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**BIOPHYSICAL ANALYSIS OF MECHANISMS  
UNDERLYING ANTITUMOR EFFECTS OF  
NEW ANTICANCER DRUGS**

Doctoral Thesis

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## **ACKNOWLEDGEMENT**

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## **Statutory declaration**

I hereby declare that this doctoral thesis has been written solely by myself. All the sources quoted in this work are listed in the “References” section. All published or submitted results included in this work are approved by co-authors.

Olomouc, May 2012

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## ABSTRACT

The mechanism of action of anticancer platinum drugs used in the clinic, such as cisplatin and its analogues, involves coordination to purine DNA bases. The cellular responses to such DNA damage primarily lead to cell death by apoptosis or necrosis. The ability of cancer cells to recognize damage induced in DNA by platinum antitumor drugs and initiate DNA repair is an important mechanism for therapeutic resistance and has a negative impact upon therapeutic efficacy. A possible direction in designing new drugs is thus focusing on pharmacological inhibition of DNA repair. Another interesting possibility is an enhancement of therapeutic efficacy of drugs already routinely used in the clinic, e.g. by photoactivation.

As an attempt aimed at DNA repair inhibition novel antitumor dinuclear azolato-bridged Pt<sup>II</sup> complexes, such as [(*cis*-{Pt-(NH<sub>3</sub>)<sub>2</sub>})<sub>2</sub>(μ-OH)(μ-pz)]<sup>2+</sup> (AMPZ), were designed and synthesized. The AMPZ complex has exhibited markedly higher toxic effects in some tumor cell lines compared to cisplatin plus circumvention of cross-resistance to cisplatin and it has been suggested that it is a consequence of small structural changes induced in DNA by this new dinuclear Pt<sup>II</sup> complex. Supposingly, these little conformational distortions in DNA represent a structural motif recognized by DNA repair proteins less efficiently than distortions induced by cisplatin and in this way escape repair mechanisms.

Our objective was to investigate differences in the DNA interactions between cisplatin and dinuclear azole-bridged Pt<sup>II</sup> complex AMPZ at the level of high-molecular-mass mammalian and plasmid DNAs, oligonucleotides and cell extracts. We studied conformation and repair of polymeric natural DNA after its modification by AMPZ and in succession we further specified the energetics, conformation, and recognition of short DNA duplexes containing a major adduct of this antitumor complex. We provided experimental evidence that modification by AMPZ exhibits reduced DNA repair synthesis in human cell-free extracts and that binding of this complex to DNA induces only small conformational distortions in DNA structure. As a consequence, the AMPZ adducts are not recognized by specific damaged-DNA binding proteins such as HMGB1 and XPA which are known to be important factors modulating antitumor activity of platinum drugs used in the clinic.

We also investigated the prospects of photoactivation of carboplatin, an analogue of cisplatin also used in the clinic. It was of our interest to examine whether parent

carboplatin can be affected by irradiation with light to the extent that its DNA binding and biological, including cytotoxic, properties are altered. We have found that carboplatin is converted to species capable of enhanced DNA binding by UVA irradiation and consequently its toxicity in cancer cells is markedly enhanced. Enabled by novel medical applications of laser and fiber optic technologies, selective photoactivation of carboplatin by light of appropriate wavelength could benefit treatment outcomes and inhibit acquisition of resistance.

## ABSTRAKT

Mechanismus účinku protirakovinných léčiv na bázi cisplatiny, používaných v klinické praxi, zahrnuje vazbu na purinové báze DNA. Buněčná odezva na toto poškození DNA primárně vede ke smrti buňky apoptózou nebo nekrózou. Schopnost nádorových buněk rozpoznat poškození a zahájit opravu DNA má však za následek terapeutickou rezistenci a má negativní vliv na terapeutickou účinnost. Jedním z možných směrů ve vývoji nových léčiv je tedy farmakologická inhibice těchto DNA opravných mechanismů. Další zajímavou možností je zvýšení terapeutické účinnosti léčiv, která se již běžně používají v klinické praxi, např. fotoaktivací.

Dvojjaderné platinové komplexy obsahující azolový můstek, jako např.  $[(cis-\{Pt-(NH_3)_2\})_2(\mu-OH)(\mu-pz)]^{2+}$  (AMPZ), jsou příkladem léčiv vyvinutých s cílem inhibovat opravu DNA. Ve srovnání s cisplatinou, AMPZ je v některých nádorových liniích výrazně toxičtější a navíc nevykazuje křížovou rezistenci k cisplatině, což je považováno za důsledek malých strukturních změn, které vazba AMPZ v molekule DNA vyvolává. Nízká distorze způsobená tímto novým komplexem s protinádorovým účinkem by mohla mít za následek jeho snížené rozpoznání proteiny DNA opravných mechanismů a zvýšení terapeutické účinnosti ve srovnání s cisplatinou.

Naším cílem bylo stanovit rozdíly v interakcích AMPZ a cisplatiny s DNA. Studovali jsme konformaci a opravu polymerní DNA po její modifikaci AMPZ a následně jsme blíže specifikovali energetiku, konformaci a rozlišení krátkých duplexů DNA obsahujících hlavní adukt tohoto komplexu. Poskytli jsme experimentální důkaz toho, že DNA s navázaným AMPZ je v lidských buněčných extraktech méně opravována než je-li modifikována cisplatinou. Dále jsme ukázali, že vazba AMPZ ve struktuře DNA vyvolává pouze malé změny, které nejsou rozpoznávány proteiny specificky se vážícími k poškozené DNA, jako jsou HMGB1 a XPA, které hrají důležitou roli v protinádorové aktivitě platinových léčiv používaných v klinice.

Naším cílem bylo rovněž zjistit, zda je klinicky používaná karboplatina vhodným kandidátem na fotoaktivovatelné léčivo. Po ozáření UVA došlo k výraznému zvýšení rychlosti vazby na DNA a ke zvýšení toxicity v nádorových buňkách. Selektivní fotoaktivace karboplatiny, umožněná vývojem laserových a optovláknových technologií, by tedy mohla zvýšit dosavadní terapeutickou účinnost karboplatiny a předejít vzniku rezistence.

## LIST OF PUBLICATIONS

The thesis is based on following papers, which are referred to in the text by Roman numerals I-III.

- I. **Mlčoušková J**, Kašpárková J, Suchánková T, Komeda S, Brabec V (2012) Conformation and Repair of Polymeric Natural DNA Damaged by Antitumor Azolato-bridged Dinuclear Pt<sup>II</sup> Complex. *J. Inorg. Biochem., In press*
- II. **Mlčoušková J**, Malina J, Novohradský V, Kašpárková J, Komeda S, Brabec V (2012) Energetics, conformation, and recognition of DNA duplexes containing a major adduct of antitumor azolato-bridged dinuclear Pt<sup>II</sup> complex. *Biochim Biophys Acta, Submitted*
- III. **Mlčoušková J**, Štěpánková J, Brabec V (2012) Antitumor carboplatin is more toxic in tumor cells when photoactivated: Enhanced DNA binding. *J Biol Inorg Chem., Submitted*

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## STATUTORY DECLARATION

## ABSTRACT

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## LIST OF ABBREVIATIONS

## APPENDIX

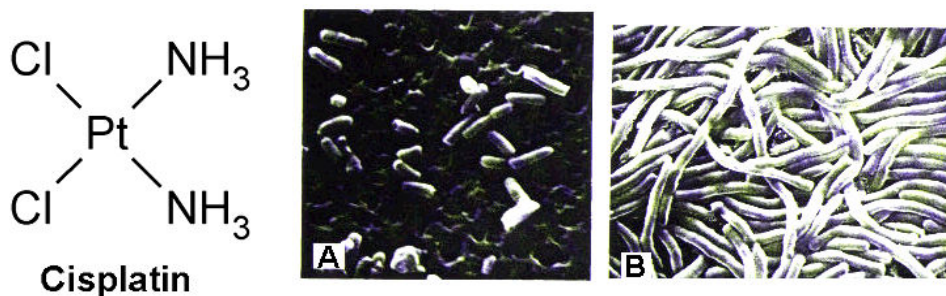
# 1. INTRODUCTION

## 1.1. The discovery of cisplatin as an anticancer drug

Malignant neoplasm, commonly referred to as cancer, has been accompanying (not just) mankind for ages. Metastatic cancer had been reported in dinosaur fossils and e.g. rectal cancer had been diagnosed in an Egyptian mummy<sup>[1]</sup>. Today, in industrialized societies, cardiovascular disease is the most frequent cause of death followed by cancer.

Various methods have been developed to recognize and subsequently treat cancer. In 1965, Barnett Rosenberg came with an unintended discovery of a simple platinum complex which was able to inhibit the division of *E. coli* bacterial cells<sup>[2]</sup> (Fig.1). Tests performed on various other bacteria followed and another step was administering the compound to mice bearing murine transplantable tumor, sarcoma-180, when marked tumor regression was achieved<sup>[3]</sup>. In 1971, first patients were treated with this compound, *cis*-[PtCl<sub>2</sub>(NH<sub>3</sub>)<sub>2</sub>] (*cis*-diamminedichloridoplatinum(II)), originally synthesized and described in 1845 and known as Peyrone's chloride<sup>[4]</sup>. Today, generally referred to as cisplatin, this complex serves as a drug widely used in the clinic. Cisplatin is highly effective for treatment of testicular and ovarian cancer and is used for a variety of other carcinomas, including bladder, small lung tumors and those of head and neck<sup>[5]</sup>. However, there are certain limitations of its usage connected with various side-effects such as nephrotoxicity, neurotoxicity and ototoxicity, inherent and acquired resistance<sup>[6]</sup>.

New generations of drugs based on cisplatin have been synthesized in order to develop new anticancer drugs having better efficiency, broader spectrum of activity and minimized side-effects<sup>[7,8]</sup>. Carboplatin, oxaliplatin and nedaplatin are examples of those successfully used in the clinic. Progress in anticancer drug design led to the development of picoplatin and orally applicable satraplatin, and many strategies such as targeted delivery to tumors, combination therapy or modulation of resistance against platinum drugs are currently in clinical testing<sup>[4]</sup>. Besides platinum, other metals such as ruthenium, osmium, gallium, gold, cobalt, rhenium, palladium, iridium are involved in anticancer drug design<sup>[8,9]</sup>. The research interests in understanding the mechanism of action of metallodrugs are of much importance particularly with the respect of designing new candidates for cancer treatment.



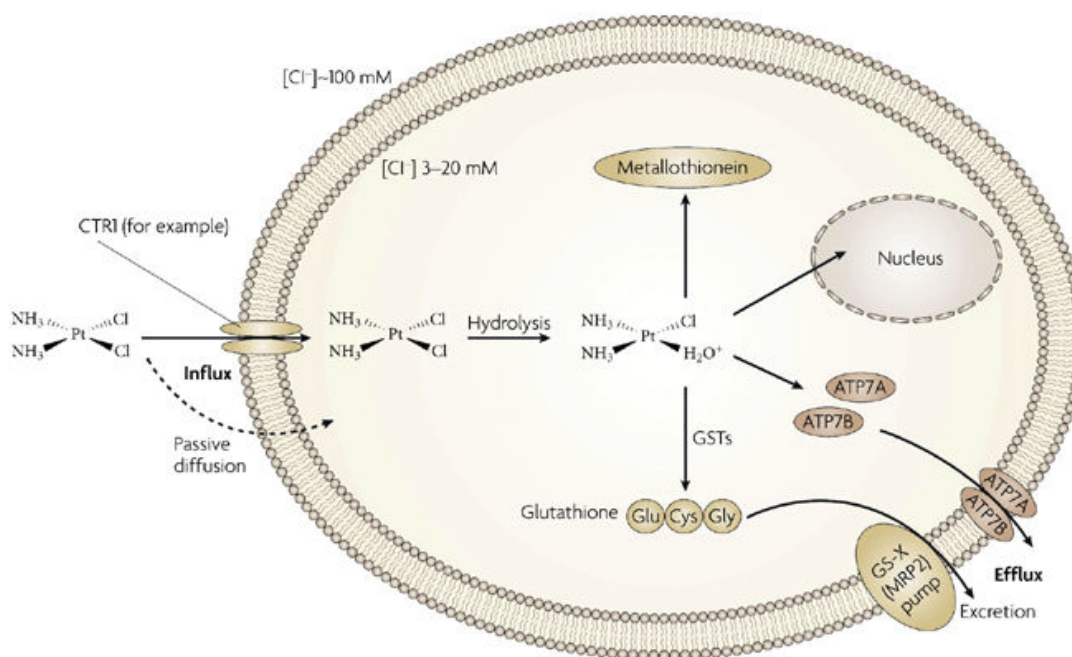
**Figure 1** Structure of cisplatin, whose potential of an anticancer drug was revealed during Barnett Rosenberg's experiments on *E. coli* bacteria, is shown left. Picture 1A presents scanning electron microphotograph of normal *E. coli*. Growing *E. coli* in cisplatin containing medium has inhibited cell division, but not growth, leading to long filaments (B). Adapted from <http://chemcases.com/cisplat/cisplat01.htm>

## 1.2. Mechanism of action of cisplatin

The molecular details of the mechanism of action of cisplatin and related compounds have been extensively studied. There is a large body of experimental evidence that their success in killing tumor cells mainly results from their ability to form various adducts on DNA<sup>[10, 11]</sup>. The early steps of the process triggering cell death initiated by administering of these drugs can be presented in four stages. These are the uptake of the platinum agents by both passive and/or active transport (1); activation of the platinum(II) complex (2); binding to nucleic acids to form a variety of Pt-DNA adducts (3); and the cellular response to DNA damage (4)<sup>[12]</sup>.

