universität wien

MASTERARBEIT

Titel der Masterarbeit

Synthesis, *in vitro* antitumor activity and DNA interactions of guanidine platinum(II) complexes

angestrebter akademischer Grad

Master of Science (MSc)

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Wien, im Juli 2010

Acknowledgements

The author is deeply grateful to all those people who directly or indirectly were involved or contributed to this work.

I would like to thank O. Univ.-Prof. Dr. Dr. Bernhard K. Keppler for giving me the opportunity to carry out my Master Thesis in his multidisciplinary and perfectly equipped group, for his encouragement, financial support and supervising the work.

I am very grateful for the assistance of Dr. Michael Jakupec for direct management, careful and critical supervision of the work at all stages, verification and discussion of results and thesis editing.

The synthetic part of the work was performed at the Chemistry Department of St. Petersburg State University (2009). The author thanks his supervisor PhD Bokach N.A. for the overall management during the synthetic part of the work.

The author is sincerely grateful to all members of the research group for their, moral support and participation in the discussion of the results. Special thanks to MSc Gerhard Muehlgassner, Dipl.-Ing. (FH) Caroline Bartel, Dipl.-Ing. (FH) Robert Trondl, BSc Maria Novak, Mag. Seied-Mojtaba Valiahdi, colleagues Simone Goeschl and Michaela Hejl for their constant support, collaboration and fellowship.

Besides the people who I worked with at the University, I want to thank my family and friends for supporting me throughout the period of my study.

Abstract

Recently it was discovered that platinum iminoether and amidine complexes have a high inhibitory potency on cancer cell proliferation and viability and probably possess a mechanism of action different from classic platinum drugs, as the compounds with *trans*-configuration are more active than *cis*-isomers.

In this work we investigated related platinum(II) complexes with the structural element $(guanidine)_2Pt^{II}$, prepared by nucleophilic addition of ammonia to the push-pull nitrile ligands in *cis*- and *trans*-$[PtCl_2(RCN)_2]$ (R = NMe_2, NEt_2, NC_5H_{10}). The neutral complexes *cis*-**1**–**3** and *trans*-**1**–**3** as well as the cationic complexes *cis*-**4**–**6** and *trans*-**4**–**6**, which contain ammine instead of chloro ligands, were synthesized and characterized by Tyan M. supervised by Prof. Dr. Kukushkin V. at the Chemistry department of St. Petersburg University. In the presented work we have synthesized cationic guanidine compounds *trans*-$[Pt(NH_2Me)_2\{NH{=}C(NHMe)NR\}_2](Cl)_2$ (R = NEt_2, NC_5H_{10}) (*trans*-**7**,**8**) by nucleophilic addition of methylamine to cyanamide platinum(II) complexes. These compounds were purified and characterized by various physical and chemical methods (elemental analysis, ESI mass spectrometry, IR, ^1H NMR and $^{13}C\{^1H\}$ NMR). We have studied the biological activity of the entire set of guanidine compounds (*cis*-**1**–**6** and *trans*-**1**–**8**), using various bioanalytical methods.

Cytotoxic activity of guanidine platinum(II) complexes was determined using the colorimetric MTT assay in two human cancer cell lines: CH1 (ovarian carcinoma) and SW480 (colon cancer). IC_{50} values of these compounds vary within a broad range of micromolar concentrations. We could confirm the expectation that the cytotoxicity of several *trans*-configured complexes is higher than that of *cis*-congeners. The analysis showed that there is a correlation between the cytotoxic potency of the compounds and the length of the variable residue on the guanidine ligand. Moreover, cytotoxicities of the cationic complexes are mostly lower, but in some cases even comparable to those of the corresponding neutral complexes, despite the lack of distinctly labile ligands.

In order to figure out whether cytotoxicity correlates with cellular uptake of the compounds cellular platinum levels were analyzed by ICP-MS upon treatment of adherent SW480 cells. Modified cellular uptake experiments (non-adherent state) were performed

alternatively to determine the cellular levels of those compounds that have a much stronger affinity to the polystyrene of the culture plates than to the cells.

Since the difference in cytotoxic potency between cisplatin and transplatin has been ascribed to different capacities of DNA binding, DNA interactions of several compounds were studied in order to find hints for the possible reasons for their different activities. For this purpose, an electrophoretic mobility shift assay indicating the impact on the secondary structure of plasmid DNA in cell-free conditions was employed. Furthermore, DNA platination was quantified in treated SW480 cells by ICP-MS. In the latter method platinum concentration was measured together with phosphorus concentration to determine amount of platinum relative to the nucleotides. The results obtained from the electrophoretic mobility shift assay, cellular uptake and DNA platination experiments are consistent with the assumption that DNA is the main target for guanidine platinum(II) complexes.

Zusammenfassung

In jüngster Zeit wurde entdeckt, dass Platin-Iminoether- und -Amidine-Komplexe ein starkes Vermögen, die Proliferation von Krebszellen zu hemmen, und wahrscheinlich einen von klassischen Platinverbindungen abweichenden Wirkmechanismus aufweisen, da die Verbindungen mit *trans*-Konfiguration aktiver sind die als die *cis*-Isomere.

In der vorliegenden Arbeit wurden nahe verwandte Platin(II)-Komplexe mit dem Strukturelement $(Guanidin)_2PtII$, welche mittels nukleophiler Addition von Ammoniak an die „Push-Pull"-Nitrilliganden in *cis*- und *trans*-$[PtCl_2(RCN)_2]$ ($R = NMe_2$, NEt_2, NC_5H_{10}) hergestellt werden, untersucht. Die neutralen Komplexe *cis*-**1**–**3** und *trans*-**1**–**3** sowie die kationischen Komplexe *cis*-**4**–**6** und *trans*-**4**–**6**, welche Ammin- anstelle von Chloro-Liganden enthalten, wurden von M. Tyan unter der Betreuung von Prof. Dr. V. Kukushkin am Department für Chemie der Universität St. Petersburg, Russland, synthetisiert und charakterisiert. In der vorliegenden Arbeit wurden die kationischen Guanidin-Verbindungen *trans*-$[Pt(NH_2Me)_2\{NH{=}C(NHMe)NR\}_2]Cl_2$ ($R = NEt_2$, NC_5H_{10}) (*trans*-**7**,**8**) mittels nukleophiler Addition von Methylamin an Cyanamid-Platin(II)-Komplexe synthetisiert. Diese Verbindungen wurden gereinigt und mittels verschiedener physikalischer und chemischer Methoden (Elementaranalyse, ESI-Massenspektrometrie, IR-Spektroskopie, ^1H- und $^{13}C\{^1H\}$-NMR-Spektroskopie) charakterisiert. Die biologische Aktivität wurde an dem gesamten Reihe von Guanidin-Verbindungen (*cis*-**1**–**6**, *trans*-**1**–**8**) mittels verschiedener bioanalytischer Methoden untersucht.

Die Zytotoxizität der Guanidin-Platin(II)-Komplexe wurde anhand des kolorimetrischen MTT-Tests in zwei humanen Krebs-Zellinien bestimmt: CH1 (Ovarialkarzinom) und SW480 (Kolonkarzinom). Die IC_{50}-Werte dieser Verbindungen variieren in einem großen Bereich mikromolarer Konzentrationen. Dabei konnte die Erwartung, dass die Zytotoxizität mehrerer *trans*-konfigurierter Komplexe jene der *cis*-Analoga übertrifft, bestätigt werden. Weiters wurde eine Korrelation zwischen der zytotoxischen Potenz und der Länge des variablen Rests am Guanidin-Liganden festgestellt. Die Zytotoxizitäten der kationischen Komplexe sind zwar überwiegend geringer, in einigen Fällen aber auch vergleichbar jenen der korrespondierenden neutralen Komplexe, trotz des Fehlens ausgesprochen labiler Liganden.

Um herauszufinden, ob die Zytotoxizität mit der zellulären Aufnahme der Verbindungen korreliert, wurden die zellulären Platingehalte nach Behandlung von adhärenten SW480-Zellen mittels ICP-MS analysiert. Modifizierte Experimente zur zellulären Aufnahme (im nicht-adhärenten Zustand) wurden alternativ durchgeführt, um auch die zellulären Gehalte jener Verbindungen, die eine wesentlich höhere Affinität zum Polystyrol der Kulturplatten als zu den Zellen aufweisen, zu bestimmen.

Da der Unterschied der zytotoxischen Potenzen von Cisplatin und Transplatin mit dem unterschiedlichen Vermögen, an DNA zu binden, erklärt wird, wurden die DNA-Interaktionen mehrerer Verbindungen untersucht, um Hinweise auf mögliche Gründe für die unterschiedlichen Aktivitäten zu finden. Für diesen Zweck wurde ein Electrophoretic Mobility Shift Assay, welcher die Auswirkung auf die Sekundärstruktur einer Plasmid-DNA unter zellfreien Bedingungen veranschaulicht, herangezogen. Weiters wurde das Ausmaß der DNA-Platinierung in behandelten SW480-Zellen mittels ICP-MS quantifiziert. Dabei wurden Platin und Phosphor zugleich gemessen, um den Gehalt an Platin in Relation zur Anzahl der Nukleotide angeben zu können. Die Ergebnisse des Electrophoretic Mobility Shift Assay, der zellulären Aufnahme und der DNA-Platinierung stehen im Einklang mit der Annahme, dass die DNA das Haupt-Target von Guanidin-Platin(II)-Verbindungen ist.

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1. INTRODUCTION

1.1 Epidemiology of Cancer

Cancer is one of the frequently talked about and most frightening diseases that have increased in incedence, rather rapidly, in the past several decades. The disease which was for a long time dubbed as the death sentence for the person diagnosed with it has now become more manageable, thanks to the fervent efforts of researchers and oncologists. Science has made rapid strides to provide many preventive and curative strategies to fight with cancer diseases.

There were about 3.2 million new cases of cancer and 1.7 million deaths from cancer in Europe in 2008 (**Fig. 1.1**). The most common types of cancer disease were colorectal cancer (436,000 cases, 13.6% of the total), breast cancer (421,000, 13.1%), lung cancer (391,000, 12.2%) and prostate cancer (382,000, 11.9%). The most common death causing cancers were lung cancer (342,000 deaths, 19.9% of the total), colorectal cancer (212,000 deaths, 12.3%), breast cancer (129,000, 7.5%) and stomach cancer (117,000, 6.8%) [1].

Around 36,000 people are diagnosed with cancer each year in Austria, with more than half being diagnosed with intestinal, lung, breast or prostate cancer. The risk of a person being diagnosed with one of these cancers before the age of 75 is approximately 10% in 2010. The risk of cancer in general before the age of 75 is as high as 27% and is markedly higher for men than for women. 33 in 100 men will be diagnosed with cancer before the age of 75, compared to 22 in 100 women.

Since 1994, the most common cancer for men has been prostate cancer, with 78.4 cases per 100,000 (4,986 cases in absolute terms) in 2007. Previously, the most frequently diagnosed cancer was lung cancer. A particularly sharp decline can be seen in malignant neoplasms of the stomach, while other types of cancer show fairly small fluctuations or increasing incidence (e.g., lymphoma, melanoma). The increase in the annual number of new cases of cancer in men during the observation period can be attributed for the most part to the growing number of prostate cancers. The most common cancer site in women continues to be breast (www.statistik.at).

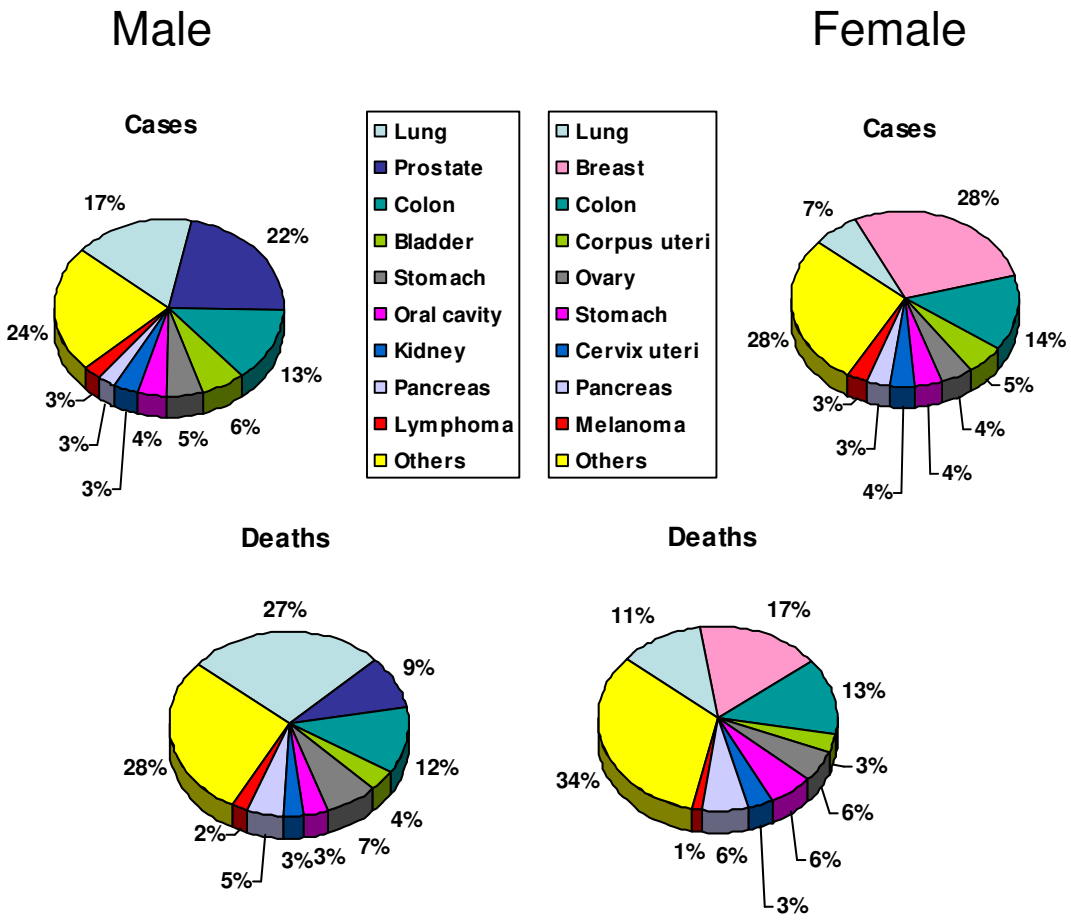


Figure 1.1. Distribution of the estimated cases and deaths for the 10 most common cancers in Europe in 2008. For each sex, the area of the segment of the pie chart reflects the proportion of the total number of cases or deaths [1].

1.2 Carcinogenesis

Cell division is a physiological process that occurs in almost all tissues and under many circumstances. Under normal circumstances, the balance between proliferation and programmed cell death, usually in the form of apoptosis, is maintained by tightly regulating both processes to ensure the integrity of organs and tissues. Mutations in DNA that lead to cancer (only certain mutations can lead to cancer and the majority of potential mutations will have no bearing) disrupt these orderly processes by disrupting the regulation of these processes.

Carcinogenic mutations results in uncontrolled cell division. The uncontrolled and often rapid proliferation of cells can lead to benign tumors first; some of these may turn into malignant tumors (cancer) by additional mutations. Benign tumors do not spread to other parts of the body or invade other tissues, and they are rarely a threat to life unless they compress vital structures or are physiologically active, for instance, producing a hormone. Malignant tumors can invade other organs by actively penetrating and destroying the surrounding tissue structures, spread to distant locations (metastasis) via the vascular and lymphatic systems and become life-threatening [2].

More than one mutation is necessary for carcinogenesis. In fact, a series of several mutations to certain classes of genes is usually required before a normal cell will transform into a cancer cell. Molecular analyses of human tumors carried out in the last decade have revealed that the genetic alterations of cancer cells are much more numerous and unstable than previously thought [3]. For instance, by sampling colorectal premalignant polyp and carcinoma cell genomes, Stoler et al. [4] found that the mean number of genomic changes per carcinoma cell was approximately 11,000. In addition, much evidence has accumulated stating that mutations (changes in the DNA sequence) are not the only cause of the altered gene expression of cancer cells. Epigenetic alterations (heritable and reversible changes other than the DNA sequence) and aneuploidy (numerical and structural abnormalities in chromosomes) are common alterations in tumor cells, which modify gene expression and may also play a crucial role in carcinogenesis [5,6,7].

It is well known that uncontrolled cell proliferation is the most relevant feature of cancer. It is also recognized that the genetic defects of cancer cells result in an altered gene expression and in the production of signals that make these cells proliferate in an uncontrolled fashion.

Equally important for cell proliferation is that the dividing cell duplicates all its cellular components to create two daughter cells [8]. To do this, proliferating cells must take nutrients, including micro- and macromolecules from the blood and use them to synthesize all components required for the formation of a new cell. Therefore the cellular uptake of different molecules including glucose from the blood is required for the synthesis of these macromolecules and cellular components (**Fig. 1.2**).

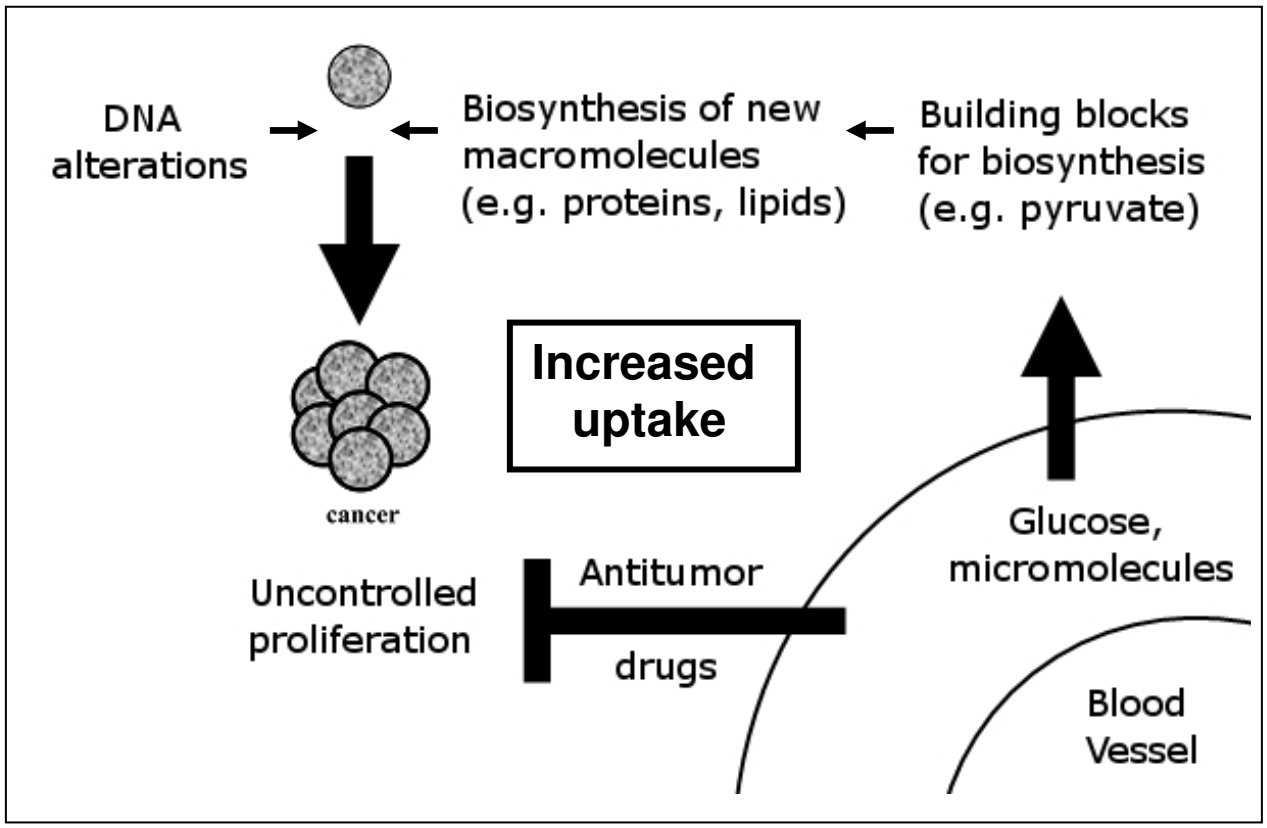


Figure 1.2. Cancer development requires both DNA alterations and a change in the metabolism. The higher rate of cancer cell proliferation leads to an increase in macro- and micro-molecule uptake, which may facilitate accumulation of anticancer agents.

On the one hand the increased cellular uptake gives cancer cells an opportunity to divide fast; on the other hand it facilitate cancer treatment with metal-based or other therapeutic agents due to increased active transport [8,42] and increased membrane fluidity [43].

