NEW METHODOLOGIES FOR STUDYING THE BIOLOGICAL ACTIVITY OF PLATINUM COMPLEXES

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Submitted to the Department of Chemistry in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Biological Chemistry
at the
Massachusetts Institute of Technology
September 1998

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ABSTRACT

A review of existing approaches to platinum drug discovery highlighted the need for new methods to perform mechanism-based, high-throughput screening of platinum complexes.

A new solution phase screening method was based on the importance of high mobility group (HMG) domain proteins in the cytotoxicity of cisplatin. Platinum compounds were evaluated based on their ability to form DNA adducts that bind specifically to HMG-domain proteins. A mixture of platinum amino acid complexes was prepared and combined with DNA to form platinum adducts, some of which were recognized by HMG1 in a gel shift assay. In order to identify the platinum complex(es) responsible for this behavior, a sublibrary synthesis and screening approach was employed. After three iterations of sublibrary synthesis and screening, [Pt(lysine)Cl_2] (Kplatin) was identified as an (N,O)-chelated platinum(II) complex with DNA adducts recognized by HMG-domain proteins. Kplatin and analogous (N,O)-chelates were less toxic towards HeLa cells than cisplatin, presumably because of charge considerations.

A solid-phase assay was envisioned in which platinum-modified DNA could be covalently linked to a solid support, and the binding of a fluorescently labeled HMG-domain protein measured to predict the cytotoxicity of the platinum complexes. A fluorescent HMG-domain protein was generated by expressing HMG1 as a fusion with the green fluorescent protein (GFPuv). The product HMG1-GFPuv retained the fluorescence and DNA-binding properties of its protein components.

To screen platinum complexes for biological activity in human cancer cells, a transcription inhibition assay was developed. A stable HeLa cell line was generated with doxycycline-inducible enhanced green fluorescent protein (EGFP). The effect of platinum complexes and other cytotoxic agents on EGFP expression was assessed. Most cytotoxic agents including trans platinum complexes stimulated EGFP transcription, whereas cis platinum complexes inhibited EGFP expression in a dose-dependent fashion. Transient transfection of the HeLa cells with testis-specific HMG enhanced the inhibitory effect of cisplatin. A second reporter gene assay using a fluorescent β-lactamase substrate demonstrated the inhibition of gene expression by cisplatin but not by ineffective platinum complexes.

Thesis Supervisor: Stephen J. Lippard
Chairman, Department of Chemistry and
Arthur Amos Noyes Professor of Chemistry

Acknowledgments

First and foremost I must acknowledge my thesis advisor Stephen J. Lippard. Although I came to MIT intending to join Steve's lab, I didn't know exactly why until one morning in September 1993, in his bioinorganic chemistry class, when he proposed the radical idea of using combinatorial chemistry to discover new platinum drugs. The idea of applying the then-novel concept of molecular diversity to the perplexing issue to platinum drug discovery enticed me, and I knew that I had found the right advisor, laboratory and project. I wanted to learn from someone who was not afraid to cross traditional scientific boundaries and apply any and every new technique to the quest for knowledge. Steve has been for me an example of scientific fearlessness, of not abandoning a promising lead just because the territory ahead is unfamiliar and threatening. He has shown tremendous confidence in my abilities and support for my research even when others have been less enthusiastic.

There are advantages to working with Steve beyond even the opportunity to learn from an excellent scientist. Indeed, the Lippard lab is an institution like no other, and I must recognize each and every past and current member for their contributions. In particular, I thank Stefanie Kane for being an exceptional role model, mentor and friend. I also thank Rajesh Manchanda, Andy Gelasco, Sudhakar Marla, Megan McA'Nulty, Besty Jamieson, Qing He, and David Coufal for lots of encouragement and friendship over the years. This thesis would not exist were it not for the extensive contributions of Deborah Zamble, who spent countless hours giving me advice, assistance and understanding. She is an excellent scientist and a great friend.

This work was greatly helped by the contributions of several members of the lab, especially Chris Ziegler and Cindy Liang. Chris' enthusiasm for platinum chemistry helped to answer some critical questions in my research and gave me the opportunity to focus on my molecular biology interests. Cindy's incredible dedication to her research, in addition to yielding valuable results, was a great inspiration to a weary senior graduate student! I also thank Peter Fuhrmann for helping me with x-ray crystallography, and Christina Eng for sharing tissue culture advice and materials. It also has been a pleasure to collaborate with Gregor Zlokarnik of Aurora Biosystems.

I have benefited tremendously from discussions with several members of the MIT faculty, especially in the last few months. I thank Bevin Engelward, Peter Dedon, and Graham Walker for thoughtful discussions of my research. I also thank my thesis committee members John Essigmann and Larry Stern. The members of the 6th floor toxicology labs were extremely hospitable to me.

On a more personal level I thank my aunt, Kaethe Sandman, who has been a tremendously supportive role model for many years. I also thank Mom, Dad, Suzanne, Chris, Amanda, Dan and the Toronto and New York Mulgunds for all of their encouragement and patience during the past five years. Special thanks go to Kristen Bohlke and Joyce Dietrich for their continuing friendship and for reminding me that there is a world beyond chemistry!

My cat, the Bubbleboy, stayed by my side every day while I wrote this thesis and I thank him for his loyalty and devotion. Most of all, I thank my husband Sandeep Mulgund for his unfaltering love and dedication during the past five years. After finishing his own doctorate, he chose to support me through the long hours and stress of my graduate school experience, and for that I cannot thank him enough. He has always given me something to look forward to, from coming home to join him and the Bubbleboy reclining on the couch to all of our plans for life after MIT. In more practical terms, his extensive knowledge of Microsoft Word was quite helpful in preparing this dissertation. In every way he has made it possible for me to finish my thesis and have a happy life at the same time.

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Overview

This dissertation consists of three chapters and one appendix. Chapter 1 reviews methods for screening platinum complexes for cytotoxicity. Chapter 2 describes a solution-phase screen of platinum-amino acid complexes. Chapter 3 introduces two cell-based methods for rapidly assessing platinum cytotoxicity. Finally, Appendix A describes the fluorescent labeling of an HMG-domain protein.

Chapter 1 evaluates various methods, including those fully described in subsequent chapters, for their utility in screening combinatorial libraries of platinum complexes. It introduces the major in vitro, cell-based and in vivo approaches to assessing the cytotoxicity of platinum complexes and describes the challenges in improving screening methods.

Chapter 2 outlines the current understanding of the mechanism of action of cisplatin with an emphasis on the role of HMG-domain proteins. A screening protocol exploiting the importance of the platinum-DNA-HMG interaction for cytotoxicity is proposed. The results of screening a mixture of platinum-amino acid complexes for the binding of their DNA adducts to HMG1 are presented. The identification of the (N,O)-chelated [Pt(lysine)Cl_2] compound Kplatin, and subsequent studies of the in vitro and cytotoxic behavior of Kplatin and analogues, are described.

Chapter 3 describes the use of reporter genes to predict and understand the cytotoxicity of platinum complexes. After reviewing the effect of DNA damage on reporter gene expression in mammalian cells, several new screening methods based on the inhibition of gene expression by active platinum complexes are described. It is shown that platinum complexes inhibit inducible EGFP transcription in HeLa Tet-On

cells and that this inhibition is well correlated with cytotoxicity. The enhancement of EGFP transcription by other cytotoxic agents, the effect of tsHMG expression on the assay, and the mechanistic implications of the results are also discussed. A second platinum screening method using β-lactamase as a reporter gene with a fluorescent substrate is also described.

Appendix A reports several attempts to fluorescently label HMG-domain proteins for use in solid-phase in vitro screening methodologies. The cloning, expression and purification of the fusion of HMG1 with the GFPuv is described. The fluorescence and DNA-binding activity of the resulting HMG1-GFPuv is also demonstrated.

Abbreviations

AC_{50}:	concentration at which 50% of cells are apoptotic
CMV:	cytomegalovirus
DDP:	diamminedichloroplatinum(II)
DMEM:	Dulbecco's modified Eagle medium
EGFP:	enhanced green fluorescent protein
FBS:	fetal bovine serum
FITC:	fluorescein isothiocyanate
GFP:	green fluorescent protein
GFPuv:	UV-optimized green fluorescent protein
HMG:	high mobility group
HMG1:	high mobility group protein 1
HMGdomB:	HMG-domain B of HMG1
IC_{50}:	concentration at which reporter gene expression is 50% of control
LC_{50}:	concentration at which 50% of cells are killed
MMS:	methyl methanesulfonate
PBS:	phosphate-buffered saline
r_b:	bound platinum to nucleotide ratio
r_f:	formal platinum to nucleotide ratio
tsHMG:	testis-specific high mobility group protein

Chapter 1

Methods for Screening the Potential Antitumor Activity of Platinum Compounds in Combinatorial Libraries*

1.1 Introduction

The success of cisplatin and carboplatin in treating cancer, combined with the intrinsic and acquired resistance of many tumors to traditional platinum chemotherapy, have generated considerable interest in developing next-generation platinum drugs. Since the discovery of the antitumor activity of cisplatin, researchers have reported the synthesis, characterization, and antitumor activity of thousands of platinum compounds [1,2]. Recent developments include novel multinuclear platinum(II) and orally active platinum(IV) complexes [3,4].

The advent of combinatorial chemistry has had a major impact on drug discovery [5-7], but has not yet facilitated the search for new cisplatin analogues. To date there is only one published report of a molecular diversity approach to platinum drug discovery [8]. One reason for this situation is that the field of combinatorial chemistry has evolved almost exclusively from organic chemistry [6,9-11]. Methods for combinatorial synthesis of inorganic compounds, with the exception of solid-state materials, have been developed only in a few laboratories [8,12]. It should be possible, however, to apply the advances made by organic chemists in parallel synthesis, automation and chemoinformatics to the generation of libraries of platinum complexes.

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* A slightly modified form of this chapter was submitted for publication *in 30 Years of Cisplatin—Chemistry and Biochemistry of a Leading Anticancer Drug*; B. Lippert, ed., Verlag Helvetica Chimica Acta: Basel.

In order for new platinum drugs to be discovered by a combinatorial approach, there must be suitable screening protocols to identify "hits" from platinum libraries [5,7]. The best screening methods accurately predict activity, are fast and simple, and can be run in a high-throughput environment. A mechanism-based screen that detects the interaction with a target molecule is ideal, but mechanistic studies of platinum drugs have not yet identified a single critical target. Aside from general agreement that DNA binding is required for cisplatin activity, there is little consensus about the biologically relevant interactions that cause tumor cell death. It has therefore been a challenge for researchers to develop a rational strategy for the synthesis of platinum compounds having better anticancer activity than cisplatin.

There are three major approaches to assessing the potential activity of a chemotherapeutic compound prior to human clinical trials. In vitro assays, either solution- or solid-phase, are often used early in the screening process, especially when a biologically relevant molecular interaction has been identified. Studies in cultured mammalian cells are employed to predict the activity of a compound. If a compound shows promise in such in vitro and cell-based assays, then it is tested extensively in animals before proceeding to humans. The remainder of this chapter discusses these three methods as potential routes to identifying active platinum compounds from combinatorial libraries.

1.2 In vitro screening methods

In vitro methods are not widely used to predict the antitumor activity of platinum compounds. Until it is determined what factors render a specific platinum-DNA adduct cytotoxic, the in vitro screens for platinum drugs will be limited. Because a good platinum drug must form DNA adducts, preliminary screening methods have been employed which measure DNA binding alone as a measure of potential antitumor activity. In one such study [13], the DNA binding of palladium and platinum complexes having intercalative ligands was monitored by the quenching of ethidium bromide fluorescence [14]. Upon metal-DNA binding, the intercalative ligand displaced ethidium from DNA, causing a decrease in fluorescence. From the change in fluorescence, DNA binding constants were determined for a series of fourteen palladium(II)/phenanthroline/amino acid complexes. There was a correlation between

DNA binding and both in vitro cytotoxicity and in vivo antitumor activity for the compounds tested. The results are summarized in Table 1.1.

Table 1.1: Comparison of DNA-binding, cytotoxicity and antitumor activity of a series of palladium(II) compounds [data from ref 13].

Complex	Pd-DNA binding constant K x 10^{-6} (L/mol)	Cytotoxicity Index[a]	Antineoplastic Ratio[b,c]
[Pd(phen)(leu)]Cl	5.10	96.7	nd
[Pd(phen)(lys)]Cl	7.96	96.5	56
[Pd(phen)(met)]Cl	5.16	95.0	nd
[Pd(phen)(ser)]Cl	2.93	93.8	nd
[Pd(phen)(arg)]Cl	4.52	90.3	50
[Pd(phen)(his)]Cl	1.45	87.6	nd
[Pd(phen)(asn)]Cl	5.84	86.9	nd
[Pd(phen)(trp)]Cl	3.35	83.7	nd
[Pd(phen)(gln)]Cl	2.25	82.8	nd
[Pd(phen)(pro)]Cl	1.00	78.3	48.2
[Pd(phen)(tyr)]Cl	4.90	77.9	nd
[Pd(phen)(ala)]Cl	1.01	73.8	nd
[Pd(phen)(gly)]Cl	1.67	70.2	nd
[Pd(phen)(asp)]	0.75	37.8	nd

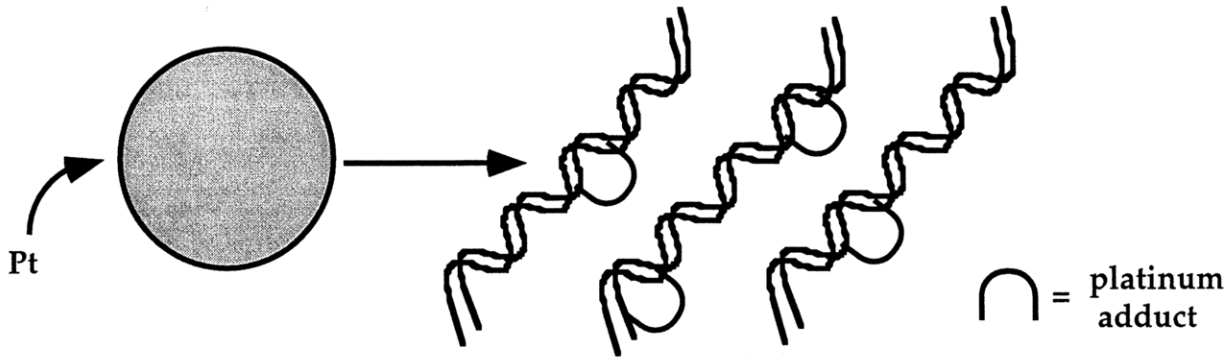
[a] Cytotoxicity index measures the percent dead MCF-7 cells counted by using the trypan blue dye exclusion assay.

[b] Pure 615 mice implanted with sarcoma 180 were treated for nine days with 20 mg/kg/day. The ratio refers to the percent increase in lifespan, relative to untreated controls.

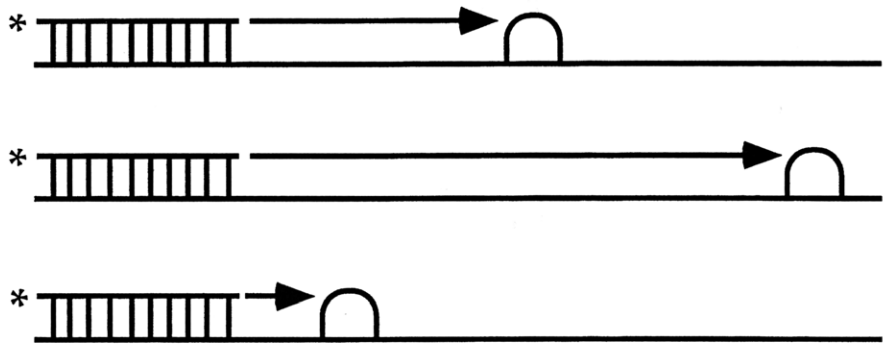
[c] nd: Not determined.

Another report reveals, however, that in vitro DNA-binding assays are insufficient to predict platinum antitumor activity [15]. Primer extension (Figure 1.1) was used to identify specific adducts formed by platinum complexes on DNA in HeLa cells. The DNA adduct profile correlated well with in vivo antitumor activity for *cis*- and *trans*-DDP, [Pt(en)Cl_2], and two acridine-tethered platinum complexes. When the complexes were allowed to react with purified DNA in solution, there were no substantial differences in adduct profiles between active and inactive compounds. This result demonstrates that cell-based assays can be better predictors of in vivo activity than in vitro assays, particularly when the in vitro screen does not require a unique, mechanism-based molecular interaction.

A. Isolate the DNA from platinum-treated cells.



B. Denature the DNA and PCR-amplify a specific gene with a radioactively labeled primer. Platinum adducts block the DNA polymerase.



C. Resolve the fragments on a denaturing gel.
Run sequencing lanes to identify the specific platinum adducts.

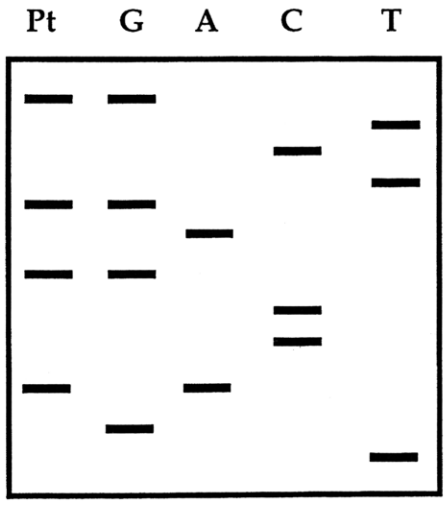


Figure 1.1: Identifying platinum-DNA adducts by primer extension.

Proteins that recognize cisplatin-modified DNA may effect the cytotoxicity of cisplatin [16]. In particular, high-mobility group (HMG) domain proteins, which specifically bind 1,2-intrastrand platinum-DNA cross-links, can mediate cisplatin cytotoxicity by shielding the adducts from repair. One strategy for screening potential platinum drugs, therefore, would be to evaluate a compound based on its ability to form DNA adducts that bind specifically to HMG-domain proteins. This approach was evaluated by screening a mixture of platinum-amino acid complexes for their ability to bind DNA and, subsequently, HMG1 in a gel mobility shift assay [8]. The mixture was prepared by combining potassium tetrachloroplatinate with two equivalents of an equimolar mixture of 17 amino acids in water; the pH was maintained at 6 and, after several days, the product mixture was evaluated without purification. The mixture was combined with DNA to form platinum adducts, as confirmed by atomic absorption spectroscopy. As illustrated in Figure 1.2, the mixture contained components that shifted the platinated DNA upon addition to HMG1 in a gel shift assay.

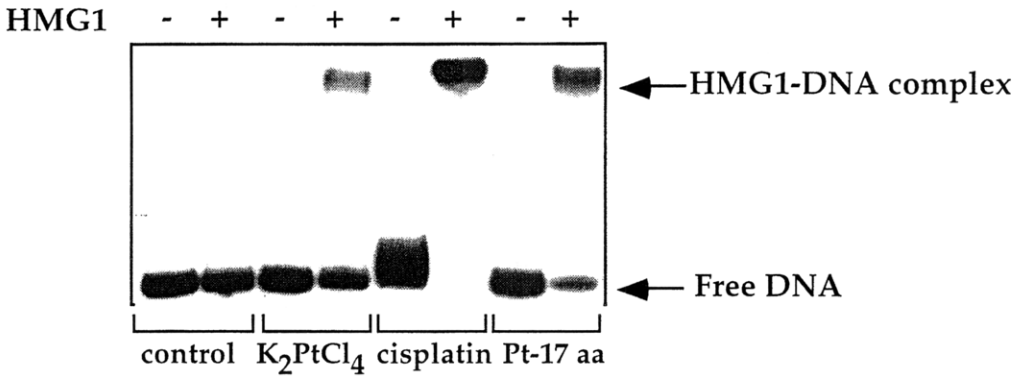


Figure 1.2: Native polyacrylamide gel demonstrating the specific binding of HMG1 to a radiolabeled, platinated 123-bp DNA fragment. The DNA was modified with platinum compounds or mixtures as indicated.

In order to identify the platinum complex or complexes responsible for this behavior, a sublibrary synthesis and screening approach was employed. Five pools of four amino acids each were used to prepare new mixtures to be screened by the gel shift assay. After three iterations of sublibrary synthesis and screening, the platinum-lysine complex Kplatin, shown in Figure 1.3, was selected. Kplatin has in vitro DNA-binding properties similar to those of cisplatin. Its toxicity towards HeLa cells, on the other hand, is almost 100-fold lower than that of the parent compound. Subsequent studies with analogous (N, O)-chelated platinum-amino acid complexes further revealed that

the ability of a platinum compound to form a ternary Pt-DNA-HMG1 complex does correlate well with its toxicity towards HeLa cells [17].

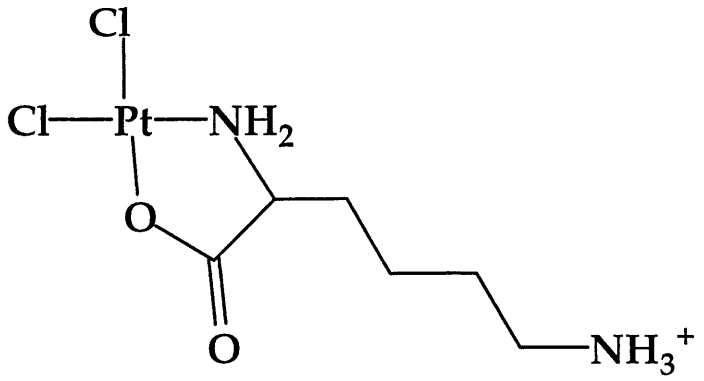


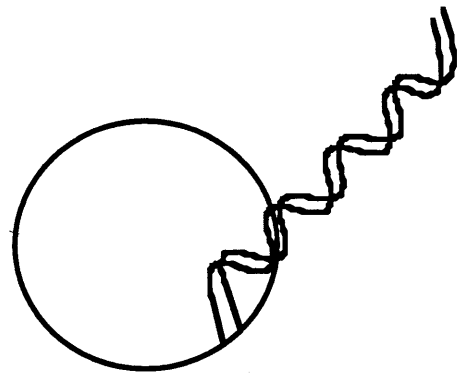
Figure 1.3: Structure of dichloro(L-lysine)platinum(II), or Kplatin.

Although the platinum-amino acid complexes do not show much promise as cytotoxic agents, these results demonstrated the utility of in vitro screening methods to survey the DNA-binding properties platinum compounds in a combinatorial manner. Assuming that HMG-domain proteins are involved in the cisplatin mechanism of action, then screening based on the Pt-DNA-HMG1 complex formation would be mechanism-based. The gel shift assay, however, is neither fast nor convenient, so alternative protein-DNA binding assays are required to adapt the method for high-throughput screening.

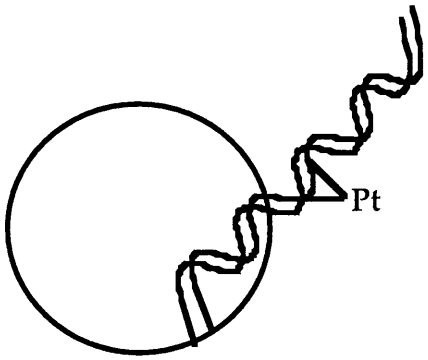
A better approach would be to screen the molecular libraries on solid phase supports [6,10,11]. Solid phase methods offer several advantages, allowing compounds to be identified by immobilization and position in a binding assay. Solid-phase screening can also be performed with the aid of robotics to increase throughput [7]. As indicated in Figure 1.4, a fluorescently labeled HMG-domain protein would facilitate the search for Pt-DNA-HMG binding by solid phase methodologies.

Accordingly, the Pt-DNA-HMG binding screen was implemented in the solid phase by constructing a fusion of HMG1 with the green fluorescent protein (GFPuv) [17]. As illustrated in Figure 1.5, the fusion protein HMG1-GFPuv binds specifically to cisplatin-modified DNA covalently attached to a nylon membrane. The binding is easily monitored by using a fluorescent microplate reader. The fluorescence readout, corresponding to bound HMG1-GFPuv, correlates well with cytotoxicity for a series of (N,O)-chelated platinum-amino acid complexes. The solid phase Pt-DNA-HMG1 binding method, or similar methods using other proteins that recognize platinated

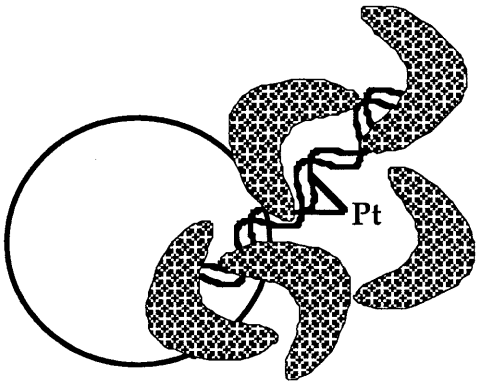
DNA, could be adapted to a high-throughput setting for the purposes of mechanism-based screening of potential platinum antitumor drugs.



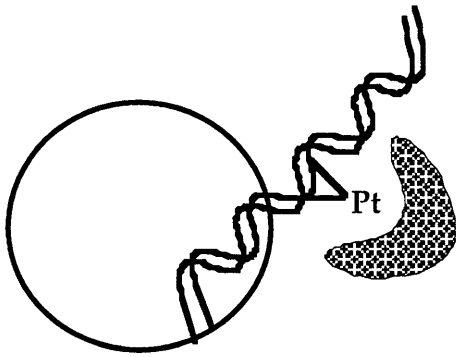
A. Covalently link DNA to solid support.



B. Allow platinum complex to react with DNA.



C. Add fluorescently labeled HMG-domain protein.



D. Wash away unbound protein and measure fluorescence.

Figure 1.4: A solid-phase approach to screening platinum compounds for specific Pt-DNA-HMG interactions.

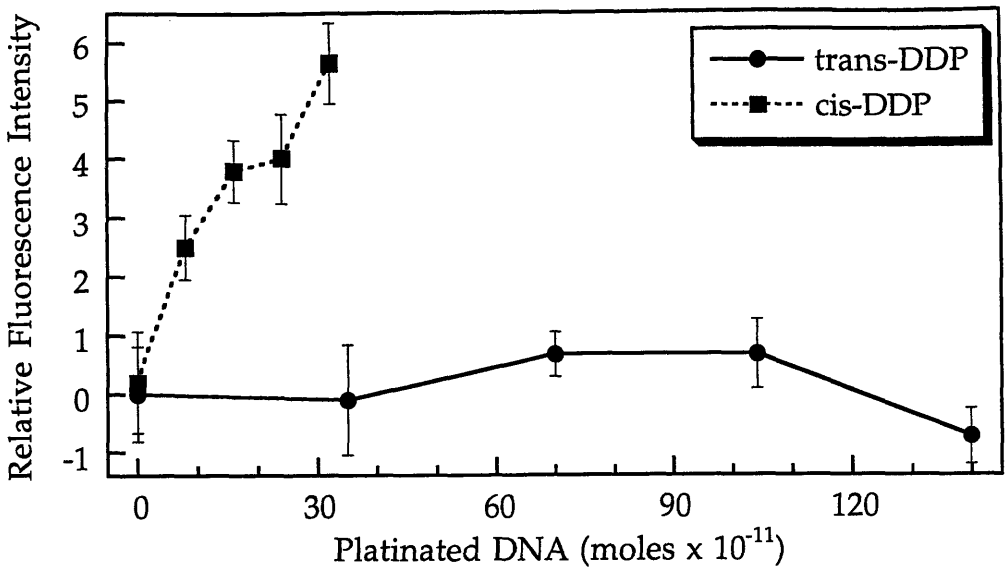


Figure 1.5: Results of the solid-phase assay for Pt-DNA-HMG1 binding. A 19-base pair DNA duplex was modified with *cis*- or *trans*-DDP and covalently linked to a nylon membrane. The membrane was incubated with HMG1-GFPuv and washed extensively. Bound protein was determined by measuring fluorescence retained on the membrane.

1.3 Cell-based assays

Cell-based assays, particularly those using human tumor cell lines, provide a relatively fast and inexpensive way to assess the in vivo activity of new compounds. The success of a cell-based assay depends in part on the nature of the chosen cell line. The NCI anticancer drug screening program tests compounds against a panel of 60 human tumor cell lines [18]. For practical reasons, individual laboratories have generally selected only a few relevant cell lines for screening. There is no general agreement about which cytotoxicity assay is the best predictor of anticancer activity.

In a clonogenic, or colony counting, assay, cells are treated with a prospective anticancer compound, and their viability is determined by measuring the resulting colonies. The clonogenic assay is widely used, partly because the ability to prevent cell division is taken as an essential property of anticancer drugs [18]. The disadvantage of the clonogenic assay is that it is time- and labor-intensive. It often takes more than one week for visible colonies to form, and manual colony-counting is quite tedious [19]. Although computer-assisted colony counting methods are available, the clonogenic assay is not particularly suited to high-throughput screening. Aside from time considerations, in order to see subtle differences in percent survival, at least 100 cells

must initially be seeded in an area large enough for individual colony formation. Thus, the clonogenic assay cannot be miniaturized to the extent necessary for conveniently screening large libraries of platinum compounds.

Several rapid, convenient cytotoxicity assays have been developed as alternatives to the clonogenic assay. The neutral red assay measures uptake of a dye into cells, with only viable cells binding the dye [20]. The microtetrazolium (MTT) assay correlates cell count with the reduction of the yellow tetrazolium salt to purple formazan, a reaction which occurs in the mitochondria of viable cells only [19,21]. Sulforhodamine B (SRB), which is used in the NCI anticancer drug screening program, is an anionic aminoxanthene dye that measures cell count by binding to cellular proteins [21]. In one study MTT and SRB assays yielded quite similar cytotoxicity profiles for cisplatin and other anticancer drugs in seven human tumor cell lines [21].

All three of the foregoing rapid cytotoxicity assays are suited to high-throughput measurements by microplate reading, but it is necessary to wait several days following treatment in order to measure cell viability. Moreover, these assays measure short-term, 2-3 day, delays in cell growth, rather than the inhibition of cell division. In one report, there was little correlation between results of the MTT and colony counting assays for non-small cell lung cancer cell lines treated with mitomycin C or cisplatin [18]. In another, very good correlation among the SRB, MTT and colony counting assays was observed for 16 human ovarian carcinoma cell lines treated with cisplatin [19].

Screening platinum complexes for cytotoxicity is not a mechanism-based approach. Another cell-based screening method [22] is predicated on the observation that mismatch-repair-deficient cells are less sensitive to cisplatin than wild type cells [22,23]. A review of the role of mismatch repair proteins in cisplatin cytotoxicity reveals that tumors resistant to cisplatin sometimes have mutations in one or more genes encoding for such proteins [16]. A mismatch repair-deficient cell line constitutively expressing GFP was mixed with a repair-proficient line and the heterogeneous population treated with DNA-damaging agents. Five days following exposure, both cisplatin and carboplatin enriched the GFP-expressing mismatch repair-deficient cell population. The potential cytotoxicity of any platinum compound might therefore be assessed by measuring the enrichment of GFP-expressing, mismatch repair-deficient cells in the population. This method has a direct fluorescent readout that could be

readily adapted to high-throughput conditions, but the 5-day delay necessary to see population enrichment cannot be shortened. Because the method is based on a proposed mechanism of resistance to platinum antitumor drugs, it is an advance over methods that merely measure cell death.