A schematic diagram showing penetration and distribution of cisplatin in the cell is shown in Fig.2. Cisplatin had long been thought to enter the cells primarily through passive diffusion, however a growing body of evidence suggests a role in cisplatin accumulation is played by active uptake by membrane proteins, such as the copper transporter CTR1<sup>[13]</sup>. The activation of cisplatin involves replacement of the chloride ligands with water molecules (hydrolysis) which is driven by a drop in chloride ion concentration as the compound crosses the cell membrane<sup>[14]</sup>. In the cytoplasm, cisplatin preferentially reacts with species containing high sulphur levels (cysteine or methionine amino acids) such as glutathione or metallothioneins, which results in partial inactivation of the drug<sup>[4]</sup>. In some platinum-resistant cancer cells, concentrations of glutathione and metallothionein are relatively high, so the drug is effectively inactivated before DNA binding can occur, thereby causing resistance. Additional active export of cisplatin from the cells through

copper exporters ATP7A and ATP7B as well as through glutathione S-conjugate export GS-X pump (also known as MRP2) can further contribute to platinum drug resistance [4]. Once in the nucleus the aquated forms of cisplatin bind DNA at the N7 position of purine bases to form primarily 1,2-intrastrand adducts (IAC) between adjacent guanosine residues or adenosine and guanosine residues [15]. Such DNA damage may disrupt several cellular processes including transcription and replication and lead to cell cycle arrest. As the cell protects itself, the Pt lesions are either removed by nucleotide excision repair or desired apoptosis (programmed cell death) is triggered [16].



**Figure 2** Cellular accumulation of cisplatin and its targets. Adapted from [4].

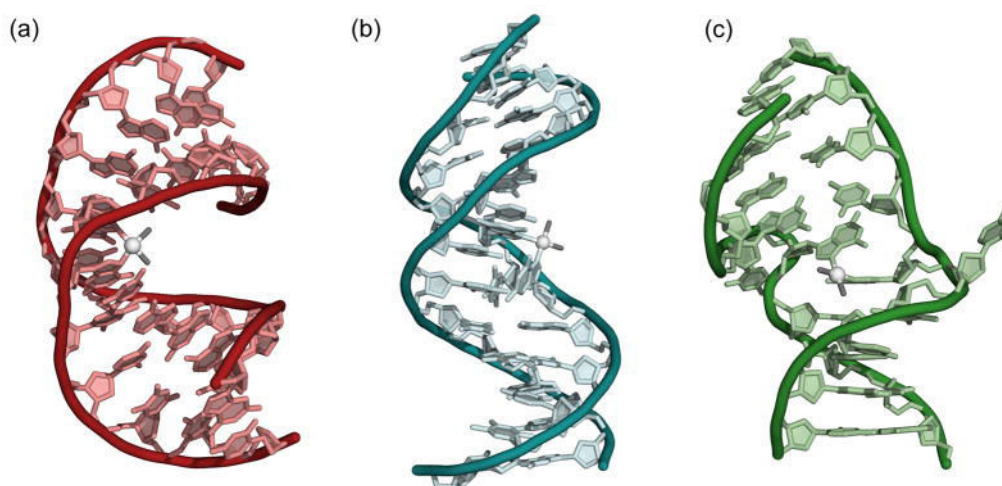
### 1.2.1. DNA adducts formed by cisplatin and related platinum compounds

When bound to DNA, cisplatin forms a spectrum of intra- and interstrand DNA cross-links, which have been identified both *in vitro* and *in vivo* [17-20]. The major adduct, comprising ~65% of total products, is a 1,2-GG intrastrand cross-link, formed between two adjacent guanine residues within one strand of the DNA duplex. Other minor products include 1,2-AG (~25%) and 1,3-GXG intrastrand adducts (5-10%; X = A, C, T), as well as a smaller number of interstrand cross-links (ICL) and monodentate adducts. The adducts of cisplatin induce changes in secondary structure of DNA. E.g. the formation of major 1,2-GG or 1,2-AG intrastrand cross-links leads to helix unwinding of ~13° and bending of

$\sim 34^\circ$  toward major groove while the interstrand cross-link unwinds the helix by  $\sim 79^\circ$  relative to B-DNA and bends it of  $45^\circ$  toward minor groove<sup>[21-24]</sup> (Fig.3).

Carboplatin forms the same type of adducts as cisplatin, with slightly different sequence preference<sup>[25]</sup>. The major carboplatin adduct formed in vitro was 1,2-GG intrastrand cross-link (58%).

As the isomer of cisplatin, *trans*-diamminedichloridoplatinum(II) or transplatin, has been found incapable of forming 1,2-intrastrand cross-links on DNA and as its antitumor activity in cells was insignificant, these intrastrand adducts were first considered to be responsible for the cytotoxicity of platinum agents in cancer cells<sup>[26-28]</sup>. However, further studies reinforced the fact that also interstrand cross-links formed by various Pt compounds of biological significance contribute to antitumor efficiencies of these drugs<sup>[29]</sup>. To clarify the mechanism by which each specific cross-link formed by Pt agent in DNA elicits its biological response, additional detailed studies are still required.



**Figure 3** X-ray crystal and NMR structures of double stranded DNA containing adducts of cisplatin. (a) 1,2-GG intrastrand cross-link; (b) 1,3-GXG intrastrand cross-link; (c) interstrand cross-link. Adapted from [16].

### 1.2.2. Effects and consequences of cisplatin binding on DNA function

The ability of cisplatin to bind to DNA and distort its structure suggested interference of DNA function in cells. As DNA replication and transcription are essential for cell division and protein production, any disruption in these processes is supposed to be accompanied by cytotoxic effects. For these reasons, the effects of platination on DNA function have

been extensively investigated with the aim of better understanding the biological activity of this drug.

Cisplatin has been shown to inhibit DNA replication and thus block the synthesis of new DNA required for cell division by prokaryotic and eukaryotic DNA polymerases both *in vivo* and *in vitro* [30-32]. However, it has been demonstrated that the DNA polymerases are able to bypass the adduct – the ability of which differs for various polymerases [32]. Through such a replication bypass, the compound can become mutagenic which is a very important factor for drugs in clinical use as it may lead to the development of secondary tumors. Although inhibition of DNA replication undoubtedly comprises an important part of the mechanism underlying antitumor effects of cisplatin, it cannot fully explain the antitumor efficiency of this drug thus indicating that the mechanism is more complex [33]. Cisplatin-induced inhibition of transcription, an essential step in protein synthesis whereby mRNA is produced from a DNA template, has also been studied [34-36]. The RNA polymerases have been shown to react differently at various platinum adducts. Bifunctional adducts of cisplatin strongly inhibited transcription of DNA while the monofunctional adducts were entirely bypassed by the RNA polymerases [37]. It has been suggested that platinum adducts next to constituting a physical barrier to the enzyme movement alongside the template also specifically alter the properties of transcription complexes as a consequence of conformational changes they induce in template DNA [37].

### 1.2.3. Cellular resistance and repair of cisplatin-DNA adducts

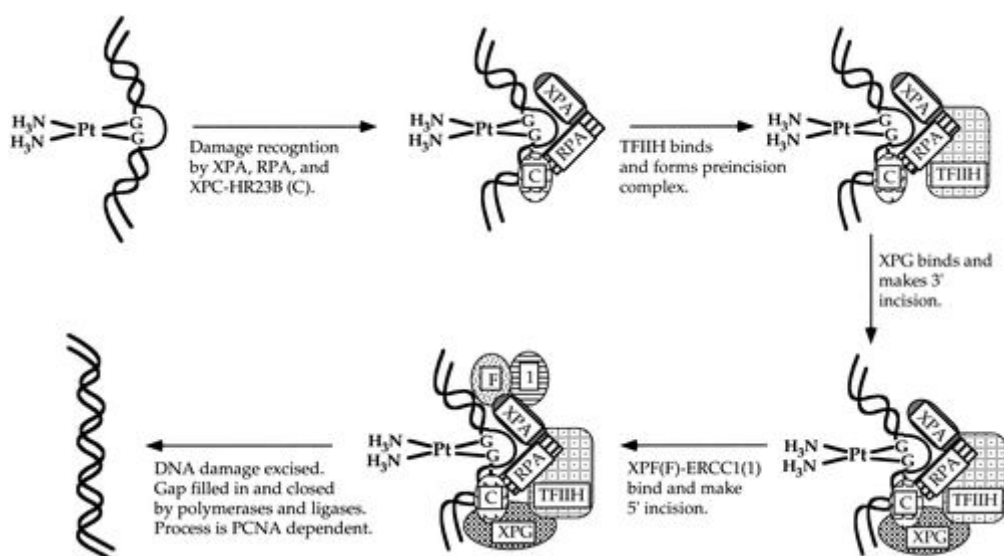
The clinical use of cisplatin has its limitations associated with resistance of tumor cells, both intrinsic and acquired through exposure to the drug. Resistance to cisplatin is multifactorial and in general it may consist of mechanisms either limiting the formation of DNA adducts and/or operating downstream of the interaction of cisplatin with DNA to promote cell survival. The formation of cisplatin-DNA adducts can be modulated by changes in intracellular accumulation of the drug (e.g. negatively by enhanced drug efflux) or increased production of intracellular thiols (see also Chapter 1.2. and Fig. 2). The cells can also be capable of enhanced repair of cisplatin-DNA adducts and increased tolerance of the resulting DNA damage.

Cisplatin-DNA adducts are repaired in cells primarily through the nucleotide excision repair (NER) pathway [38]. This process involves many proteins and is used to repair a

variety of DNA lesions, including damage caused by UV radiation, by excision of poly- or oligonucleotide fragments. It has been found using cell-free extracts or a reconstituted NER system that 1,2- and 1,3-intrastrand cross-links of cisplatin are efficiently repaired [39]. Schematic diagram of NER acting against 1,2-GG-IAC of cisplatin is shown in Fig. 4. In the first step, the DNA damage is recognized which implies binding of XPA (for more details on this protein, see Chapter 1.2.4.2.), RPA, and XPC–HR23B (C) proteins. Afterwards, transcription factor II H binds forming a preincision complex. Following steps include binding of XPG which makes an incision to the 3'-side of the damage, and XPF–ERCC1, which is responsible for an incision to the 5'-side of the damage [40]. Once the piece of damaged DNA has been excised, the protein complex dissociates, and the gap is filled in and closed by DNA polymerases and ligases in a PCNA dependent process.

It has been previously shown that members of the high mobility group (HMG) of non-histone proteins participate in the molecular events of repair of Pt-DNA lesions. For example the cisplatin major adducts 1,2-IACs are specifically recognized by HMG-domain proteins. This strong recognition and binding of HMG-domain proteins to the sites of DNA adducts might block the repair processes, e.g. by shielding the DNA adducts against repair mechanisms. It is thought that also other proteins play a key role in mechanism of antitumor action of platinum agents ([41]; see Chapter 1.2.4.)

Besides NER there are other cellular mechanisms, that can affect the cytotoxicity of cisplatin adducts, such as homologous recombination or mismatch repair (MMR), and translesion synthesis. One of the main pathways regulating cell survival following DNA damage also leads through tumor suppressor protein p53 which is a nuclear phosphoprotein involved in the control of cell cycle, DNA repair and apoptosis [42]. For more details on these topics, see [43].



**Figure 4** A schematic diagram of nucleotide excision repair of a 1,2-GG-intrastrand cross-link of cisplatin. Adapted from [11].

#### 1.2.4. Proteins binding to Pt-DNA adducts

A number of cellular proteins have been identified that bind to platinum-DNA adducts with specificity over unmodified DNA, including those associated with DNA repair (e.g. xeroderma pigmentosum group proteins, RPA, MutS $\alpha$ ), HMG-domain proteins, and others (e.g. TATA binding protein) [44]. Following two chapters will be dedicated to the representatives of the two major groups, the HMGB1 and XPA proteins.

