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DISSERTATION

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KURZZUSAMMENFASSUNG

Krebs ist eine der häufigsten Todesursachen weltweit und die Behandlung ist weiterhin eine Herausforderung für die heutige Medizin. Neben den häufig angewandten organischen Medikamenten, werden auch der Metallkomplex Cisplatin und seine Derivate in der Behandlung eingesetzt. Nichtsdestotrotz kann deren Anwendung zu schweren Nebenwirkungen oder Zytostatika-Resistenzen führen. Insofern müssen Alternativen gefunden werden, die weniger Nebenwirkungen aufweisen oder die auftretenden Resistenzen überwinden können. Gold Verbindungen sind eine sehr vielversprechende Alternative da sie einen anderen Wirkmechanismus aufweisen. Im Gegensatz zu Cisplatin, werden Schwefel- oder Selenhaltige Enzyme gehemmt. Deshalb wurde für viele Gold Komplexe die Thioredoxinreduktase als potenzielles Ziel identifiziert, die in einigen Krebszellen überexprimiert ist.

In dieser Arbeit wurden Gold(I) *bis*-*N*-heterozyklische Carben Komplexe als potenzielle antikrebs Verbindungen synthetisiert. Durch die Verwendung von diesen Komplexen lassen sich gewisse Resistenzen überwinden und durch zwei NHC Liganden wird eine ausreichende Stabilität unter physiologischen Bedingungen gewährleistet. Außerdem haben frühere Studien gezeigt, dass sich kationische Au(I) *bis*-NHC Komplexe bevorzugt im Mitochondrium von Krebszellen anreichern können, was zu einer gewissen Selektivität führen kann. Zusätzlich sollen die NHCs mit funktionellen Gruppen versehen werden um eine Kopplung von Biomolekülen, die zielgerichtet Krebszellen adressieren (sog. „targeted therapy“), zu ermöglichen.

In einer vorherigen Arbeit wurde ein Isomerengemisch von dinuklearen Au(I) hydroxy-brückenfunktionalisierten *bis*-NHC Komplexen synthetisiert.[1] Im Laufe dieser Arbeit wurde das Isomerengemisch getrennt und die unterschiedliche Reaktivität gegen Cystein untersucht. Das *anti*-Isomer zeigt eine erhöhte Reaktivität was sich auch in einer erhöhten Affinität gegen TrxR widerspiegelt. Interessanterweise zeigen die Isomere keine nennenswerten Unterschiede in der antiproliferativen Aktivität gegen eine Vielzahl von Krebszellen. Der Komplex mit Mesityl *N*-Substituenten zeigte antiproliferative Aktivitäten gegen eine Vielzahl von Krebszelllinien mit IC_{50} Werten im niedrigen mikromolaren Bereich ($IC_{50} < 10$ µM) und eine nennenswert niedrigere Aktivität gegen die gesunde Zelllinie V79. Die höchste Selektivität wurde für MCF7 erreicht, mit einem Selektivitätsindex von 19. Weiterführende Verteilungsstudien mittels ICP-MS und Kernmikroskopie zeigten eine Anreicherung von Gold in der mitochondrialen Membran. Die nennenswerte antiproliferative Aktivität, die hohe Stabilität und besonders die hohe Selektivität würden weitere Studien, wie zum Beispiel *in vivo* Tests, sehr vielversprechend machen. Um weitere Lokalisationsstudien zu ermöglichen, wurde an die Hydroxy Gruppe des Komplexes mit *iso*-Propyl *N*-Substituenten ein Fluoreszenz Label mittels

Veresterungsreaktion angebracht. Durch eine Postmodifikationsroute war es möglich nur eine Anthracengruppe zu konjugieren, was zu einer deutlichen Erhöhung der antiproliferativen Aktivität führt und zu einer gewissen Selektivität gegen Krebszellen. Durch die Quantenausbeute von 18% sollte es möglich sein weiterführende „Live Cell Imaging“ Experimente mittels Fluoreszenzmikroskopie durchzuführen.

In einem weiteren Teil dieser Arbeit wurden Au(I) *bis*-NHC Komplexe auf Basis von Histidin synthetisiert und auf ihre Wirkung gegen Krebszellen näher untersucht. Die Verwendung von Histidin als Carben Vorläufer, stellt eine einfache und elegante Möglichkeit dar, funktionelle Gruppen im Rückgrat des Carbens einzubringen, die anschließend für weitere Modifikationen verwendet werden können. Eine Modifikation an dieser Stelle, sollte weder die Stabilität der Gold-Carben Bindung beeinträchtigen noch die sterische Abschirmung des Goldkerns verändern. Die Verwendung von Benzyl- *N*-Substituenten und eines -Esters oder -Amids statt einer freien Carbonsäure, resultierte in guten antiproliferativen Aktivitäten mit IC_{50} Werten im niedrigen mikromolaren Bereich (IC_{50} < 10 µM) gegen eine Vielzahl von Krebszelllinien. Die verbleibende Aminogruppe wurde nicht verändert, um eine ausreichende Wasserlöslichkeit zu gewährleisten. Außerdem konnte eine hohe Stabilität gegen Glutathion beobachtet werden, was darauf schließen lässt, dass Deaktivierungsmechanismen im Blut, bei weiterführenden *in vivo* Versuchen, verhindert werden könnten.

ABSTRACT

Cancer is one of the most leading causes of death worldwide and still a challenging task to treat. Among the frequently applied organic chemotherapeutic drugs, the metal complex cisplatin and its derivatives are still in clinical use today. Despite of that, the DNA targeting agent causes severe side effects or cancer might become resistant against it. Thus, research is ongoing to find alternatives to overcome drug resistances or to develop compounds with less side effects. Gold compounds emerged as a promising alternative due to its different mechanism of action. In contrast to cisplatin, usually sulphur or selenium containing enzymes are preferably targeted. Thioredoxin reductase, which is overexpressed in some cancer cells, was identified as main target for many gold complexes.

This thesis focuses on the synthesis and characterization of novel Au(I) *bis*-NHC complexes as potential anticancer candidates. The application of Au(I) might help to overcome drug resistances and due to the utilization of two NHC ligands a sufficient stability is ensured. Moreover, cationic Au(I) *bis*-NHC complexes have shown to accumulate in the mitochondria of cancer cells, which might lead to a certain selectivity. In addition, NHC ligands bearing functional groups are applied, which would enable bioconjugation to address specifically cancer cells (so called “targeted therapy”).

In a previous work an isomer mixture of dinuclear Au(I) hydroxyl bridge functionalized *bis*-NHC complexes with different *N*-substituents were synthesized.[1] Consequently in this thesis, the isomers were separated and their different reactivity against cysteine was investigated, showing an increased reactivity for the *anti*-isomer, which also leads to a higher affinity against TrxR. Interestingly no notable difference of the isomers in the overall antiproliferative activity was observed. The complex with mesityl *N*-substituents shows IC_{50} values in the low micromolar range (IC_{50} < 10 µM) against various cancer cell lines and most important, a notable lower activity against the healthy cell line V79. The highest selectivity was reached for the MCF7 cell line with a selectivity index of 19. Further distribution studies, including uptake studies *via* ICP-MS and nuclear microscopy, revealed an accumulation of the complex in the mitochondrial membrane. Due to the notable antiproliferative activity, the high selectivity and stability, these complexes are promising candidates for further studies, e.g. *in vivo* testing. To enable further localization experiments, the hydroxyl group of the complex with *iso*-propyl *N*-substituents was conjugated to a fluorescence label *via* esterification, which would allow for cell imaging *via* fluorescence microscopy. Due to a postmodification of the gold complex only one anthracene molecule can be conjugated, which leads to a significant improvement in the antiproliferative activity and to a notable selectivity against cancer cells. Moreover, a quantum yield of 18% should be enough for further cell imaging studies.

In another part of this thesis, a series of histidine derived Au(I) *bis*-NHC complexes was synthesized and tested against various cancer cell lines. Utilization of histidine as NHC precursor, is an efficient and elegant way towards NHCs with functional groups in the backbone, which can be then used for further modifications. Bioconjugation in the backbone of the NHC should neither affect the stability of the gold-carbene bond, nor the sterical shielding of the gold nucleus. The application of benzyl- *N*-substituents and an -ester or -amide instead of the free carboxylic acid in the backbone, leads to notable antiproliferative activities with IC_{50} values in the low micromolar range in various cancer cell lines. The amino group was not further converted, to ensure sufficient water solubility of the complexes. Furthermore, a high stability against glutathione is observed, which should prevent deactivation mechanism in the blood, if further *in vivo* experiments are conducted.

LIST OF ABBREVIATIONS

ASK 1	apoptosis signal-regulating kinase
Bn	benzyl
Dppe	2-b*is* (diphenylphosphino)-ethane
CLL	chronic lymphocytic leukemia
CML	chronic myelogenous leukemia
Cp	cyclopentadienyl
CuAAC	copper catalyzed azide-alkyne cycloaddition; “click-reaction”
Dipp	2,6-diisopropylphenyl
DLC	delocalized lipophilic cation
DNA	deoxyribonucleic acid
Et al.	and others (lat. et alii)
E. coli	*Escherichia coli*
ESI-MS	electrospray ionization mass spectrometry
FDA	food and drug administration
GpG	1,2 guanosine crosslinks
GR	glutathione reductase
GSH	glutathione
HSA	human serum albumin
IC_{50}	half maximal inhibitory concentration
ICP-MS	inductively coupled plasma-mass spectrometry
^{i}Pr	isopropyl
LDA	lithium diisopropylamide
Log P	logarithm of the n-octanol–water partition coefficients
Me	methyl
MMC	4-methylene-7-methoxy-coumarin
MMP	mitochondrial membrane permeabilization
MOF	metal organic frameworks
n-BuLi	*n*-butyllithium
NER	nucleotide excision repair
NHC	*N*-heterocyclic carbene
NADPH	nicotinamide adenine dinucleotide phosphate reduced form
$NADP^{+}$	oxidized form
NMR	nuclear magnetic resonance
PET	positron emission tomography
PLL	prolymphocytic lymphoma
Prx	peroxiredoxin
PSMA	prostate-specific membrane antigen
PIXE	proton induced X-ray emission
ROS	reactive oxygen species
RP-HPLC	reversed phase high performance liquid chromatography
S. aureus	*Staphylococcus aureus*
SI	selectivity index
SC-XRD	single crystal x-ray diffraction
SLL	small lymphocytic lymphoma
Trx	thioredoxin
TrxR	thioredoxin reductase
Mes	2,4,6-trimethyl phenyl
WHO	world health organization

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1 INTRODUCTION

1.1 Cancer – Definition and Statistics

Cancer is currently one of the most common causes of death worldwide. In the United States cancer is the second leading cause of death and worldwide in the top 10 according to the world health organization (WHO).[2, 3] Moreover, the incidence of cancer worldwide has increased from 12.7 million in 2012 to 14.1 million in 2018 the trend is still rising.[4] However, it is not completely accurate to consider cancer as one single disease. Cancer is defined as an uncontrollable cell growth leading to malignant tumors and can be found in nearly any tissue, e.g. lung, breast or prostate.[5] The formation of tumor cells, called tumorigenesis, involves several steps and is still not fully understood.[6] The most common cancer types are lung, breast-, colorectum-, prostate- and stomach-cancer. Among men, prostate cancer is the second leading cancer type, among females breast cancer has the highest incidence.[4] The mortality and incidence rate are not necessarily correlating, strongly depending on the type and stage of cancer. While breast cancer has the highest incidence rate for females, the mortality is quite low, which is also obtained for prostate cancer (Figure 1).[3] Despite that, the mortality for lung cancer is quite high.[7]

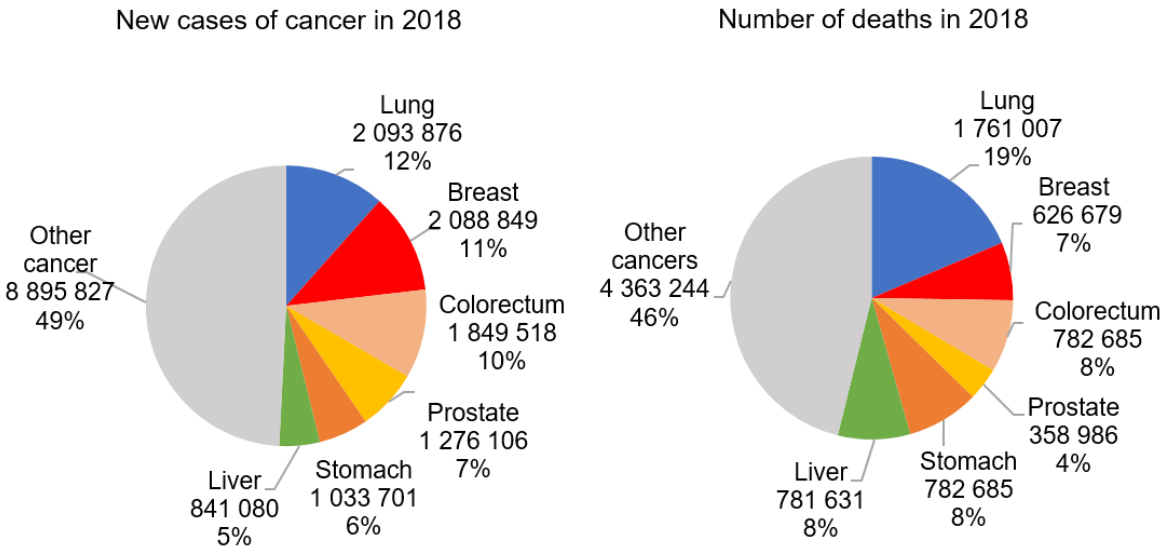


Figure 1: Number of cancer infections and cancer deaths in 2018.[8]

Due to its high mortality and incidence, a great effort is put into developing various cancer therapies. The most common types of anticancer therapy are surgery[9], radiotherapy[10], chemotherapy[11] and targeted therapy[12] amongst more specific therapies like immune-[13] or hormonal-therapy[14]. The chances of cure strongly depend on the stage of cancer and its type. In general, the earlier the cancer is detected, the higher the chances of an effective cure. When cancer is detected early enough, the tumors can often be completely removed by surgery. In

later stages surgery can be applied to remove parts of the cancer tissues (e.g. metastasis). This is often the instance for prostate and breast cancer, where the tumors can be removed completely, which also explains the high survival rates.

1.2 Cancer treatment

Surgery is the oldest oncological therapy. First surgery methods were described in the ancient age. However, survival rates were quite low then since there was no anesthesia or asepsis.[9] Moreover, without sufficient diagnostic methods surgery was difficult and only applicable for a few types of cancer (e.g. breast cancer). Of great importance was the discovery of X-rays by Wilhelm Conrad Röntgen, who was awarded with the Noble Price in 1901.[15] This discovery enabled locating the tumors. Caused by this discovery, surgery became widely applicable in most of the major cancer types. Nowadays different methods are applied to remove the tumors, for example by temperature, lasers or light.[16-18] However, if the cancer forms metastases, which spread over the whole body, removing the whole cancer tissue *via* surgery is difficult or has to be combined with other therapies, to ensure complete removal of the malignant tissue.[9]

Another approved clinically application is radiation therapy. Here the cancer cells are destroyed or at least the cancer is shrank by high-energy radiation, before other treatments (e.g. surgery) are applied. Upon radiation, the deoxyribonucleic acid (DNA) is damaged, preventing cell division and therefore usually leading to cell death *via* apoptosis or mitotic catastrophe.[10, 19] Since cell proliferation is influenced by radiation, cancer cells are more affected due to faster growth rates.[20] Nevertheless, healthy tissues, which are adjacent to the cancer cells or in the pathway of irradiation, can be also damaged. Indeed, normal cells exhibit faster DNA repair rates, leading to higher stability against radiation.[21] Therefore, the efficiency of radiation therapy strongly depends on location of the tumor and on the selectivity of radiation.[22] In the early stages a few types of cancer can be cured with radiation therapy alone, for example skin cancers, lymphomas, cervix-, lung-, prostate-, head- and neck- carcinomas. In later stages or different types of cancer radiation therapy is often combined with other treatments.[10]

Primary tumors might possess in later stages the ability to spread in other parts of the body and form secondary tumors, so-called metastasis.[23] At this stage chemotherapy is frequently used since the treatment has to be systemic to target tumor cells.[23] Chemotherapy is defined as the treatment of diseases with chemical drugs. It was invented by Paul Ehrlich, who synthesized a drug called Salvarsan to treat syphilis.[24] Most of the chemotherapeutic drugs interact in different ways with the DNA. They can act as DNA-modifying agents, anti-metabolites, spindle poisons, or topoisomerase inhibitors.[23] After DNA modification (e.g. *via* alkylation or DNA adduct formation) the cell division is inhibited due to DNA defects. This type

of drugs are not specific to one cell cycle phase, since they are only damaging the DNA in general and not depending on DNA synthesis.[25]

Anti-metabolites are molecules that are very similar to compounds occurring in the body, therefore they can easily take part in the metabolism and interfere with it. Many anti-metabolites mimic the role of pyrimidine bases, are consequently incorporated into DNA and thus inhibiting the DNA synthesis. A prominent example is 5-fluorouracil, containing a fluorine atom instead of a methyl group to imitate the nucleobase thymine (Figure 2).[26]

Spindle poisons prevent the assembly of the microtubule, which are essential for cell division in the M-phase. A clinically applied drug is paclitaxel[27] representing another class of chemotherapeutics that inhibits the topoisomerase, which is responsible for DNA transcription and replication.[23] Figure 2 depicts a few examples for each type of chemotherapeutic drug, mentioned before.

