental analysis, found: C, 47.49; H, 8.13; N, 10.34.Calcd for $C_{11}H_{22}N_2O_6$: C, 47.47; H, 7.97; N, 10.07. ^{1}H NMR in D_2O: δ = 0.90 (d, $^3J_{H,H}$ = 7.5 Hz, 3H, H-7), 1.36-1.46 (m, 1H, H-5), 1.58 (m, 1H, H-5`), 1.65-1.81 (m, 3H, H-3, H-6, H-3`), 1.84-2.10 (m, 2H, H-6`,H-4), 3.36 (m, 1H, H-1), 3.54 (m, 1H, H-2), 4.24 (s, 2H, CHOH). ^{13}C NMR in D_2O: δ = 17.5 (C-7), 23.7 (C-6), 25.4 (C-4), 27.4 (C-5), 33.5 (C-3), 48.3 (C-2), 50.9 (C-1), 73.8 (CHOH), 178.5 (COO).

***(SP*-4-3)-Dichlorido[(*1R,2R,4R*)-4-methyl-1,2-cyclohexanediamine-κ²*N,N`*]platinum(II) 2a**

A solution of (*1R,2R,4R*)-4-methyl-1,2-cyclohexanediaminium L-tartrate (0.3 g, 1.1 mmol) in water (10 mL) was mixed with a solution of K_2PtCl_4 (0.45 g, 1.1 mmol) in water (15 mL). The pH of the reaction mixture was kept at ca. 7 over 24 h by addition of NaOH solution (0.25 M). The yellow product was filtered off, washed with HCl (2M, 2 × 5 mL) and water (3 ×10 mL), and dried in vacuum over P_2O_5. Yield: 0.33 g (78%), m.p. > 400 °C (decomp.). Elemental analysis, found: C, 21.41; H, 3.93; N, 6.89. Calcd for $C_7H_{16}N_2PtCl_2$: C, 21.33; H, 4.09; N, 7.11. MS (ESI$^+$) *m/z* 417 $[M+Na]^+$, $[\alpha]_{20}^D$ = +100 (c = 0.5, DMSO). ^{1}H NMR in DMF-d_7: δ = 0.84-0.97 (m, 1H, H-5ax; d, $^3J_{H,H}$ = 6.5 Hz, 3H, H-7), 1.22 (q, $^3J_{H,H}$ = $^2J_{H,H}$ = 12 Hz, 1H, H-3ax), 1.41 (m, 1H, H-4ax), 1.48-1.60 (m+m, 2H, H-6ax, H-5eq), 1.99-2.11 (m+m, 2H, H-3eq, H-6eq), 2.46-2.66 (m+m, 2H, H-1ax, H-2ax), 5.01 (m, 2H, N^1-H/N^2-H), 5.58 (m, 2H, N^1-H/N^2-H). ^{13}C NMR in DMF-d_7: δ =20.6 (C-7), 31.0 (C-6), 31.5 (C-4), 33.0 (C-5), 40.2 (C-3), 63.2 (C-2), 63.4 (C-1). ^{15}N NMR in DMF-d_7: δ = –21.3. ^{195}Pt NMR in DMF-d_7: δ = –644.

***(SP*-4-3)-Dichlorido[(*1S,2S,4S*)-4-methyl-1,2-cyclohexanediamine-κ²*N,N`*]platinum(II) 2b**

Following the same procedure as described for **1b**, complex **2b** was obtained from (*1S,2S,4S*)-4-methyl-1,2-cyclohexanediaminium D-tartrate (0.3 g, 1.1 mmol). Yield: 0.35 g (83%), m.p. > 400 °C (decomp.). Elemental analysis, found: C, 21.23; H, 3.87; N, 6.89. Calcd for $C_7H_{16}N_2PtCl_2$: C, 21.33; H, 4.09; N, 7.11. MS (ESI$^+$) *m/z* 417 $[M+Na]^+$, $[\alpha]_{20}^D$ = -100 (c = 0.5, DMSO). ^{1}H NMR in DMF-d_7: δ = 0.84-0.97 (m, 1H, H-5ax; d, $^3J_{H,H}$ = 6.5 Hz, 3H, H-7), 1.22 (q, $^3J_{H,H}$ = $^2J_{H,H}$ = 12 Hz, 1H, H-3ax), 1.41 (m, 1H, H-4ax), 1.48-1.60 (m+m, 2H, H-6ax, H-5eq), 1.99-2.11 (m+m, 2H, H-3eq, H-6eq), 2.46-2.66 (m+m, 2H, H-1ax, H-2ax), 5.00 (m, 2H, N^1-H/N^2-H), 5.58 (m, 2H, N^1-H/N^2-H). ^{13}C NMR in DMF-d_7: δ =20.6 (C-7), 31.0 (C-6),

31.5 (C-4), 33.0 (C-5), 40.2 (C-3), 63.2 (C-2), 63.4 (C-1). ^{15}N NMR in DMF-d_7: δ = –21.4. ^{195}Pt NMR in DMF-d_7: δ = –644.

(*SP*-4-3)-Dichlorido[(*1R,2R,4S*)-4-methyl-1,2-cyclohexanediamine-κ²*N*,*N*`]platinum(II) 2c

Potassium tetrachloroplatinate (8.255 g, 0.02 mol) was dissolved in distilled water (50 mL) and filtered through a glass fiber filter. A solution of (*4S*)-methyl-(*1R,2R*)-cyclohexane-diaminium L-tartrate (5.566 g, 0.02 mol) in 50 mL of distilled water was added. The mixture was stirred for 24 h, whereby the pH of the reaction mixture was kept at 7.0 via addition of 0.25M NaOH. After the supernatant had become almost colorless and the pH was rising rapidly upon further addition of the base, the yellow precipitate was filtered off via a G4 glass sinter filter, washed twice with 2M HCl, then three times with distilled water and dried in vacuum over P_2O_5. Yield: 7.33 g (93%), elemental analysis, found: C, 21.17; H, 3.85; N, 7,15. Calcd for $C_7H_{16}N_2PtCl_2$: C, 21.33; H, 4.09; N, 7.11. ^{1}H NMR in DMF-d_7: δ = 0.96 (d, $^3J_{H,H}$ = 7.5 Hz, 3H, H-7), 1.37 (m, 1H, H-5eq), 1.45 (m, 1H, H-5ax), 1.69 (m, 1H, H-3ax), 1.76 (m, 1H, H-6ax), 1.85-1.97 (m+m+m, 3H, H-3eq, H-4eq, H-6eq), 2.48 (m, 1H, H-1ax), 2.74 (m, 1H, H-2ax), 4.97 (m, 2H, N^1-H/N^2-H), 5.47 (m, 1H, N^2-H), 5.60 (m, 1H, N^1-H). ^{13}C NMR in DMF-d_7: δ =17.2 (C-7), 26.7 (C-6), 27.6 (C-4), 30.0 (C-5), 37.5 (C-3), 58.7 (C-2), 63.9 (C-1). ^{15}N NMR in DMF-d_7: δ = –20.8 (N^2), –19.9 (N^1). ^{195}Pt NMR in DMF-d_7: δ = –657.

(*1R*,*2R*,*4R*)-*N,N´*-Bis(benzyloxycarbonyl)-4-methyl-1,2-cyclohexanedicarbamate 6

(*1R*,*2R*,*4R*)-4-Methyl-1,2-cyclohexanediamine was recovered from the respective diaminium tartrate **1a** (0.5 g) by reaction with a NaOH solution (5 M, 25 mL) and extraction with dichloromethane (3 × 20 mL). Triethylamine (0.68 mL, 4.87 mmol) was added to the combined CH_2Cl_2 fractions and the reaction mixture was treated with benzyloxycarbonylchloride (0.57 mL, 4.05 mmol) under vigorous stirring at 0–5 °C. Then it was allowed to warm up to room temperature and stirred for another 3 h (TLC control, hexane : ethyl acetate = 3 : 1). The reaction mixture was diluted with CH_2Cl_2 (10 mL), washed with brine (2 × 15 mL), and dried over Na_2SO_4. The solvent was removed to give a slightly yellow crude product which was purified by recrystallization from MeOH. Yield: 0.2 g (30%), m.p. 178–179 °C, $[\alpha]_{20}^{D}$ = -24 (c = 0.5, acetone). Elemental analysis, found: C, 69.45; H, 7.18; N, 7.36. Calcd for $C_{23}H_{28}N_2O_4$: C, 69.66; H, 7.12; N, 7.07. MS (ESI^+) *m/z* 419 $[M+Na]^+$. ^1H NMR in $CDCl_3$: δ = 0.94 (d, $^3J_{H,H}$ = 6.5 Hz, 3H, H-7), 1.08–0.93 (m, 2H, H-3, H-5), 1.32 (m, 1H, H-6), 1.54 (m, 1H, H-4), 1.72 (m, 1H, H-5´), 2.09–2.05 (m, 2H, H-3´, H-6`), 3.46-3.36 (m, 2H, H-2, H-1), 5.12–5.04 (m, 6H, OCH_2, NH), 7.34 (s, 10H, H-ar). ^{13}C NMR in $CDCl_3$: δ = 22.0 (C-7), 31.9 (C-4), 32.6 (C-5), 33.7 (C-6), 41.6 (C-3), 55.5 (C-2), 55.7 (C-1), 67.0 (OCH_2), 128.3 (C-ar), 128.4 (C-ar), 128.9 (C-ar), 137.0 (Cq-ar), 157.2 (C=O).

(*1S*,*2S*,*4S*)-*N,N´*-Bis(benzyloxycarbonyl)-4-methyl-1,2-cyclohexanedicarbamate 7

Following the same procedure as described for **6**, compound **7** was obtained from **1b** (0.1 g,). Yield: 0.76 mg (53%), m.p. 179–180 °C, $[\alpha]_{20}^{D}$ = +24 (c 0.5, acetone). Elemental analysis, found: C, 69.46; H, 7.26; N, 7.08. Calcd for $C_{23}H_{28}N_2O_4$: C, 69.66; H, 7.12; N, 7.07. MS (ESI^+) *m/z* 419 $[M+Na]^+$. ^1H NMR in $CDCl_3$: δ = 0.94 (d, $^3J_{H,H}$ = 6.5 Hz, 3H, H-7), 1.08–0.93 (m, 2H, H-3, H-5), 1.32 (m, 1H, H-6), 1.54 (m, 1H, H-4), 1.72 (m, 1H, H-5´), 2.09–2.05 (m, 2H, H-3´, H-6`), 3.46-3.36 (m, 2H, H-2, H-1), 5.12–5.04 (m, 6H, OCH_2, NH), 7.34 (s,

10H, H-ar). ^{13}C NMR in $CDCl_3$: δ = 22.1 (C-7), 31.9 (C-4), 32.6 (C-5), 33.6 (C-6), 41.5 (C-3), 55.5 (C-2), 55.7 (C-1), 67.0 (OCH_2), 128.3 (C-ar), 128.4 (C-ar), 128.9 (C-ar), 137.0 (Cq-ar), 157.2 (C=O).

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PAPER

Solid-phase synthesis of oxaliplatin–TAT peptide bioconjugates

Sergey Abramkin,[a] Seied M. Valiahdi,[a] Michael A. Jakupec,[a] Markus Galanski,*[a] Nils Metzler-Nolte[b] and Bernhard K. Keppler*[a]

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Platinum-based drugs play a crucial role in the fight against cancer. Oxaliplatin, which is used in the treatment of colorectal carcinoma, was the last platinum-based agent to be approved worldwide. However, the efficiency of the therapy is limited for example by a low accumulation of the drug in cancer cells. Cell-penetrating peptides (CPPs) are known to ease the cellular membrane transport and are used as vectors for low-molecular-weight drugs and drug carriers; of them, TAT peptides are the best-studied group. In this work, a TAT-peptide fragment (YGRKKRRQRRR) was for the first time conjugated to a platinum(IV) analog of oxaliplatin as a vehicle for membrane penetration. Solid-phase peptide synthesis and subsequent coupling with the platinum complex afforded mono- and difunctionalized conjugates, which were separated by preparative HPLC and characterized by analytical HPLC, ESI-MS, and ^{1}H NMR spectroscopy. Both conjugates are active in the low micromolar range in CH1 and SW480 human cancer cells, requiring much lower concentrations than the untargeted analogs for equal effects.

Introduction

From their beginning with cisplatin, whose anticancer properties were discovered in the 1960s,[1,2] the structures of platinum agents underwent considerable changes in a long history of drug development, resulting in 2nd (carboplatin) and 3rd (oxaliplatin, Fig. 1) generations of platinum-based drugs.[3–6] But the core, a platinum atom with two coordinated N-donor functions, remained the same together with the major shortcomings of parental cisplatin—adverse side effects and limited efficiency of platinum-based therapy.