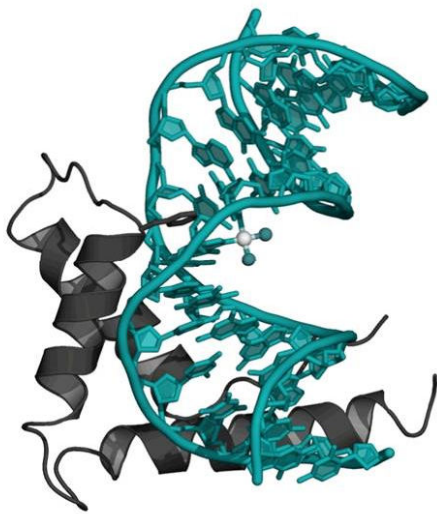
##### 1.2.4.1. High-mobility group box protein 1

High-mobility group box protein 1 (HMGB1) is a very abundant non-histone chromosomal protein, which is known to have a regulatory effect on many cellular processes involving DNA, including chromatin remodeling, recombination, replication, and transcription [45-46]. HMGB1 has been shown to selectively bind cisplatin-DNA adducts protecting them from NER [41].

HMGB1 consists of two tandem HMG domains, A and B (HMGB1a and HMGB1b), and a C-terminal acidic tail. The domains are linked by a short lysine-rich region (the A/B linker). The binding affinity of domain A for DNA containing the 1,2-GG-IAC is dependent on the flanking nucleotide sequence and it is generally higher than the affinity



of domain B <sup>[47]</sup>. Domain A thus plays main role in the the full-length protein binding to the cisplatin intrastrand cross-link <sup>[48]</sup>. The structure of a complex formed between a 16mer duplex DNA containing a centralized 1,2-GG intrastrand cross-link of cisplatin and the HMGB1a domain was solved by X-ray crystallography <sup>[49]</sup> (Fig. 5). Here, the HMG domain binds the adduct in the widened minor groove opposite the cross-link located in the major groove. A phenylalanine residue intercalates into a hydrophobic notch created by the adduct. Distortions such as unwinding, prebending and preformation of a hydrofobic notch potentiate the recognition and affinity of HMGB1 and other damaged-DNA binding proteins.



**Figure 5** X-ray crystal structure of HMGB1 domain A bound to a cisplatin 1,2-GG intrastrand cross-link. Adapted from [16].

#### 1.2.4.2. Xeroderma pigmentosum group A protein

Xeroderma pigmentosum group A protein (XPA) is an essential subunit of the mammalian NER system which has been implicated in recognition of DNA damaged by cisplatin <sup>[50]</sup>. Moreover, DNA repair (excision) is crucially dependent on the assembly of XPA protein with a sharp bending angle in the damaged DNA substrate. XPA is a 31 kDa protein with a zinc finger motif, consisting of 273 amino acids <sup>[51]</sup>.

XPA belongs to a group of proteins which are absent in patients with NER deficiency characteristic of disease xeroderma pigmentosum (XP) <sup>[52]</sup>. Because of the inability to perform nucleotide excision repair, individuals with XP are extremely sensitive to UV

radiation and have a predisposition toward skin cancer. XP has seven different genetic complementation groups, XP-A through XP-G, and a variant form, XP-V. XP cells have an increased sensitivity to cisplatin treatment, providing further evidence that this pathway is important in the cellular processing of the drug <sup>[53-55]</sup>.

### 1.3. Cisplatin analogues routinely used in the clinic

Since the introduction of cisplatin, many bifunctional analogues have been designed and synthesized with the aim to become potential new anticancer agents. However, only three structurally-related platinum drugs have entered widespread clinical use. These are carboplatin and oxaliplatin (see also Chapter 1.4.1. and Fig. 6).

Carboplatin (*cis*-diammine-[1,1-cyclobutanedicarboxylato]platinum(II)) differs from cisplatin by the nature of the leaving group, cyclobutyldicarboxylate (CBDCA) instead of the two chlorides. Carboplatin exhibits lower toxicity and better administration than cisplatin, however it is still effective in the same spectrum of tumors and does not overcome cross-resistance to cisplatin <sup>[56]</sup>.

Oxaliplatin, or 1R,2R-diaminocyclohexane oxalatoplatinum(II), on the other hand, has no cross-resistance with cisplatin and in combination with 5-fluorouracil and leucovorin it is active for the treatment of colorectal cancer <sup>[57]</sup>.

Other cisplatin analogues, such as nedaplatin and lobaplatin, are registered for use in cancer treatment in Japan or China, respectively <sup>[58]</sup>.

Summarized, none of cisplatin analogues demonstrates substantial advantages over cisplatin, although oxaliplatin has shown potential for use in some cisplatin-resistant tumors if administered in combination with 5-fluorouracil and folinic acid <sup>[57]</sup>.

### 1.4. Strategies of improvement in Pt metallodrugs design and effectivity

As much progress has been made in elucidating the mechanism of action of cisplatin, new ways of improvement in platinum drug design and effectivity have opened. The major strategies are based on synthesis of new drugs and targeted delivery of drugs to tumors. A greater specificity of drug action can also be achieved by selected photoactivation. Furthermore, co-administration of platinum drugs with pharmacological modulators of

resistance mechanisms or combination of platinum drugs with agents targeting specific molecular abnormalities that are characteristic of cancer show to be promising <sup>[4]</sup>.

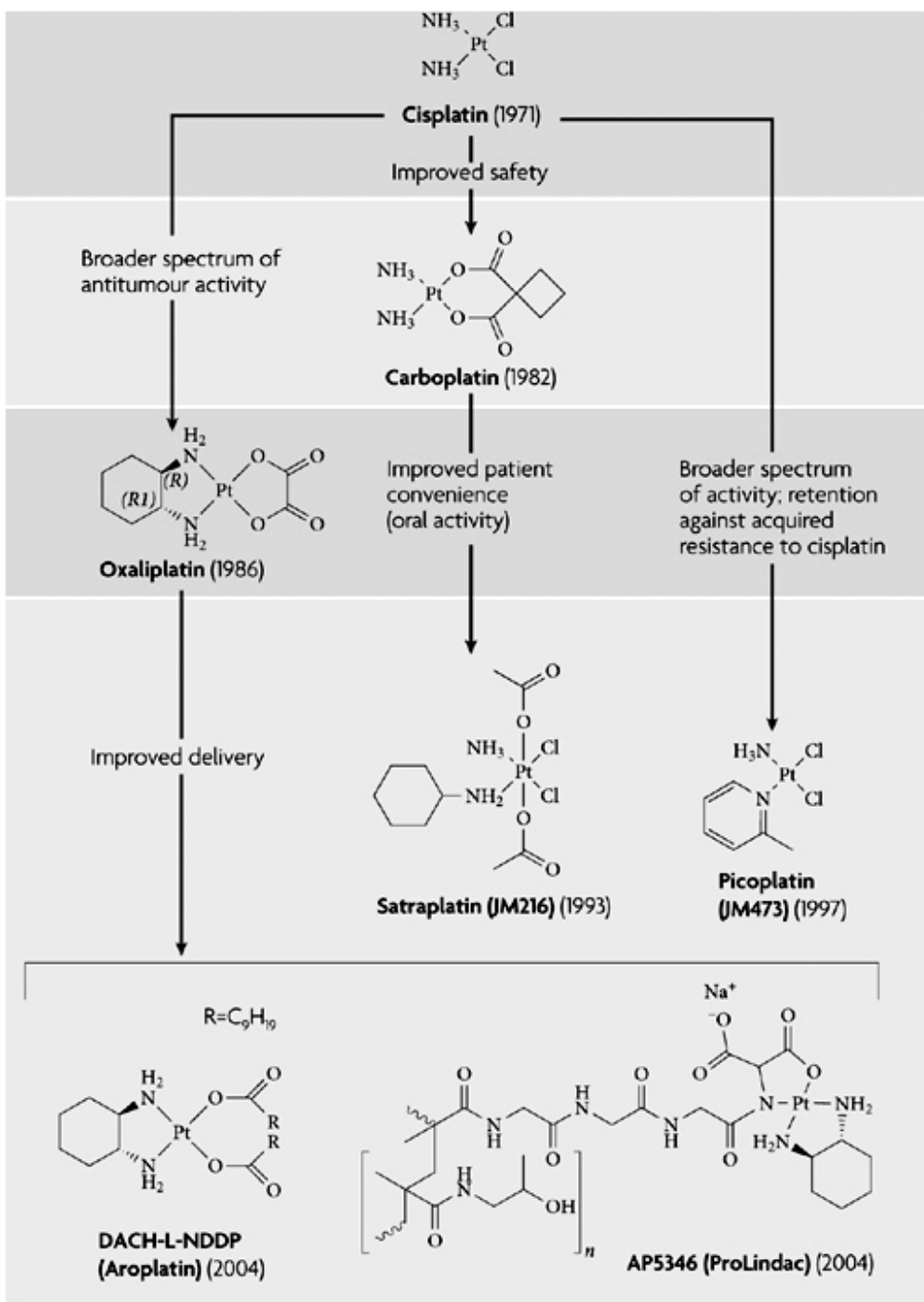
#### 1.4.1. Synthesis of new platinum drugs

Since the discovery of cisplatin, tens of thousands platinum complexes have been designed and synthesized. Currently, there are still a large number of cisplatin analogous modifications still being pursued worldwide. This direction has been encouraged by successful approval of oxaliplatin by American Food and Drug Administration (FDA) in 2002. Further examples that are doing well in clinical trials are satraplatin and picoplatin <sup>[4]</sup>. Satraplatin, or bis-acetato-ammine-dichloro-cyclohexylamine platinum(IV), is used to treat prostate cancer and its major advantage over its predecessors is oral administration. Picoplatin, or *cis*-amminedichloro(2-methylpyridine) platinum(II), exhibits broader spectrum of activity compared to cisplatin and circumvents acquired resistance to cisplatin. A schematic overview of platinum drugs routinely or experimentally used in the clinic is shown in Fig. 6.

Both satra- and picoplatin are examples of a rational drug design based on the knowledge of mechanism of action of cisplatin and its analogues. Satraplatin was developed to be an orally active version of carboplatin, while picoplatin was designed to provide steric bulk around the platinum centre which was shown to lead to a relative reduction in inactivation by thiol-containing species such as glutathione and metallothionein <sup>[59-60]</sup>.

The strategies of design of new cisplatin analogues are mostly based on altering the amine ligand which is responsible for the structure of the adducts formed upon interacting with DNA, or the halide leaving group that influences tissue and intracellular distribution of the platinum complexes and improves the drug's toxicity profile. Other approaches have focused on applying platinum(IV) complexes or utilizing ligands bearing other donor atoms than nitrogen (e.g. S, P, O) <sup>[7]</sup>.

However, as cisplatin and its analogues exhibit very similar patterns of antitumor efficacy, novel, unrelated structures such as *trans*-platinum(II) or polynuclear platinum complexes have been synthesized and evaluated. As an example of an attempt of such kind, the pyrazolato-bridged dinuclear platinum(II) complex (AMPZ) was introduced.

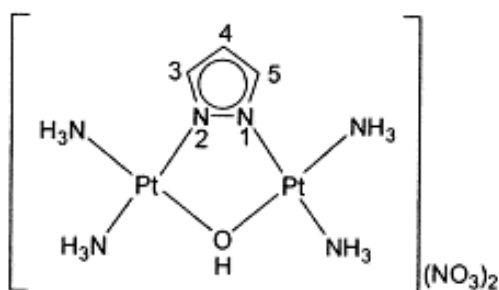


**Figure 6** A scheme of platinum drugs routinely used or currently successful in clinical trials. Years indicating when each drug was first given to patients are given in parenthesis. The platinum drugs lobaplatin and nedaplatin are not included owing to their limited worldwide use (China or Japan, respectively). Adapted from [4].

#### 1.4.1.1. Pyrazolato-bridged dinuclear platinum(II) complex (AMPZ)

The pyrazolato-bridged dinuclear platinum(II) complex  $[(cis-\{Pt(NH_3)_2\})_2(\mu-OH)(\mu-pz)]^{2+}$  (pz=pyrazolate) (for structure see Fig. 7) has demonstrated remarkably improved cytotoxicity over cisplatin in several human tumor cell lines and, moreover, circumvention of cross-resistance to cisplatin <sup>[61-62]</sup>. AMPZ was rationally designed with following structural features: hydroxide acting as a leaving group, the bridging rigid pyrazole occupying the correct distance between the two Pt atoms to enable binding of two neighboring guanines, and some flexibility to induce minimal distortion in its 1,2-intrastrand adducts formed on DNA <sup>[61]</sup>.