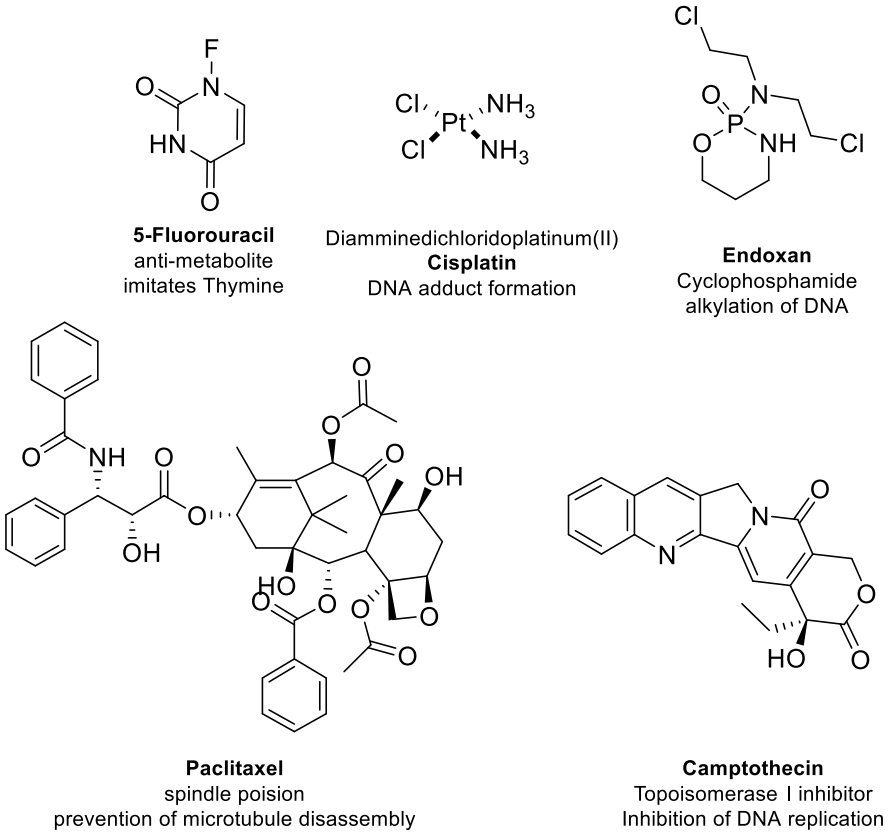


Figure 2: Examples of several applied chemotherapeutic drugs.

Since most common chemotherapeutics influence the cell cycle, rapidly growing cells are more affected. Due to the increased proliferation of cancer cells, a certain selectivity is obtained. However, also rapidly growing healthy cell lines are targeted, causing several side effects, e.g. myelosuppression, alopecia and nausea amongst others. Moreover, drug resistance is becoming an increasing problem.[28] A huge effort is made in academic and clinical studies to design more selective drugs.[23]

One current approach is the so-called targeted therapy. Here the drug interacts with certain enzymes, genes or peptides in general, which are overexpressed or mutated only in malignant cells.[12, 23] By designing drugs that can interact selectively with these molecules the cancer can be targeted without attacking healthy cells. The targeted therapy agents can be divided into three different cases. One common approach is the use of small molecules that can overcome the cell membrane and interact with the target inside the cell, for example by inhibiting kinases. Currently, many small molecule kinase inhibitors are clinically approved, for example imatinib (Gleevec®) (Figure 3). Imatinib inhibits the tyrosine kinase which is overexpressed in chronic myelogenous leukemia (CML) caused by the BCR-ABL gene.[29]

Monoclonal antibodies are also applied for targeted therapy. They cannot cross the cell membrane but interact with certain receptors on the cell surface to block signaling pathways or trigger an immune response. Trastuzumab (Herceptin®) was one of the first successful applied antibodies for targeted therapy. It is used against breast cancer, which overexpresses the HER-2 gene, by inhibiting cancer growth.[30]

Moreover, monoclonal antibodies can be coupled to toxic or radioactive agents to selectively navigate them to cancer cells. In targeted radionuclide therapy ^{177}Lu complexes are directed to prostate cancer *via* an inhibitor of the prostate specific membrane antigen (PSMA) (Figure 3). For diagnostic applications combinations with ^{68}Ga and certain antibodies are used.[31] Research is ongoing to identify new targets for targeted therapy and also to find suitable inhibitors for those. For example, only around 30% of breast cancers are HER-2 positive and can be targeted by antibodies like Trastuzumab, for other cancers new targets must be identified.

Here, a constant challenge remains as the rise of new resistances against certain inhibitors is ongoing.[32]

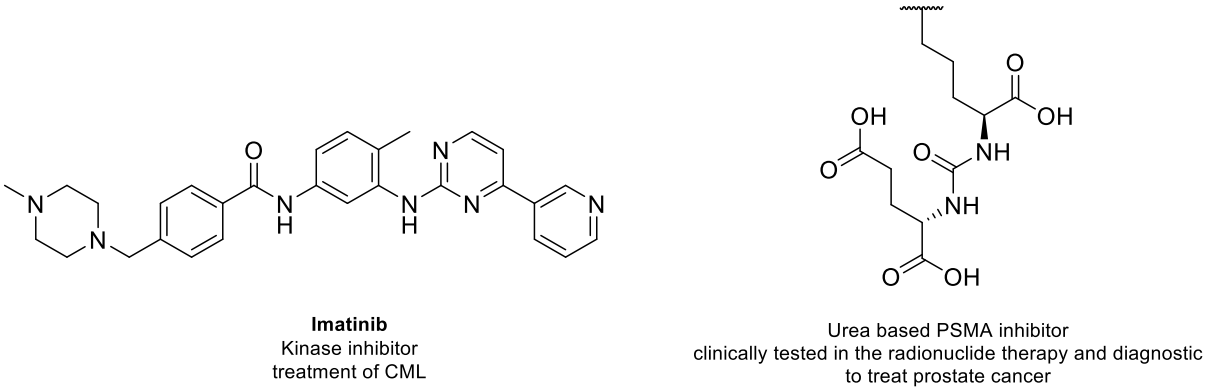


Figure 3: Examples of currently applied motifs or drugs for targeted therapy.

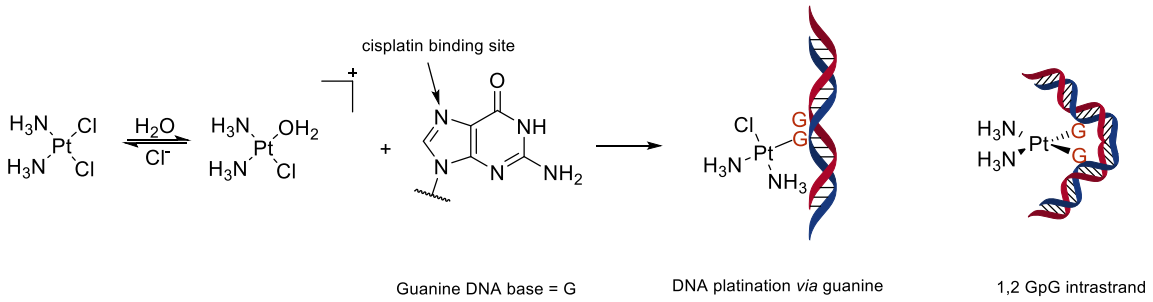
1.3 Platinum based Complexes as Anticancer Drugs

Although there are numerous publications about transition metal complexes as antitumor agents the examples of metal complexes that are clinically approved or in clinical trials are, indeed, very scarce. The most prominent compound is cisplatin. The antiproliferative activity against *Escherichia coli* (*E. coli*) was accidentally discovered in 1965 by Rosenberg *et al*..[33] They investigated the influence of an electromagnetic field on the proliferative activity of *E. coli.,* using platinum electrodes, considering them as inert. Further studies revealed that the electrodes reacted and the resulting platin complex inhibits cell growth. The complex was identified as *cis*-diamminedichloridoplatinum(II) and named cisplatin.[34] These results prompted Rosenberg to investigate its antiproliferative activity in general. The complex shows antiproliferative activity against sarcoma 180 and leukemia L1210 in mice,[35] which led to the clinical approval by the food and drug administration (FDA) in 1978.[36]

The mechanism of action of cisplatin is well studied today and involves four different steps. First, the uptake of cisplatin into the cell. In earlier times it was discussed if it passes the cell membrane only *via* passive diffusion or *via* an active mechanism.[37] Nowadays it is known that membrane proteins and especially copper transporters (e.g. CTR1) or organic cationic transporters (OCT) 1-3 mediate the uptake.[36, 38-40]

After the cellular uptake cisplatin hydrolyzes to the mono-aqua-complex $[Pt(NH_3)_2(H_2O)Cl]^+$ and the respective di-aqua complex $[Pt(NH_3)_2(H_2O)_2]^{2+}$, which can then reach the nucleus (Scheme 1). Hydrolysis is suppressed in the blood due to the high chloride concentration (104 mM), where inside the cell the chloride concentration is significantly lower (4 mM – 10 mM) leading to hydrolysis.

After the hydrolysis and uptake into the nucleus, the highly electrophilic complex interacts with the DNA strand. Since the N7 position of guanine and adenine shows the highest basicity, these DNA bases are preferably platinated.[36] Consequently, another DNA base can replace the remaining leaving group (chloride or H_2O), leading to an irreversible crosslink between the DNA strand. Crosslinks primarily appear within the same DNA strand, causing 1,2 intrastrands, but also 1,3 intrastrands or interstrands can occur (Scheme 1). However, it was shown that the development of 1,2 guanosine crosslinks (GpG) is the most common.[36, 41] After platination and crosslinking of the DNA, the strands exhibit a bent structure.



Scheme 1: Mode of action of DNA inhibition with cisplatin.

After recognition of damaged DNA, the cells halt the progression of the cell cycle and try to repair the damage, to prevent the replication of damaged DNA, which otherwise might lead to mutations. Cisplatin-DNA adducts can be removed by nucleotide excision repair (NER) machinery, to prevent apoptosis of the cell. An enhanced NER activity can lead to cisplatin resistance of the cancer cells.[42, 43] However, cisplatin can interact with proteins shielding the DNA lesion and preventing DNA repair mechanisms. One example of those proteins are high mobility box proteins.[36] If the DNA lesion is not repaired, usually the cell is destroyed by a programmed cell death to prevent the multiplication of damaged DNA, the so-called apoptosis. In the case of cisplatin the cell cycle stops at the G2/M phase and if the DNA damage cannot be repaired the transcription *via* RNA polymerases is blocked, triggering apoptotic mechanisms.[36, 41] Although the DNA is the main target of cisplatin, the latter also interacts with certain proteins to inhibit DNA repair mechanisms.[41, 44] However, repair mechanisms are not exclusive deactivation or decomposition pathways for cisplatin. Since it is administered intravenously, it can interact with human serum albumin (HSA), which is the most abundant protein in human blood.[45] Its thiol containing residues like cysteine or methionine can interact with cisplatin and deactivate it.[46] It was shown that interaction with methionine residues of HSA is the main decomposition pathway rather than interaction with cysteine residues.[47] Once in the cell, cisplatin can be sequestered by the thiol containing peptide glutathione (GSH). The resulting cisplatin-GSH adduct is consequently removed during the cellular detoxification program *via* export pumps. An overexpression of these pumps, including ATP7B can lead to cisplatin resistance.[36, 42] Figure 4 summarizes the uptake, deactivation and mode of action pathways of cisplatin.

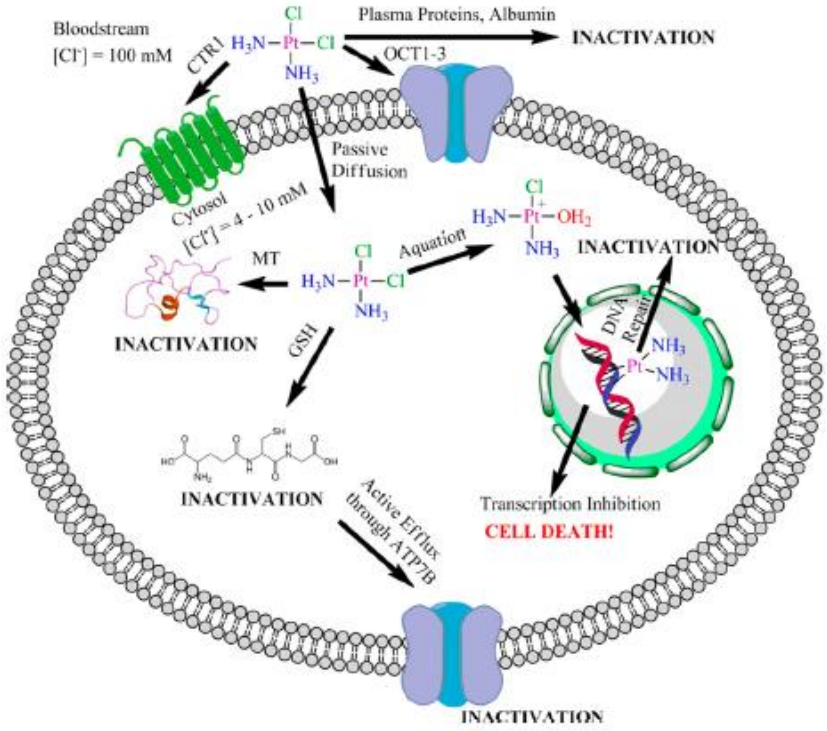


Figure 4: Schematic mode of action of cisplatin, including cell uptake, inactivation, externalization and DNA inhibition. Reprinted with the permission of reference 36.

Due to severe side effects of cisplatin several analogues have been developed and partially applied for anticancer therapy. The goal is to find suitable drugs which are less toxic or able to overcome cisplatin resistances. Until now there are three cisplatin-like antitumor drugs approved worldwide with the general structure L_2PtX_2 (L = amine ligand, X = anionic leaving group) (Figure 5). In the case of carboplatin the chloride leaving groups are replaced by a dicarboxylato-ligand leading to an increased water stability and therefore to less side effects.[48, 49] However, after hydrolysis the same intermediate, like in the case of cisplatin, is formed, thus similar resistances are observed.[49] As a result, other amine ligands were applied to overcome cisplatin resistances. Oxaliplatin is currently applied as anticancer agent, here the NH_3-ligands are replaced by a diaminocyclohexane-ligand. Although oxaliplatin also forms predominantly 1-3 GpG adducts, the bulkier ligand points into the DNA major groove and prevents the interaction with DNA repair proteins, thus overcoming certain cisplatin resistances.[49] Additionally, three more cisplatin like drugs are applied in some countries. Nedaplatin is approved in Japan, bearing *cis*-ammine ligands and a glycolate ligand as leaving group for increased water solubility. Heptaplatin and Lobaplatin are approved in South Korea and China, respectively. Both bear chelating ligands as leaving groups and additionally chelating amino ligands.[36]

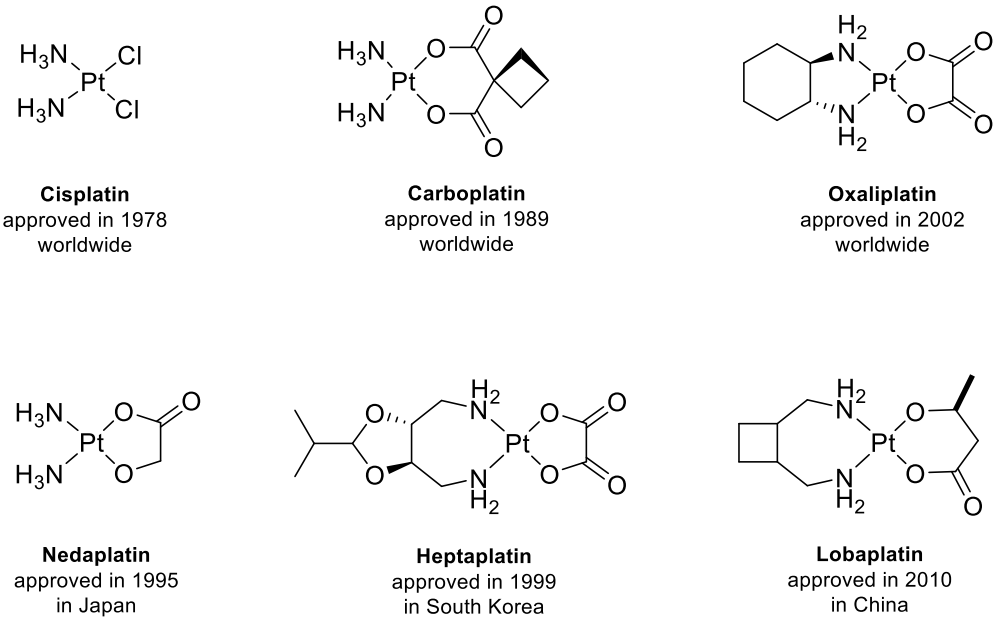


Figure 5: Cisplatin and its approved derivatives.