Nuclear DNA is believed to be the ultimate target for cisplatin and analogs. Reacting with N-7 of guanine and forming intrastrand cross-links, cisplatin causes DNA bending and unwinding,[7] followed eventually by apoptosis.

The latest successfully approved platinum complex, oxaliplatin, has a slightly different mode of action and is used in metastatic colorectal cancer, which is inherently resistant to cis- and carboplatin. The chelating oxalate ligand is more inert as leaving group compared to chlorides, lowering the aquation rate and thus the risk of undesired side reactions. Additionally, oxaliplatin-derived DNA adducts are processed differently by the cellular machinery in comparison to those of cis- and carboplatin. Pt(IV) complexes have significantly lower ligand exchange rates, compared to their Pt(II) counterparts, which might be of interest in order to ameliorate systemic toxicity.

It is widely accepted that platinum(IV) complexes act as prodrugs which have to be reduced to the corresponding platinum(II) analogs before displaying their anticancer potential (activation by reduction). With the octahedral geometry of platinum(IV) complexes, two extra ligand sites are introduced, which may be used for drug targeting strategies. In this context it is worthwhile mentioning that upon reduction of Pt(IV) to Pt(II) these extra ligands (*e.g.* targeting groups) are lost, producing the original cytotoxic platinum(II) complex.

Short amino acid sequences have already been used as targeting moieties for Pt(II)[8] and Pt(IV)[9] cisplatin analogs. However,

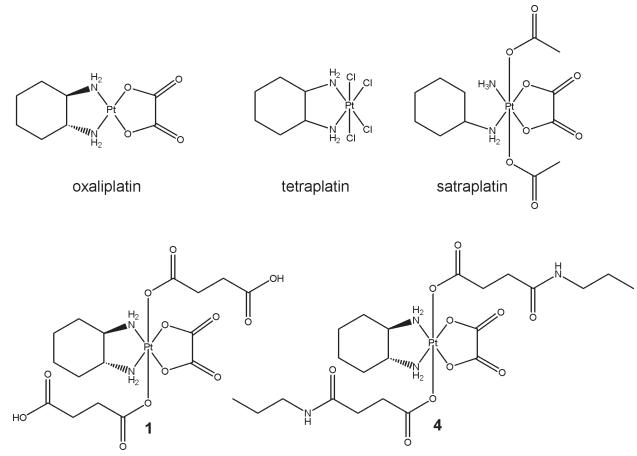
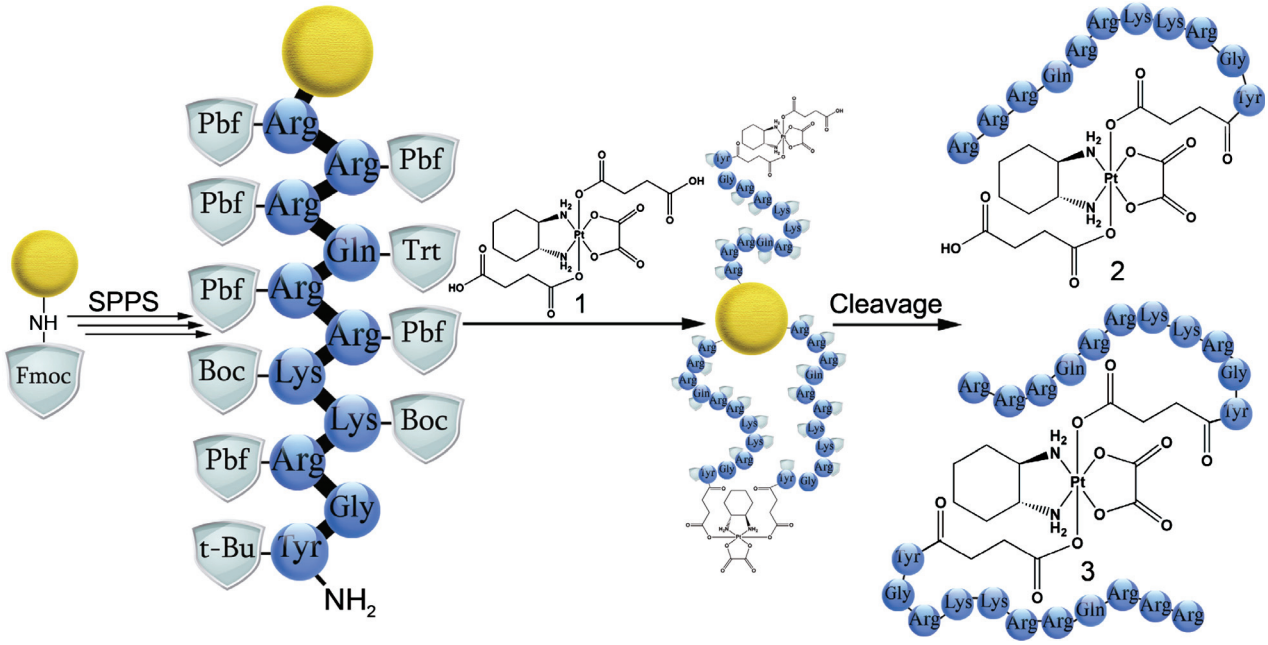


Fig. 1 Chemical structures of oxaliplatin, tetraplatin, satraplatin, and complexes **1** and **4**.

[a]*Institute of Inorganic Chemistry, University of Vienna, WährингerStrasse 42, A-1090 Vienna, Austria. E-mail: markus.galanski@univie.ac.at; Fax: +43 1 427752680*
[b]*Lehrstuhl für Anorganische Chemie I – Bioanorganische Chemie, Fakultät für Chemie und Biochemie, Ruhr-Universität Bochum, Universitätsstrasse 150, D-44801 Bochum, Germany*



Scheme 1 Preparation of conjugates **2** and **3**.

coupling of different peptides to other clinically approved anticancer agents such as oxaliplatin is still of high interest. Depending on the amino acid sequence, not only basic parameters such as lipophilicity or water solubility can be changed, but more complex interactions of the drug with the microenvironment may be influenced. One of the authors has reported the synthesis of peptide conjugates with organometallic complexes,[10–14] for example for intracellular delivery[15–17] and targeting peptide receptors.[18–21] In one case, enhanced permeation through the blood–brain barrier was observed for an enkephalin conjugate with the iron-containing ferrocene moiety.[22] Low cellular accumulation is one of the main reasons for drugs to be ineffective,[23] and the uptake itself depends on the ability of molecules to penetrate the cell membrane. In this respect cell-penetrating peptides may be very useful. These are a group of peptides capable of translocating across the cellular membrane through diverse pathways.[24] Among these are several lysine and arginine rich sequences. The TAT-peptide especially is well-known and has been studied in detail.

The TAT protein (Trans-Acting Activator of Transcription) of HIV-1 was discovered in 1987. The minimal region required for membrane translocation was determined, namely residues 47–57 (YGRKKRRQRRR). This peptide facilitates intracellular delivery of various cargos like drug agents with limited cell permeability, larger biomolecules and even carriers such as liposomes and nanoparticles.[25] Later on, another important advantage was found, namely that TAT-coupled proteins may overcome the main obstacle in brain cancer treatment, the blood–brain barrier.[26,22] Consequently, the focus of the present work was to combine the properties of the TAT peptide with the cytotoxicity of an established anticancer drug, oxaliplatin. As synthetic platform, the corresponding platinum(IV) complex should be used.

Results and discussion

Synthesis and characterization

The platinum precursor **1** was chosen because of several reasons. Recently, the cytotoxicity of non-targeted Pt(IV) complexes was tested in our group, and the derivatives of oxaliplatin showed moderate antiproliferative activity compared to cisplatin analogs.[27] Targeting of a less active molecule should demonstrate a more pronounced effect than targeting of a complex that already has inherently high cytotoxicity. Furthermore, the choice of oxalate as leaving group seemed advantageous. The stability of platinum(IV) complexes in the organism plays a crucial role, especially in the case of attached targeting moieties, which must not be released too early (before reaching the tumor).

It is known that tetraplatin (two equatorial and two axial chlorido ligands) is reduced within seconds in the blood stream. When replacing the axial chlorido ligands by carboxylates, the reduction is slowed down as found for satraplatin (6.3 min).[28] Upon replacing the equatorial chlorido ligands with carboxylates, the reduction potential again decreases[29] and the resulting compound (*e.g.* oxaliplatin derivative) should be equipped with a higher stability. Besides kinetics, the oxaliplatin derivative was chosen with consideration of future RP-HPLC purification of the products. The lipophilic *R*,*R*-cyclohexane-1,2-diamine ligand in oxaliplatin (in comparison to the ammonia ligands in cisplatin) was preferred for the synthesis of TAT-conjugates in order to facilitate the separation of multiply positively charged molecules (TAT has six arginine and two lysine residues) on RP-HPLC. The peptide was synthesized manually by a solid-phase method according to Scheme 1.

In contrast to a recently published procedure,[9] coupling of activated **1** with the deprotected peptide was not performed after

cleavage of the peptide in solution due to the undesired reaction of the free amino groups of lysine with the platinum center. Rather, the conjugation step was also performed using the polymer-bound peptide with protected side chains. The platinum precursor complex was activated with CDI as described for the synthesis of non-targeted analogs.[27,30,31] The complex is readily soluble in DMF and can therefore be used as a building-block in solid-phase peptide synthesis without any problems. According to the chosen strategy, two products are expected, namely the monoconjugate **2** and the diconjugate **3**. Their ratio will *inter alia* depend on the distance between the peptide chains on the resin beads. Indeed, both conjugates could be isolated after cleavage and deprotection using reverse-phase HPLC.

The hydrophilic properties of the TAT-peptide required a prolonged running time for separation of mono- and diconjugates. The chromatograms are shown in Fig. S1 in the ESI.†

The purity of the conjugates was confirmed by analytical HPLC (Figures S1 and S2, ESI†). Characterization using ESI-MS was troublesome because multiply charged species were formed, for example, in the case of the monoconjugate $(M+2H^+)^{2+}$, $(M+3H^+)^{3+}$ and for the diconjugate $(M+6H^+)^{6+}$, $(M+7H^+)^{7+}$, $(M+8H^+)^{8+}$, $(M+9H^+)^{9+}$ ions were observed. Besides signals of the conjugates, signals of the succinate–TAT fragment were also observed, for both conjugates **2** and **3** (Figures S3 and S4, ESI†). The latter species turned out to be characteristic in mass-spectra of the conjugates not only when the electro-spray ionization method was used. In MALDI-TOF spectra the same ion (but monocharged) was found (Figure S5, ESI†). Differentiation between mono- and diconjugate could also be accomplished *via* their ^{1}H NMR spectra; especially the CH–N methine protons of the cyclohexanediamine unit, the methylene protons of the succinate moiety and the Cα-protons of the peptide were very useful in this respect. The characteristic regions of mono- and diconjugate (2.25–5.0 ppm) are shown in Fig. 2. The two CH–N protons of the coordinated cyclohexanediamine ligand resonate at 2.79 (**2**) and 2.77 (**3**) ppm, respectively. Obviously, as expected, the pattern of the CH_2 protons of the succinate moieties, which can be found in the region between 2.35 and 2.6 ppm, is markedly different. In the case of the diconjugate **3**, two multiplets were detected, since both succinate units are identical. However, in the monoconjugate **2**, only one of the succinates was derivatized, resulting in three or four partly overlapping resonances. Additionally, the amounts of mono- and diconjugation can also be calculated from the integration ratios of the Cα-protons (3.6–4.5 ppm) relative to the CH–N and the succinate CH_2 protons. For monoconjugate **2** a ratio of 12 : 2 : 8 was found, whereas for the diconjugate **3**, it was 24 : 2 : 8.