NMR studies of oligonucleotide duplex cross-linked at the two adjacent guanines by AMPZ and molecular dynamics simulations have revealed unwinding of the DNA helix by approximately 15°, that is, to a similar extent as cisplatin, but, in contrast to the latter, no significant bending in the helix axis <sup>[63]</sup>. A hypothesis which needed further experimental evidence has been proposed, that low distortions caused by 1,2-intrastrand adducts of AMPZ could be a trigger to induce cytotoxic effects and be favorable for escaping from the damaged-DNA recognition and repair systems in the cells <sup>[61, 63]</sup>.



**Figure 7** Structure of  $[(cis-\{Pt(NH_3)_2\})_2(\mu-OH)(\mu-pz)](NO_3)_2$ . The numbering of the aromatic protons is indicated.

#### 1.4.2. Targeted delivery of drugs to tumors

An attractive strategy seems to be selective transport of antitumor agent to cancer cells. This strategy represents a challenge of not only having to deliver platinum to the tumor in a relatively inactive form, but also subsequent need to achieve good release and activation. The liposomally bound and targeted DACH-L-NDDP (Aroplatin) <sup>[64]</sup> and AP5346

(ProLindac) linked to hydroxypropylmethacrylamide (HPMA) <sup>[65]</sup> (see Fig. 6), both based on the DACH stable ligand found in oxaliplatin, are currently tested in clinical trials.

### 1.4.3. Photoactivated chemotherapy

Recently, platinum complexes were shown to be particularly attractive as potential photochemotherapeutic anticancer agents <sup>[66]</sup>. Selective photoactivation of platinum drugs by irradiation of exclusively cancer cells may avoid enhancement of toxic side-effects, but may increase toxicity selectively in cancer cells and extend application of photoactivatable platinum complexes also to resistant cells and to a wider range of cancer types.

The strategy of photoactivation is based on photoreduction of Pt(IV) complexes to the cytotoxic Pt(II) species <sup>[7]</sup>. However, photoactivation may not only apply to newly designed compounds activable by UVA <sup>[67-68]</sup> or visible <sup>[69-70]</sup> light. The clinically ineffective transplatin [*trans*-diamminedichloridoplatinum(II) ] is almost as cytotoxic as cisplatin when treated cancer cells are irradiated with UVA and the cytotoxicity of photoactivated transplatin is mainly due to enhanced formation of DNA CLs <sup>[71]</sup>.

#### 1.4.3.1. Carboplatin as a potential photoactivable drug

Carboplatin has already been introduced as a less toxic analogue of cisplatin routinely used in the clinic (Chapter 1.3.). The activation of Pt<sup>II</sup> drugs such as cis- and carboplatin involves hydrolysis (Chapter 1.2.). In comparison to cisplatin, carboplatin contains the kinetically less labile cyclobutanedicarboxylate (CBDCA), which is a poorer leaving group than chloride when undergoing hydrolysis <sup>[72]</sup>. To achieve the same level of platination, markedly higher concentrations of carboplatin are required <sup>[73]</sup>. The reduced toxic effect in tumor cells is attributable to slower hydrolysis and thus lower reactivity of carboplatin with nucleophiles, on the other hand so is the more acceptable side-effect profile <sup>[4]</sup>.

The differences between dose levels of cisplatin and carboplatin required to obtain equal levels of DNA platination upon *in vitro* incubations appeared to be much larger than those resulting in equitoxicity in cultured cells or in tumor cells of patients treated with these Pt<sup>II</sup> drugs <sup>[74-75]</sup>. A theory has been suggested that the relatively strong effect of carboplatin in cells is due to some activation mechanism resulting in conversion of the parent carboplatin molecules to species capable of DNA binding either through a direct attack by nucleophilic

bases in the DNA or through aquation <sup>[76]</sup>. Thiourea, glutathione, methionine-containing peptides, the sulfur-containing compounds or other nucleophiles present in cytoplasmic extracts of tumor cells, oxygen free radicals produced by a system containing the Fe-EDTA chelate and ascorbate are examples of agents capable of conversion of carboplatin to reactive species able to bind to DNA <sup>[76-78]</sup>. However, none of the foregoing activation mechanisms would be effective if applied *in vivo* to enhance DNA binding capability of carboplatin and consequently its toxicity selectively in tumor cells without affecting normal cells and thereby also increasing unwanted side effects.

Previous photochemical studies on cisplatin, transplatin, and [Pt(en)Cl<sub>2</sub>] have reported that irradiation induces substitution of chloride ligands by solvent molecules, either H<sub>2</sub>O or DMSO <sup>[79-80]</sup>. It has also been shown that clinically ineffective transplatin [*trans*-diamminedichloridoplatinum(II) ] is almost as cytotoxic as cisplatin when treated cancer cells are irradiated with UVA <sup>[71]</sup>. Thus, carboplatin could also be a potential candidate of a photoactivable drug. In that case, selective photoactivation by light of appropriate wavelength, enabled by novel medical applications of laser and fiber optic technologies, could benefit treatment outcomes and inhibit acquisition of resistance in patients treated by carboplatin.

## 2. AIMS

The main aims of this thesis are:

- 1) To study conformation and repair of polymeric natural DNA after its modification by antitumor azolato-bridged dinuclear Pt<sup>II</sup> complex (AMPZ).
- 2) To further specify the energetics, conformation, and recognition of DNA duplexes containing a major adduct of antitumor azolato-bridged dinuclear Pt<sup>II</sup> complex.
- 3) To investigate the enhancement of DNA binding capability and cytotoxicity of antitumor carboplatin by photoactivation.



### 3. MATERIALS AND METHODS

#### 3.1. Chemicals

Cisplatin, carboplatin and transplatin (purity was  $\geq 99.9\%$  based on elemental and ICP trace analysis) were obtained from Sigma (Prague, Czech Republic). The azolato-bridged dinuclear Pt<sup>II</sup> complex AMPZ was synthesized according to published procedures [61,81]. [PtCl(dien)]Cl was a generous gift of Prof. G. Natile from the University of Bari. For structures, see Fig. 8. Stock solutions of the platinum complexes [ $5 \times 10^{-4}$  M in NaClO<sub>4</sub> (10 mM)] were stored in the dark at 277 K. The concentrations of platinum in the stock solutions were determined by flameless atomic absorption spectrometry (FAAS).

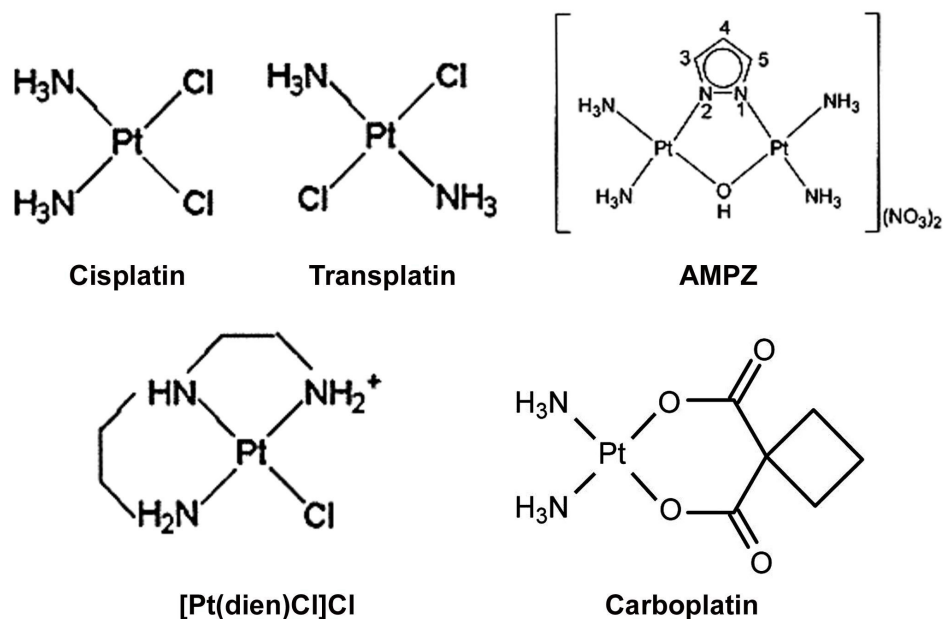
Calf thymus (CT) DNA (42% G + C, mean molecular mass ca. 20 000 kDa) was prepared and characterized as described previously [82-84]. The plasmids, pUC19 (2686 bp), pSP73KB (2455 bp) and pBR322 (4361 bp), were isolated according to standard procedures. The synthetic oligodeoxyribonucleotides were purchased from VBC-GENOMICS (Vienna, Austria) or DNA Technology (Aarhus, Denmark). The purity of the oligonucleotides was verified by either high-pressure liquid chromatography (HPLC) or gel electrophoresis. The molar concentrations of the single-stranded oligonucleotides or duplexes are related to the oligomers (not to the monomer content) or double-stranded molecules, respectively. Molar extinction coefficients for the single-stranded oligonucleotides (related to the 12–23-mer strands) were determined by phosphate analysis [84].

Restriction endonucleases *EcoRI*, *SspI*, *BamHI*, *HindIII*, *NdeI*, and *PvuI*, T4 DNA ligase, Klenow fragment of DNA polymerase I, and T4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA). DNA polymerase  $\eta$  (Pol $\eta$ ) was from EnzyMax, LLC (Lexington). Proteinase K was from Boehringer (Mannheim, Germany). Riboprobe Gemini System II for transcription mapping containing SP6 and T7 RNA polymerases was purchased from Promega (Madison, WI).

The N-terminal His6-tagged xeroderma pigmentosum group A (XPA) protein was obtained by expressing the plasmid DNA pET15b/XPA template [85] in RTS 500 Escherichia coli HY (Roche) and purified on Ni<sup>2+</sup>-NTA agarose and by hydroxyapatite chromatography [86]. The plasmid DNA pET15b/XPA was kindly provided by Richard D. Wood. Expression and purification of domains A (residues 1–84 [87]) and B (residues 85–180 [87])

(HMGB1a and HMGB1b, respectively) of recombinant rat full-length HMGB1 protein (HMG = high mobility group) were carried out as described [87-89].

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was from Calbiochem (Darmstadt, Germany). RPMI 1640 medium, fetal bovine serum (FBS), trypsin/EDTA and DMEM medium were from PAA (Pasching, Austria). Gentamycin was from Serva (Heidelberg, Germany). Acrylamide, bis(acrylamide), EtBr, NaCN, dithiothreitol, and urea were from Merck KgaA (Darmstadt, Germany). Sodium dodecyl sulfate (SDS) was from Serva (Heidelberg, Germany). PEG (molecular mass 6000), dimethyl sulfate (DMS) and  $\text{KMnO}_4$  were from Sigma (Prague, Czech Republic). Sephadex G-50 (Coarse) was from Sigma-Aldrich (Prague, Czech Republic). Deoxyribonucleotide triphosphates (dNTPs) were from Roche Diagnostics, GmbH (Mannheim, Germany). Nonidet P-40 was from Fluka (Prague, Czech Republic). Radioactive products were from Amersham (Arlington Heights, IL, USA). The CFE was prepared from the repair-proficient HeLa S3 cell line as reported previously [90].



**Figure 8** Overview of platinum compounds used in this work.

### 3.2. Irradiation

DNA samples in cell-free media and cells were irradiated using the LZC-4V illuminator (photoreactor) (Luzchem, Canada) with temperature controller and with UVA tubes (4.3

mW cm<sup>-2</sup>;  $\lambda_{\max} = 365$  nm). DNA samples in cell-free media were also irradiated using LUXEON Star/O source (Light Emitting Diode) (Quadica Developments Inc., Brantford, Ontario, Canada ) with optic that allows to aim the light source onto the sample (65 mW cm<sup>-2</sup>,  $\lambda_{\max} = 458$  nm).

### **3.3. Kinetics of binding to calf-thymus DNA, platination reactions**

#### **3.3.1. AMPZ**

CT DNA was incubated with AMPZ at an  $r_i$  value of 0.08 ( $r_i$  is defined as the molar ratio of free platinum complex to nucleotide phosphates at the onset of incubation with DNA) in NaClO<sub>4</sub> (10 mM) at 310 K for 48 hours. At various time intervals, an aliquot of the reaction mixture was withdrawn, dialyzed against 1 M NaCl and the number of molecules of the platinum compound coordinatively bound per nucleotide residue ( $r_b$  value) was determined by flameless atomic absorption spectrophotometry (FAAS).

The oligonucleotides were treated in stoichiometric amounts with AMPZ in deionized water at 298 K. The platinated oligonucleotides were purified by ion-exchange HPLC with linear 0.2 to 0.6 M NaCl gradient in 10 mM Tris-Cl (pH 7.4). It was verified that the modified oligonucleotides contained two platinum atoms by platinum FAAS. It was also verified by dimethyl sulphate footprinting of platinum on DNA that in the platinated strands, one molecule of AMPZ was coordinated to both G residues.

