9.6: Mapping the Major Adducts of cis- and trans-DDP on DNA; Sequence Specificity

As we have seen, the antitumor activity of cisplatin is most likely the result of its DNA-binding properties. But what are the adducts? The human genome has more than a billion nucleotides. Does platinum recognize any special regions of the DNA or any particular sequences? In other words, is binding simply random or is there at least a regioselectivity? In this section, we discuss the best strategies for answering these questions, strategies that evolved in pursuit of learning how *cis*-DDP binds to DNA. We also illustrate their power in elucidating the DNA-binding properties of other metal complexes of interest to bioinorganic chemists.

a. Early Strategic Approaches

The first experiments to imply the sequence preferences of *cis*-DDP binding to DNA employed synthetic polymers.[108,109] Specifically, the buoyant density of poly(dG)•poly(dC), poly(dG•dC), and their *cis*-DDP adducts was studied in the analytical ultracentrifuge. The greatest shift in buoyant density was seen for the platinum adducts of poly(dG)•poly(dC), from which it was concluded that platinum forms an intrastrand crosslink between two neighboring guanosine nucleosides on the same strand. This interpretation was suggested by the known preference of metal ions, and especially platinum, for binding at the N7 position on the guanine base (Figure 9.9), information available from model studies of metal-nucleobase chemistry. Although other interpretations of the buoyant-density shift were possible, especially since the amount of platinum bound was not quantitated, the conclusion proved to be correct, as confirmed by later investigations. Interestingly, *trans*-DDP did not selectively increase the buoyant density of poly(dG)•poly(dC).

Following these initial experiments, the regioselectivity of *cis*-DDP binding was investigated by studying the inhibition of enzymatic digestion of platinated DNA. For example, the platinum complex inhibits the cleavage of DNA by restriction enzymes that recognize specific sequences and cut both strands of the double helix.[110] The resulting fragments are readily identified on electrophoresis gels. One such restriction enzyme is Bam HI. As shown by the arrows in Scheme (9.11), Bam HI cleaves a six-bp palindromic sequence at the phosphodiester bonds between two guanosine nucleosides. Formation of an intrastrand crosslink between the two adjacent guanosine nucleosides inhibits digestion by the enzyme. Another method, termed exonuclease mapping, involves digestion of the strands of duplex DNA from its 3'-ends.[111,112] When the enzyme encounters a bound platinum atom, it is unable to proceed further. Analysis of the digestion products by gel electrophoresis reveals the presence of discrete bands caused by the inhibition of digestion by bound platinum at specific sequences. Results from experiments of this kind were the most definitive at this time in demonstrating the profound regioselectivity of cisplatin for adjacent guanosines, and strongly supported the earlier conclusion that the drug was making an intrastrand d(GpG) crosslink.

Figure 9.18. Platinum is first bound to a single-stranded DNA template, in this example from bacteriophage M13mp18, to which is next annealed a short, complementary oligonucleotide termed a "primer" for DNA synthesis. Addition of the large (Klenow) fragment of *E. coli* DNA polymerase I and deoxynucleoside triphosphates, one of which bears a ^{32}P label, [α-^{32}P]dATP, initiates replication. When the enzyme encounters a platinum adduct, the chain is terminated. By running out the newly synthesized DNA strands on a sequencing gel, the sites of platinum binding can be detected by comparing the positions of the radiolabeled fragments with those obtained from sequencing ladders. The results of this procedure, which has been termed "replication mapping," confirmed that *cis*-DDP binds selectively to $(dG)_n$ ($n \geq 2$) sequences. In addition, they showed that *trans*-DDP blocks replication, in a much less regioselective manner, in the vicinity of sequences of the kind d(GpNpG), where N is an intervening nucleotide. These data afforded the first clear insight into the sequence preferences for *trans*-DDP on DNA. A control experiment run with DNA platinated by the monofunctional complex [Pt(dien)Cl]$^+$ gave the interesting result that DNA synthesis was virtually unaffected.

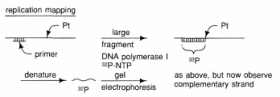

Figure 9.18 - Diagram illustrating the replication mapping experiment. To a single-stranded, platinated template is annealed a short primer for DNA synthesis using DNA polymerase I (Klenow fragment) and radiolabeled nucleotides. Sites of platinum binding are revealed as bands on gel electrophoresis where chain termination occurs (see text for details).