Cytotoxicity in cancer cell lines

The cytotoxicity of conjugates **2** and **3** in comparison to precursor **1** and non-targeted amide derivative **4** (Fig. 1) was examined in four human cancer cell lines derived from ovarian carcinoma (CH1, SK-OV-3), colon carcinoma (SW480) and lung adenocarcinoma (A549) by using the MTT assay. The results are presented in Table 1, concentration-effect curves are shown in Figure S6 in the ESI.†

Both novel conjugates, **2** and **3**, showed promising antiproliferative activity with IC_{50} values in the low micromolar range (1.4–31 μM). Notably, they were significantly more active than the non-targeted analogs **1** and **4**. The carboxylic acid derivative **1** showed the lowest cytotoxicity. This is readily explained by its low lipophilicity due to two carboxylic acid groups, resulting in a low cellular accumulation. In contrast, the cytotoxic potency of the non-targeted amide derivative **4** is three to five times higher than that of **1**. This finding is in accord with previous investigations of analogous platinum(IV) complexes.[32,33] The highest cytotoxicity was observed for targeted conjugates **2** and **3** in CH1 cells; with the monoconjugate **2** being up to 39 and 8 times more cytotoxic than the non-targeted carboxylate **1** or amide **4**, respectively. In general, all IC_{50} values of the monoconjugate were slightly lower in all cell lines compared to those of the diconjugate. In that context it is worthwhile mentioning that the TAT peptide alone features a rather low cytotoxic potency in A549, HeLa, or CHO cancer cells.[34]

Experimental section

Materials and instrumentation

All chemicals and solvents were used as received from commercial suppliers. (*OC*-6-33)-Bis(3-carboxypropanoato)(*trans*-(1*R*,2*R*)-diaminocyclohexane)oxalatoplatinum(IV), complex **1**,

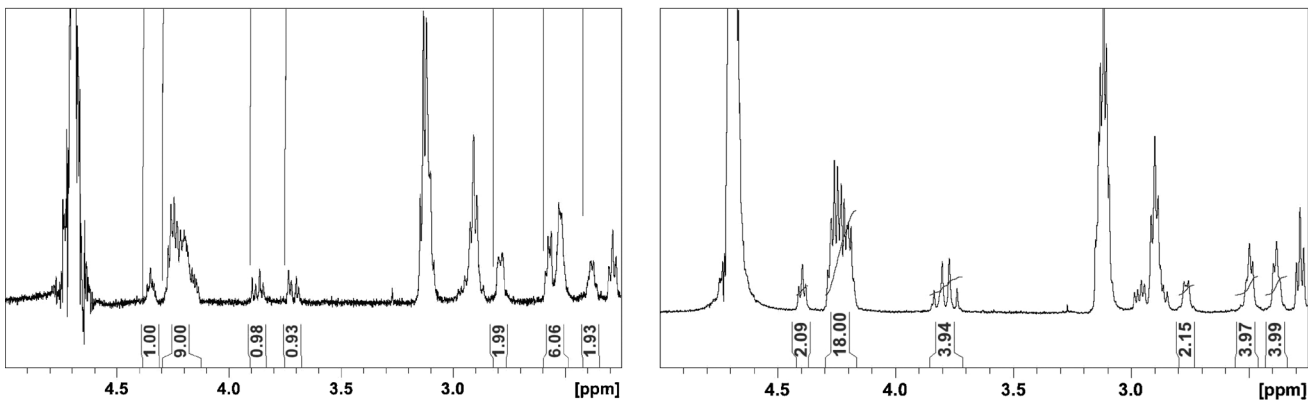


Fig. 2 ^{1}H NMR spectra of **2** (left) and **3** (right) in D_2O, showing the region between 2.25 and 5.0 ppm.

Table 1 Cytotoxicity of conjugates **2** and **3**, precursor complex **1**, and its amide derivative **4** in four human cancer cell lines

	IC_{50} [μM][a]			
Compound	CH1	A549	SW480	SK-OV-3
1[b]	55 ± 28	n.d.	44 ± 9	142 ± 26
2	1.4 ± 0.3	11 ± 2	3.2 ± 0.4	22 ± 3
3	2.5 ± 0.6	23 ± 4	4.8 ± 1.6	31 ± 4
4[b]	11 ± 2	n.d.	12 ± 5	44 ± 10

[a] 50% inhibitory concentrations in CH1, A549, SW480 and SK-OV-3 cells in the MTT assay, 96 h exposure. Values are the means ± standard deviations obtained from three independent experiments. [b] data taken from ref. 27.

was synthesized according to a literature procedure.[27] The Fmoc-Rink-Amide AM resin (0.59 mmol g^{-1}), 1-hydroxybenzotriazole monohydrate (HOBT monohydrate), 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU), *N*,*N*,*N*-diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA) and protected acids (Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-L-Tyr(tBu)-OH) were purchased from Iris Biotech (Germany). *N*,*N*′-Carbonyldiimidazole (CDI), piperidine and phenol were purchased from Sigma–Aldrich Handels GmbH, extra dry DMF was purchased from Acros Organics. Syringe reactors for the peptide synthesis were purchased from MultiSynTech GmbH. Conjugates were purified using a preparative GROM Saphir C18 column (25 mm × 250 mm) on an Agilent 1200 series HPLC instrument. HPLC analysis was performed on a Dionex Summit system controlled by the Dionex Chromeleon 6.60 software using Agilent ZORBAX Bonus-RP column (4.6 mm × 250 mm).

^{1}H NMR spectra were recorded with a Bruker Avance III 500 MHz NMR spectrometer at 500.32 MHz in D_2O at room temperature, using the solvent residual peak for ^{1}H as internal reference. Electrospray ionization mass spectrometry was performed on a Bruker Esquire 3000 instrument. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectra were measured on a Bruker Daltonics Autoflex with sinapinic acid as matrix.

Synthesis of conjugates

The Fmoc-protected TAT-peptide was manually synthesized on a Rink amide resin (0.2 mmol, 339 mg) according to the standard Fmoc procedure with a 4-fold excess of amino acids (0.8 mmol).[35] TBTU (257 mg, 0.8 mmol) with addition of HOBT (123 mg, 0.8 mmol) and DIPEA (340 μL, 3.46 mmol) in 2–2.5 mL DMF were used to activate the protected amino acids. Deprotection of the coupled amino acids was performed using 20% piperidine in DMF. After completion of the sequence, the resin was deprotected, washed with DMF and coupled with **1**.

CDI was used to activate the carboxylic groups. Complex **1** (0.4 mmol, 253 mg) was dissolved in 1.5 ml of dry DMF and heated to 60 °C. A solution of CDI (136 mg, 0.84 mmol) in 2 mL of dry DMF was added and the mixture was stirred at 60 °C for 10 min. The mixture was cooled to room temperature under slow argon flushing; it was transferred into a reactor containing the protected polymer-bound peptide and stirred for 24 h. After the conjugation, the excess of activated precursor was removed, the resin was washed with DMF and dichloromethane and dried in vacuo. The conjugates were cleaved from the resin using a TFA–water–phenol (95 : 2.5 : 2.5) mixture (2 h) and precipitated with cold ether.

Crude products were dissolved in a mixture of 0.1% TFA in water and 0.1% TFA in methanol (80 : 20) and purified using a HPLC (trace and gradient conditions are shown in Figure S1 in the ESI†). The monoconjugate was eluted at 32 min, followed by the diconjugate at 35 min. The fractions, containing the products, were collected and lyophilized, yielding 7.3 mg of the monoconjugate **2** and 34 mg of the diconjugate **3** (1.7% and 4.6% with respected to the resin loading). Purity was confirmed by analytical HPLC (Figures S2 and S3 in the ESI†).

ESI-MS spectra of **2** and **3** are shown in Figures S3 and S4 in the ESI,† and the following characteristic *m*/*z* values were found: monoconjugate **2**: 1086.5 $(M+2H^+)^{2+}$, 724.7 $(M+3H^+)^{3+}$; diconjugate **3**: 619.5 $(M+6H^+)^{6+}$, 531.1 $(M+7H^+)^{7+}$, 464.9 $(M+8H^+)^{8+}$. In MALDI-TOF MS measurements, only the TAT-succinate moieties were found: monoconjugate **2** 1659.9; diconjugate **3** 1660.0. calculated: $(+H)^+$ 1658.9 (Figure S5 in the ESI†).

Cell lines and culture conditions

CH1 (ovarian carcinoma, human) cells were donated by Lloyd R. Kelland (CRC Centre for Cancer Therapeutics, Institute of Cancer Research, Sutton, U.K.). A549 (non-small cell lung cancer, human) and SW480 (colon carcinoma, human) cells were kindly provided by Brigitte Marian (Institute of Cancer Research, Department of Medicine I, Medical University of Vienna, Austria), and SK-OV-3 (ovarian carcinoma, human) cells by Evelyn Dittrich (General Hospital, Medical University of Vienna, Austria). Cells were grown in 75 cm^2 culture flasks (Iwaki/Asahi Technoglass) as adherent monolayer cultures in Minimal Essential Medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, and 4 mM L-glutamine and 1% v/v non-essential amino acids (from 100x stock solution) (all purchased from Sigma–Aldrich) without antibiotics. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air.

Cytotoxicity tests in cancer cell lines

Cytotoxicity in the cell lines mentioned above was determined by the colorimetric MTT assay (MTT = 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide, purchased from Fluka). Cells were harvested from culture flasks by trypsinization and seeded in 100 μL aliquots in complete culture medium into 96-well microculture plates (Iwaki/Asahi Technoglass) in the following densities, to ensure exponential growth of untreated controls throughout the experiment: 1.5 × 10^3 (CH1), 3.5 × 10^3 (SK-OV-3), 4.0 × 10^3 (A549), and 2.5 × 10^3 (SW480) viable cells per well. Cells were allowed to settle and resume exponential growth in drug-free medium for 24 h, followed by the addition of dilutions of the test compounds in 100 μL/well of the same medium. After continuous exposure for 96 h, the medium

was replaced by 100 μL/well RPMI 1640 medium (supplemented with 10% heat-inactivated fetal bovine serum and 4mM L-glutamine) plus 20 μL/well solution of MTT in phosphate-buffered saline (5 mg mL^{-1}) (all purchased from Sigma-Aldrich). After incubation for 4 h, medium–MTT mixtures were removed, and the formazan product formed by viable cells was dissolved in DMSO (150 μL/well). Optical densities at 550 nm were measured with a microplate reader (Tecan Spectra Classic), using a reference wavelength of 690 nm to correct for unspecific absorption. The quantity of viable cells was expressed as a percentage of untreated controls, and 50% inhibitory concentrations (IC_{50}) were calculated from concentration-effect curves by interpolation. Evaluation is based on means from three independent experiments, each comprising six replicates per concentration level.

Conclusions

Mono- and difunctionalized conjugates of an oxaliplatin prodrug were prepared for the first time by solid-phase peptide synthesis and characterized by ^{1}H NMR spectroscopy, ESI-MS and analytical HPLC. Remarkably, inhibition of cancer cell proliferation requires only low micromolar concentrations of the conjugates. Compared to its non-targeted platinum(IV) analogs, the monoconjugate was up to a factor of 39 more cytotoxic. These results are very promising and warrant further studies *in vitro* and especially *in vivo*.

Acknowledgements

We would like to thank David Köster from the Ruhr-University of Bochum for help with the solid phase synthesis and measuring MALDI spectra. The solid-phase peptide synthesis was performed in Bochum during a short-time scientific mission supported by COST Action D39. Financial support by COST, FFG, Austrian Science Fund FWF (P20683-N19), the Austrian Council for Research and Technology Development, and the Deutsche Forschungsgemeinschaft DFG (through grant FOR630) is gratefully acknowledged.