Another, related method [24] uses a HeLa Tet-on cell line stably transfected with the enhanced green fluorescent protein (EGFP) gene under the control of the tetracycline-responsive element (TRE). Upon induction with doxycycline, the cells express EGFP. Treatment with cisplatin and other effective platinum complexes caused a dose-dependent decrease in EGFP expression. Treatment with *trans*-DDP and other DNA-damaging agents led to a sharp increase in EGFP expression. Figure 1.6 shows typical results of a 13.5-hour co-treatment of HeLa cells with platinum and doxycycline, and Figure 1.7 plots platinum cytotoxicity versus EGFP inhibition. The method exploits the apparent difference in cellular response to cisplatin adducts compared to other forms of DNA damage. Although the somewhat low levels of EGFP expression require cell lysis in order to quantitate fluorescence, with the use of improved fluorescent reporter gene systems and better detection methods this approach could be adapted for high-throughput work. The advantage of the EGFP induction assay is speed; the results can be obtained overnight.

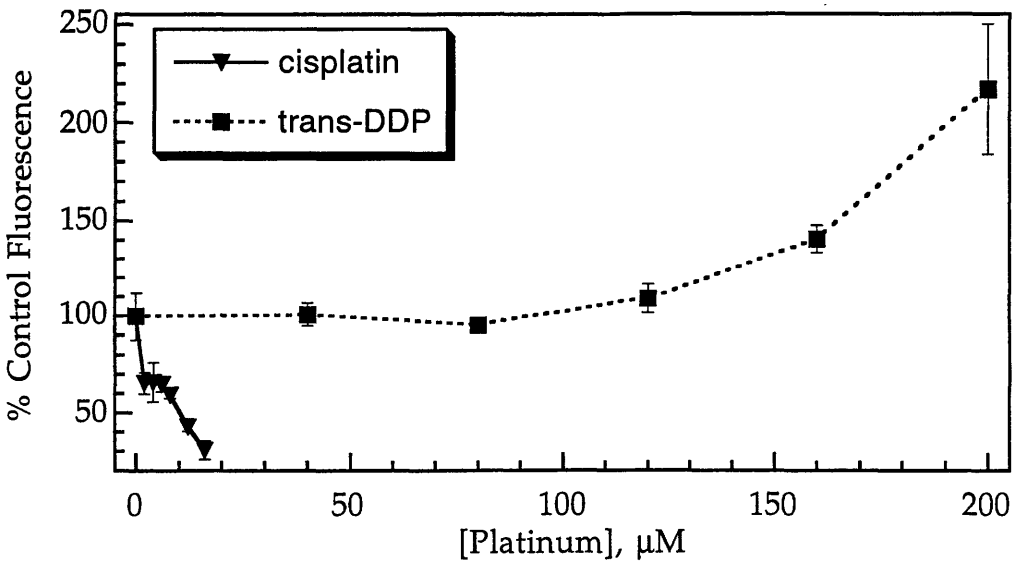


Figure 1.6: Effect of a 13.5-hour *cis*- or *trans*-DDP treatment on doxycycline-inducible EGFP expression in HeLa Tet-on cells. The fluorescence values were divided by total protein to correct for variations in cell count.

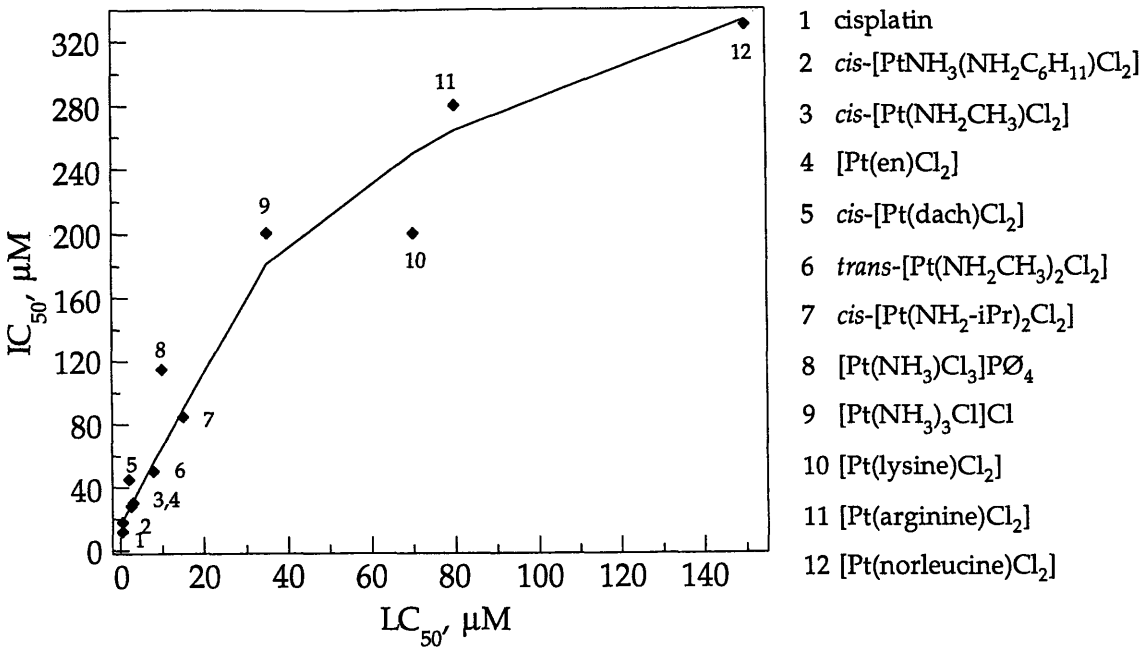


Figure 1.7: IC_{50} versus LC_{50} values for HeLa cells treated with a series of platinum complexes. LC_{50} was determined by a colony counting assay. IC_{50} was the platinum concentration at which EGFP expression was reduced to 50% of control.

Another reporter gene assay has also been used to screen platinum compounds. The fluorescent compound CCF2-AM, depicted in Figure 1.8, emits green fluorescence when intact but blue light upon cleavage with β-lactamase [25]. The compound is readily taken up by cells and, because of the enzymatic amplification, very low concentrations of the β-lactamase reporter gene can be detected. Figure 1.9 reveals that *cis*-DDP, to a much greater extent than *trans*-DDP, inhibits inducible β-lactamase expression as measured by CCF2 cleavage in a Jurkat cell line. This assay is performed in an automated high-throughput setting, and the results can also be obtained overnight [24].

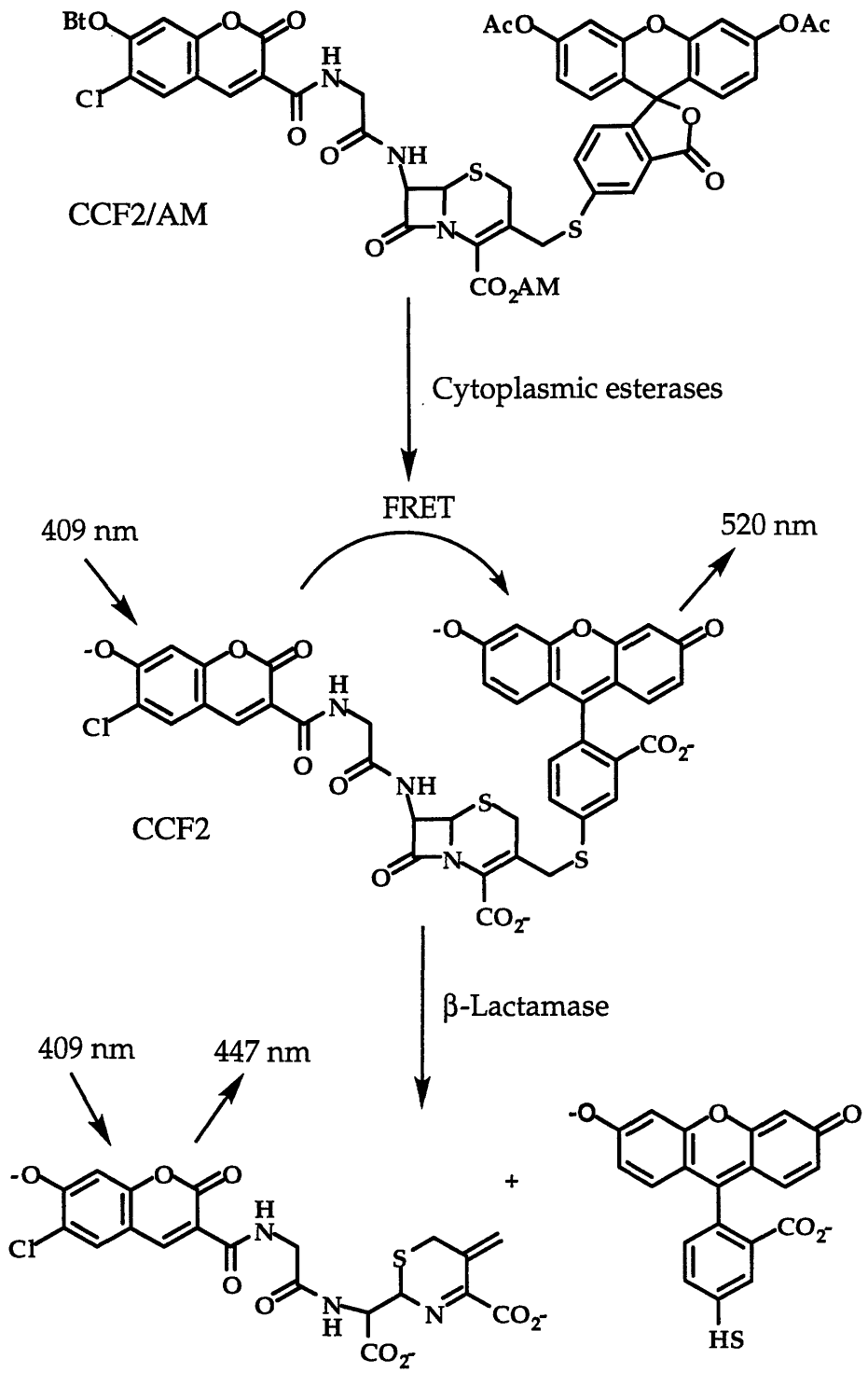


Figure 1.8: Cleavage of the fluorescent reporter CCF2-AM in cells expressing β-lactamase.

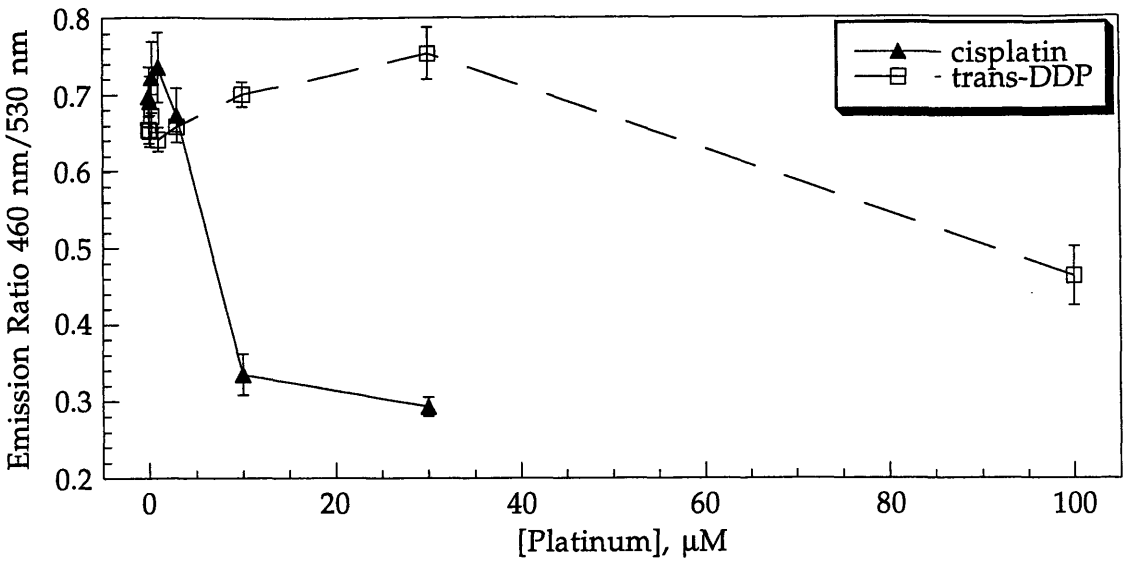


Figure 1.9: Effect of a 20-hour *cis*- or *trans*-DDP treatment on carbachol-inducible β-lactamase expression in Jurkat cells. The gene expression was determined by measuring the cleavage of the fluorescent dye CCF2-AM.

These new reporter gene cell-based techniques represent the beginning of a new era in platinum drug screening in cells. Instead of merely measuring cytotoxicity, the new methods screen compounds for cellular effects specific to biochemical changes induced by platinum antitumor drugs. The methods are also convenient and readily adaptable to high-throughput settings. In both the mismatch repair and reporter gene induction assays, the major disadvantage is that stable cell lines expressing reporter genes must be constructed. Because it can take up to several months to obtain stably transfected cell lines, it is necessary to choose cell types judiciously before proceeding with screening.

1.4 In vivo screening methods

Animal models are frequently employed to assess the antitumor activity of new platinum compounds. Often, mouse tumors such as mammary, lung or colon carcinomas are used. In other cases human solid tumor xenografts are grown in immune-deprived mice for drug screening. Although it can be argued that the efficacy of a compound against tumors in mice may not predict its activity in humans, the pharmacokinetic and toxicity properties of a new compound must be evaluated in animals before proceeding with human clinical trials [26].

Although animal models can provide important information regarding the bioavailability and pharmacology of potential anticancer drugs in mammals, they are

not always accurate predictors of activity against human tumor cells. In one report [27], the activity of a series of isomeric [1,2-bis(difluorophenyl)ethylenediamine]dichloroplatinum(II) compounds was evaluated in MXT murine mammary carcinomas in vivo; the same compounds were also tested against several human cell lines in culture. The in vivo screen revealed a 2,6-difluorosubstituted compound to be the most active, whereas the 2,4-difluorosubstituted compound was most active against the human breast cancer cell lines. It was concluded that the mouse mammary carcinoma is not an appropriate model for human breast cancers. Extreme caution must be employed when animal tumor results are used to predict activity in human tumors.

Xenografts in mice of human cancer cell lines can serve as good models for human tumors. In one report [28], a panel of eight human ovarian carcinoma cell lines and companion xenografts were used to screen cisplatin, carboplatin, tetraplatin and iproplatin. Overall, there was good correlation between the cell-based and xenograft assays. This report not only demonstrates that xenografts can be good models for human tumors, but also that human cell line assays can predict the activity of a platinum compound approximately as well as xenograft studies. In most cases, it would seem prudent to use human cell line assays as preliminary screens of platinum compounds before moving on to animal studies.

Although it is essential to test promising compounds in mice and other animal models prior to human trials, it is economically, ethically and often scientifically preferable to use cell-based and in vitro approaches to eliminate inactive compounds before commencing animal trials. Clearly, animal models are not an appropriate screen for combinatorial libraries of platinum complexes; they should be used to study further the promising leads identified by high-throughput methods.

An alternative in vivo approach to screening platinum complexes was recently described [29]. A series of diamine platinum(II) complexes was applied to cucumber or maize roots; the root growth inhibition indicated the cytotoxicity of the compound. Because the root growth inhibition results correlated with the antitumor activity of the complexes in mice, it was proposed that plant roots would afford inexpensive and rapid screens for platinum compounds. Although the plant-based screening method is unique and inexpensive, it is not an ideal approach to identifying platinum drugs to treat human cancers. Given that even the selection of human tumor cell lines can affect the results of a cytotoxicity assay, it seems unlikely that any plant cell line would

suitably model human cancer. This method could be used as a very preliminary screen for general cytostatic activity, but it is more laborious than any of the high-throughput cell-based assays; the root length must be measured by hand daily for three days.

1.5 Conclusions

Although the field of medicinal chemistry has been revolutionized by the advent of combinatorial synthesis and high-throughput screening, application of these methodologies for platinum drug discovery and lead optimization is in its infancy. In part, new methods for inorganic combinatorial synthesis must be devised. This process can be accelerated by exploiting the recent advances in organic small-molecule combinatorial chemistry. Also, new mechanism-based methods for screening platinum compounds must be developed with a focus on speed and automation. The three reporter gene assays described in this chapter [22,24], as well as the Pt-DNA-HMG1 binding assays [8,17], represent the beginning of a new trend toward mechanism-based screens for platinum compounds. It would be especially useful to have more solution- or solid-phase in vitro approaches to predicting platinum cytotoxicity. In order to develop screening methods, however, a greater understanding of the biochemistry of platinum antitumor activity is essential. When there is compelling evidence for the role of a specific platinum-DNA-protein interaction in platinum cytotoxicity, for example, then a high-throughput, mechanism-based screening method will surely follow.

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Chapter 2

A Mechanism-Based, Solution Phase Method for Screening Combinatorial Mixtures of Potential Platinum Anticancer Drugs*

2.1 Introduction

Cisplatin is used to treat testicular and other tumors. Its mechanism of action is not entirely understood, but it is generally accepted that the drug binds primarily to DNA, inhibiting replication and transcription and triggering programmed cell death [1]. The major cisplatin-DNA adducts are the 1,2-d(GpG) and -d(ApG) intrastrand cross-links, which bend and partially unwind the double helix [2,3]. An x-ray crystal structure of duplex DNA containing the major d(GpG) cisplatin adduct was recently obtained at 2.6 Å resolution [4]. Two solution NMR structures of duplex DNA containing the major d(GpG) adduct were also reported [5,6]. The resulting altered structure serves as a recognition site for the binding of cellular proteins, many of which contain one or more high-mobility group (HMG) domains [7]. Results from our laboratory and others indicate that most HMG-domain proteins bind specifically to cisplatin-modified DNA [8-15], further bending the duplex [16,17]. The platinum-DNA-protein complex could in principle interfere with the natural function of the HMG-domain protein [11]. There is convincing evidence that HMG-domain proteins can block the excision repair of the major cisplatin adducts in vitro [18,19]. Moreover, studies in *Saccharomyces cerevisiae* have demonstrated that Ixr1, an HMG-domain

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* This chapter has been published in a slightly altered form in Journal of Biological Inorganic Chemistry **1998**, *3*, 74-80.

protein, mediates the sensitivity of yeast to cisplatin and that this property is a consequence of shielding of the adducts from excision repair [10,20]. This repair shielding mechanism is supported by a recent report that overexpression of HMG2 sensitizes the lung adenocarcinoma cell line PC-14 to cisplatin [21].

Because the significance of HMG-domain proteins in mediating cisplatin cytotoxicity is supported by several lines of experimental evidence, we decided to use the binding of HMG1 to platinated DNA as a method to screen new platinum compounds for cytotoxic and, ultimately, anticancer activity. To initiate this work, a pool of seventeen naturally occurring amino acids was chosen. Platinum-amino acid chemistry has been extensively investigated [22-24]. Amino acids coordinate to platinum through nitrogen, oxygen and sulfur donors, and a single amino acid can bind to platinum(II) in a monodentate, bidentate or tridentate fashion. The binding of more than one amino acid to a single platinum atom and the potential formation of geometric isomers further contribute to the molecular diversity of this class of compounds. *A priori* it seemed likely that some platinum-amino acid complexes would react with DNA to induce favorable interactions with HMG-domain proteins and therefore be potential antitumor drug candidates. There have been only a few studies of the antitumor properties of platinum-amino acid compounds [25-32]. A comprehensive investigation of the potential activity of this class of compounds has not been carried out.

Since it is laborious to screen hundreds of individually prepared platinum-amino acid complexes for their ability to induce DNA-HMG domain binding, we applied a molecular diversity approach to search for appropriate candidates. There have been several reports of screening mixtures of peptides for metal-binding activity [33-36] but to our knowledge, there have been no published reports of the screening of pools of metal complexes for biological activity. Molecular diversity approaches to drug discovery often apply parallel solid-phase syntheses of large numbers of organic compounds, followed by the simultaneous screening of all of the compounds for the desired activity [37-39]. An alternate approach involves the solution-phase synthesis and screening of a large mixture of compounds, followed by several rounds of sub-mixture synthesis and screening to identify active components [40-42].

Because platinum-amino acid compounds can be prepared easily in aqueous solution, mixtures of the complexes are well-suited to solution-phase synthesis. The current understanding of the mechanism of action of platinum antitumor drugs

underscores the importance of the ternary platinum-DNA-HMG-domain interaction for anticancer activity. We thus employed a solution-phase, mechanism-based method to screen platinum-amino acid compounds for the in vitro markers of anticancer activity. In the first phase of the screening, a pool of platinum-amino acid complexes was formed and used to modify DNA in aqueous solution. This pool was then screened by the gel electrophoretic mobility shift assay to detect Pt-DNA-HMG-domain binding. In the second phase, sub-mixture synthesis and screening were employed to identify, by the same assay, the compounds present in the ternary complex.

2.2 Experimental

Cisplatin was obtained as a gift from Johnson-Matthey. Deionized water, purified with a MilliQ system (Millipore), was used for all aqueous procedures. Recombinant rat HMG1 [8], HMG1 domain B (HMGdomB) [43], HeLa cell-free extract [44], recombinant mouse testis-specific HMG [14,45] and a 3'-end labeled 123-bp DNA fragment [43] were prepared as described. The DNA was radiolabeled after modification by platinum.

2.2.1 Physical Measurements.

Electronic spectra were recorded on a Varian Cary 1E UV-visible spectrophotometer. Infrared spectra were measured by using KBr pellets on a BioRad FTS-7 FT-IR 3200 spectrophotometer. ^{195}Pt NMR spectroscopy was performed on a Varian VXR-300 spectrometer, and referenced to K_2PtCl_4 (0.1 M in 1 M HCl) as an external standard at δ -1624. Platinum concentrations were determined with a Varian atomic absorption spectrophotometer AA-1475, equipped with a graphite tube atomizer GTA-95. Standard solutions of potassium hexachloroplatinate(IV) were used to calibrate the instrument. High performance liquid chromatography was performed with a Perkin Elmer Series 4 liquid chromatograph, equipped with an LC-95 UV/visible spectrophotometer detector set to 260 nm. MALDI-TOF mass spectra were obtained by C. Costello and K. Tang (Boston University Medical School Mass Spectrometry Resource) using a Finnigan MAT Vision 2000 instrument operated in the positive ion reflectron mode, at 5 kV accelerating voltage, with 4 kV postacceleration at the detector. The detector was equipped with a 337 nm LSI nitrogen laser, and the 3-hydroxypicolinic acid matrix was doped with ammonium citrate. X-ray

crystallography was performed by Dr. Peter Fuhrmann on a Siemens SMART/CCD X-ray diffractometer with Mo Kα radiation (λ = 0.71073 Å). The structure was solved by direct methods and refined on F^2 by using the SHELXTL software package.

2.2.2 Synthesis of Mixtures of Platinum-Amino Acid Complexes.

In a typical procedure, an 80 mM aqueous solution of K_2PtCl_4 was prepared, and an equimolar mixture of L-amino acids (Sigma) was added to yield a 160 mM total amino acid concentration. The solution was stirred at room temperature for 5-7 days; sodium hydroxide solution was added 4-6 times daily to maintain a pH of 6. The reactions were terminated by freezing the solutions when the pH was stable at 6 for 12 hours. The product mixtures were used without purification.

2.2.3 Preparation of Kplatin.

An aqueous solution of 160 mM L-lysine and 80 mM K_2PtCl_4 was stirred at room temperature. The pH of 6 was maintained by the addition of sodium hydroxide solution several times daily, and after one week the solution was cooled to 0° C to precipitate the yellow product. The precipitate was recrystallized from water by slow evaporation. FTIR (cm^{-1}, KBr pellet): 3242, 3189, 3125, 1643, 1613, 1514. UV-vis (λ_{max}, nm, H_2O): 290 sh, 325, 391 sh. ^{195}Pt NMR (δ, H_2O): -1634. Anal. Calcd for $PtCl_2C_6O_2N_2H_{14}\cdot 2.5\ H_2O$: C, 15.76; H, 4.19; N, 6.13. Found: C, 15.53; H, 3.36; N, 6.01.

2.2.4 Modification of DNA with Platinum Compounds.

The 123-bp DNA was dissolved in 10 mM sodium phosphate, pH 6.8, at a nucleotide concentration of 60 μM. Platinum compounds or mixtures were dissolved in water and cleared by centrifugation at 10,000 x g; the resulting platinum solution was added to DNA at the appropriate r_f (formal platinum: nucleotide ratio) and the reaction was incubated at 37°C for 14 h. The DNA was precipitated with ethanol and the pellet was washed to remove unbound platinum. The r_b (bound platinum: nucleotide ratio) was determined by atomic absorption and UV-visible spectrophotometry.

2.2.5 Modification of a Pentadecanucleotide with Kplatin.

A 15-mer, d(CCTCTCTGGTTCTTC), was modified with cisplatin, purified and 5'-end-labeled as previously described [43]. To prepare a Kplatin-modified 15-mer, the

oligonucleotide was incubated with 1.2 equiv of Kplatin in water for 24 h at 37°C. The products were purified by anion-exchange HPLC on a Dionex Nucleopac PA-100 column (9 x 250 mm) with a solvent system of 25 mM ammonium acetate, pH 6.0, in 10% aqueous acetonitrile and a 30 min, 1 mL/min, gradient of 0.25-0.35 M NaCl. The products, designated 15K1 and 15K2, were eluted from the column at 12.28 and 13.29 min, respectively. The two products were extensively dialyzed against water in Spectra/Por 7 tubing, 1000 MW cutoff (Spectrum).

2.2.6 Gel Mobility Shift Assays.

A mixture of ^{32}P-labeled 123-bp DNA (10^4 cpm) with 2 μg of unmodified, unlabeled chicken erythrocyte DNA was diluted to a total volume of 10 μL in a buffer of 4% (v/v) glycerol, 10 mM magnesium chloride, 50 mM potassium chloride, 1 mM EDTA, 1 mM spermidine, 0.05% (v/v) Nonidet P40, 0.2 mg/mL bovine serum albumin, and 10 mM HEPES pH 7.9. HMG1 (1 μg) was present as indicated. Samples were incubated on ice for 20 min, followed by the addition of 0.5 μL of loading dye, yielding a final concentration of 5% (v/v) glycerol with 0.01% (w/v) bromophenol blue and 0.01% (w/v) xylene cyanol. Samples were loaded onto pre-run, pre-equilibrated (4°C) 6% native polyacrylamide gels (29:1 acrylamide:bis) and run in 45 mM Tris borate, 1 mM EDTA, pH 8.3 at 300 V for 3 h. After electrophoresis, gels were dried, visualized by autoradiography (Kodak X-Omat film) and quantitated by using a PhosphorImager (Molecular Dynamics). The mobility shift assay was performed on the 15-mer essentially as described [43]; the double stranded 15-mer was used at a final concentration of 10 nM.

2.2.7 Sequencing, Digestion and Extension of DNA Fragments.

A singly 5'-end labeled 82-bp DNA fragment was prepared from 123-bp DNA as previously described [43]. An aliquot of the 82-bp fragment was analyzed by Maxam-Gilbert sequencing. The remaining radioactive 82-bp fragment was combined with carrier calf thymus DNA and cisplatin (r_f 0.035) or Kplatin (r_f 0.06) in 10 mM sodium phosphate, pH 6.8. The reactions were incubated for 14 h at 37°C and precipitated with ethanol. The platinum-DNA adducts were characterized by T4 DNA polymerase digestion followed by sequenase extension [46]. The final products of the reactions

were dissolved in formamide loading dye and separated on a denaturing polyacrylamide gel.

2.2.8 Modified Western Blots.

Modified western blots were performed as previously described [47] with the following modifications. HMG1 and HeLa cell-free extract were separated on an SDS/PAGE 12% gel. Binding buffer was 30 mM HEPES-NaOH, pH 7.5, 10 mM $MgCl_2$, 0.25% (w/v) nonfat dry milk. Platinated or unplatinated ^{32}P-labeled chicken erythrocyte DNA (1 x 10^6 cpm; 100-300 pmol) was used as a probe; unlabeled, unmodified chicken erythrocyte DNA (10 μg/mL) was added as a competitor.

2.2.9 Cytotoxicity Assays Using HeLa Cells.

All tissue culture reagents were purchased from Gibco BRL. HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% certified fetal bovine serum. In preparation for the cytotoxicity assay, 200 cells in 4 mL of platinum-free media were distributed to each 6 cm tissue culture plate. The cells were incubated for 18-24 h and then treated with platinum solutions. Two or more plates were prepared for each data point. After a 24 h treatment, the media were gently aspirated and the cells were washed with phosphate-buffered saline. The cells were incubated in 4 mL of complete media for 7-10 days; the colonies were then stained with 0.1% methylene blue (Fluka) in 50% ethanol and counted.

A sample of Kplatin was submitted to the National Cancer Institute Developmental Therapeutics Program In-Vitro Screen to be tested against a panel of 60 human tumor cell lines.

2.2.10 Modification of DNA with Kplatin analogs.

The Kplatin analogs [Pt(Arg)Cl_2] or Rplatin, [Pt(N-acetyl-lysine)Cl_2] or NacKplatin, and [Pt(norleucine)Cl_2] or Norplatin were synthesized by Dr. Christopher Ziegler. For each platination reaction, 7 x 10^6 cpm of radiolabeled 123-bp DNA was combined with 1 μg of chicken erythrocyte DNA. The DNA was diluted in 200 μL of 20 mM sodium phosphate buffer, pH 6.8. The platinum complexes were dissolved in water and added at the appropriate r_f, and the reactions were incubated overnight at

37°C. The DNA was then precipitated with ethanol and redissolved in water. A set of nonradioactive reactions was run in parallel to measure r_b.

2.3 Results

2.3.1 Synthesis and Screening of Platinum-Amino Acid Mixtures.

The gel mobility shift assay was used to probe the ability of DNA modified with different pools of platinum amino acid complexes to bind to HMG1. A platinum-amino acid mixture composed of 17 L-amino acids was prepared; C, M and H were excluded in the initial screening because of their known ability to bind strongly to metal ions. Figure 2.1 demonstrates that some of the DNA modified with the reaction product(s) of $[PtCl_4]^{2-}$ with the 17-amino acid mixture bound specifically to HMG1. To identify the platinum complexes most responsible for this interaction, five platinum-amino acid sub-mixtures, each containing four randomly selected L-amino acids, were synthesized and assayed in a similar manner. Table 2.1 summarizes the results. The amino acid ligands in the sub-mixtures which induced the most specific protein-DNA binding were KFRQ and YSPG. An additional round of sub-mixture synthesis was then performed by using combinations of three amino acids derived from these two pools. Since the KFRQ sub-mixtures induced more specific DNA-HMG1 binding than the YSPG sub-mixtures, with pools containing K being the best (Table 2.1), the three lysine-containing sub-mixtures were further divided into two-amino acid sub-mixtures. The resulting compounds were very promising in their ability to modify DNA in such a manner as to cause HMG1 to bind selectively. The KK sub-mixture in particular, prepared from K_2PtCl_4 plus 2 equivalents of lysine, was comparable to cisplatin in its ability to form a ternary platinum-DNA-protein complex. From the platinum-lysine mixture, we recovered crystals of the active complex, $[Pt(Lys)Cl_2]$ hereafter referred to as Kplatin, a known compound [48,49] having the structure illustrated in Figure 2.2 and summarized in Table 2.2. Additional structural information is available as supplementary material at http://link.springer.de/link/service/journals/00775/supp/list98.htm.