In yet another approach to the problem, DNA containing *cis*- or *trans*-DDP adducts was electrostatically coupled to bovine serum albumin, to enhance its antigenicity, and injected into rabbits.[113,114] The resulting antisera and antibodies were then studied for their ability to recognize and bind specifically to platinated DNAs having defined sequences, such as poly(dG)•poly(dC) and poly[d(GC)]•poly[d(GC)].

From experiments of this kind, the major *cis*-DDP adduct recognized by the antibody was found to be *cis*-[$Pt(NH_3)_2${d(GpG)}], in accord with the findings of the enzymatic mapping experiments. Unplatinated DNA was not recognized, nor was DNA platinated with *trans*-DDP. On the other hand, the antibody recognized DNA platinated with antitumor-active compounds [$Pt(en)Cl_2$] and [Pt(DACH)(CP)], where DACH = 1,2-diaminocyclohexane and CP = 4-carboxyphthalate. This result revealed that the antibody recognized the structural change in DNA that accompanies formation of d(GpG) intrastrand crosslinks, irrespective of the diamine ligand in the coordination sphere of the platinum atom. The antibody is also capable of distinguishing adducts formed by active versus inactive platinum complexes. Most importantly, DNA isolated from the cells of mice bearing the L1210 tumor five hours after cisplatin injection, was recognized.[113,115] Subsequent studies[116] revealed that these antibodies could detect cisplatin-DNA adducts formed in the white blood cells of patients receiving platinum chemotherapy. Thus, the antibody work linked the regiospecificity of platination chemistry *in vitro* with that occurring *in vivo* and in a clinically relevant manner.

Additional studies with monoclonal antibodies generated using DNA platinated with *cis*- or *trans*-DDP further confirmed and extended these results.[117] This later work indicated that intrastrand crosslinked d(ApG) and d(GpG) sequences possess a common structural determinant produced by *cis*-DDP platination, and that carboplatin is also capable of inducing the same DNA structure. For *trans*-DDP-platinated DNA, a monoclonal antibody was obtained that appeared to have the intrastrand d(GpTpG) adduct as its major recognition site. In all these studies, the primary structural determinant appears to be DNA duplex opposite the site of platination, since fairly major stereochemical changes could be made in the amine ligands with no appreciable effect on antibody binding.

b. Degradation, Chromatographic Separation, and Quantitation of DNA Adducts

Experiments in which DNA platinated with *cis*-DDP is degraded to chromatographically separable, well-defined adducts have been invaluable in revealing the spectrum of products formed. In a typical experiment, platinated DNA is digested with DNAse I, nuclease P1, and alkaline phosphatase. These enzymatic digestions degrade DNA into nucleosides that can be readily separated by high-performance liquid chromatography (HPLC). Detection of the adducts can be accomplished by the UV absorption of the nucleoside bases at 260 nm or, for platinum complexes containing a radioactively labeled ligand such as [^{14}C]ethylenediamine,[118] by monitoring counts. In addition to peaks corresponding to dA, dC, dG, and dT, the chromatographic trace contains additional peaks corresponding to specific platinum nucleobase adducts such as *cis*-[$Pt(NH_3)_2(dG)_2$]. The precise nature of these adducts was established by comparison with chemically synthesized compounds structurally characterized by NMR spectroscopy.[118-121] An alternative method for identifying the adducts employed antibodies raised against specific platinum-nucleobase complexes.[122]

This approach has revealed the relative amounts of various adducts formed by a variety of platinum complexes; selected results are summarized in Table 9.4. Usually, for cisplatin, the relative amounts of the various adducts formed varies according to the series *cis*-[$Pt(NH_3)_2${d(pGpG)}] > *cis*-[$Pt(NH_3)_2${d(pApG)}] > *cis*-[$Pt(NH_3)_2${d(GMP)}$_2$] > monofunctional adducts. Only when the total incubation time was short, less than an hour, were the monofunctional adducts more prevalent, as expected from the kinetic studies of *cis*-DDP binding to DNA discussed previously. It is noteworthy that no d(pGpA) adducts were detected. This result, which is consistent with information obtained by enzymatic mapping, can be understood on stereochemical grounds.[123] If the guanosine nucleoside N7 position is the most-preferred binding site on DNA, closure to make an N7,N7 intrastrand crosslink between two adjacent purine nucleotides is more feasible in the 5' direction along the helix backbone (N7•••N7 distance of ≈ 3 Å) than in the 3' direction (N7•••N7 distance ≈ 5 Å). In addition, molecular-mechanics modeling studies[124] indicate that a highly unfavorable steric clash occurs between the 6-amino group of the 3'-adenosine residue in a d(pGpA) crosslink and the platinum ammine ligand, whereas in the platinated d(pApG) sequence, the 6-oxo group forms a stabilizing hydrogen bond to this ligand. A 28 kJ mol^{-1} preference of *cis*-DDP for binding d(pApG) over d(pGpA) was calculated.