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Supporting Information

Solid-Phase Synthesis of Oxaliplatin-TAT Peptide Bioconjugates

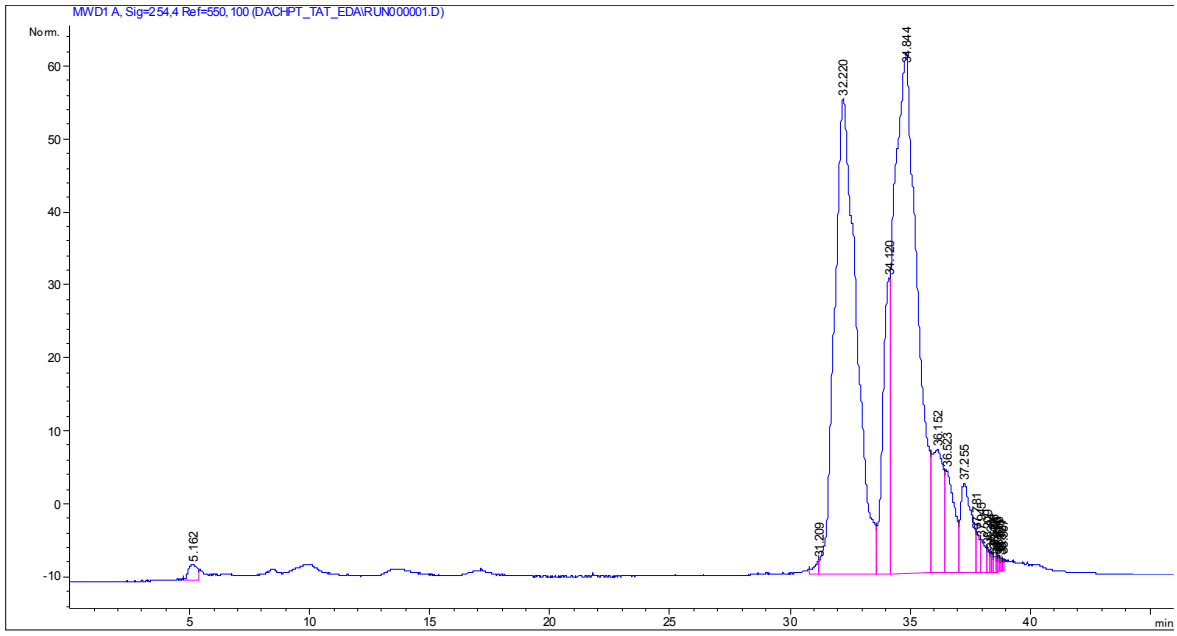
Sergey Abramkin[a], Seied M. Valiahdi[a], Michael A. Jakupec[a], Markus Galanski* [a], Nils Metzler-Nolte[b] and Bernhard K. Keppler* [a]

[a]Institute of Inorganic Chemistry, University of Vienna, Währinger Strasse 42, A-1090 Vienna, Austria

[b]Lehrstuhl für Anorganische Chemie I – Bioanorganische Chemie, Fakultät für Chemie und Biochemie, Ruhr-Universität Bochum, Universitätsstrasse 150, D-44801 Bochum, Germany

Contents

Figure S1. HPLC purification of of conjugates **2** and **3**. Column: GROM Saphir C18 column (25 mm × 250 mm), eluents: 0.1% TFA in water and 0.1% TFA in methanol. Monoconjugate **2** was eluted at 32 min, diconjugate **3** at 35 min.

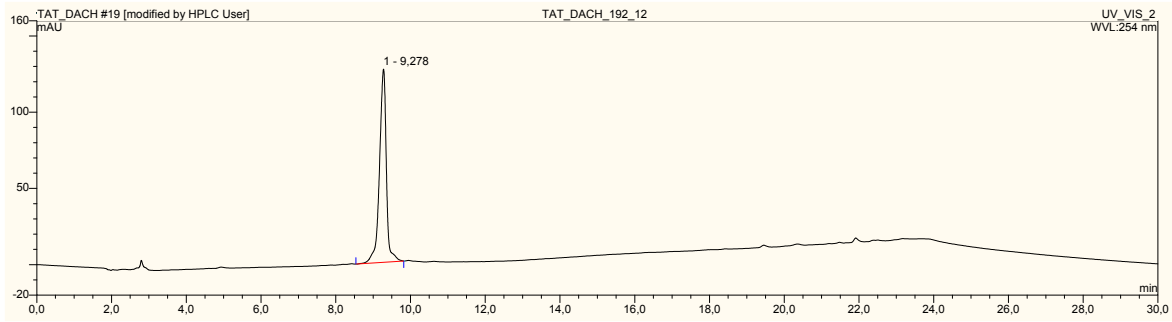


Gradient conditions:

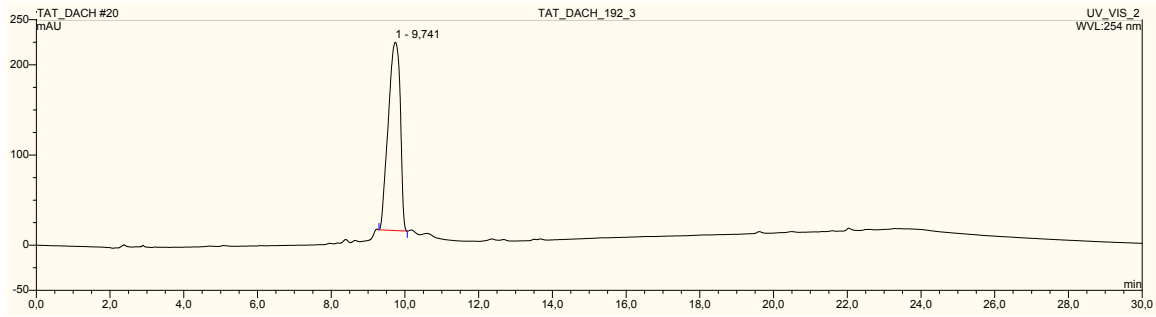
time, min	% MeOH	Rate, ml/min
0	20	20
25	20	20
35	40	20
45	20	20

Figure S2. Analytical HPLC traces of purified conjugates **2** (A, 9.28 min) and **3** (B, 9.74 min). Column : Agilent ZORBAX Bonus-RP (4.6 mm × 250 mm), eluents: 0.1% TFA in water and 0.1% TFA in methanol.

A Monoconjugate **2**:



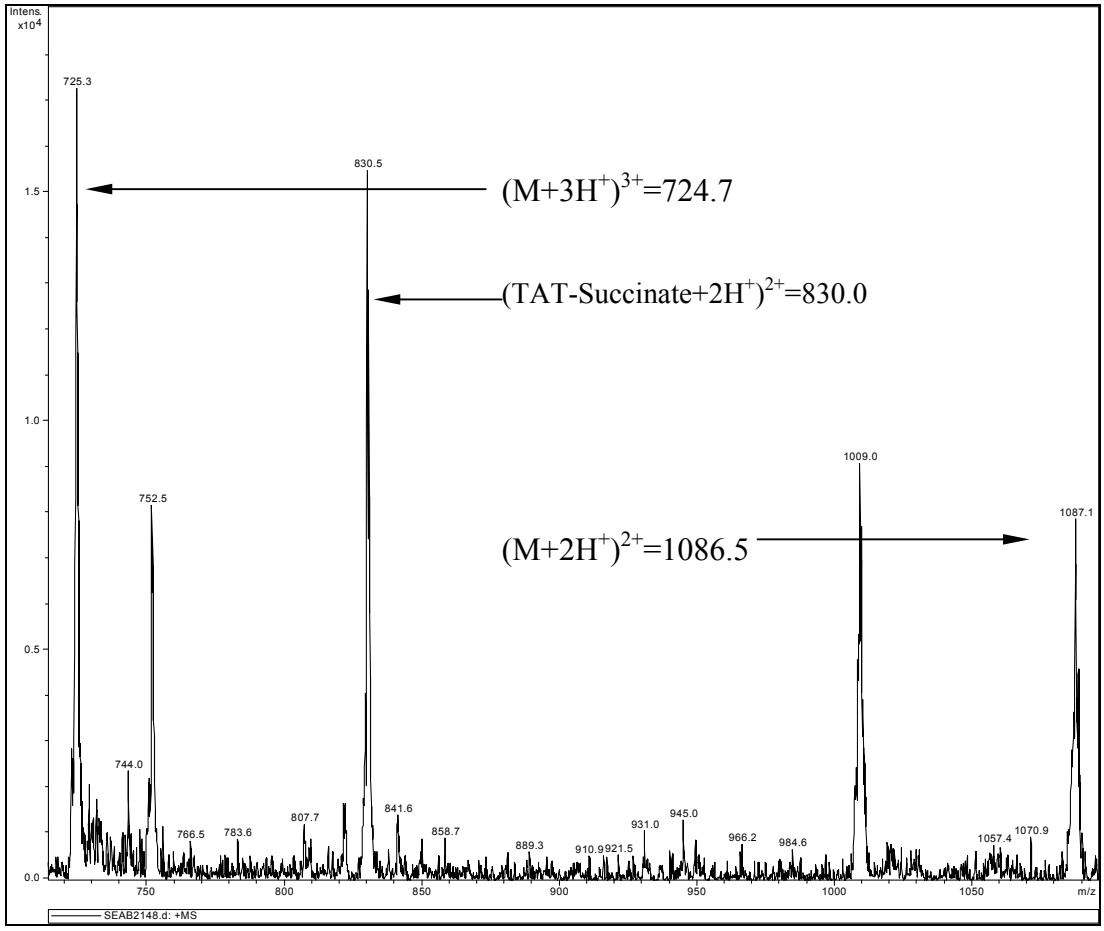
B Diconjugate **3**:



Gradient conditions:

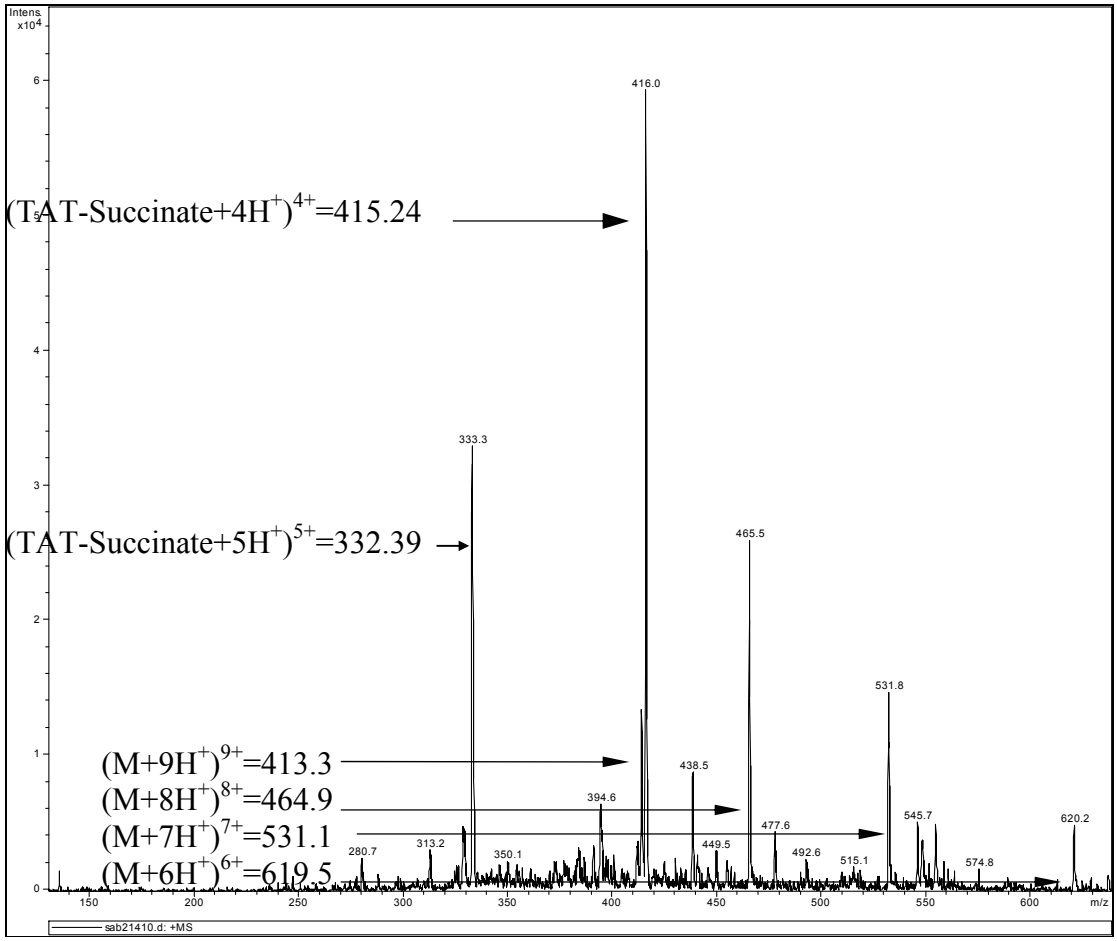
time, min	% MeOH	Rate, ml/min
0	5	0.8
20	95	0.8
3	5	0.8

Figure S3. ESI-MS characterization of conjugate **2**.



Monoconjugate **2**: Exact Mass: 2171.05
TAT-Succinate: Exact Mass: 1657.98

Figure S4. ESI-MS characterization of conjugate **3**.



Diconjugate **3**: Exact Mass: 3711.01
TAT-Succinate: Exact Mass: 1657.98

Figure S5. MALDI-TOF characterization of conjugates **2** and **3**.