#### **3.3.2. Carboplatin**

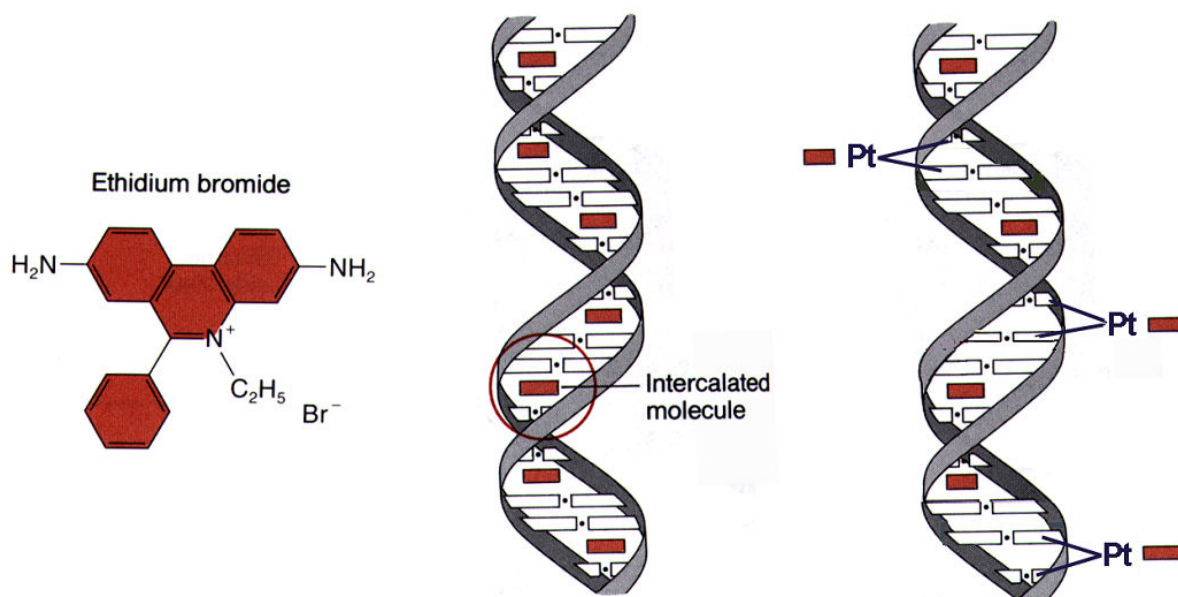
CT DNA (0.2 mg mL<sup>-1</sup>) was mixed with carboplatin (50  $\mu$ M) in NaClO<sub>4</sub> (10 mM) and immediately irradiated (UVA,  $\lambda_{\max} = 365$  nm, or visible light,  $\lambda_{\max} = 458$  nm) for indicated time (periods of irradiation longer than ~5 h were not used due to avoiding DNA damage by UVA light) or kept in the dark at 310 K. The  $r_i$  value was 0.08. Aliquots were removed at various time intervals, quickly filtered using a Sephadex G-50 column to remove free (unbound) Pt. The Pt content in these DNA samples ( $r_b$ , defined as the number of the molecules of platinum complex coordinated per nucleotide residue) was determined by FAAS.

### 3.4. DNA melting

The melting curves of unmodified or platinated CT DNA ( $32 \mu\text{g mL}^{-1}$ ) were recorded on Shimadzu RF 40 spectrofluorophotometer by measuring the absorbance at 260 nm in the medium containing  $\text{NaClO}_4$  (0.01 or 0.07 M) with Tris-HCl (1 mM, pH 7.4)/EDTA (0.1 mM). The value of  $T_m$  was determined as the temperature corresponding to a maximum on the first-derivative profile of the melting curves.

### 3.5. Ethidium bromide fluorescence measurements

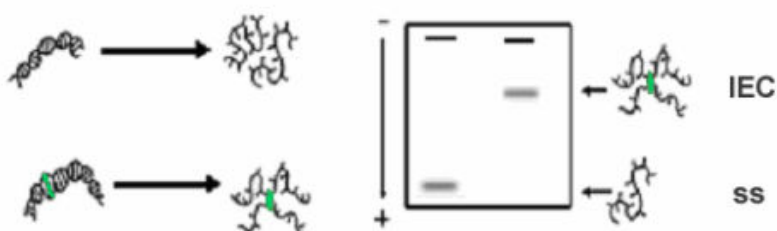
EtBr as a fluorescent probe can be used to characterize DNA binding of small molecules such as platinum antitumor drugs<sup>[91]</sup>. The fluorescence of EtBr is markedly enhanced as a consequence of its intercalation into DNA, but binding of EtBr to DNA by intercalation is blocked in a stoichiometric manner by a wide spectrum of DNA-binding platinum drugs (Fig. 9), causing decrease of EtBr fluorescence intensity as compared with that for non-platinated DNA. EtBr measurements were performed on a Shimadzu RF 40 spectrofluorophotometer using a 1-cm quartz cell. The excitation wavelength was set on 546 nm and the emitted fluorescence was analyzed at 590 nm. The fluorescence intensity was measured at 298 K in NaCl (0.4 M) to avoid secondary binding of EtBr to DNA<sup>[92-93]</sup>. The concentrations were  $0.01 \text{ mg mL}^{-1}$  for DNA and  $0.04 \text{ mg mL}^{-1}$  for EtBr, which corresponded to the saturation of all sites of EtBr in DNA<sup>[93]</sup>.



**Figure 9** Blocking of EtBr intercalation to DNA by bifunctional adduct of cisplatin.

### 3.6. Interstrand cross-link assay

This assay is based on gel electrophoresis of linearized plasmid DNA under denaturing (alkaline) conditions. If the studied platinum complex forms interstrand cross-links on modified DNA, the two strands of the duplex are not separated by denaturation and they appear as a slowly migrating band (IEC fraction) while denatured DNA migrates as a faster band of single stranded (ss) fraction (Fig. 10).



**Figure 10** Principle of interstrand cross-link assay.

#### 3.6.1. AMPZ

AMPZ or cisplatin at varying concentrations were incubated with 2  $\mu\text{g}$  of pUC19 DNA after it had been linearized by *EcoRI*. The platinated samples were precipitated by ethanol and analyzed for DNA interstrand CLs in the same way as described in previously

published articles [36, 94]. The linear duplexes were first 3'-end labeled by means of Klenow fragment of DNA polymerase I and [ $\alpha$ - $^{32}$ P]dATP. The samples were deproteinized by phenol, precipitated by ethanol and the pellet was dissolved in 18  $\mu$ l of NaOH (30 mM) with EDTA (1 mM), sucrose (6.6 %) and bromophenol blue (0.04 %). The amount of interstrand CLs was analyzed by electrophoresis under denaturing conditions on alkaline agarose gel (1 %). After the electrophoresis was completed, the intensities of the bands corresponding to single strands of DNA and slowly migrating interstrand cross-linked duplex were quantified by means of a Molecular dynamics phosphor imager (Storm 860 system with ImageQuant software). The frequency of interstrand CLs, F (the number of interstrand CLs per adduct), was calculated as  $F = XL/5372 \cdot r_b$  (pUC19 plasmid contained 5372 nucleotide residues). XL is the number of interstrand CLs per one molecule of the linearized DNA duplex which was calculated assuming Poisson distribution of the interstrand CLs as  $XL = -\ln A$ , where A is the fraction of molecules running as a band corresponding to the non-cross-linked DNA [94].

### 3.6.2. Carboplatin

Plasmid DNA pSP73KB was linearized by NdeI and 3'-end labeled by means of Klenow fragment of DNA polymerase I and [ $\alpha$ - $^{32}$ P]dATP. Carboplatin at the concentration of  $9 \times 10^{-7}$  M (under irradiation conditions) or  $5.5 \times 10^{-6}$  M (in the dark) was mixed with 1  $\mu$ g of linear pSP73KB in 10 mM NaClO<sub>4</sub> (hence,  $r_i$  was 0.003 or 0.018, respectively) and irradiated ( $\lambda_{max} = 365$  nm) or kept in the dark for 0 - 5 h. Samples were withdrawn at various time intervals (0.5, 1, 3, 4, 5 h). The amount of interstrand CLs in these samples was analyzed by electrophoresis under denaturing conditions on alkaline agarose gel (1%) and  $r_b$  value was determined by FAAS. After the electrophoresis was completed, the intensities of the bands corresponding to single strands of DNA and interstrand cross-linked duplex were quantified. The number of interstrand CLs per adduct (%ICL/Pt) was calculated as  $\%ICL/Pt = XL/4910 \times r_b$  (pSP73KB plasmid contained 4910 nucleotide residues).

### 3.7. Restriction enzyme cleavage inhibition assay

These experiments were performed with three different restriction endonucleases cleaving the DNA between two adjacent guanine residues (*Bam*HI), adjacent G and A (*Eco*RI), and

adjacent T and A residues (*SspI*). Formation of Pt-DNA adducts inhibits the DNA cleavage by these enzymes in accordance with growing level of modification and preferential binding sites of the complex. Studied on electroforetic agarose gel, the inhibition of enzyme cleavage reflects in growing intensity of non-cleaved DNA fraction.

pSP73KB DNA was first linearized by the PvuI endonuclease. After this cleavage reaction the restriction enzyme was removed by phenol/chloroform extraction. The linearized fragments were modified by the Pt<sup>II</sup> complex to various  $r_b$  values. Subsequent digestions were performed after ethanol precipitation by incubating the non-modified or platinated primary digests of pSP73KB DNA with *Bam*HI, *Eco*RI or *Ssp*I. The amount of the enzyme used in the secondary digestions (performed in the media recommended and supplied by the manufacturer) was that necessary to cut 1  $\mu$ g of linearized DNA in 1 h at 310 K. The resulting DNA fragments were subjected to electrophoresis in agarose (1 % gel) in TAE buffer [Tris-acetate (0.04 M) + EDTA (0.04 M, pH 7.0)] at 198 K with a potential drop of 0.8 V/cm. The gel was stained with EtBr and samples visualized and photographed under ultraviolet light. The intensities of the bands were quantified by means of a Molecular dynamics phosphor imager (Storm 860 system with ImageQuant software). Importantly, pSP73KB DNA only contains one cleavage site of *Bam*HI (Fig. 11), *Eco*RI or *Ssp*I at the positions 45, 24 and 2014, respectively (these numbers correspond to the nucleotide numbering in the sequence map of pSP73KB plasmid).



**Figure 11** Complex of restriction endonuclease *Bam*HI and DNA. Adapted from [95]. Recognition site of the enzyme is shown above.

### 3.8. Flow linear dichroism

Binding of AMPZ to CT DNA was also monitored by linear dichroism (LD) spectroscopy, which is a technique primarily used to study the functionality and structure of molecules. LD can be defined as the difference in absorption of light polarized parallel and perpendicular to an orientation axis <sup>[96]</sup>. This technique is applicable in systems which are either intrinsically oriented, or orientable under certain experimental conditions. Long molecules, such as DNA (minimum length of ~250 bp), can be oriented in a flow Couette cell. The flow cell consists of a fixed outer cylinder and a rotating solid quartz inner cylinder, separated by a gap of 0.5 mm, giving a total path length of 1 mm. The LD spectra of flow-oriented DNA at the concentration of 0.1  $\mu\text{g mL}^{-1}$  modified by the Pt<sup>II</sup> complexes were recorded at 298 K in NaClO<sub>4</sub> (10 mM) plus NaCl (20 mM) and sodium cacodylate (10 mM, pH 7.0) <sup>[96-97]</sup>. The spectra were collected on Jasco J-720 spectropolarimeter.

### 3.9. Liquid crystalline dispersions of DNA

The cholesteric liquid crystalline dispersions of DNA can be used as an *in vivo* model system simulating the principal properties of DNA molecules within cells, such as their spatial ordering in condensed and packed state <sup>[98]</sup>. In the presence of PEG water-containing salt solutions, linear double-stranded DNA is condensed, forming helically twisted liquid crystalline dispersions of DNA molecules (the left-handed helicoidal structure of cholesteric phase from the right-handed DNA molecules). The occurrence of the cholesteric liquid crystalline form of DNA can be observed by measuring circular dichroism spectra as it is accompanied by an intense negative CD band at *ca.* 275 nm <sup>[99]</sup>. The decrease in the amplitude of this band in the CD spectra of liquid crystalline dispersions of DNA modified by antitumor platinum complexes is associated with the disappearance of the helical twist of the liquid crystalline microphase due to alterations of DNA secondary structure, such as disturbances in the stacking interactions of bases. Thus, the CD spectra of liquid crystalline microphases of DNA modified by platinum compounds are very sensitive to the extent of conformational alterations the platinum compounds induce in DNA <sup>[100]</sup>.