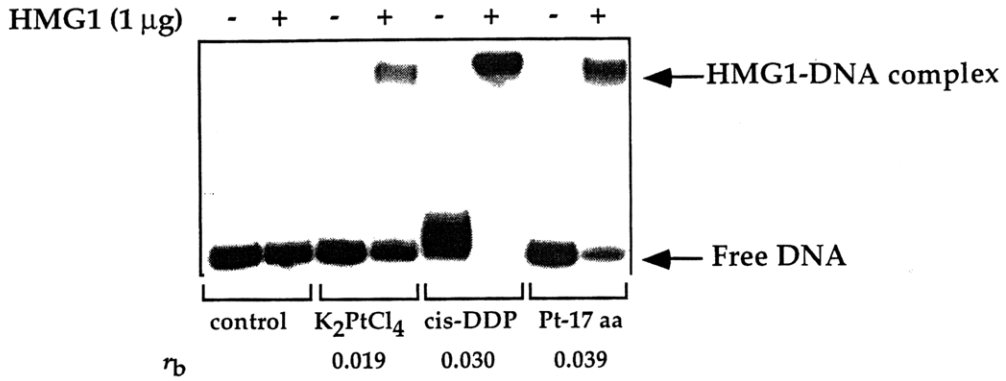

Figure 2.1: Gel mobility shift assay demonstrating the binding of HMG1 to a 123-bp DNA fragment modified with a platinum-amino acid library.

Table 2.1: Binding of HMG1 to 123-bp DNA Modified with Various Platinum Compounds and Mixtures.

Platinum compounds[a]	r_b	% Specific Binding[b,c]
none	0	0
K_2PtCl_4	0.019	1.7
17-aa mixture	0.039	45.7
cisplatin	0.030	74.2
none	0.000	0
WINC	0.043	<0
LMHD	0.066	<0
TVAE	0.012	1.9
KFRQ	0.030	17.4
YSPG	0.014	18.9
cisplatin	0.030	77.0
none	0.000	0
SPG	0.011	5.2
YSG	0.011	6.4
YPG	0.016	19.2
YSP	0.014	18.5
YSPG	0.014	11.9
Cisplatin	0.030	74.4
None	0.000	0
KFRQ	0.030	23.0
FRQ	0.051	25.4
KFR	0.024	30.5
KRQ	0.036	34.0
KFQ	0.041	52.3
cisplatin	0.030	77.6
none	0.000	0
KR	0.022	17.1
KQ	0.036	14.8
KFQ	0.026	28.4
KF	0.032	33.6
KK	0.045	44.5
cisplatin	0.021	54.4

[a] Platinum-amino acid mixtures are indicated by the one-letter code for the component amino acids.

[b] Using ImageQuant phosphorimager software, volumes were measured for each full lane (V_f) and for the upper portion (shifted band) of the lanes (V_u) where protein-DNA complexes were normally observed. % shift = $(V_u/V_f) \times 100\%$.

% binding = $(\%\ \text{shift})_{\text{sample + HMG1}} - (\%\ \text{shift})_{\text{sample-HMG1}}$.

[c] % specific binding = $(\%\text{binding})_{\text{sample}} - (\%\text{binding})_{\text{unmodified DNA}}$.

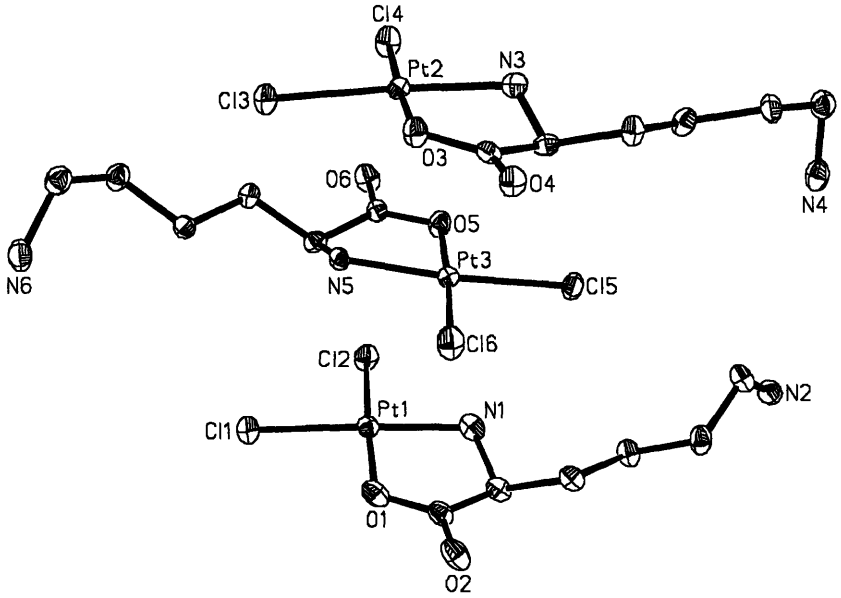

Figure 2.2: ORTEP of Kplatin showing the 50% probability thermal ellipsoids and atom labeling scheme for three molecules in the asymmetric unit.

Table 2.2: Crystal data and structure refinement for Kplatin.

Empirical formula	$C_6H_{14}C_{12}N_2O_2Pt$ and 8 H_2O per asymmetric unit
Formula weight	1380.67
Temperature	188(2) K
Wavelength	0.71073
Crystal system	orthorhombic
Space group	$P2_12_12_1$
Unit cell dimensions	a=9.7825(2) Å b=18.1650(3) Å c=21.9086(3) Å
Volume, Z	3893.14(12) $Å^3$, 12
Density (calculated)	2.356 g/cm^3
Linear absorption coefficient	11.228 mm^{-1}
Absorption correction	analytical
T_{min}/T_{max}	0.0936/0.3391
F(000)	2624
Crystal size	0.30 x 0.24 x 0.10 mm^3
Theta range for data collection	1.46° to 28.26°
Limiting indices	$-12\leq h\leq 12$, $-24\leq k\leq 14$, $-28\leq l\leq 28$
Reflections collected	23791
Independent reflections	8986 [R_i=0.0325]
Refinement method	Full-matrix least-squares on F^2
Data/restraints/parameters	8986/16/488
Goodness-of-fit on F^2	1.104
Observed reflections [$I>2\sigma(I)$]	8133
Final R indices [$I>2\sigma(I)$]	R1=0.0267, wR2=0.0529
R indices (all data)	R1=0,0327, wR2=0.0556
Absolute structure parameter	-0.006(6)
Largest diff. peak and hole	0.494 and –2.555 e/$Å^3$

2.3.2 *Stability of Kplatin in aqueous solution.*

To measure the effects of temperature and incubation time on the stability of Kplatin in aqueous media, a ^{195}Pt NMR study was performed. The results are summarized in Table 2.3. The major peak at δ -1634, corresponding to the $[Pt(Lys)Cl_2]$ species, and a secondary peak at δ-1410, corresponding to the $[Pt(Lys)Cl(H_2O)]^+$ species, were observed in all of the aqueous and phosphate-buffered solutions. The peak at δ -1057, corresponding to $[Pt(Lys)(H_2O)_2]^{2+}$, was not observed in any aqueous or phosphate-buffered solution. A subsequent study [50] indicated that the dichloride is also the major Kplatin species present after incubation in either DMEM or DMEM with 10% fetal bovine serum.

Table 2.3: ^{195}Pt NMR analysis of Kplatin solutions (49 mM).

Solvent	Temp (°C)	Time (h)	Percent[c] $[Pt(Lys)Cl_2]$ (δ –1634)	Percent $[Pt(Lys)Cl(H_2O)]^+$ (δ –1410)	Percent $[Pt(Lys)(H_2O)_2]^{2+}$ (δ –1057)
dH_2O	37	24	86.7	13.3	0
dH_2O	37	48	90.6	9.4	0
dH_2O	70	24	89.9	10.1	0
dH_2O	70	48	86.1	13.9	0
NaPi[a]	25	0	88.2	11.8	0
NaPi	25	24	90	10	0
NaPi	25	48	87.2	12.8	0
NaPi	37	24	89.6	10.4	0
NaPi	37	48	90.3	9.7	0
NaPi	55	24	91.5	8.5	0
NaPi	55	48	85.6	14.4	0
NaPi	70	24	90.5	9.5	0
NaPi	70	48	52.1	47.9	0
dH_2O + 1 equiv Ag[b]	25	24	36.4	63.6	0
dH_2O + 1 equiv Ag	70	24 (after 24 h activation)	39.7	60.3	0
dH_2O + 2 equiv Ag	25	24	0	29.6	70.4
dH_2O + 2 equiv Ag	70	24 (after 24 h activation)	16.9	25.0	58.1

[a] NaPi: 10 mM sodium phosphate buffer, pH 6.8.

[b] Kplatin solutions were activated by adding silver nitrate in water and incubating in the dark for 24 h at 25°C. The silver chloride precipitate was removed by centrifugation.

[c] Percentages were determined by approximate integrations of NMR peaks. Because the relaxation time of the ^{195}Pt nucleus is rapid and varies with ligand environment [51], these results should not be considered exact quantitations of Kplatin species.

2.3.3 Interaction of Kplatin with DNA.

To determine whether and how Kplatin modifies DNA, the complex was synthesized, purified, and allowed to react with pBR322 DNA at an r_f value of 0.09 for various time periods. After incubation, the platinated DNA was purified on G50 Sephadex and the r_b was measured by atomic absorption spectrophotometry. Figure 2.3 plots the amount of bound platinum per nucleotide as a function of time. After 24 h at 37°C, the reaction was complete and $r_b = r_f$. An exonuclease mapping analysis [52] using T4 polymerase [46] was then undertaken to determine the types of DNA adducts formed by Kplatin, the results of which are presented in Figure 2.4.

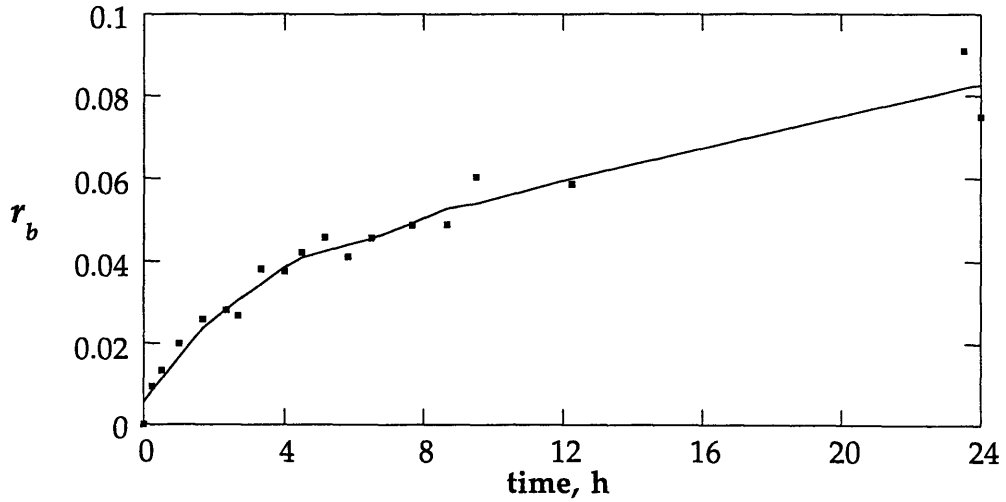

Figure 2.3: Time course of the reaction of Kplatin with pBR322 DNA. DNA (0.22 mM) was combined with Kplatin, r_f=0.09, and incubated in a 1 mM sodium phosphate, 3 mM sodium chloride buffer, pH 6.8, at 37°C. At the specified times, reactions were terminated by the addition of 0.2 M NaCl. Unbound platinum was removed by spin filtration through G50 Sephadex.

Because the 3' to 5' exonuclease activity of T4 DNA polymerase is inhibited at the site of bifunctional platinum adducts, bands appear in the gel corresponding to stop sites at the 3′ side of the lesions. The unmodified 82-bp DNA fragment has some intrinsic stop sites, but modification with either Kplatin or, in a control experiment, cisplatin resulted in the appearance of new stop sites on the sequencing gel. The major new fragments generated by cisplatin on the 82-mer correspond to the d(AGG) and d(GGAGG) sites; Kplatin induced stop sites at all of the d(AG) and d(GG) sites on the 82-mer. A different assay employing sequenase was used to test for the presence of interstrand cross-links [46]. Briefly, an interstrand cross-link would covalently tether

both strands of DNA after T4 polymerase digestion, allowing sequenase to extend them back to their full lengths. The sequenase extension step did not notably alter the appearance of any bands on the sequencing gel, however. Interstrand adducts therefore do not comprise a significant fraction of Kplatin lesions on DNA.

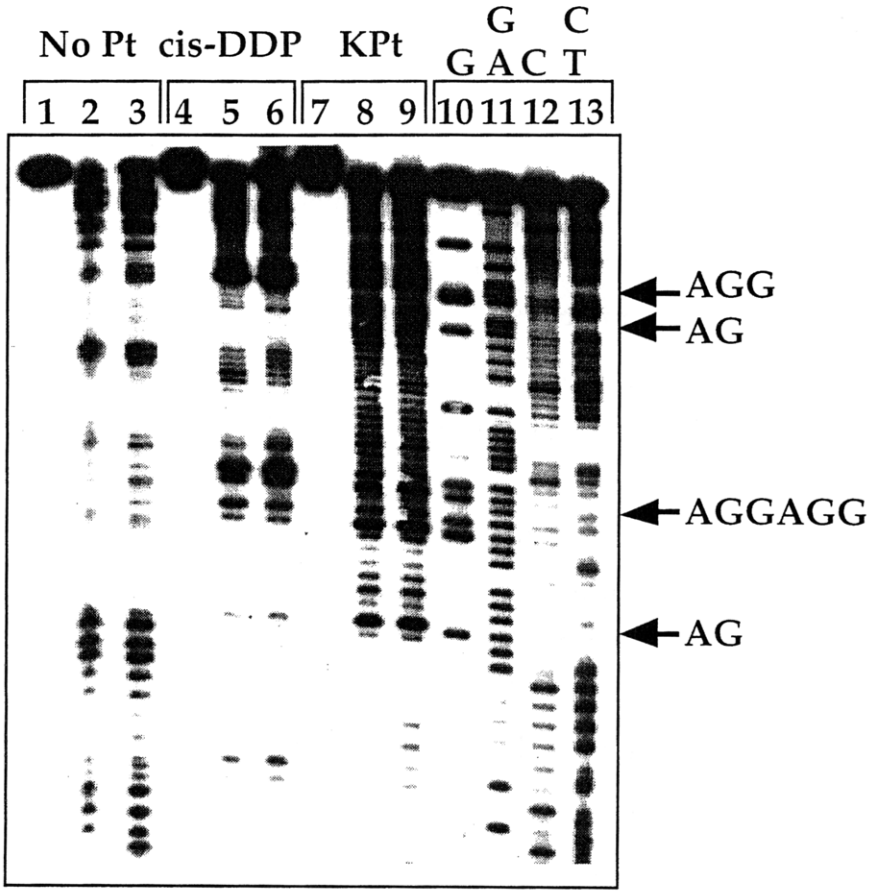

Figure 2.4: Denaturing 12% polyacrylamide gel revealing products of exonuclease digestion of a platinum-modified 156-bp DNA fragment. Lanes 2, 5 and 8: DNA digested by T4 DNA polymerase. Lanes 3, 6 and 9: DNA digested by T4 DNA polymerase followed by Sequenase extension. Lanes 10, 11, 12 and 13: Maxam Gilbert sequencing reactions performed on unmodified DNA.

Because the T4 DNA polymerase digestions indicated that Kplatin modifies d(GpG) and d(ApG) sites, the products of the reaction of Kplatin with a 15mer containing a single d(GpG) site were analyzed by ion exchange HPLC. The products were eluted from the column in two distinct peaks, 15K1 and 15K2. Maxam Gilbert sequencing of the oligonucleotides [53,54] confirmed that the two adjacent guanine sites on 15K1 and 15K2 were modified. MALDI-TOF mass spectrometric analysis gave *m/z* values of 4801 and 4809 for 15K1 and 15K2, respectively; the error for external calibration was ±0.1%. The mass spectrometric data therefore match the expected *m/z*

value of 4805 for an adduct formed by hydrolysis of the Kplatin chloride ligands followed by coordination of the resulting $\{Pt(Lys)\}^{2+}$ fragment to the N7 atoms of adjacent guanosines. It was expected that Kplatin would form two orientational isomers [55] at a d(GpG) site on DNA because of its asymmetry. In these isomers, the lysine side chain would be oriented toward either the 3′ or the 5′ end of the oligonucleotide.

2.3.4 Interaction of Kplatin-Modified DNA with HMG-Domain Proteins.

The binding of Kplatin-modified DNA to various HMG-domain proteins was assessed in gel mobility shift assays. Figure 2.5 shows that Kplatin-modified 123-bp DNA bound to HMG1 nearly as well as cisplatin-modified DNA and that the binding increased with increasing r_b. Similar results were observed with HMGdomB (Figure 2.6), tsHMG (Figure 2.7), and with DNA modified with Kplatin prepared from D-lysine (Figure 2.8). The binding of duplex 15K1 and 15K2 to HMGdomB was quantitated as shown in Table 2.4; the two isomers of Kplatin-modified DNA have approximately the same affinity for the HMG domain. A modified western blot (Figure 2.9) revealed the binding of cisplatin- and Kplatin-modified DNA to proteins present in a HeLa cell-free extract. Both cisplatin- and Kplatin-modified DNA bound to HMG1 and several other proteins in the cell-free extract. The control blot, probed with unmodified DNA, had no bands (data not shown). Although Kplatin was selected from combinatorial libraries based solely on its ability to induce the binding of DNA to HMG1, the compound is comparable to cisplatin in causing DNA to bind to other proteins.

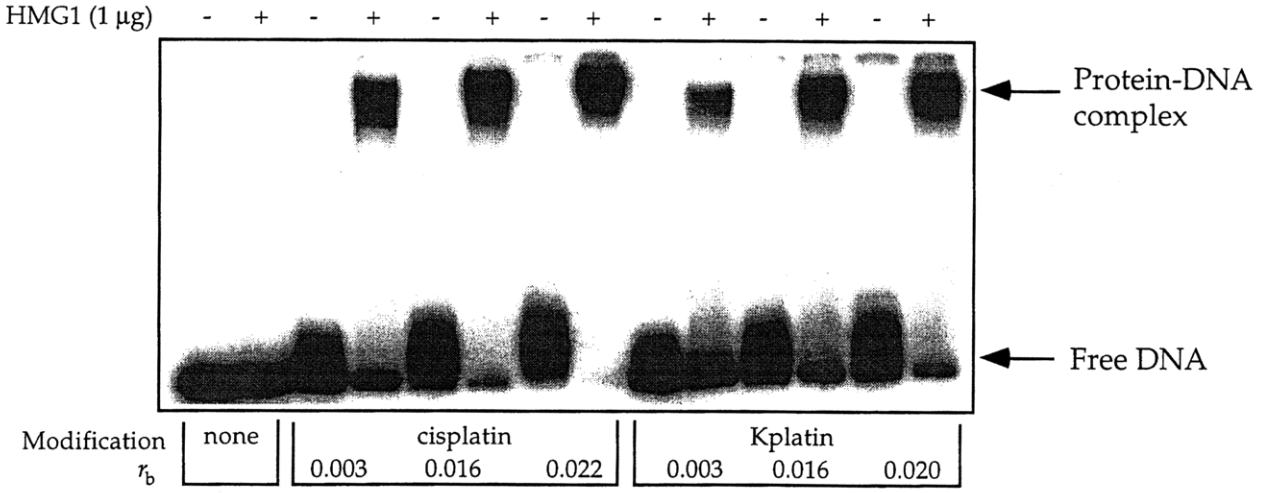

Figure 2.5: Gel mobility shift assay comparing the binding of HMG1 to 123-bp DNA modified with varying levels of cisplatin and Kplatin.

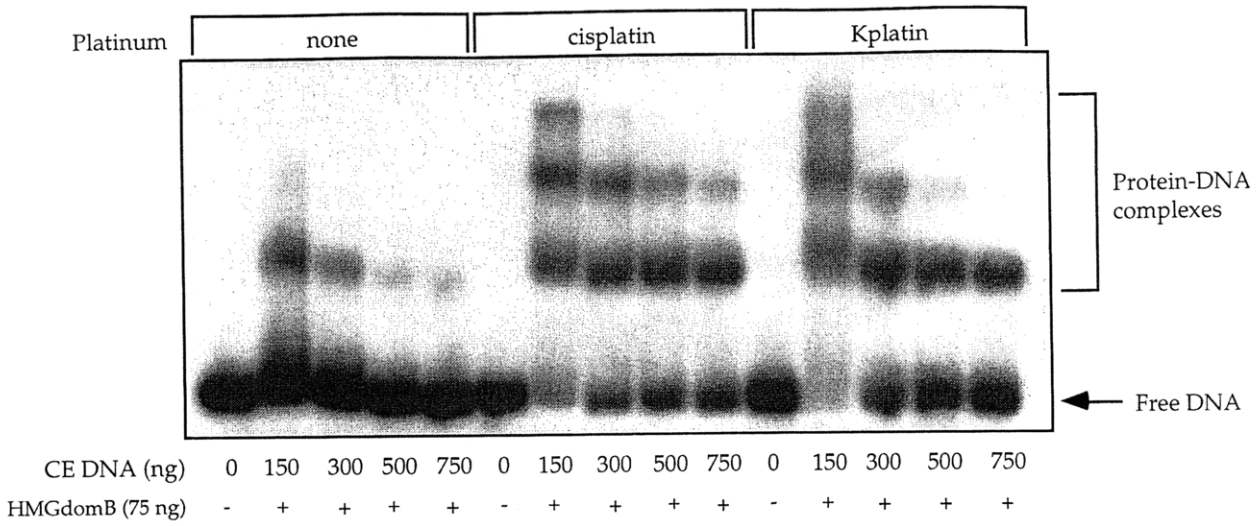

Figure 2.6: Gel mobility shift assay comparing the binding of HMGdomB to 123-bp DNA modified with cisplatin and Kplatin.

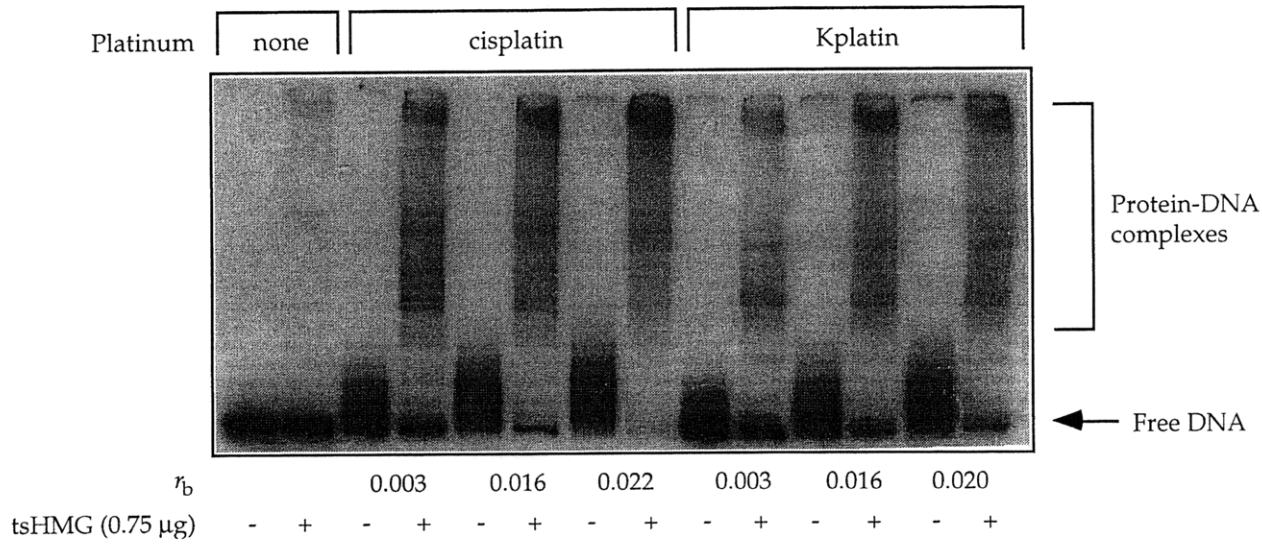

Figure 2.7: Gel mobility shift assay comparing the binding of tsHMG to 123-bp DNA modified with varying levels of cisplatin and Kplatin.

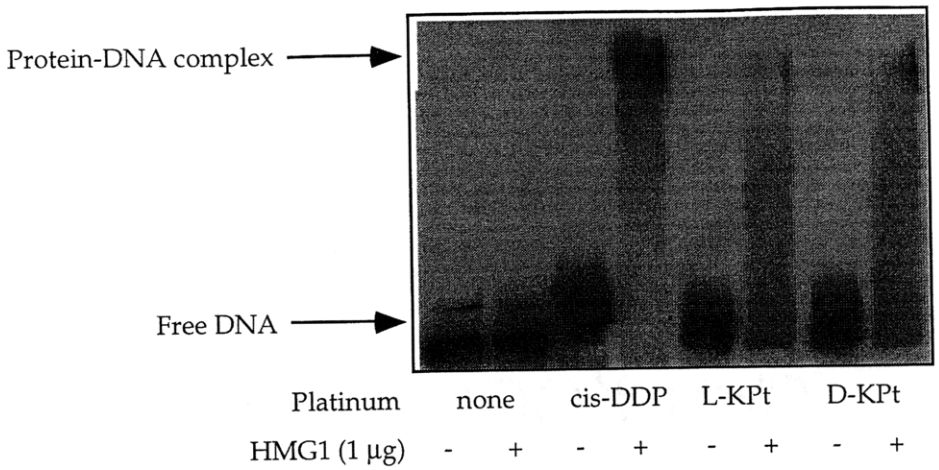

Figure 2.8: Gel mobility shift assay comparing the binding of HMG1 to 123-bp DNA modified with cisplatin, L-Kplatin and D-Kplatin.

Table 2.4: Binding of HMGdomB to Duplex 15mer Site-Specifically Modified with Cisplatin and Kplatin.

15mer	[HMGdomB], nM	% Specific Shift[a]
Cisplatin-modified	0	0
	50	36.8
	100	39.2
	400	44.4
15K1	0	0
	50	20.9
	100	29.6
	400	36.8
15K2	0	0
	50	24.9
	100	26.7
	400	34.9

[a] % specific shift=$(\%\ \text{shift})_{\text{sample}}$-$(\%\ \text{shift})_{\text{unmodified DNA}}$

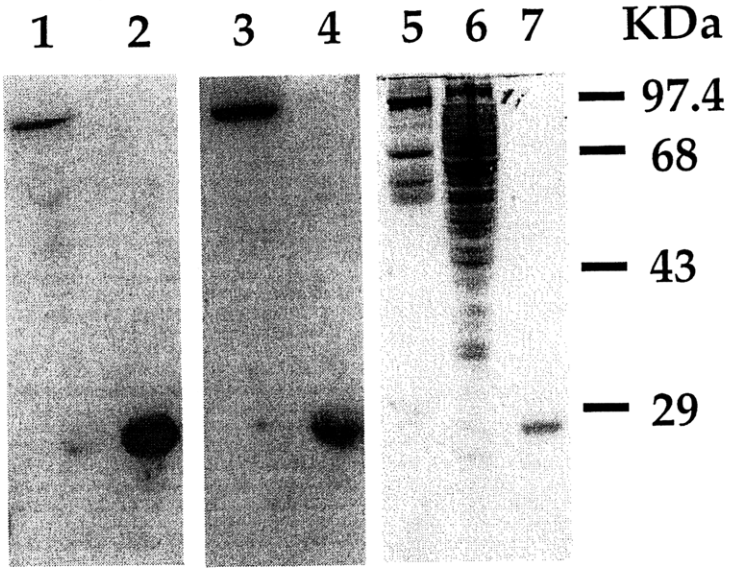

Figure 2.9: Modified western blot analysis of platinated chicken erythrocyte DNA binding to HeLa cell-free extract proteins. Lanes 1-4 are autoradiographs of the blots and lanes 5-7 are Coomassie-stained proteins separated by SDS-PAGE. Lanes 1-2 were probed with cisplatin-modified DNA, r_b=0.021. Lanes 3-4 were probed with Kplatin-modified DNA, r_b=0.016. Lanes 1, 3 and 6: HeLa cell-free extract. Lanes 2, 4 and 7: HMG1. Lane 5: molecular weight markers. A control blot using an unmodified chicken erythrocyte DNA probe revealed no nonspecific protein-DNA binding (not shown).

2.3.5 Kplatin Cytotoxicity Studies.

Because Kplatin induces the same protein-DNA interactions as cisplatin, it was anticipated that Kplatin would be cytotoxic. A 24-h treatment of HeLa cells with Kplatin did induce cell death, as shown in Figure 2.10. For a 24-h cisplatin treatments the average LC_{50} = 0.50 ± 0.05 μM; for Kplatin the average LC_{50} = 59.2 ± 7.8 μM, where LC_{50} is the dose that killed 50% of the treated cells. As Figure 2.11 demonstrates, similar results were obtained when HeLa cells were treated with Kplatin prepared from D-Lys, although the stocks of HeLa cells used in the experiments differed in overall platinum sensitivity. Additionally, cisplatin-resistant HeLa cells [56] exhibited cross-resistance to Kplatin.

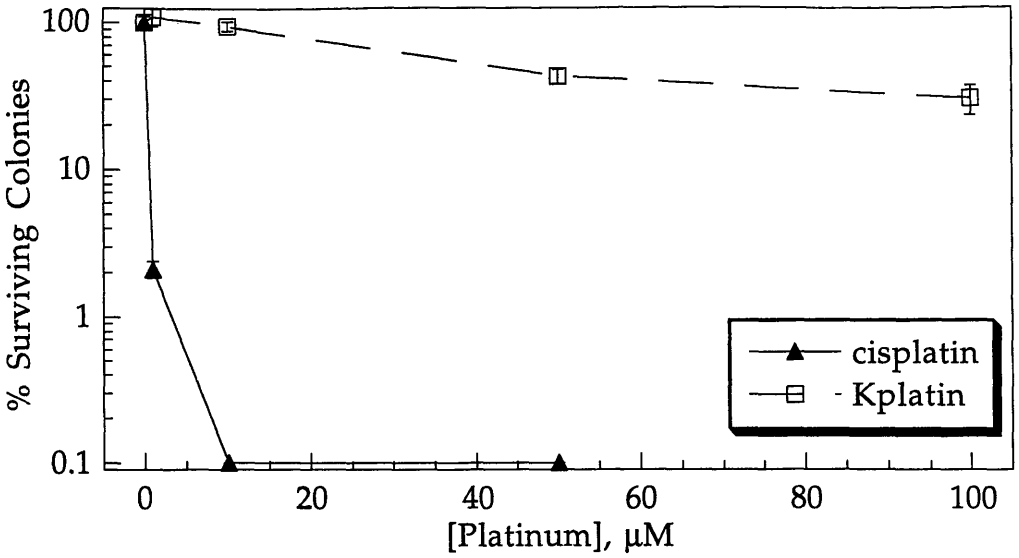

Figure 2.10: Colony-counting assay comparing the toxicity of cisplatin and Kplatin toward HeLa cells.