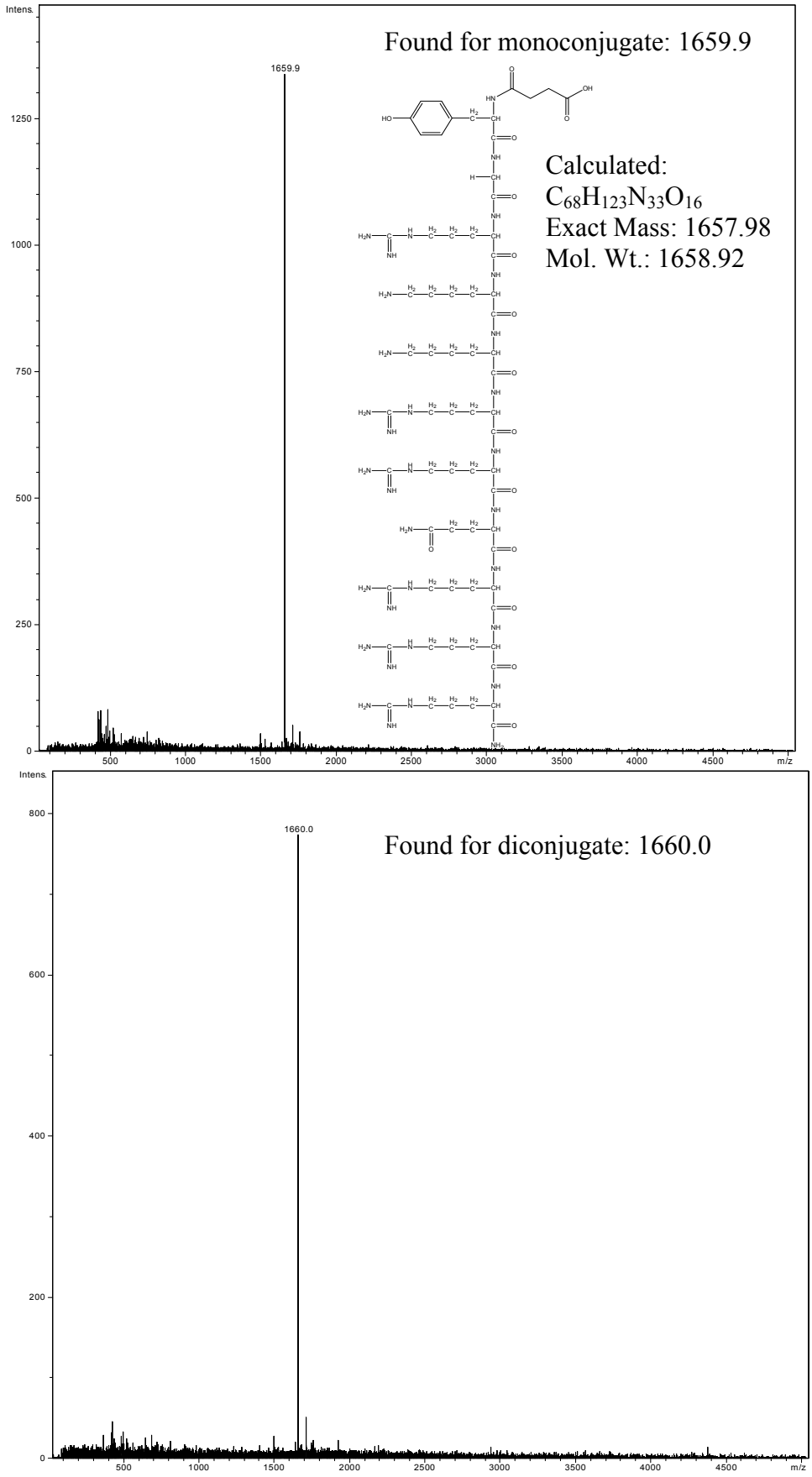
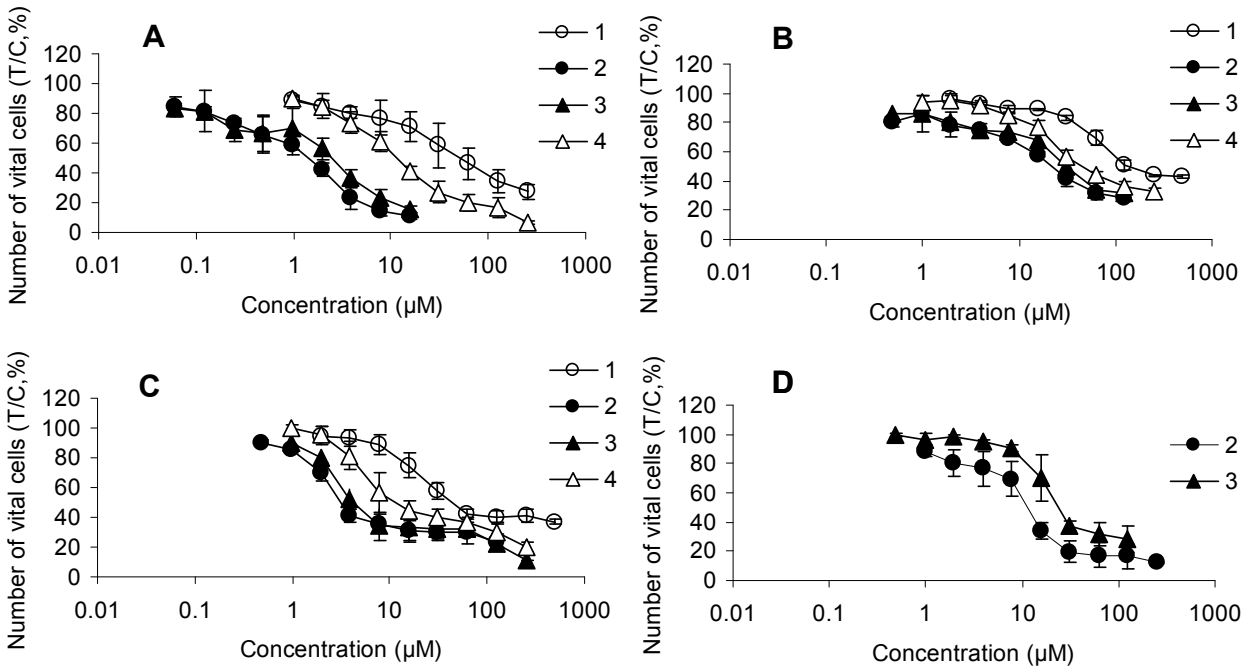


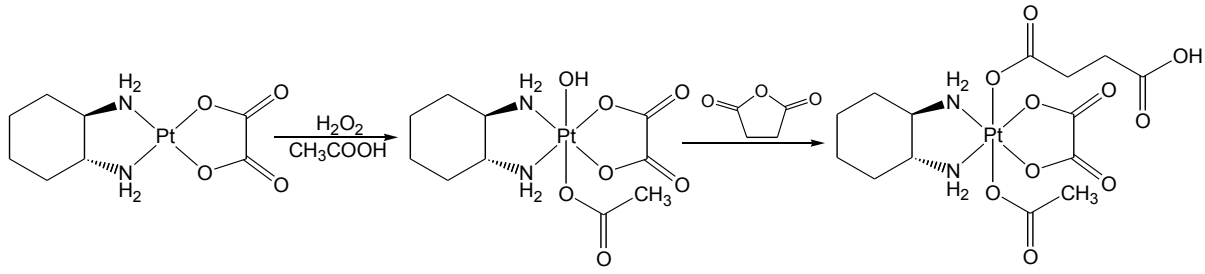
Figure S6. Concentration-effect curves of conjugates **2** and **3** and the precursor complex **1** and its amide derivative **4** in CH1 (A), SK-OV-3 (B), SW480 (C) and A549 (D) cell lines obtained by the MTT assay (96 h exposure)



Optimization of the Coupling Strategy of Pt(IV) Complexes to Peptides

The ability of appended peptides to modify cytotoxic properties of platinum compounds was demonstrated with Pt(II) and Pt(IV) complexes. Both mono- and bisconjugates with the TAT peptide were more active, compared to the starting compound and an analog without a targeting group. The vectors increased the cytotoxicity of the complexes, other peptides, containing the RGD motif (a sequence, able to bind to receptors on the cell surface, integrins) increased their selectivity towards some cell lines.

The comparable activity of both conjugates suggests, that the synthetic strategy can be aimed at obtaining a single product. A platinum complex with one peptide moiety is preferred, the other route wastes one peptide chain. This goal can be achieved by using monofunctionalyzed Pt building blocks. One of the axial positions of Pt(IV) complexes can be blocked on the stage of oxidizing Pt(II) complexes. When the reaction is performed in non-aqueous medium, the unsymmetrical monohydroxido monosolvato complexes are formed, according to the following equation:



Initially synthesis of Pt(IV) conjugates with shorter sequences was performed in solution, but it would be troublesome for longer sequences containing numerous basic amino acids. Besides reaction with side-chain amino groups, there was another disadvantage of the method. Both activated carboxylic groups of starting platinum compounds could react. Later, the solution-state derivatization of the cisplatin analogs with a longer, 36 amino acid peptide chlorotoxin was developed, based on different basic properties of terminal amino groups of the peptide and ε-aminogroup of lysines.[1] The platinum complexes were taken in excess (5:1 or 10:1) to find better conditions for the synthesis.

It's obvious that a straightforward solution-state strategy for selective conjugation is necessary for the development of more complex conjugates. This will allow avoiding cleavage under aggressive conditions and application of scavengers like triisopropylsilane (TIS) and ethanedithiol. Reaction of thiols with maleimide is a widely used methodology for effective crosslinking under milder conditions. Equipment of platinum precursors with a

maleimide functionality opens up a way to append different peptides via an additional cysteine. The only prerequisite of this strategy is the absence of cysteine in the peptide chain. The reaction is normally performed in a slightly basic environment in phosphate buffer, but other bases can be used instead.[2]

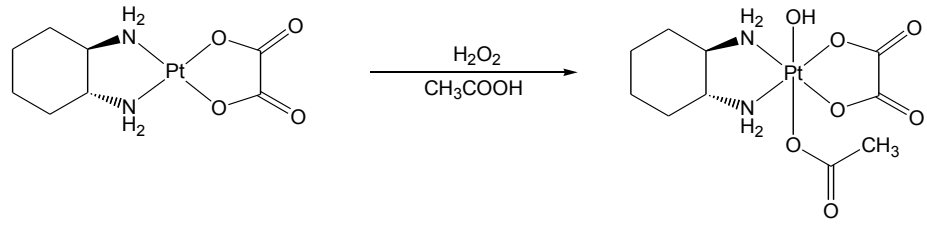
Bombesin was chosen as a delivery vector for the cytotoxic complex. It is a neuropeptide with many biological functions, its receptors are overexpressed in many tumors including breast, prostate, intestine, pancreas and brain tumors. Previously, it was used for diagnostic purposes, but its potential could be helpful in therapy as well.[3]

Oxaliplatin was synthesized as described in the literature.[4]

Oxidation of oxaliplatin with hydrogen peroxide in acetic acid afforded the corresponding Pt(IV) complex with the oxaliplatin core and the axial acetato and hydroxido ligands. The symmetrical dihydroxido byproduct could be separated because of the lower solubility in a methanol/ethyl acetate mixture; traces of the diacetato complex were inert in the next step and were removed after the acylation step.

Two carboxylic acid anhydrides were used to prepare the platinum precursors for the conjugate synthesis. The complex with free carboxylic acid group was obtained via reaction with succinic anhydride as described for the symmetrical dihydroxido complexes. The symmetric anhydride for the synthesis of the maleimido-derived complex was prepared from the corresponding carboxylic acid and triphosgene.