Table 9.4 - Geometric features of the platinum coordination spheres of *cis*- [$Pt(NH_3)_2${d(pGpG)}].

a) Bond distances are in Angstroms and angles are in degrees.

b) Conventions used for assigning Base/Base and Base/PtN4 dihedral angles can be found in J. D. Orbell, L. G. Marzilli, and T. J. Kistenmacher, *J. Am. Chem. Soc.* **103** (l981), 5126. The numbers in square brackets refer to the corresponding N(ammine)•••O6 distance, in Å (see text).

Bond Distances and Angles[a]	Molecule 1	Molecule 2	Molecule 3	Molecule 4
Pt-N1	2.03(2)	2.01(2)	2.08(2)	2.08(2)
Pt-N2	2.03(3)	2.09(2)	2.04(3)	2.06(3)

Bond Distances and Angles[a]	Molecule 1	Molecule 2	Molecule 3	Molecule 4
Pt-N7A	2.01(2)	2.02(2)	1.91(3)	1.93(3)
Pt-N7B	2.05(2)	1.95(3)	2.00(3)	2.06(3)
N7A-Pt-N1	88.6(9)	90.3(9)	91.0(1)	88.4(9)
N7A-Pt-N2	179(1)	173.3(8)	178(1)	177(1)
N7A-Pt-N7B	89.1(9)	90.0(1)	85(1)	89(1)
N1-Pt-N2	92(9)	90.8(9)	91(1)	93(1)
qN1-Pt-N7B	176.5(9)	179.0(1)	173(1)	175(1)
N2-Pt-N7B	90.3(9)	89.0(1)	93(1)	89(1)

Dihedral Angles[b] Molecule	3'-Gua/5'-Gua	5'-Gua/PtN_4	3'-Gua/PtN_4
1	76.2(5)	110.6(5) [3.30(3)]	86.1(5)
2	81.0(5)	110.8(5) [3.49(3)]	95.5(5)
3	86.8(6)	81.0(6)	58.0(6) [3.11(4)]
4	80.6(5)	76.6(6)	59.6(6) [3.18(4)]

There are two likely sources of *cis*-[$Pt(NH_3)_2${d(GMP)}$_2$] in the spectrum of adducts. This species could arise from long-range intrastrand crosslinks, where the two coordinated guanosines are separated by one or more nucleotides. In support of this possibility is the fact that digestion of chemically synthesized *cis*-[$Pt(NH_3)_2${d(GpNpG)}], where N = C or A, led to *cis*-[$Pt(NH_3)_2${d(Gua)}-{d(GMP)}] and mononucleotides.[118,119,121] The other source of this product is interstrand crosslinked DNA, known to occur from the alkaline elution studies.

As indicated in Table 9.4, in all the experiments there was platinum that was unaccounted for in the quantitation procedures, which employed either antibodies, platinum atomic absorption spectroscopy, or a radiolabeled ethylenediamine ligand. Some of this material was assigned to oligonucleotides having high platinum content, resistant to enzymatic degradation.

Two important points emerge from the quantitation of adducts by this method. One is that intrastrand d(GpG) and d(ApG) crosslinks constitute the major adducts (>90 percent of total platination) made by cisplatin on DNA *in vivo*. Because they were identified by an antibody specific for their structures, no chemical change brought about by cellular metabolism has occurred. Secondly, the preponderance of these adducts far exceeds the frequency of adjacent guanosine or guanosine/adenosine nucleosides in DNA. This latter result implies a kinetic preference for, or recognition of, d(pGpG)- and d(pApG)-containing sequences by cisplatin.

c. Postscript: A Comment on Methodologies

With few exceptions, none of the experimental studies described in this section could have been carried out in 1969, when Rosenberg first demonstrated the anticancer activity of *cis*-DDP. The techniques of DNA sequencing, monoclonal antibody formation, oligonucleotide synthesis, HPLC, FPLC, and many of the higher resolution gel electrophoresis methodologies employed were the result of later developments driven by rapid advances in the fields of molecular biology and immunology. Future progress in elucidating the molecular mechanisms of action of cisplatin and other inorganic pharmaceuticals will no doubt benefit from new technological discoveries and inventions of this kind yet to come.