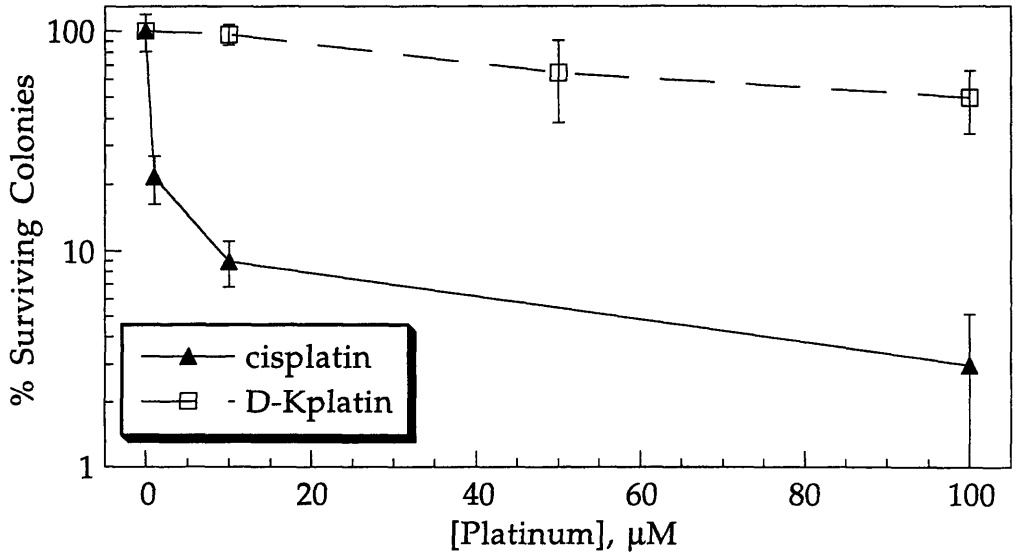

Figure 2.11: Colony-counting assay comparing the toxicity of cisplatin and D-Kplatin toward HeLa cells.

The results of the National Cancer Institute Developmental Therapeutics Program In-Vitro Screen indicated that for all but one human tumor cell line, the LC_{50} value of Kplatin was greater than 0.1 mM; in the exceptional case, a colon tumor cell line KM12, LC_{50} for Kplatin was 74 μM.

Table 2.5 shows the effect of serum content in the medium on the cytotoxicity of Kplatin. The cytotoxicity was fairly constant between 10%-50% fetal bovine serum, but the survival doubled when the cells were treated in serum-free DMEM.

Table 2.5: Effect of serum content on Kplatin cytotoxicity in HeLa cells.

Percent Serum (v/v)	Percent Survival[a]
0	26.9
10	12.1
20	10.0
30	13.5
40	16.5
50	11.7

[a] Cells were treated with 100 μM Kplatin for 24 hours in DMEM with the indicated percent fetal bovine serum. Cells were then washed with PBS and incubated in DMEM with 10% serum.

2.3.6 Pt-DNA-HMG1 binding activity of Kplatin analogs

Table 2.6 compares the calculated r_f and measured r_b values for chicken erythrocyte DNA modified with the platinum complexes depicted in Figure 2.12. Cisplatin reacted readily with DNA, whereas Kplatin and Rplatin were moderately reactive, and NacKplatin was only slightly reactive. Subsequent studies [57] indicated that NacKplatin and Norplatin were equally reactive with DNA. Figure 2.13 shows the results of the gel shift assay using 123-bp DNA modified with four platinum complexes. As expected, cisplatin- and Kplatin-modified DNA specifically bound HMG1. Rplatin-modified DNA also bandshifted whereas NacKplatin, at r_b=0.004, did not. When DNA was treated with a large excess of NacKplatin or Norplatin, the resulting platinated DNA did bind HMG1 [57].

Table 2.6: Comparison of r_f and r_b values for chicken erythrocyte DNA treated with platinum complexes.

Pt complex	r_f	r_b
cisplatin	.025	0.019
Kplatin	.025	0.007
Rplatin	.025	0.007
NacKplatin	.025	0.004

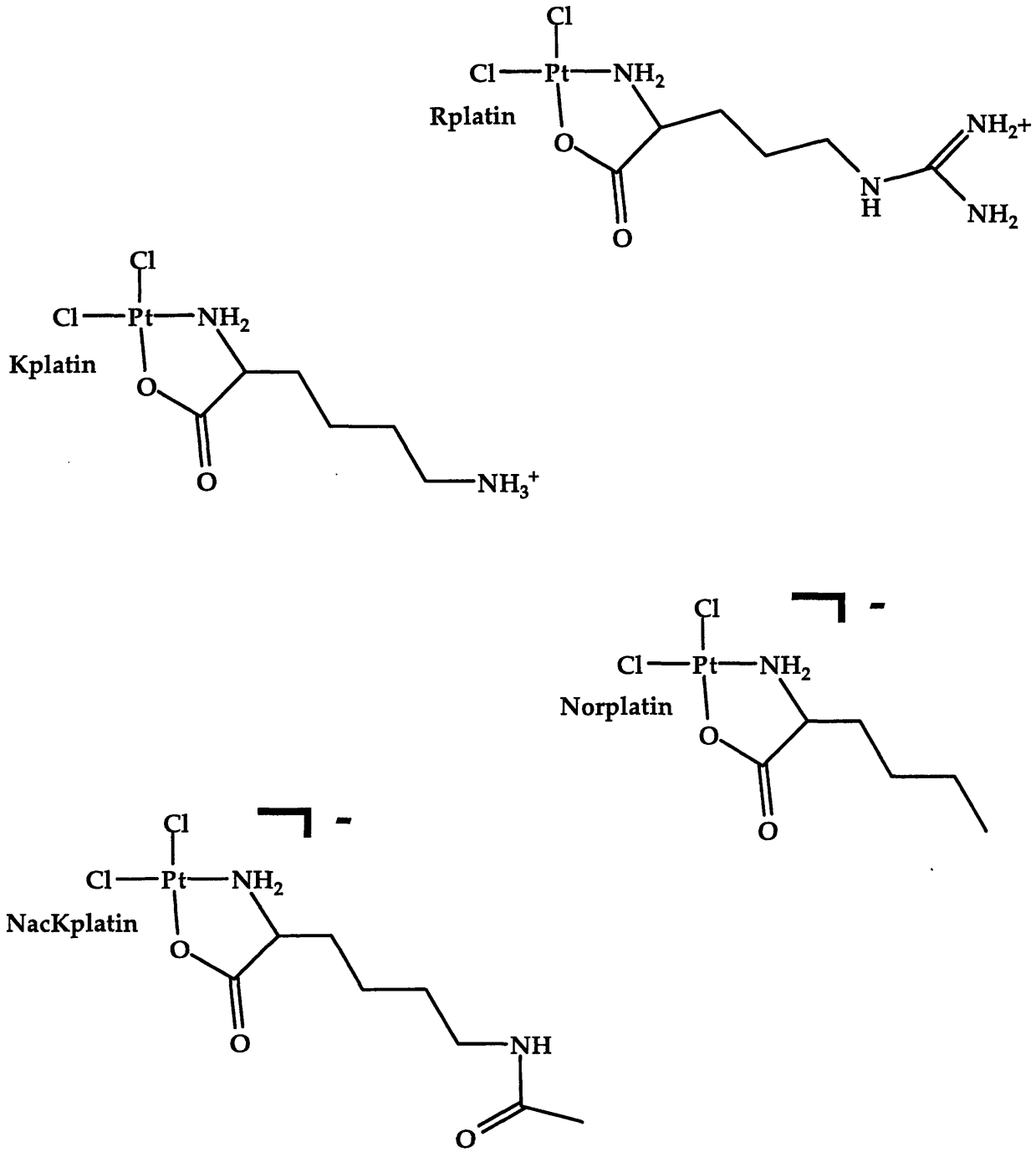

Figure 2.12: Structures of (N,O)-chelated platinum(II) amino acid complexes.

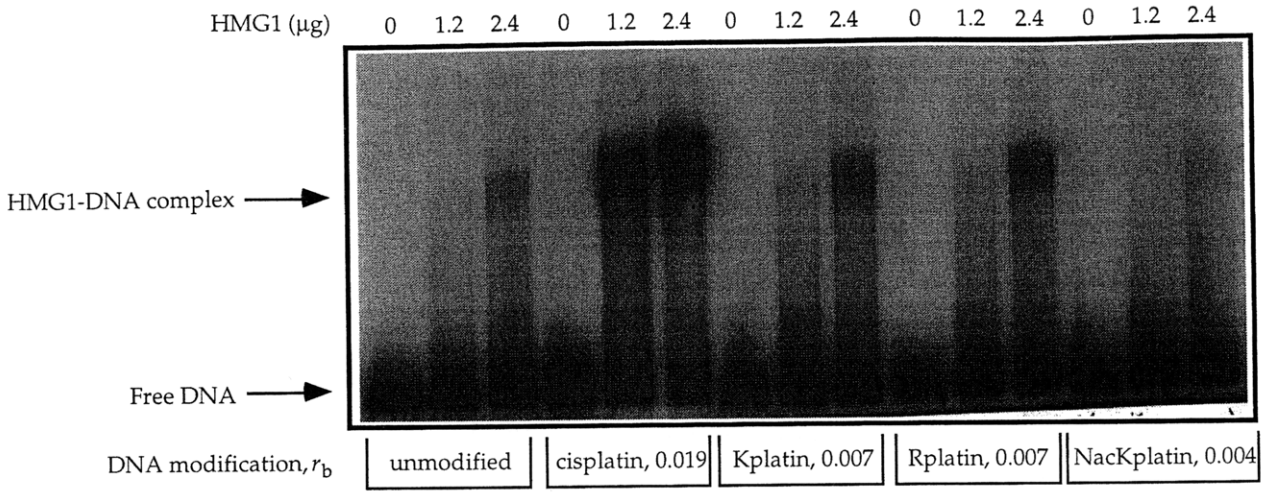

Figure 2.13: : Gel mobility shift assay comparing the binding of HMG1 to 123-bp DNA modified with cisplatin and (N,O)-chelated platinum(II) amino acid complexes.

2.3.7 Cytotoxicity of Kplatin analogs

Figure 2.14 shows that Kplatin and Rplatin were nearly equitoxic towards HeLa cells, with LC_{50} values of about 25 μM for a 24 h treatment. NacKplatin had no apparent effect on HeLa cell division. The HeLa stock used for this experiment was more sensitive than the stocks used in Figures 2.10 and 2.11. In a subsequent study, using HeLa C27 cells (described in Chapter 3), Norplatin had an LC_{50} of 150 μM, compared to 70 μM for Kplatin.

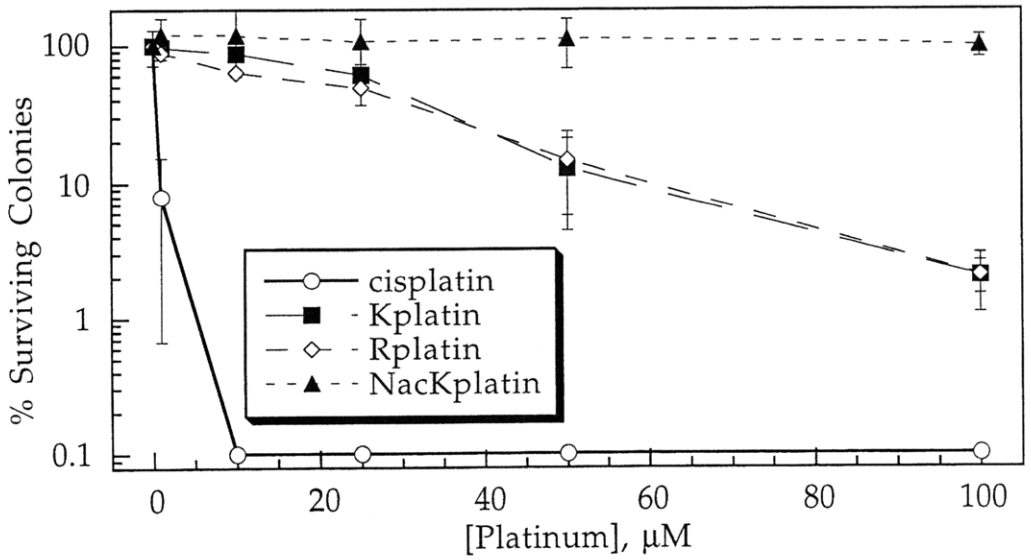

Figure 2.14: Colony-counting assay comparing the toxicity of cisplatin and (N,O)-chelated platinum(II) amino acid complexes toward HeLa cells.

2.4 Discussion

The first goal of our study was to develop a mechanism-based screening method to exploit molecular diversity for selecting new platinum anticancer drug candidates. Based on the current understanding of the mechanism of action of cisplatin, we screened a solution-phase mixture of platinum-amino acid compounds on the basis of their ability to modify DNA and induce binding to HMG1. After several iterations of sub-mixture synthesis and screening, a single platinum-lysine compound emerged, the DNA-HMG-domain binding activity of which approached that of cisplatin.

The compound selected by the screen, [Pt(Lys)Cl_2] or Kplatin, differs structurally from the known effective platinum antitumor agents in that it is an (N,O) chelate rather than a bis(amine). A related class of compounds contain the *cis*-[Pt(NH_3)$_2$]$^{2+}$ moiety coordinated by bis(phosphonomethyl)-aminoacetate [58], but this ligand is lost upon DNA binding unlike lysine in [Pt(Lys)Cl_2]. Pure Kplatin had been previously synthesized and structurally characterized [48,49]. Kplatin is stable for several days in aqueous solution at physiological pH and temperature. Like cisplatin, in a reaction with DNA it loses two chloride ligands and modifies purine bases. Because of their asymmetry, intrastrand d(GpG) Kplatin cross-links afford two linkage isomers, a property previously encountered for another asymmetric platinum compound, *cis*-[Pt(NH_3)($NH_2C_6H_5$)Cl_2] [55]. This complex similarly forms site-specific orientational isomers with oligonucleotides. Kplatin was selected for its ability to induce binding between DNA and HMG1, but like cisplatin, it also causes DNA to bind specifically to both tsHMG and HMGdomB. Because HMG-domain proteins generally recognize bent and otherwise distorted DNA structures [59], it is likely that Kplatin bends DNA in a manner similar to that of cisplatin.

Another objective of this research was to evaluate the cytotoxic properties of newly identified anticancer drug candidates. Although its interactions with DNA and HMG-domain proteins mimic those of cisplatin, Kplatin is 100-fold less toxic than cisplatin toward HeLa cells, and its activity against other human tumor cell lines is comparably low. Kplatin has a different DNA adduct profile than cisplatin, forming fewer specific d(GG) and d(AG) adducts required for HMG-domain protein binding.

Another possibility is that Kplatin is not efficiently transported into cells. Although the compound is charge neutral, its dipolar nature and the dangling ε-amino group of the lysine side chain may prevent diffusion through the cell membrane. Preliminary cell uptake studies (K.E.S. and S.J.L., unpublished results) indicate that, at equitoxic doses, Kplatin is present at much higher concentrations in whole cell digests than cisplatin. This result suggests that the complex may be concentrated in the cell membrane where it is unable to form toxic DNA adducts. Alternatively, if Kplatin were to enter the cell, the amino acid ligand could be metabolized, resulting in a different and possibly less active platinum compound. The similar activity of both D- and L-Kplatin would discount this latter hypothesis, however, since the two enantiomers are unlikely to undergo the same metabolic transformations. It is also possible that Kplatin decomposes under physiological conditions, or that it reacts with components of the cell growth medium. The ^{195}Pt NMR results, however, indicate that dichloride species predominates in aqueous solutions and in media with or without serum. Additionally, serum enhances Kplatin cytotoxicity, discounting the possibility that serum components irreversibly bind and inactivate the platinum.

The studies of the Kplatin analogs provide further insight into the cytotoxic behavior of the (N,O) chelates. The (N,O)-chelated platinum-arginine complex Rplatin resembles Kplatin in its ability to form DNA adducts that bind HMG1. Kplatin and Rplatin were also equally toxic toward HeLa cells. Because they are both charge neutral, (N,O)-chelated amino acid platinum complexes with positively charged side chains, the amine and guanidinium residues of Kplatin and Rplatin probably do not contribute significantly to the biological activity of the complexes. To test the effect of side chain charge, NacKplatin and Norplatin were investigated. Both of these complexes have a neutral side chain, but their overall negative charge inhibits binding to anionic DNA. NacKplatin had no toxic effect on HeLa cells, whereas Norplatin was about half as toxic as Kplatin. The DNA affinity of the two complexes is equal, but the hydrophobic norleucine side chain may enhance the cellular uptake, and thus the cytotoxicity, of Norplatin. The utility of the (N,O) chelates as cytotoxic agents is limited because of charge considerations. A cationic side chain yields a neutral platinum

complex that binds DNA, but the dipolar nature of the complex may inhibit cellular uptake. On the other hand, a neutral side chain yields an anionic complex that does not readily form the DNA adducts that are essential for cytotoxicity.

2.5 Conclusions

We have demonstrated that a solution-phase molecular diversity approach can be used to identify small inorganic compounds that induce protein-DNA binding. After several rounds of sub-mixture synthesis and screening, a moderately cytotoxic compound having the desired in vitro binding activity was identified. The platinum-lysine compound Kplatin is an (N,O) chelate, in contrast to the canonical platinum antitumor drug family comprising cisplatin and carboplatin. Subsequent studies of analogous (N,O) chelates indicated that the electrostatic properties of these complexes limit their cytotoxicity.

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Chapter 3

Expression of Inducible Reporter Genes in Human Cancer Cells in Response to Treatment with Cytotoxic Agents

3.1 Introduction

A critical component of platinum antitumor drug discovery is the evaluation of the cytotoxicity of new complexes [1]. To facilitate the screening of combinatorial libraries of platinum complexes, fast, high-throughput assays are needed. Methods utilizing cultured cancer cells are desirable because they provide essential information about the uptake and metabolism of new compounds. The commonly used cytotoxicity assays, however, are not ideal. Clonogenic or colony counting methods can take over one week to yield results and are not suited for high-throughput screening [1]. Assays employing dyes such as neutral red [2], microtetrazolium (MTT) [3] or sulforhodamine B [4] can be performed with the aid of robotics for high throughput, but cells must be incubated for several days before quantitation. The goal of this research was therefore to develop rapid cell-based assays, suitable for high-throughput screening, that accurately predict the cytotoxicity of platinum complexes.

Transcription, a key step in gene expression, seemed at the outset to be a suitable focal point for assay development. The in vitro inhibition of replication and transcription by platinum-DNA adducts is well-documented [5-8]. Platinum complexes can inhibit promoter-driven gene expression in mammalian cells. In one study [9], plasmids encoding the β-galactosidase reporter gene under the control of the CMV or SV40 early region (SVER) promoter, were modified with *cis*- or *trans*-DDP and transfected into mammalian cell lines. Cisplatin inhibited β-galactosidase transcription

2-3 fold better than *trans*-DDP, an effect attributed to more efficient bypass of *trans*-DDP adducts by RNA polymerase. In a series of transient transfection studies of CV-1 monkey cells, *cis*- and *trans*-DDP both inhibited reporter gene expression from various promoters [10]. Cisplatin, unlike *trans*-DDP, better inhibited expression from strong than weak promoters, suggesting that the targeting of highly expressed genes may contribute to the cytotoxicity of the cis isomer. In mouse tumor cells cisplatin, but not an equitoxic dose of *trans*-DDP, blocked transcription from the mouse mammary tumor virus (MMTV) promoter [11]. It was suggested that structural features of cisplatin-DNA adducts prevent chromatin remodeling and transcription factor binding, both essential for transcriptional activation, consistent with the results of recent x-ray and NMR studies [12-14].

Cisplatin and other DNA-damaging agents can also stimulate gene expression from promoters implicated in resistance or apoptosis pathways. In mammalian cells, DNA damage can induce p53-dependent apoptosis or an SOS-type response that results in cell cycle arrest, DNA repair, and enhanced survival [15,16]. In mouse erythroleukemia cells, c-*myc*-driven chloramphenicol transferase (CAT) expression was enhanced by cisplatin [17]. Activation of c-*myc* has been implicated in cisplatin resistance [18]. In melanoma cells, cisplatin and UV light both induce c-*jun* promoter activity, which is correlated with apoptosis in many cell lines [19]. A review of the effect of cisplatin on genes involved in the regulation of cell growth is available [20].

DNA damage also induces gene expression from promoters with no obvious role in cytotoxicity or resistance. Transient and stable transfections of appropriate vectors in HeLa cells revealed that cisplatin inhibited CAT expression from the SVER promoter and the adenovirus 2 major late promoter (MLP), but induced expression from the HIV-1 long terminal repeat (LTR) and adenovirus 5 E3 promoters [21]. Similarly, in HeLa cells, mitomycin C and UV light increased HIV-1 LTR-promoted gene expression by up to 150 fold [22]. In human and rat fibroblasts, cisplatin stimulated CAT expression from the HIV-1 LTR promoter [23].

The promoter-specific effects of *cis*- and *trans*-DDP in mammalian cells imply that a reporter gene assay could be used to screen novel platinum complexes for potential cytotoxic activity. In addition to offering speed and convenience, a reporter gene assay would measure responses to DNA damage, rather than the endpoint of cell death. Such an assay could therefore be used for mechanistic studies as well as drug discovery.

Until recently, reporter gene assays were not ideal for high-throughput screening. The popular CAT and luciferase assays, for example, both require cell lysis followed by substrate addition to monitor enzyme activity. A recent and widely used reporter gene is the *Aequoria victoria* green fluorescent protein, or GFP [24-26]. Post-translational cyclization of a Ser-Tyr-Gly tripeptide results in a stable, species-independent fluorophore that requires no substrates or cofactors. GFP is a 238 amino acid protein that emits 509 nm light when excited at 395 nm. Mutagenesis of the glycine and three neighboring amino acids has generated functional GFP mutants with unique fluorescence properties. The commercially available GFP variants such as the EGFP (enhanced green fluorescent protein) contain humanized codons and are visible in intact cells and tissues. Because GFP is not an enzyme, however, very low levels of GFP cannot be detected over background autofluorescence. Also, nonexpressing cells cannot be detected by fluorescence, so they must be measured by some other method in order to correct GFP values for variations in cell plating. To overcome such problems associated with GFP and other reporters, a fluorescence-based method using β-lactamase as a reporter enzyme was recently developed [27]. The fluorogenic substrate CCF2-AM is readily taken up into the cytoplasm, where esterases convert it to the membrane-impermeant substrate CCF2. Upon excitation with violet light, intact CCF2 emits green fluorescence, whereas the product of β-lactamase-catalyzed CCF2 hydrolysis emits blue light. It is thus possible to distinguish expressing blue cells from nonexpressing green cells by their fluorescence color. The ratio of blue to green fluorescence intensities is a measure of β-lactamase expression.

There have been only a few reports linking cytotoxicity and reporter gene expression. One proposed screening method utilized mouse cells lines constitutively expressing firefly luciferase or β-galactosidase [28]. Upon death the cell membranes ruptured and released the reporter enzyme into the growth medium, where it could be quantitated by adding substrate. Although this method detected cell death, it did not take into account the influence of cytotoxic agents on reporter gene expression. In another study [29], a series of antimycobacterial agents were screened for the ability to inhibit GFP expression in *M. tuberculosis*. The results demonstrated that GFP inhibition could be used to predict the activity of potential tuberculosis drugs. Another screening method [30] is based on the observation that mismatch-repair-deficient cells are more

resistant to some forms of DNA damage than wild type cells [30,31]. A mismatch repair-deficient cell line constitutively expressing GFP was combined with a repair-proficient line and the heterogeneous population treated with platinum complexes and other DNA-damaging agents. Within five days of exposure, cisplatin and carboplatin enriched the GFP-expressing mismatch repair-deficient cell population. The cytotoxicity of any platinum compound might therefore be predicted by measuring the enrichment of GFP-expressing, mismatch repair-deficient cells in the population.

To observe the effects of platinum complexes on reporter gene expression, we utilized both constitutive and inducible promoters. For strong constitutive gene expression, the CMV promoter was placed upstream of EGFP and β-lactamase in HeLa and Jurkat cells, respectively. For inducible EGFP expression in HeLa cells, a Tet-On system (Figure 3.1) was chosen [32], where the gene of interest (EGFP) was cloned into a plasmid downstream of the minimal early promoter of the CMV (P_{minCMV}). In the presence of doxycycline, the tet-responsive transcriptional activator (rtTA) binds and activates the tetracycline-responsive element (TRE), which in turn activates the CMV. In that manner, the addition of doxycycline to the growth medium induces EGFP expression. Inducible β-lactamase expression in Jurkat cells was achieved by using a previously reported [27] cell line with the NF-AT-β-lactamase reporter and the G-protein coupled muscarinic receptor under the control of the CMV promoter (M_1-GPCR/NFAT-bla or C2 Jurkat). Treatment of the cells with the muscarinic agonist carbachol induced β-lactamase expression.

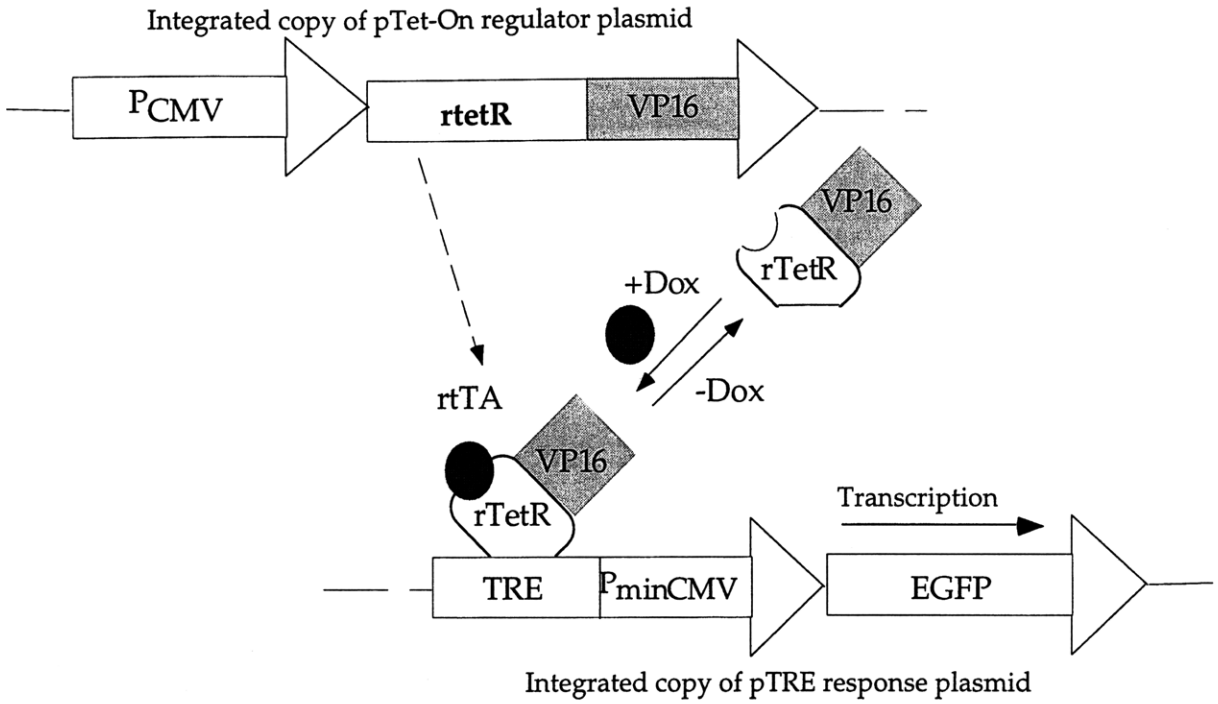


Figure 3.1: Tet-On System for Inducible Gene Expression

3.2 Experimental

3.2.1 Materials

Tissue culture media and antibiotics were purchased from Gibco BRL. Tet System Approved FBS, pEGFP-1, pTRE, pTK-Hyg, HeLa Tet-On cells and anti-GFP antibody were obtained from Clontech. All restriction enzymes and T4 DNA ligase were from New England Biolabs. Cisplatin and JM335 were obtained from Johnson-Matthey. All other platinum complexes were provided by S. J. Lippard and coworkers. Calicheamicin was donated by P. Dedon (MIT).

3.2.2 Physical measurements

Fluorescence spectra were recorded on a Hitachi F-3010 Fluorescence spectrophotometer. All EGFP fluorescence microplate readings were obtained using a Molecular Devices Fmax instrument equipped with a 485±14 nm excitation and a 538±25 nm emission filter. CCF2 fluorescence was measured using a Cytofluor 4000 microtiter plate fluorimeter with a 395±10 nm excitation filter and emission filters of 460±20 nm (blue fluorescence) and 530±15 nm (green fluorescence). In both cases the emission values were corrected by subtracting readings from blank wells. Automated

DNA sequencing was performed on an ABI Prism system at the MIT Biopolymers Laboratory. Platinum concentrations, relative to a potassium hexachloroplatinate(IV) standard, were determined with a Varian atomic absorption spectrophotometer AA-1475, equipped with a graphite tube atomizer GTA-95. Phosphorimager analysis was performed on a BioRad Molecular Imager.

3.2.3 *Stable transfection of HeLa with a constitutive EGFP expression vector*

The mammalian vector pEGFP-N1 was amplified in *E. coli* and purified by using a Qiagen-500 kit. The plasmid was further purified by digestion with Plasmid-Safe DNase (Epicentre Technologies). The pEGFP-N1 was linearized with EcoO109I, and the reaction mixture was extracted with phenol and precipitated with ethanol.

HeLa cells were plated on 10-cm plates and grown to 60% confluence in DMEM supplemented with 10% FBS, 100 μg/mL penicillin and 100 U/mL streptomycin. Two hours before transfection, the cells were given fresh media.

To form calcium phosphate-DNA complexes [33], three aliquots of linearized pEGFP-N1 were precipitated with ethanol; they contained 10, 25 and 50 μg of DNA, respectively. The DNA pellets were air-dried and each was dissolved in 0.5 mL of 0.25 M calcium chloride. Each DNA solution was then added, dropwise with agitation, to 500 μL of 2x Hepes-buffered saline (HBS) solution. The DNA precipitates were incubated at room temperature for 20 min and then distributed over the HeLa cells. After a 16 h 37°C incubation, the cells were washed twice with PBS and given fresh media. They were incubated for about 32 h before selection was begun.

To select for transfectants, the HeLa cells were maintained in media containing 0.8 mg/mL geneticin; the media was replaced every 48 h. After 4 days of selection, the cells were split 1:10. After 16 days of selection, 14 resistant colonies were transferred to a 24-well plate. The clones were maintained in selective medium and expanded until frozen stocks could be prepared from the nine most viable cell lines. Two of the putative transfected HeLa clones, and the parental HeLa cell line, were grown to confluence and examined by fluorescence microscopy using an FITC filter.

3.2.4 *Cisplatin treatment of EGFP-expressing HeLa cells*

A pEGFP-N1-transfected HeLa clone and parental HeLa cells were grown to 70% confluence on 10-cm plates. Cisplatin was dissolved in water and added to the cells for

a 24 h treatment, after which the medium from each plate was collected. The cells were washed with PBS, which was combined with the collected media. The cells were then trypsinized and added to the appropriate collection tubes, which were centrifuged for 5 min at 350 x g. The cell pellets were resuspended in 1 mL of PBS and re-centrifuged twice; the final pellets were each suspended in 1 mL of PBS with 10 μg/mL each of pepstatin, leupeptin and aprotinin and 400 μg/mL of Pefablock. The cells were then counted by using a hemocytometer, and their emission spectra at 509 nm were recorded (395 nm excitation).