(*OC*-6-44)-Acetato(*R*,*R*-1,2-diaminocyclohexane)hydroxido(oxalato)platinum(IV)



1.19g (3 mmol) of oxaliplatin were suspended in 20 mL of acetic acid, and 0.25 mL of 30% H_2O_2 were added. The mixture was stirred for 30 min, then the next 0.25 mL portion of hydrogen peroxide was added. After 30 minutes a third portion was added (0.75 mL). In 1.5 hours the solution turned yellowish and almost clear. The mixture was stirred overnight. Acetic acid was removed on a rotary evaporator and the oily residue was suspended in water (40 mL), forming a turbid solution. Addition of water, suspending and removal of water on a rotary evaporator was performed 2 times more till the product became solid. The product was suspended in ethyl acetate, methanol was added stepwise till the solution turned almost clear and the addition of a next portion of methanol did not change the appearance. The mixture was filtered through a glass sinter filter, the solvent was removed on a rotary evaporator, and the white, slightly yellowish product was dried in vacuum

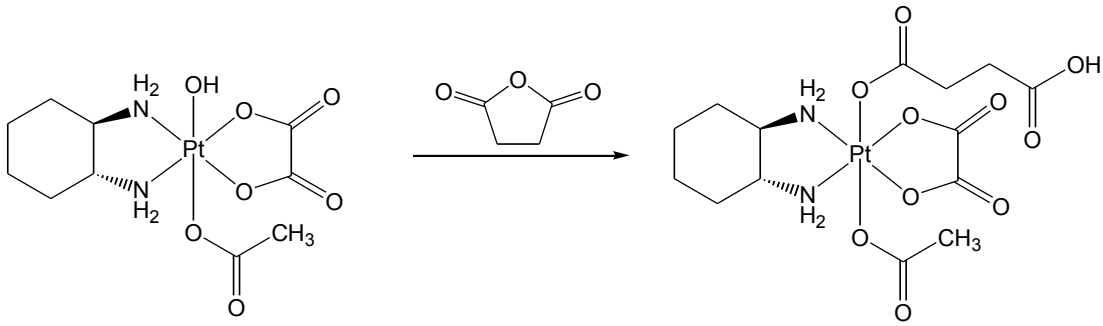
Yield: 0.975g (69%)

^{1}H NMR (DMSO): δ = 8.70 – 8.48 (bs, 1H, N*H*$_2$), 8.26 – 8.06 (bs, 1H, N*H*$_2$), 7.89 – 7.68 (bs, 1H, N*H*$_2$), 7.19 – 7.00 (bs, 1H, N*H*$_2$), 2 protons superimposed by DMSO signal, 2.13 – 2.01 (m, 2H, chxn), 1.92 (s, 3H, CH_3), 1.54 – 1.41 (3H, chxn), 1.39 – 1.30 (1H, chxn), 1.24 (s, 1H, O*H*), 1.15 – 1.09 (m, 2H, chxn) ppm

^{13}C NMR (DMSO): δ = 180.3 (*C*(O)CH_3), 164.3 (2C, *C*(O), oxalate), 61.9, 60.5 (2C, *C*H, (chxn)), 31.4, 31.2 (2 C, *C*H_2, (chxn)), 24.4 (*C*H_3, acetato), 24.1, 24.1 (2C, *C*H_2, (chxn)) ppm

^{195}Pt NMR (DMSO): δ = 3033 ppm

(*OC*-6-44)-Acetato (3-carboxypropanoato) (*R,R*-1,2-diaminocyclohexane) oxalatoplatinum(IV)



0.770 g (1.65 mmol) of the hydroxido complex was mixed with 0.400 g (4 mmol) of succinic anhydride and suspended in 15 mL of DMF. The mixture was stirred overnight at 50°. DMF was removed under high vacuum, the oil was suspended in 200 mL of acetone, and methanol was added stepwise till the amount of insoluble solid did not change upon addition of the next portion of methanol. The solution was filtered, the solvents were removed on a rotary evaporator and the residue was dissolved in acetone. Then, diethyl ether was added under vigorous stirring till the addition did not cause further precipitation (about 500 mL). The product was filtered, washed with ether and dried in vacuum.

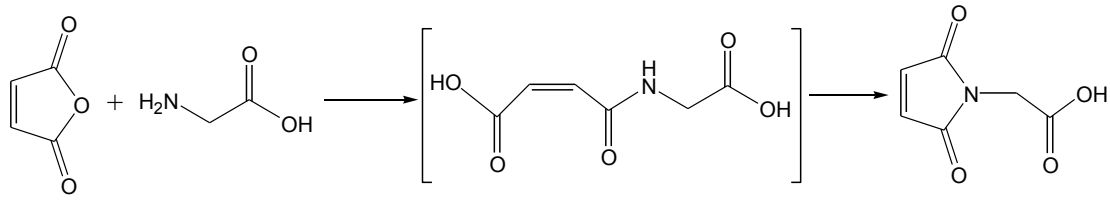
Yield: 0.805 g (90%) of unpurified product

^{1}H NMR (DMSO): δ = 12.1 (bs, 1 H, COO*H*), 8.52 – 8.07 (br, 4 H, NH_2), 2.63 – 2.57 (m, 2H, chxn), 2.44 – 2.34 (4H, succinato), 2.17 – 2.06 (4H, chxn), 1.96 (s, 3H, CH_3), 1.54 – 1,47 (m, 2H, chxn), 1.46 – 1.33 (m, 2H, chxn), 1.19 – 1.12 (m, 2H, chxn) ppm

^{13}C NMR (DMSO): δ = 180.1, 179.1 (2C, *C*(O)CH_3, *C*OO), 174.2 (*C*OOH), 163.9, 163.8 (2C, *C*(O), oxalate), 61.4 (2C, *C*H, (chxn)), 31.4, 31.3(2C, (chxn)), 31.0, 30.0 (2C, (succinato)), 24.0, 23.90 (2C, (chxn)), 23.4 (*C*H_3, acetato) ppm;

^{195}Pt NMR (DMSO): δ = 3239 ppm

(2,5-Dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)acetic acid

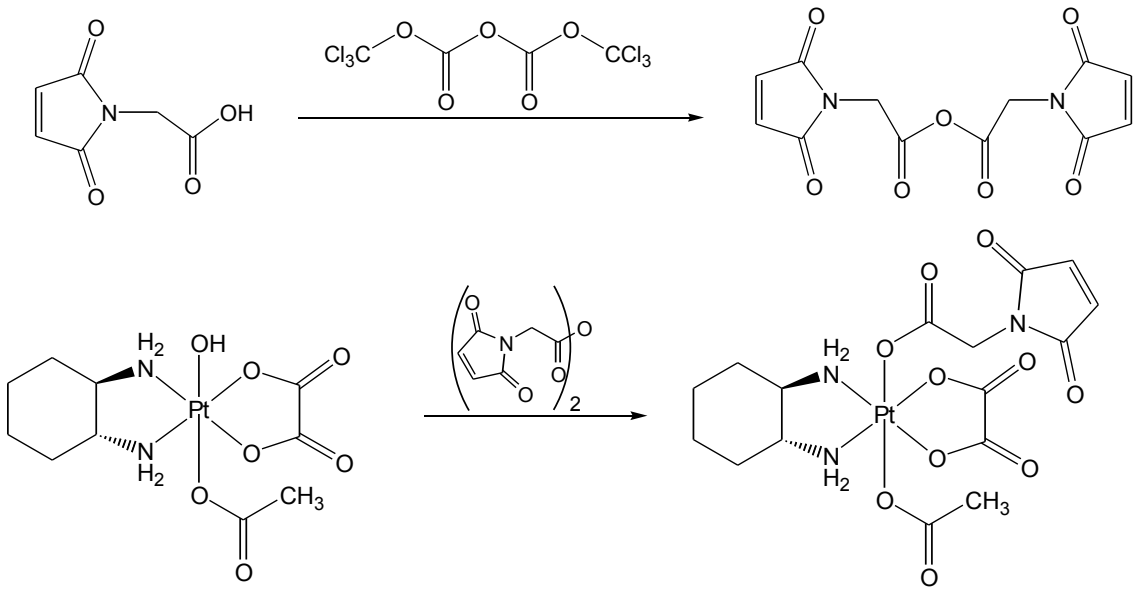


A suspension of maleic anhydride (10 g, 0.102 mol) and glycine (7.732 g, 0.103 mol) in acetic acid (100 mL) was heated at reflux for 1.5 hours, till the white solid was completely dissolved. Acetic acid was removed on a rotary evaporator, the residue was dissolved in 100 mL of water and extracted with ethyl acetate (3 × 100 mL). The extracts were dried over magnesium sulfate, the solvent was removed on a rotary evaporator, the product was recrystallized in ethyl acetate.

Yield: 6.3 g, 40%

^{1}H NMR (DMSO): δ = 13.15 (br, 1H, COO*H*), 7.13 (s, 2H, C=C–*H*), 4.15 (s, 2H, CH_2).

(*OC*-6-44)-Acetato(*R*,*R*-1,2-diaminocyclohexane)[(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl) acetato]oxalatoplatinum (IV)



A solution of maleimidoacetic acid (155 mg, 1 mmol) and 200 µL of triethylamine in 15 mL of absolute THF was cooled in a ice/salt bath. Triphosgene (60 mg, 0.2 mmol) was added as solid. The mixture turned dark-brown and soon a white precipitate formed. After 3 hours the solid was filtered off and washed with tetrahydrofurane. The solution was concentrated on a rotary evaporator, dissolved in chloroform and the Pt complex (118 mg, 0.25 mmol) was added. The heterogeneous mixture was refluxed overnight.

The product was filtered off, washed with chloroform and purified chromatographically (Ethyl acetate : Ethanol, 5:1, R_f=0.35)

Yield: 38 mg (25%)

ESI-MS: 633.0754 (Calculated for M+Na^+: 633.0772)

^{1}H NMR (DMSO): δ = 8.33 (br, 2H, NH_2), 8.28 (br, 1H, NH_2), 7.78 (br, 1H, NH_2), 7.09 (s, 2H, =C−*H*), 4.24 (d, $^2J_{H,H}$ = 17.3 Hz, 1H, CH_2,), 4.19 (d, $^2J_{H,H}$ = 17.3 Hz, 1H, CH_2,) 2.56 (m, 2H, CH, chxn), 2.12 (m, CH_2, chxn), 2.10 (m, CH_2, chxn), 1.96 (s, CH_3, acetato), 1.44 (m, CH_2, chxn), 1.16 (m, CH_2, chxn),

^{13}C NMR (DMSO): δ = 179.1 (*C*(O)CH_3), 173.3 (*C*(O)CH_2 (glycine)), 171.0 (*C*(O)(maleimide)), 164.1, 164.0 (*C*(O), oxalate), 135.3 (*C*H=*C*H (maleimide)), 61.7, 60.8 (*C*H, (DACH)), 39.3 (*C*H_2, (glycine)), 31.4, 31.2, 24.1, 24.0 (*C*H_2, (DACH)), 23.4 (*C*H_3, acetato) ppm;

^{195}Pt NMR (DMSO): δ = 3253.73ppm.

General procedure of the solid-phase peptide synthesis

The resin was weighed in a glass vial and allowed to swell in DMF for 30 minutes under shaking, then it was transferred into a reactor (10 mL syringe with PET filter). The swollen resin was deprotected.

Deprotection procedure:

20% solution of piperidine in DMF was taken up into the syringe and shaken for 15 minutes. The solution is removed and resin is washed 5 times with 4 mL DMF.

The activated amino acid was taken up into the syringe.

Activation procedure:

TBTU ((1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate) was mixed with HOBT (1-hydroxybenzotriazole) (4-fold excess in respect to the resin loading) and dissolved in DMF in a glass vial. This solution was added to a solid amino acid, followed by 10 equivalents DIPEA (diisopropylethylamine). After stirring, the solution turned yellow, clear and homogeneous (reaction time is about 2 minutes).

The coupling time depended on the amino acid. For amino acids with side chain protective groups it was 30 min, for amino acids without side chain protection – 20 min. Arginine(Pbf) required a longer time (1 hour). After the coupling step, the resin was washed with DMF 5 times. After reaction, the Kaiser test was performed to estimate completeness of the coupling. If the test had been positive, the coupling was repeated.

Kaiser test procedure:[5]

Several resin beads were transferred into an eppendorf vial and washed two times with ethanol. Several drops of each solution were added:

KCN in pyridine (2 ml 0.001 M KCN in 98 ml)

Ninhydrin, 6% in ethanol (w/v)

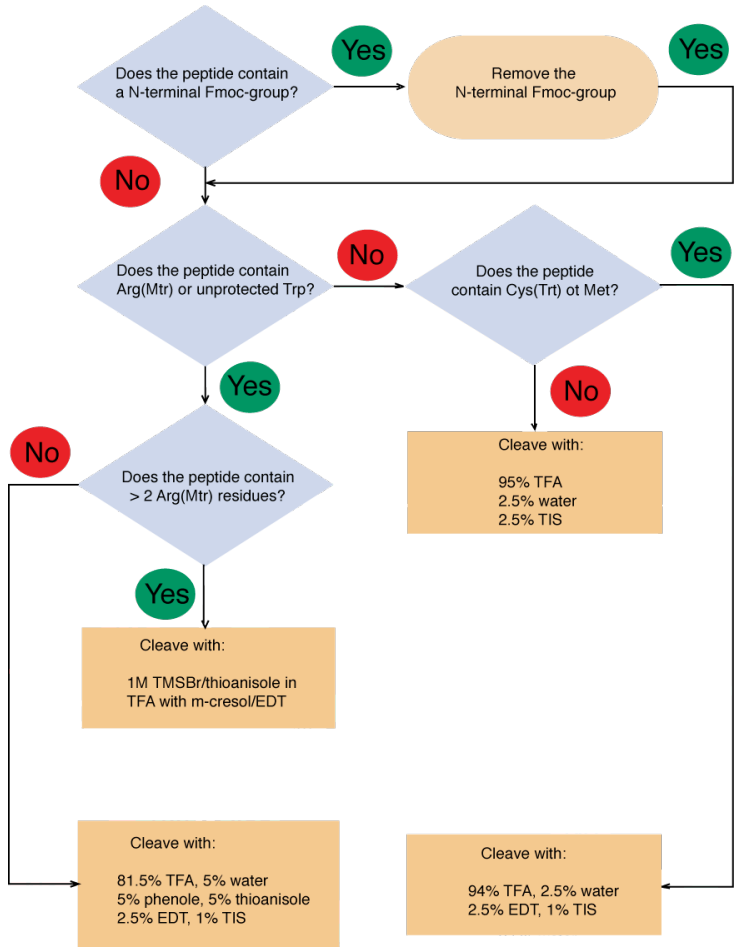
Phenol in ethanol, 80% (w/v)

The resulting solution was carefully mixed and heated at 100-120°C for 4-6 minutes. If the solution or the resin beads are turning blue, free amino groups were present and the coupling had to be repeated.