To overcome drug resistance and to minimize side effects, many different platin complexes have been investigated. Besides cisplatin-like complexes, also different motifs are synthesized for example trans-, polynuclear- or monofunctional platin(II) complexes and also platin(IV) complexes have gained attention.[36] The most advanced platin(IV) anticancer agent is Satraplatin, which is currently in clinical phase III (Figure 6).[50] It can be applicated orally and shows milder toxicity towards healthy cells than cisplatin. Moreover, anticancer activity can be obtained against cisplatin resistant tumors.[51] Satraplatin shows reduced expression in the intestines to platinum(II), releasing the axial acetate-ligands.[52] Since Satraplatin has to be reduced to form the active species, it is assigned to the group of prodrugs. Moreover, drug delivery systems were investigated to enhance the selective uptake into cancer cells, for example metal organic frameworks (MOFs), carbon nanotubes or polymeric micelles.[36]

1.4 Metal Complexes in Clinical Trials for the Treatment of Cancer

To overcome drug resistance related to cisplatin-like drugs, different metal complexes were evaluated towards their anticancer properties, aiming different mechanism of cell death to overcome certain drug resistances.[53] In the field of academic research a variety of different metal complexes (e.g. containing Ti, Fe, Cu, Tc, Ru, Pd, Ag, Pd, Pt, Au, Os) were synthesized.[54-56] Only a few of these complexes are tested in clinical trials. In Figure 6 a few of those metal complexes are depicted which have entered clinical trials for anticancer studies, containing ruthenium, titanium, platinum, gallium and gold.[53, 57-60]

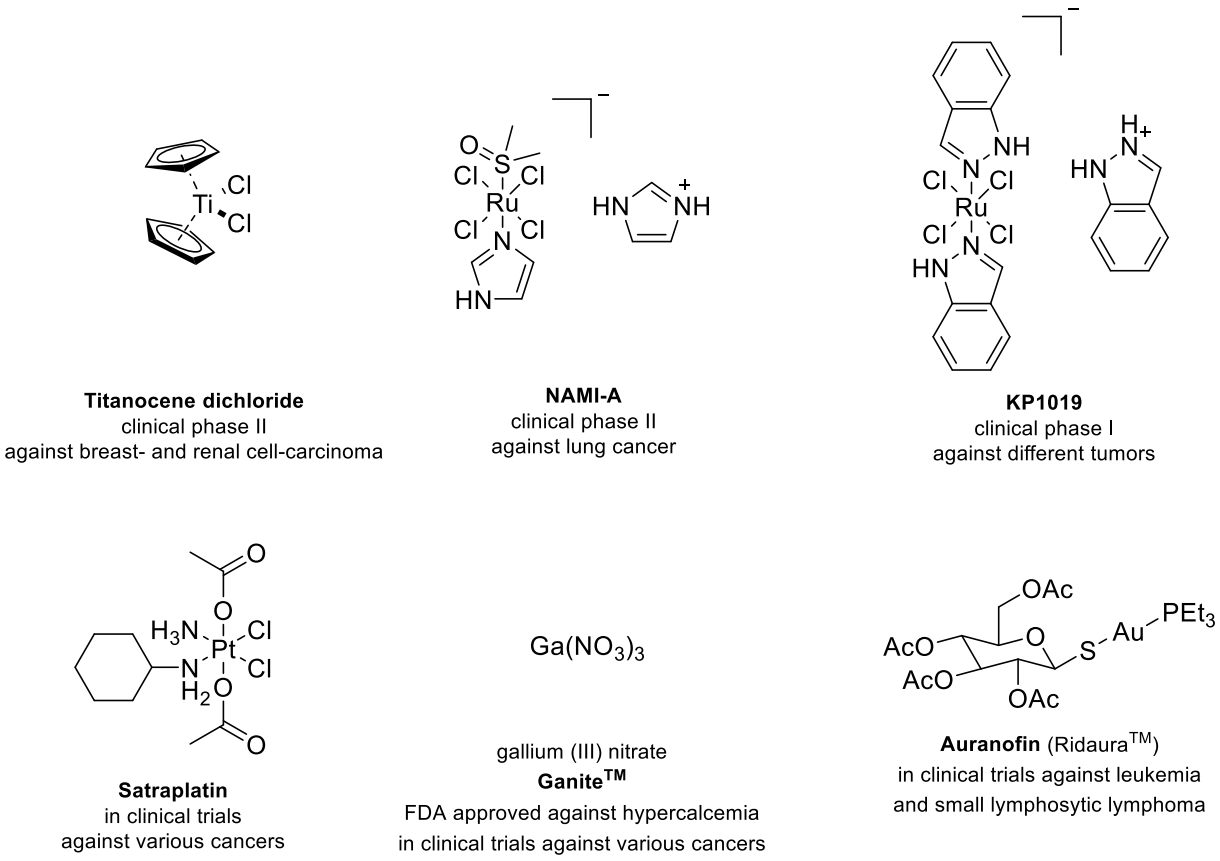


Figure 6: Examples of different metal complexes that are in clinical trials against different cancers.

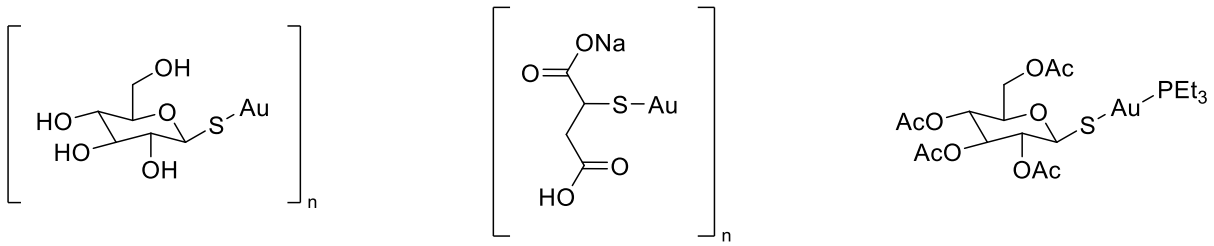
Titanocene dichloride, the simplest titanium anticancer complex, forms Ti-DNA adducts, leading to cell cycle arrest at the late S/early G2 phase and thus triggering apoptotic cell death.[61] Although it reached the clinical phase II for treatment of breast- and renal cell-carcinomas, it failed at this stage due to its insufficient efficacy and insufficient hydrolysis stability.[53, 62] To increase its stability different ligands were tested. One of the first examples was Budotitane, in which the cyclopentadienyl- (Cp) ligands are replaced by a diketonato (1-phenylbutane-1,3-dione)-ligand and the chlorides with ethoxy groups. This compound reached clinical phase I against various cancers, however the stability issues were still substantial.[63] Currently various titanium complexes are investigated as well, for example new metallocene complexes (mainly *ansa*-metallocene), different diketonato complexes and nowadays also various phenolato complexes.[63]

Another promising metal is ruthenium, due to its redox properties, for example the Ru complex NAMI-A (Figure 6). It is proposed that Ru(III) complexes are "activated by reduction" in the reductive environment of the cancer cells (Ru(III) → Ru(II)), which should lead to less cytotoxic side effects.[64] Moreover, due to its high affinity to serum proteins like albumin and transferrin, the tumor targeting potential is increased.[65] The reactive Ru(II)-complexes can bind directly to DNA, which leads consequently to apoptosis.[66] Especially for the next generation of these drugs KP1019 and its respective sodium salt (NKP-1339), it was shown that primarily DNA binding is not the major mechanism of action.[65] Instead, the complexes can interfere with the

redox balance of the cell and participate in Fenton like reactions, generating reactive oxygen species (ROS).[67] This can lead to G2/M cell cycle arrest and consequently to apoptosis *via* the mitochondrial pathway.[65] Despite the promising results in previous studies, NAMI-A did not pass the clinical phase II against non-small lung cancer in combined therapy with gemcitabine. The combination of gemcitabine and NAMI-A did not lead to a significant improvement, compared to gemcitabine alone.[66] However, KP1019 and especially its sodium salt NKP-1339 are still promising candidates for a clinical application (Figure 6).[65, 68]

Interestingly, a very simple metal compound is currently in clinical application, gallium(III) nitrate, which is at present applied against cancer associated hypercalcemia.[58] Patients suffering from hypercalcemia exhibit a higher calcium level in the blood, which can be caused by metastatic cancer targeting bone tissue. These bone metastases can cause osteoclastic bone resorption, which lead to the observed high levels of calcium.[69] Besides for the treatment of hypercalcemia, different gallium complexes or salts are in clinical trials against various cancer types.[58, 70] Due to its similarity to Fe(III), Ga(III) can act as an iron mimetic and thus influence iron related processes.[58] Except for its therapeutic application gallium can be also used for diagnostic applications. ^{67}Ga(III) citrate was already used as a tumor imaging agent for several decades, however nowadays it gets replaced by newer imaging methods, e.g. positron emission tomography (PET).[70] However, new imaging agents based on ^{68}Ga complexes are under investigation as well.[71, 72]

In the research field of gold, Auranofin (Ridaura®) is nowadays the most advanced drug. The Au(I) drug contains a 1-β-D thioglucose tetraacetate- and a triethylphosphine- ligand. It was developed by B. Sutton *et al.* in the 1970s and early 1980s and approved for clinical use in 1985.[73-75] Auranofin was a promising alternative for two injectable Au(I) drugs aurothiomalate and aurothioglucose, which were applied against rheumatoid arthritis (Figure 7). In contrast to the other gold drugs, Auranofin can be administered orally and shows higher anti-inflammatory effects *in vitro*, which made this drug an ideal substitute.[76]



aurothioglucose
Solganol

sodium aurothiomalate
Myochrysine

Auranofin
Ridaura

Figure 7: Historically and currently applied gold drugs against rheumatoid arthritis.

Nowadays there are more potent anti-rheumatoid arthritis drugs, which makes Auranofin less applied against this disease.[76, 77] However, research is focusing on other medicinal applications for Auranofin. Its anticancer activity *in vitro* and *in vivo* were described in earlier publications and it is by now in clinical phase II against chronic lymphocytic leukemia (CLL), small

lymphocytic lymphoma (SLL) and prolymphocytic lymphoma (PLL).[60, 78-80] It is also applied as antiparasitic, antibacterial or antiviral agent.[76] In contrast to cisplatin and many other metal complexes, gold complexes do not mainly interact with the DNA, but show a high affinity to thiol containing enzymes or peptides, leading to a significantly different mechanism of action.[81, 82] Since gold compounds are emerging as promising anticancer agents, it is worth to give a short overview about them.

1.5 Gold compounds in Medicinal Chemistry

During the past decades, gold compounds have drawn the attention of scientists as anticancer agents, including gold nanoparticles, molecular Au(I) and Au(III) complexes.[81, 83, 84] The number of publications containing "gold" and "anticancer" is continuously rising. Only in 2019 over 500 publications were reported containing these keywords (Figure 8). Moreover, the number of publications has been still rising over the last years, proving the high interest in this topic. Before discussing the current scientific results of gold based anticancer agents, it is worth to give a short historical overview of their development.

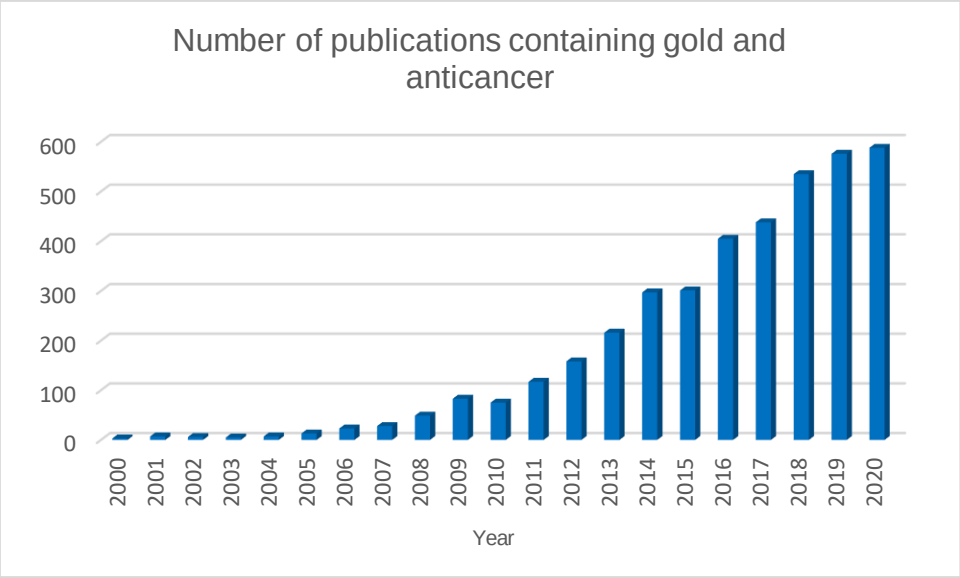


Figure 8: Number of publications containing gold and anticancer; publication report was created by Web of Science (last accessed: 11.01.2021, keyword search: gold* anticancer OR gold* anti-cancer).

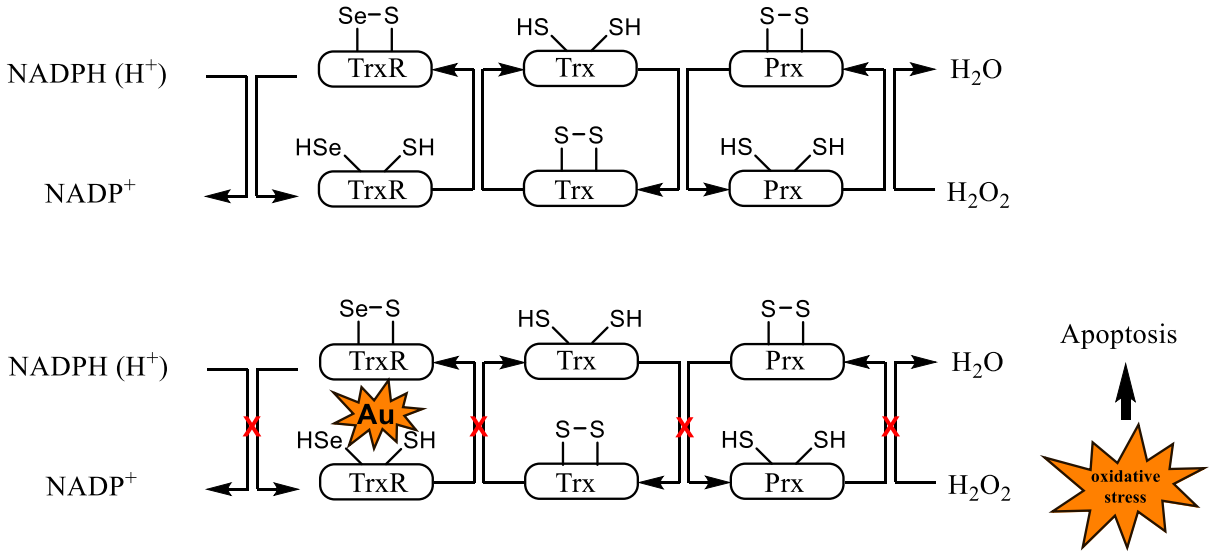
The first application of gold compounds in medicine dates back to ancient China around 2500 BC, but also other ancient cultures report the medicinal use of elemental gold.[85] It was used to "seek longevity", treat furuncles or sores. But it was also stated that gold relieves joint pain.[85] The first evidence for the therapeutic effects of gold compounds was made by Robert Koch in 1890 when he found out that gold cyanide exhibits antiproliferative activity against *Tubercle bacillus*, a tuberculosis bacteria strain. However, no activity was observed in animals.[75, 86] Later it was reported that $KAuCl_4$ and sodium aurothiomalate are active against tuberculosis and

syphilis, which led to the extensive use of intravenously administered gold salts as tuberculosis therapy.[74] Although, later studies showed no efficacy of gold salts against tuberculosis. Nevertheless, relieved joint pain of patients who received aurothiomalate, motivated Jacques Forestier to further study the efficacy of gold salts against rheumatoid arthritis.[74] This led to the development and approval of different intravenously administered gold drugs. The first orally administrable gold drug Auranofin was discovered by B. Sutton *et al.* in the 1970s and early 1980s and afterwards approved in 1985 for the treatment of rheumatoid arthritis.[75] A few years later the antiproliferative activity of Auranofin against tumor cells *in vitro* and also a limited activity in a mouse model was reported.[78, 79]

Nowadays the pharmaceutical industry has a great interest in drug repurposing, which is defined as the identification of new therapeutic targets of FDA approved drugs. Approved drugs can be usually considered as safe for humans and therefore clinical studies are often less time and cost expensive. Because of that, Auranofin gained attention as a potential anticancer drug. It reached clinical trials against a variety of different types of cancer, mainly lymphoma or leukemia.[60] Additionally, the anticancer activity of Auranofin and the mechanism of action is extensively studied in academic research.[87, 88]

The major mechanism of action for Auranofin and similar gold complexes can be considered as “DNA-independent” in contrast to the previously described cisplatin analogs.[87] Due to the high affinity of gold to selenium and sulphur targets containing these moieties were identified as cysteine, selenocysteine and methionine. Therefore, thiol containing enzymes, which are overexpressed in cancer cells are the ideal targets for gold drugs. Thioredoxin reductase (TrxR) was identified as the potentially most relevant target, however, interaction with other thiol containing enzymes may also play an crucial role (e.g. glutathione reductase and cysteine protease).[88, 89] TrxR is a pyridine nucleotide-disulfide oxidoreductase, which is, together with glutathione reductase (GR), essential for controlling the cellular redox balance.[87, 90] There are two isomers of thioredoxin (Trx), which are located in the cytosol (Trx1) and the mitochondria (Trx2), respectively. Inhibition of these enzymes leads to apoptosis *via* the mitochondrial pathway.[87] The regulation of hydrogen peroxide *via* the TrxR pathway involves three different enzymes. Trx, TrxR and peroxiredoxin (Prx). All of these enzymes bear a Cys-XX-Cys or a Cys-XX-Sec motif, which can be oxidized to form disulfide bridges. ROS (e.g. H_2O_2) are reduced by Prx and consequently, Prx is oxidized forming a disulfide bridge. The reduced form of Trx can then transfer electrons to Prx to reduce it again. Reduction of Trx is conducted by TrxR and the oxidized TrxR is reduced by nicotinamide adenine dinucleotide phosphate (reduced form: NADPH, oxidized form: $NADP^+$) and consequently $NADP^+$ is formed. If at least one of these enzymes is inhibited, the cellular ROS cannot be properly regulated anymore and therefore the cell starts apoptosis to prevent further damage to the organism (Scheme 2).[91] In the case of Auranofin or similar gold compounds, TrxR is usually inhibited,

due to the higher affinity to selenocysteine compared to cysteine.[87] Binding to the Sec or Cys groups prevents the formation of a disulfide bridge and thus preventing the reduction of the Trx, which leads to an increasing amount of ROS in the cell. The inhibition of Trx2 leads again to apoptosis *via* the mitochondrial pathway.[87] Under stress the permeability of the inner mitochondrial membrane increases, which results in a disruption of oxidative phosphorylation and osmotic swelling of the mitochondrial matrix. Moreover, the permeabilization of the outer mitochondrial membrane is raised. All of the mentioned factors lead to the release of pro apoptotic proteins such as cytochrome c, which trigger apoptosis *via* caspase-dependent and -independent mechanisms.[91, 92] In addition, reduced Trx1 can bind to apoptosis signal-regulating kinase 1 (ASK 1) which is an inhibitor for apoptosis. The oxidized Trx1 is not able to bind to ASK 1 and therefore no inhibition occurs, thus, triggering apoptotic mechanisms.[87]



Scheme 2: Thioredoxin system for the mediation of the ROS reduction (top) and inhibition of the Trx system by gold compounds such as Auranofin (bottom).[87]

However, the high thiophilicity of gold is also a major drawback. Since gold complexes can irreversibly bind to blood transporters like HSA or GSH, the complexes might decompose in the blood not reaching their targets - the cancer cells. This is the main reason why many gold complexes show lower or insufficient activity *in vivo* and do not reach clinical trials.[75] To balance the reactivity and stability of the gold complexes ligand design is crucial. After the initial discovery of Auranofin as a potential anticancer drug, a variety of different Au complexes was synthesized, aiming to higher stability *in vivo*, a better selectivity against cancerous cells or a different mode of action. Different types of stabilizing ligands, such as phosphines, thiolates or *N*-heterocyclic carbenes (NHCs) were reported for Au(I) complexes. Also, the anticancer properties of various Au(III) complexes were investigated, bearing cyclometalated complexes or pyridine ligands amongst others.[83, 93]