1.3 Chemotherapy and Pt-based Therapeutic Agents

Chemotherapy is the general term for any treatment involving the use of chemical agents to stop cancer cells from growing. Chemotherapy can eliminate cancer cells at sites remote from the primary tumor site. Therefore, chemotherapy is considered a ***systemic*** treatment. More than half of all people diagnosed with cancer receive chemotherapy. Cancer treatments are tailored to the specific needs of the patient. Increasingly, it is common to use several treatments or modalities at the same time or in sequence in order to prevent both local recurrence and recurrence throughout the body. When modalities are combined to treat cancer, this is referred to as a multi-modality treatment. In addition to chemotherapy, other modalities include:

- surgery (physical removal);
- radiation therapy (exposure to high energy particle beams);
- biologicals therapy (monoclonal antibodies);
- hormonal therapy (interference with hormone-dependent growth);
- immunomodulating therapy (modulation of immune system, e.g. interferons).

Chemotherapy works by destroying cancer cells, unfortunately, it cannot tell the difference between a cancer cell and some healthy cells. Chemotherapeutic agents frequently eliminate not only the fast-growing cancer cells but also other fast-growing normal cells in the body, including, hair follicles and hematopoietic cells. An undesirable consequence of chemotherapy affecting patient's body which is not related to the cancer is referred to as a complication of treatment, or a *side effect*. Most of these side effects induced by tolerable doses of the drugs are reversible after treatment. Even though a relatively high burden of side effects is usually accepted in the treatment of this life-threatening disease, great efforts are being made to develop drugs that are better tolerated by the patients.

In the last several decades many biochemical and molecular biology discoveries have been made. These discoveries help to solve the urgent problems of medicine and pharmacology, which led to the broad investigations and the development of new industry for novel therapeutic agents. The spectrum of currently used chemotherapeutics is extremely wide, one of the main streams is the invention of novel metal based drugs which are capable of binding to the most common biomolecular targets of the cells – DNA, proteins, amino acids *etc*.

One of the most interesting classe of substances, which specifically bind to different biomolecules of the living cells are the platinum based complexes. Today several platinum based drugs are being used in the clinics and play a leading role in cancer chemotherapy all over the world.

The most well know and widely spread currently is the neutral complex *cis*-diamminedichloroplatinum(II) (CDDP, cisplatin), whose biological activity was discovered by Rosenberg B. in 1965 [9]. The principal drawbacks of CDDP are high toxicity, mutagenic effects and low selectivity between cancer and normal cells. These shortcomings led to the investigations of complexes with platinum and other transition elements [10]. Main goal of current research is to synthesize novel substances with a better ratio «cytotoxic activity/side effects».

Right after the discovery of the cytotoxic effect of cisplatin (**Fig. 1.3**), many laboratories all over the world started the investigations of CDDP's structural analogues. Unfortunately very few discovered drugs have a better therapeutic index (ratio of antitumour activity/toxicity to the patient's body). One of the most successful drug recently – carboplatin – wins due to fewer side effects.

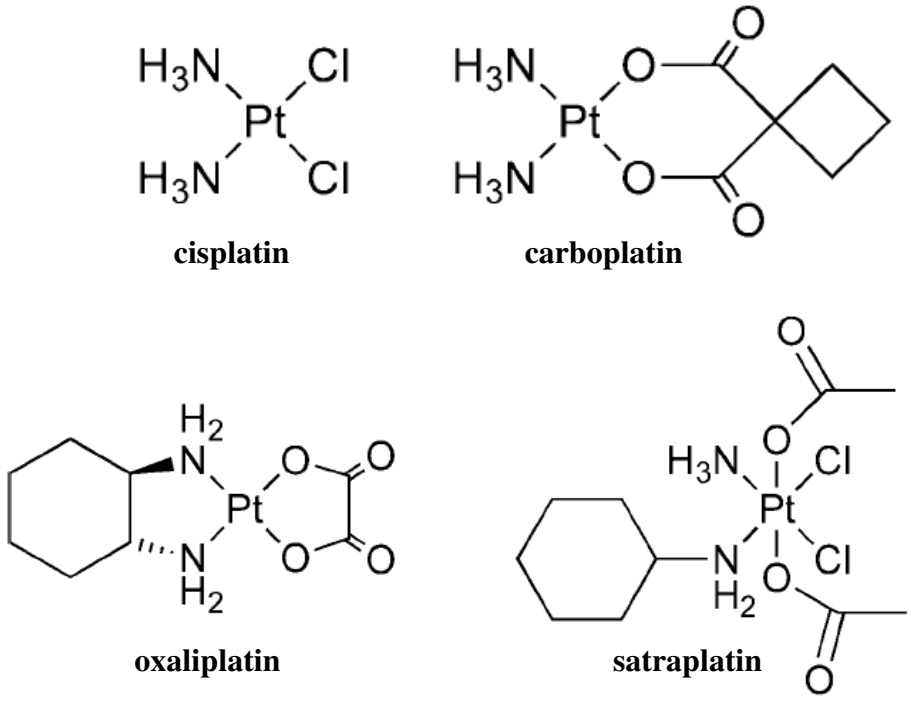


Figure 1.3. Clinically approved platinum(II) complexes (cisplatin, carboplatin, oxaliplatin) and satraplatin – the platinum(IV) complex being in decisive clinical trials currently.

The success of cisplatin has imposed a certain direction on the investigation of novel substances, in the first instance excluding compounds that may possess a different and probably unique mode of action.

Today we know a lot of biologically active compounds which violate the basic «structure – activity» relationship announced by Cleare and Hoeschele for platinum based compounds [23,29,30,33]. This work, which is devoted to synthesis, characterization and analysis of the biological activity of guanidine complexes, whose mode of cytotoxic activity is probably different from that of cisplatin, contributes to the knowledge about these non-classic platinum drugs.

1.3.1 Pt-based anticancer drugs in clinical use

Cisplatin, carboplatin, and oxaliplatin (**Fig. 1.3**) are currently the only metal-containing antitumor compounds that have broad application in cancer chemotherapy. About 50% of chemotherapeutic treatments are based on the application of these complexes. However, significant side effects, as well as acquired or intrinsic resistance of cancer cells are the main shortcomings of this type of chemotherapy.

In the past several decades a large number of innovative platinum anticancer drugs was synthesized. The mechanisms of action of these compounds and their cytotoxic activity were investigated in tests *in vitro* and *in vivo*. Consistently two main strategies in the synthesis of platinum complexes were chosen. The first strategy is the establishment of cytotoxic drugs containing ligands exhibiting the specific function of improving tumor selectivity. Thus, these compounds are designed to have a selective effect on cancer cells without significant side effects on the patient's organism. The second strategy is the development of non-classic platinum compounds which do not resemble the structure of cisplatin [12].

1.3.2 Platinum compounds with improved tumor selectivity

The selectivity against cancer cells can be achieved through several approaches based on the differences between normal and cancer cells. One difference arises from the rapid growth of cancerous tumors that force the process of angiogenesis to occur in an uncontrolled manner,

which leads to defects in the formation of the vascular endothelium. Violation of intercellular communication in the vessels causes increased expression of mediators. This leads to increased permeability of macromolecules through the blood vessels and the retention of antitumor drugs in the area of malignancy (EPR effect) [13]. In order to exploit this effect and to increase the accumulation of cytotoxic platinum drugs at the tumour site, macromolecular constructs have been synthesised. Some macromolecular copolymers (e.g. AP5280) were already investigated in patients [14]. Other examples of such macromolecular constructs are liposomal formations of cisplatin (lipoplatin) [15], and oxaliplatin (lipoxal) [16].

Another approach in cancer treatment is based on targeting of cytotoxic agents using organ-specific or receptor-specific carriers. The liver can be nicely targeted by platinum complexes containing the residues of galactose [17], or bile acids [18,19] in their structure. On one hand the expression of galactose receptors is particularly high on the cytoplasmic membrane of parenchymal liver cells and on the other hand the bile acids are synthesized in the liver and stored in the gallbladder and therefore have quite strict localization.

Despite the extensive angiogenesis the blood flow to the inner parts of the solid tumors is insufficient which leads to hypoxia – oxygen deficiency of large tumors. Therefore, compounds that can be reduced in such conditions will be selective to the cancerous tissue. It has been shown that the octahedral complexes of platinum(IV) can be reduced to the corresponding planar complexes of platinum(II) accompanied by the removal of axial ligands in such conditions. These compounds serve as prodrugs – the forerunner of a pharmaceutical agent turning in the active drug only inside the tumor [20]. Satraplatin (**Fig. 1.3**), undergoing the phase III of clinical trials, is the most promising compound of this group [21]. Moreover, in contrast to the cisplatin-like complexes which can only be introduced intravenously, satraplatin is safe for enteral application.

Due to insufficient supply of growing tumors with oxygen – the cancer cells adopt an anaerobic metabolism, which leads to an increase in proton concentration in the intercellular space and thus contributes to the lowering of pH (i.e. pH 6.0, with the normal pH 7.4). This provides grounds for the directed synthesis of compounds exhibiting cytotoxic activity in a weakly acidic environment. One of the most well-known compounds of this type is bis-(O-dithiocarbonate)-platinum(II) [22].

1.3.3 Non-classic platinum complexes as antitumor agents

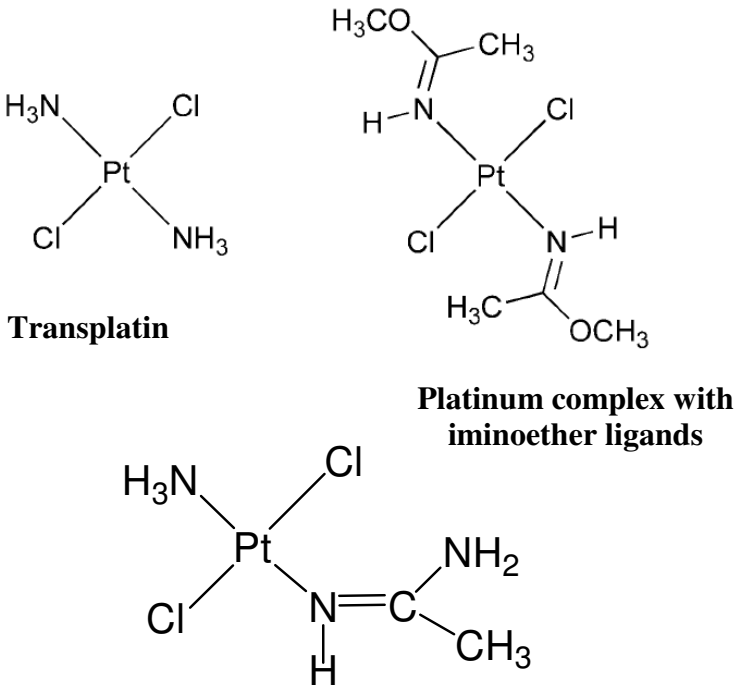
Two years after cisplatin was established in the clinical trials Cleare and Hoeschele investigated a series of platinum complexes and proposed the classic «structure – activity» relationship for platinum based drugs [23]:

1. Platinum complexes must contain 2 (or 1 bidentate) labile ligands, as well as 2 (or 1 bidentate) kinetically inert amino-containing ligands;
2. All the ligands must be coordinated to platinum(II);
3. Complexes should be neutral and of *cis* geometry.

According to Cleare and Hoeschele platinum compounds not complying with these requirements (non-classic compounds), e.g. *trans* configured platinum(II) complexes, should be less active or do not possess cytotoxic activity. The main target of platinum complexes with any geometry is DNA. The difference in cytotoxicity of compounds with various configurations is generally due to the form and strength of their adducts to biomolecules. The common prototype for non-classic platinum complexes is transplatin (*trans*-DDP), which possesses lower cytotoxic activity in comparison with its structural analogue – cisplatin (**Fig. 1.4**). This fact is even more remarkable because Sadler et al. have shown the increase of transplatin's cytotoxic effect in case the treated cells were irradiated with UVA light. The UVA photo–activation results in formation of a large number of interstrand DNA adducts, while in the normal conditions only CDDP is capable of forming such kind of adducts [24].

1.3.4 *Trans*-configured platinum complexes as anticancer drugs

Complexes with iminoether ligands (**Fig. 1.4**) were investigated thoroughly. It has been shown that complexes exhibit significant antitumor activity on leukemia cells and cancerous tumors in mice [25]. Moreover, these compounds are capable of forming stable monofunctional adducts (with a single strand of DNA helix) with purine DNA bases and thereby stop the DNA and RNA polymerases which are of great necessity for gene expression [26]. Promising results obtained for complexes with iminoether ligands led to further research in this direction. New compounds with other isosteric ligands have been described, e.g. complexes with amidine ligands (**Fig. 1.4**) which are coordinated to platinum with imine nitrogen [27].



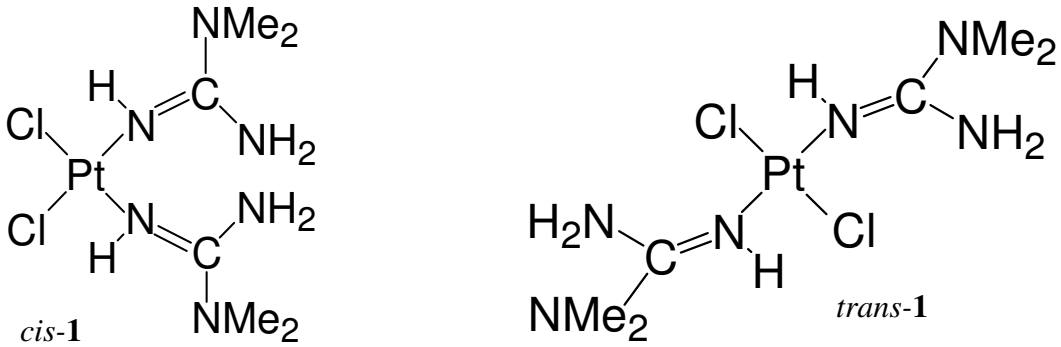
Transplatin

Platinum complex with iminoether ligands

Platinum complex with amidine ligand

Figure 1.4. *Trans*-configured non-classic platinum(II) complexes.

Currently, considerable attention is devoted to the guanidine platinum complexes. This is partly due to the discovery of biological and pharmacological (anticancer) activity of these complexes [28]. These compounds also belong to non-classic platinum(II) complexes, as *in vitro* studies have shown that cytotoxic activity of *trans*-configured complexes is higher than of corresponding complexes of *cis*-geometry. In this work the synthesis of new cationic guanidine complexes is reported, together with the analysis of biological activity of *trans*- and *cis*-configured guanidine platinum(II) complexes (**Fig. 1.5**).



cis-**1**

trans-**1**

Figure 1.5. Guanidine platinum(II) complexes.

1.4 Cytotoxicity Analysis

Determination of cytotoxic activity of antitumor agents is conducted usually using a colorimetric method with MTT reagent {(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide} used as the dye [36].

This method is based on the fact that the respiratory chain and electron transport systems of the living cells are able to reduce MTT reagent (yellow solution in PBS) to water-insoluble formazan (forming purple crystals inside the cells) (**Fig. 1.6**) [37].

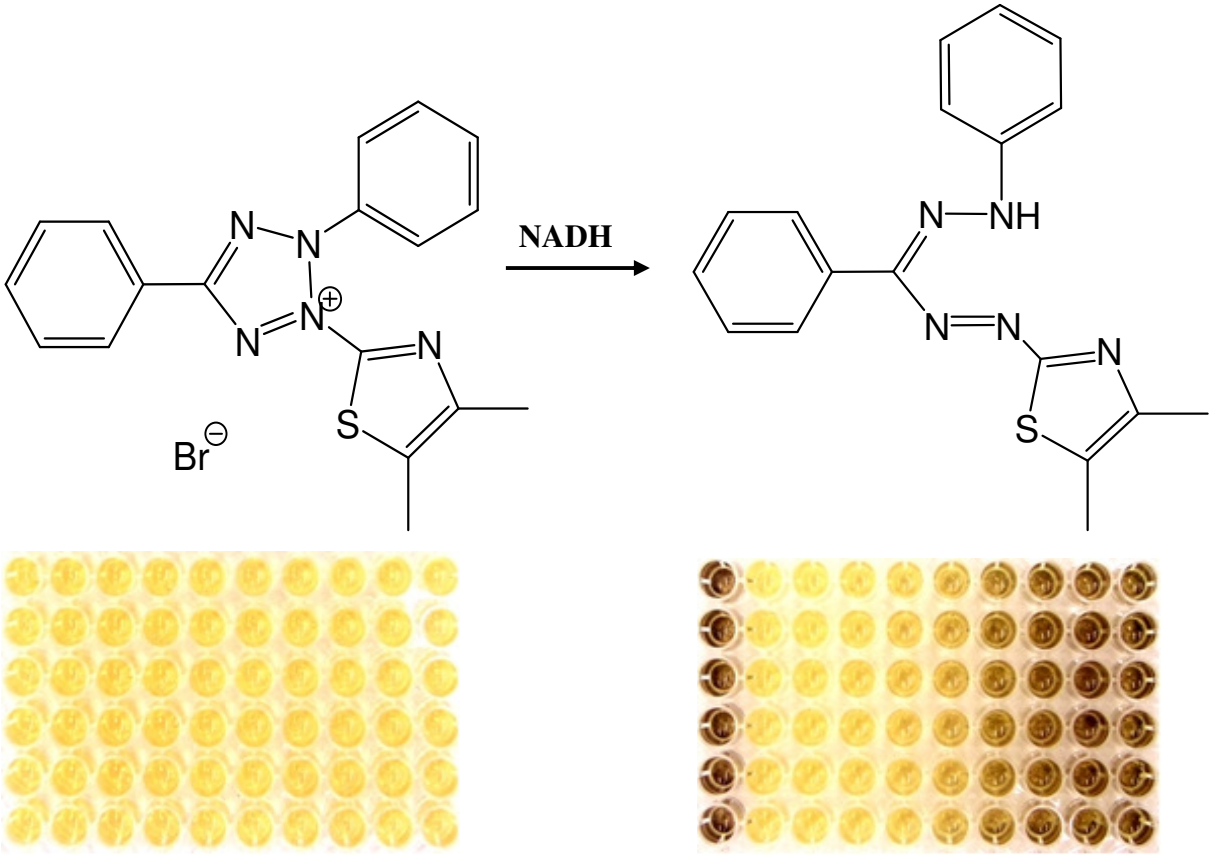


Figure 1.6. MTT reduction to formazan crystals under consummation of NADH and the corresponding change in color produced by viable cells in the medium.

Purple formazan crystals formed inside living cells are usually dissolved using organic solvents such as dimethyl sulfoxide to obtain purple formazan solution (**Fig. 1.7**).

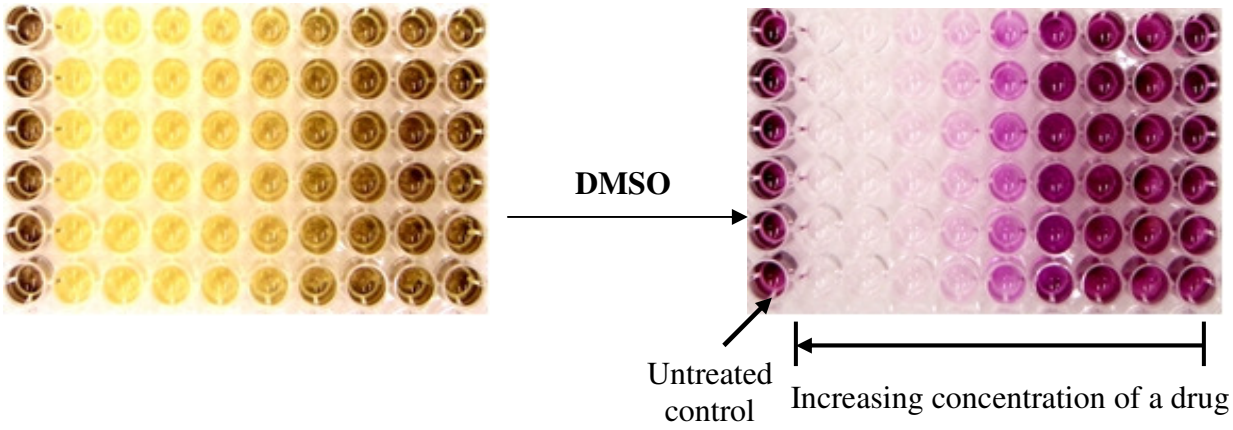


Figure 1.7. Dissolution of formazan crystals in dimethyl sulfoxide.

The intensity of light absorption of colored solution can be measured with a spectrophotometer at a specific wavelength (usually corresponding to the absorption maximum of ~ 550 nm). The optical densities allow assessing the percentage of living cells in the sample in relation to untreated control microcultures.

An alternative method for cytotoxicity testing is based on a different colorimetric method using MTS reagent (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) as a dye in presence of phenazine methosulfate (PMS). The main difference of this method is that the resulting formazan product produced in the living cells is water-soluble and has a maximum absorption at 490–500 nm wavelength in phosphate buffer [31]. The main advantages of this method are: the reagent pair MTS + PMS is reduced more effectively than its MTT analog forming a water-soluble formazan product, whose level of toxicity to the cells is lower than of insoluble crystals (in the case of MTT). The main disadvantage of this method is that the reagents are more expensive.