The sequence of deprotection/coupling steps was repeated subsequently for all amino acids. The last Fmoc-group was removed as usual, the resin was washed 5 times with DMF, transferred into a fresh syringe, washed 5 times with methylene chloride and dried in vacuum.

The peptide was cleaved from the resin with a suitable mixture (Scheme 1). The syringe containing the acid was shaken for 2 hours, afterwards the solution was pushed out of the

syringe into a flask, the resin in reactor was washed once with TFA. The combined TFA solutions were concentrated under vacuum with the help of a membrane pump and with a liquid nitrogen trap. After concentration, diethyl ether was added to precipitate the product.



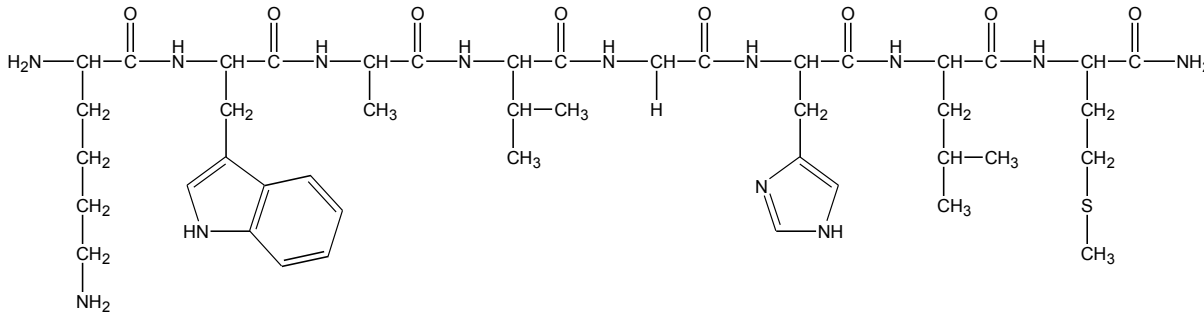
Scheme 1,Taken from ref. 6

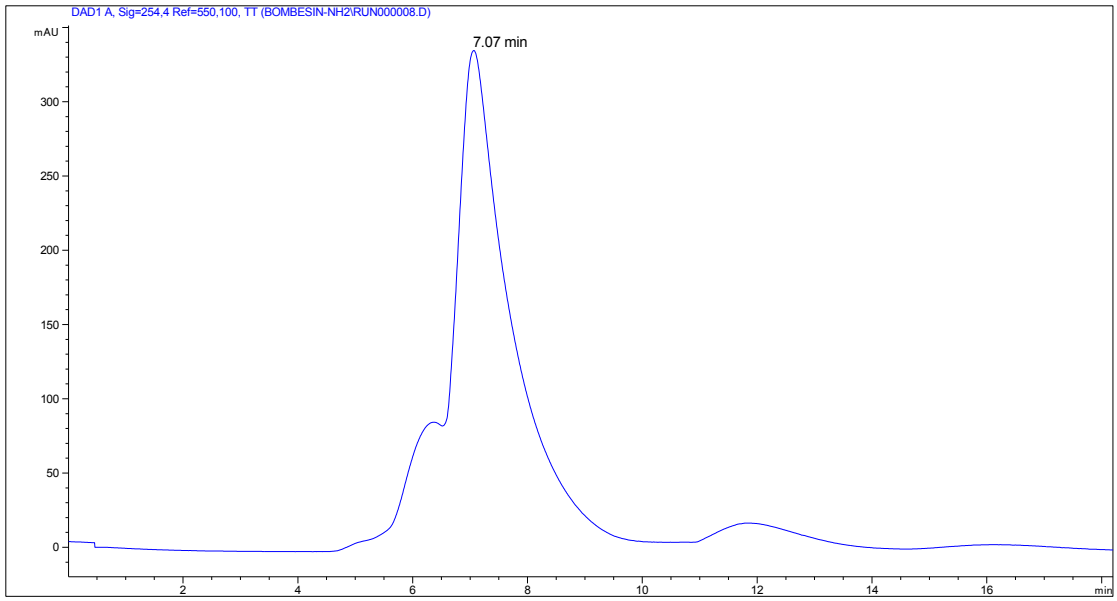
The fraction containing the crude peptide was cooled with liquid nitrogen and centrifuged. Ether was decanted, the solid is suspended in ether, cooled and centrifuged again. The procedure was repeated 2 times.

The solid is dissolved in a suitable solvent (water, methanol, acetonitrile), filtered through a 20 µm syringe filter and purified via RP-HPLC.

Glutaminyltryptophylalanylvalylglycylhistidylleucylmethioninamide

octapeptide (Bombesin)





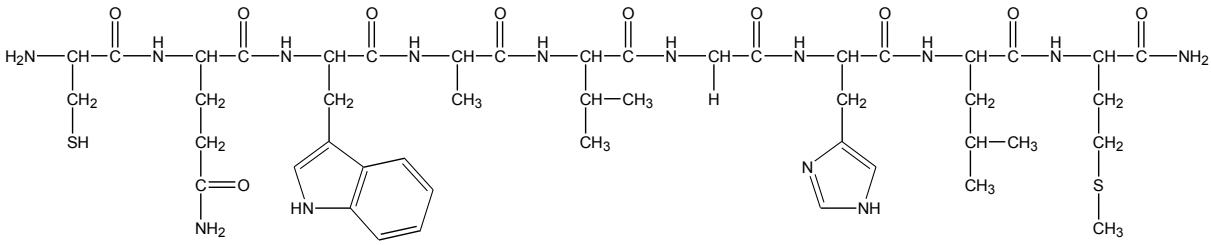
The peptide was synthesized according to the standard procedure in two reactors with 0.4 mmol equivalents of resin in each of them. Cleavage was performed with a TFA/water/EDA (95/2.5/2.5) mixture. The raw peptide was purified on a C-18 RP HPLC Grom Saphir column in an isocratic mode with 50:50 ratio of 0.1% TFA in methanol and water.

Yield: 238 mg (32%).

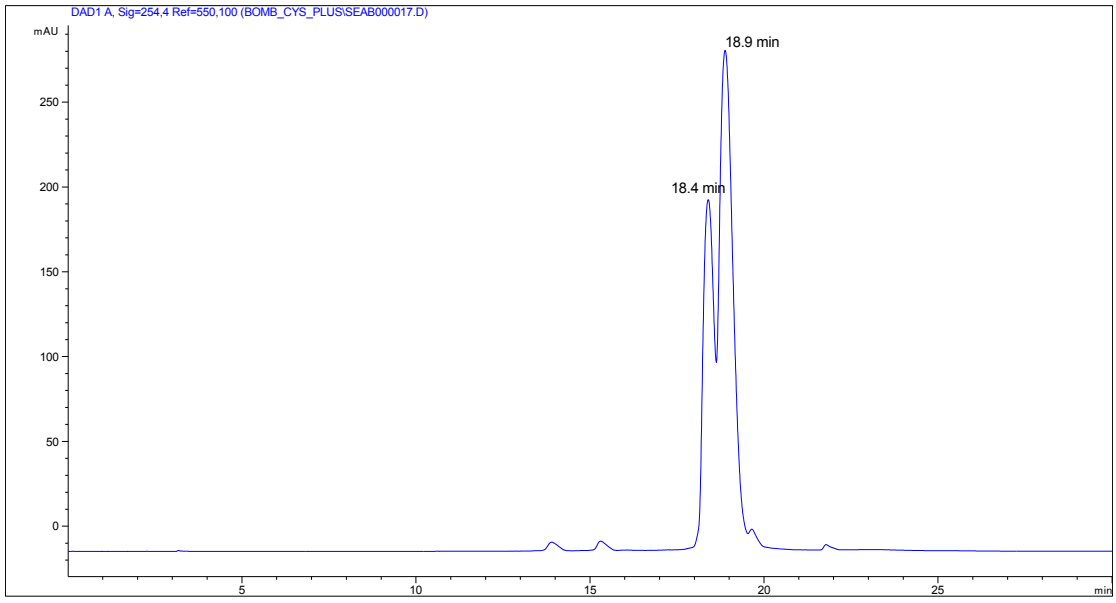
Calculated mass for $C_{43}\ H_{65}\ N_{13}\ O_9\ S$ $+H^+$=941.49

ESI-MS: 941.7, 963.5 ($M+Na^+$)

Cysteinylglutaminyltryptophylalanylvalylglycylhistidylleucylmethioninamide nonapeptde (Cysteinylbombesin)



The peptide was synthesized according to the standard procedure in two reactors with 0.3 mmol equivalents of resin in each of them. Cleavage was performed with a TFA/water/TIS (triisopropylsilane) mixture (95/2.5/2.5), but surprisingly the purified product (isocratic, 50/50, ~10 min) showed a signal in the mass spectrum M+244, indicating the incomplete removal of Trt group.



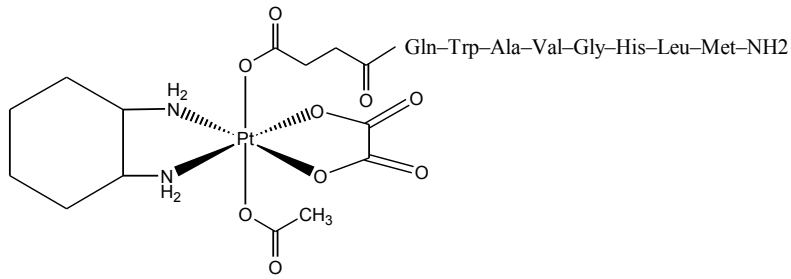
Separation conditions: 0.1% TFA in water and methanol

Time	% Methanol	Rate,mL/min
0.00	5.0	20
20.00	95.0	20
30.00	5.0	20

The solid was dissolved in 10 mL TFA and several drops of TIS were added to the yellow solution. It became colorless immediately, indicating Trt cation scavenging; the precipitation step was repeated once more.