After the success of Auranofin, Mirabelli *et al.* studied the structure-activity relation of 63 different Au(I)-phosphine complexes with the general structure LAuX (L = thiolates, sulfides, pyridines, amines and phosphines, X = halides, nitriles, phosphines, thiolates).[94, 95] The highest *in vitro* and *in vivo* activity was observed for the Auranofin derivatives with L = substituted phosphines and X = thiosugars. Also, other ancillary ligands depicted high antiproliferative activities, but the phosphine ligands were crucial for superior antiproliferative activities.[94] Later delocalized lipophilic cations (DLC), namely Au(I) phosphine complexes were reported bearing chelating phosphine ligands (e.g. 2-*bis*(diphenylphosphino)ethane (dppe)), to significantly increase the *in vivo* cytotoxicity due to higher stability against GSH.[96] Further derivatives were synthesized by replacing the phenyl substituents of the phosphine with pyridines (Figure 9).[97, 98]

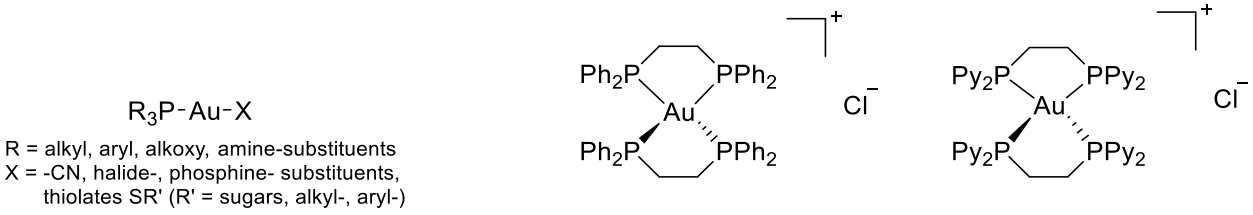


Figure 9: Examples of Au(I) phosphine complexes, which antiproliferative activities were studied

As expected, these type of complexes exhibits high stability against GSH and good *in vitro* and *in vivo* anticancer activity. Furthermore, a correlation between cytotoxicity/selectivity and lipophilicity was observed. The higher the lipophilicity, the higher the cytotoxicity but the lower the selectivity.[96] This trend can be explained by the higher cellular uptake. The more lipophilic the complex, the higher the cellular uptake, which leads in consequence to higher cytotoxicity. However, higher lipophilicity might lead to unselective accumulation and severe side effects.[97, 99] Therefore, ligand design is a challenging task with the aim to stabilize the complexes to prevent deactivation during the transport and ensure selective cellular uptake.
Since the phosphine ligands themselves are cytotoxic and might be oxidized under physiological conditions, they are often replaced by other ligands.[80] One promising type of ligands are NHCs. Carbenes in general are defined as neutral carbon atoms containing a six-electron valence shell, which usually leads to a high reactivity due to their incomplete electron octet.[100] However, carbenes can be stabilized by adjacent heteroatoms, e.g. nitrogen, the so-called *N*-heterocyclic carbenes.[100]

1.6 *N*-Heterocyclic Carbenes as Alternative Ligands to Phosphines

The first metal NHC complexes were reported by Wanzlick and Öfele who synthesized mercury and chromium NHC complexes, respectively.[101, 102] Moreover, Wanzlick *et al.* attempted to synthesize a free NHC *via* an elimination reaction of a trichloromethyl dihydroimidazole. Instead of the free carbene the formation of a dimer was observed, the so-called “Wanzlick-

type-dimer".[103] Arduengo *et al.* synthesized the first isolable free NHC, containing an imidazolium salt with two sterically demanding adamantyl *N*-substituents, which stabilize the free carbene.[104] A few years later, more synthetic routes were discovered to synthesize metal carbene complexes for example by reacting the Wanzlick-type-dimer with a platinum precursor.[105-107] Figure 10 summarizes the first metal NHC complexes and synthetic routes towards them.

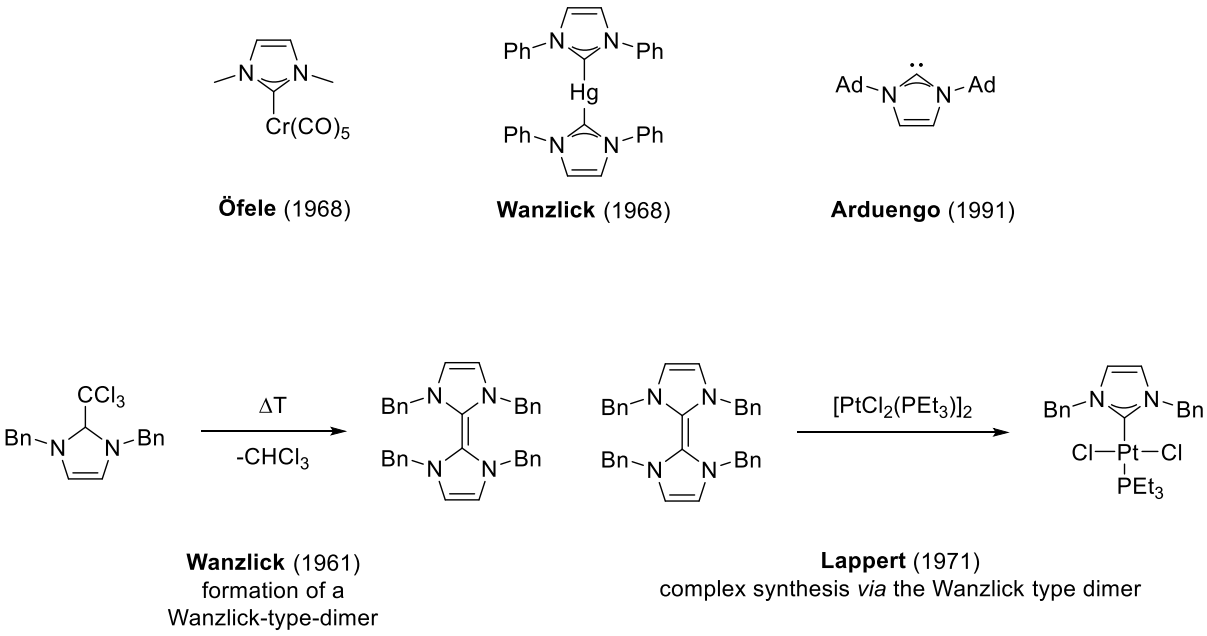


Figure 10: Historical overview of the first reports in the field of metal NHC chemistry.

The electronic structure of NHCs can be described as singlet carbene, with the lone pair in one sp^2-hybrid orbital and an unoccupied p_z-orbital. The free carbene is stabilized by the so-called push-pull effect. The lone pair of the carbene is stabilized by the electron withdrawing adjacent nitrogen atoms (-I effect) and additionally by donating electron density *via* the +M effect to stabilize the p-orbital (Figure 11).[100]

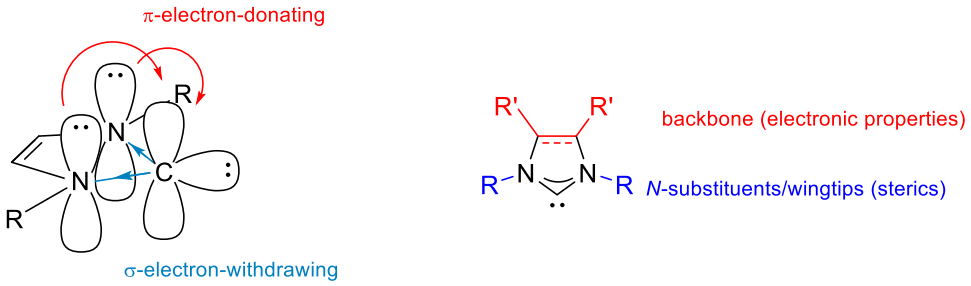


Figure 11: Schematic representation of the stabilization of imidazol-2-ylidenes (push-pull effect) and the modifiable sites of the NHCs.

Although the push-pull effect can be observed for every NHC, it strongly depends on the type of NHC and the *N*-substituents.[100]

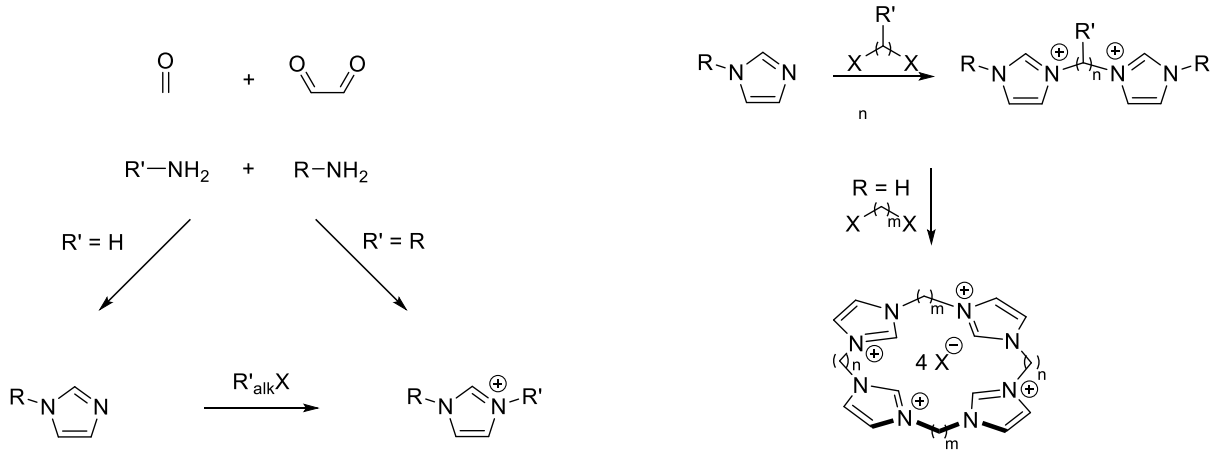
Upon reacting with a metal, the electron lone pair interacts with an unoccupied d-orbital of the metal, resulting in a strong σ-bond. Although π-bonding interactions (π-donation

and -backbonding) are possible, these interactions are rather low compared to the σ-donation.[100, 108, 109]

Compared to their phosphine counterparts, which are also considered as σ-donors, the metal-ligand bond is usually shorter. Also a higher bond dissociation energy is observed for NHCs, thus, leading to higher stability.[100]

Since the most applied NHCs are imidazolylidenes, in the following a short overview of the commonly applied synthetic strategies for the ligand precursors is given.

Depending on the *N*-substituents of the imidazolium salt different strategies are applied. Alkyl *N*-substituents are usually obtained *via* nucleophilic substitution reaction of an alkyl halide with an imidazole. If reacted with a substituted imidazole two different *N*-substituents can be introduced. Aryl substituted imidazoles or imidazolium salts are usually synthesized *via* condensation reaction of glyoxal, formaldehyde and the corresponding amine (Scheme 3, left). Depending on the alkyl halide, also dimeric, oligomeric or even macrocyclic imidazoles or imidazolium salts can be achieved, when the imidazole is reacted with a dihalide compound (e.g. CH_2Br_2) (Scheme 3, right).[110, 111]



Scheme 3: Synthetic pathways for mono-imidazolium salts (left) and dimeric or cyclic imidazolium salts (right). Counterions are omitted for a better overview.

A plethora of mono-imidazolium salts are known in literature, differing in their *N*-substituents and the substituents of the backbone. Additionally, *bis*-imidazolium salts are differing in the length of the bridge, a few of them bear a functional group at it (e.g. hydroxyl, carboxyl or chloride) or are macrocyclic.[112-115]

Besides the most used imidazolium salts as NHC precursors, there are different other carbene precursors used in metalorganic chemistry. Per definition, NHCs contain a carbene incorporated in a heterocycle bearing at least one nitrogen and may bear another heteroatom.[100, 112] Moreover, NHCs can be differentiated in the location of the carbene.[100] Usually the carbene is formed between the two nitrogen atoms at the C^2 position due to the stabilization effect of the nitrogen atoms. However, in certain cases also deprotonation at the C^5 position is possible, those carbenes are usually described as “abnormal” or “mesoionic” and

considered as stronger electron donors.[116] Figure 12 summarizes the most common types of NHCs.

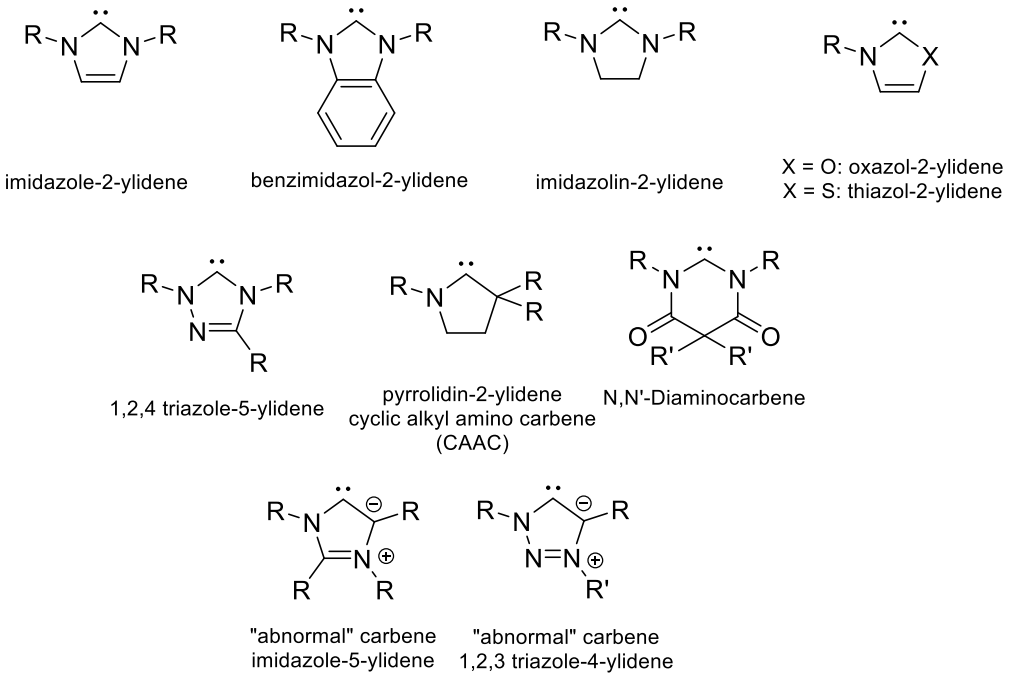
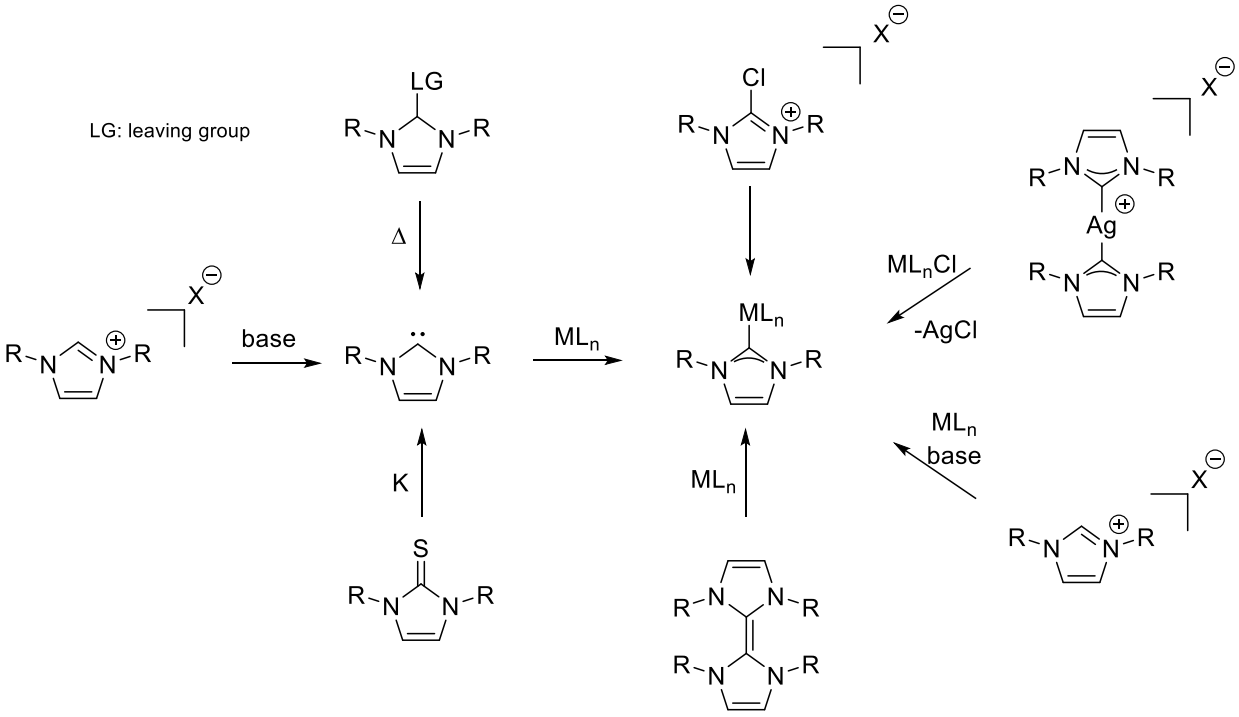


Figure 12: Structures of the commonly applied N-heterocyclic carbenes.