There are methods for determining the number of dead cells (as long as they are not degraded), with a use of Trypan Blue as a dye e.g., which selectively stains dead tissues or cells in blue color. This substance, which belongs to the azo-dyes (R–N=N–R´, where R, R´ – aryl groups) is unable to stain living cells, as it can not penetrate through intact plasma membranes, but it is easily absorbed by dead cells, because their membranes are damaged. The blue staining of dead cells can be observed under the microscope. This method does not depend on the metabolic activity of the cells, however, it is very time-consuming, which is limiting factor when large number of samples are analyzed [39].

The reduction of tetrazolium salts (MTT, MTS) to formazan occurs only in living cells. Thus, the measurement of the optical density of the samples can be used to determine cell viability. However, sometimes different results can be obtained while using these methods, because many external factors can lead to the increase or decrease of metabolic activity of living cells. Changes in metabolic activity can cause significant fluctuations in optical density parameter, while the number of living cells remains unchanged. In this regard, it is important to maintain constant favorable conditions (5% CO_2 pressure, 37 ºC temperature, humid atmosphere) during cells cultivation. The quantity of vital cells is usually expressed in terms of T/C values by comparison with untreated control microcultures, and 50% inhibitory concentrations (IC_{50}) are calculated from concentration-effect curves by interpolation (**Fig. 1.8**).

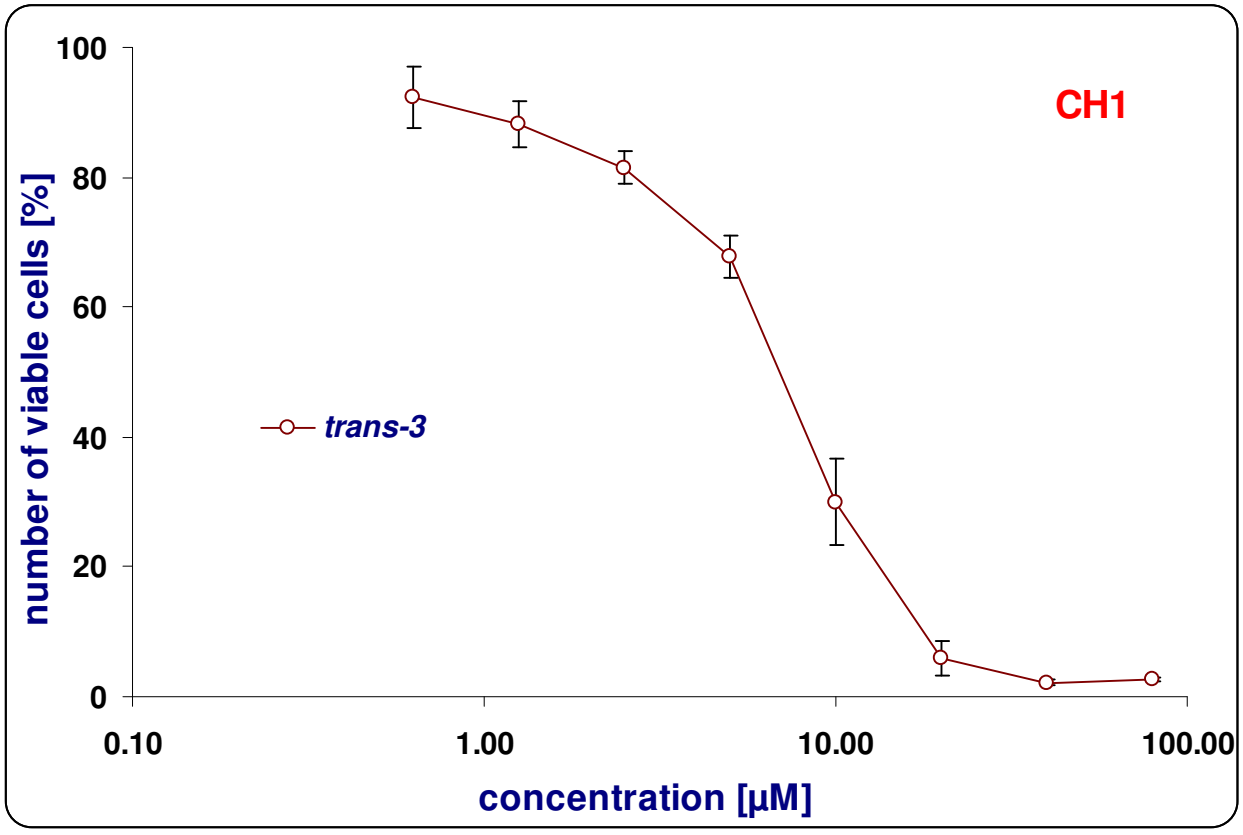


Figure 1.8. Concentration-effect curve showing the effect of *cis*-**6** complex on ovarian carcinoma cells (CH1).

1.5 Aims and Goals

The neutral compounds *cis*- and *trans*-[$PtCl_2${NH=C(NH_2)NR_2}$_2$], as well as cationic complexes *cis*- and *trans*-[$Pt(NH_3)_2${NH=C(NH_2)NR_2}$_2$]$(Cl)_2$ represent promising couples for study of biological activity, which includes the investigation of the antitumor activity and the determination of the main cellular targets. These compounds are the nitrogen analogues of a new class of anticancer drugs based on amidine platinum(II) complexes [21,22,23,41].

The main aim of this work was the directed synthesis of the cationic guanidine complexes *trans*-[$Pt(NH_2Me)_2${NH=C(NHMe)NR_2}$_2$]$(Cl)_2$ (R = NEt_2 [*trans*-**7**], NC_5H_{10} [*trans*-**8**]), and the determination of the structure–activity relationship for the entire group of guanidine platinum(II) complexes (*cis*-**1**–**6** and *trans*-**1**–**8**).

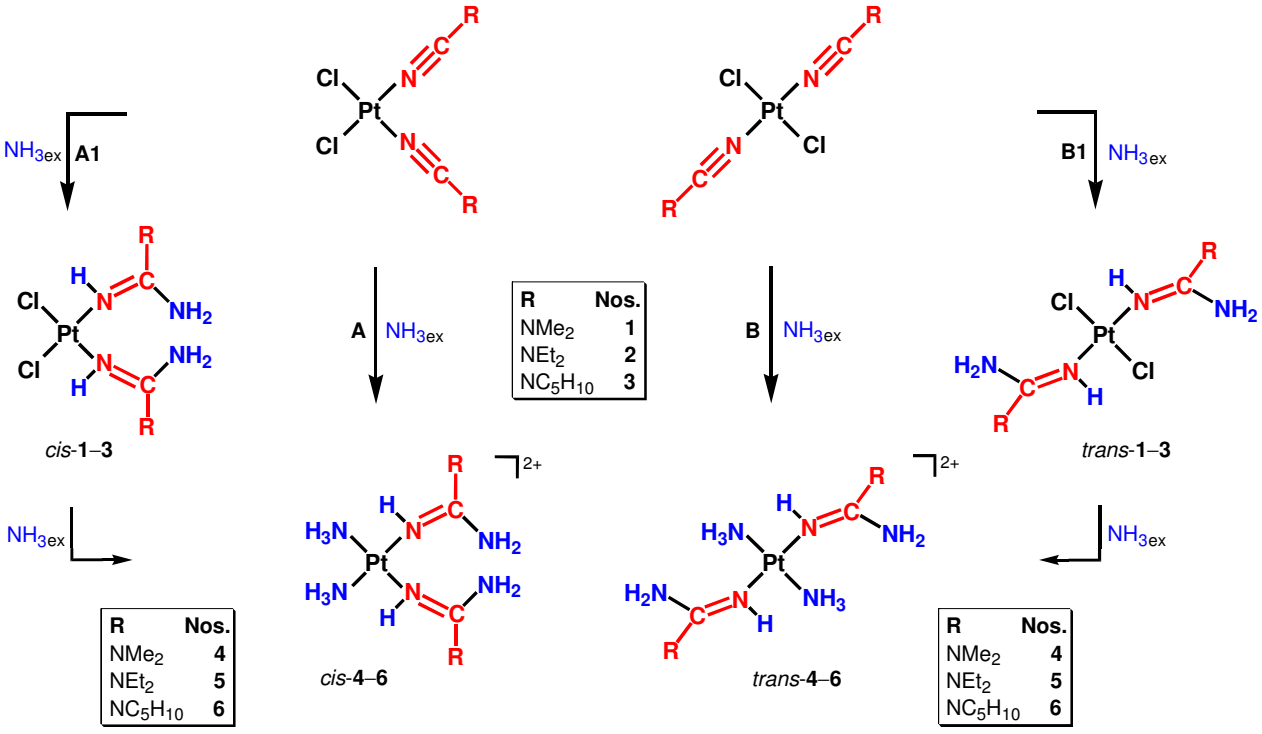
To achieve the aim several goals were established:

- To synthesize new guanidine platinum(II) complexes by metal-mediated nucleophilic cyanamide-amino coupling;
- To analyze the antitumor activity of a whole set of guanidine platinum(II) complexes using the MTT assay;
- To determine whether DNA is one of the main targets of the complexes by means of electrophoretic mobility shift assay, cellular uptake and DNA platination experiments.

2. MATERIALS AND METHODS

Synthesis. In this work we investigated the biological activity of platinum(II) complexes with the structural element (guanidine)$_2$Pt^{II}, prepared by nucleophilic addition of ammonia to the push-pull nitrile ligands in *cis*- and *trans*-[$PtCl_2(RCN)_2$] (R = NMe_2, NEt_2, NC_5H_{10}) platinum(II) complexes. Complexes *cis*-**1–6** and *trans*-**1–6** were synthesized and characterized by Tyan M. supervised by Prof. Dr. Kukushkin V. at the Chemistry department of St. Petersburg University. In addition, we have carried out the nucleophilic addition of methylamine to cyanamide platinum(II) complexes (*trans*-**7**,**8**). The products were purified and characterized by various physical and chemical methods. We have studied the biological activity of the entire set of guanidine compounds (*cis*-**1–6** and *trans*-**1–8**), using various bioanalytical methods.

Pt^{II}-mediated cyanamide-amino coupling. The work carried out by Tyan M. showed that the absorption of gaseous ammonia solutions by *cis*-and *trans*-[$PtCl_2(RCN)_2$] (R = NMe_2, NEt_2, NC_5H_{10}) complexes at 20–25 °C for 25–30 min led to the formation of neutral *cis*-and *trans*-[$PtCl_2\{NH{=}C(NH_2)NR_2\}_2$] (*cis*-**1–3** and *trans*-**1–3**) (**Scheme 1, A1, B1**) and cationic *cis*-and *trans*-[$Pt(NH_3)_2\{NH{=}C(NH_2)NR_2\}_2](Cl)_2$ (*cis*-**4–6** and *trans*-**4–6**) complexes [11]. All the neutral complexes are soluble in DMSO.

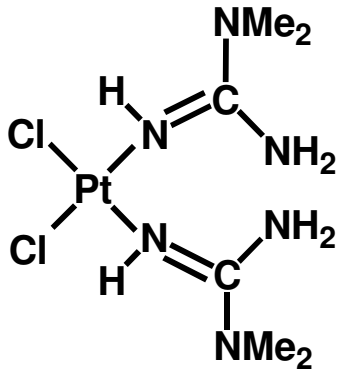


Scheme 1

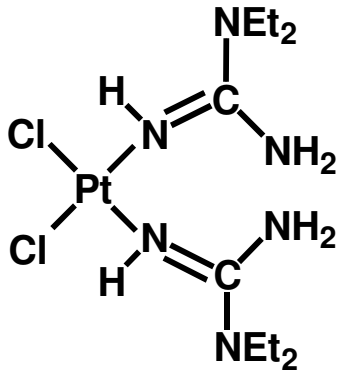
Individual cationic guanidine complexes *cis*- and *trans*-- $[Pt(NH_3)_2\{NH=C(NH_2)NR_2\}_2](Cl)_2$ (*cis*-**4**–**6** and *trans*-**4**–**6**) were obtained due to longer amination of initial push-pull platinum(II) complexes for 12 h at room temperature (**Scheme 1**, **A**, **B**) and isolated with yields of 73–97%. All the cationic complexes are well soluble in water and medium.

List of compounds. *cis*-**1**–**6** and *trans*-**1**–**6** complexes prepared by Tyan et al. [11].

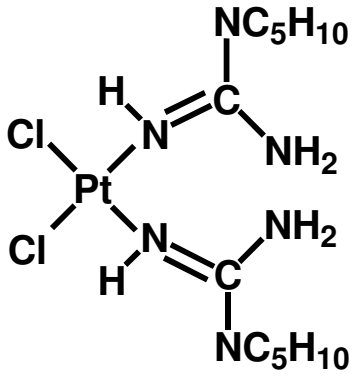
***cis*-[$PtCl_2${NH=C(NH_2)NMe_2}$_2$]** (*cis*-**1**) MW = 440.2; solubility: DMSO



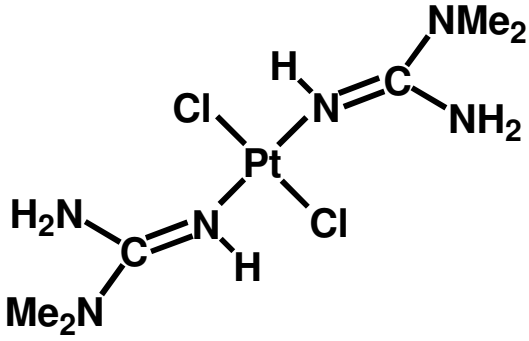
***cis*-[$PtCl_2${NH=C(NH_2)NEt_2}$_2$]** (*cis*-**2**) MW = 496.3; solubility: DMSO, acetone (poor)



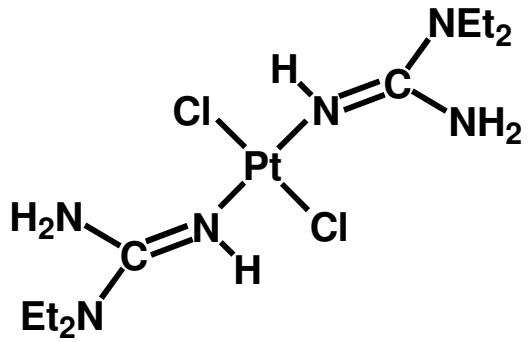
***cis*-[PtCl$_2${NH=C(NH$_2$)NC$_5$H$_{10}$}$_2$]** (*cis*-**3**) MW = 520.4; solubility: DMSO, acetone (poor)



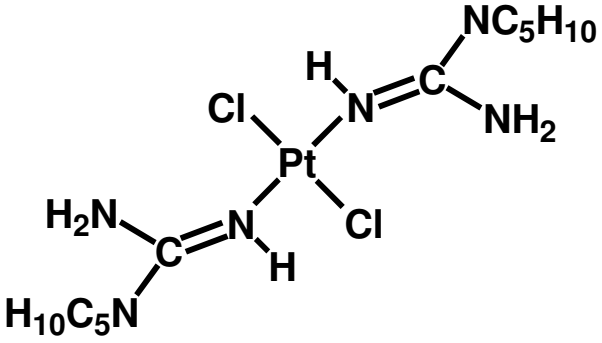
***trans*-[PtCl$_2${NH=C(NH$_2$)NMe$_2$}$_2$]** (*trans*-**1**) MW = 440.2; solubility: DMSO, acetone (poor)



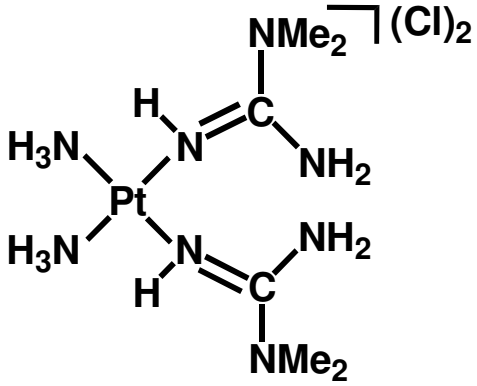
***trans*-[PtCl$_2${NH=C(NH$_2$)NEt$_2$}$_2$]** (*trans*-**2**) MW = 496.3; solubility: DMSO, acetone (poor), H_2O (poor)



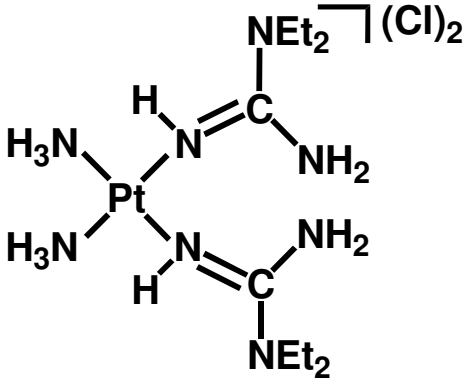
***trans*-[PtCl$_2${NH=C(NH$_2$)NC$_5$H$_{10}$}$_2$]** (*trans*-**3**) MW = 520.4; solubility: DMSO, acetone (poor), H_2O (poor)



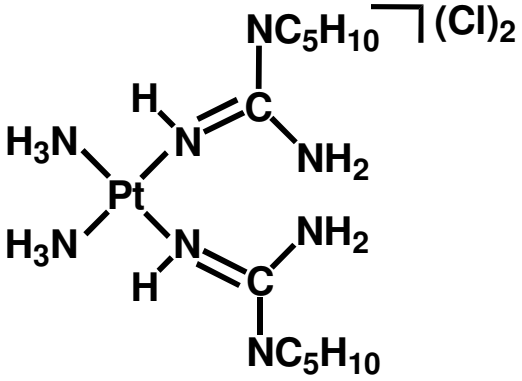
***cis*-[Pt(NH$_3$)$_2${NH=C(NH$_2$)NMe$_2$}$_2$](Cl)$_2$** (*cis*-**4**) MW = 474.3; solubility: DMSO, MeOH, EtOH, H_2O



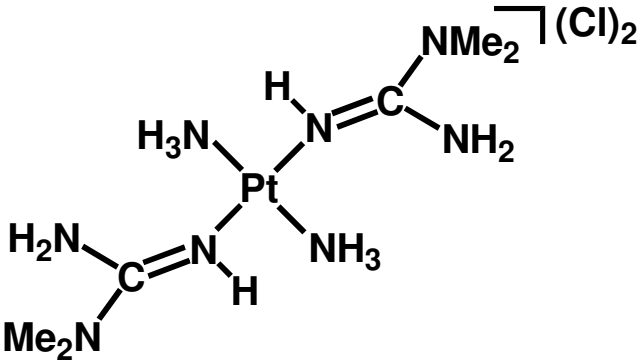
***cis*-[Pt(NH$_3$)$_2${NH=C(NH$_2$)NEt$_2$}$_2$](Cl)$_2$** (*cis*-**5**) MW = 530.4; solubility: DMSO, MeOH, EtOH, H_2O



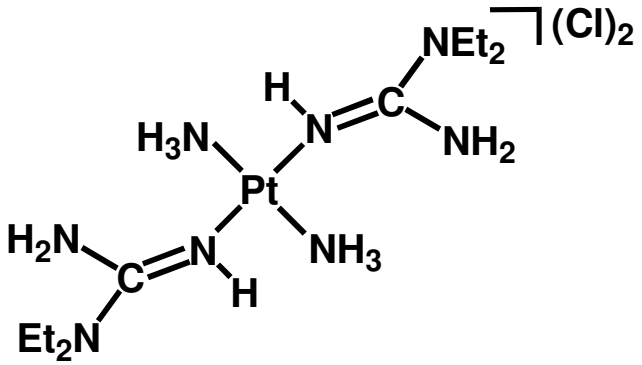
***cis*-[Pt(NH$_3$)$_2${NH=C(NH$_2$)NC$_5$H$_{10}$}$_2$](Cl)$_2$** (*cis*-**6**) MW = 554.4; solubility: DMSO, MeOH, EtOH, H_2O



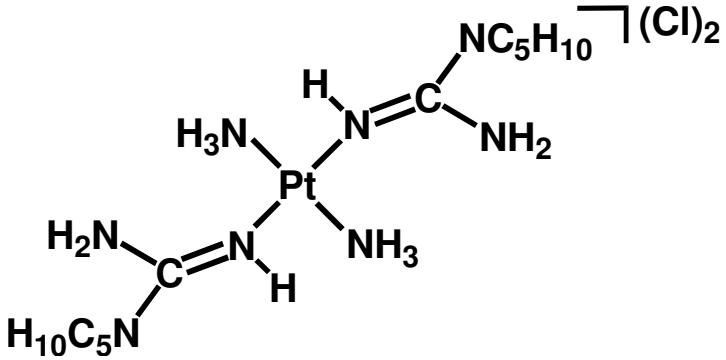
***trans*-[Pt(NH$_3$)$_2${NH=C(NH$_2$)NMe$_2$}$_2$](Cl)$_2$** (*trans*-**4**) MW = 474.3; solubility: DMSO, MeOH, EtOH, H_2O