3.2.5 Measurement of EGFP half-life in stably transfected HeLa cells

A HeLa clone constitutively expressing EGFP was grown to 70% confluence on 10-cm plates. Each plate was washed with 5 mL of DMEM deficient in methionine, cysteine and glutamine. Each plate was then starved by treatment with 3 mL of DMEM (no M, C, Q) with 15% dialyzed FBS (1000 MWCO, Sigma). After 30 min, 0.4 mCi of ^{35}S methionine/cysteine (Dupont NEN) and 2 mM L-glutamine were added to each plate for a 90 min incubation. Each plate was then washed and given 10 mL of complete 10% FBS DMEM.

For each time point, one plate of cells was washed with 5 mL of cold PBS. The cells were then incubated on ice for 30 min in 1 mL of lysis buffer (50 mM Tris, pH 8, 150 mM NaCl, 0.5% NP40, 1 mM DTT, 10 μg/mL each of aprotinin, leupeptin, and pepstatin, and 0.5 mg/mL Pefablock). The lysate was centrifuged for 10 min at 10,000 x g at 4°C. The supernatant was flash-frozen in liquid nitrogen and stored at -80°C.

(Note: All of the following procedures were carried out at 4°C and all centrifugations were at 10,000 x g.)

The protein concentration in each cell extract was determined by using the BioRad DC protein assay. The extracts were diluted to 0.64 mg/mL in lysis buffer. Each sample was combined with 6 μL each of normal rabbit and mouse sera and 100 μL of washed staph A (donated by M. Bogyo, H. Pleough laboratory). The tubes were rocked for 45 min and then centrifuged for 10 min. The supernatant fractions were transferred to new tubes and the procedure was repeated, but with 11 μL each of rabbit and mouse serum. The cell extracts were thus depleted of components that nonspecifically bound protein A and/or antibodies. The cleared cell extracts were then combined with 2 μL of polyclonal anti-GFP antibody and rocked overnight. Each extract was then combined

with 80 µL of washed staph A, rocked for 1 h and spun for 30 s. The pellets were resuspended in 200 µL of NET buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% NP40). After four cycles of centrifugation and resuspension, the suspensions were transferred to new tubes and centrifuged for 30 s. The pellets were resuspended in 100 µL of SDS loading dye, boiled for 5 min and loaded on a 4.5% stacking, 10% separating SDS polyacrylamide gel. The gel was soaked in Amplify (Amersham) to intensify the signal for autoradiography.

3.2.6 Cloning of pTRE-EGFP

The gene encoding the enhanced green fluorescent protein (EGFP) was PCR-amplified from pEGFP-1 to incorporate EcoRI and XbaI sites. The primers were as follows, with restriction sites indicated in boldface:

Forward (EcoRI) primer:

5'-TCT CGA GCT CAA GCT TC**G AAT TC**T GCA GTC GAC GGT-3'

Reverse (XbaI) primer:

3'-ATT TCG CCG GCG CTG **AGA TCT** AGT ATT AGT CGG TAT-5'

The PCR reaction was performed in an EZStart 100 reaction tube (Molecular Bio-Products). To the prepared reaction tube, 0.2 µg of template DNA (pEGFP-1) and 0.5 µg of top and bottom primers were added, with 5 U of Pfu polymerase (Stratagene) in a total volume of 50 µL. The reaction cycles were as follows:

1 cycle: 94°C, 45 s.

25 cycles: 94°C, 45 s; 55°C, 45 s; 72°C, 120 s.

1 cycle: 72°C, 600 s.

The PCR product was precipitated with ethanol and digested with EcoRI and XbaI. After heat-inactivation of the enzymes, the DNA was precipitated with ethanol.

The plasmid encoding the tetracycline-responsive element, pTRE, was amplified in *E. coli* and purified with a Qiagen 500 column. The plasmid was then digested with EcoRI and XbaI; the linearized vector was purified on a 1% agarose gel.

The EcoRI/XbaI digest of EGFP was combined with the EcoRI/XbaI digest of pTRE and incubated for 16 h at 16°C with T4 DNA ligase in the presence of 10 µM ATP. The ligation mixture was transformed into chemically competent XL1-Blue cells and plated on LB ampicillin agar plates. The resultant colonies were inoculated into LB ampicillin media and grown to saturation; the plasmids were isolated by the boiling

lysis method. The plasmid DNA was digested with BamHI and analyzed on a 1% agarose gel. The two samples that possessed the expected 750-bp insert were purified with a Qiagen 500 kit and digested with a series of restriction enzymes to verify that the EGFP gene had been properly inserted. The products were analyzed on a 1% agarose gel. The two plasmids were then submitted to the MIT Biopolymers facility for automated DNA sequencing on the ABI Prism system. The primers were as follows:
Forward: 5'-ATA GAA GAC ACC GGG ACC GA-3'
Reverse: 3'-CT ACT CAA ACC TGT TTG GTG-5'

3.2.7 Transfection of HeLa Tet-on cells with pTRE-EGFP

The plasmids pTRE-EGFP and pTK-Hyg were amplified in *E. coli*, purified on Qiagen 500 columns and linearized with SspI and HindIII, respectively. HeLa Tet-on cells were cultured in DMEM with 10% FBS, 100 μg/mL of penicillin, 100 U/mL of streptomycin, and 400 μg/mL of geneticin. The cells were transfected using the calcium phosphate method described in Section 3.2.3. To confer hygromycin resistance, each plate of cells was transfected with 2 μg of linearized pTK-Hyg in addition to pTRE-EGFP. The selective medium contained 0.4 mg/mL geneticin and 0.2 mg/mL hygromycin. After 11 days of selection, 70 resistant colonies were transferred to 24-well plates and expanded.

Forty-eight of the putative pTRE-EGFP transfected HeLa Tet-on clones, and the parental HeLa Tet-On cell line, were grown to confluence on 35-mm plates and induced with 4.3 μM doxycycline. Sixteen hours after induction, the cells were examined by fluorescence microscopy using an FITC filter. Frozen stocks were prepared from twelve green fluorescent clones.

Five of the selected clones were grown to 70% confluence on 10-cm plates. After a 16 h 4.3 μM doxycycline induction, each plate of cells was washed with PBS and lysed in 250μL of PBS with 2% SDS and 15 μg/mL of pepstatin, leupeptin and aprotinin. Each lysate sample was passed five times through a 25 G 5/8 needle, boiled for five min, flash-frozen in liquid nitrogen and stored at -80°C. The total protein was quantitated by using the BioRad DC assay, and a 100 μg sample of each extract was boiled in SDS loading buffer and run on a 4.5% stacking, 10% separating SDS polyacrylamide gel. The proteins were transferred to nitrocellulose and probed with the polyclonal anti-GFP antibody by Western blotting. For visualization, the blot was

washed with an anti-rabbit antibody conjugated to horseradish peroxidase; the chemiluminescent substrate luminol was then applied, and the blot was immediately exposed to x-ray film for 2 min.

3.2.8 EGFP expression assay in HeLa C27

The selected HeLa C27 cells were maintained in DMEM supplemented with 10% FBS, 100 μg/mL penicillin, 100 U/mL streptomycin, 0.4 μg/mL geneticin and 0.2 μg/mL hygromycin. Twenty-four hours before induction, the cells were distributed in 6-well plates such that they would be 60-70% confluent upon induction. Aqueous 0.4-2.0 mM platinum solutions were prepared no more than 2 h before use, and platinum concentrations were confirmed by atomic absorption spectroscopy. In a typical co-treatment experiment, the platinum complexes were added to each well together with 21.6 μM doxycycline. After a 13.5 h incubation, the cells were washed with PBS and lysed by vigorous shaking in 125 μL of PBS with 0.5% SDS. The lysates were transferred, using wide-bottomed pipet tips, to a black 96 well microplate (Corning Costar) for fluorescence plate reading. A 4-μL aliquot of lysate was removed from each sample and used in the Nano-Orange protein assay (Molecular Probes). The EGFP fluorescence values were divided by their respective protein assay readings to correct for variations in cell plating. The corrected fluorescence values were then normalized relative to the control (unplatinated) samples. All samples were prepared in triplicate, and the data was averaged with error bars representing one standard deviation.

3.2.9 Northern blotting of RNA from HeLa C27 extracts

For Northern blotting analysis, HeLa C27 cells were plated on 10 cm tissue culture dishes and grown to 70% confluence. Parallel samples for the EGFP expression assay were prepared in triplicate on 6-well plates. The cells were treated with the appropriate cytotoxic agents and induced for 13.5 h with 21.6 μM doxycycline. RNA was isolated from cell extracts by using the Biotecx Ultraspec-II kit. After agarose-formaldehyde gel electrophoresis, the RNA was transferred to nitrocellulose and processed according to standard procedures [34]. The blot was probed with random primer-labeled DNA encoding EGFP and, as a control, human GAPDH (donated by D. Housman laboratory, MIT). The blot was quantitated by phosphorimager analysis with a BioRad Molecular

Imager, and EGFP values were corrected for loading by normalizing to the ubiquitously expressed GAPDH.

3.2.10 Transient transfection of HeLa C27 with pcDNA3.1-tsHMG

The pcDNA3.1-tsHMG plasmid, and the control plasmid pcDNA3.1, were obtained from Christina Eng, amplified in *E. coli* and purified on Qiagen 500 columns. HeLa C27 cells were distributed on 6-well plates 24 h prior to transfection. Two hours before transfection, when the cells were about 25% confluent, the growth medium was changed. The cells were transfected with the circular plasmids by the calcium phosphate method, with either 6 or 10 μg of plasmid per well. The precipitate was washed away after 16 h, and the cells were incubated in fresh media for 48 h. The cells were then co-treated for 13.5 h with cisplatin and doxycycline in fresh media, and the EGFP expression assay was performed as described in Section 3.2.8.

3.2.11 Cytotoxicity and apoptosis assays

Jurkat C2 cells, obtained from Gregor Zlokarnik (Aurora Biosystems), were maintained at 10-50 x 10^4 cells/mL in RPMI 1640 medium supplemented with 10% FBS, 100 μg/mL penicillin and 100 U/mL streptomycin. To prepare for cytotoxicity and apoptosis assays, the cells were diluted to 10 x 10^4 cells/mL and distributed in 24-well plates with 0.5 mL/well. The cells were treated with aqueous solutions of platinum complexes for 19.5 h, then transferred to microcentrifuge tubes, pelleted and resuspended in 20 μL of PBS. A 5 μL aliquot was combined with 5 μL of trypan blue (0.4% in PBS) and counted under a light microscope. The remaining 15 μL sample was fixed in 0.5 mL of cold methanol for 10 minutes and then pelleted. After a 5 min DAPI (1 μg/mL in PBS) treatment, the cells were pelleted and mounted on glass slides in 10 μL of Mowiol mounting medium. The stained nuclei were counted under a fluorescence microscope. All samples were prepared in duplicate.

For HeLa C27, colony counting cytotoxicity assays were performed as previously described [35]. All samples were prepared in triplicate, and platinum concentrations were verified by atomic absorption spectroscopy. For apoptosis assays, HeLa C27 cells were distributed on 24-well plates 24 h prior to platinum treatment. After the treatment, the supernatant medium was transferred to microcentrifuge tubes, and the cells were washed with PBS, which was transferred to the same tubes. The cells were

trypsinized, added to the tubes, pelleted and stained in the same manner as the Jurkat C2 cells.

3.2.12 β-Lactamase expression assay in Jurkat cells

The β-lactamase assay was performed by Dr. Gregor Zlokarnik of Aurora Biosystems according to the following protocol. Jurkat C2 cells in RPMI medium were distributed into black 96 well clear-bottom microtiter plates at 100,000 cells per well. The platinum complexes were dissolved at a concentration of 1 mM in water. Serial dilutions of these solutions were prepared in water and added in 10% of final volume to the cells. After a 20 h platinum treatment, the cells were induced with 100 μM carbachol for 4 h. Cells were then loaded with 1 μM CCF2/AM at room temperature for 1 h prior to fluorescence plate reading. The corrected signal from the blue channel was divided by the signal from the green channel to obtain the final blue to green intensity ratio. With the gain settings used for this experiment, a population with >95% of the cells expressing β-lactamase gave a ratio of greater than 3.0 and a population of with no cells expressing β-lactamase gave a ratio of 0.1-0.2. Jurkat cells constitutively expressing β-lactamase under the control of the CMV early promoter (CMV-bla Jurkat) were treated in the same manner as the C2 clone, but were kept 24 h in the incubator without any induction period prior to dye loading. All samples were prepared in quadruplicate, and the data were averaged with error bars representing standard error (= standard deviation/square root of number of multiples).

3.3 Results

3.3.1 Constitutive EGFP expression in HeLa cells

The two putative pEGFP-N1 transfected HeLa clones appeared bright green under a fluorescence microscope equipped with FITC filters, whereas the parental HeLa cell line was invisible. It was concluded that both clones were constitutively expressing EGFP under the control of the CMV promoter. To determine the effect of cisplatin on CMV-driven constitutive EGFP expression, the fluorescence of the cells was measured after cisplatin treatment. Table 3.1 shows that even toxic cisplatin concentrations did not cause a marked decrease in EGFP fluorescence after 24 h. The half-life of the EGFP in HeLa cells was determined by ^{35}S labeling of proteins, followed by immuno-precipitation. The autoradiogram in Figure 3.2 shows that the EGFP half-life is 20-30 h.

Table 3.1: Relative fluorescence intensity (RFI) at 509 nm (395 nm excitation), corrected for cell count, for HeLa cells after a 24 h 37°C cisplatin treatment.

[Cisplatin], μM	RFI/cells x 10^{-6}: parental HeLa	RFI/cells x 10^{-6}: pEGFP-N1-transfected HeLa
0	1.03	5.89
1	1.47	6.56
5	1.88	5.87
10 (some cells dead)	1.80	5.98
40 (all cells dead)	1.33	4.93

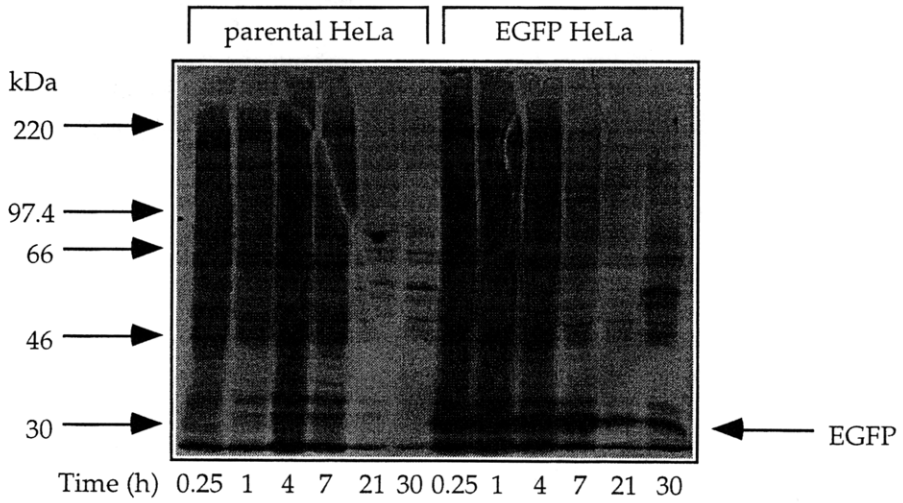


Figure 3.2: SDS/PAGE analysis of immunoprecipitated ^{35}S-labeled proteins from parental and pEGFP-N1-transfected HeLa cells. The cells were pulsed with ^{35}S and then incubated for the indicated times. The extracts were precipitated with an anti-GFP antibody.

3.3.2 Inducible EGFP expression in HeLa Tet-On cells

To generate HeLa cells with inducible EGFP expression, the pTRE-EGFP plasmid was first cloned. Figure 3.3 shows that restriction digestion of pTRE-EGFP resulted in fragments of the expected lengths. Automated DNA sequencing confirmed that the desired plasmid had been obtained. HeLa Tet-On cells were transfected with pTRE-EGFP, with the hygromycin resistance plasmid pTK-Hyg added to allow selection. By fluorescence microscopy it appeared that at least 12 of the hygromycin-resistant clones possessed doxycycline-inducible EGFP expression. The Western blot in Figure 3.4 confirmed that clones 27, 28 and 29 all expressed EGFP in the presence of doxycycline. HeLa C27 was used for most subsequent EGFP induction studies. The time course of doxycycline induction of EGFP fluorescence in HeLa C27 is plotted in Figure 3.5. A 13.5 h induction period provided sufficiently high fluorescence readings, which were independent of doxycycline concentration over a range from 2.16 to 216 μM.

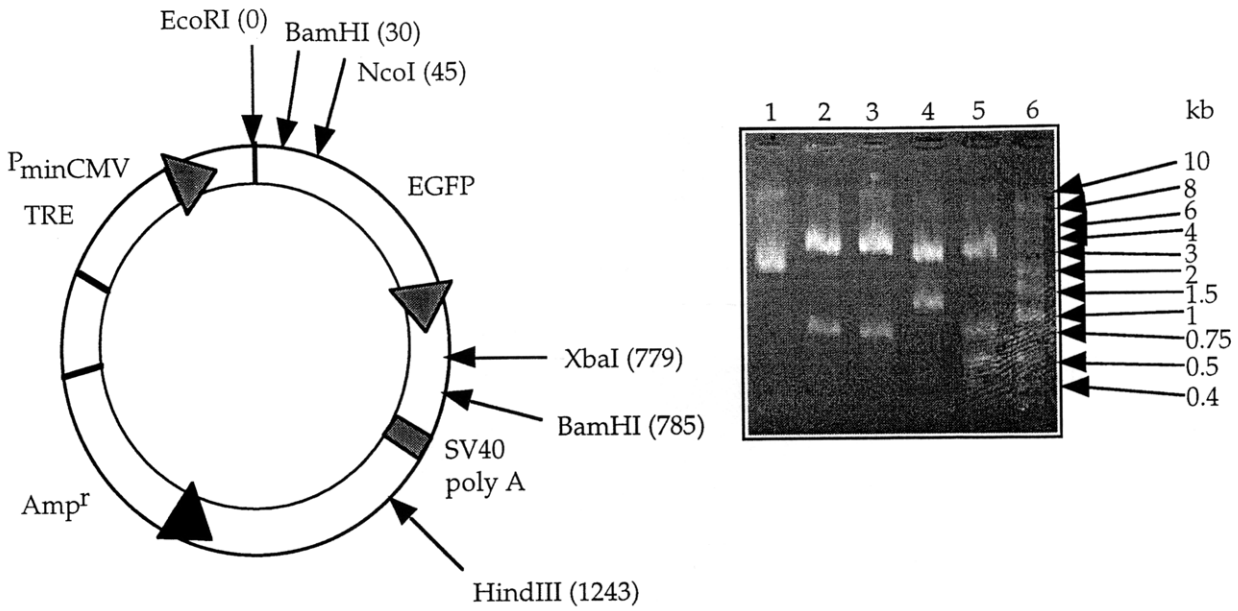


Figure 3.3: Plasmid map and ethidium bromide stained agarose gel showing the products of restriction digestion of pTRE-EGFP. Lane 1: Undigested plasmid. Lane 2: EcoRI/XbaI digest; 0.8 and 3.1 kb expected. Lane 3: BamHI digest; 0.8 and 3.1 kb expected. Lane 4: NcoI/HindIII digest; 1.2 and 2.7 kb expected. Lane 5: BamHI/HindIII digest; 0.5, 0.8 and 2.6 kb expected. Lane 6: DNA size standards.

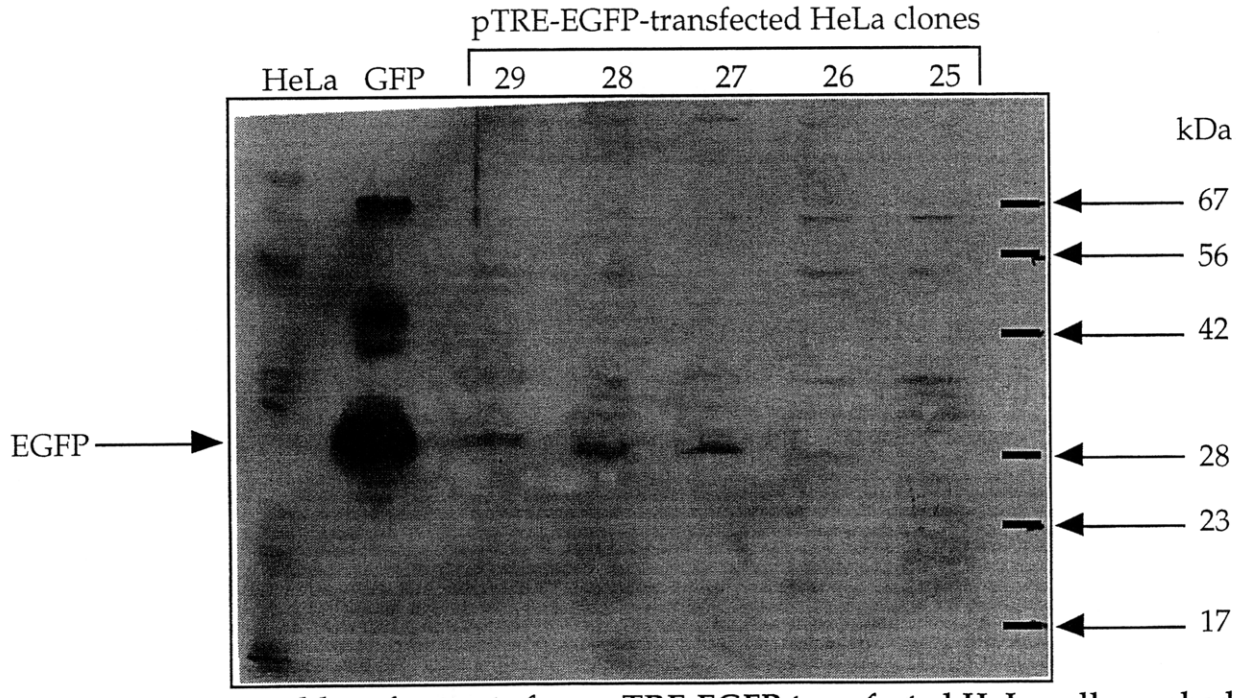


Figure 3.4: Western blot of extracts from pTRE-EGFP-transfected HeLa cells, probed with an anti-GFP antibody. Parental HeLa cells and crude recombinant GFPuv were used as controls.

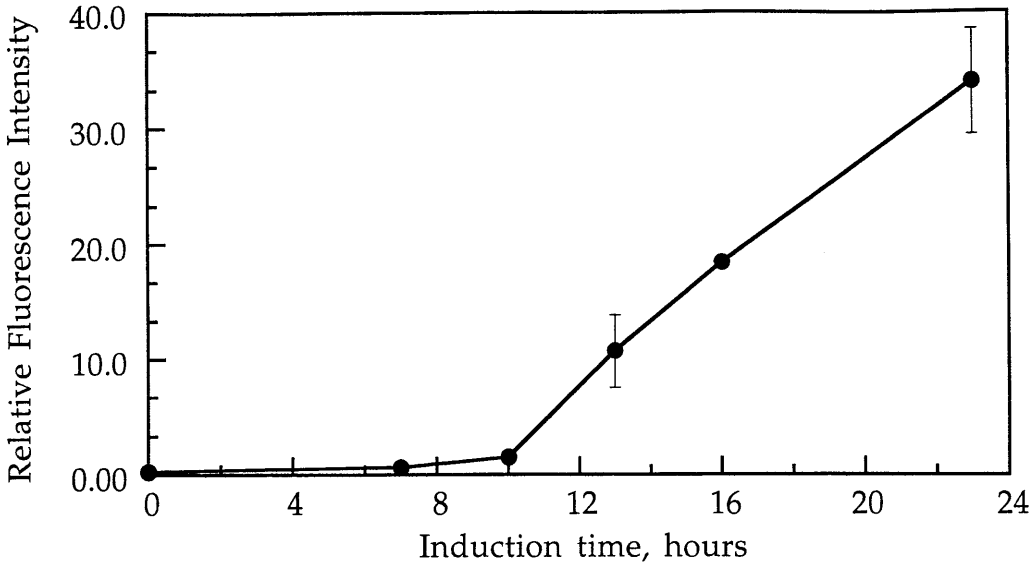


Figure 3.5: Time course of EGFP induction in HeLa C27. Cells were treated with 21.6 μM doxycycline.

3.3.3 Effect of platinum complexes on EGFP expression in HeLa C27

HeLa C27 was initially exposed to platinum complexes under various condi-tions to determine their effect on EGFP expression. For a 13.5 h induction, platinum was added either 2 h before doxycycline ("pre-treatment") or at the same time as the doxycycline ("co-treatment"). In a third protocol the cells were treated with platinum for 2 h, washed, and then induced ("pre/wash"). Figure 3.6 shows that all three methods of cisplatin treatment caused a dose-dependent decrease in EGFP expression. The pre/wash condition required higher doses than pre-treatment or co-treatment to obtain the same EGFP inhibition, as expected since platinum was not present in the media for any of the 13.5 h induction period. Figure 3.7 shows that *trans*-DDP affected EGFP expression in a manner distinct from cisplatin. At low *trans*-DDP concentrations, there was a dose-dependent increase in EGFP, followed by a sharp decrease at very high concentrations. To determine whether the dose-response results were unique to HeLa C27, the 13.5 h co-treatment assay was repeated in HeLa Tet-On C28. Figure 3.8 shows that similar results were obtained in C28.

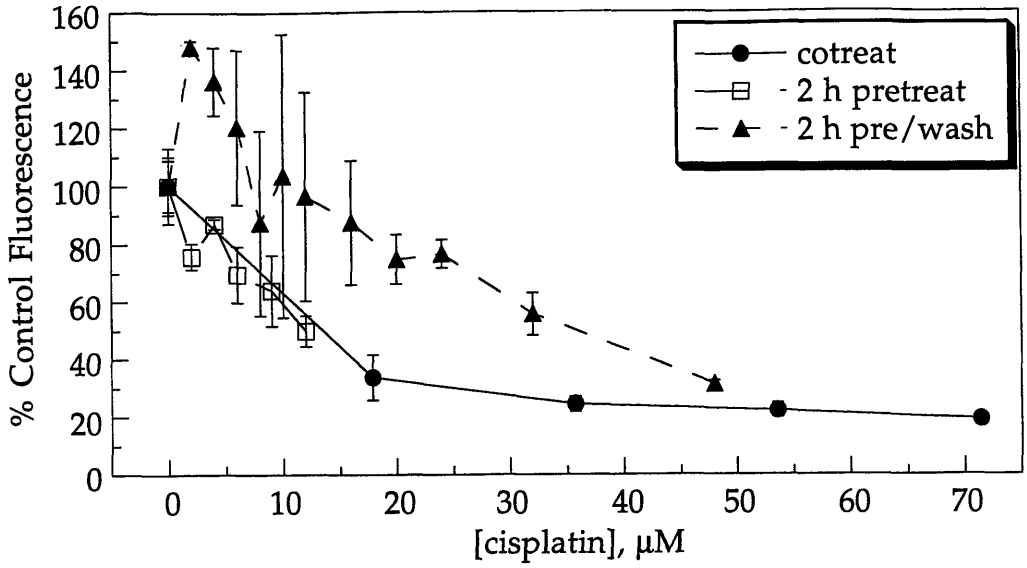


Figure 3.6: Effect of cisplatin treatment on EGFP expression in HeLa C27. All samples were induced with 21.6 μM doxycycline for 13.5 h. Fluorescence readings were divided by total cellular protein and normalized to induced, unplatinated control (100%).

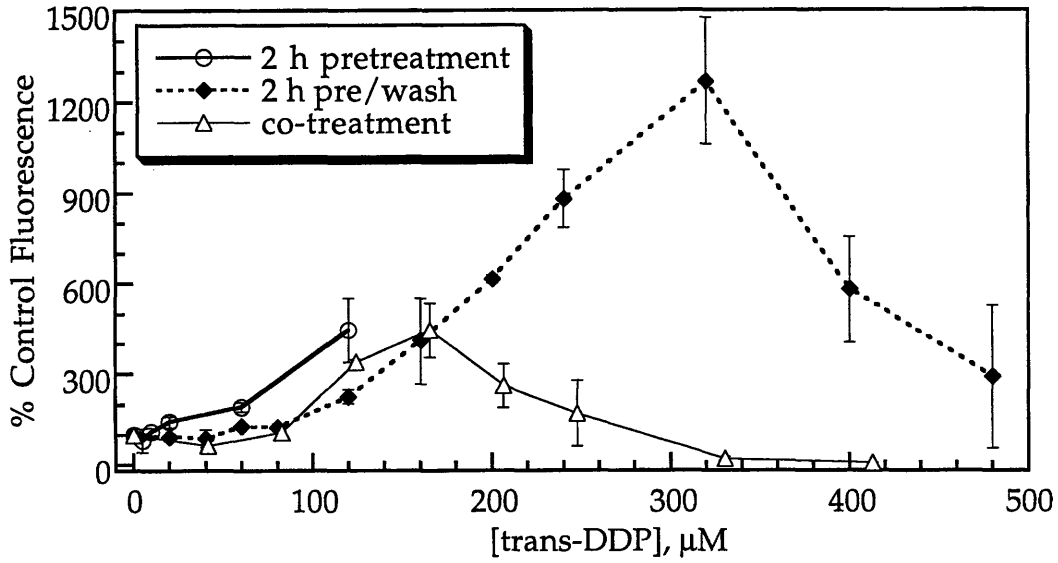


Figure 3.7: Effect of *trans*-DDP treatment on EGFP expression in HeLa C27. All samples were induced with 21.6 μM doxycycline for 13.5 h.

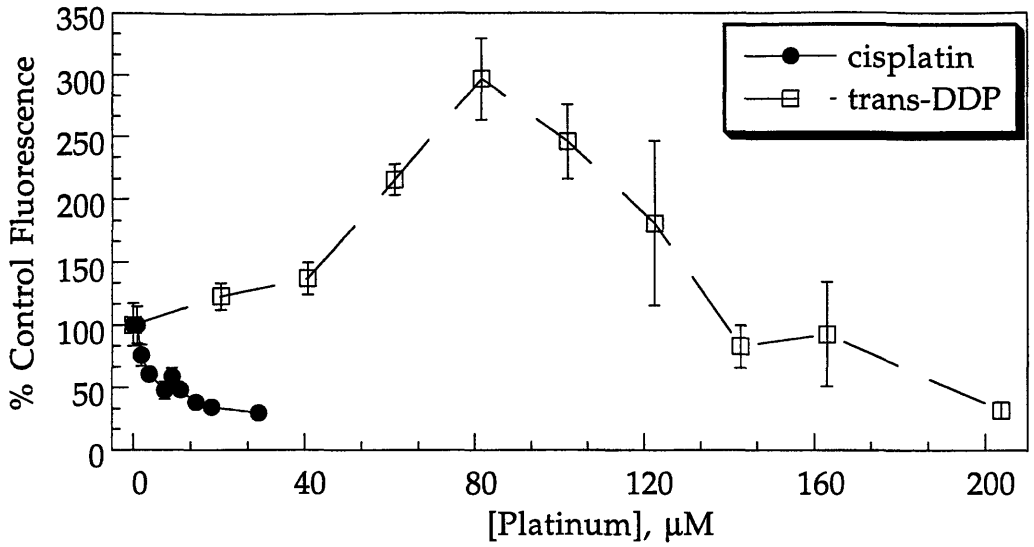


Figure 3.8: Effect of *cis*- or *trans*-DDP co-treatment on EGFP expression in HeLa C28.