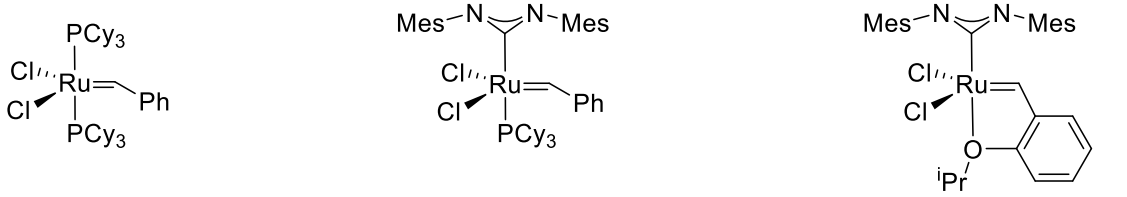
Although the different NHC precursors vary in their accessibility, electronic and steric properties, the synthesis routes for their late transition metal complexes are quite similar. In general, there are two routes to obtain late transition metal NHC complexes. They can be synthesized *via* the reaction of the metal with the free carbene (*in situ* generated or isolated) or *via* direct complex formation.[117] The free carbene is usually generated by deprotonation of the corresponding imidazolium salt with strong bases (e.g. *n*-butyllithium (*n*-BuLi) or lithium diisopropylamide (LDA)). Less common is the reduction of a thione with potassium or an α-elimination to form the free carbene.[112, 118]

Direct metal complex formation usually involves weaker bases to form the metal-NHC complex. Internal bases are commonly used, which means the use of metal precursors that bear a basic ligand (e.g. palladium(II) acetate or iron(II) bis(trimethylsilyl)amide). The addition of external bases to the metal precursor is also possible, for example, alkali-carbonates, -hydroxides or -alkoxides. The last commonly applied route is the so called “silver-route”, where Ag_2O is used as an internal base to form the corresponding Ag(I) NHC complex. The *in situ* generated or isolated silver complexes can subsequently be treated with a metal precursor bearing a halide. Thus, leading to the formation of insoluble silver halide and a carbene transfer to the other metal (transmetalation route). Less common are oxidative additions to form a metal NHC complex or complex formation *via* the Wanzlick-type-dimer.[112] Scheme 4 summarizes the different synthetic approaches to obtain metal-NHC complexes.



Scheme 4: Common approaches to obtain metal-NHC complexes.

As mentioned previously, NHCs bear similarities to phosphines, for example, the strong σ-donor properties or the relatively low π-acceptor properties. Therefore, they are considered as an alternative for phosphines.[119, 120] Due to their stronger σ-donor properties and their higher stability, various catalytic applications are reported in the literature, for example, cross-coupling-, hydroformylation-, hydrosilylation-, polymerization- and olefin metathesis-reactions.[119] In the latter, initially two different phosphines were used as ligands for the Ru alkylidene complex, leading to the "Grubbs first generation" olefin metathesis catalysts (Figure 13). The larger and more basic phosphine accelerates the dissociation of the other phosphine, due to the *trans*-effect, which is proposed as a critical step in the olefin metathesis.[121] However, by replacing the phosphine with a stronger σ-donating NHC, the *trans*-effect is increased, which leads to a higher activity compared to two phosphines or two NHCs.[119, 122] This finding led to the synthesis of different olefin metathesis catalysts, bearing different phosphine and NHC ligands. A further improvement was made by introducing an alkoxy chelate, which further increases the activity.[123,124] Since olefin metathesis is of great interest in organic chemistry (e.g. syntheses of pharmaceuticals or natural products), is environmentally friendly and highly efficient and it was awarded with the noble prize in 2002.[125]



Grubbs catalyst 1st generation | Grubbs catalyst 2nd generation | Grubbs-Hoveyda catalyst

Figure 13: First Ru-based metatheses catalysts developed by Grubbs and Hoveyda.

Due to the stronger σ-donating properties of NHCs compared to phosphines, the metal carbene bond is strengthened.[119] Moreover, NHC ligands are not as prone to oxidation as phosphine ligands. These properties result in highly stable metal-NHC complexes. For example, a Pd(II) *bis*-NHC is reported to retain catalytic activity in methane oxidation under very harsh conditions (potassium persulfate in trifluoracetic acid at 100 °C).[126]

1.7 Au(I) NHC Complexes applied as potential Anticancer Agents

The above mentioned advantages of NHCs did not only lead to their application in catalysis, but also in the field of bioinorganic chemistry.[100] In this field the complexes must show sufficient stability under physiological conditions, making NHCs ideal ligands. Moreover, gold, among other transition metals, is a promising alternative to overcome current issues in chemotherapy, for example overcoming resistance issues. Therefore, gold NHC complexes are promising anticancer agents.[81, 89]

Although the first Au(I) NHC complex was synthesized in 1989, the first pioneering studies about the cytotoxicity of Au(I) NHC complexes were reported by Berners-Price *et al.* in 2004 and 2008.[127-129] They evaluated the mechanism of action of different dinuclear Au(I) bidentate *bis*-NHC complexes in biological systems (Figure 14). The complexes induce mitochondrial membrane permeabilization (MMP) and thus, leading to mitochondrial swelling and apoptosis.[128] In the following studies the mechanism of action of mononuclear Au(I) DLCs was investigated. [129, 130] DLCs can rapidly pass the mitochondrial membrane due to the high membrane potential $\Delta\Psi_m$ and additionally accumulate in the mitochondria of cancer cells due to a higher $\Delta\Psi_m$ which is observed in many cancer cells.[131, 132]

In another study, the correlation between MMP and lipophilicity of similar Au(I) *bis*-NHC complexes was reported (Figure 14). Six-linear Au(I) *bis*-NHC complexes with different alkyl *N*-substituents, varying in their lipophilicity, were synthesized. The lipophilicity, which can be estimated from the logarithm of the n-octanol–water partition coefficients (log P) vary from -1.09 to 1.73, while the complex with methyl (Me) *N*-substituents depicts the lowest lipophilicity, the one with cyclohexyl displays the highest one. The lipophilicity directly correlates with the MMP, *i.e.* the higher the lipophilicity, the higher is the antimitochondrial activity.[130]

In the following study, the detailed mechanism of action was revealed.[129] The evaluated compounds are cytotoxic against the breast cancer cell line MDA-MB-23 and MDA-MB-468 but significantly less toxic to the healthy epithelial cell line HMEC. The selectivity and cytotoxicity correlate with the lipophilicity and the most promising results were observed for the moderately lipophilic complex with the NHC ligand with isopropyl-(^{i}Pr)-wingtips. Moreover, TrxR was identified as a potential target for these complexes. At 5 µM concentration of this complex, TrxR is inhibited by ~50%, while the closely related GR is not inhibited. These enzymes vary in their redox active site, while TrxR bears a –Cys-Sec– active site, GR bears a –Cys-Cys– active center.[133] Further ^{1}H-NMR (nuclear magnetic resonance) spectroscopy experiments revealed a potential mechanism of action, involving stepwise NHC ligand dissociation and Cys/Sec coordination to the metal and finally forming [AuSec]$^{-}$ or [AuCys]$^{-}$. The ligand exchange rate constant is 20-80-fold higher for Sec compared to Cys, which is in accordance with the observed high affinity to TrxR compared to GR.[129]

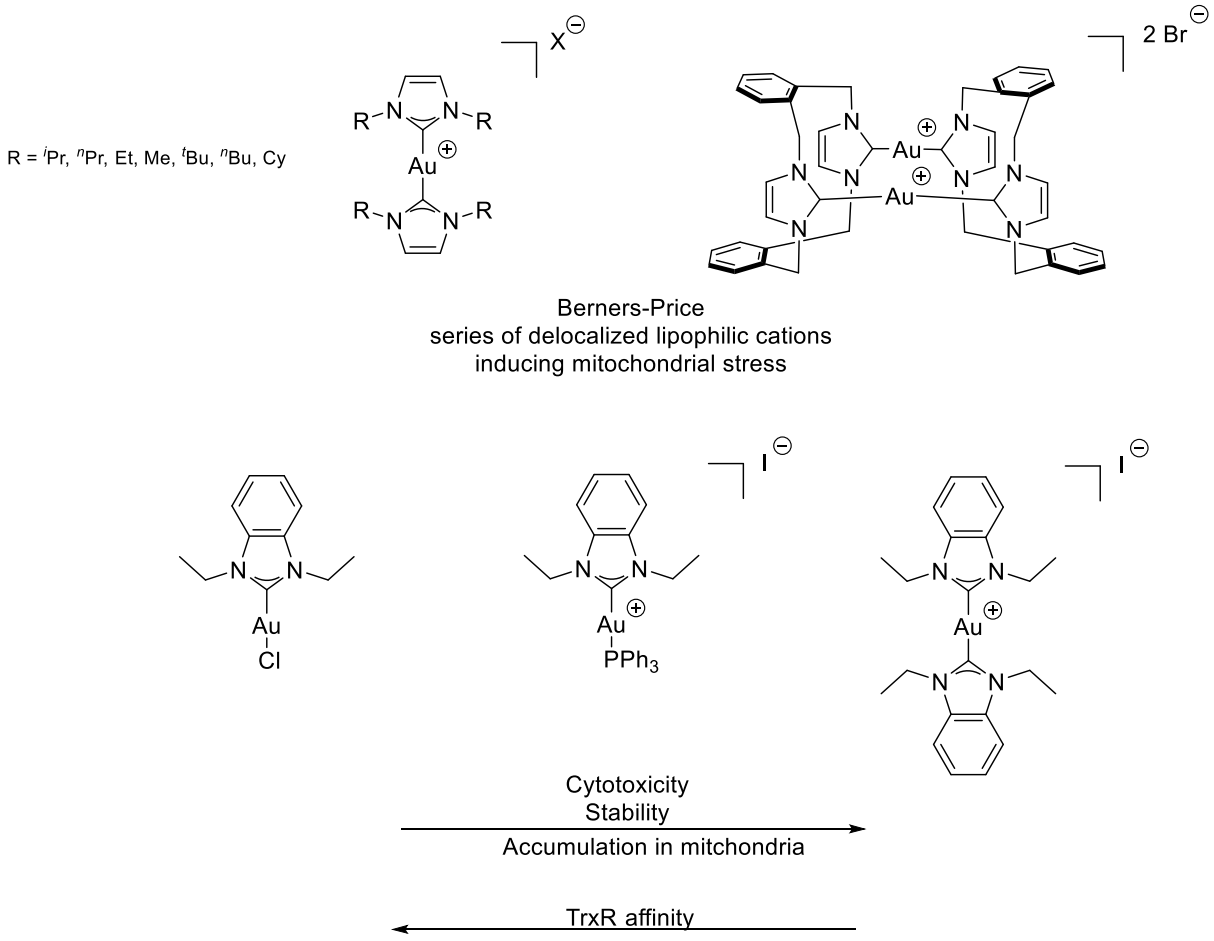


Figure 14: Several Au(I) NHC-complexes which were investigated as potential anticancer agents. Top: series of delocalized lipophilic cations synthesized by Berners-Price et al..[128-130], bottom: a comparative study by Ott *et al.* with benzimidazole-type ligands and different ancillary ligands.[134]

In conclusion, these key studies revealed that DLC Au(I) NHC complexes can selectively target the mitochondria of cancer cells and induce apoptosis. Later studies confirmed TrxR as a potential target for various other Au(I) complexes.[87-89, 135] Since inhibiting TrxR activity *via*

ligand exchange reactions is one of the most common mechanisms of action for gold compounds, the ancillary is often changed to a more labile ligand. Ott *et al.* synthesized a series of benzimidazolylidene Au(I) complexes with chloride as a second ligand (Figure 14, bottom). Due to the more labile chloride ligand, ligand exchange is enhanced and thus, TrxR inhibition is increased.[136] However, it is important to retain substantial stability in the presence of thiols otherwise ligand exchange might lead to inactivation or metabolism reactions.[134, 137] To enhance cellular uptake and mitochondrial accumulation the formation of cationic DLCs might be beneficial. Ott *et al.* compared the previously discussed neutral benzimidazolylidene Au(I) complexes with their cationic analogs bearing a phosphine or NHC as a second ligand.[134] Since many deactivation and metabolism processes of gold complexes involve binding to serum albumin, the affinity against serum albumin was investigated.[137, 138] Auranofin shows a fast reaction and after 6 h 73% of the gold is bound to serum albumin. The neutral complex reacted similarly, after 6 h 66% of gold is bound. As expected, the more stable *bis*-NHC complexes shows a lower affinity to serum albumin. The amount of bound gold is below 20% after 6 h. With a phosphine as a second ligand, the binding to serum albumin is slower compared to the corresponding chloride ligand, but also reaches overall higher values, compared to the *bis*-NHC complex. The TrxR inhibition is in agreement with the previously described stability against thiols. The more labile the second ligand, the higher the inhibition of TrxR. Thus, the *bis*-NHC complex shows a still notable but lower TrxR inhibition, compared to the other described complexes. Although TrxR inhibition is reduced, mitochondrial accumulation and cellular uptake are increased for the cationic species, presumably due to the DLC character. Overall, higher cytotoxicity is observed compared to the neutral NHC complex.[134]

Except for chlorides, phosphines and NHCs, a greater variety of different ancillary ligands for Au(I) is possible. Since phosphine alkynyl Au(I) complexes showed a good antiproliferative *in vitro* and also *in vivo,* similar alkynyl Au(I) NHC complexes were synthesized.[139-142] An alkynyl Au(I) NHC complex was developed by Casini *et al.* showing a good antiproliferative activity in various cancer cells and interestingly no cytotoxicity in healthy rat kidney tissues.[142] In the same study a thiosugar was applied as an ancillary ligand, to synthesize an Auranofin NHC-derivative. Although a slight selectivity is observed, the alkynyl complex is significantly more selective. Further studies with model thiol substrates showed that the weaker alkynyl ligand is replaced by the thiol and depending on the NHC ligand, also the NHC ligand undergoes ligand exchange.[143]

Moreover, a variety of different Au(I) NHC complexes with thiols or phosphines as ancillary ligands were reported with the aim to synthesize heterobimetallic complexes.[89] Upon coordination of a thiol or phosphine ligand, which additionally coordinates to another metal, different heterobimetallic complexes can be realized. *Via* this procedure a titanocene moiety

was attached to an Au(I) complex *via* a thiol ligand or a Ru-cymene motif was conjugated *via* a terminal phosphine coordinating to Au(I).[144, 145] Both types of heterobimetallic complexes showed a synergistic effect on the antiproliferative activity.

Another approach to synthesize heterobimetallic complexes is the attachment of the second moiety to the wingtip. *Via* this procedure ferrocenyl/Au(I) *bis*-NHC complexes were synthesized and a synergistic effect of both metals was observed, too.[146] Moreover, other moieties can be conjugated *via* the wingtip to synthesize Au(I) NHC complexes with various properties, e.g. luminescence[147-150], increased water solubility[151], chelating NHCs[150] or dinuclear complexes.[1, 128, 152] Although a variety of different Au complexes being active in *in vitro* models, the number of NHC-complexes showing *in vivo* efficacy is quite scarce.[152-157]

In summary, it is important that sufficient stability is ensured, to prevent deactivation and metabolism of the complexes. NHC ligands represent ideal ligands to ensure the stability of the complexes. Moreover, sterically demanding wingtips shield the gold and additionally hinders attack of the nucleophilic thiol groups.[158]

1.8 Targeted Therapy with Metal Complexes – Concepts and Examples

Besides stability issues, many complexes also exhibit selectivity issues, not allowing for selectively targeting cancer cells or tissues. Despite the well-known “targeting approach” in anticancer therapy based on organic molecules, the number of inorganic complexes featuring rational targeting of cancer cells is quite scarce.[159, 160] Even if there is a selectivity, it is usually not obtained *via* rational targeting of certain overexpressed targets in cancer cells, which is commonly used in anticancer therapy for organic molecules.

One useful approach is the conjugation of carbohydrates to the complex. Since cancer cells are more energy demanding than healthy cell lines, the uptake of sugars is increased in cancer cells.[161, 162] Thus, a certain selectivity towards cancer tissues might be achieved in some cases using this so called *Warburg effect*.[163] The conjugation of sugars to metal complexes is a quite known topic, for example, different Pt(II) or Pt(IV) complexes were synthesized bearing carbohydrates.[164-168] Lippard *et al.* investigated the mechanism of action and showed that the uptake proceeds *via* glucose or organic cation transporters, both overexpressed in cancer cells, which leads to an accumulation of them in malignant cells.[168] Moreover, a variety of different Auranofin analogs was reported and their medicinal properties were studied.[94, 169] Also a few carbohydrate Au(I) NHC conjugates are reported, where the sugars are applied as an ancillary ligand or attached as wingtip of the NHC.[142, 170-172] However, in the reports described herein, only Casini *et al.* investigated the *ex vivo* selectivity of the sugar containing complex. Since only a slightly increased selectivity was observed no detailed mechanistic studies were conducted.[142] As mentioned before, the *N*-substituents and the ancillary ligands stabilize the

complex by steric shielding or by electronic effects. In the case of Auranofin rapid metabolism is observed, which might be related to the thiosugar ligand and also count for the other complexes bearing a sugar as an ancillary ligand.[137]

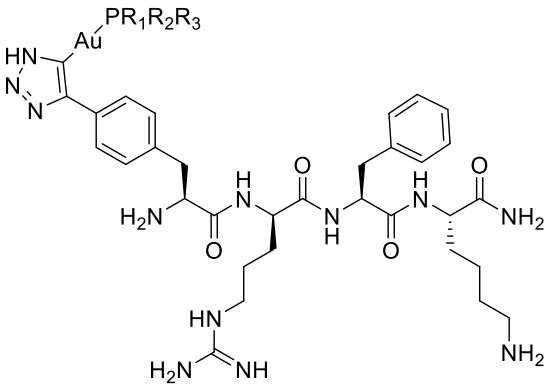
Another approach to increase the selectivity is the conjugation of certain peptides that can target selectively cancer cells.[173] In general metal peptide conjugates can be obtained *via* the conjugation of the metal complex to a peptide at its side chain or to the *C*- or *N*-terminus of the peptide. Chemo- and regioselectivity must be carefully evaluated, since the complex must be stable during the reaction but also side reactions of the functional groups of the peptide must be prevented.[160] As a proof of principle, a variety of different metal complexes was conjugated to different peptides, most of these metals bear carbonyl-, aryl, phosphine or cyclopentadienyl ligands.[160] Except conjugation to a peptide, also direct incorporation of the complex into the peptide is possible. In this case, certain residues of the peptide can be modified and act as ligands for the metal. Regarding the proteogenic amino acids, eight amino acids can potentially act as ligands. There are different suitable amino acids, which contain carboxylates (asparagine and glutamine), amines (lysine), thiols (cysteine), aromatic rings (phenylalanine, tryptophan and tyrosine) and imidazoles (histidine). The residues can be directly utilized as ligands or they can be modified to act as ligand. For example, thiol or amine residues can directly act as ligands, while imidazole residues are usually converted to an imidazolium salt which then can be applied as carbene ligand.[160] The latter method was initially described by Erker *et al.* for histidine itself and then expanded by Albrecht *et al.* who synthesized new complexes containing histidine or small peptides, mainly for catalytic applications.[174-178]