Yield 26 mg, 4%

Coupling of (*OC*-6-44)-Acetato (3-carboxypropanoato) (*R,R*-1,2-diaminocyclohexane) oxalatoplatinum(IV) with bombesin



Bombesin (154 mg, 0,2 mmol) and 230 mg of the platinum precursor (230 mg, 0.4 mmol) were mixed in 5 mL of DMF, then EDC (81 mg, 0.42 mmol) and 100 µL DIPEA were added. The mixture was stirred for 24 hours, DMF was removed in vacuum and the mixture was dissolved in a water:methanol mixture. The solution was filtered with a membrane filter (20 µm pore) and the filtrate was purified with HPLC. 0,1% TFA in water and methanol were used as mobile phase

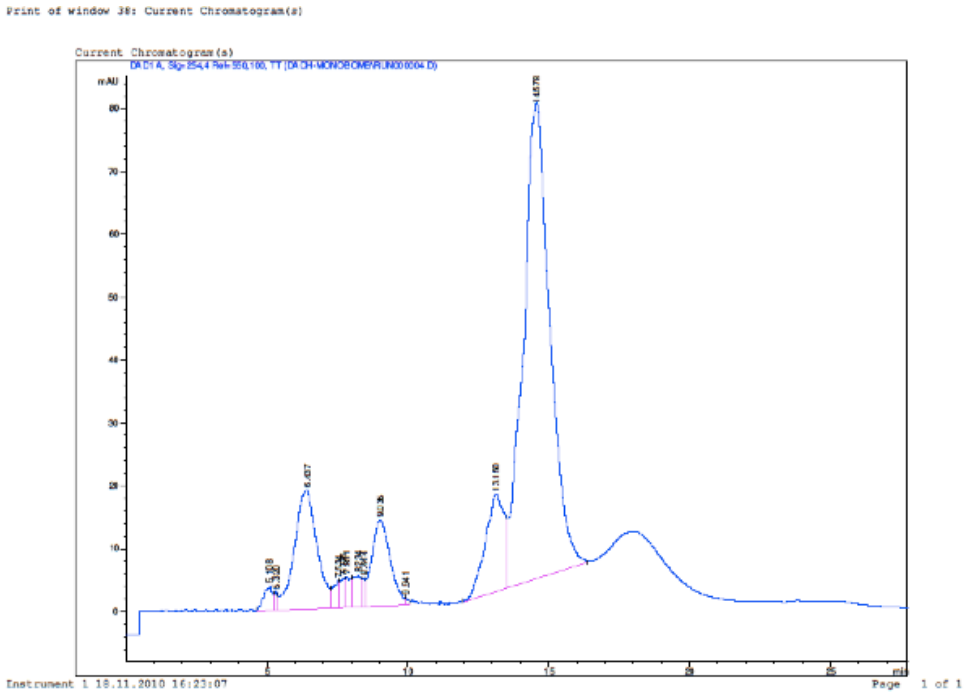
Time	% Methanol	Rate,mL/min
0.00	5.0	20
20.00	95.0	20
30.00	5.0	20

The product was eluted at 14.6 min

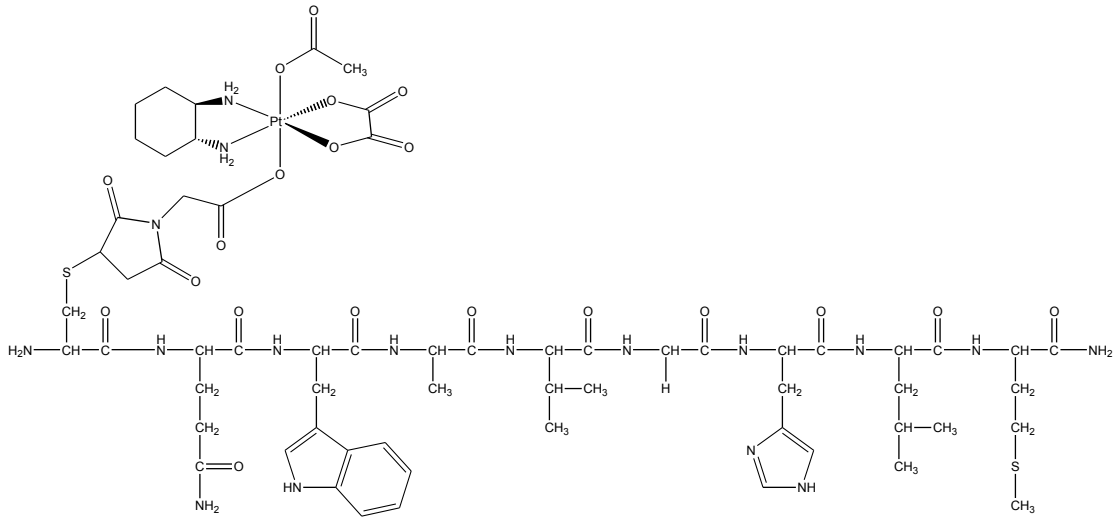
Calculated mass of product (C57 H85 N15 O18 S1 Pt1) + H^+ = 1495.56:

ESI-MS: 1494.7 and 1040.6 (Peptide+succinate residue, M=1040.49)

Yield: 13 mg (0.87%)



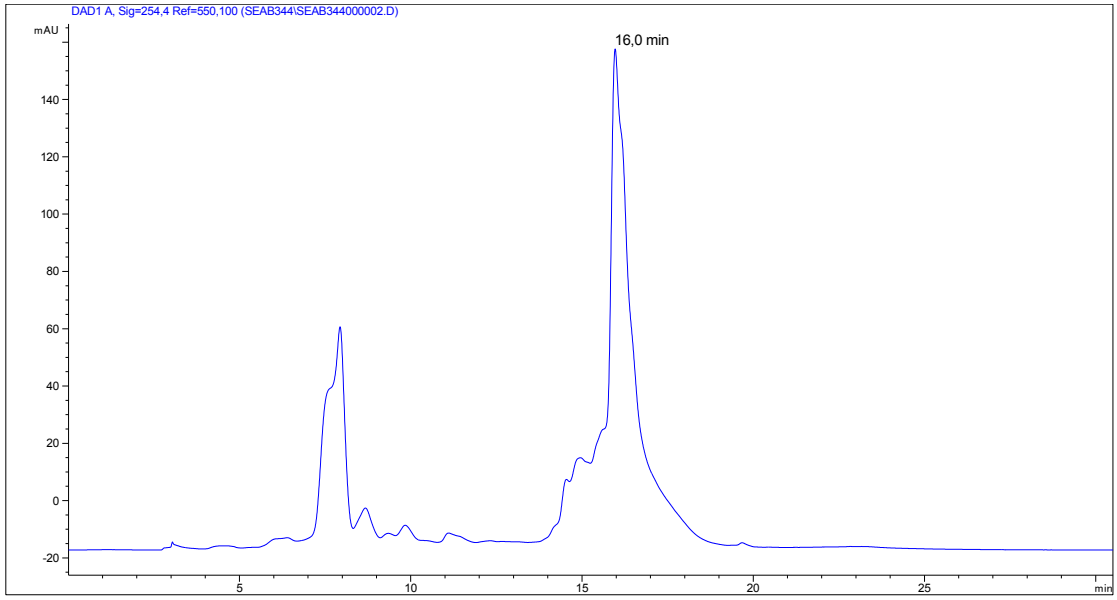
Coupling of (*OC*-6-44)-acetato(*R*,*R*-1,2-diaminocyclohexane)[(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)acetato]oxalatoplatinum (IV) with cysteinylbombesin



(*OC*-6-44)-acetato(*R*,*R*-1,2-diaminocyclohexane)[(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)acetato]oxalatoplatinum (23 mg, 0.038 mmol) was dissolved in 2 mL of methanol and added to a solution of bombesin (26 mg, 0.025 mmol) in 1 mL of methanol. Potassium carbonate on tip of the spatula was dissolved in 200 µL of a 1:1 water methanol mixture and added. The solution was stirred 2 days at room temperature under argon. A white precipitate was formed. The mixture was dissolved in 0.1% TFA, the product was purified via RP-HPLC on a C-18 column, eluted at 16 min.

Time	% Methanol	Rate,mL/min
0.00	5.0	20
20.00	95.0	20
30.00	5.0	20

Yield: 5.1 mg (12%)



ESI-MS (Low resolution):

1654.0 (Calculated for $[C_{62}\ H_{91}\ N_{17}\ O_{20}\ Pt\ S_2 + H]^+$=1653.5794)

Axial ligand: 1198.2 (Calculated for $[C_{52}\ H_{76}\ N_{15}\ O_{14}\ S_2 + H]^+$=1198.5)

High resolution MS: 1653.5749

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III. Conclusions

Different types of novel oxaliplatin derivatives are described in the present dissertation.

The first project focused on modification of the non-leaving cyclohexanediamine ligand. Two synthetic pathways were developed for selective synthesis of different isomers of the analogs. *SP*-4-3{(1*R*,2*R*,4*R*)-4-Methyl-1,2-cyclohexanediamine}oxalatoplatinum(II), the isomer with equatorial orientation of the methyl substituent could be prepared from the corresponding dichlorido complex via activation with silver nitrate. Racemic 4-methylcyclohexene was used as precursor for synthesis of the non-leaving methylcyclohexanediamine ligand. The alkene was converted into the *trans*-diol; on the next step, OH- groups were mesylated and substituted with azide. Reduction with hydrogen/palladium afforded the corresponding diamine, which could be separated into *S*,*S*- and *R*,*R*- isomers by recrystallization of the L-tartaric acid salt. The analog featuring an axial methyl group, *SP*-4-3{(1*R*,2*R*,4*S*)-4-methyl-1,2-cyclohexanediamine} oxalatoplatinum(II), required a more complex synthetic pathway. The enatiomerically pure starting compound (*S*)-4-methylcyclohexene was obtained from natural (*S*)-citronellal via Wittig reaction and ring-closure metathesis. The catalyst for metathesis was synthesized *in situ* from tungsten oxychloride and tetraethyl lead. (*S*)-4-Methylcyclohexene was converted into the diazide in one step using manganese triacetate and sodium azide, futher transformations were the same.

The enantiomeric composition of the isomer mixtures was determined using a chiral HPLC with prior CBZ-derivatization of the diamines. Biological activity of both isomers was evaluated *in vitro* and *in vivo*. The isomer with an axial methyl group showed a better effect then its isomer exhibiting an equatorial methyl group and oxaliplatin itself in cytotoxicity test on all cell lines, but investigation of the activity in a leukemia mouse model brought opposite results. Application of the analog with an axial methyl group resulted in an increased anticancer activity and significantly lower toxicity compared to oxaliplatin. 5 out of 6 mice survived for a long time under application of the maximum tolerated dose (which was also higher then the one of oxaliplatin) what makes the compound worthy for further investigations.

In the second part, Pt(IV) analogs of oxaliplatin were obtained and conjugated to the TAT peptide via amide bond formation. According to the structure of the starting platinum

complex, two products could be expected, 1:1 and 1:2 in ratio of platinum:peptide. The isolated complexes were characterized with NMR, mass spectrometry and analytical HPLC. Both substances were tested active in cytotoxicity experiments in human cell lines CH1, SK-OV-3, (ovarian carcinoma), SW480 (colon carcinoma) and A549 (nonsmall-cell lung cancer). In all these experiments, the conjugates showed higher activity compared to the precursor acid and an analog without appended peptide. This proved the supposition, that peptides can work as delivery vectors for platinum complexes, facilitating their cellular transport.

The third project focused on optimization of the conjugation strategy. Oxaliplatin was oxidized in acetic acid to keep only one hydroxo group for further reactions. The asymmetric complex was acylated with a symmetric anhydride, containing a maleimide fragment. Reaction of maleimide and a cysteine-derived peptide afforded the desired complexes under mild conditions and better yields.

For that purpose, the octapeptide bombesin was chosen as tumor-homing vector, its receptors are expressed in many cancer cells. One conjugate was prepared using standard peptide coupling of the free amino group of peptide and carboxylate on the platinum precursor, another one was prepared using the maleimide-cysteine reaction. The conjugates were characterized with NMR, ESI-MS and analytical HPLC.

IV. Curriculum Vitae

Personal Data:	
Name:	Sergey Abramkin
Date of birth:	05. 08. 1983
Nationality:	Russia
Current occupation:	Scientific staff member, University Vienna
Address:	Höfergasse 1A/1/17 A-1090 Wien, Austria
Telephone:	0043 676 745 02 03
email:	sergey.abramkin@univie.ac.at
Main research fields and interests:	Platinum antitumor complexes, coordination chemistry, medicinal chemistry, bioinorganic chemistry, peptide conjugates
Education:	
2012	**PhD, Chemistry** - Specialization in Bioinorganic Chemistry; PhD topic: Synthesis and Chracterization of Novel Oxaliplatin Analogs, University of Vienna, Austria; Supervised by Prof. B. K. Keppler.
2006	**Master of Science**, Chemistry - Specialization in Organic and Analytical Chemistry; Master thesis: "Synthesis of alkynil-substituted benzonitriles and their cobalt complexes for study of 1,3-dipolar cycladdition reactions", University of Vienna, Austria and University of Saint-Petersburg, Russia; Supervised by Prof. V. Yu. Kukushkin.

2004	**Bachelor of Science**, Chemistry, Bachelor thesis:"Synthesis and analysis of reactions of heterocyclization of *ortho*-tetradcadiynylbenzoic acid and its derivatives: hydrazide and hydroxamic acid" Supervised by Prof. I. A. Balova
2000	A-Levels (Matura)
1989 – 2000	Elementary- and highschool, Russia

International experience

2008	Ruhr-Universität Bochum, Germany: Three months studies and research in the field of peptide and conjugate chemistry; Supervised by Prof. N. Metzler-Nolte

Publications in international journals

Jungwirth, U., Xanthos, D. N.; Gojo, J.; Bytzek, A. K., Körner, W.; Heffeter, P., Abramkin, S. A.; Jakupec, M. A., Hartinger, C. G., Windberger, U.; Galanski, M.; Keppler, B. K. and Berger, W. Anticancer activity of methyl-substituted oxaliplatin analogs. Mol. Pharmacol. **2012**, 81, 719

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2009 Abramkin, S.; Galanski, M. and Keppler, B. K. Synthesis and Ccharacterization of peptides for subsequent Pt derivatization. COST D39 Working Group Meeting Debrezen, Hungary. Oral communication

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