The effect of a 13.5 h co-treatment with two major types of platinum complexes was tested. The cis platinum complexes have chloride ligands in the cis configuration and mimic cisplatin by inhibiting EGFP expression in a dose-dependent manner (Figure 3.9). The trans platinum complexes, with chlorides in the trans configuration, induced EGFP expression at low doses and inhibited it at high doses in a manner similar to *trans*-DDP (Figure 3.10).

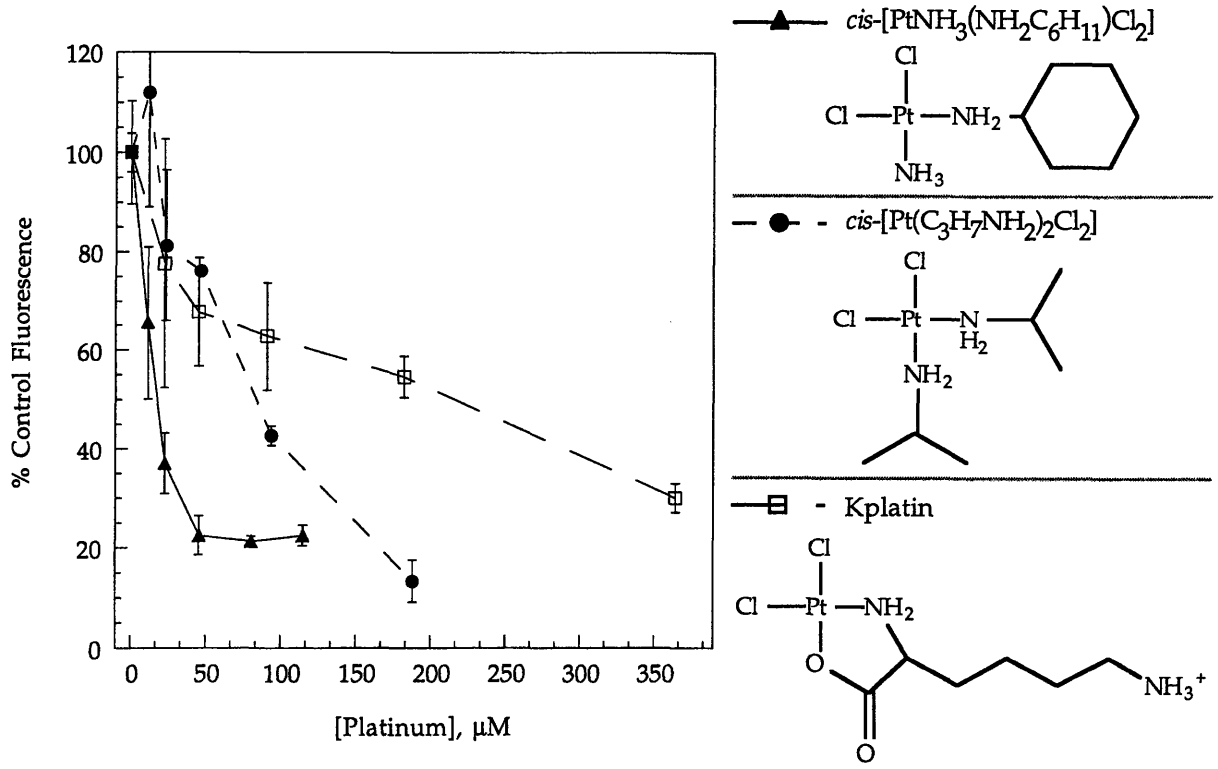


Figure 3.9: Effect of cis platinum complexes on EGFP expression in HeLa C27.

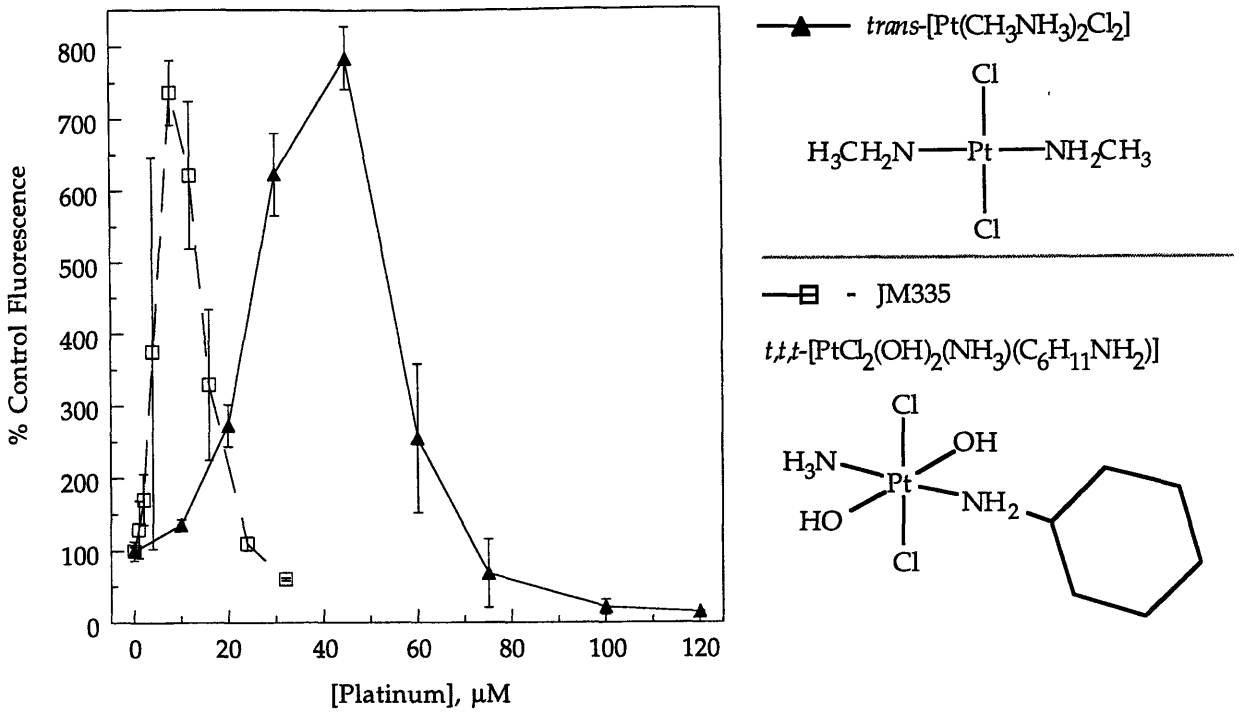


Figure 3.10: Effect of trans platinum complexes on EGFP expression in HeLa C27.

DAPI staining revealed that a 16.5 h treatment with 20 μM cisplatin induced apoptosis in 50% of the C27 cells, *e.g.*, AC_{50}=20 μM. After a 20 h treatment, the AC_{50} for cisplatin was 16 μM. Although trypan blue staining showed that a 20 h treatment with 360 μM *trans*-DDP killed more than 90% of the cells, under no conditions did *trans*-DDP induce apoptosis in more than 15% of the HeLa C27 cells.

3.3.4 Effect of other cytotoxic treatments on EGFP expression in HeLa C27

To assess the generality of the results with platinum, HeLa C27 was treated with several non-platinum cytotoxic agents. MMS, an alkylating agent, behaved like *trans*-DDP, stimulating EGFP expression at low (<1 mM) doses and inhibiting it at high (>3 mM) doses (Figure 3.11). Similarly, a short (15-90 min) 45°C heat shock caused a time-dependent increase in EGFP expression, whereas a 120 min treatment caused a decrease (Figure 3.12). Calicheamicin, on the other hand, was similar to cisplatin in causing only a decrease in EGFP expression (Figure 3.13).

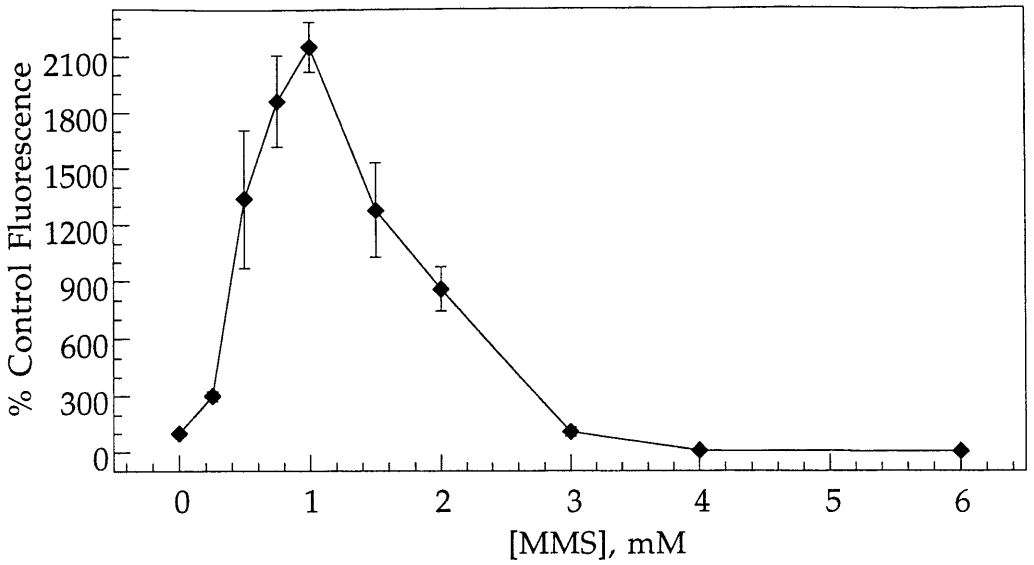


Figure 3.11: Effect of MMS on EGFP expression in HeLa C27. Cells were treated with MMS in serum-free medium for 1.5 h, then washed and induced in complete medium.

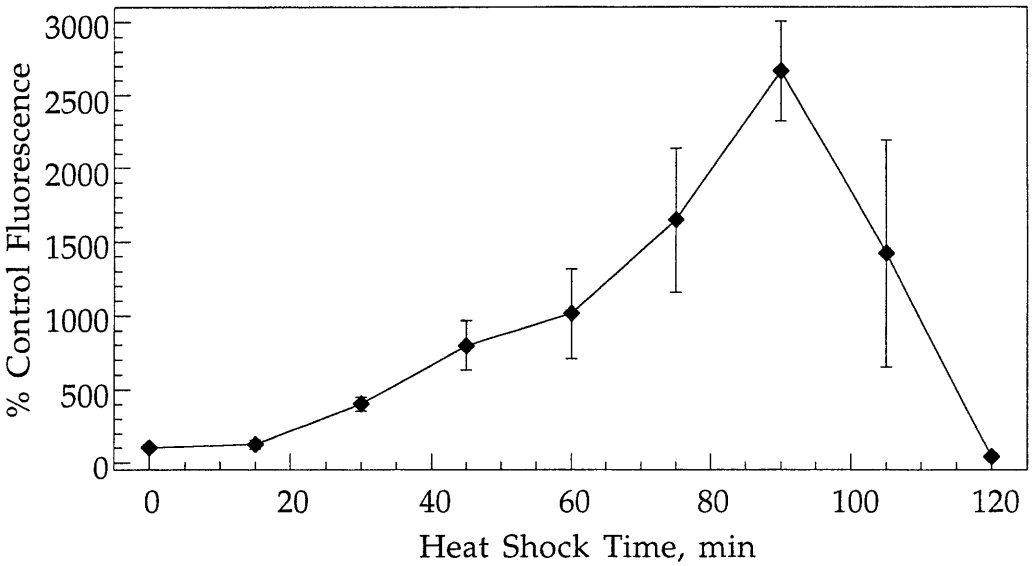


Figure 3.12: Effect of 45°C heat shock on EGFP expression in HeLa C27. After the heat shock the cells were induced at 37°C.

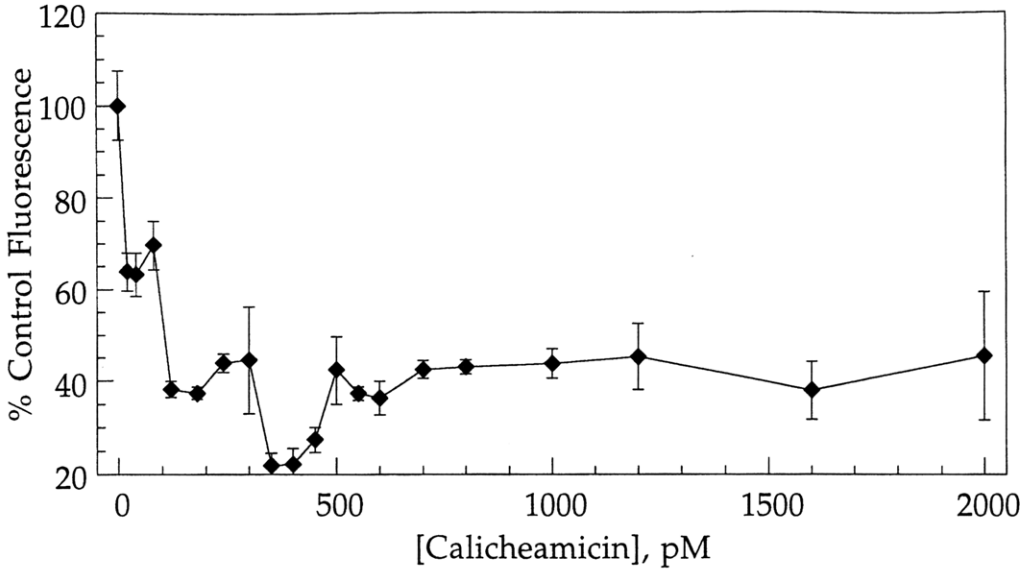


Figure 3.13: Effect of calicheamicin on EGFP expression in HeLa C27. A methanol solution (<1% total volume) of calicheamicin was added to the cells in PBS for a 15 min treatment. The cells were then washed and induced in complete medium.

3.3.5 Northern analysis of EGFP transcription in HeLa C27

To determine whether changes in EGFP expression occur at the transcriptional or post-transcriptional level, HeLa C27 cells were treated with cytotoxic agents and subjected to Northern analysis. Parallel cell samples were assayed for EGFP fluorescence. A separate control was prepared for MMS, with cells treated in serum-free media for 1.5 h and then given fresh complete media before induction. As Figure 3.14 reveals, EGFP transcription was greatly enhanced by *trans*-DDP, MMS and heat shock treatment and blocked by treatment with cisplatin. In addition to specific inhibition of the EGFP, there was also an overall decrease in transcription in the cisplatin-treated cells. The results summarized in Table 3.2 show that transcriptional effects account for the changes in EGFP expression.

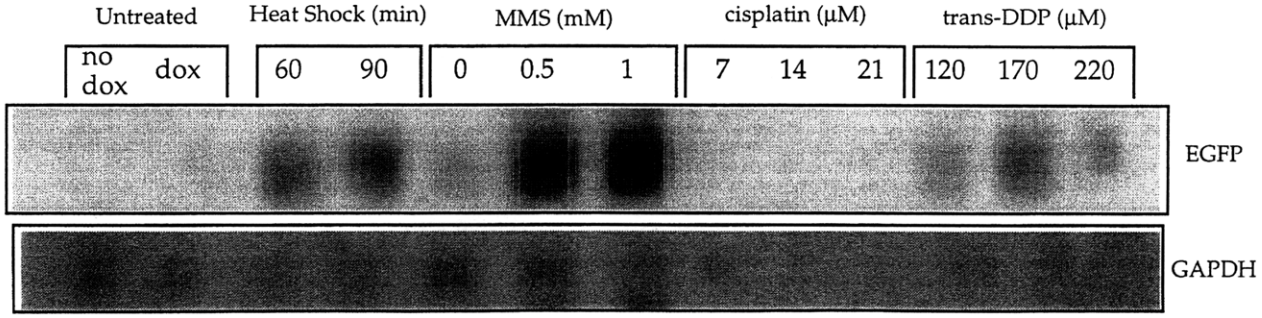


Figure 3.14: Northern blot of RNA prepared from HeLa C27 cells treated with various cytotoxic agents.

Table 3.2: Summary of Northern blot results. Parallel HeLa C27 samples were treated and analyzed separately for EGFP fluorescence and RNA.

Treatment	Relative EGFP fluorescence[a]	Relative EGFP RNA[b]
None	1.0	1.0
Heat shock, 60 min	5.1	18.6
Heat shock, 90 min	1.4	16.3
None, MMS conditions	1.0	1.0
0.5 mM MMS	9.4	36.1
1.0 mM MMS	13.6	73.0
7 μM cisplatin	0.8	0
14 μM cisplatin	0.7	0
21 μM cisplatin	0.7	0
120 μM *trans*-DDP	2.5	5.4
170 μM *trans*-DDP	7.0	24.0
220 μM *trans*-DDP	4.2	2.6

[a] EGFP fluorescence samples were prepared in triplicate. Fluorescence readings were divided by total protein to correct for uneven cell plating.

[b] RNA was quantitated by phosphorimager analysis of the Northern blot. All readings were normalized for loading by using GAPDH results.

3.3.6 Effect of tsHMG on the cisplatin response of HeLa C27

Transient transfection was used to study the effect of an HMG-domain protein on the cellular response to cisplatin. First, HeLa C27 cells were transfected with the pcDNA3.1-tsHMG expression plasmid and induced with doxycycline to determine whether transient transfection and doxycycline induction were compatible procedures. Fluorescence measurements (data not shown) and Western analysis (Figure 3.15) confirmed that tsHMG and EGFP could be simultaneously expressed. Next a 13.5 h cisplatin co-treatment assay was performed. Cells were transfected both with pcDNA3.1-tsHMG or with pcDNA3.1 as a control. Figure 3.16 shows that tsHMG enhanced the inhibitory effect of cisplatin on EGFP expression.

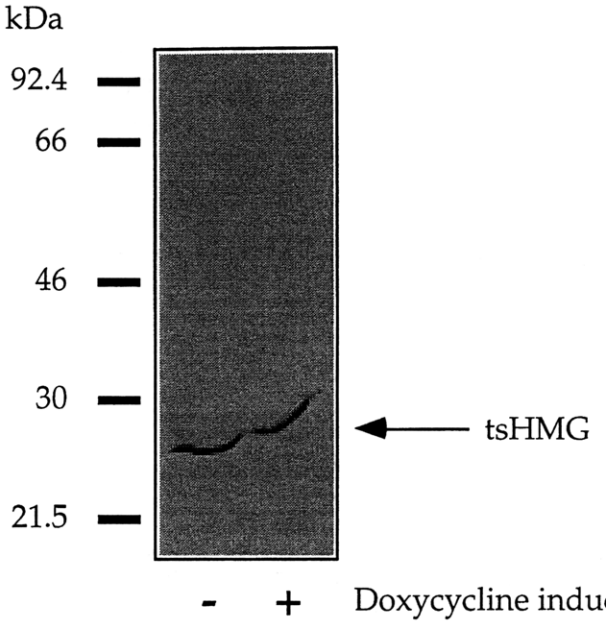


Figure 3.15: Western blot of HeLa C27 extracts after transient transfection with pcDNA3.1-tsHMG (50 μg/10 cm plate), probed for tsHMG expression with an anti-mtTFA antibody.

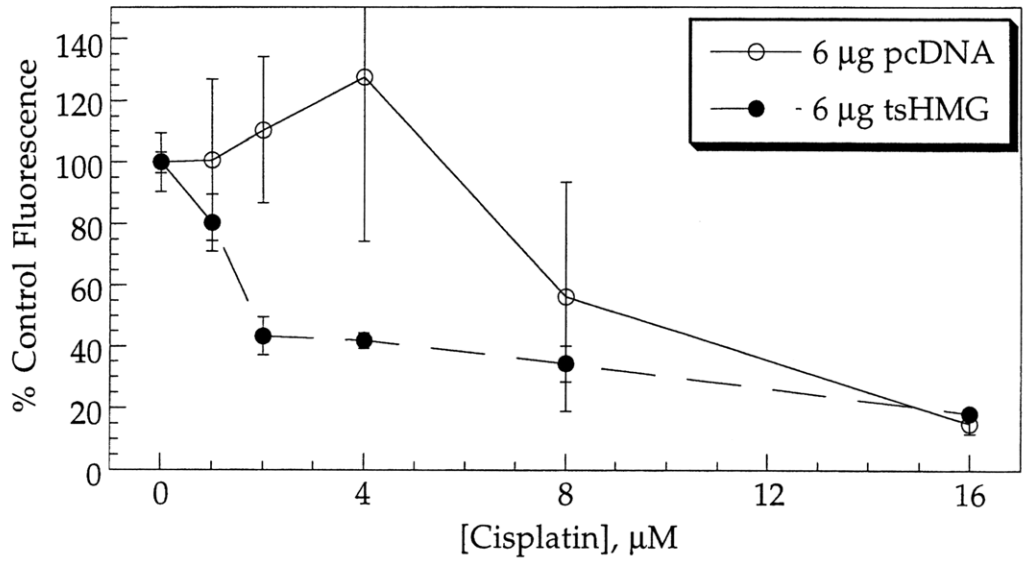


Figure 3.16: Effect of tsHMG on cisplatin inhibition of EGFP expression in HeLa C27. Cells were transfected with 6 μg of pcDNA3.1-tsHMG, or as a control, pcDNA3.1.

3.3.7 Effects of low-dose cisplatin treatment on EGFP expression

Although cisplatin treatment generally resulted in a dose-dependent decrease of EGFP expression, Figure 3.17 shows that low concentrations of the drug caused a modest increase in EGFP expression. The increase was two-fold after a 13.5 h co-treatment with 1.7 μM cisplatin, falling to less than 1.5-fold after a 17.5 h co-treatment.

Repetitions of this experiment showed consistent results but also consistently high error. To study other time-dependent effects, HeLa C27 cells were treated with low doses of cisplatin for short periods, then washed and incubated for a short time before induction. The results shown in Figure 3.18 demonstrate the difficulties of such an experiment. EGFP expression is highly dependent on the freshness of the growth medium, with a difference of two hours changing expression by up to 30%.

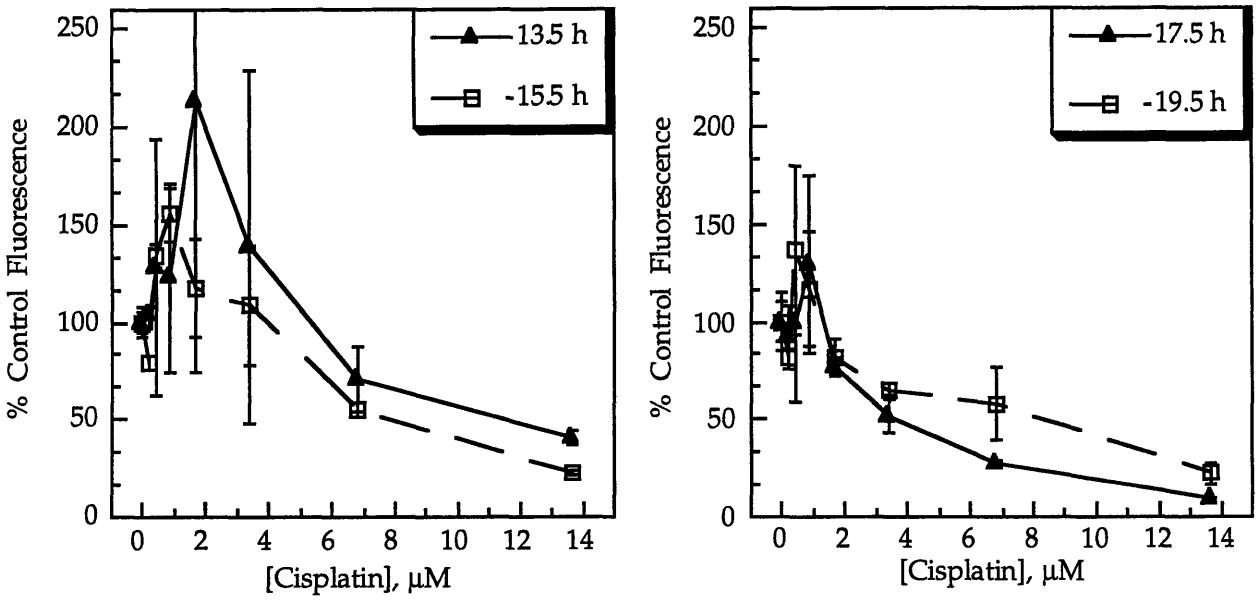


Figure 3.17: Increases in EGFP expression caused by low doses of cisplatin. HeLa C27 cells were cotreated with doxycycline and cisplatin and induced for the indicated time.

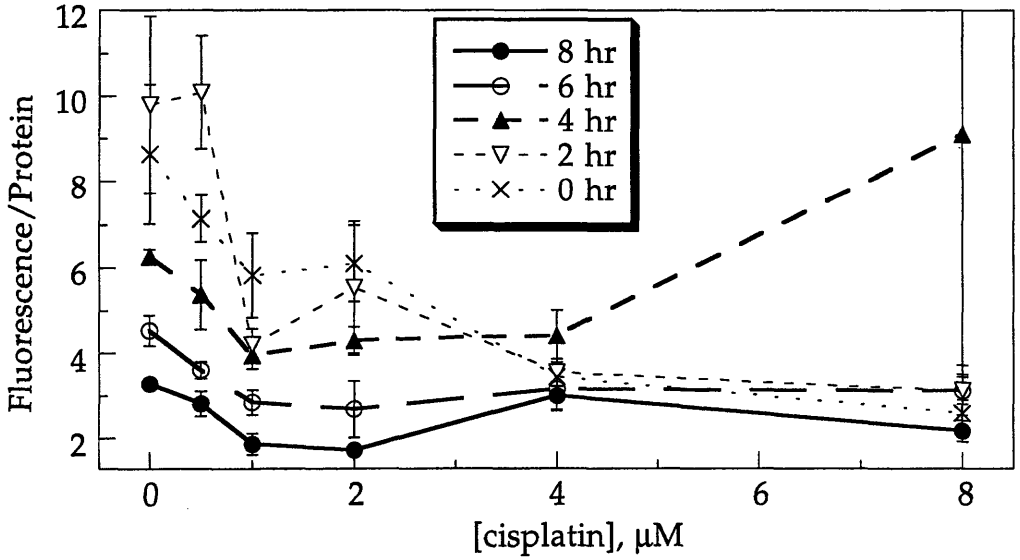


Figure 3.18: Effect of media changing on EGFP expression in HeLa C27. After a 4 hour cisplatin treatment, the cells were given fresh media and incubated for the indicated time before the 13.5 hour doxycycline induction.

3.3.8 Screening of platinum complexes by reporter gene assays

To test the potential utility of the EGFP assay as a screening method for new drugs, the 13.5 h co-treatment protocol was employed to evaluate a series of known platinum complexes. The compounds shown in Figure 3.19 are comparable in cytotoxicity to cisplatin, and they all inhibited EGFP expression at low doses. Higher concentrations of the moderately cytotoxic complexes shown in Figure 3.20 were required to inhibit EGFP expression. The inactive platinum complexes shown in Figure 3.21 had little or no effect on EGFP expression.

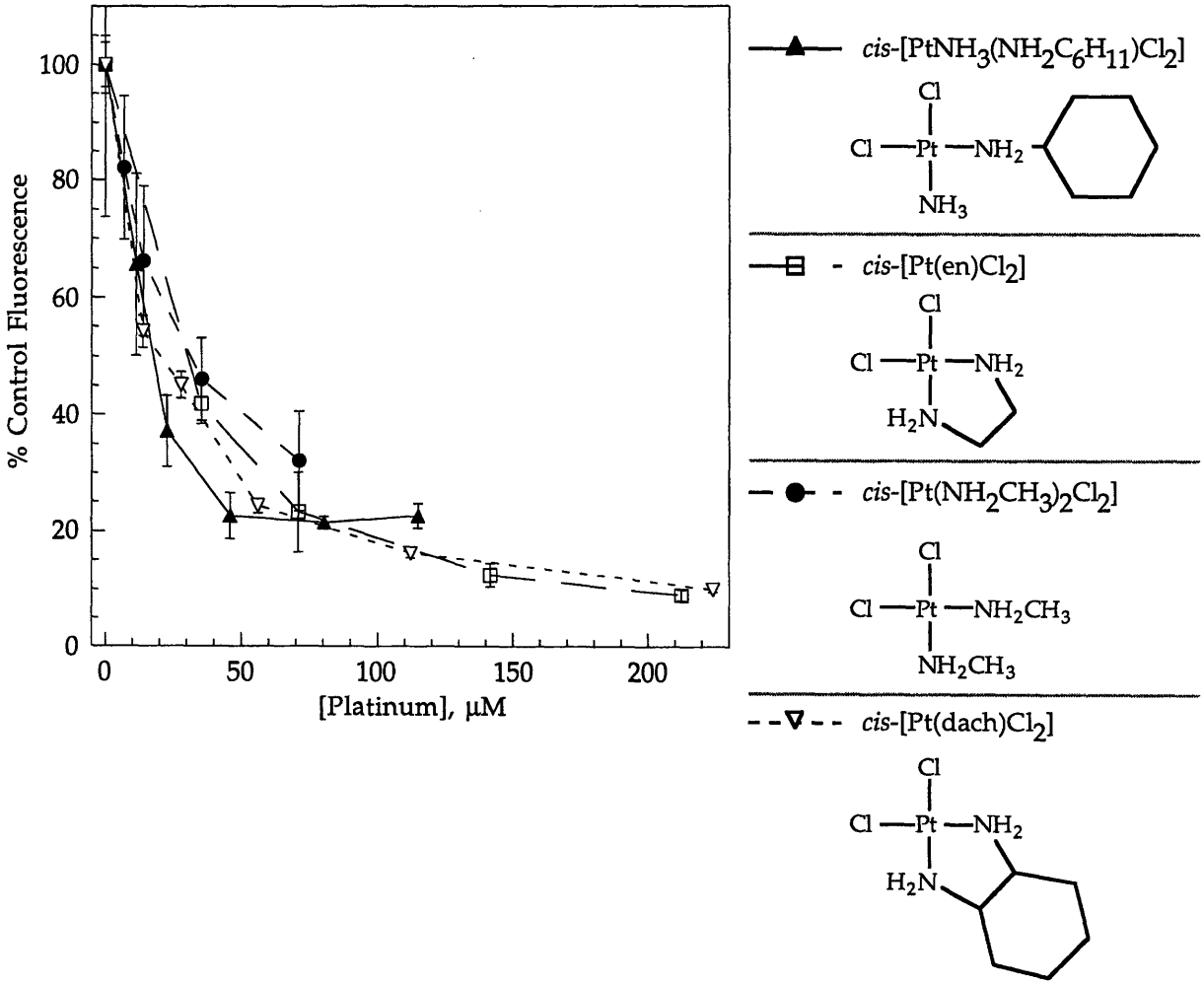


Figure 3.19: Results of EGFP expression assay using highly cytotoxic platinum complexes.