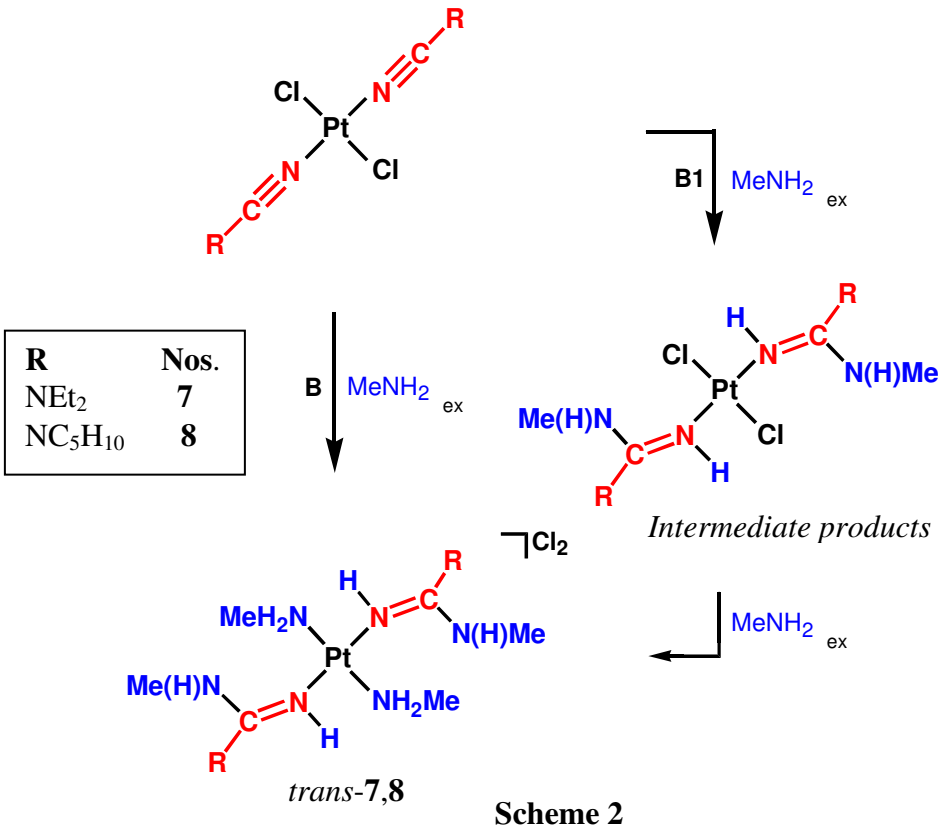
***trans*-[Pt(NH$_3$)$_2${NH=C(NH$_2$)NEt$_2$}$_2$](Cl)$_2$** (*trans*-**5**) MW = 530.4; solubility: DMSO, MeOH, EtOH, H_2O



***trans*-[Pt(NH$_3$)$_2${NH=C(NH$_2$)NC$_5$H$_{10}$}$_2$](Cl)$_2$** (*trans*-**6**) MW = 554.4; solubility: DMSO, MeOH, EtOH, H_2O



In continuation of the work, we have synthesized cationic guanidine compounds *trans*-[Pt(NH$_2$Me)$_2${NH=C(NHMe)NR}$_2$](Cl)$_2$ (R = NEt$_2$, NC$_5$H$_{10}$) (*trans*-**7**,**8**) (**Scheme 2**, **B**, **B1**). Initial cyanamide platinum(II) complexes [PtCl$_2$(RCN)$_2$] (R = NEt$_2$; NC$_5$H$_{10}$) were prepared as a mixture of geometric *cis*- and *trans*-isomers from K$_2$[PtCl$_4$] and 4 equivalents of the respective cyanamides. *Cis*- and *trans*-complexes were separated by column chromatography on SiO$_2$ Merck 60 F$_{254}$ (eluent: Me$_2$CO:CHCl$_3$ = 1:10, by volume). *trans*-**7**,**8** were prepared by prolonged amination of push-pull platinum(II) complexes with methylamine (10x excess) for 4 h at room temperature. Total yield amounted 83–87%. Both novel complexes are well soluble in water and medium.



Scheme 2

All the substances were identified and characterized by several physical and chemical methods of analysis (see chapter **3.2**).

Synthetic and characterization data for *trans*-7,8 complexes. *trans*-[Pt(NH$_2$CH$_3$)$_2${NH=C(NHCH$_3$)N(C$_2$H$_5$)$_2$}$_2$] (*trans*-**7**) Anal. Calcd for $C_{14}H_{40}N_8Cl_2Pt$: C, 28.67; H, 6.87; N, 19.11. Found: C, 28.42; H, 6.90; N, 19.37. ESI$^+$-MS, *m/z*: 1137.49 $[2M - HCl]^+$ (calc.: 1137.51), 551.26 $[M - HCl]^+$ (calc.: 551.27), 520.22 $[M - HCl - NH_2CH_3]^+$ (calc.: 520.22), 452.20 $[M - 2NH_2CH_3 - 2HCl]^+$ (calc.: 452.20), 257.65 $1/2[M - 2Cl]^{2+}$ (calc.: 257.65). IR (KBr, selected bands, cm^{-1}): 3269 (m), 3194 (m), 3092 (m), *ν*(N–H); 2970 (m-w), 2936 (m-w), 2888 (m-w), *ν*(C–H); 1591 (s), *ν*(C=N); 1282 (m-w), *ν*(C–N). ^{1}H NMR [$CDCl_3$, *δ*]: 7.43 (s, br, *H*, =NH–), 5.58 (s, br, *H*, –NH–), 4.88 (s, br, 2*H*, –NH$_2$–), 3.31 (q, $^3J_{HH}$ 6.9 Hz, 4*H*, –CH$_2$– [in NEt$_2$]), 3.01 (d, $^3J_{HH}$ 4.5 Hz, 3*H*, –CH$_3$ [in NHCH$_3$]), 2.30 (t, $^3J_{HH}$ 6.2 Hz, 3*H*, –CH$_3$ [in NH$_2$CH$_3$]), 1.14 (t, $^3J_{HH}$ 6.9 Hz, 6*H*, –CH$_3$ [in NEt$_2$]). ^{13}C{^{1}H} NMR [$CDCl_3$, *δ*]: 164.72 (C=N), 43.61 (–CH$_2$– [carbons in NEt$_2$]), 33.18, 31.62 (–CH$_3$ [carbons in NHCH$_3$/ NH$_2$CH$_3$]), 13.53 (–CH$_3$ [carbons in NEt$_2$]).

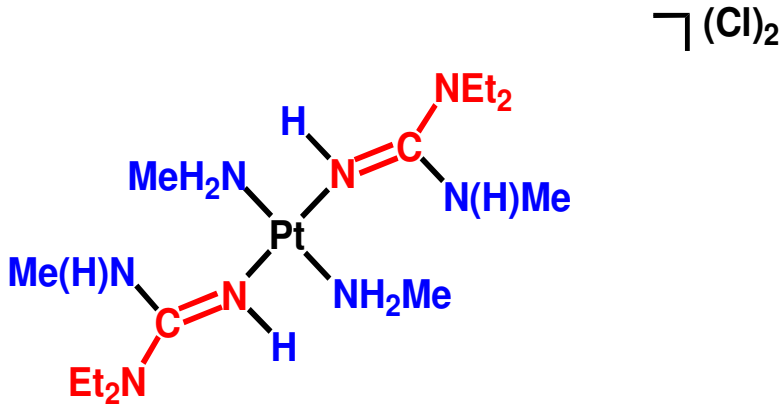


Figure 2.1. Structure of *trans*-**7** complex.

Obtained through prolonged amination of the undried CH_2Cl_2 solutions (2 mL) of the *trans*-$[PtCl_2(NCNEt_2)_2]$ complex (0.205 mmol; 120 mg) by gaseous methylamine (10x excess) for 4–5 h at room temperature led to formation of the cationic *trans*-$[Pt(NH_2CH_3)_2\{NH{=}C(NHCH_3)N(Et)_2\}_2](Cl)_2$ complex (**Fig. 2.1**). The color changing of solution from yellow to colorless and the precipitation of colorless product was observed. The precipitate was isolated by filtration, dried and then analysed thoroughly. The total yield of *trans*-$[Pt(NH_2CH_3)_2\{NH{=}C(NHCH_3)N(C_2H_5)_2\}_2]$ amounted to about 87% (125.2 mg). **Sol**: $CHCl_3$; MeOH; H_2O. MW = **M** = 586,5; $\mathbf{M^0}$ = 515,5 (MW – 2Cl).

***trans*-$[Pt(NH_2CH_3)_2\{NH{=}C(NHCH_3)N(C_5H_{10})\}_2]$** (*trans*-**8**) Anal. Calcd for $C_{16}H_{40}N_8Cl_2Pt$: C, 31.47; H, 6.60; N, 18.35. Found: C, 31.14; H, 6.71; N, 18.46. ESI$^+$-MS, *m/z*: 1185.49 $[2M - HCl]^+$ (calc.: 1185.50), 575.26 $[M - HCl]^+$ (calc.: 575.27), 544.22 $[M - HCl - NH_2CH_3]^+$ (calc.: 544.22), 538.27 $[M - 2HCl]^+$ (calc.: 538.27), 475.20 $[M - 2NH_2CH_3 - 2HCl - H]^+$ (calc.: 475.20), 269.64 $1/2[M - 2Cl]^{2+}$ (calc.: 269.65). IR (KBr, selected bands, cm^{-1}): 3418 (m), 3277 (m), 3089 (m), ν(N–H); 2936 (m-w), 2854 (m-w), ν(C–H); 1584 (s), ν(C=N); 1256 (m-w), ν(C–N). ^{1}H NMR [$CDCl_3$, δ]: 7.62 (s, br, *H*, =NH–), 5.42 (s, br, *H*, –NH–), 4.81 (s, br, 2*H*, $-NH_2-$), 3.22 (s, 4*H*, α–CH_2– [in NC_5H_{10}]), 2.95 (d, 3*H*, $-CH_3$ [in $NHCH_3$]), 2.28 (t, 3*H*, $^3J_{HH}$ 6.3 Hz, $-CH_3$ [in NH_2CH_3]), 1.59 (s, br, 6*H*, β, γ $-CH_2-$ [in NC_5H_{10}]). $^{13}C\{^1H\}$ NMR [$CDCl_3$, δ]: 166.07 (C=N), 49.55 (α–CH_2– [carbons in NC_5H_{10}]), 32.96, 31.36 ($-CH_3$ [carbons in $NHCH_3$/ NH_2CH_3]), 26.10, 24.55 (β, γ $-CH_2-$ [carbons in NC_5H_{10}]).

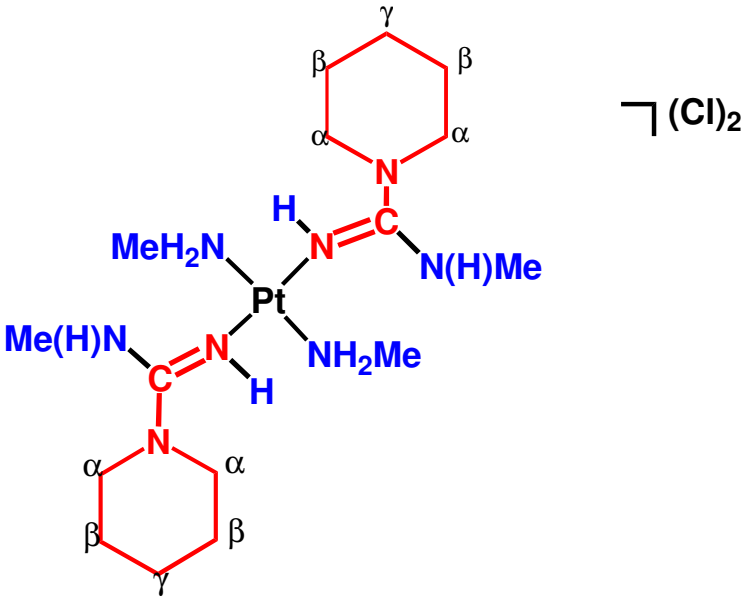


Figure 2.2. Structure of *trans*-**8** complex.

Obtained through prolonged amination of the undried CH_2Cl_2 solutions (2 mL) of the *trans*-$[PtCl_2(NCNC_5H_{10})_2]$ complex (0.213 mmol; 130 mg) by gaseous methylamine (10x excess) for 4-5 hours at room temperature led to formation of the cationic *trans*-$[Pt(NH_2CH_3)_2\{NH{=}C(NHCH_3)N(C_5H_{10})\}_2](Cl)_2$ complex (**Fig. 2.2**). The color changing of solution from yellow to colorless and the precipitation of colorless product was observed. The precipitate was isolated by filtration, dried and then analysed thoroughly. The total yield of *trans*-$[Pt(NH_2CH_3)_2\{NH{=}C(NHCH_3)N(C_5H_{10})\}_2]$ amounted to about 83% (135.2 mg). **Sol**: $CHCl_3$; MeOH; H_2O. MW = **M** = 610,53; $\mathbf{M^0}$ = M – 2Cl = 539,62.

Solvents were obtained from commercial sources and used without further purification and drying. Initial cyanimide platinum(II) complexes $[PtCl_2(Et_2NCN)_2]$ and $[PtCl_2(C_5H_{10}NCN)_2]$ were prepared according to published methods [31].

Nitrogen, Hydrogen and Carbon Analysis was carried out at the Department of Organic Chemistry, St. Petersburg State University by burning the substance in a stream of oxygen in the device 185V Carbon Hydrogen Nitrogen Analyzer, Hewlett Packard.

Infrared Spectra were recorded on a Shimadzu FTIR-8400S spectrophotometer (range 4000–400 cm^{-1}). The samples were pelletized with KBr.

^{1}H NMR and ^{13}C{^{1}H} NMR spectra were measured on a Bruker DPX 400 spectrometer at room temperature. Chemical shifts in ^{1}H NMR spectra were measured relatively to the signals of the solvent ($CDCl_3$ 7.27). Chemical shifts in ^{13}C{^{1}H} NMR spectra were measured relatively to the signals of the solvent ($CDCl_3$ 77.4).

Electrospray Mass Spectra were recorded on the instrument Bruker micrOTOF, equipped with an electrospray ion source type (ESI or ES^+). Resolution of the instrument was 10000 at half of the peak. For the mass spectrometric studies samples were dissolved in MeCN. The upper boundary of the solution concentration was 5–15 mg/ml. The resulting solution was supplied to the electrospray capillary using a KDScientific syringe pump. The voltage generated at the final electrode, and the voltage on the capillary were –500 V and –4500 V (ESI^+-MS) or 3500 V (ESI^--MS), respectively. The sample solution flow through the electrospray capillary was 3 ml/min. The output voltage of the capillary was ± 70 or ± 150 V. ESI^+ and ESI^- mass spectra were recorded in the range 50 to 3000 m/z. Drying gas pressure in the spray was 0.4 bar, flow rate and temperature of the drying gas were 4.0 l/min and 180°C, respectively.

Thin Layer Chromatography was performed on Merck 60 F_{254} aluminum plates with inflicted layer of silica gel.

Cell Lines and Cultivation Conditions. The cytotoxicity test was performed on two cancer cell lines: CH1 (ovarian carcinoma – cells are more sensitive to cisplatin) and SW480 (colon carcinoma – cells are more resistant to cisplatin), kindly provided by Lloyd R. Kelland (CRC Centre for Cancer Therapeutics, Institute of Cancer Research, Sutton, UK) and Brigitte Marian (Institute of Cancer Research, Medical University of Vienna, Austria), respectively. Cell monolayer adherent cultures were grown in 75 cm^2 culture flasks (Iwaki/Asahi Technoglass, Gyouda, Japan) in complete medium [i.e., minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, and 1% nonessential amino acids (all purchased from Sigma-Aldrich, Austria)]. Cell cultures were incubated at 37 °C in a moist atmosphere containing 5% CO_2.

Cytotoxicity in Cancer Cell Lines. Cytotoxic activity of guanidine platinum(II) complexes was determined using the colorimetric MTT assay. The cells were collected from flasks by trypsinization and seeded into 96-well microculture plates (Iwaki/Asahi Technoglass,

Gyouda, Japan) in densities of 1500 (for CH1) and 2500 (for SW480) cells per well. After 24 h preincubation of the cells the test compounds were dissolved in complete medium, and 200 µl of solution was added per well. Those compounds which were insufficiently soluble in medium were dissolved in dimethyl sulfoxide and then diluted not to exceed the concentration of 0.5% DMSO in the applied solution. The exact data about the solubility of the substances is presented in chapter **3.1**. The cells were exposed to test compounds for 96 h. At the end of exposure, drug solutions were replaced with 150 µl RPMI 1640 culture medium (supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine) plus 20 µl MTT solution in phosphate-buffered saline (5 mg/ml). After 4 h incubation the RPMI/MTT mixtures were removed and formazan crystals formed in the vital cells were dissolved in 150 µl of dimethyl sulfoxide per well. Optical densities were measured at 550 nm wavelength with a microplate reader (Tecan Spectra Classic). The quantity of vital cells was expressed in terms of T/C values by comparison with untreated control microcultures, and 50% inhibitory concentrations (IC_{50}) were interpolated from dose-dependent curves. Evaluation was based on means from at least three independent experiments, each comprising at least six microcultures per concentration level [32].

Electrophoretic Mobility Shift Assay was used to determine the drug-DNA interactions. The pPUC19 DNA plasmid (Fermentas, UK) was incubated with test compounds for different periods of time (3 h – 15 min). The electrophoresis was performed in 1% agarose gel for 2 h at 80 V and 55 mA in 1x TBE buffer (0.45 M Tris borate, 0.01 M EDTA, pH 8.3, purchased from Eppendorf AG, Germany). PEQLAB electrophoresis chambers were used. The staining was performed in EtBr solution in 1x TBE (0.2 µg/ml) for 20 minutes. Images were taken under UVE light in the GelDoc-It Imaging System (UVP).

Cellular Uptake Experiments (adhesive state) were performed to determine the intracellular drug concentration. Cells were seeded in 6-well plates in a total volume of 2.5 ml complete medium in densities about 3×10^5 cells per well. Cell microcultures were incubated at 37 °C for 24 h prior to exposure to the test compounds. The cell number was determined using Trypan Blue staining in parallel microcultures during the 2 h exposure period. After the exposure the medium was removed, cells were washed with PBS and consecutively lyzed with 0.5 ml sub-boiled HNO_3 (conc.) per well for 1 h. Pt concentration was quantified by ICP-MS in presence of 0.5 µM In standard. Results are based on three independent experiments, each consisting of three replicates. The method is thoroughly described in Egger et al. [40].

Cellular Uptake Experiments (non-adherent state) were performed alternatively to determine the intracellular concentration of those compounds that have a much stronger affinity to the polystyrene of the culture plates than to the cells (in this case, cells can be easily collected without the need for measuring adsorption blanks). The cells were harvested from the culture flasks, counted and seeded to 15 ml tubes in amounts of approximately 3×10^5 cells per tube which corresponds with cell concentration in adhesive state experiments. Cells were exposed to the drugs for 2 h immediately after seeding and the samples were shaken carefully every 15 minutes during the incubation to keep the cells in suspension. After the exposure the cells were centrifuged, the medium was removed, cells were washed with PBS and consecutively transferred to 1.5 ml tubes where they were lyzed with 0.5 ml sub-boiled HNO_3 (conc.) per tube for 1 h. Pt concentration was quantified by ICP-MS in the presence of 0.5 μM In standard. Results are based on three independent experiments, each consisting of three replicates.

DNA Platination levels were determined as a platinum-phosphorus ratio by ICP-MS in the presence of In/Be standard. In order to characterize DNA platination, cells were seeded into Petri dishes and grew until 80% confluence, subsequently samples were treated with test drug in 10 μM concentration for 24 h. After the exposure cells were washed in TBS (0.0027 M KCl, 0.137 M NaCl, 0.025 M Tris Base, pH 7.4) and harvested with trypsin. The DNA was extracted using lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 20 μg/mL Rnase, 0.2 % Triton X-100, pH 7.4), precipitated with isopropanol and extensively washed with 70% ethanol. The DNA pellets were dried and hydrolyzed in 2% sub-boiled HCl over night at room temperature.