Liquid crystalline dispersions of double-helical DNA (pSP73KB plasmid linearized by *Nde*I) modified by the platinum complexes were formed by mixing DNA and PEG solutions as described earlier <sup>[98,100]</sup>. Briefly, 1 mL of non-modified DNA or DNA



modified by the platinum complex dissolved in NaClO<sub>4</sub> (0.01 M) at the concentration of 0.06 mg mL<sup>-1</sup> was mixed vigorously with 3 mL PEG (200 mg mL<sup>-1</sup>; dissolved in 0.3 M NaClO<sub>4</sub>) for 1 hr. CD spectra of liquid crystalline dispersions of plasmid DNA modified by the platinum complexes were recorded after 24 hours at 298 K using a JASCO J-720 spectropolarimeter. The cell path length was 1 cm. Spectra were recorded in the range 220-350 nm in 0.5-nm increments with an averaging time of 1 s.

### 3.10. Chemical probing of DNA conformation

As a chemical probe of DNA conformation for 22 bp duplex platinated by AMPZ or cisplatin, a chemical agent KMnO<sub>4</sub> was used. KMnO<sub>4</sub> can be used for mapping of structural changes in the vicinity of thymine residues as it reacts preferentially with thymine residues in single-stranded DNA and in distorted double-stranded DNA [101, 102].

The reaction of the platinated oligonucleotide duplexes with KMnO<sub>4</sub> was performed as described previously [101]. The top strand of the oligonucleotide duplexes was 5'-end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP and T4 polynucleotide kinase. In the case of the platinated oligonucleotides, platinum was removed after reaction of the DNA with the probe by incubation with NaCN (0.2 M, pH 11) at 318 K for 16 h in the dark.

### 3.11. Ligation and electrophoresis of oligonucleotides

The top strands of 16- or 22-base pairs (bp) oligonucleotide duplexes were designed to contain only one high-affinity platinum binding site, the two adjacent guanine bases of the intrastrand CL. The sequences were also designed to leave a 1 bp overhang at its 5'-ends in double-stranded form. These overhangs facilitate polymerization of the monomeric oligonucleotide duplexes by T4 DNA ligase in only one orientation, and maintain a constant interadduct distance throughout the resulting multimer. The 22-bp sequence repeat yields 22-bp phasing of platinum adducts corresponding approximately to two helical repeats, allowing eventual bends to add constructively. The 16-bp phasing of the adducts corresponds approximately to one-and-a-half-helical repeat, i.e. the adducts are almost perfectly dephased and any directed bends will add destructively, preventing any appreciable anomalous mobility shifts.

The 16 or 22 bp duplexes unplatinated or containing single, 1,2-GG intrastrand CL of AMPZ or cisplatin were 5'-end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP by using T4 polynucleotide

kinase. The duplexes were allowed to react with T4 DNA ligase. The resulting samples were subsequently examined on 8% native polyacrylamide (PAA) [mono:bis(acrylamide) ratio 29:1] electrophoresis gels. Other details of these experiments were as described in previous papers [23,103].

### **3.12. Electrophoretic mobility shift assays**

The recognition of 1,2-GG intrastrand CL of AMPZ and cisplatin by HMGB1 domain and XPA proteins was studied by means of agarose gel electrophoresis. The protein binding appeared as a shift in electrophoretic mobility corresponding to the protein complex with modified DNA.

#### **3.12.1 HMGB1 domain proteins**

Radioactively labeled 23-bp DNA probe with blunt ends were titrated with HMGB1a or HMGB1b proteins. The duplexes (10 nM) were incubated with the proteins in 10  $\mu$ L sample volumes in a buffer composed of HEPES (10 mM, pH 7.5),  $MgCl_2$  (10 mM), LiCl (50 mM), NaCl (0.1 M), spermidine (1 mM), bovine serum albumin (0.2 mg.mL<sup>-1</sup>), and Nonidet P40 (0.05% v/v). For all gel mobility shift experiments, samples were incubated on ice for 1 h and made 7% in sucrose and 0.017% in xylene cyanol before loading on running, precooled (277 K), prerun (300 V, 1-2 h) 5% native PAA gels [29:1 acrylamide:bisacrylamide, 0.5x Tris borate-EDTA (TBE) buffer (Tris-HCl (45 mM), boric acid (45 mM), and EDTA (1 mM, pH 8.3)]. Gels were electrophoresed at 277 K and 300 V for ~1.5 h, dried, exposed to a molecular imaging plate, and analyzed on a Fujifilm bioimaging analyzer. The radioactivities associated with the bands were quantitated with the AIDA image analyzer software. Other details have been published previously [87,104].

#### **3.12.2. XPA**

<sup>32</sup>P-labeled DNA substrates (300 pM) and the amount of XPA indicated was incubated at 293 K in 15- $\mu$ L reactions containing 25 mM HEPES-KOH (pH 8.3), KCl (30 mM),  $MgCl_2$  (4 mM), EDTA (1 mM), dithiothreitol (0.9 mM), bovine serum albumin (45  $\mu$ g mL<sup>-1</sup>), and glycerol (10%). To assess binding at equilibrium, reactions were stopped after 30 min by cooling the samples to 273 K. Following addition of gel loading buffer (3  $\mu$ L) containing

Tris-HCl (100 mM, pH 8.3), glycerol (10%), and orange G (0.05%), the extent of binding was determined on native 5% PAA gels. Electrophoresis was performed for 50 min at 277 K; gels were dried, exposed to a molecular imaging plate, analyzed on a FUJIFILM bioimaging analyzer, and the radioactivities associated with bands were quantitated with the AIDA image analyzer software.

## 4. RESULTS AND DISCUSSIONS

This PhD. thesis is based on 3 papers accepted or submitted for publication in international journals. These papers with comments on author's contribution are enclosed in the Appendix part. In this section, the overall results obtained are briefly summarized.

### 4.1. Conformation and repair of polymeric natural DNA after modification by antitumor AMPZ complex (paper I)

The design of new antitumor platinum drugs is currently also focused on compounds which form on DNA major adducts hardly removable by DNA repair systems. As an attempt of this kind, novel antitumor azolato-bridged dinuclear Pt<sup>II</sup> complexes, such as [*cis*-Pt(NH<sub>3</sub>)<sub>2</sub>]<sub>2</sub>(μ-OH)(μ-pyrazolate)]<sup>2+</sup> (AMPZ), have been designed and synthesized. AMPZ exhibits markedly higher cytotoxicity in several tumor cell lines than clinically well-established mononuclear cisplatin and moreover, appears to circumvent cross-resistance to cisplatin [61,62].

Our primary objective was a deeper insight into the mechanism of action of AMPZ. Thus, it was necessary to delineate differences in the interactions of AMPZ and cisplatin with natural, high-molecular-mass DNA. We examined the kinetics of binding of AMPZ to DNA, major adducts formed on DNA, and the nature of distortions caused by these adducts. We also studied the repair synthesis of plasmid DNA after its modification by AMPZ.

#### 4.1.1. The DNA binding mode of AMPZ

The DNA binding experiments carried out in a cell-free medium indicated that modification reactions resulting in the irreversible coordination of AMPZ were considerably slower than that of cisplatin. The time at which the binding reached 50% (*t*<sub>50%</sub>) was ~30 h for AMPZ (Appendix, Fig. S1, paper I) while *t*<sub>50%</sub> for the reaction of mononuclear cisplatin with DNA under comparable conditions was 2 h [105]. In addition, determination of DNA interstrand cross-linking efficiency of AMPZ revealed that AMPZ forms only a very small amount of interstrand adducts (~2% compared to 6% for cisplatin

[36]) (Appendix, Fig. 4, paper I). Even less percentage, characterized by incorporation of [<sup>14</sup>C]thiourea, was found for monofunctional adducts of AMPZ.

The transcription mapping experiment (Appendix, Fig. 3, paper I), based on inhibition of RNA synthesis by T7 RNA polymerase in the vicinity of platinum adducts, indicated that preferential DNA binding sites of AMPZ correspond to those of cisplatin. In addition, platinated plasmid DNA was digested with three different restriction endonucleases cleaving the DNA between adjacent guanine residues (*Bam*HI), adjacent G and A (*Eco*RI), and adjacent T and A residues (*Ssp*I). The results suggested that AMPZ preferentially binds to natural DNA with a random sequence to neighboring guanine residues in one strand of DNA (Appendix, Fig. 2, paper I).

#### 4.1.2. Characterization of distortions in calf-thymus DNA globally modified by AMPZ

The main feature predicted for AMPZ was that it induces only low distortions in DNA structure, and these were proposed to be favorable for escaping from the damaged-DNA recognition and repair systems in the cells [61, 63]. Thus, we were interested in monitoring the distortions caused by AMPZ on CT DNA globally modified by this compound.

The melting curves (Appendix, Fig. 5 and Fig. S2, paper I) revealed different melting behaviour of CT DNA modified by AMPZ and cisplatin. In general, three major factors affect the thermal stability of DNA modified by Pt<sup>II</sup> complexes [106]. These are (i) a destabilizing effect of conformational distortions due to the formation of CLs induced in DNA by platinum coordination; (ii) stabilizing effects of DNA interstrand CLs which prevent dissociation of DNA strands; (iii) stabilizing effect of the positive charge on the Pt<sup>II</sup> centers. As AMPZ is a positively charged compound, it introduces additional stabilization of the DNA duplex by decreasing electrostatic repulsion of negative charges of phosphate groups located at the complementary strands. However, cations present in the medium also stabilize DNA against thermal denaturation in this way [107] and the stabilization effect of the positive charge on the Pt<sup>II</sup> centers can thus be suppressed by choosing experimental conditions of high ionic strength (in our case 0.07 M Na<sup>+</sup>). Furthermore, as AMPZ formed only 2% of interstrand CLs (Appendix, Fig. 4, paper I) it was reasonable to assume that the stabilizing effect of interstrand CLs was negligible. Thus, the remaining factor which might affect the melting temperature of DNA modified

by AMPZ under the conditions of high ionic strength (0.07 M Na<sup>+</sup>) was destabilizing effect of conformational distortions induced by lesions of AMPZ. The effect of these lesions on  $T_m$  in the medium of high ionic strength was negligible whereas the lesions of cisplatin under identical conditions pronouncedly decreased  $T_m$  (Appendix, Fig. 5B, paper I). Hence, the results of DNA melting experiments are consistent with the thesis that major DNA adducts of AMPZ distort and destabilize double helix of DNA markedly less than major DNA adducts of cisplatin.

The capability of AMPZ to prevent EtBr from intercalation to DNA was also studied. The intercalation of EtBr to DNA leads to a marked enhancement of EtBr fluorescence, however the approach of this fluorescent probe to DNA can be blocked by adducts formed by wide spectrum of DNA-binding platinum drugs. The molecules of dinuclear bifunctional AMPZ bound to CT DNA sterically hindered the molecules of EtBr from intercalation, which lowered EtBr fluorescence in comparison with the experiment in which unplatinated DNA was used (Appendix, Fig. 6A, paper I). For comparison, the clinically ineffective monofunctional complex [PtCl(dien)]Cl was also used, which resulted in only a slight decrease of EtBr fluorescence intensity. Comparison with monofunctional [PtCl(dien)]Cl suggests that the conformational distortion induced in DNA by the adducts of AMPZ is somewhat more delocalized, but extends over considerably less base pairs around the platination sites than in the case of the adducts of mononuclear bifunctional complexes such as cisplatin. Thus, the results shown in Figure 6A (Appendix, paper I) are consistent with capability of AMPZ to form DNA adducts which either span less base pairs around the binding sites or induce conformational distortions in DNA which extend over considerably less base pairs than in case of major CLs of cisplatin. Since both cisplatin and AMPZ form mainly the identical type of major DNA adducts, namely 1,2-intrastrand CLs, so that major adducts of both platinum complexes span only two base pairs, the latter eventuality, i.e. that lesions induced in DNA by AMPZ are considerably less distorting DNA conformation than cisplatin lesions, is more likely.

The latter view that adducts of AMPZ distort DNA conformation considerably less than adducts of cisplatin was also corroborated by the terbium fluorescence data (Appendix, Fig. 6B, paper I) and the results describing formation of liquid crystalline dispersions of DNA (Appendix, Fig. 7, paper I). Terbium ion (Tb<sup>3+</sup>) fluorescence is used to investigate local perturbations induced in conformation of double-helical DNA by various agents including cisplatin. The assay is based on the observation that Tb<sup>3+</sup> fluorescence is strongly enhanced when the ion is bound to G residues in distorted DNA regions [108,109]. DNA

modification by cisplatin resulted in marked enhancement of  $Tb^{3+}$  fluorescence, whereas changes in fluorescence signal after DNA modification by AMPZ were negligible (Appendix, Fig. 6B, paper I). We also recorded CD spectra of the liquid crystalline dispersions of linearized pSP73KB plasmid in the presence of PEG, unmodified or modified by AMPZ, cisplatin and  $[PtCl(dien)]^+$  at the same  $r_b$  value = 0.05 (Appendix, Fig. 7, paper I). An intense negative CD band at around 275 nm was observed for control, the unmodified DNA, and this band was remained almost unaffected for DNA modified by non-distorting monofunctional  $[PtCl(dien)]^+$ . The amplitude of this band somewhat decreased as a consequence of the modification of DNA by AMPZ, whereas the entirely band disappeared when DNA was modified by cisplatin. The results indicate that in contrast to cisplatin AMPZ disturbs DNA liquid crystals only weakly, although somewhat more than monofunctional  $[PtCl(dien)]^+$ .