Despite many of these metal peptide conjugates are tested as anticancer agents, mainly the influence of the peptides on the antiproliferative activity is investigated. Only a small number of peptides is conjugated to target certain receptors to enhance the uptake into cancer cells (Figure 15).[159, 160]

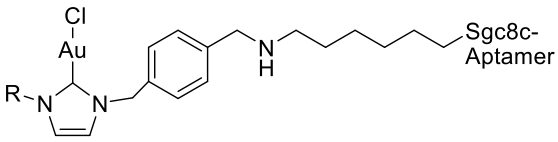
Metzler-Nolte *et al.* incorporated an Au(I) phosphine complex bearing an azide into a peptide, upon azide alkyne cycloaddition (Figure 15).[179] The peptide used herein is based on previously described peptide sequences which are known to effectively cross the cell membrane and accumulate in the mitochondria.[180, 181] The complexes show good antiproliferative activities and more importantly also good selectivity against cancer cells, compared to non-malignant fibroblast cells. Moreover, cisplatin resistance could be overcome.[179]

Veige *et al.* conjugated an Au(I) NHC complex to a CCRF-CEM-leukemia specific aptamer (sgc8c) *via* an amidation reaction (Figure 15).[182] The conjugation of this aptamer leads to specific recognition and internalization of the complex into these leukemia cells. Other cells are not affected. Conjugation of random aptamers led to a loss in selectivity and also to a loss in activity, proving that sgc8c is responsible for effective delivery.[182]

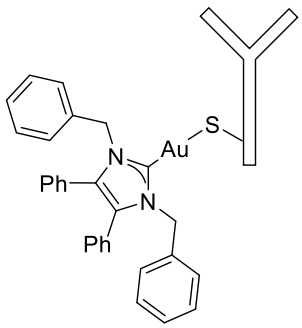
Very recently, Tacke and Bernardes *et al.* conjugated an Au(I) NHC complex to albumin and the clinically applied antibody Trastuzumab (Figure 15). Conjugation was conducted *via* a free cysteine site of albumin or the antibody.[183] Unfortunately, no significant improvement in selectivity or cytotoxicity is observed compared to the Au(I) complex itself. Lewis and Contel *et al.* conjugated an Au(I) phosphine complex to Trastuzumab with an additional linker at the lysine or cysteine side chains (Figure 15). These conjugates show enhanced cytotoxicity compared to Trastuzumab.[184]



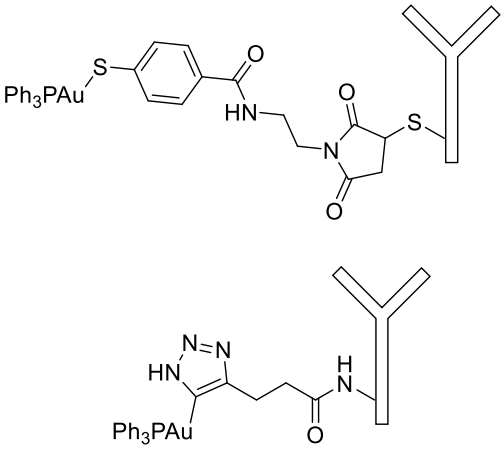
Metzler-Nolte (2012)
conjugation of a peptide to increase mitochondrial uptake



Veige (2016)
conjugation of a aptamer to target specifically CCRF-CEM leukemia cells



Tacke and Bernardes (2018)
conjugation of a Au(I) NHC to Trastuzumab or serum albumin *via* the Cys-site chains



Lewis and Contel (2019)
conjugation of a Au(I) phosphine complex to Trastuzumab *via* the Cys-or Lys site chains

Figure 15: Au(I) complexes conjugated to different biomolecules for enhanced selectivity towards cancer cells.

Although there are a few more publications for platinum drugs utilization of certain biomolecules for targeted therapy (e.g. estrogen vectors[185], peptides[186, 187], sugars[168] or bisphosphonates[188]), the amount of gold complexes is quite scarce, despite the above mentioned.[159]

In summary the Au(I) complexes should exhibit certain stability under biological conditions especially against thiols to prevent decomposition and metabolism. Besides, they should show good cytotoxicity to justify further modifications. Considering the properties of NHCs (e.g. stability or modifiability) are quite suitable to hit these criteria. Moreover, NHCs with certain functional groups enable the conjugation of specific biomolecules for targeted anticancer therapy, a field which is currently barely explored.

2 OBJECTIVE

Since cancer is one of the leading causes of death worldwide, cancer treatment is one of the most important tasks today.[3] Among other therapies, chemotherapy represents one of the common anticancer treatments.[23] However, most of the currently applied drugs cause severe side effects or cancer cells can cultivate resistances. Therefore, new promising anticancer agents must be found. Due to their unique and different properties to common organic drugs, metal complexes have drawn the attention of researchers. Although cisplatin and a few derivatives are approved as anticancer drugs, research is ongoing to find better alternatives.[36]

Metal complexes for bioinorganic applications need to exhibit certain stability under biological conditions, for example against thiols to prevent decomposition and metabolism. Besides, they should show good cytotoxicity to justify further studies. Considering the theoretical background NHCs ligands are quite suitable meeting these criteria.

Gold compounds are promising attributed to their different mode of action compared to other compounds. Many gold complexes inhibit the in malignant cells overexpressed enzyme thioredoxin reductase which consequently leads to controlled cell death (so-called apoptosis).[87, 88] In the seminal works of Berners-Price a correlation between delocalized lipophilic cations (Au(I) *bis*-NHC complexes) and selective accumulation in mitochondria of cancer cells was shown.[129] Additionally, applying *bis*-NHC complexes should lead to ensured stability, especially against thiols, which is one of the most common decomposition pathways for gold complexes.[137] Other studies show a positive effect of bidentate *bis*-NHC complexes to further increase stability. This led to the development of a stable Au(I) NHC complex with sufficient stability *in vivo*.[152] Moreover, NHCs with certain functional groups enable conjugation of certain biomolecules for targeted anticancer therapy, a field which is currently barely explored.

In this thesis new metal NHC complexes, especially Au(I) *bis*-NHC complexes are synthesized, and their applicability as potential anticancer agents is investigated (e.g. selectivity, stability and antiproliferative activity). Moreover, mechanistic studies of the most promising candidates are investigated to see if the complexes are applicable for further studies (e.g. *in vivo* studies.) If promising properties are observed, the complexes can be further conjugated with biomolecules to increase their affinity to cancer cells or enable imaging and distribution studies. To pursue this strategy NHC ligands with functional groups are applied allowing conjugation in the backbone or the bridge of *bis*-NHCs. This should not strongly affect the stability of the complexes considering recent studies.

3 Results – Publication Summaries

In this chapter, the most important publications that originated from this thesis are summarized. The published articles, the bibliographic data and the reprint permissions can be found in the appendix.

3.1 Antiproliferative Activity of Functionalized Histidine-derived Au(I)*bis*-NHC Complexes for Bioconjugation

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In this article, the synthesis and antiproliferative activity of different histidine derived Au(I) *bis*-NHC complexes are reported, varying in their *N*-substituents and the functional groups in the backbone (Figure 16). Additionally, preliminary cellular uptake studies were conducted using proton induced X-ray emission (PIXE) microscopy.

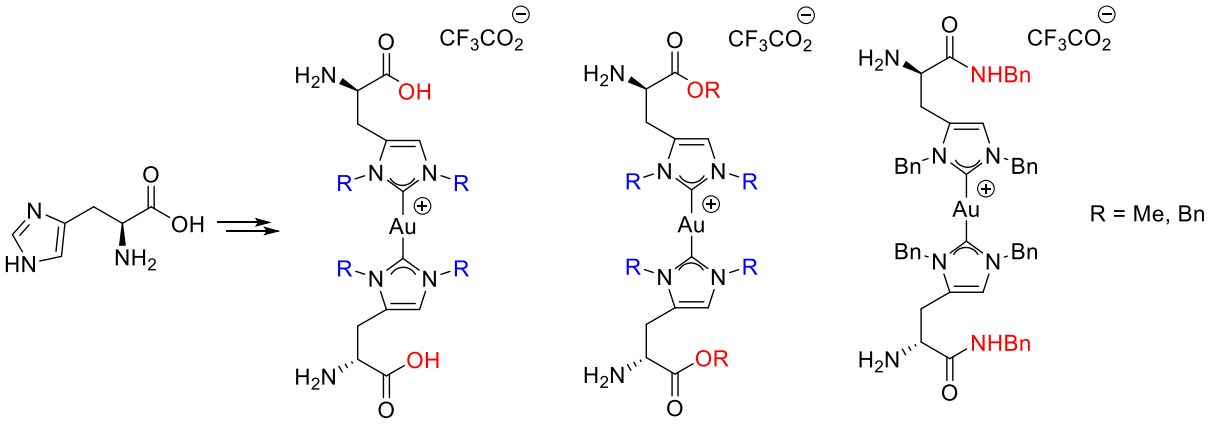

Figure 16: Series of different histidine derived Au(I) *bis*-NHC complexes.

As mentioned in the chapters before, the amount of gold complexes applied in targeted therapy is quite scarce. Usually, NHC complexes can be modified by changing the ancillary ligand (e.g. phosphines or thiols) or the wingtip. However, both strategies affect the stability of the complexes by changing the electronic or steric properties. Introducing functional groups in the backbone of the NHC is comparably difficult. Therefore, the naturally derived NHC precursor L-histidine is applied to synthesize a new group of Au(I) *bis*-NHC complexes for further bioconjugation. Strategies to obtain different functional groups in the backbone are presented and different *N*-substituents are applied. A strong dependence of the wingtips and functional groups on the antiproliferative activity of these complexes is observed. With an amino acid functionality (amine and carboxylic acid) the complexes are inactive (IC_{50} > 100 µM),

independent of the wingtip. In the other cases, the Me-wingtips do not show any activity, too. However, with benzyl (Bn) *N*-substituents a comparable activity to cisplatin is detectable in various cancer cell lines (breast, prostate, ovarian, cervix) and also a certain selectivity is observed with selectivity indices (SIs) against MCF7 up to 5.9 for the amide and 4.1 for the ester. Despite stability against GSH and cysteine of the complex with the Bn ester functionality, a slow hydrolysis in aqueous media (RPMI cell medium) is observed. Although the ester slowly hydrolyzes to the inactive compound, only a slightly lower activity is observed, compared to the more stable amide. This might indicate that the uptake is faster than the hydrolysis of this compound. PIXE microscopy indicates that both complexes might have different intercellular distribution profiles.

In conclusion, these histidine-derived gold complexes are suitable candidates for further bioconjugation. Due to two NHC ligands, the stability against thiols is ensured and the complexes with Bn wingtips show a sufficient antiproliferative activity. Bioconjugation at the backbone should not influence the stability but, depending on the biomolecule, should increase the selectivity or cellular uptake.

3.2 Mechanisms underlying the cytotoxic activity of syn/anti-isomers of dinuclear Au(I) NHC complexes

Bruno Dominelli‡, Christian H.G. Jakob‡, Jens Oberkofler, Pauline J. Fischer, Eva-Maria Esslinger, Robert M. Reich, Fernanda Marques, Teresa Pinheiro, João D.G. Correia and Fritz E. Kühn*

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In the last years dinuclear Au(I) complexes also attracted attention as potential anticancer candidates. For example, Che *et al.* reported a dinuclear Au(I) NHC complex with high stability against blood thiols and a sufficient anticancer activity *in vivo*.[152] In this work detailed studies are reported concerning the mechanism of action of previously reported dinuclear Au(I) *bis*-NHC complexes.[1] The main focus lies on the separation of the two different main isomers (*syn* and *anti*, regarding the position of the hydroxyl group) and their influence on the antiproliferative activity, their reactivity against thiols and their TrxR affinity (Figure 17). Furthermore, cellular uptake studies were conducted *via* inductively coupled plasma mass spectrometry (ICP-MS) and nuclear microscopy.

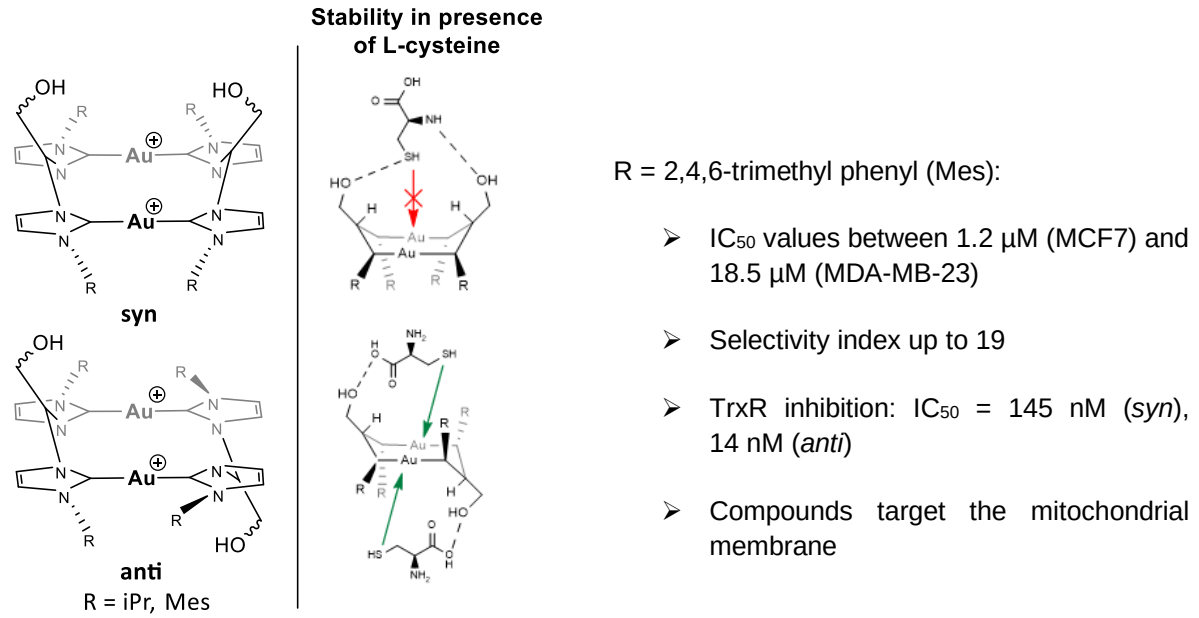

Figure 17: The two different major isomers of the hydroxyl bridge functionalized Au(I) *bis*-NHC complexes and their biological properties.

The isomeric mixture of these complexes was separated by reversed phase high performance liquid chromatography (RP-HPLC), the first fraction can be assigned to the *anti*-isomer, the second one to the *syn*-isomer. Reactivity studies against cysteine with ^{1}H-NMR spectroscopy and electrospray ionization mass spectrometry (ESI-MS), show a dependence on the wingtip (^{i}Pr and Mes) and the isomer. In the case of ^{i}Pr wingtips, only the *anti*-isomer reacts at 37 °C with cysteine. ESI-MS shows a single ligand exchange with cysteine. When increasing the

temperature to 50 °C the *syn*-isomer reacts similarly. Density functional theory (DFT) calculations were conducted to understand this reactivity behavior. In the case of the *syn*-isomer the complex is bent, thus shielding the gold nuclei from one site. Moreover, due to the *syn*- arrangement of the hydroxyl groups, two hydrogen bonds are formed with cysteine, which prevents a nucleophilic attack. In the case of the *anti*-isomer, nucleophilic attack can occur from both sides and additionally only one hydrogen bond is formed to the cysteine, directing the sulphur to the gold which enhances the reactivity. In the case of Mes wingtips, the reactivity is reduced, indicating a stabilization caused by steric shielding.

Regarding the antiproliferative activity, no notable difference between the isomers is observed. However, with Mes *N*-substituents the complexes show good activity, while with ^{i}Pr the complexes are inactive. Interestingly, the TrxR affinity strongly depends on the isomers. The TrxR activity is nine times higher for the *anti*-Mes complex than for the *syn*-Mes complex (IC_{50}: 16 nM vs. 145 nM), which is in accordance with the previously described reactivity against cysteine. Cellular distribution studies of *syn*-Mes show that the complex accumulates in the membranes of the cell, including the mitochondrial membrane, which counts for 40% of total membranes in mammalian cells.[189] Altogether, these studies indicate that inhibition of mitochondrial TrxR is the main mechanism of action. Further studies about the antiproliferative activity show that *syn*-Mes might become an ideal candidate for potential *in vivo* studies. Good IC_{50} values are observed in various cancer cell lines and a high selectivity towards the breast cancer cell line MCF7 is observed with an SI around 19.

3.3 Improved Antiproliferative Activity and Fluorescence of a Dinuclear Gold(I) Bisimidazolylidene Complex via Anthracene-Modification

Christian H. G. Jakob‡, Bruno Dominelli‡, Jonas F. Schlagintweit, Pauline J. Fischer, Franziska Schuderer, Robert M. Reich, Fernanda Marques, João D. G. Correia and Fritz E. Kühn*

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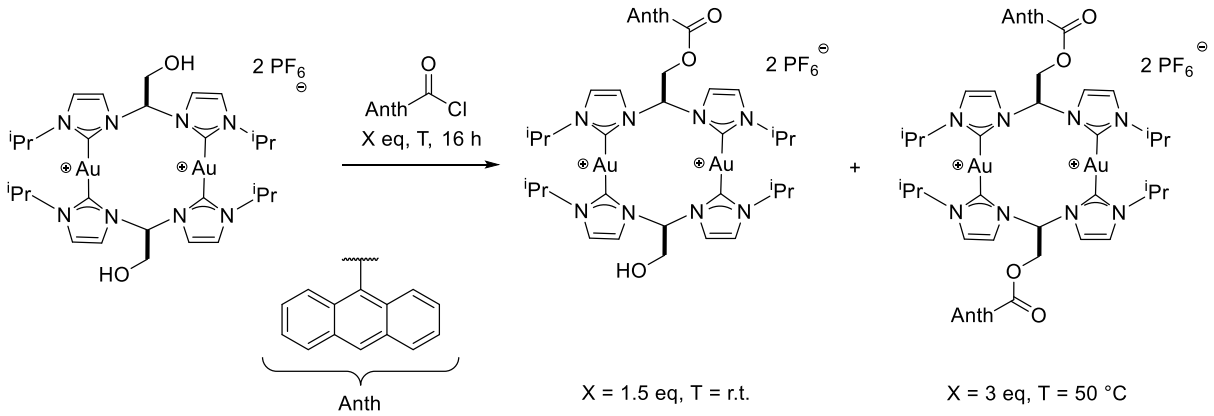
Among bioconjugation at the *N*-substituent or backbone of NHCs also the bridge can be utilized in the case of dinuclear Au(I) NHC complexes. On the one hand, the influence of a sterically demanding group at the bridge on the isomer ratio is investigated, on the other hand, the applicability of a postmodification is studied. As a proof of principle 9-anthracene carboxylic acid is used (Scheme 5).