3. RESULTS AND DISCUSSION

3.1 Identification and Characterization of Compounds

Prior to the amination reactions we have synthesized cyanamide platinum(II) complexes: $[PtCl_2(Et_2NCN)_2]$ and $[PtCl_2(C_5H_{10}NCN)_2]$. *Cis*- and *trans*- compounds were separated using column chromatography on silica gel (SiO_2 Merck 60 F_{254}). Stability pre-test of the starting compounds was conducted using a two-dimensional chromatography method on aluminum Merck 60 F_{254} plates; also it helped us to select an optimal system for elution. Monitoring of the reactions was carried out using the TLC method in the presence of reference substances – the original substances in the same conditions. The conclusion about the reaction stage was based on the appearance of new spots on TLC plates and the disappearance of initial spots. The TLC method helps to indicate the selectivity of the reactions by the number of spots on the plate after the elution; it helps to predict the possible geometrical structure of the product due to retention indexes, and in some cases the possible application of chromatographic methods for isolation and purification of the reaction products [33].

After the isolation of the products in the solid state the IR spectra were recorded in mid-IR range (4000–400 cm^{-1}). We could assess the direction and extent of the reactions due to the absence of some characteristic absorption bands of the initial cyanamide complex and the appearance of absorption bands specific to the reaction products, and due to relative intensity of the bands in the IR spectrum [34]. The characteristic C≡N bands of the initial cyanamide Pt^{II} complexes are observed in the IR spectrum in the range 2250–2350 cm^{-1} (**Fig. 3.1**).

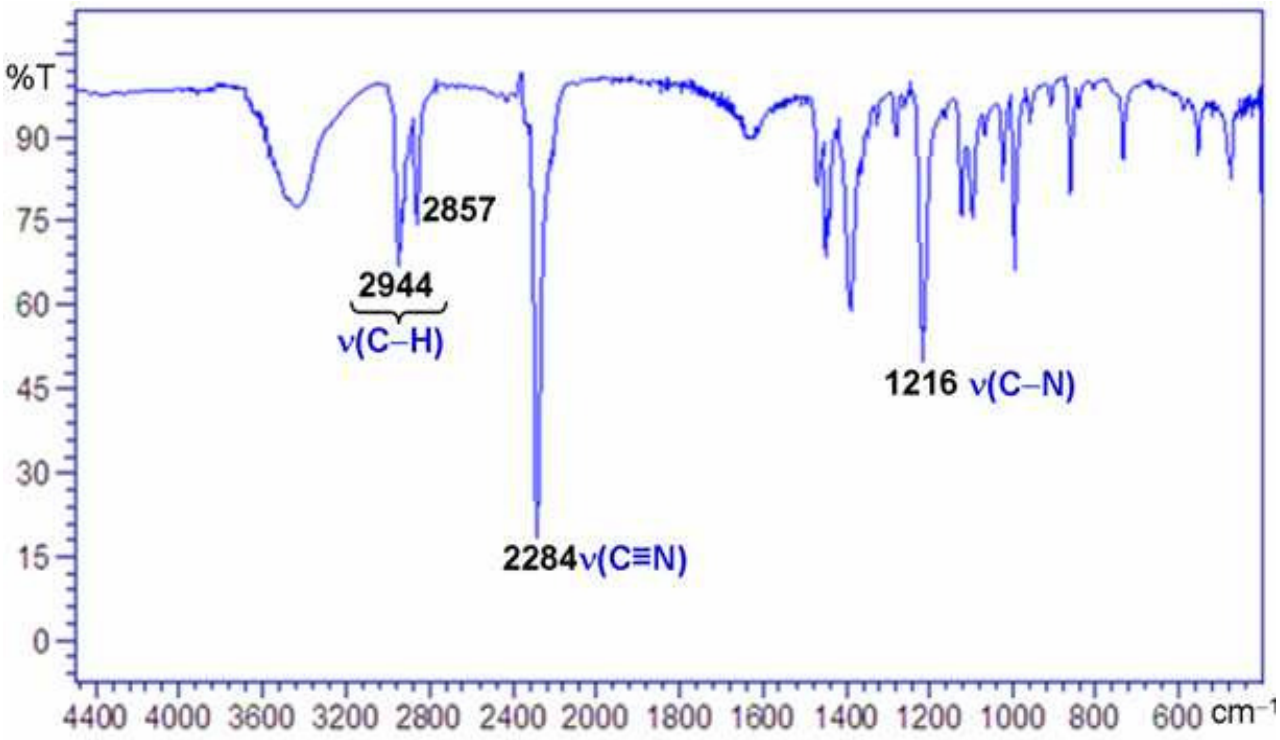


Figure 3.1. Mid-range IR-spectrum of *cis*-$[PtCl_2(NCNC_5H_{10})_2]$ complex (KBr).

When the nucleophilic cyanamide-methylamine coupling takes place, a characteristic C=N vibration band appears in the range 1700–1500 cm^{-1} and characteristic bands of the imine hydrogen atoms appear at frequencies of 3300–3000 cm^{-1} in the IR spectrum. One can also observe the absence of a characteristic C≡N vibration band in the range of 2350–2250 cm^{-1} (**Fig. 3.2**).

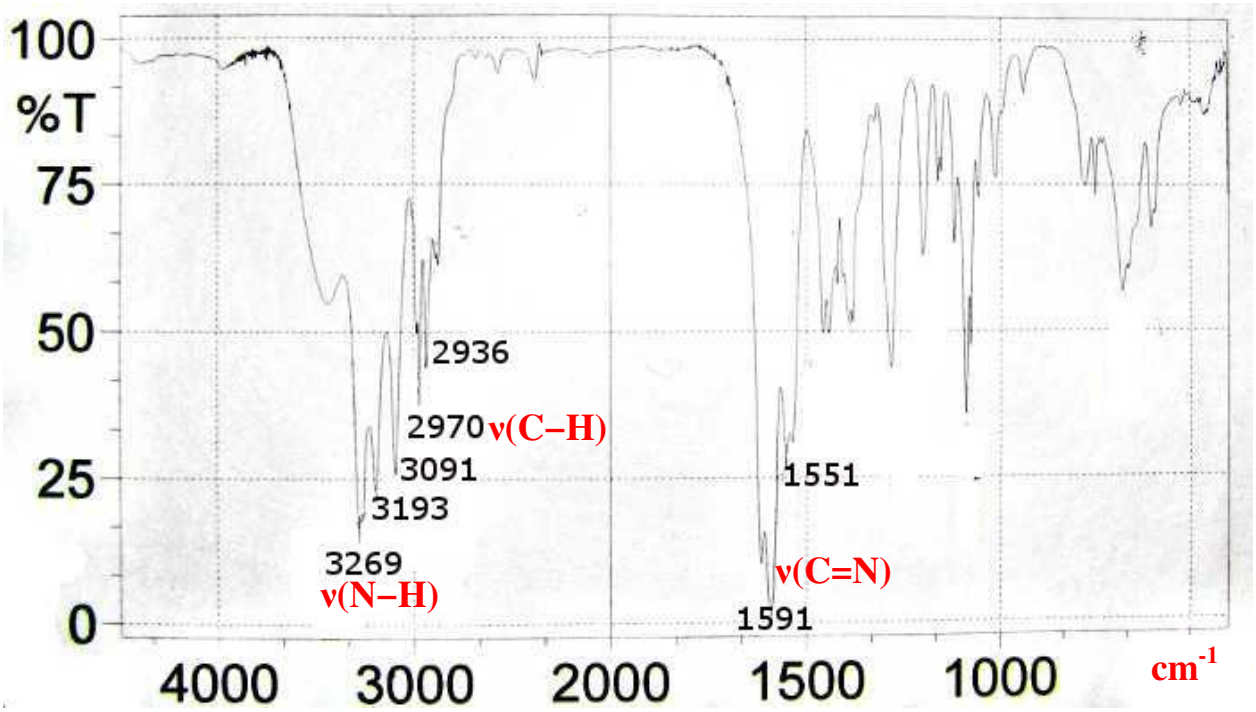


Figure 3.2. Mid-range IR-spectrum of *trans*-**7** complex (KBr).

In the ES^+ mass spectra characteristic molecular ion peaks and peaks corresponding to the fragmentation of the analyzed compounds are observed. The most frequent peaks are $[M + H]^+$, $[M + K + 2H - 2NH_3 - Cl]^+$, $[M - NH_3 - Cl]^+$, $[M - 2NH_3 - 2Cl - H]^+$, $[M - 2Cl - H]^+$ for the reaction products of nucleophilic addition of ammonia and $[2M - H]^+$, $[M - H - Cl - NH_2Me]^+$, $1/2[M - 2Cl]^{2+}$ for the reaction products of nucleophilic addition of methylamine. Additionally the fragmentation of the complexes with the removal of the ligand and the formation of multiply charged ions can occur. However, in practice this happens rarely due to the fact that in the ES^+ mass spectrometry quite a soft ionization method is used: the ionization runs at atmospheric pressure under the influence of the potential difference [35].

Thus, the methods of IR spectroscopy and ES^+ mass spectrometry allow to establish the direction of the reactions and to confirm the presence of some fragments in the molecule of the product.

In case of high purity of the compounds, we recorded the ^1H NMR and $^{13}C\{^1H\}$ NMR spectra. The assignment of signals based on the analysis of chemical shifts and on integration of

signals in the ^{1}H NMR spectrum helps to determine the number of protons of each type and to suggest a possible structure and the configuration of the complex.

$^{13}C\{^{1}H\}$ NMR spectra allow to complete the overall picture with additional structural information and to clarify the compound's formula. In the $^{13}C\{^{1}H\}$ NMR spectrum we have recorded the appearance of the C=N group carbon atom signal, which is typical for the product of nucleophilic addition to cyanamide ligands. Changes in chemical shifts corresponding to all carbon atom signals of the products were observed.

After the spectroscopic characterization of the substances the elemental analysis (C, H, N) was performed. The match of the obtained results with the theoretically calculated, within the error of the method, is additional proof of the product's purity and assumed chemical formula.

In the next chapter more detailed characterization data for the compounds derived from cyanamide–methylamine coupling will be presented. Characterization data for the reaction products with ammonia have been described in the thesis of Tyan M. and in the related article [11].

3.2 Characterization of Novel Cationic Guanidine Platinum(II) Complexes

The composition of *trans*-**7** and *trans*-**8** complexes is consistent with the data of C, H, N elemental analysis. ES^+ mass spectrometry data was obtained for these compounds (**Fig. 3.3**).

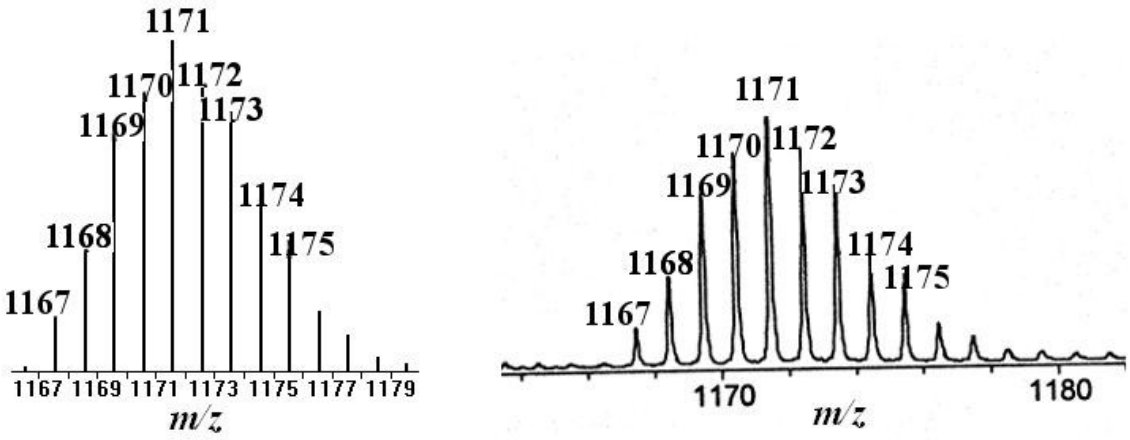


Figure 3.3. Signal of *trans*-**7** complex, corresponding to the quasi-ion $[2M - H]^+$. Calculated (left) and recorded using ES^+ mass spectrometry method (right).

In the IR spectra *trans*-**7** and *trans*-**8** complexes give average intensity bands corresponding to N–H vibrations, which appear at frequencies of 3300–3000 cm^{-1}, and a high intensity ν(C=N) band in the range from 1639 to 1515 cm^{-1} (**Fig. 3.4**).

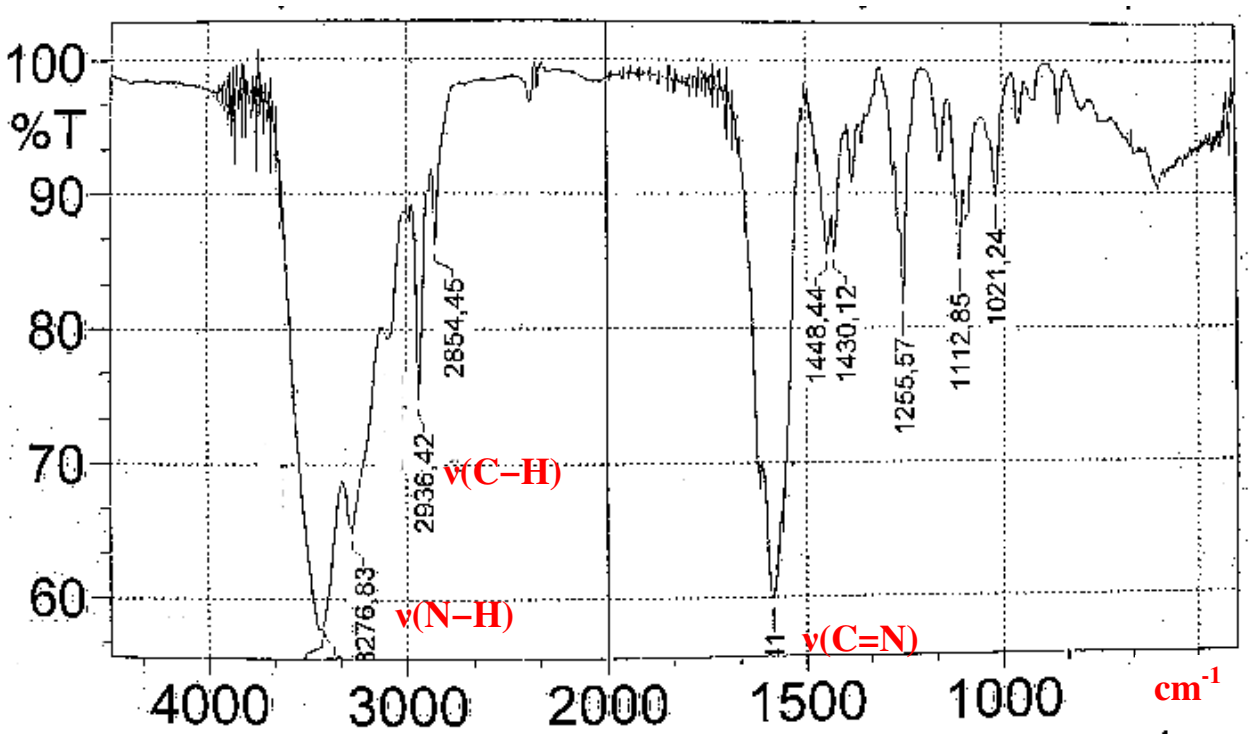


Figure 3.4. IR spectrum of the *trans*-**8** complex in the mid-IR range (KBr).

The characteristic feature of *trans*-**7** and *trans*-**8** ^{1}HNMR spectra (**Fig. 3.5**) is the presence of three singlets in 1:1:2 ratio at 7.51-7.35 for imine proton (***H*N=C**), at 5.69-5.48 for amide proton of guanidine group (***H*N**–Me) and at 5.12-4.69 for amine protons (***H*$_2$N**–Me) of methylamine coordinated to platinum, respectively.

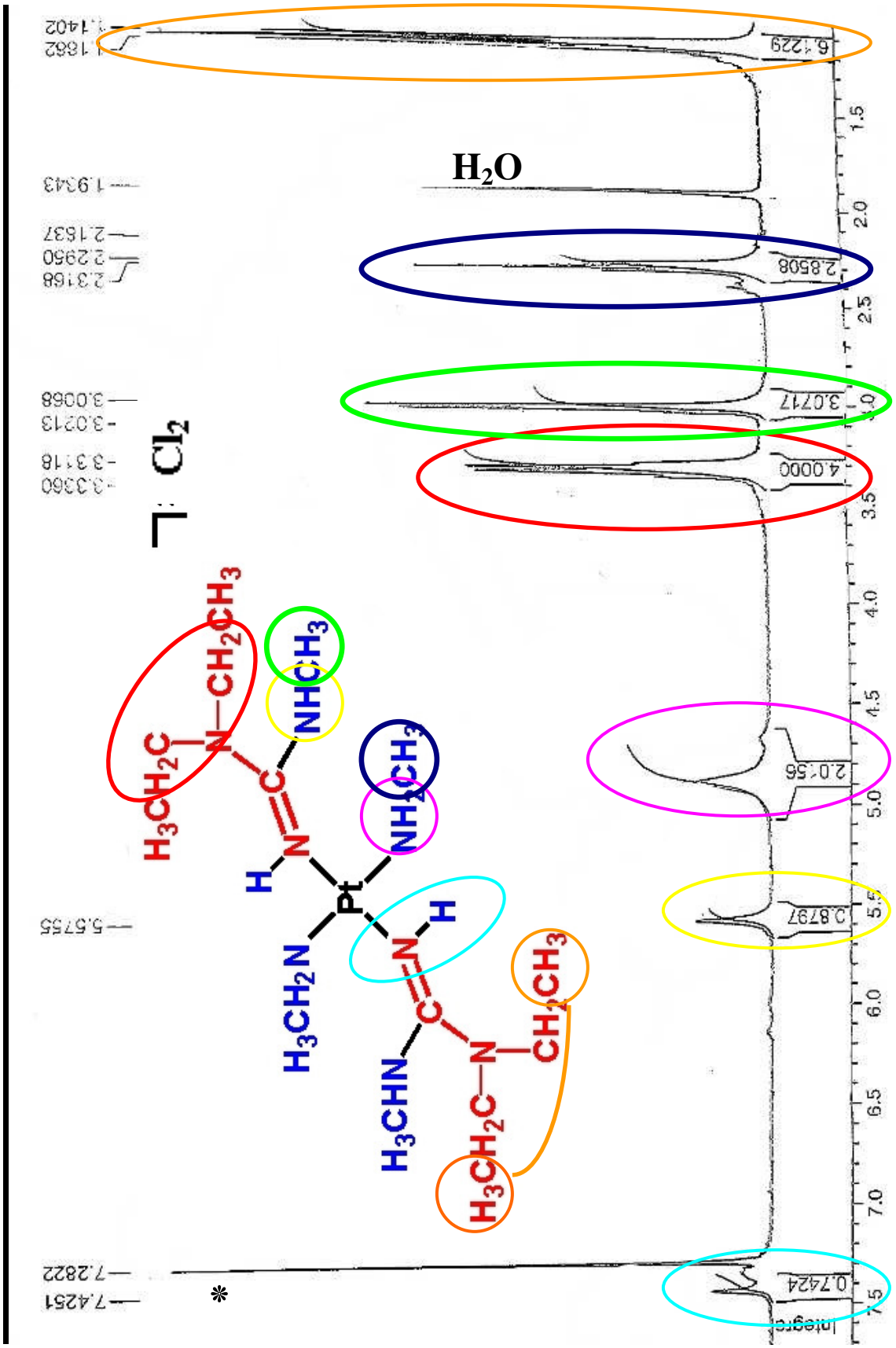


Figure 3.5. ^{1}H NMR spectrum of *trans*-**7** complex in $CDCl_3$. Solvent peak is marked with *.

In the $^{13}C\{^{1}H\}$ NMR spectra of *trans*-**7** and *trans*-**8** compounds the N=***C***–N carbon atom signals are observed in the range 166.85–162.33; these signals are arranged in the characteristic area for C=N carbon atoms (**Fig. 3.6**).