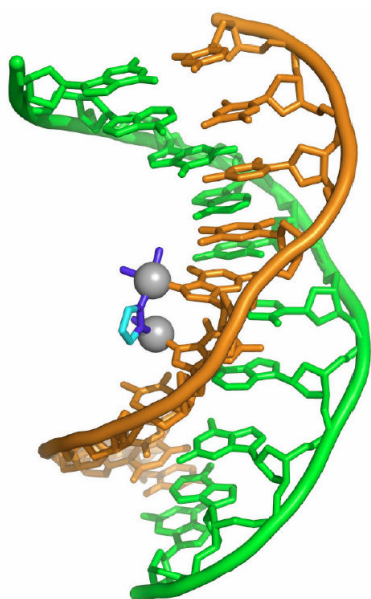
#### 4.1.3. DNA repair synthesis

Recent clinical studies suggest that high levels of expression of proteins associated with nucleotide excision repair of cisplatin-DNA adducts result in tumor resistance and, ultimately, are responsible for the low efficacy of classical platinum-based regimens [110,111]. Nucleotide excision repair system (NER) most efficiently recognizes and removes irreversible DNA adducts that severely distort and destabilize double-helical DNA. Thus, based on the outcome of our experiments in cell-free systems, the adducts produced by AMPZ should be poor substrates for nucleotide excision repair. The relative resistance to DNA repair would explain why AMPZ show major pharmacological advantages over cisplatin in several cancer cell lines [61].

To support this, we performed a DNA repair synthesis assay on plasmid DNA randomly modified by AMPZ and cisplatin. The results (Appendix, Fig. 8, paper I) demonstrated that the damage-induced DNA repair synthesis detected in the plasmid modified with AMPZ was considerably lower than that found for cisplatin at the same level of modification.

## 4.2. Energetics, conformation, and recognition of DNA duplexes containing a major adduct of antitumor AMPZ complex (paper II)

Our previous results (paper I) demonstrated that modification by AMPZ distorts double helical structure of polymeric DNA much less than modification by cisplatin. To further characterize the antitumor mechanism of action of AMPZ, we expanded our study of some important biophysical and biochemical aspects of the alterations induced in short synthetic DNA duplexes uniquely and site-specifically modified by the major DNA adduct of AMPZ, namely 1,2-GG intrastrand CL (Fig. 12). We compared these biophysical and biochemical properties with those obtained under identical conditions for the same adduct of cisplatin. A particular attention was paid to details of conformational distortions induced by the adducts of AMPZ and cisplatin, associated alterations in the thermodynamic stability of the duplexes containing these adducts, and recognition of these adducts by two specific damaged-DNA binding proteins known as important factors modulating the antitumor effects of platinum drugs already used in clinic.



**Figure 12** Structure of a double stranded DNA decamer d(CTCTGGTCTC)-d(GAGACCAGAG) containing a major adduct of AMPZ, the 1,2-GG-IAC, derived from NMR combined with molecular modeling. Adapted from [8].



#### 4.2.1. Conformational distortions induced by 1,2-GG-intrastrand cross-links of AMPZ

To obtain information on how major adducts of AMPZ affect DNA conformation, oligodeoxyribonucleotide duplexes containing a site-specific 1,2-GG intrastrand CL of AMPZ or cisplatin were analyzed by a chemical probe of DNA conformation and by a phase-sensitive gel electrophoresis bending assay.

The platinated 22 bp duplex was treated with  $\text{KMnO}_4$ , a chemical agent which preferentially reacts with thymine residues in single-stranded DNA and in distorted double-stranded DNA. Gel electrophoresis of piperidine-induced specific strand cleavage at  $\text{KMnO}_4$ -modified bases in the unplatinated 22 bp duplex or the duplex containing the single 1,2-GG-IAC of AMPZ or cisplatin (Appendix, Fig. 2A, paper II) revealed that the conformational distortion induced by the 1,2-GG-IAC of AMPZ is delocalized, extending over at least five base pairs around the adduct (Appendix, Fig. 2B, paper II). Importantly, the distortion induced by the adduct of AMPZ appears to be significantly less pronounced than that induced by the adduct of cisplatin.

The oligonucleotide duplexes for the phase-sensitive gel electrophoresis bending assay were designed and prepared as described in Materials and methods (Chapter 3.11.). In accord with previously published data <sup>[21-22, 112]</sup>, a significant retardation was observed for the multimers of 22-bp duplexes containing single, 1,2-GG intrastrand CL of cisplatin and a relatively very small retardation was observed for the multimers of cisplatinated 16-bp duplexes (Appendix, Fig. 5B, paper II). In contrast, almost negligible retardation was observed for the multimers of both 16-bp and 22-bp duplexes modified by the 1,2-intrastrand CL of AMPZ (Appendix, Fig. 5B, paper II). The relative mobility of the multimers is expressed as the *K* factor, which is defined as the ratio of calculated length of a multimer and its real length. The higher this ratio, the more bent is a given oligomer. A *K* value of 1 means that the oligomer migrates as though it had no bends <sup>[23]</sup>. The *K* value obtained for the multimers of the duplex 22 bp long and containing the intrastrand CL of cisplatin (Appendix, Fig. 5C, paper II) increases dramatically with DNA length, resembling the electrophoretic behavior of bent DNA molecules in which the sequence repeat closely matches the double helix screw repeat <sup>[113]</sup>. Furthermore, the mobility was relatively unaltered in cisplatinated duplex DNA molecules containing platinum atoms repeated at 16-bp intervals, representing a phasing of ~1.5 helical turns. This latter control

assures that the gel mobility changes are not simply due to charge or localized frictional effects of the  $[\text{Pt}(\text{NH}_3)_2]^{2+}$  adduct. In contrast, the plots obtained for the multimers of the duplexes 16 and 22 bp long and containing the intrastrand CL of AMPZ (Appendix, Fig. 5D, paper II) appear as an almost horizontal line passing through the value  $K = 1$ , indicating no curvature. Thus, our observations show that DNA modified by 1,2-GG intrastrand CLs of AMPZ is not bent, which is consistent with a  $^1\text{H}$  NMR and molecular modeling studies of the 10-bp duplex containing 1,2-GG intrastrand CL of AMPZ [8, 63] (Fig.12).

#### 4.2.2. Thermodynamic stability of DNA duplexes modified by AMPZ

The chemical probing by  $\text{KMnO}_4$  indicated that the adducts of both AMPZ and cisplatin should thermodynamically destabilize DNA, however, the destabilization should be significantly more pronounced in case of the adduct of cisplatin. Therefore, we examined the thermodynamical parameters by means of differential scanning calorimetry (DSC), a technique based on measuring the difference in the amount of heat required to increase the temperature of a sample and reference as a function of temperature.

The thermodynamic stability of the DNA duplex is expressed as the free energy of duplex melting,  $\Delta G^0$ . We examined changes in thermodynamic stability of the unmodified duplex ( $\Delta\Delta G^0_{298}$  values) after the introduction of the 1,2-GG intrastrand CL of AMPZ and cisplatin. The thermodynamic parameters derived from DSC data (Appendix, Table 1 and Fig. 3, paper II) revealed that the adduct of cisplatin destabilized the double helix markedly more than the CL of AMPZ, as indicated by a  $18.6 \text{ kJ mol}^{-1}$  versus only  $4.0 \text{ kJ mol}^{-1}$  decrease in the Gibbs free energy for dissociation of duplex containing the CL of cisplatin and AMPZ, respectively, at 298 K.

These results are consistent with the presumption that major adduct of AMPZ, in contrast to the same adduct of cisplatin, decreases thermodynamic stability of DNA only negligibly. The difference in the efficiency of the CLs of cisplatin and AMPZ to thermodynamically destabilize DNA is associated with a different capability of these adducts to distort the DNA conformation.

### 4.2.3. Protein recognition of 1,2-GG-intrastrand cross-links of AMPZ

HMG-domain proteins, such as HMGB1, have already been introduced as proteins selectively binding cisplatin-DNA adducts protecting them from NER (Chapters 1.2.3. and 1.2.4.1.). Our experiments demonstrated that 1,2-GG intrastrand CL of AMPZ is not recognized by both A and B domains of HMGB1 (Appendix, Fig. 7, paper II), presumably as a consequence of small extent of bending in DNA modified with AMPZ. This suggests that binding of proteins that have a high affinity for the rigidly bent DNA does not play a role in the mechanism of action of AMPZ. This notion was further corroborated by the observation that the major 1,2-GG intrastrand CLs of AMPZ are recognized by the XPA protein considerably less (~8-fold) than the same adducts of cisplatin (Appendix, Fig. 8, paper II). The XPA protein has been introduced in Chapter 1.2.4.2. as an essential subunit of the mammalian NER system implicated in recognition of DNA damaged by cisplatin [50]. We suggest that our findings may also apply to other damaged-DNA recognition proteins belonging to the NER system.

The lack in recognition of a major adduct of AMPZ by damaged-DNA binding proteins such as XPA significantly contributed to our previous results (paper I), which suggest that the adducts produced by AMPZ should be poor substrates for nucleotide excision repair and thus demonstrate a major pharmacological advantage over cisplatin.

### 4.3. Photoactivation of carboplatin (paper III)

Carboplatin is a second-generation analogue of cisplatin, also used in the clinic. Compared to cisplatin, carboplatin exhibits a more acceptable side-effect profile attributable to its lower reactivity with nucleophiles, since the CBDCA ligand is a poorer leaving group than chlorides in cisplatin. However, the toxic effects in tumor cells are also reduced and markedly higher concentrations of carboplatin are required to achieve a level of platination equivalent to that of cisplatin.

Recently, platinum complexes have been shown to be particularly attractive as potential photochemotherapeutic anticancer agents (see also Chapter 1.4.3.). Selective photoactivation of platinum complexes by irradiation of cancer cells may increase desired toxicity without enhancement of toxic side-effects, and extend application of photoactivatable platinum complexes also to resistant cells and to a wider range of cancer

types. Therefore, it was of our interest to examine whether carboplatin can be affected by irradiation with light to the extent that its DNA binding and cytotoxic properties are altered.

#### 4.3.1. DNA-binding mode of photoactivated carboplatin

The CT DNA binding experiments carried out in a cell-free medium revealed that the modification reaction resulting in the irreversible coordination of UVA ( $\lambda_{\text{max}} = 365 \text{ nm}$ ) photoactivated carboplatin to DNA was markedly faster than that of carboplatin in the dark (Appendix, Fig. 2, paper III). No enhancement of the binding rate was observed for irradiation by light from the visible region ( $\lambda_{\text{max}} = 458 \text{ nm}$ ).

To further characterize the DNA binding of UVA-irradiated carboplatin, the fluorescent probe EtBr (see Chapter 3.5.) was used. The extent to which carboplatin bound to DNA lowered EtBr fluorescence (Appendix, Fig. 3A, paper III) was dependent on the time of UVA irradiation. Short times of irradiation (30 min) resulted in DNA adducts which decreased EtBr fluorescence only slightly so that in this case DNA adducts of photoactivated carboplatin could be coclustered, from the viewpoint of their capability to inhibit EtBr fluorescence, rather with monofunctional DNA adducts of  $[\text{PtCl}(\text{dien})]\text{Cl}$ . A continuing irradiation of carboplatin bound to DNA led to further decrease of EtBr fluorescence and the irradiation for 4 h resulted in a decrease of EtBr fluorescence which was identical to that caused by the same amount of bifunctional adducts formed by cisplatin. Hence, the results are consistent with capability of photoactivated carboplatin to form bifunctional CLs in DNA similarly as cisplatin in a two-step process, forming first monofunctional adducts of diammine Pt(II) species with monodentate CBDCA followed by a second step involving displacement of monodentate CBDCA resulting in the formation of bifunctional CLs. A similar two-step process was observed in the reaction of monomeric 5'-GMP with carboplatin in the dark <sup>[72]</sup> and irradiation-promoted substitution of leaving chloride groups by water was reported in previous studies on cisplatin and transplatin <sup>[71, 80]</sup>. Our results suggest that a similar mechanism of photoactivation might be effective when carboplatin is irradiated by UVA, i.e. that leaving ligand of carboplatin (CBDCA) is lost upon irradiation and substituted by water markedly more readily than in the dark.