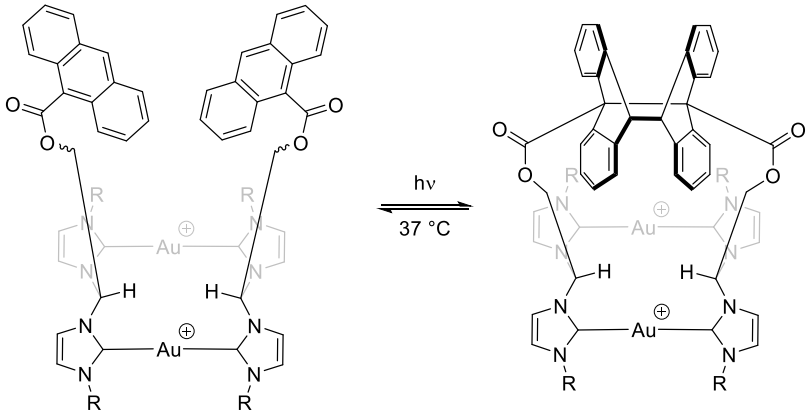
Scheme 5: Different modification routes with 9-anthroyl chloride towards luminescent Au(I) *bis*-NHC complexes.

The anthracene moiety is coupled to the complex *via* reacting the imidazolium salt or the complex with anthroyl chloride. The imidazolium salt is further converted to the corresponding Ag(I) and Au(I) complexes. While the silver complex shows only one major isomer (*anti*), during transmetalation to Au(I) different isomers are obtained. Starting from the unmodified *syn*-Au(I) complex the mono- and the di-substituted complexes can be synthesized. Coupling of two hydrophobic anthracene molecules lead to a decreased water solubility and precipitation in the cell medium. Also, an anion exchange to chloride, which usually increases the water solubility was not successful, since hydrolysis of one ester group is observed. Therefore, this complex is excluded from further biological studies. Since the mono-ester shows sufficient water solubility, the antiproliferative activity is studied against cervix- (HeLa) and breast-(MCF7) carcinoma. The complex shows good antiproliferative activity in both cell lines and also an improved selectivity with SIs of 8.8 and 8.0, respectively. Comparing these IC_{50} values to the ones of the unmodified complexes a considerable improvement is observed, which might be related to the higher lipophilicity. Moreover, due to the coupling of anthracene, these complexes show luminescence. Interestingly, the external quantum yield of the mono ester is

18%, while the quantum yield of the di-ester amounts only to 8%. This might be related to the intramolecular [2+2] photocycloaddition of the two anthracenes upon irradiation (Scheme 6).

Ex. quantum yields Φ

Imidazolium salt	28%
Mono-ester	18%
Di-ester	8%



Scheme 6: Quantum yields (left) and reversible intermolecular [2+2] photocycloaddition (right).

In conclusion, a possible postmodification route at the bridge of the *bis*-NHC ligand *via* esterification is presented which can be then transferred to more complex biomolecules, for targeting specifically certain receptors of cancer cells. Moreover, fluorescence microscopy is beneficial to investigate the mode of action.

3.4 Dinuclear Gold(I) Complexes Bearing N,N'-Allyl-Bridged Bisimidazolylidene Ligands

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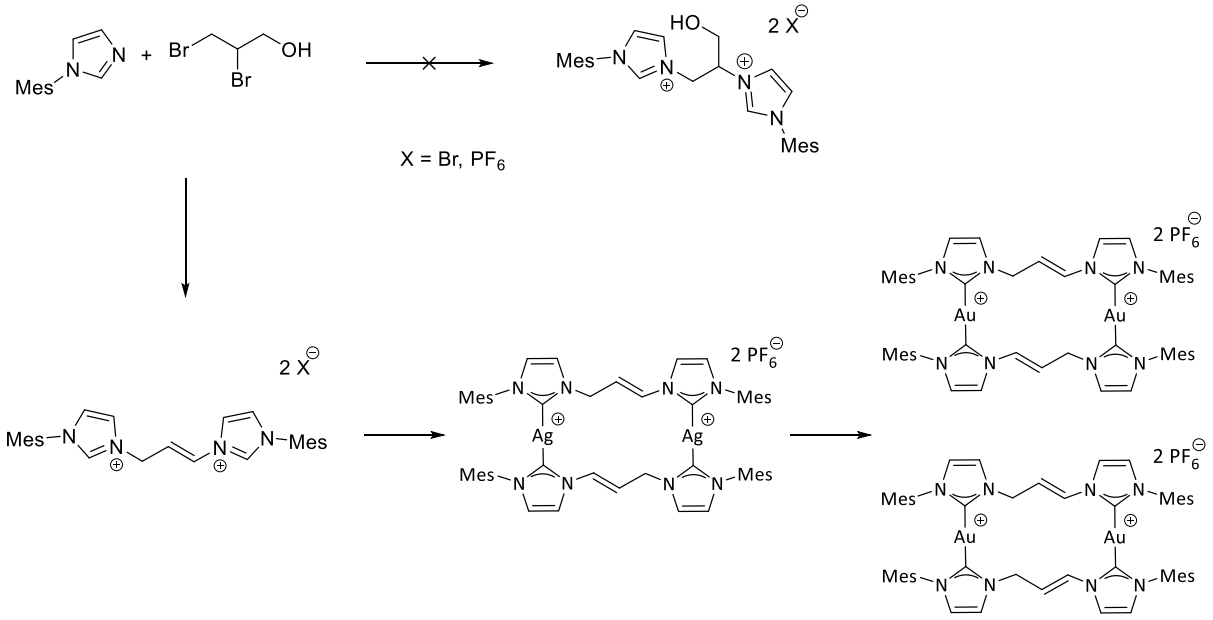
In this publication, the synthesis and characterization of a bridge functionalized *bis*-imidazolium salt and their corresponding Ag(I) and Au(I) complexes are reported. Initially, it was intended to synthesize asymmetric hydroxyl functionalized *bis*-imidazolium salts. However, the formation of an allylic bridge is observed (Scheme 7).



Scheme 7: Synthesis routes to the initially desired asymmetric *bis*-imidazolium salt (top) and the allylic bridged *bis*-imidazolium salt and its corresponding Ag(I) and Au(I) *bis*-NHC complexes.

^{1}H-NMR spectroscopy indicates the formation of the E-configuration of the olefin with coupling constants around 14 Hz. Single crystal x-ray diffraction (SC-XRD) of the complexes prove unambiguously the E-configuration and show a twisted shaped structure due to the rigid olefinic bridge. Moreover, during the transmetalation to the Au(I) complex two different isomers are formed, regarding the position of the double bond. ^{1}H-NMR spectroscopy indicates the formation of the C_2-symmetric and the point symmetric complex. In the case of the Ag(I) complex only the point symmetric isomer is observed.

Further reactions are investigated to synthesize heterometallic complexes *via* the formation of a Pd-allyl bond. The reaction of the Au(I) complex with different Pd(II) precursors and bases did not result in the formation of the desired product. Upon reacting the *bis*-imidazolium salt under the same conditions, the formation of a Pd-allyl is observed. This indicates that the

unsuccessful formation is caused by the rigid shaped structure of the Au(I) complex and is not a matter of base strength. Moreover, a photo reactivity of the *bis*-imidazolium salt and the Au(I) complex is detected. Upon irradiation at 366 nm the olefins of the bridge of the Au(I) complex isomerizes to the Z-configuration, which is indicated by lower coupling constants in the ^{1}H-NMR spectrum ($^{3}J \approx 8\text{-}9$ Hz). However, a mixture of the different isomers occurs (E/E, E/Z, Z/Z) and no intermolecular [2+2] photocycloaddition of the olefins is observed.

3.5 Anticancer and Antibacterial Properties of Trinuclear Cu(I), Ag(I) and Au(I) Macrocyclic NHC/Urea Complexes

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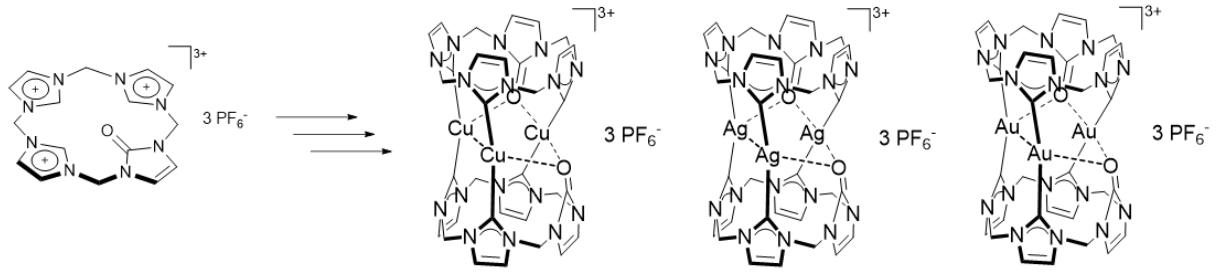
In this work, novel macrocyclic trinuclear Ag(I) and Au(I) complexes are synthesized bearing an urea moiety instead of one of the carbenes. Additionally, the antibacterial and the antiproliferative activity of these two complexes and the previously described Cu(I) complex are elaborated (Scheme 8).[190] Potential luminescence due to d^{10}-d^{10} interactions is investigated by fluorescence spectroscopy.



- Photoluminescence (Ag: $\lambda_{em,max}$ = 481 nm; Au: $\lambda_{em,max}$ = 334 nm)
- Antibacterial activity (*E. coli* and *S. aureus*: MIC: ≈ 30 µM)
- Antiproliferative activity (MCF7 and HeLa: IC_{50}: ≈ 3 µM (Ag); 12.22 µM – 25.1 µM (Cu)

Scheme 8: Synthesis of the trinuclear urea/NHC complexes and their biological and photophysical properties. Reprinted with the permission of reference [191].

Since trinuclear macrocyclic complexes are quite scarce, the structural identification is interesting. Moreover, the ligand bears a urea moiety which might influence the biological and photophysical properties. The Au(I) complex is inactive against the bacteria strains *Staphylococcus aureus* (*S. aureus,* Gram positive) and *E. coli* (Gram negative) and inactive against two cancer cell lines (HeLa and MCF7). Comparing the new complex with the previously described tetranuclear Au(I) NHC complex, which shows only moderate activities (IC_{50} = 44.5 ± 20 µM and 97.7 ± 22 µM, after 48 h incubation time), a loss in activity is observed.[192] It seems that the urea moiety has no beneficial influence on the biological properties and lowering the number of gold leads to a loss of activity. The Cu(I) complex is only moderately active against the cancer cell lines and shows no antibacterial properties. However, the Ag(I) complex shows moderate antibacterical activities and promising anticancer activities (IC_{50} ≈ 3 µM in both cancer cell lines).

The Ag(I) and Au(I) complexes show luminescence with emission maxima $\lambda_{em,max}$ = 481 nm and $\lambda_{em,max}$ = 334 nm, respectively. Interestingly, the previously described corresponding tetranuclear Ag(I) complex does not show luminescence.[193]

3.6 Fluorescent palladium(II) and platinum(II) NHC/1,2,3-triazole complexes: antiproliferative activity and selectivity against cancer cells

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Beside Au(I) NHC complexes, other transition metals are widely applied, too.[55] Despite the variety of different metal complexes, which are investigated at an academic stage, mainly platinum complexes are clinically applied as anticancer metallodrugs.[36] Due to the above mentioned side effects of common chemotherapeutics, various alternatives are tested. In this work previously synthesized Pd and Pt complexes bearing NHC/triazole hybrid ligands and 2,6-diisopropylphenyl (Dipp) wingtips are tested against their antiproliferative activity.[194] Depending on the synthetic conditions, two different coordination modes can be obtained. On the one hand one ligand is coordinating in a tetradentate fashion, with two NHCs and two triazoles as donor ligands. On the other hand, two ligands are coordinating in a bidentate manner, with only the NHCs coordinating to the metal. Additionally, the Dipp *N*-substituents are replaced by 4-methylene-7-methoxycoumarin (MMC), with the aim to apply these complexes in fluorescent microscopy to investigate the mechanism of action (Figure 18).

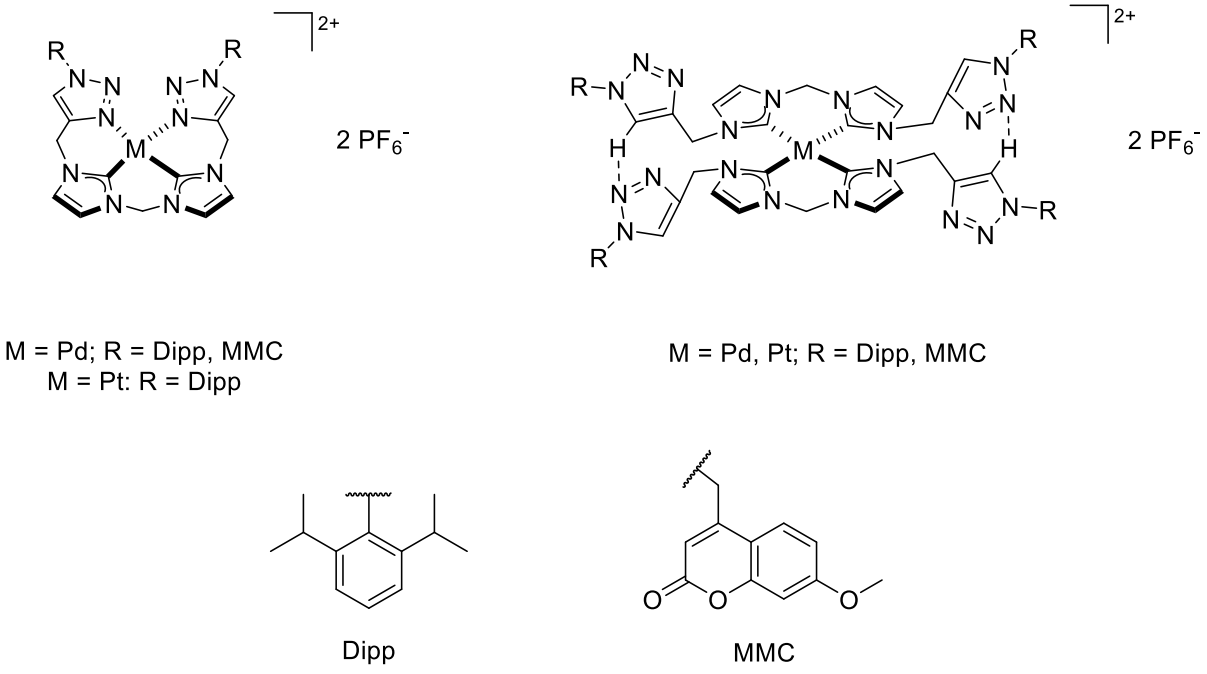

Figure 18: Complexes tested for their antiproliferative activity against cancer and helthy cells.

The preligands are synthesized *via* reacting the methylene *bis*-imidazolium salt with alkyne *N*-substituents with the respective azide in a copper catalyzed azide-alkyne cycloaddition

reaction (CuAAC, “click-reaction”). Similar routes, which are applied for the Dipp or MMC substituents can be easily transferred to more biological relevant groups.

The antiproliferative activity against HeLa and MCF7 cancer cell lines and against healthy HaCaT cell line strongly depends on the coordination mode and on the wingtips. With Dipp groups the complexes exhibit a higher activity than the corresponding complexes with MMC. In addition, the complexes bearing one ligand are only moderately active or not active, depending on the R group. These observations are presumably related to the different lipophilicity, the higher the lipophilicity, the higher the antiproliferative activity. While the metal has no significant influence on the Dipp substituted complexes, a strong influence is noted with the MMC ones. In the case of the MMC complexes only the Pd complex with two ligands is active in HeLa, while the isostructural Pt complex is inactive. Interestingly, the MMC complex is also inactive in the healthy cell line HaCaT, amounting to a SI of >16.

The MMC complexes exhibit external quantum yields between 7% and 9% with a absorption maxima at $\lambda_{abs,max} \approx 324$ nm. Interestingly depending on the metal, the wavelength of the emission maximum is shifted. The Pd complexes show the maximum at $\lambda_{em,max} \approx 401$ nm, and the maximum of the Pt complex is shifted to $\lambda_{em,max} = 425$ nm.

The quantum yields are sufficient for fluorescence microscopy and consequently the complexes are applied in cell imaging studies in HeLa and HaCaT. The difference in the activity can be related to the uptake of the compounds into the cells. While the tetradentate ones are not imported into the cells, the bidentate Pd complex is readily imported. The active Pd-MMC complex is presumably located in late endosomes and lysosomes and no fluorescence is observed in the nucleus, which suggests that the DNA is not the main target. Interestingly, in HaCaT a similar behavior is observed, but no antiproliferative activity is obtained.

The Pd complex bearing two ligands with MMC groups, is a promising candidate for further studies, since the complex is exclusively active in HeLa and not in a healthy cell line. Furthermore, detailed mechanistic investigations are necessary to find an explanation for the difference in the antiproliferative activity.

4 CONCLUSION AND OUTLOOK

In this thesis different metal NHC complexes were synthesized, characterized and evaluated as potential anticancer agents. Due to the high stability of the NHC ligand, mainly *bis*-NHCs were applied to ensure sufficient stability under biological conditions (e.g. water or thiols). A strong focus lies on the synthesis of Au(I) complexes, due to the different mechanism of action in comparison to currently applied metallodrugs (e.g. cisplatin) which potentially allow overcoming current resistances. The herein described complexes bearing a functional group allow for further bioconjugation to selectively address cancer cells. All suitable complexes are tested for their antiproliferative activity *in vitro* and with the most promising complexes, further studies are carried out to investigate the mechanism of action.