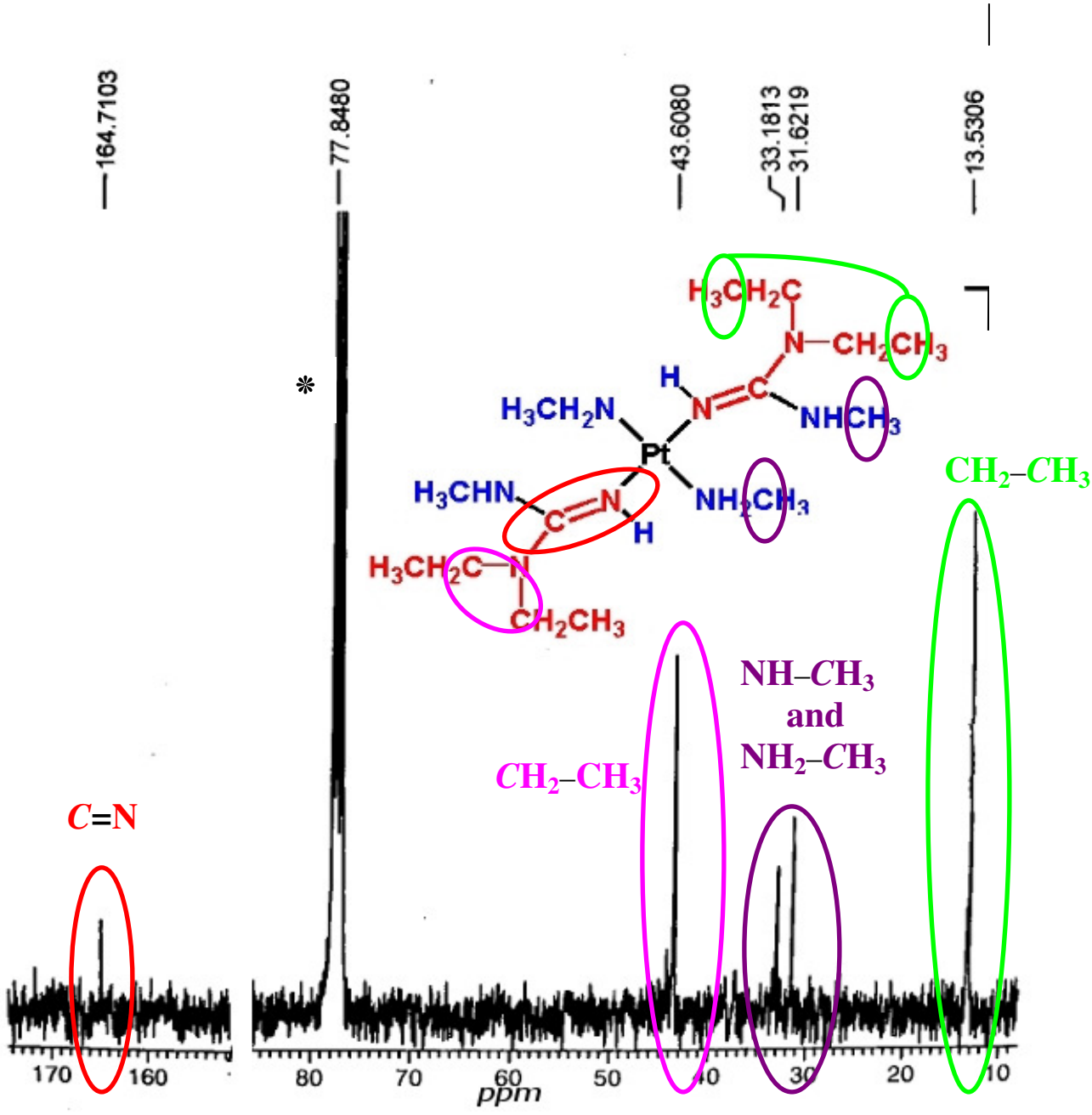


Figure 3.6. $^{13}C\{^{1}H\}$ NMR spectrum of *trans*-**7** complex in $CDCl_3$. The peak of the solvent is marked with an asterisk.

3.3 Cytotoxic Activity of Guanidine Platinum(II) Complexes

Cytotoxicity tests were performed *in vitro* on two cancer cell lines (CH1 and SW480). The inhibitory potency on cell proliferation and viability was characterized using the spectrophotometric MTT method (MTT assay). To compare the activity of different compounds the IC_{50} value was determined. IC_{50} values for each compound were obtained from three independent experiments.

The *in vitro* antitumor activity of complexes *cis*-**1**–**6** and *trans*-**1**–**8** was investigated (**Tab. 1**) and structure-activity relationships were inferred from the data.

Table 1. IC_{50} values (μM; mean ± standard deviation) of complexes *cis*-**1**–**6** and *trans*-**1**–**8** on two cell lines (CH1 and SW480).

		CH1	SW480
1	*cis*	2.7 ± 0.4	36 ± 2
	trans	5.2 ± 1.1	36 ± 10
2	*cis*	3.0 ± 0.4	32 ± 7
	trans	23 ± 4	62 ± 5
3	*cis*	4.4 ± 0.8	32 ± 5
	trans	7.4 ± 1.3	19 ± 3
4	*cis*	21 ± 6	374 ± 40
	trans	209 ± 65	>640
5	*cis*	39 ± 12	42 ± 6
	trans	25 ± 4	111 ± 22
6	*cis*	40 ± 2	46 ± 2
	trans	8.2 ± 2	55 ± 1
7	*trans*	135 ± 42	241 ± 3
8	*trans*	325 ± 58	>640
cisplatin		0.14 ± 0.03	3.3 ± 0.4
transplatin		15 ± 2	19 ± 3

		CH1	SW480
1	*cis*	2.7 ± 0.4	36 ± 2
2	*cis*	3.0 ± 0.4	32 ± 7
3	*cis*	4.4 ± 0.8	32 ± 5
1	*trans*	5.2 ± 1.1	36 ± 10
2	*trans*	23 ± 4	62 ± 5
3	***trans***	7.4 ± 1.3	**19 ± 3**
4	*trans*	209 ± 65	>640
5	*trans*	25 ± 4	111 ± 22
6	***trans***	**8.2 ± 2**	55 ± 1
4	*cis*	21 ± 6	374 ± 40
5	*cis*	39 ± 12	42 ± 6
6	***cis***	**40 ± 2**	46 ± 2
7	*trans*	135 ± 42	241 ± 3
8	*trans*	325 ± 58	>640
cisplatin		0.14 ±0.03	3.3 ± 0.4
transplatin		15 ± 2	19 ± 3

The most interesting relationship is shown for substances *trans*-**4**,**5**,**6** (**Fig. 3.7**). The antitumor activity of these compounds correlates with the length of the side residue (IC_{50} varies from 209 μM for R = NMe_2 to 8.2 μM for R = NC_5H_{10}). Moreover, the cytotoxicity of complex *trans*-**6** on the CH1 cells is significantly higher than that of *cis*-**6** (**Fig. 3.8**).

It is also worth mentioning that the most active compound on the SW480 cell line is complex *trans*-**3** with the largest side residue R (R = NC_5H_{10}, IC_{50} = 19 μM). Antitumor activity of this compound exceeds the corresponding value for the most active complex of *cis*-configuration (*cis*-**3**, IC_{50} = 32 μM) twice (**Fig. 3.9**).

Taking into account the similarity of the structure of platinum(II) complexes with guanidine ligands and cisplatin/transplatin and the cytotoxicity of the latter couple we might expect the opposite outcome. Regarding these findings guanidine platinum(II) complexes are interesting objects for further investigation. Since the differences in cytotoxic potency between cisplatin and transplatin have been ascribed to different capacities of DNA cross-linking, analysis of DNA interactions of compounds *cis*-**1**,**3**,**4**,**6** and *trans*-**1**,**3**,**4**,**6**,**7** was done in order to find hints for the possible reasons for their different activities (see section **3.5** and **3.6**).

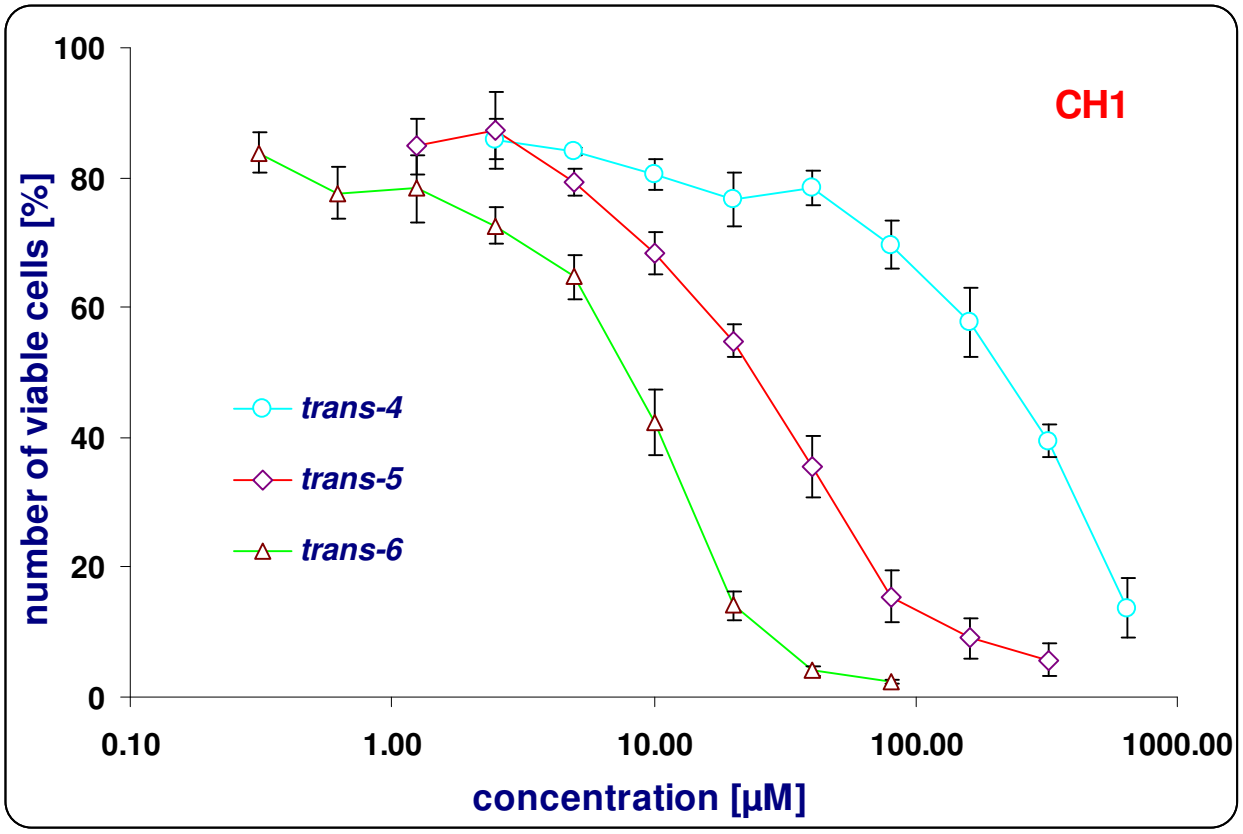


Figure 3.7. The relationship between the *in vitro* antitumor activity of substances *trans*-**4**,**5**,**6** and the length of the side residue (R = NMe_2 in *trans*-**4**, NEt_2 in *trans*-**5**, NC_5H_{10} in *trans*-**6**) in CH1 cells.

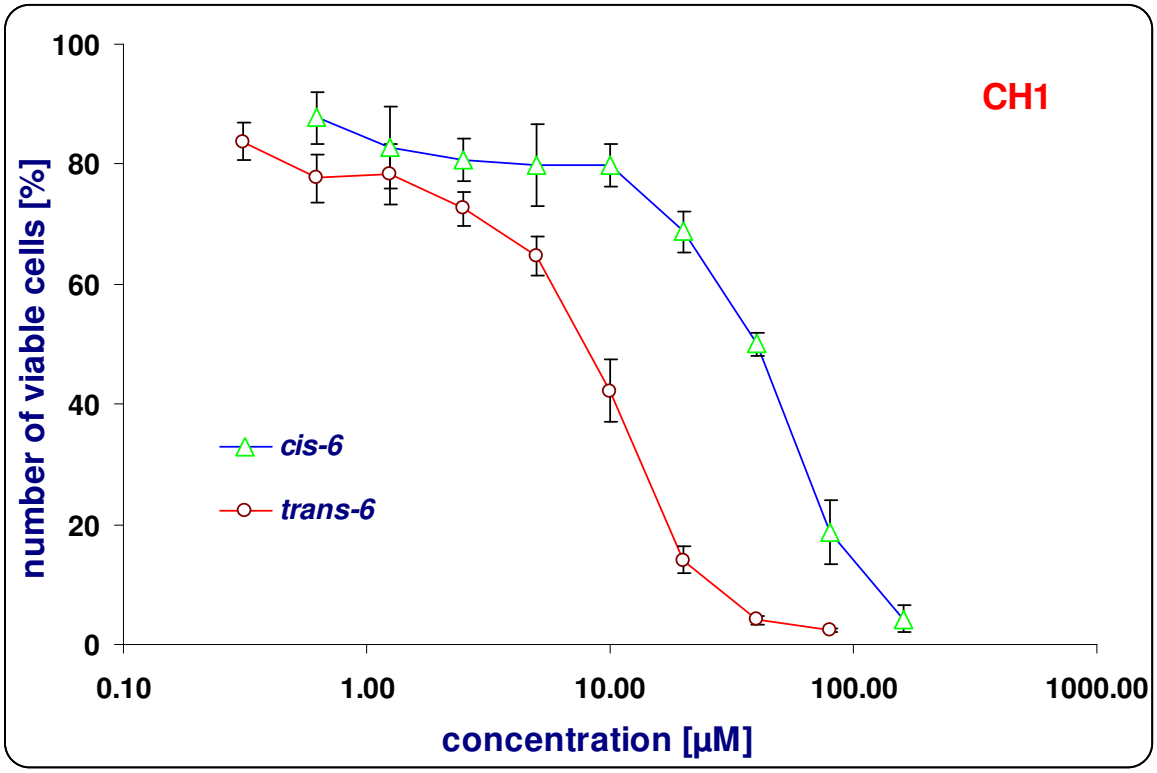


Figure 3.8. Comparison of cytotoxic activity of compounds *trans*-**6** and *cis*-**6** in CH1 cells; IC_{50} = 8.2 µM (*trans*-**6**), IC_{50} = 40 µM (*cis*-**6**).

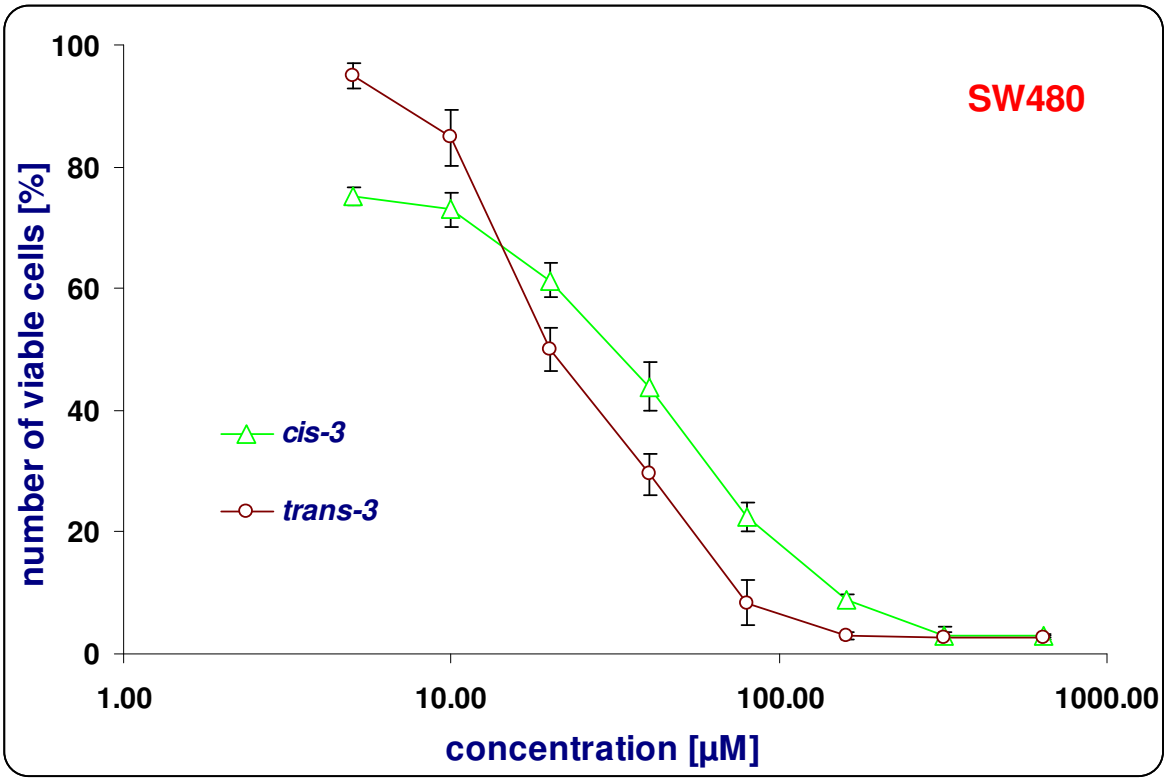


Figure 3.9. Comparison of cytotoxic activity of compounds *trans*-**3** and *cis*-**3** in SW480 cells; IC_{50} = 19 µM (*trans*-**3**), IC_{50} = 312 µM (*cis*-**3**).

The IC_{50} values of complexes *cis*-**1**–**6**, *trans*-**1**–**8** were compared with the couple cisplatin/transplatin. All the compounds are inferior in activity to cisplatin (IC_{50} = 0.1 μM for CH1 cell line, IC_{50} = 3.3 μM for SW480 cell line). However, the deviation is not always high, e.g. *trans*-**3** (IC_{50} = 18.6 μM for SW480 cell line, **Fig. 3.13**). Cytotoxic activity of the majority of the samples exceeds or is comparable to that of transplatin (IC_{50} = 15 μM for CH1 cell line, IC_{50} = 19 μM for SW480 cell line, **Fig. 3.10–3.13**).

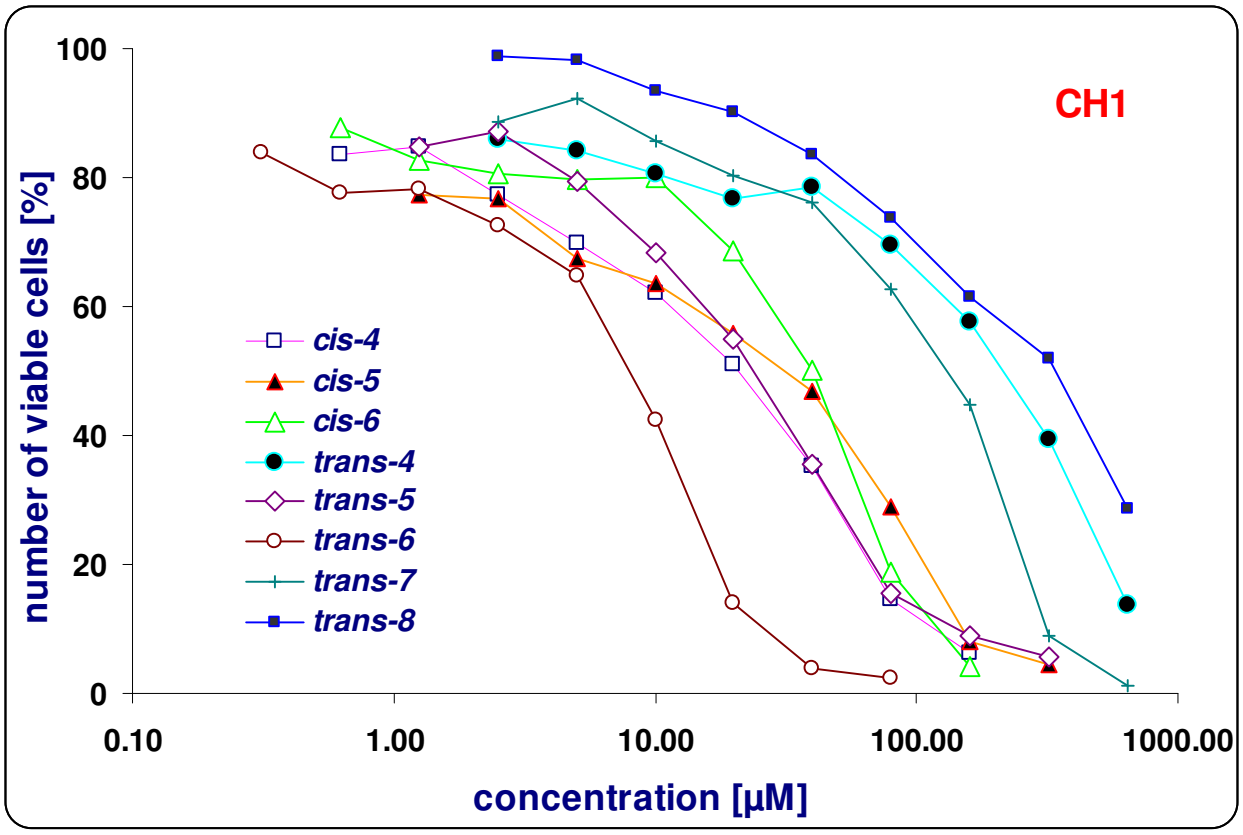


Figure 3.10. Cytotoxicity of cationic *cis*- and *trans*- guanidine complexes in CH1 cells.