An intriguing aspect of photoactivation of carboplatin was that the frequency of interstrand cross-links (% of cross-links formed between the two strands of DNA duplex per one molecule of the Pt complex; see also Chapter 3.6.) was substantially increased (~5-fold; Appendix, Fig. 4, paper III) compared to IEC frequency of carboplatin in the dark which was only 1-2 %. The interstrand CLs pose a special challenge to repair enzymes because they involve both strands of DNA. Thus, they would not be repaired so readily as intrastrand lesions because the repair enzymes would require the information in the complementary strand for resynthesis. In this way, the interstrand CLs might persist considerably longer than intrastrand adducts, which would result in higher cytotoxicity of the interstrand lesions. Thus, next to markedly enhanced rate of binding, higher DNA interstrand cross-linking efficiency of photoactivated carboplatin might be another factor leading to potential enhancement of carboplatin toxicity in tumor cells.

#### 4.3.2. Cytotoxicity studies upon photoactivation

The cytotoxicity of photoactivated carboplatin was studied on A2780 ovarian carcinoma cell line (Appendix, Fig. 5, paper III). The  $IC_{50}$  values (the drug concentration required for 50% inhibition of cell growth) were more than 10-fold lower for photoactivated carboplatin compared to carboplatin in the dark. Thus, photoactivated carboplatin was markedly more toxic to A2780 cells than carboplatin in the dark, which confirmed our expectations that changes in DNA-binding mode of photoactivated carboplatin such as faster kinetics of DNA reactivity and enhanced interstrand cross-linking efficiency would result in enhancement of cytotoxicity in tumor cells.

## 5. CONCLUSIONS

With respect to the aims raised in this thesis, we conclude that:

- Our results revealed that AMPZ forms in polymeric DNA a spectrum of adducts similar to that formed by cisplatin, with 1,2-GG-intrastrand cross-link being the major adduct.
- We also demonstrated that AMPZ induces only little conformational distortions in highly polymeric DNA with a random nucleotide sequence.
- We experimentally proved that DNA modified by AMPZ was much less repaired compared to cisplatin. Thus, we concluded that low distortions caused by AMPZ represent a structural motif recognizable by DNA repair systems less efficiently than distortions induced by cisplatin.
- We have shown that a major adduct of AMPZ, the 1,2-GG-intrastrand cross-link, induces no bending in DNA oligonucleotide duplexes and causes only negligible decrease in the thermodynamic stability of the modified duplex.
- Furthermore, the 1,2-GG-intrastrand cross-link was not recognized by damaged-DNA binding proteins such as XPA, which significantly contributed to our suggestion that the adducts produced by AMPZ should be poor substrates for nucleotide excision repair and thus demonstrate a major pharmacological advantage over cisplatin.
- Thus, DNA adducts of a novel group of antitumor azolato-bridged dinuclear Pt<sup>II</sup> complexes such as AMPZ can escape repair mechanisms more easily than those of cisplatin, which may potentiate their antitumor effects in cancer cells.
- We investigated carboplatin as a potential photoactivable drug. We have found that carboplatin is converted to species capable of enhanced DNA binding by UVA irradiation and consequently its toxicity in cancer cells is markedly enhanced. Given that recent advances in laser and fiber optic technologies make it possible to irradiate also internal organs with light of highly defined intensity and wavelength, the clinically already established carboplatin is a proper candidate for use in photoactivated cancer chemotherapy.

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## LIST OF ABBREVIATIONS

A	Adenine
AMPZ	$[(cis-\{Pt-(NH_3)_2\})_2(\mu-OH)(\mu-pz)]^{2+}$ (pz = pyrazolate)
ATP	Adenosine triphosphate
C	Cytosine
Carboplatin	<i>cis</i> -diammine-[1,1-cyclobutanedicarboxylato]platinum(II)
CBDCA	Cyclobutyldicarboxylate
CD	Circular dichroism
CFE	Cell-free extract
Cisplatin	<i>cis</i> -diamminedichloridoplatinum(II)
CL	Cross-link
CT	Calf-thymus
DACH	Diaminocyclohexane
DMS	Dimethyl sulfate
dNTP	Deoxyribonucleotide triphosphate
DSC	Differential scanning calorimetry
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
F	Frequency of interstrand cross-links
FAAS	Flameless atomic absorption spectrometry
FDA	Food and Drug Administration
G	Guanine
GMP	Guanosine monophosphate
HeLa S3	Human epithelial carcinoma cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMGB1	High-mobility group protein B1
HPLC	High-performance liquid chromatography
HPMA	Hydroxypropylmethacrylamide
IAC	Intrastrand cross-link
ICL	Interstrand cross-link
LC	Liquid crystal(line)

LD	Linear dichroism
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	Nuclear magnetic resonance
PAA	Polyacrylamide
PCNA	Proliferating cell nuclear antigen
PEG	Polyethylene glycol
Pol $\eta$	DNA polymerase $\eta$
$r_b$	Number of molecules of the platinum compound coordinatively bound per nucleotide residue
$r_i$	Molar ratio of free platinum complex to nucleotide phosphates at the onset of incubation with DNA
RPA	Replication protein A
SDS	Sodium dodecyl sulfate
T	Thymine
TBE	Tris/Borate/EDTA buffer
TFIIH	Transcription factor II H
$T_m$	Melting temperature
Transplatin	<i>trans</i> -diamminedichloridoplatinum(II)
TRIS	Tris(hydroxymethyl)aminomethane
XL	Number of interstrand CLs per one molecule of the linearized DNA duplex
XP	Xeroderma pigmentosum
XPA, C, F, G	Xeroderma pigmentosum group proteins (A,C,F,G)

## **APPENDIX**

PAPER I

**DNA conformation and repair of polymeric natural DNA  
damaged by antitumor azolato-bridged dinuclear Pt<sup>II</sup> complex**

J. Mlčoušková, J. Kašpárková, T. Suchánková, S. Komeda, V. Brabec

*Journal of Inorganic Biochemistry (accepted April 2012)*

I declare that my role in preparation of this paper was as following:  
Experimental realization of kinetics of binding, restriction enzyme cleavage inhibition  
assay, DNA melting, ethidium bromide fluorescence measurements, liquid crystalline  
dispersions of DNA. Preparation of the manuscript.

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# DNA conformation and repair of polymeric natural DNA damaged by antitumor azolato-bridged dinuclear Pt<sup>II</sup> complex

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## ABSTRACT

Design of new antitumor Pt drugs is currently also focused on those new Pt complexes which form on DNA major adducts that can be hardly removed by DNA repair systems. An attempt of this kind has already been done by designing and synthesizing new antitumor azolato-bridged dinuclear Pt<sup>II</sup> complexes, such as [*cis*-Pt(NH<sub>3</sub>)<sub>2</sub>]<sub>2</sub>(μ-OH)(μ-pyrazolate)]<sup>2+</sup> (AMPZ). This new Pt<sup>II</sup> complex exhibits markedly higher toxic effects in some tumor cell lines than conventional mononuclear cisplatin. The primary objective in the present study was to further delineate differences in the interactions of AMPZ and cisplatin with natural, high-molecular-mass DNA using a combination of biochemical and molecular biophysics techniques. The results demonstrate for the first time that little conformational distortions induced by AMPZ in highly polymeric DNA with a random nucleotide sequence represent a structural motif recognizable by DNA repair systems less efficiently than distortions induced by cisplatin. Thus, DNA adducts of azolato-bridged dinuclear Pt<sup>II</sup> complexes can escape repair mechanisms more easily than those of cisplatin, which may potentiate antitumor effects of these new metallodrugs in cancer cells.

Keywords: DNA damage; DNA conformation; DNA repair; dinuclear platinum; antitumor

## 1. Introduction

The mechanism of action of anticancer platinum drugs used in the clinic, such as *cis*-Pt(NH<sub>3</sub>)<sub>2</sub>Cl<sub>2</sub> (cisplatin, Fig. 1) and its analogues, involves coordination to purine DNA bases, the cellular responses to the DNA damage which primarily lead to cellular

PAPER II

**Energetics, conformation, and recognition of DNA duplexes  
containing a major adduct of anticancer azolato-bridged  
dinuclear Pt<sup>II</sup> complex**

**J. Mlčoušková, J. Malina, V. Novohradský, J. Kašpárková, S. Komeda, V. Brabec**

*Biochimica et Biophysica Acta (submitted)*

I declare that my role in preparation of this paper was as following:  
Experimental realization of chemical probing of DNA conformation, ligation and  
electrophoresis of oligonucleotides, flow linear dichroism, electrophoretic mobility shift  
assays with HMGB1 domain and XPA proteins. Preparation of the manuscript.

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# Energetics, conformation, and recognition of DNA duplexes containing a major adduct of anticancer azolato-bridged dinuclear Pt<sup>II</sup> complex

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*Abbreviations:* AMPZ, [ $\text{cis-}\{\text{Pt}(\text{NH}_3)_2\}_2(\mu\text{-OH})(\mu\text{-pyrazolate})\}^{2+}$ ]; cisplatin [*cis*-diamminedichloridoplatinum(II)]; bp, base pair; CL, cross-link; CT, calf thymus; DSC, differential scanning calorimetry; EMSA, electrophoretic mobility shift assay; FAAS, flameless atomic absorption spectrometry; DMS, dimethyl sulfate; dNTP, deoxyribonucleotide triphosphate; HPLC, high-pressure liquid chromatography; HMG, high mobility group; HMGB1a, domain A of full length HMGB1 protein; HMGB1b, domain B of full length HMGB1 protein; LD, linear dichroism;  $T_m$ , melting temperature; NER, nucleotide excision repair; PAA, polyacrylamide; Pol $\eta$ , DNA polymerase  $\eta$ ;  $r_b$ , the number of molecules of the platinum complex bound per nucleotide residue; SDS, sodium dodecyl sulfate; XPA, xeroderma pigmentosum group A.

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## ABSTRACT

*Background:* Design of anticancer metallodrugs is currently also focused on platinum complexes which form on DNA major adducts that can be hardly removed by DNA repair systems. Hence, antitumor azolato-bridged dinuclear Pt<sup>II</sup> complexes, such as [ $\text{cis-}\{\text{Pt}(\text{NH}_3)_2\}_2(\mu\text{-OH})(\mu\text{-pyrazolate})\}^{2+}$  (AMPZ), were designed and synthesized. These complexes exhibit markedly higher toxic effects in tumor cell lines than mononuclear conventional cisplatin.

**PAPER III**

**Antitumor carboplatin is more toxic in tumor cells when  
photoactivated: Enhanced DNA binding**

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*Journal of Biological Inorganic Chemistry (accepted May 2012)*

I declare that my role in preparation of this paper was as following:  
Experimental realization of kinetics of binding, ethidium bromide fluorescence  
measurements, DNA interstrand cross-linking assay. Preparation of the manuscript.

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# Antitumor carboplatin is more toxic in tumor cells when photoactivated: Enhanced DNA binding

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**Abstract** Carboplatin, an analogue of "classical" cisplatin, is a widely used second-generation platinum anticancer drug. Cytotoxicity of cisplatin and carboplatin is mediated by platinum-DNA adducts. Markedly higher concentrations of carboplatin are required, and the rate of adduct formation is considerably slower. The reduced toxic effects in tumor cells and a more acceptable side-effect profile are attributable to the lower reactivity of carboplatin with nucleophiles, since the CBDCA ligand is a poorer leaving group than chlorides in cisplatin. Recently, platinum complexes were shown to be particularly attractive as potential photochemotherapeutic anticancer agents. Selective photoactivation of platinum complexes by irradiation of cancer cells may avoid enhancement of toxic side effects, but may increase toxicity selectively in cancer cells and extend application of photoactivatable platinum complexes also to resistant cells and to a wider range of cancer types. Therefore, it was of interest to examine whether carboplatin can be affected by irradiation with light to the extent that its DNA binding and cytotoxic properties are altered. We have found that carboplatin is converted to species capable of enhanced DNA binding by UVA irradiation and consequently its toxicity in cancer cells is markedly enhanced. Recent advances in laser and fiber optic technologies make it possible to irradiate also internal organs with light of highly defined intensity and wavelength. Thus, carboplatin is a candidate for use in photoactivated cancer chemotherapy.

**Key words** Anticancer Platinum · Cytotoxicity · Photoactivation · DNA adducts · UVA

## Abbreviations

bp	base pair
carboplatin	<i>cis</i> -diammine(1,1-cyclobutyldicarboxylato)platinum(II)
CBDCA	cyclobutyldicarboxylate
Cisplatin	<i>cis</i> -dimminedichloridoplatinum(II)
CL	cross-link
CT	calf-thymus