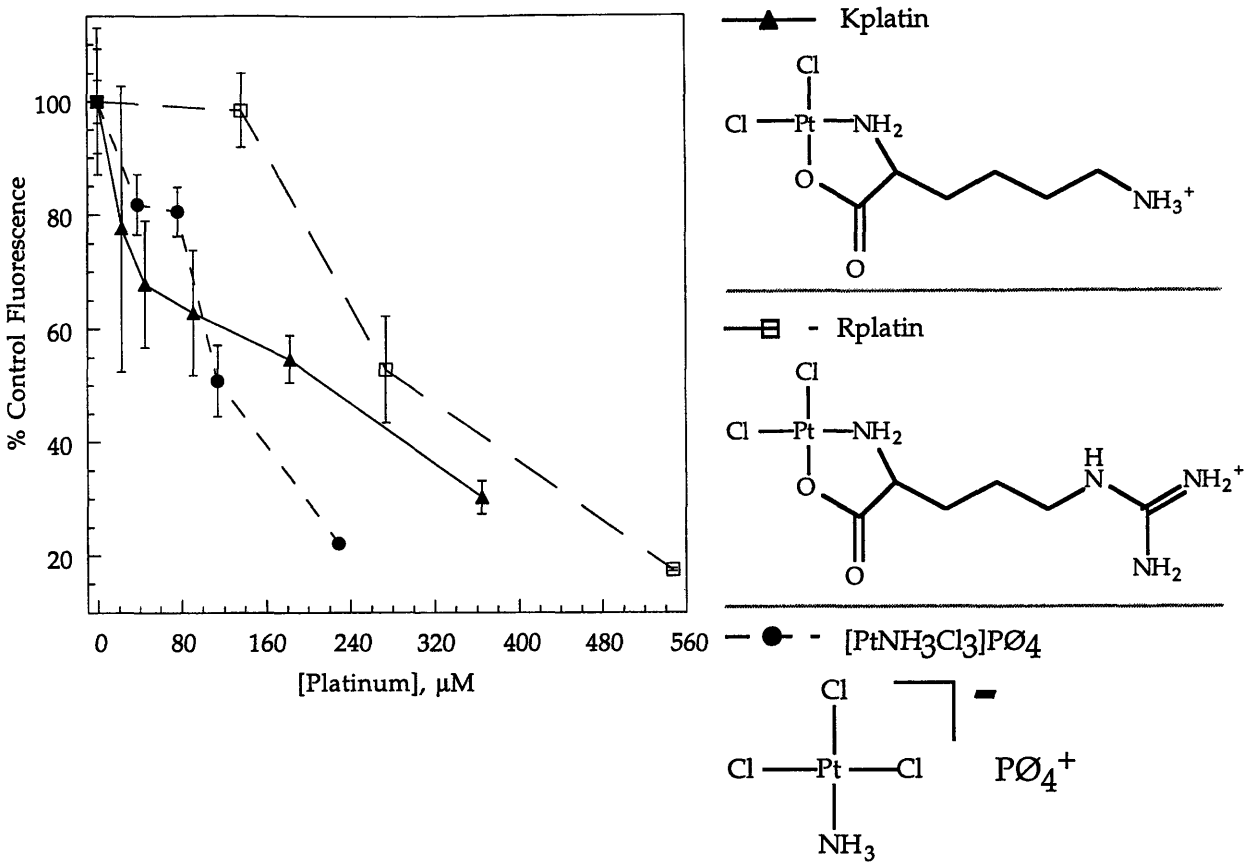


Figure 3.20: Results of EGFP expression assay using moderately cytotoxic platinum complexes.

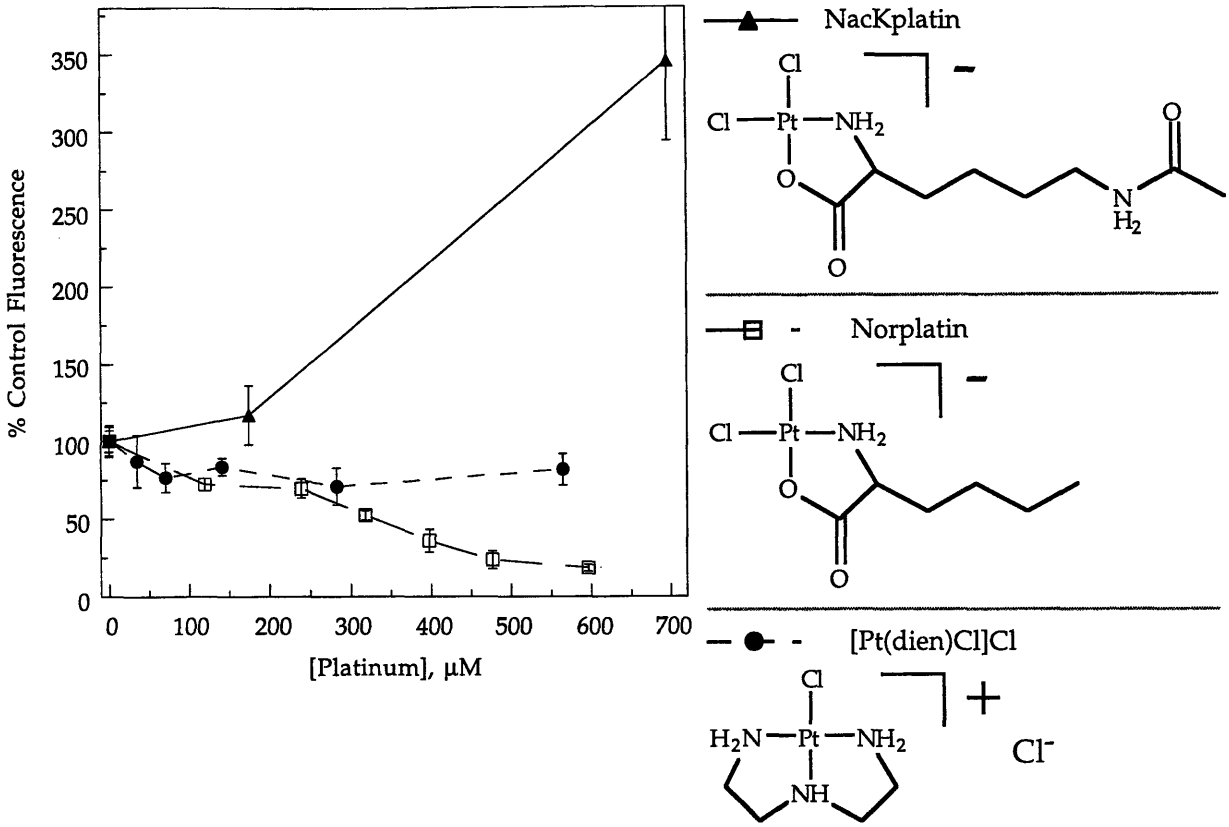
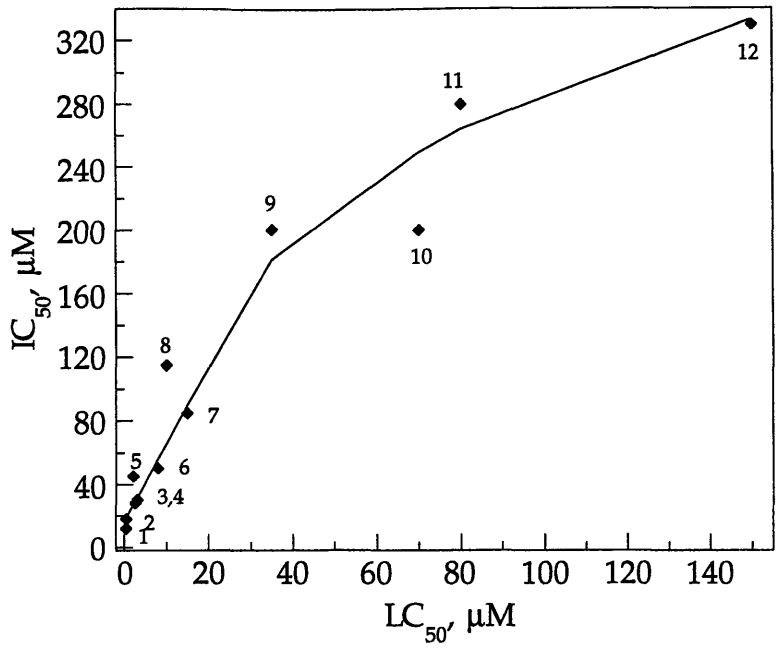


Figure 3.21: Results of EGFP expression assay using minimally cytotoxic platinum complexes.

The 13.5 h co-treatment method was employed to measure IC_{50} values for various platinum complexes, where IC_{50} is the platinum concentration at which EGFP expression was reduced to 50% of control. Colony counting cytotoxicity assays were used to measure the LC_{50} values for the same complexes, where LC_{50} is the concentration at which 50% of the cells failed to form colonies. Figure 3.22 plots the correlation between LC_{50} and IC_{50} for the tested compounds.



1 cisplatin
2 *cis*-[$PtNH_3(NH_2C_6H_{11})Cl_2$]
3 *cis*-[$Pt(NH_2CH_3)Cl_2$]
4 [$Pt(en)Cl_2$]
5 *cis*-[$Pt(dach)Cl_2$]
6 *trans*-[$Pt(NH_2CH_3)_2Cl_2$]
7 *cis*-[$Pt(NH_2\text{-}iPr)_2Cl_2$]
8 [$Pt(NH_3)Cl_3$]$P\emptyset_4$
9 [$Pt(NH_3)_3Cl$]Cl
10 [Pt(lysine)Cl_2]
11 [Pt(arginine)Cl_2]
12 [Pt(norleucine)Cl_2]

Figure 3.22: Comparison of IC_{50} versus LC_{50} for a series of platinum complexes.

To study the activity of platinum complexes with another reporter gene assay, Jurkat C2 cells were treated with *cis*-DDP, *trans*-DDP and $K_2[PtCl_4]$. The carbachol-dependent induction of β-lactamase was quantitated by measuring the ratio of intact and cleaved CCF2-AM substrate (Figure 3.23a). The IC_{50} was 8 µM for cisplatin, but neither *trans*-DDP nor tetrachloroplatinate blocked β-lactamase induction at concentrations up to 100 µM. The sensitivity of Jurkat C2 cells to *cis*- and *trans*-DDP was measured by trypan blue and DAPI staining. A 19.5 h cisplatin treatment resulted in an LC_{50} of 30 µM and an AC_{50} of 15 µM. *trans*-DDP had an LC_{50} of 100 µM after 19.5 h, but never caused apoptosis in more than 35% of the cells. The CCF2 cleavage assay was repeated (Figure 3.23b) in the constitutively expressing CMV-bla-Jurkat cell line. In that case, the IC_{50} was 10 µM for cisplatin, 100 µM for *trans*-DDP, and tetrachloroplatinate did not affect β-lactamase expression.

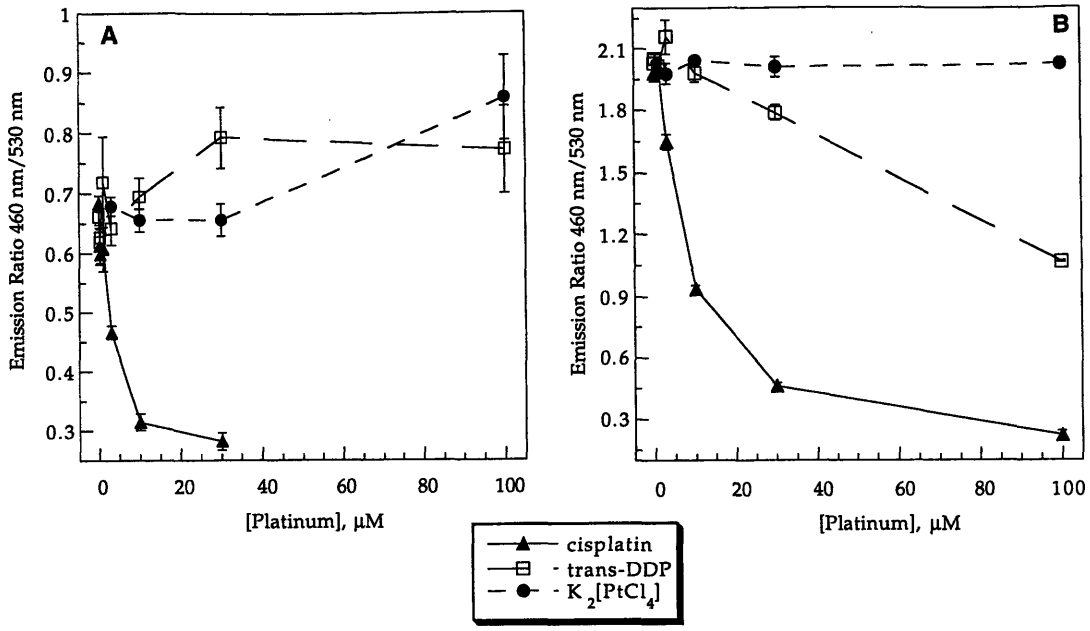


Figure 3.23: Effect of platinum treatment on β-lactamase expression, as measured by CCF2 cleavage, in two Jurkat cell lines. A: C2 Jurkat cells were treated with platinum for 20 hours and then induced with carbachol for 4 hours. B: CMV-bla Jurkat cells were treated with platinum for 24 hours.

3.4 Discussion

3.4.1 Constitutive expression of EGFP in HeLa cells

In the cloned HeLa cell lines that constitutively expressed EGFP, no change in EGFP fluorescence was observed upon treatment with cisplatin, even at concentrations that killed the cells within 24 h. Because these cell lines express high levels of EGFP with a half life of 20-30 h, the residual EGFP concentrations in the cells prior to cisplatin treatment are probably sufficient to obscure any effect from platinum treatment. Recently, a series of GFP mutants with short (2-3 h) half-lives was made commercially available. These mutants may permit the use of constitutively expressed EGFP as a reporter of platinum cytotoxic activity.

3.4.2 Inducible expression of EGFP in HeLa Tet-On cells

The effects of cytotoxic agents on EGFP expression in HeLa C27 cells were surprising in that different agents yielded two distinct types of response. For one class of cytotoxic agent, including *trans*-DDP, other trans platinum complexes, and, to a greater extent, MMS and heat shock, EGFP expression rose sharply, reached a

maximum, and then fell with increasing levels of damage. In the other class were cisplatin, other cis platinum complexes, and calicheamicin, where a dose-dependent decrease in EGFP expression occurred. Very low (<2 μM) concentrations of cisplatin caused an approximately 200% increase in EGFP expression relative to the untreated control, quite modest compared to the maximal values of 800%, 2100% and 2500% for trans platinum complexes, MMS and heat shock, respectively. Northern analysis revealed a correlation between EGFP fluorescence and transcription of the gene; furthermore, cisplatin-treated cells had lower total RNA content than others, indicating an overall transcriptional decrease. Although various forms of DNA damage can increase or decrease reporter gene expression, there are no reports in which different cytotoxic agents caused opposite effects on transcription from the same promoter. There also are no examples of a single cytotoxic agent both enhancing and inhibiting gene expression from the same promoter in a dose-dependent manner.

The contrasting effects were not a function of overall cytotoxicity. For example, *trans*-[$Pt(NH_2CH_3)_2Cl_2$] caused a dose-dependent increase in EGFP expression, from one to five times its LC_{50} value of 8 μM. By contrast, [Pt(lysine)Cl_2] brought about a dose-dependent decrease at values ranging from 0.3-5 times its 70 μM LC_{50} value. Apoptosis cannot explain the different effects either. Apoptosis is not induced by *trans*-DDP, but it is induced by heat shock [36,37], MMS [38], and JM 335, the platinum(IV) complex shown in 3.9 [39], all of which stimulate EGFP expression. The results are also unlikely to be a consequence of the specific site of integration of the TRE-EGFP construct into the host genome since similar results were obtained with HeLa C28.

The upregulation of the EGFP by heat shock as well as DNA damaging agents suggests that it is a general stress response. The mammalian stress response involves the induction of many types of genes including heat shock proteins [40,41], stress activated protein kinases (JNK SAPK) [42,43], the p53-dependent cell cycle arrest and apoptosis pathway proteins, and DNA repair enzymes [15,16]. Although the Tet-On gene expression system is not endogenous to mammalian cells, this artificial construct could nonetheless also be activated by stress. The damage induced by cis platinum complexes and calicheamicin must be different from other forms of stress. It is not surprising that calicheamicin blocks gene expression, since it is a highly toxic compound that enters cells, cleaves DNA and induces apoptosis in less than 4 h [44,45]. The inhibition of EGFP transcription by many cis platinum complexes, of variable toxicity

but all capable of forming 1,2-intrastrand DNA cross-links, suggests a structural effect. The distortion of DNA (reviewed in [46]) induced by the 1,2-intrastrand cross-link could lead to differential protein recognition, and thus transcriptional consequences of cis platinum adducts compared to most other types of damage. The modest increase in EGFP at low doses of cisplatin could result from the conflicting influences of stress response and the unique structural consequences of its DNA adducts.

Cisplatin-DNA adducts are recognized by a variety of proteins [47], including those involved in nucleotide excision repair (NER), DNA mismatch repair, and the high-mobility group (HMG)-domain proteins. It seems unlikely that either of the repair systems is responsible for the different transcriptional effects, since NER processes *trans*-DDP intrastrand cross-links [48] and the mismatch repair pathway recognizes MMS damage [49]. The HMG-domain proteins, which specifically recognize DNA damaged with cis platinum complexes but not any of the other agents tested, could be involved. HMG-domain proteins may contribute to cisplatin cytotoxicity by blocking the repair of cisplatin-DNA adducts [50-53]. Repair blockage would cause cis platinum adducts to be more persistent than other forms of DNA damage. If upregulation of the EGFP is a response to stress in HeLa C27, however, then persistent DNA adducts might be expected further to induce rather than to block EGFP expression, unless the shielding is so effective that this damage is completely unrecognized.

It has also been proposed that cisplatin adducts could titrate HMG-domain proteins away from their natural binding sites [54]. Because many HMG-domain proteins serve as transcription factors, such an event could lead to cell death through the misregulation of transcription [55]. If the transcription factors necessary for EGFP induction specifically recognize cisplatin-DNA adducts, then stress- and doxycycline-induced EGFP transcription would be prevented by cisplatin and its structural analogs. This mechanism would also explain the modest increase in EGFP expression at low levels of cisplatin. Only when a sufficient number of adducts formed would the titration be effective.

The titration hypothesis also applies to non-HMG-domain transcription factors. It was recently shown that the basal transcription factor TATA binding protein-TFIID (TBP) recognizes both cisplatin- and UV-damaged DNA [56]. In a subsequent study [57], DNA modified with a series of damaging agents was assayed for TBP binding and for transcription inhibition in HeLa whole-cell extracts. For each type of adduct tested,

TBP binding correlated with transcription inhibition, with cisplatin and *cis*-[Pt(dach)Cl_2] having strong effects. In some experiments, *trans*-DDP-modified DNA bound TBP and inhibited transcription slightly, whereas MMS and [Pt(dien)Cl]Cl did neither. It was proposed that the kinked DNA structure formed by cis platinum adducts resembles the TBP binding site and can thus titrate the protein away from its normal transcriptional activator region. These results are entirely consistent with the present EGFP expression results, and the observation that *trans*-DDP enhances EGFP expression to a lesser extent than MMS or heat shock could be explained by low-level transcription factor binding, as observed in the TBP study.

3.4.3 Support for the repair shielding hypothesis

As mentioned in the previous section, HMG-domain proteins can mediate cisplatin cytotoxicity by shielding the adducts from repair. This behavior was observed in NER assays in which HMG-domain proteins inhibited the repair of the major 1,2-d(GpG) and d(ApG) intrastrand cisplatin-DNA adducts in vitro [51,53]. In *Saccharomyces cerevisiae*, Ixr1, an HMG-domain protein, modulated the sensitivity of yeast to cisplatin in a manner consistent with the shielding of the adducts from excision repair [50,52]. It has also been reported that HMG2 overexpression sensitized lung adenocarcinoma cells to cisplatin [58]. A recent study showed that expression of tsHMG enhanced cisplatin-induced apoptosis in HeLa Tet-On cells [59]. This protein is particularly interesting because cisplatin is especially effective against testicular tumors, and tsHMG recognizes [60] and blocks the NER [53] of cisplatin adducts with unusually high proclivity. To examine further the role of tsHMG in cisplatin cytotoxicity, the EGFP induction assay was performed on HeLa C27 cells expressing tsHMG. Transient transfection of C27 to express tsHMG augmented the cisplatin-dependent inhibition of EGFP expression, especially at low platinum concentrations. Because tsHMG is not normally expressed in HeLa cells, it is highly unlikely that this result is a consequence of additional transcription factor hijacking. The results are best explained by the repair shielding hypothesis. If the recognition of cisplatin-DNA adducts by transcription factors prevented gene expression, then repair blockage by tsHMG could augment that effect by allowing the damage to persist, as illustrated in Figure 3.24. As stated previously [61], the two mechanisms are not mutually exclusive and may, in fact, be synergistic.

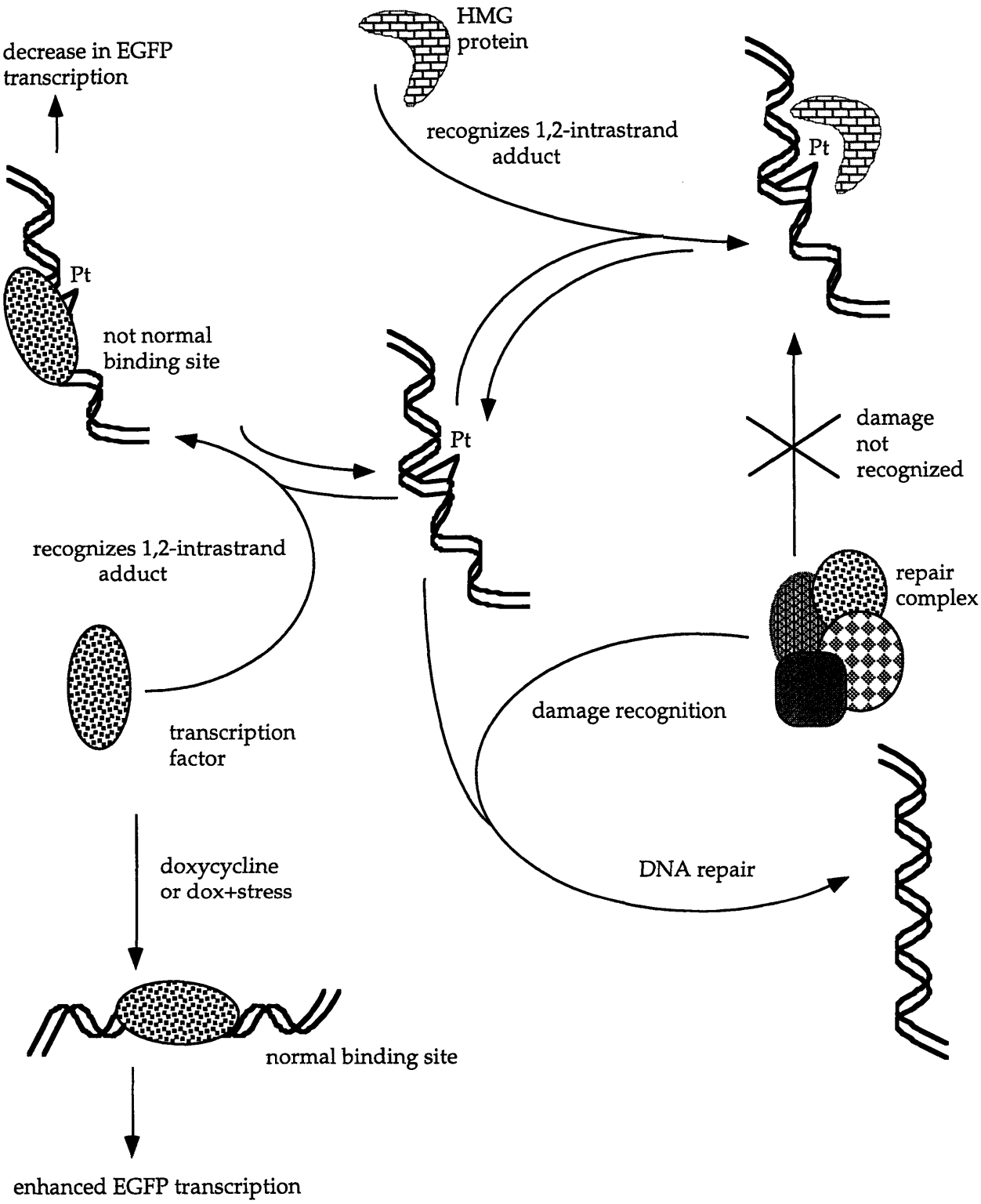


Figure 3.24: Proposed scheme to explain the effects of cytotoxic agents on EGFP expression.

Because the EGFP induction assay provides insight into the cellular processing of platinum damage, it was expected that it could be used to study the time course of damage recognition and repair. Technical considerations prevented the successful execution of such experiments, however. Because the timing of media changes greatly affected EGFP induction, it was difficult to compare the results of timed treatments and recovery periods. Although the inducible reporter gene system was ideal for many of the experiments reported in this chapter, a constitutively expressed reporter could eliminate several variables for repair and recovery experiments.

3.4.4 The use of reporter gene assays for screening platinum complexes

The two reporter gene assays used in this chapter are both good methods for screening platinum complexes to predict cytotoxicity. The EGFP induction assay yielded a good correlation between LC_{50} and IC_{50} values for a series of known platinum complexes. Although the EGFP method is much faster than any currently available cytotoxicity assay, with results available 13-14 h after platinum treatment, it is not yet suitable for high-throughput work. Modest levels of EGFP expression necessitate the lysis of cells from a 35-mm well in a small volume of buffer, followed by transferal of the lysate to smaller wells in order to intensify the fluorescence signal. With improvements in inducible gene expression, GFP fluorescence intensity and fluorescence detection methods, however, the assay could be adapted for high-throughput screening. The β-lactamase CCF2/AM assay, which showed a dose-dependent decrease in gene expression upon treatment with cisplatin but not with ineffective platinum complexes, also surpasses existing cytotoxicity assays in speed. The results are available within 24 h of platinum treatment, and the assay is performed with the aid of robotics for high-throughput screening. The promising initial results have inspired a collaborative effort to screen combinatorial libraries of platinum complexes with the β-lactamase assay [62].

An additional advantage to both reporter gene assays is that they screen platinum complexes for a biochemical effect, inhibition of gene expression, rather than measuring cell death as an endpoint. In that sense, they are mechanism-based screening methods. The major disadvantage is that both methods require stable cell lines expressing the desired reporter genes, whereas cytotoxicity assays can be performed on any cultured

cell line. Because it can take several months to clone stable transfectants, it is essential to choose relevant cell lines for platinum drug screening.

3.5 Conclusions

The work described in this chapter was undertaken to develop new screening methodologies for combinatorial libraries of platinum complexes. As the results for both the EGFP and β-lactamase assays indicate, that goal was achieved with two fast and convenient assays, one of which is already performed in a high-throughput environment. These assays represent a new approach to cell-based screening of platinum complexes, focusing on mechanistic events rather than cytotoxic endpoints. In addition to developing new screening methods using reporter genes, this research yielded some information on the mechanism of action of platinum anticancer drugs. The results imply that the upregulation of EGFP transcription is a general stress response in HeLa C27, and that the 1,2-intrastrand platinum-DNA cross-link is recognized by one or more proteins in a manner that blocks the stress response and down-regulates transcription. Although the identity of the recognition element(s) is not known, the results are consistent with transcription factor binding to 1,2-intrastrand platinum-DNA adducts. Whatever the mechanistic significance of the transcriptional decrease may be, it is clearly a cellular response to cisplatin, so the effect of an HMG-domain protein on that response was determined. The results, showing that tsHMG steepened the dose-response curve, support the concept that HMG-domain proteins block the repair of cisplatin adducts.

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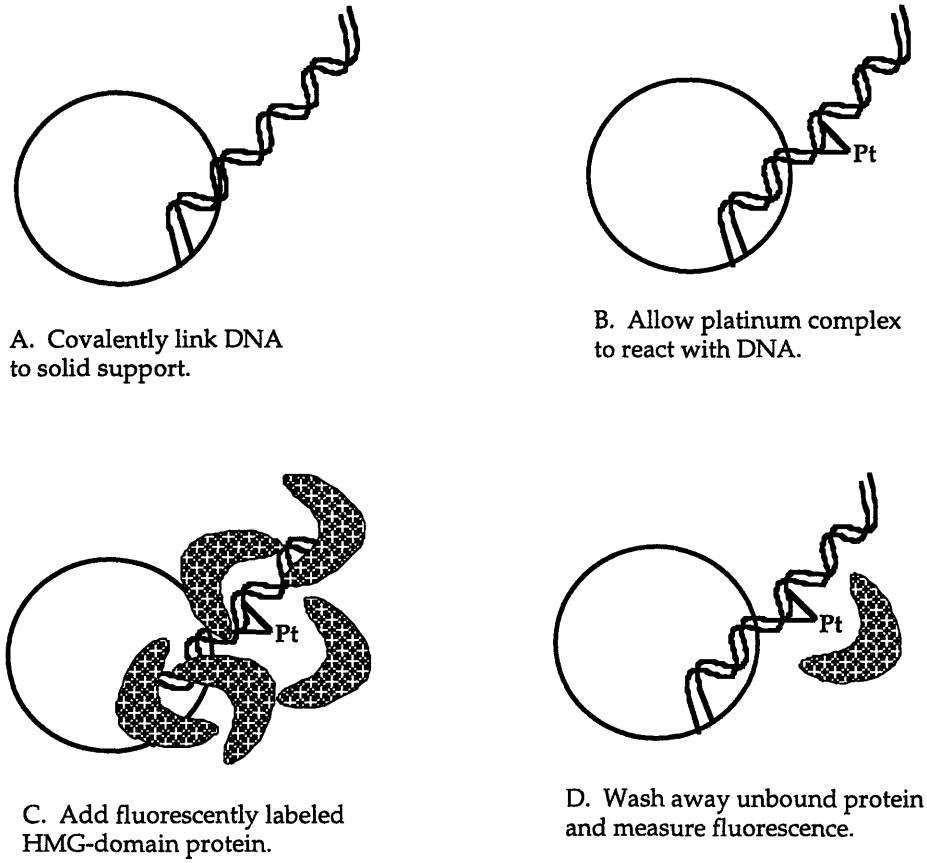
Appendix A

Cloning and Purification of the Fusion Protein HMG1–GFPuv

A.1 Introduction

The solution-phase assay described in Chapter 2 used a gel mobility shift assay to identify from platinum-amino acid mixtures a compound, Kplatin, that forms 1,2-intrastrand DNA cross-links specifically recognized by HMG1. The gel shift assay effectively quantitates protein-DNA binding but has several limitations in its application as a screening method for cytotoxic platinum complexes. For each set of platinum complexes, the DNA must be platinated, precipitated and quantitated. After the modified DNA is radiolabeled, a series of protein-DNA samples must be electrophoresed and the results analyzed with a phosphorimager. Not only are some of these steps labor-intensive, but cost and safety considerations preclude the use of radioactivity in high-throughput screening. A better approach would be to screen platinum complexes on solid phase supports [1-3]. Solid phase methods allow compounds to be identified by immobilization and position in a binding assay, and they can be performed with the aid of robotics to increase throughput [4]. Macromolecular binding assays frequently employ fluorescence [5] because fast and accurate detection methods are available. Small fluorescent moieties often can modify proteins and nucleic acids without disrupting structure and function. The scheme shown in Figure A.1 was envisioned, whereby an HMG-domain protein could be labeled, and DNA covalently

linked to a solid support, such that the binding of the fluorescent HMG-domain protein to platinated DNA could be quantitated.



A. Covalently link DNA to solid support.

B. Allow platinum complex to react with DNA.

C. Add fluorescently labeled HMG-domain protein.

D. Wash away unbound protein and measure fluorescence.

Figure A.1: Proposed solid-phase method for screening platinum compounds for Pt-DNA-HMG complex formation.