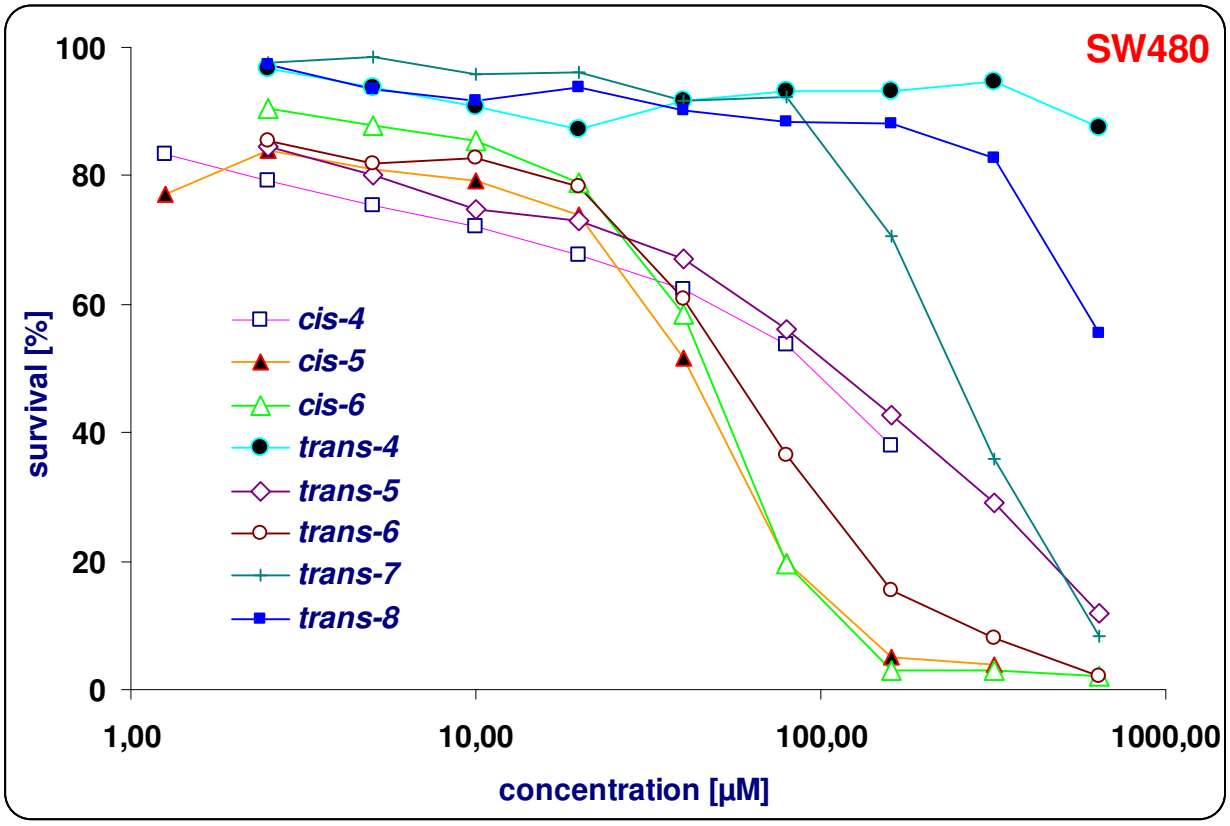


Figure 3.11. Cytotoxicity of cationic *cis*- and *trans*- guanidine complexes in SW480 cells

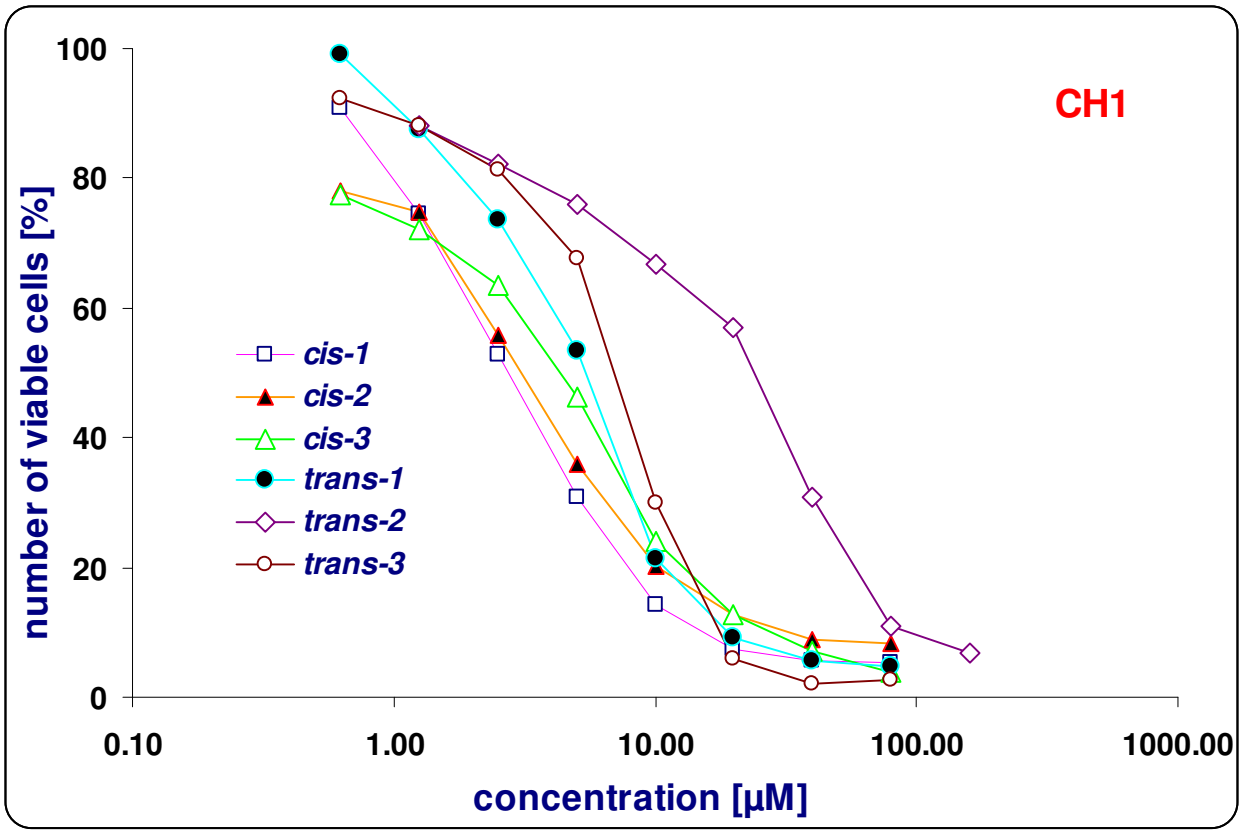


Figure 3.12. Cytotoxicity of neutral *cis*- and *trans*- guanidine complexes in CH1 cells

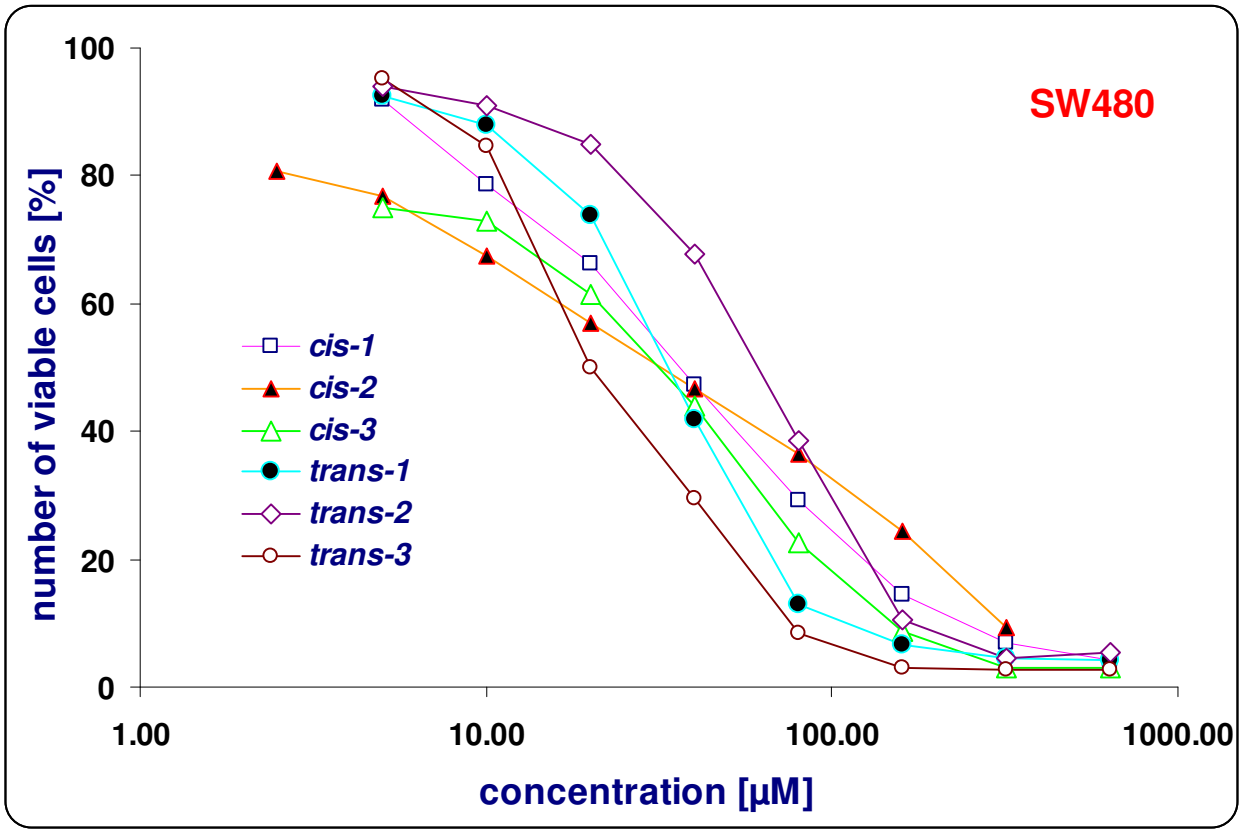


Figure 3.13. Cytotoxicity of neutral *cis*- and *trans*- guanidine complexes in SW480 cells

3.4 Cellular Uptake Experiments

In order to figure out whether cytotoxicity correlates with cellular uptake of the compounds cellular platinum levels were analyzed in treated cells by ICP-MS. Inductively coupled plasma mass spectrometry (ICP-MS) is one of the main techniques for sensitive qualitative and quantitative analysis of metals and non-metals. The main advantages of this method are rapidity, high sensitivity (which is important due to small concentration of some substances in the cells), multi-elemental analysis (which is crucial for DNA platination experiments where Pt concentration has to be measured together with P concentration to determine the amount of platinum relative to the number of nucleotides) and good control of interferences.

The total amount of cellular Pt, which is taken up by human cancer SW480 cell line during 2 h exposure to a 50 µM of *cis*-**1**,**3**,**4**,**6**, *trans*-**1**,**3**,**4**,**6**,**7**, as well as the reference compounds was measured using ICP-MS (**Fig 3.14**). As described by Egger et al. [40]

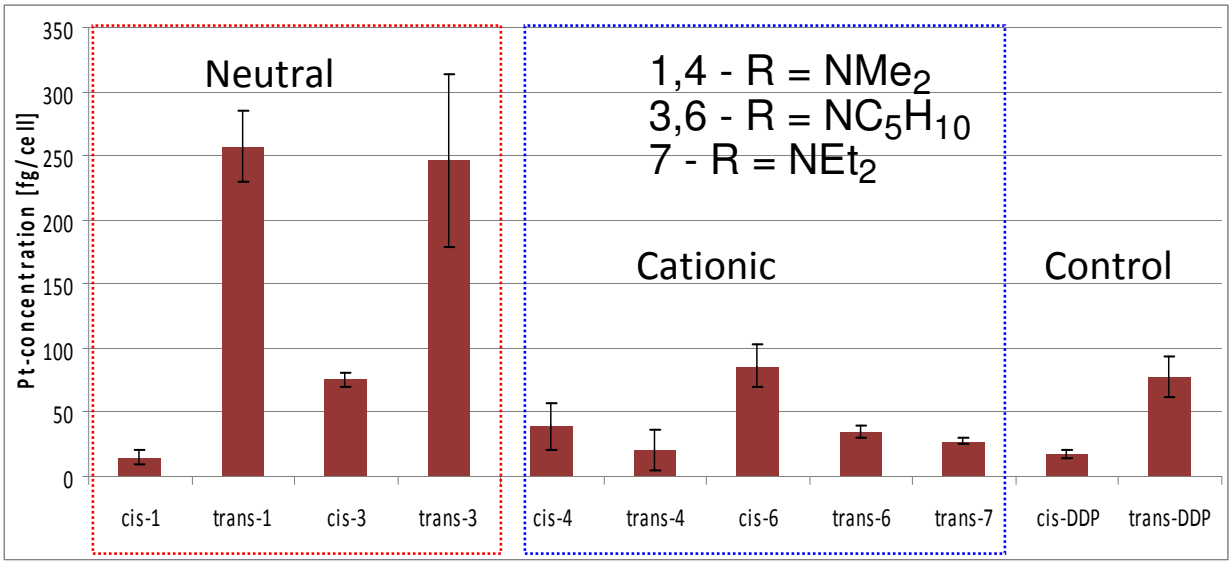


Figure 3.14. Cellular uptake in adherent SW480 cells during 2 h exposure with 50 μM *cis*- and *trans*-configured Pt complexes.

The dependence of cellular uptake on coordination geometry in the case of neutral guanidine complexes (*cis*-**1**, *cis*-**3**, *trans*-**1**, *trans*-**3**) correlates well with the reference couple (*cis*-DDP, *trans*-DDP), where *trans*-configured compounds are taken up more efficiently by the cells. For the cationic guanidine complexes (*cis*-**4**, *cis*-**6**, *trans*-**4**, *trans*-**6**) the reversed relationship is observed. During the experiment with adhesive cell microcultures we faced a problem that some of the substances (*cis*-**4**, *trans*-**4**) had high affinity to the plastics of the dishes. The control wells which were exposed to the test substances but were free from cells during all the experiment, showed a high concentration of the Pt at ICP-MS detection, meaning that big amount of drug must have been absorbed to the well's surface and became desorbed by the preparation.

To overcome the problem we decided to optimize the conditions for these two substances and performed the experiment with non-adherent cells. These helped us to avoid desorption of adsorbed compounds as we isolated the exposure and lyzing steps from each other using different vessels.

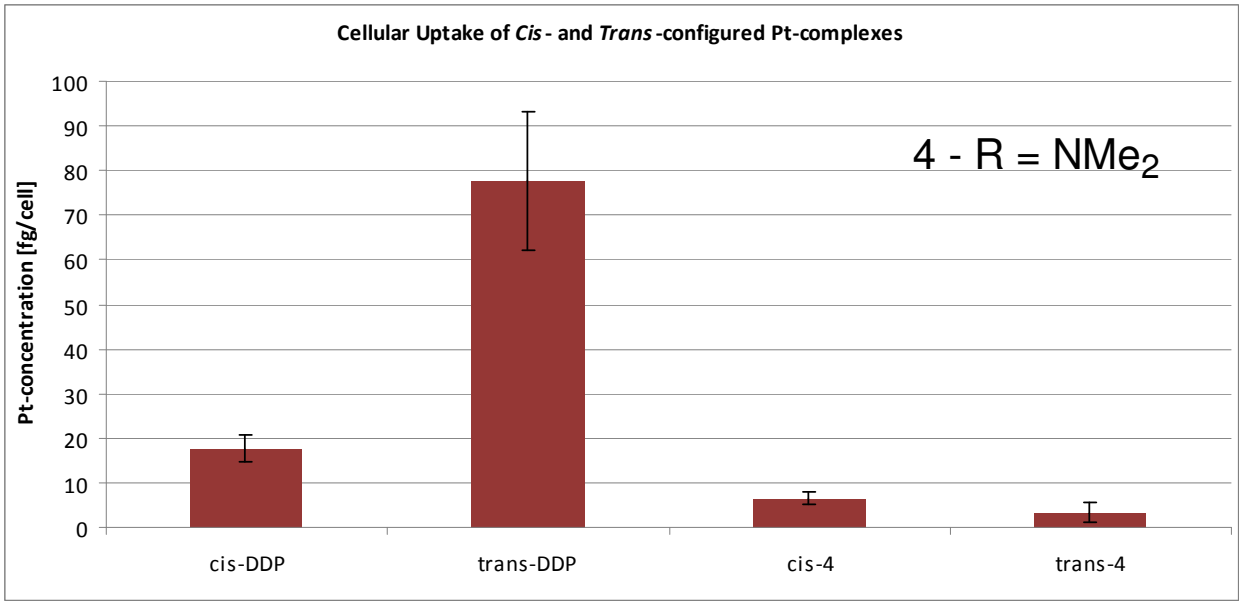


Figure 3.15. Cellular uptake of compounds *cis*-**4**, *trans*-**4** (50 µM, 2 h exposure) in non-adherent experiment compared with the *cis*-DDP/*trans*-DDP couple in SW480 cells.

The modified experiment showed rather appropriate repeatability and comparatively low deviation levels. The values obtained for *cis*-DDP and *trans*-DDP correspond well with those observed in the adherent state.

To interpret the uptake results several important features common and diverse for the cationic and neutral guanidine complexes should be mentioned:

1. On one hand the symmetry of the *trans*-configured complexes reduces the dipole moment of the molecule making them more lipophilic. Thereby neutral *trans*-compounds possess a higher ability to penetrate lipid membranes. On the other hand the dipole moment of *cis*-complexes is asymmetric, causing an increase of polarity, lower lipophilicity and lower cellular permeability as a consequence. Another important difference between *cis*- and *trans*- complexes is their three-dimensional geometry. While *trans*-complexes maintain the planar structure in the solution *cis*-complexes steric hindrance of adjacent ligands can force their rotation out of the coordination plane. The planer structure is sterically more favorable for passage through membranes which allows the *trans*-complexes to be taken up more efficiently.

2. Kinetically inert amino-containing ligands of the cationic complexes are very stable and can be hardly exchanged with H_2O. Recently we can not explain what leads to a higher

lipophilicity and better cellular permeability of *cis*-configured cationic guanidine complexes in comparison with their *trans*-isomers.

3. Talking about the relationship between different *cis*-/*trans*-couples we suggest that the permeability of complexes with larger organic side residues is higher due to their pronounced lipophilicity which helps them to enter the cells.

3.5 DNA Platination

ICP-MS was used to detect the DNA platination of SW480 cells. Pt concentration was measured together with P concentration to determine the amount of bound platinum relative to the number of nucleotides. Based on Pt and phosphorus concentrations the Pt-nucleotide ratio was calculated as a measure for the extent of DNA platination. The DNA platination is given in comparison with cellular uptake data (**Fig. 3.16**).