A series of novel histidine derived Au(I) *bis*-NHC complexes were synthesized, varying in the functional group of the carbonyl (carboxylic acid, ester, amide), showing high stability against GSH and cysteine. However, depending on the functionality of the carbonyl group slow hydrolysis of the ester bond in aqueous media is observed, while the Au(I)-carbene bond is stable over 48 h. The antiproliferative activity of the ester containing complex is only slightly lower than the one of the corresponding amide moiety, although the corresponding carboxylic acid is inactive. These observations might indicate that the cellular uptake is faster than the hydrolysis, maintaining the notable cytotoxicity. PIXE microscopy shows that the cellular distribution profile of the active complexes might be different. However, further studies about the uptake are necessary to prove this assumption. By quantifying the amount of gold in the cellular organelles by ICP-MS the distribution of the complexes can be investigated. Moreover, identifying the cellular target is crucial for understanding the mechanism of action. Although high stability against cysteine is observed, TrxR still might be the cellular target. In the seminal work of Berners-Price *et al.* it was reported that Au(I) *bis*-NHC complexes show a higher reactivity to selenocysteine, which is part of the activity site of TrxR, compared to cysteine, which is part of the active site in GR.[129] Since only a slight selectivity of the complexes against cancer cells is obtained (SIs around 4), conjugation of a biomolecule to selectively address cancer cells might be beneficial. The conjugation might be conducted at the carboxylic acid or amine groups in the backbone, since this conjugation should not strongly affect the steric or electronic properties of the NHC. Also, molecules for cellular imaging are beneficial to investigate cellular distribution.

In another part of this thesis, the mechanism of action of dinuclear hydroxyl bridged Au(I) *bis*-complexes and the influence of the isomers are investigated. During the synthesis of the Au(I) complexes two different major isomers are obtained which can be separated by RP-HPLC. SC-XRD analysis confirmed the first species as *anti*- and the second as *syn*-isomer. A different

reactivity of both isomers against cysteine is observed, especially for the complexes with the less sterically demanding *N*-substituents ^{i}Pr. While the *syn*-isomer is inert at 37 °C with cysteine, with the *anti*-isomer a single ligand exchange is observed. Increasing the temperature to 50 °C the *syn*-isomer reacts similarly. DFT calculations revealed that the hydroxyl groups can form hydrogen bonds with the functional groups of cysteine. In the case of the *anti*-isomer two cysteine molecules can interact with the complex and the sulphur is directed to the gold nuclei, enabling a nucleophilic attack. For the *syn*-isomer only one cysteine can interact *via* two hydrogen bonds, blocking the sulphur from interacting with the gold nuclei. Moreover, the second site is blocked by the *N*-substituents preventing a nucleophilic attack. This different reactivity against thiols correlates with the TrxR affinity, the *anti*-isomer with Mes wingtips shows an IC_{50} value which is nine times lower compared to the *syn*-isomer. However, the IC_{50} values are still remarkable (IC_{50}: 16 nM *anti* and 145 nM *syn*), which might be related to a higher affinity against selenocysteine, which was also observed for other gold complexes.[129] Further biological studies revealed that the complexes accumulate in the membranes of the cells, indicating that inhibition of the mitochondrial TrxR is most likely the mechanism of action. Moreover, the *syn* complex with Mes *N*-substituents shows promising antiproliferative activity against various cancer cell lines and remarkably high selectivity against breast cancer cell lines, especially against MCF7, with a SI of 19. Upcoming studies should investigate the mode of cell death. If TrxR inhibition is the only mode, exclusively apoptosis should be observed. If other mechanisms play a role, also undefined cell death (so-called necrosis) might occur. Since a high selectivity and good antiproliferative activities are achieved, further studies should investigate if the complex can overcome common drug resistances and if this is the case, further *in vivo* studies are possible.

The hydroxyl group allows postmodification to conjugate a labeling molecule for detailed distribution studies. As a proof of principle, a luminescent anthracene molecule was conjugated to the hydroxyl bridge modified Au(I) *bis*-NHC complexes *via* esterification. Depending on the amount of anthracene one hydroxyl group remains, which can be used for further conjugations. When conjugating two hydrophilic anthracene molecules to the complex with ^{i}Pr substituents, the water solubility is significantly decreased, which leads to precipitation in aqueous media. However, conjugating one anthracene the complex is sufficiently water soluble, and the antiproliferative activity is significantly increased compared to the unmodified complex. Moreover, a certain selectivity with SIs around 8 is observed. Additionally, the mono-ester exhibits an external quantum yield of 18%, which should be sufficient for further fluorescent microscopy studies to investigate the cellular distribution *in vitro*. Due to the commonly observed lability of esters in aqueous media, the hydroxyl group should be converted to an amine, to conduct an amidation reaction under similar conditions. Afterwards coupling a chelator for radioactive labeling would allow for *in vivo* imaging.

5 APPENDIX

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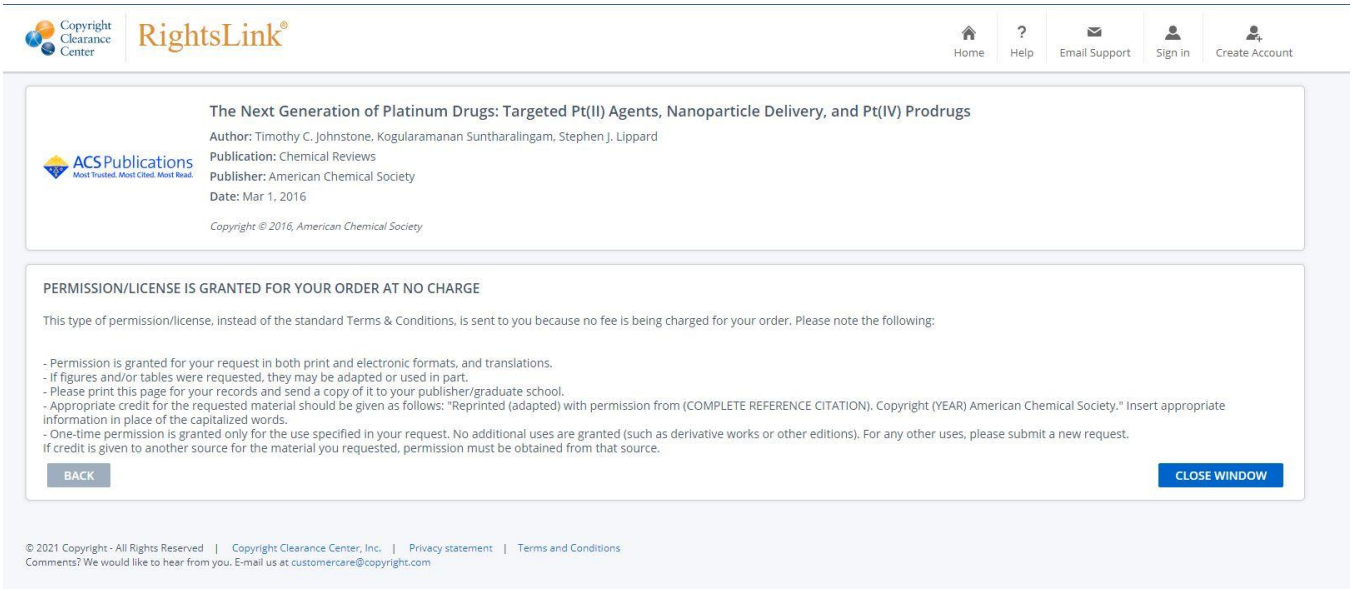

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Title: Dinuclear Gold(I) Complexes Bearing N,N'-Allyl-Bridged Bisimidazolylidene Ligands

Published in: *Chem Asian J.* **2020***, 15,* 1848–1851*.*

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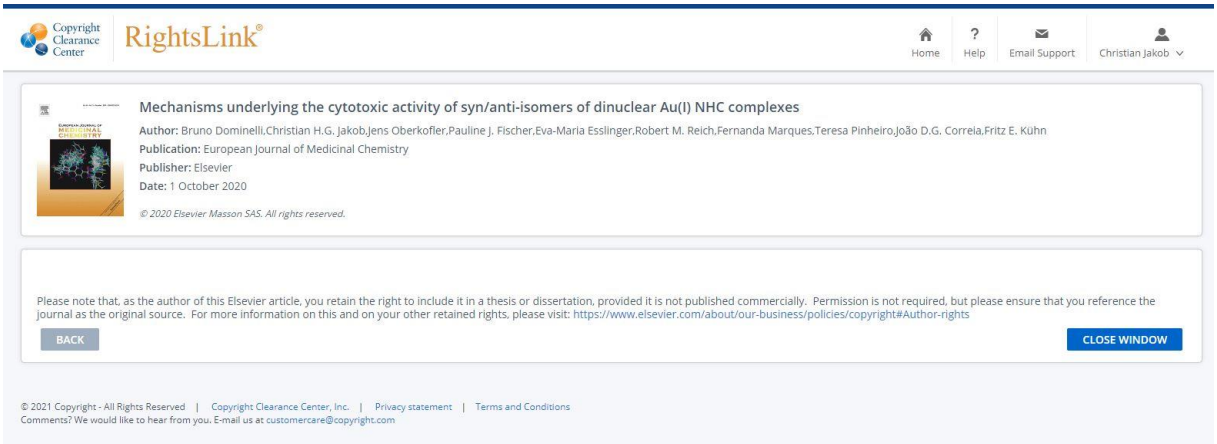

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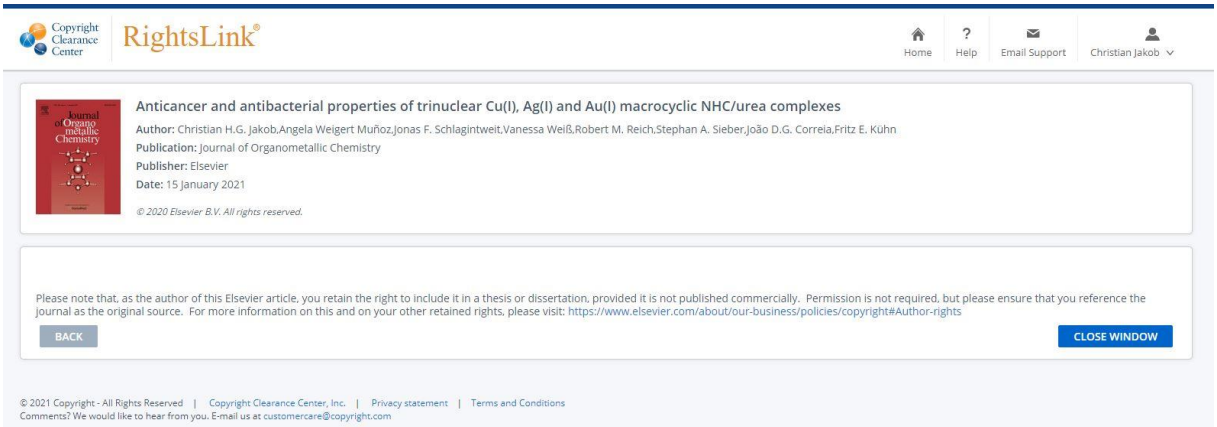

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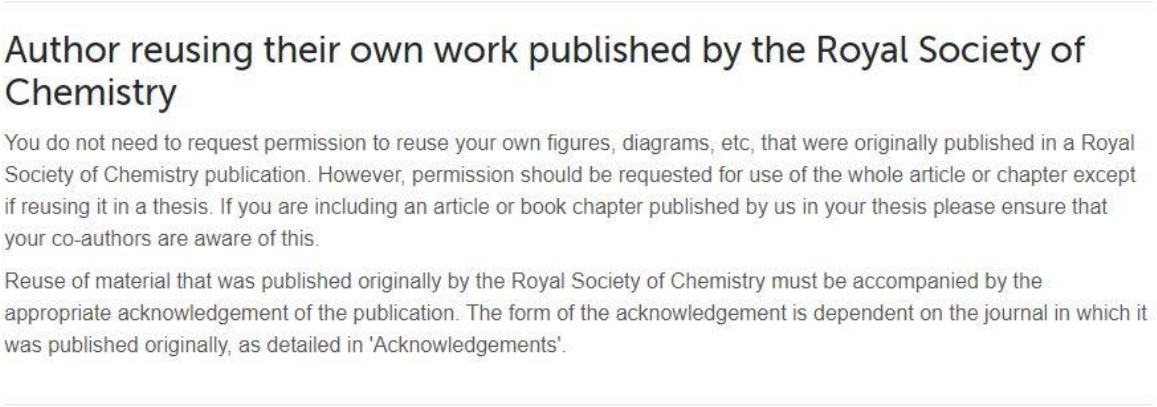

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5.2 Bibliographic Data of Complete Publications

Antiproliferative Activity of Functionalized Histidine-derived Au(I)bis-NHC Complexes for Bioconjugation

Christian H. G. Jakob,[a] Bruno Dominelli,[a] Eva M. Hahn,[a] Tobias O. Berghausen,[a] Teresa Pinheiro,[b] Fernanda Marques,[c] Robert M. Reich,[a] João D. G. Correia[c] and Fritz E. Kühn*[a]

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*Corresponding author

Chem. Asian J. **2020***, 15,* 2754–2762*.* Direct Link: 10.1002/asia.202000620

Mechanisms underlying the cytotoxic activity of syn/anti-isomers of dinuclear Au(I) NHC complexes

Bruno Dominelli‡,[a] Christian H. G. Jakob‡,[a] Jens Oberkofler, Pauline J. Fischer,[a] Eva-Maria Esslinger, [a] Robert M. Reich, [a] Fernanda Marques,[b] Teresa Pinheiro,[c] João D. G. Correia[c] and Fritz E. Kühn*[a]

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‡ These authors contributed equally to this work

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Eur. J. Med. Chem., **2020**, *203*, 112576-12586. Direct Link: 10.1016/j.ejmech.2020.112576

Improved Antiproliferative Activity and Fluorescence of a Dinuclear Gold(I) Bisimidazolylidene Complex via Anthracene-Modification

Christian H. G. Jakob‡,[a] Bruno Dominelli‡,[a] Jonas F. Schlagintweit,[a] Pauline J. Fischer,[a] Franziska Schuderer,[a] Robert M. Reich,[a] Fernanda Marques,[b] João D. G. Correia[b] and Fritz E. Kühn*[a]

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Chem Asian J. **2020***, 15,* 4275–4279*.* Direct Link: 10.1002/asia.202001104

Dinuclear Gold(I) Complexes Bearing N,N'-Allyl-Bridged Bisimidazolylidene Ligands

Christian H. G. Jakob‡,[a] Bruno Dominelli‡,[a] Julia Rieb,[a] Christian Jandl,[a] Alexander Pöthig,[a] Robert M. Reich,[a] João D. G. Correia[b] and Fritz E. Kühn*[a]

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Chem Asian J. **2020***, 15,* 1848–1851*.* Direct Link: 10.1002/asia.202000453

Anticancer and antibacterial properties of trinuclear Cu(I), Ag(I) and Au(I) macrocyclic NHC/urea complexes

Christian H.G. Jakob‡,[a] Angela Weigert Muñoz‡,[b] Jonas F. Schlagintweit,[a] Vanessa Weiß,[a],[c] Robert M. Reich,[a] Stephan A. Sieber,[b] João D.G. Correia[d] and Fritz E. Kühn*[a]

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J. Organomet. Chem., **2021***, 932,* 121643-121646*.*

Direct Link: 10.1016/j.jorganchem.2020.121643

Fluorescent palladium(II) and platinum(II) NHC/1,2,3-triazole complexes: antiproliferative activity and selectivity against cancer cells

Jonas F. Schlagintweit‡,[a] Christian H. G. Jakob‡,[a] Kevin Meighen-Berger,[b] Thomas F. Gronauer,[c] Angela Weigert Muñoz,[c] Vanessa Weiß,[a],[d] Matthias J. Feige,[b] Stephan A. Sieber,[c] João D. G. Correia[e] and Fritz E. Kühn*[a]

[a] Molecular Catalysis, Catalysis Research Center and Department of Chemistry, Technische Universität München, Garching 85748, Germany.

[b] Cellular Protein Biochemistry, Department of Chemistry and Institute for Advanced Study Technische Universität München, Lichtenbergstraße 4, Garching 85748, Germany.

[c] Chair of Organic Chemistry II, Department of Chemistry, Technische Universität München, Lichtenbergstraße 4, Garching 85748, Germany.

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‡ These authors contributed equally to this work

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Dalton Trans., **2021***, 50*, 2158-2166.

Direct Link: 10.1039/D0DT04114A.

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5.4 Complete List of Publications

Journal Articles

[1] Christiane M. Egger‡, Christian H.G. Jakob‡, Felix Kaiser‡, Olivia Rindle, Philipp J. Altmann, Robert M. Reich and Fritz. E. Kühn, "Reactivity Studies of a Dipyridine Ethinyl Ligand with Zinc(II)", *Eur. J. Inorg. Chem.*, **2019,** *48*, 5059-5065.

[2] Christian H. G. Jakob‡, Bruno Dominelli‡, Julia Rieb, Christian Jandl, Alexander Pöthig, Robert M. Reich, João D. G. Correia, and Fritz E. Kühn, "Dinuclear Gold(I) Complexes Bearing N,N'-Allyl-Bridged Bisimidazolylidene Ligands", *Chem. Asian J.* **2020**, *15*, 1848-1851.

[3] Christian H. G. Jakob, Bruno Dominelli, Eva M. Hahn, Tobias O. Berghausen, Teresa Pinheiro, Fernanda Marques, Robert M. Reich, João D. G. Correia and Fritz E. Kühn "Antiproliferative Activity of Functionalized Histidine-derived Au(I)*bis*-NHC Complexes for Bioconjugation", *Chem. Asian J.* **2020**, *15*, 2754-2762.

[4] Jonas F. Schlagintweit‡, Florian Dyckhoff‡, Linda Nguyen, Christian H.G. Jakob, Robert M.Reich, Fritz E.Kühn Mixed tetradentate NHC/1,2,3-triazole iron complexes bearing cis labile coordination sites as highly active catalysts in Lewis and Brønsted acid mediated olefin epoxidation, *J. Catal.*, **2020**, *383*, 144-152.

[5] Bruno Dominelli‡, Christian H.G. Jakob‡, Jens Oberkofler, Pauline J. Fischer, Eva-Maria Esslinger, Robert M. Reich, Fernanda Marques, Teresa Pinheiro, João D.G. Correia and Fritz E. Kühn, "Mechanisms underlying the cytotoxic activity of syn/anti-isomers of dinuclear Au(I) NHC complexes", *Eur. J. Med. Chem.*, **2020**, *203*, 112576-12586.

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