Amine or thiol groups on proteins can be chemically modified [5]. Lysine side chains, of which there are many on HMG1, and N-terminal amines react with isothiocyanates. Cysteine thiols can be modified by reaction with iodoacetamide. Because HMGdomB contains only a single cysteine residue, the thiol should be free under reducing conditions to react with a fluorescent label. In addition to these synthetic approaches, it is also possible to label fluorescently a protein by expressing it as a fusion with the GFP [6,7]. Although GFP fusions are typically expressed without purification in cells or organisms, it was expected that an HMG1-GFP fusion could be purified and used for screening methodologies. This appendix describes several attempts to prepare fluorescently labeled HMG-domain proteins with native DNA-binding activity.

A.1 Experimental

A.1.1 Fluorescein isothiocyanate (FITC) labeling of HMG1.

A standard protocol for lysine modification was followed [8]. HMG1 was dialyzed against FITC labeling buffer (50 mM boric acid, pH 9.2, 200 mM sodium chloride) at 4°C for 48 h. The dialyzed protein was spectrophotometrically quantitated by using the approximation 1 OD_{280}=0.74 mg/mL. FITC was weighed into a dry vial and dissolved in anhydrous dimethyl sulfoxide. A mixture of 28 μM HMG1 and 214 μM FITC was stirred in the dark for 2 h; the protein was then transferred to a Microcon 10 device and washed extensively with HMG1 storage buffer (50 mM Tris pH 7.3, 50 mM sodium chloride, 5 mM 2-mercaptoethanol). The binding of modified HMG1 to 123-bp DNA was assayed as previously described [9].

A.1.2 Fluorescent labeling of HMGdomB cysteine residues.

The Molecular Probes protocol for cysteine modification was followed [5]. A 1 mg sample of HMGdB was desalted in a Microcon 3 device (Amicon) and diluted in 2.2 mL of a degassed 50 mM Tris pH 7.5 buffer. In the dark, 112 μL of a 20 mM solution of 4-iodoacetamidosalicylic acid was added dropwise to the protein solution. After the reaction was stirred for 2 h at room temperature in the dark, excess reagent was consumed by adding 5 μL of 1 M dithiothreitol (DTT). The protein was washed extensively in a Microcon 3 device with a 50 mM NaCl, 50 mM Tris pH 7.5 buffer. The protein was analyzed by UV/visible and fluorescence spectroscopy and by SDS/PAGE. The fluorescently labeled HMGdomB was used in a gel shift assay with a 15-bp probe as previously described [9]. After electrophoresis the gel was dried and quantitated on a Molecular Dynamics PhosphorImager.

A.1.3 Cloning of the HMG1-GFPuv expression plasmid.

All enzymes were purchased from New England Biolabs unless otherwise indicated. The following primers were used to amplify the HMG1 gene from the HMG1 expression plasmid.

Top HMG1-PCR: (boldface: HindIII site)

5′-GAAGGAGATAT**AAGCTT**GGGCAAAGGAGATCCTAAGAAGCCG-3′

Bottom HMG1-PCR: (boldface: SmaI site)

3′-CTACTTCTTCTTCTACTACTACTACTTG**GGGCCC**CAAGATCGCGT-5′

The PCR reaction was performed by using an EZStart 100 reaction tube (Molecular Bio-Products). To the prepared reaction tube, 0.2 μg of template DNA (pHMG1) and 0.5 μg of top and bottom primers were added, with 5 U of Pfu polymerase (Stratagene) in a total volume of 50 μL. The reaction cycles were as follows:

1 cycle: 94°C, 45 s.

25 cycles: 94°C, 45 s; 55°C, 45 s; 72°C, 120 s.

1 cycle: 72°C, 600 s.

The PCR product was precipitated with ethanol and digested with HindIII and SmaI; the enzymes were heat-inactivated and the products were used without further purification.

The pGFPuv bacterial expression vector (Clontech) was amplified in *E. coli* and purified with a Qiagen 500 plasmid purification kit. The plasmid was digested with HindIII and SmaI, purified on a 1% agarose gel and precipitated with ethanol.

The HindIII/SmaI digest of the HMG1 PCR product was combined with the HindIII/SmaI digest of pGFPuv and incubated for 16 h at 16°C with T4 DNA ligase in the presence of 10 μM ATP. The ligation mixture was transformed into chemically competent XL1-Blue *E. coli* and plated on LB ampicillin agar plates. The resultant colonies were inoculated into LB ampicillin media and grown to saturation; the plasmids were isolated by the boiling lysis method. The plasmids were digested with XhoI and NsiI and analyzed on a 1% agarose gel for the expected 1030-bp insert. Eight colonies having the insert were subcloned on LB amp plates for further study. Two of the plasmids were purified with a Qiagen-500 kit for restriction mapping and sequencing. The putative pHMG1-GFPuv plasmids were digested with a series of restriction enzymes to verify that the HMG1 gene had been properly inserted. The products were analyzed on a 1% agarose gel. The two plasmids were submitted to the MIT Biopolymers facility for automated DNA sequencing on the ABI Prism system. The primers were as follows:

Forward:
5′-ATAACAATTTCACACAGGAAACAGCTATGACCA-3′
Middle forward:
5′-GGCCGCCAAGCTGAAGGAGAAGTATGAGAAGGATATT-3′
Reverse:
3′-TACTCATTTCCTCTTCTTGAAAAGTGACCTCAA-5′
Middle reverse:
3′-GGGTTCTCCGGAGGAAGCCGGAAGAAGAACAAGACA-5′

A.1.4 Expression and purification of HMG1-GFPuv.

The pHMG1-GFPuv-1 plasmid was transformed into BL21DE3 *E. coli* by electroporation. The bacteria were plated on LB ampicillin agar plates and one resulting colony was inoculated into 250 mL of LB ampicillin media. The starter culture was grown to saturation and then inoculated into eight prewarmed 500-mL flasks of LB ampicillin. After 3 h of shaking at 37°C, the OD_{600} was 0.6 and the protein expression was induced with 0.5 mg/mL of IPTG; the cells were shaken for an additional 3 h at 37°C. The cells were harvested by centrifugation (5000 x g, 15 min) and resuspended in 100 mL of solution A (described below) containing 1mg/mL Pefablock. The bacteria were lysed by sonication and the cell debris was cleared by ultracentrifugation (40 krpm, 30 min, Beckman 45Ti rotor).

The purification method was an adaptation of a recent successful HMG1-GFPuv purification [10]. The solutions used for column chromatography all contained 2.5% (v/v) glycerol, 5 mM DTT, 0.025% (v/v) NP40, 0.025% (v/v) Tween 80. In addition, the solutions contained the following: A, 1 mM $MgCl_2$ and 10 mM NaCl; B, 300 mM $MgCl_2$ and 50 mM NaCl; C, 10 mM NaCl; D, 100 mM potassium phosphate pH 7.4 and 10 mM NaCl; E, 300 mM potassium phosphate pH 7.4 and 10 mM NaCl.

The crude protein solution was loaded at 2 mL/min onto a 12 cm long, 3 cm diameter hydroxyapatite column that was pre-equilibrated in solution A. The column-bound protein was washed with solution A until no UV absorbance was detected; it was then washed with solution B for 1 h at 2 mL/min. The column was then washed with one column volume of solution C and then with solution D. When green protein began to elute from the column, 4-mL fractions were collected; more fractions were collected as a gradient from 100:0 to 0:100 D:E gradient was run. The green fractions

were analyzed by SDS/PAGE and the gels were stained with SYPRO Orange (BioRad) and photographed with a Polaroid camera equipped with a transilluminator. All of the fractions containing an approximately 60 kDa protein were pooled, concentrated in Centriprep 10 devices (Amicon), and diluted into 30 mL of solution D.

The hydroxyapatite column was washed with 100 mL of solution E and was then equilibrated with 200 mL of solution D. The partially purified HMG1-GFPuv was loaded on the column at 2 mL/min and washed with 120 mL of solution D. The protein was then eluted first with a linear gradient from 100:0 to 30:70 D:E, 2 mL/min, over 40 min. Further elution was accomplished by a second linear gradient from 30:70 to 100:0 D:E, 2 mL/min, 100 min. The remaining protein was eluted by washing the column with solution E for 20 min at 2 mL/min. The protein was collected in 4-mL fractions; the fractions were analyzed on SDS gels stained with SYPRO Orange.

The fractions that contained a 60-kDa product were pooled, concentrated in Centriprep 10 devices and diluted in 10 mL of FPLC buffer (150 mM potassium phosphate pH 7.4, 0.025% NP40, 0.025% Tween 80, 5 mM DTT). A Superdex 75 column was washed in 400 mL of degassed FPLC buffer at 1.5 mL/min. The protein was loaded onto the column and eluted at 1.5 mL/min with 4 mL fractions collected. The fractions were analyzed on an SDS gel, and the two visibly green fractions, which contained the 60-kDa protein, were concentrated in a Centricon 30 device and exchanged into a 50 mM Tris pH 7.5, 50 mM NaCl buffer. The final protein sample was quantitated by UV/visible spectrophotometry and stored at a concentration of 10 mg/mL in frozen aliquots.

A gel shift assay to measure the binding of platinated 123-bp DNA to HMG1-GFPuv was performed as previously described [9]. The excitation and emission spectra of solutions of HMG1-GFPuv and GFPuv were recorded on a Hitachi F-3010 fluorescence spectrophotometer.

A.1.5 Expression and purification of GFPuv

The solutions used for GFPuv purification all contained 5% (v/v) glycerol, 1 mM DTT, 0.01% (v/v) NP40. In addition, the solutions contained the following: A, 1 mM $MgCl_2$ and 10 mM NaCl; B, 300 mM $MgCl_2$ and 50 mM NaCl; C, 10 mM NaCl, 10 mM $MgCl_2$ and 100 mM potassium phosphate pH 7.4. GFPuv was overexpressed from the pGFPuv plasmid in *E. coli* in the same manner as HMG1-GFPuv. The cells were lysed

in solution A. The crude protein solution was loaded at 2 mL/min onto a 12 cm long, 3 cm diameter hydroxyapatite column that was pre-equilibrated in solution A. The column-bound protein was washed with solution A until no UV absorbance was detected; it was then washed with solution B for one hour at 2 mL/min. A linear gradient from 100% B to 100% C was run, with 80 4-mL fractions collected. The green fractions were analyzed by SDS/PAGE and the gels were stained with SYPRO Orange. The fractions containing a pure 30-kDa protein were pooled and concentrated in an Ultrafree 15 device (Millipore).

A.2 Results

A.2.1 Modification of HMG1 with FITC

As Figure A.2 shows, the reaction of HMG1 with FITC inhibited its specific recognition of cisplatin-modified DNA. Even doubling the usual HMG1 concentration did not result in protein-DNA binding.

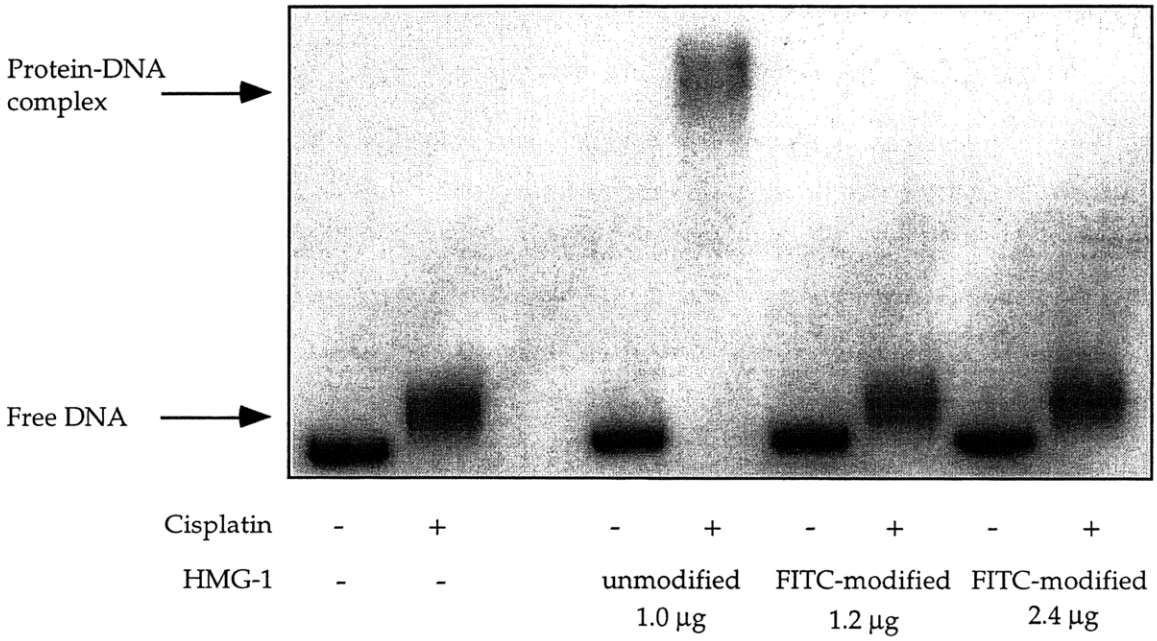


Figure A.2: Gel mobility shift assay comparing the binding of 123-bp DNA to HMG1 and lysine-modified HMG1.

A.2.2 Modification of HMGdomB with 4-iodoacetamidosalicylic acid

The UV/visible spectrum of labeled HMGdomB had a shoulder at 300 nm, where the dye absorbs. The dye: protein ratio, calculated from the relative absorbances and extinction coefficients, was 0.38. Fluorescence spectroscopy confirmed the presence

of the salicylic acid label on the protein; upon excitation at 304 nm, the protein had the expected 400 nm emission. The labeled protein ran like HMGdomB on an SDS polyacrylamide gel; this result was expected since the label should have added only 321 Da to the protein molecular weight. Figure A.3 shows the results of a gel mobility shift assay comparing the DNA binding of unmodified and modified HMGdomB. The results are quantitated in Table A.1. The DNA-binding ability of HMGdomB was inhibited by the presence of the fluorescent label; the magnitude of inhibition was comparable to the degree of labeling of the protein (38%).

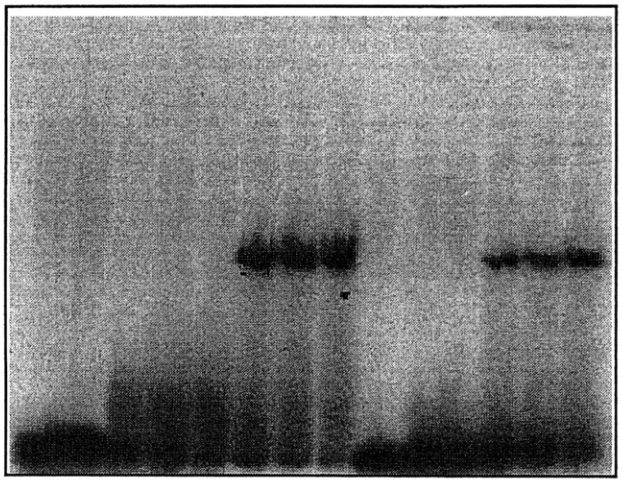


Figure A.3: Gel mobility shift assay comparing the binding of a 15-bp platinated DNA duplex to HMGdomB and cysteine-modified HMGdomB. The DNA was used at a final concentration of 20 nM.

Table A.1: Binding of HMGdB and cysteine-modified HMGdB to a 15-bp DNA duplex.

Unlabeled HMGdB, μM	Labeled HMGdB, μM	% Shift, unplatinated DNA	% Shift, cisplatin-modified DNA
0.10	0	28	52
0.20	0	34	56
0.50	0	45	59
0	0.10	7	33
0	0.20	9	37
0	0.50	13	48

A.2.3 Cloning of the HMG1-GFPuv expression plasmid.

The ligation of the HMG1 gene into HindIII/SmaI-digested pGFPuv, and subsequent transformation of the reaction mix into XL1-Blue cells, resulted in approximately 100 ampicillin-resistant colonies. The 64 colonies that were selected and grown

in 2 mL of LB ampicillin media were harvested by centrifugation and inspected under longwave ultraviolet light. Three classes of cell pellets were observed: 22 non-fluorescent, 26 bright green fluorescent, and 16 moderately green fluorescent. The plasmid DNA that was isolated from several members of each group was analyzed for the presence of the desired insert. The 1030-bp NsiI/XhoI insert that was expected for plasmids encoding the HMG1-GFPuv fusion protein was observed only in the DNA derived from moderately green fluorescent colonies.

Figure A.4 shows the results of restriction mapping of two putative pHMG1-GFPuv plasmids. The digestions with various restriction enzymes resulted in fragments of the expected lengths. Two putative pHMG1-GFPuv plasmids were submitted for sequencing with four primers: forward, reverse, middle forward, and middle reverse. It was necessary to use primers that started at the middle of the HMG1 insert in order to verify the correct sequence of the lengthy 640-bp insert. The sequencing results confirmed that pHMG1-GFPuv-7 had no mutations.

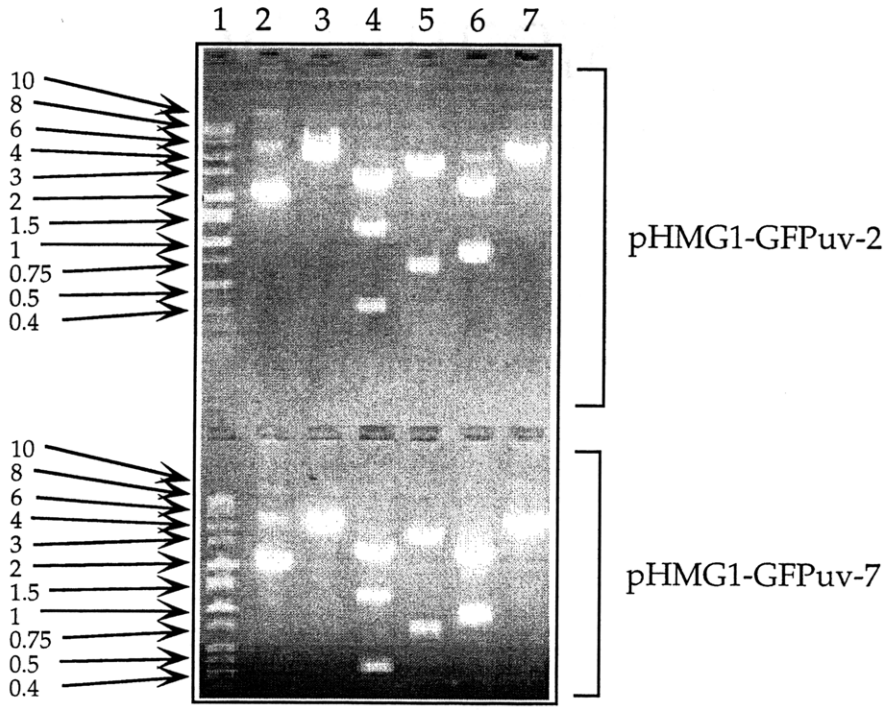


Figure A.4: Ethidium bromide-stained agarose gel showing the restriction digestion products of two HMG1-GFPuv plasmids. Lane 1: DNA size standards in kilobases. Lane 2: Undigested plasmid. Lane 3: NsiI digest; 3.9 kb expected. Lane 4: StuI/HindIII digest; 2.4 and 1.5 kb expected. Lane 5: SmaI/HindIII digest; 3.3 kb and 0.64 kb expected. Lane 6: NdeI digest; 2.3, 0.85 and 0.76 kb expected. Lane 7: BamHI digest; 3.9 kb expected.

A.2.4 Expression and Purification of the HMG1-GFPuv Fusion Protein.

Figure A.5a shows that two rounds of hydroxyapatite chromatography resulted in HMG1-GFPuv with three major impurities, all less than 45 kDa. Figure A.5b shows that gel filtration FPLC removed most of the impurities from the fusion protein; the overloaded lanes (9-10) show that the protein is more than 90% pure.

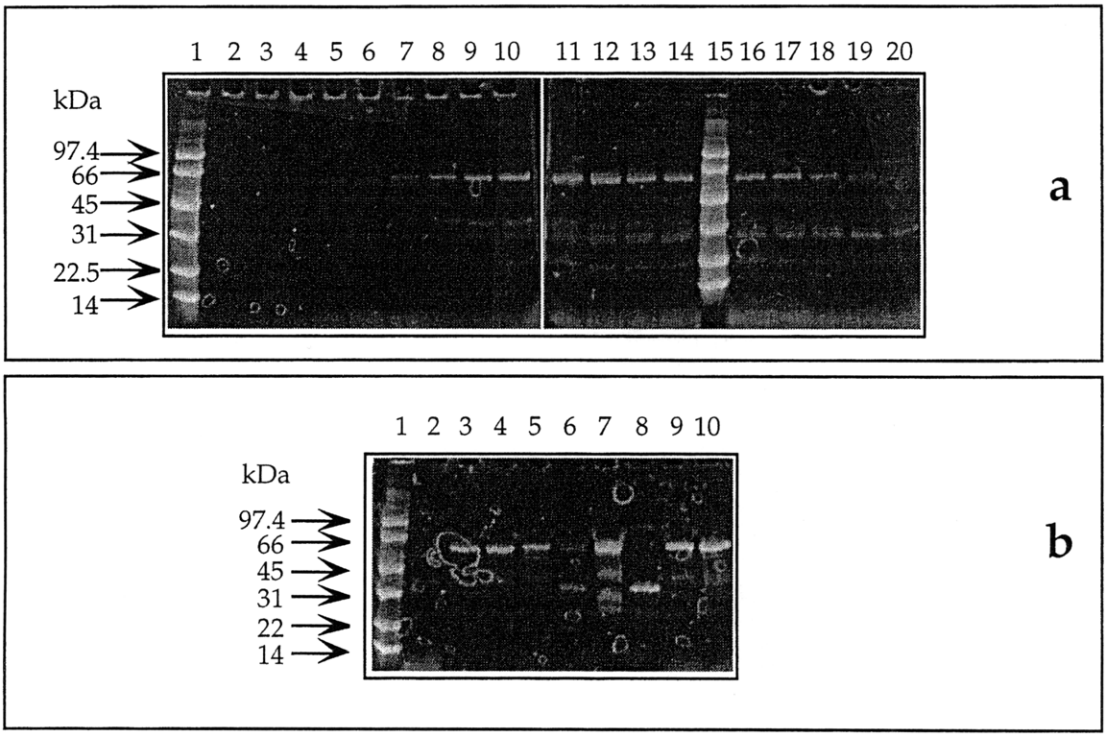


Figure A.5: SYPRO Orange-stained SDS-PAGE gels showing the purification of HMG1-GFPuv. A.5a: Results of hydroxyapatite chromatography (second round) of HMG1-GFPuv. Lanes 1 and 15: Molecular weight markers. Lanes 2-14 and 16-20: Samples of fractions eluted from the hydroxyapatite column in a phosphate gradient. The fractions in lanes 5-14 and 16-19 were pooled. A.5b: Results of Superdex 75 size exclusion FPLC chromatography of hydroxyapatite-purified HMG1-GFPuv. Lane 1: Molecular weight markers. Lanes 2-6: Samples of fractions eluted from the Superdex column. Lane 7: Previously purified HMG1-GFPuv. Lane 8: GFPuv. Lane 9: Sample from lane 3, overloaded. Lane 10: Sample from lane 4, overloaded. The fractions in lanes 3-4 were pooled.

The fluorescence spectra of HMG1-GFPuv are shown in Figure A.6. The 395 nm excitation maximum and 509 nm emission maximum were identical to those observed for GFPuv (data not shown). Like HMG1, the fusion protein bound specifically to cisplatin-modified DNA as shown in Figure A.7. A control experiment (data not shown) confirmed that GFPuv had no specific interaction with DNA.

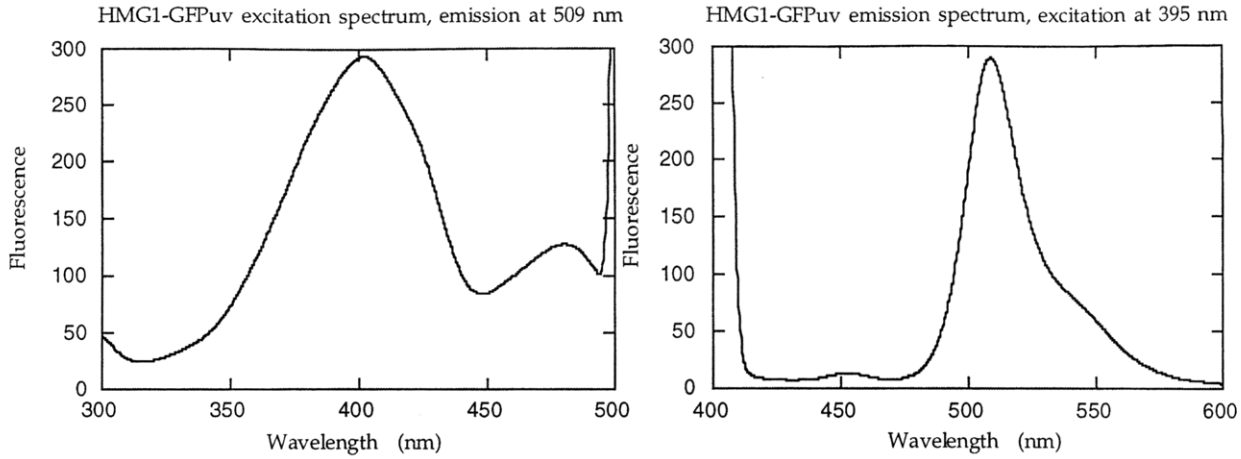


Figure A.6: Fluorescence spectra of HMG1-GFPuv.

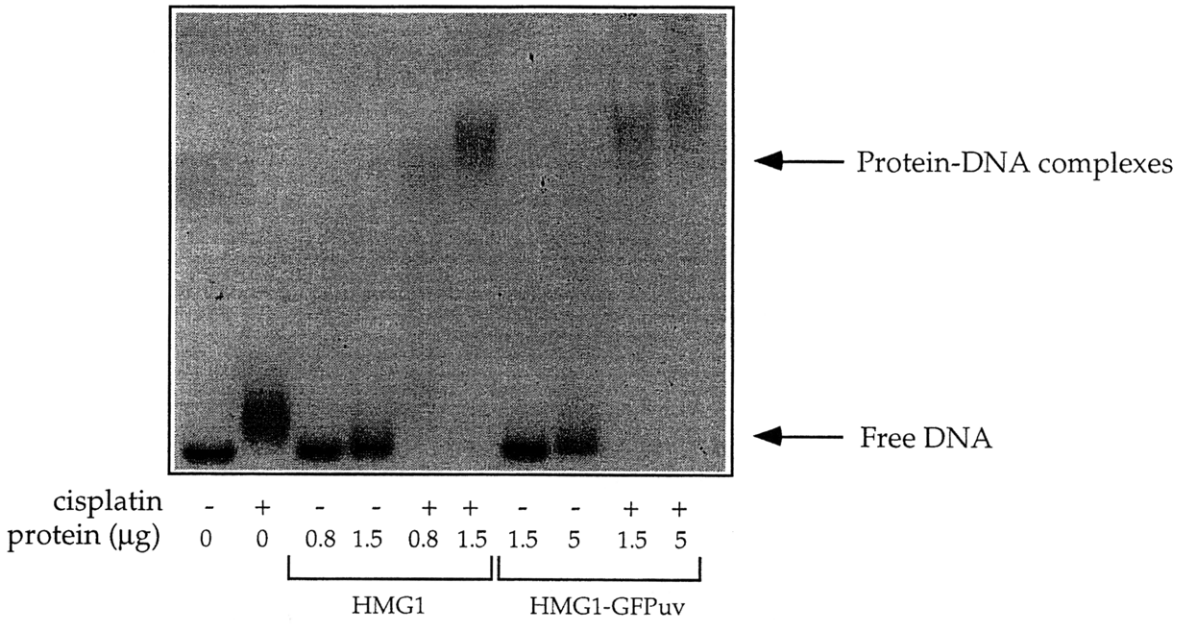


Figure A.7: Gel mobility shift assay comparing the binding of 123-bp DNA to HMG1 and HMG1-GFPuv.

A.2.5 Expression and Purification of GFPuv.

Figure A.8 shows that GFPuv was purified (>90%) by hydroxyapatite chromatography. The GFPuv was used as a control in experiments assessing the activity of HMG1-GFPuv.

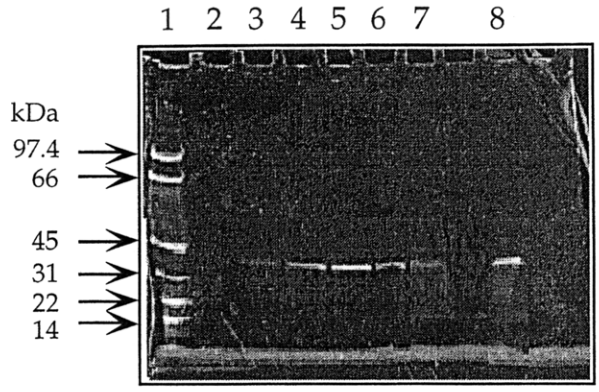


Figure A.8: SYPRO Orange stained SDS-PAGE gel showing the purification of GFPuv. Lane 1: Molecular weight markers. Lanes 2-7: Samples of fractions eluted from the hydroxyapatite column in a phosphate gradient. Lane 8: Previously purified GFPuv.

A.3 Discussion

The two attempts to modify HMG domain proteins chemically yielded products that did not recognize cisplatin-modified DNA. It seems plausible that lysine modification could inactivate HMG1 by preventing charge interactions necessary for binding anionic DNA [11]. The modification of the sole cysteine in HMGdomB could interfere with a critical structural or functional role of the residue. The expression of the HMG1-GFPuv fusion protein was more successful. HMG1 was not expected to affect the GFP fluorophore, which is well-shielded by the β-barrel structure of the surrounding protein [12]. The GFPuv was fused to the C-terminus of HMG1 in order to avoid disrupting the basic N-terminal region believed to be important for DNA binding. Early attempts to purify HMG1-GFPuv were complicated by the susceptibility of the linker region to protease degradation and also by the tendency of the GFPuv subunit to self-associate [10]. The hydroxyapatite protocol was much more successful than ion exchange, hydrophobic interaction, DNA affinity, or heparin chromatography. The resulting protein was a DNA-binding, fluorescent fusion of HMG1 and GFPuv.

As described in Section 1.2, HMG1-GFPuv has been used in a solid-phase assay to quantitate Pt-DNA-HMG1 binding [13]. Although this protocol is not yet optimized for high-throughput screening, it has demonstrated the utility of fluorescently labeled proteins in solid-phase screening methods.

A.4 References

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Biographical Note

Karen E. Sandman (1972-) received the A.B. (Chemistry, High Honors) from Princeton University in 1993. At Princeton she studied the interaction of vanadium bromoperoxidase with lipophilic substrates under the direction of Professor John T. Groves. In September 1993 she began graduate studies in Biological Chemistry at the Massachusetts Institute of Technology. She assisted in teaching undergraduate biochemistry and biotechnology courses during the 1993-1994 academic year. From 1996 to 1998, Karen was a trainee on the National Cancer Institute Cancer Training Grant at MIT.

Karen presented her research on synthesis and screening of platinum-amino acid complexes at the First Lake Tahoe Symposium on Molecular Diversity in Drug Discovery in November 1996. This work was subsequently published in the Journal of Biological Inorganic Chemistry, Volume 3, 1988. At the Metals in Biology Gordon Conference in January 1998, Karen presented her research on cell-based methods for predicting platinum cytotoxic activity. Her postdoctoral appointment is at New England Biolabs under the direction of Dr. Christopher Noren.