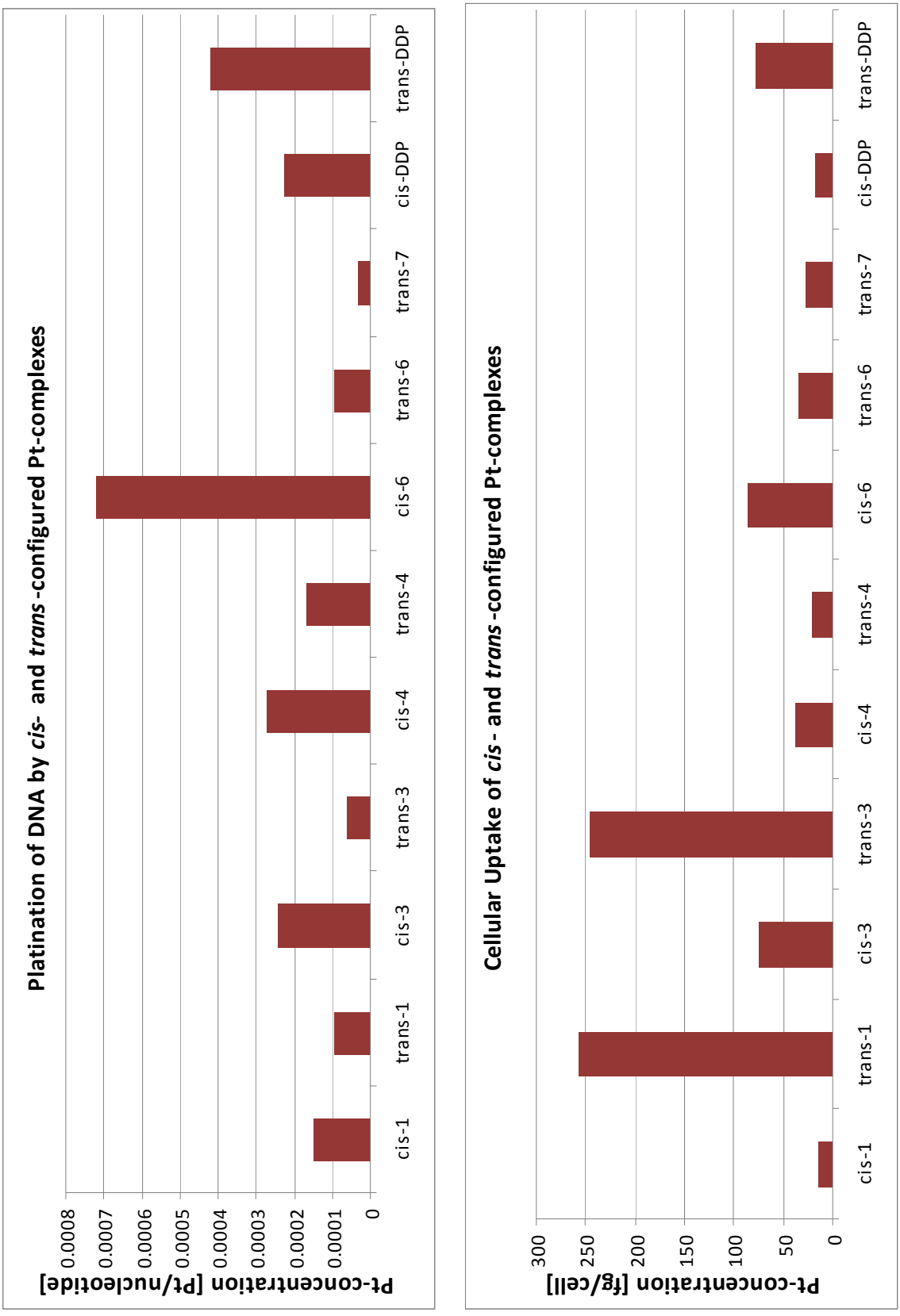


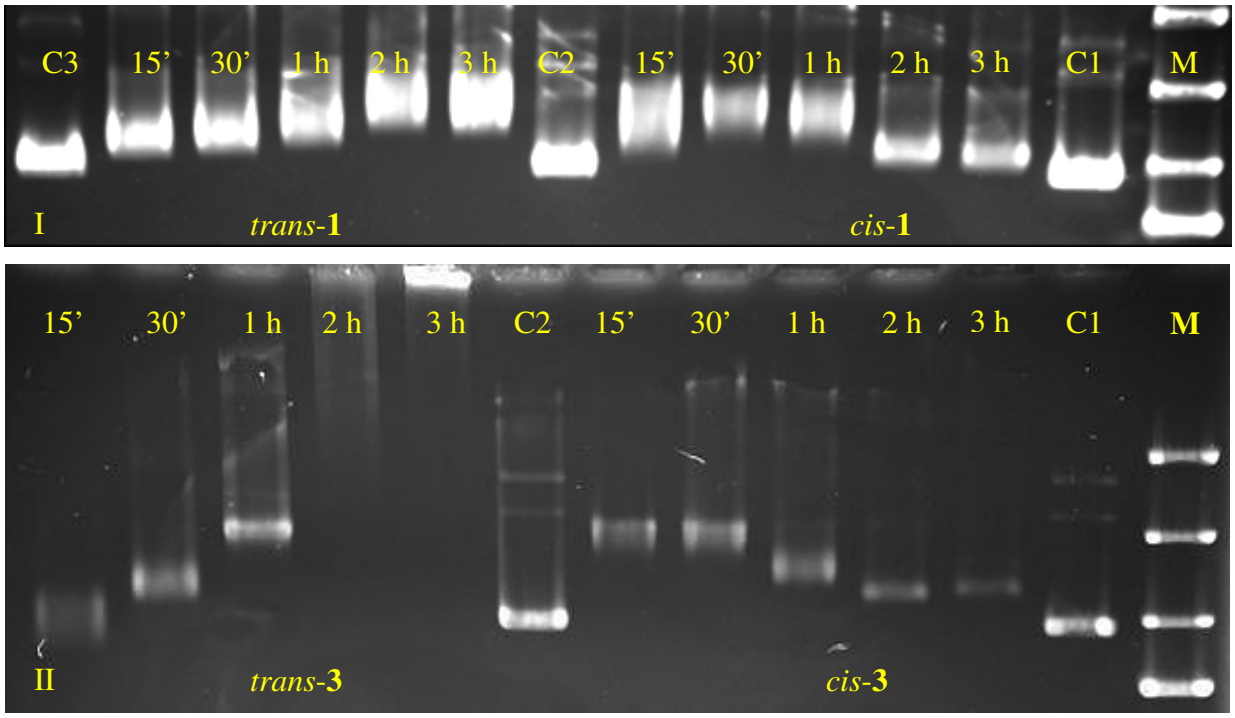
Figure 3.16. DNA platination (10 µM, 24 h exposure) and cellular uptake (50 µM, 2 h exposure) of *cis*- and *trans*-configured Pt complexes in SW480 cells.

The cellular uptake of *cis*-DDP/*trans*-DDP and cationic guanidine complexes corresponds quite well with the DNA platination values, while the *trans*-configured neutral complexes, which were taken up more effectively show lower levels of DNA binding then their *cis*-congeners.

For *cis*-configured guanidine complexes higher number of DNA interactions was shown. We assume that ligands in *cis*-position are less shielded and are capable of forming the higher number of Pt-DNA adducts. The strength and structure of the adducts differs greatly e.g. lower amount of *trans*-**3** complex in the SW480 cells nuclei is more cytotoxic then its *cis*-isomer (**Fig. 3.10**).

3.6 Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay was performed for the same set of substances. The pUC19 plasmid was exposed to the drugs for different time periods (3 h – 2 h – 1 h – 30 min – 15 min) and then the mobility of the DNA was studied in the agarose gel. The retardation of the supercoiled form plasmid in the gel allows us to judge about the impact of the compounds on DNA secondary structure (**Fig. 3.17**).



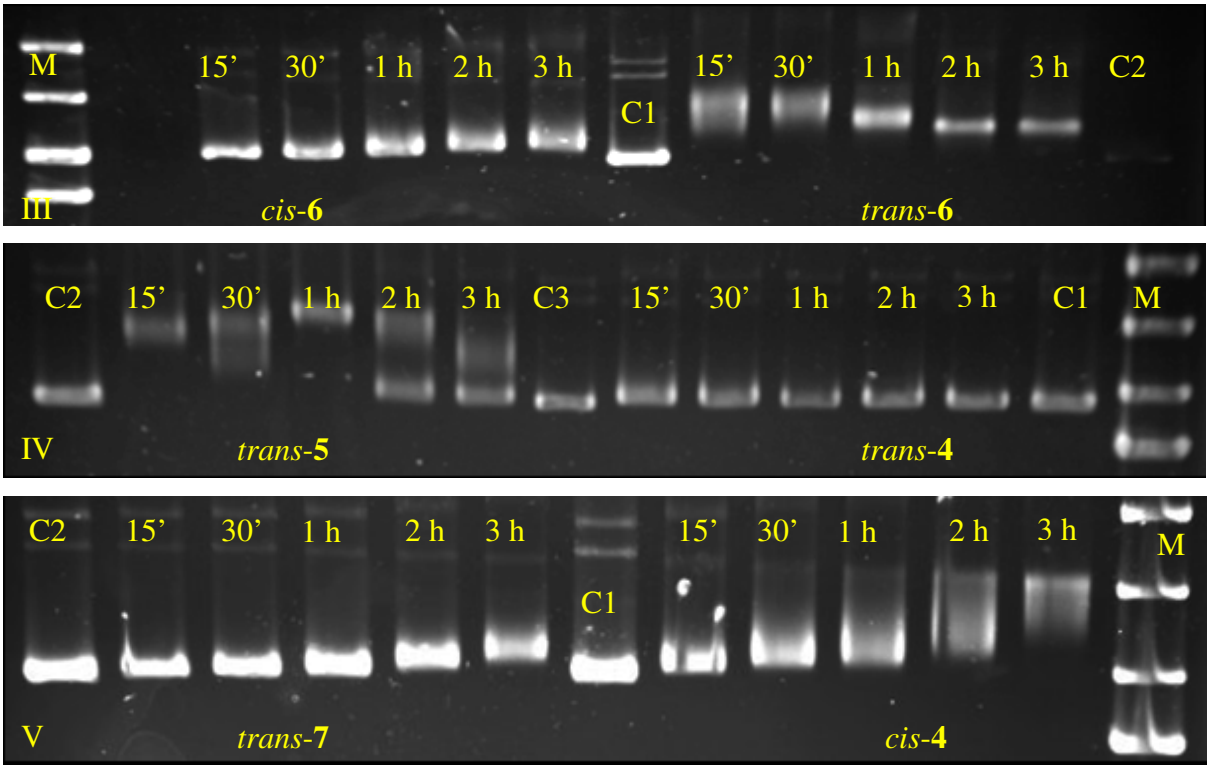


Figure 3.17 (I–V). Mobility of the plasmid pUC19 after the exposure to 100 μM of different *cis-*/*trans*- configured drugs. C – control, M – marker. Bright bands correspond to the supercoiled form of the plasmid, whereas bands of the open circular form are mostly too weak to allow interpretation.

From all the tested compounds only *trans*-**4** (**Fig. 3.17 IV**) does not alter the electrophoretic mobility of the plasmid significantly, suggesting that if this compound interacts with DNA at all it does so without altering DNA secondary structure, excluding all forms of cross-links. This is consistent with the lack of marked cytotoxicity in the MTT assay (**Fig. 3.10**, **3.11**). All the other drugs are capable of DNA binding in varying degree. Complex *trans*-**3** is also remarkable (**Fig. 3.17 II**), as it is able to bind to DNA such that DNA becomes immobile in the electric field and remains in the starting well during the electrophoresis after 3 h exposure with the drug. This might be caused by interhelical cross-linking of plasmid molecules to larger aggregates which are not able to enter the separation gel.

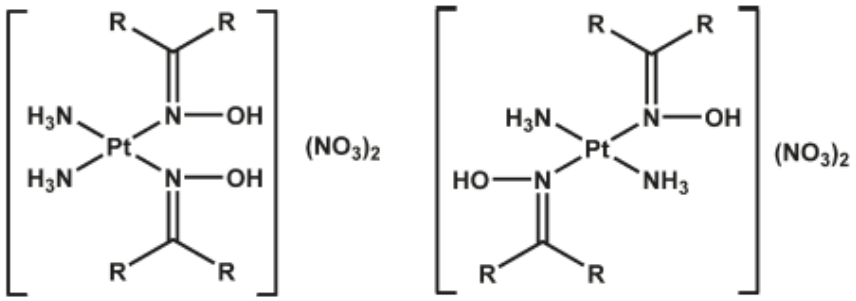
Remarkably, the cationic complexes *trans*-**5**–**7** (containing N-donor ligand only) show a capacity of altering DNA secondary structure similar to cross-linking compounds, even though the lack of readily exchangeable ligands suggests that DNA interaction of these compounds should be of an electrostatic kind primarily.

Overall, there is no clear correlation between the cytotoxicity and the impact on plasmid DNA for most of the drugs concerning both the time required for the shift and the extent of the shift. The only clearly observed relationship belongs to the compounds *trans*-**4**,**5**,**6**, whose cytotoxicity increases with the enlargement of the side residue (**Fig. 3.10**, **3.11, 3.17 III**,**IV**). The fastest DNA impact was shown to the most active complex *trans*-**6**, while least active substance *trans*-**4** does not show any significant impact on the secondary structure of the plasmid DNA.

An interesting pattern was observed simultaneously for several *cis*- and *trans*-configured complexes (*cis*-**1**,**3** and *trans*-**5**,**6**). In these cases, the initial shift of the bond becomes reversed upon longer exposure. We assume that the explanation of the plasmid DNA retardation pattern can be similar to the one described for related platinum complexes by Zorbas-Seifried et al.: untwisting of negative supercoils by platinum-DNA adducts can be followed by induction of positive supercoils [32]. In general, monofunctional and bifunctional adducts can result in retardation of the plasmid DNA due to higher mass, decrease of positive charge, untwisting of the supercoiled form or acceleration of the plasmid DNA due to bending and/or twisting. The composite action of all these factors in time can lead to the observed pattern.

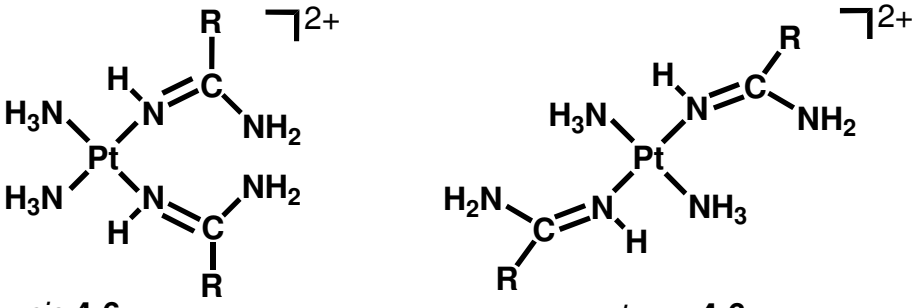
4. CONCLUSION and OUTLOOK

Novel *cis*- and *trans*-configured (guanidine)$_2$Pt^{II} complexes have been synthesized and characterized by elemental analyses, IR, electrospray ionization mass spectrometry, ^{1}H, ^{13}C NMR spectroscopy. We carried out metal-mediated nucleophilic addition of $MeNH_2$ to push-pull Pt^{II} nitriles with cyanamide ligands R_2NCN (R = NEt_2, NC_5H_{10}). The obtained *trans*-**7**,**8** compounds and the previously synthesized guanidine complexes (*cis*-**1**–**6**, *trans*-**1**–**6**) were tested for cytotoxic activity. The compounds *cis*-**1**–**6** and *trans*-**1**–**8** are promising materials for antitumor activity investigation, because they are the nitrogen analogues of a new class of potential anticancer drugs based on amidine platinum(II) complexes [29,30]. For several *cis*-/ *trans*- couples containing larger organic residues, it was reliably shown that the cytotoxic activity of *trans*-complexes is superior to those of corresponding *cis*-isomers. Moreover, according to the article by Scaffidi-Domianello et al. [41] published very recently a similar dependence of cytotoxicity on geometric isomerism was observed in the case of bis(oxime)platinum(II) complexes $[Pt(NH_3)_2(R_2C{=}NOH)_2](NO_3)_2$, where R = Me (7c/7t), Et (8c/8t), and *n*-Pr (9c/9t). These oxime complexes are structurally related to the guanidine complexes investigated here (**Fig. 3.18**). The cytotoxicity of these compounds is also comparable and shows a similar pattern (**Tab. 2**), as the platinum complexes with larger residues (R = *n*-Pr, NC_5H_{10}) and 4 nitrogen donor atoms possess higher antitumor activity in some cases.



7c R = Me
8c R = Et
9c R = *n*-Pr

7t R = Me
8t R = Et
9t R = *n*-Pr



R	Nos.
NMe_2	**4**
NEt_2	**5**
NC_5H_{10}	**6**

Figure 3.18. Platinum(II) oxime and guanidine complexes.

Table 2. IC_{50} values (μM; mean ± standard deviation) of platinum(II) oxime and guanidine complexes in CH1 and SW480 cancer cells (exposure time 96 h).

		CH1	SW480
4	*cis*	21 ± 6	374 ± 40
	trans	209 ± 65	>640
5	*cis*	39 ± 12	42 ± 6
	trans	25 ± 4	111 ± 22
6	*cis*	40 ± 2	46 ± 2
	trans	8 ± 2	55 ± 1

		CH1	SW480
7c	*cis*	21 ± 7	567 ± 39
7t	*trans*	448 ± 180	>1000
8c	*cis*	717 ± 46	>1000
8t	*trans*	>1000	>1000
9c	*cis*	162 ± 46	>1000
9t	*trans*	29 ± 5	350 ± 30

IC_{50} values of of guanidine compounds (*cis*-**1**–**6** and *trans*-**1**–**8**), vary within a broad range of micromolar concentrations. We could confirm the expectation that the cytotoxicity of several *trans*-configured complexes is higher than that of *cis*-congeners. The analysis showed that there is a correlation between the cytotoxic potency of the compounds and the length of the variable residue on the guanidine ligand. Moreover, cytotoxicities of the cationic complexes are mostly lower, but in some cases even comparable to those of the corresponding neutral complexes, despite the lack of distinctly labile ligands.

Cationic guanidine complexes *trans*-**7**,**8** – the reaction products of nucleophilic $MeNH_2$ addition to cyanamides – have shown a rather low level of *in vitro* antitumor activity (IC_{50} = 135 μM, IC_{50} = 325 μM on CH1 cells). Based on MTT assay (**Fig. 3.10**), cellular uptake (**Fig. 3.14**)

and DNA platination (**Fig. 3.16**) results. The low cytotoxicity of *trans*-**7** may be explained by the weak impact on DNA, as observed in the electrophoretic mobility shift assay, which may be due to sterical difficulties in interacting with DNA, rather than by the extent of cellular uptake, which is similar to both the weakly cytotoxic *trans*-**4** and the much cytotoxic *trans*-**6**.

Further investigations of drug-DNA interactions and other potential intracellular targets could shed some light on the mode of action of these complexes. Overall, the results obtained from the electrophoretic mobility shift assay, cellular uptake and platination experiments are consistent with the assumption that DNA is the main target for guanidine platinum(II) complexes.

Our further research can be devoted to expanding the family of $(guanidine)_2Pt^{II}$ complexes by appropriate derivatization. Their DNA interactions can be further characterized as well as studied in cells by means of the Comet Assay, and their subcellular distribution can be studied by the promising method of Secondary Ion Mass Spectrometry, which allows to observe the subcellular Pt^{II} distribution on a (sub)micrometer scale.

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6.1 **Curriculum Vitae**

Anton A. Legin

Born: January 24th, 1987

Education

A. School, High School and University

- Student, primary school | School N^{o} 639 (with an intensive learning of foreign
- Student, secondary school | languages; supervised by UNESCO), 1994–2004,
- Student, high school | St.Petersburg, Russia. Graduated with excellent records, 2004

- 2004–2008: Undergraduate Student of the Department of Biology and Pedology, specialisation: Biochemistry, St.Petersburg State University, Russia; the highest ranking student of the Department (2005–2008)
- Bachelor Degree with Honours, Department of Biology and Pedology, St.Petersburg State University, Russia, 2008
- Summer student, Institute of Inorganic Chemistry – Bioinorganic, Environmental and Radiochemistry, University of Vienna, Austria, August–October 2008. Under supervision of Professor B. K. Keppler and Dr. M. Jakupec
- 2008–2010: Undergraduate Magister Student of the Department of Chemistry, specialisation: Analytical chemistry, St.Petersburg State University, Russia; the highest ranking student of the Department (2008–2010)
- Magister Degree with Honours, Department of Chemistry, St.Petersburg State University, Russia, 2010

B. Additional Studying

- Student, The Institute of Foreign Languages, French language courses, St.Petersburg, Russia, 2000
- Student, School N^{o} 171 French language courses, St.Petersburg, Russia, 2002-2003
- In the framework of international school exchange participated in English language refinement programme, Worcestershire, England, 2003

Experience

- Bachelor Graduation Work «The assembly of "expression cassette", consisting of *NEO* gene, under control of gene's *ACT* promoter and gene's *FLD1* terminator in yeast *Pichia Pastoris*» under supervision of Dr. Marina V. Padkina, Department of Biology and Pedology, St.Petersburg State University, 2005–2008
- Summer Research Work «The functioning mechanism of antitumour drugs based on platinum complexes» under supervision of Prof. Bernhard K. Keppler and Dr. Michael Jakupec, Institute of Inorganic Chemistry – Bioinorganic, Environmental and Radiochemistry, University of Vienna, Austria, 2008
- Magister Thesis «Metal-mediated cyanamide-amine coupling and the study of antitumor activity of the resulting guanidine platinum(II) complexes» under supervision of PhD Nadezda A. Bokach, Department of Chemistry, St.Petersburg State University, 2008–2010

Honours and Awards

- The State Certificate of Honour for Excellence in Studying: English, French, Biology and Chemistry, 2004
- Diploma for persuasive knowledge in English, 2004
- "Compliment Certificate" Diploma in the school sport life contest, 2004
- Awarded Bachelor Degree Diploma with Honours as the Highest Ranking Student, Department of Biology and Pedology, St.Petersburg State University, Russia, 2008
- The Highest Record in the State Graduation Examination, Department of Biology and Pedology, St.Petersburg State University, Russia, 2008
- Awarded Magister Degree Diploma with Honours as the Highest Ranking Student, Department of Chemistry, St.Petersburg State University, Russia, 2010

Conferences

- Poster presentation at the «XXIV International Chugaev Conference on Coordination Chemistry» Saint-Petersburg, Russia, 2009;

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- Legin A. A., Tyan M. R., Kukushkin V. Yu. Platinum(II) complexes with guanidine ligands. Thesis book of XXIV International Chugaev Conference on Coordination Chemistry, Saint-Petersburg, Russia, 2009, p. 623;

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