Mechanism-Based Rational Design of Cisplatin Analogues

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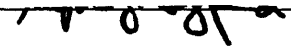

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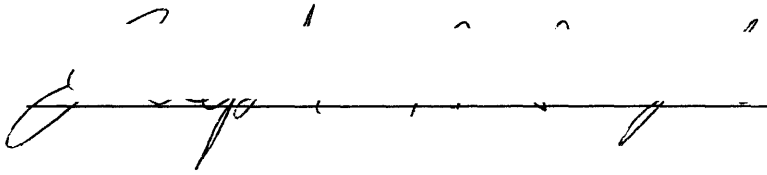

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By
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ABSTRACT

The success of cisplatin as an anticancer drug is attributed to the ability of the platinum compound to damage DNA and subsequently induce apoptosis. Details of the cellular processing of cisplatin-damaged DNA can provide invaluable insight into the rational design of cisplatin analogues or combination therapies. Chapter 1 provides a survey of recent developments in the understanding of the mechanism of cisplatin action and summarizes relevant platinum-based anticancer compounds.

Chapter 2 describes a series of estrogen-tethered platinum(IV) complexes (**BEPn, n**=1-5) that were synthesized, evaluated for their ability to upregulate HMGB1 and screened for cytotoxicity against human breast cancer cell lines. All **BEPn** complexes induced the overexpression of HMGB1 in ER(+) MCF-7 cells. **BEP3** was nearly twice as cytotoxic in ER(+) MCF-7 cells than in ER(-) HCC-1937 cells. This result suggests the possibility of using compounds in this class specifically to target ER(+) malignancies, such as breast and ovarian cancers. In addition, the series of **BEPn** compounds provide an example of a useful strategy in the development of platinum-containing anticancer agents, namely, using mechanistic insights to aid in the rational design of new complexes.

The strategy of exploiting estrogen-induced HMGB1 upregulation to sensitize ER(+) cells to platinum was further pursued in work described in chapters 3 and 4. Chapter 3 reports the synthesis and characterization of a series of platinum(IV)-estrogen conjugates derived from

carboplatin. Although these **BECPn** complexes were moderately cytotoxic in ER(+) MCF-7 human breast cancer cells, no differential cytotoxicity was observed as compared to ER(-) HCC-1937 cells. However, these compounds represent the first example of a biomolecule-tethered platinum(IV) complex that reduces to yield carboplatin in cells.

The platinum estrogen conjugate described in chapter 4 was designed not only to induce upregulation of HMGB1 but also to enter ER(+) cells selectively. Unlike the **BEP** and **BECP** compounds, **BEEP** was designed to maintain affinity for the estrogen receptor and by tethering platinum to estradiol through the 17α-position of the steroid ring. Compounds with affinity for the estrogen receptor, which is overexpressed in breast and ovarian cancers, are selectively taken up into ER(+) cells. Unexpectedly, **BEEP** had very low affinity for the estrogen receptor and was therefore equally cytotoxic in ER(+) and ER(-) human breast cancer cell lines.

A common feature of many cancers is overexpression of the folate receptor, which is responsible for the uptake of folic acid. Therefore targeting the folic receptor is an attractive method for achieving selective uptake in cancer cells. Chapter 5 describes the synthesis and biological activity of a folic acid-tethered platinum(IV) compound, which demonstrates the validity of this premise.

The nuclear protein HMGB1 has recently been discovered to function as an extracellular signaling agent. Because of oxygen deprivation, the core of a solid tumor dies by necrosis and passively releases HMGB1 into the extracellular environment. This characteristic of solid tumors leads to the hypothesis that extracellular HMGB1 is taken up by surrounding viable tumor tissue and mediates cisplatin sensitivity. The final chapter investigates the capability of exogenously administered HMGB1 to modulate the cytotoxicity of cisplatin and *trans*-DDP in human cancer cells.

The Appendix sections provide detailed experimental protocols for several useful laboratory methods. In Appendix A, a procedure for isolation of nuclei from cisplatin-treated cells is presented. The nuclei were subsequently used by our collaborators to examine the post-translational modifications of histones induced by cisplatin exposure. A protocol for isolation of protein extracts from formalin-fixed paraffin-embedded tissue is described in Appendix B. In addition, the extracts were probed by western blot analysis to examine the expression levels of HMGB1 in clinical testicular seminoma samples. Appendix C provides a solid-phase synthetic methodology for the preparation of peptide-conjugated platinum(IV) compounds.

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This thesis is dedicated to my husband, Randy.

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Abbreviations

1,2-d(GpG)	*cis*-[$Pt(NH_3)_2${d(GpG)-N7(1)-N7(2)}]
1,2-d(ApG)	*cis*-[$Pt(NH_3)_2${d(ApG)-N7(1)-N7(2)}]
AAG	3-methyladenine DNA glycosylase
ALLnL	N-acetyl leucyl-leucyl norlucinal
BEPn	*bis*-estrogen-*cis*-diamminedichloroplatinum(IV) compounds containing *n* methylene linker chain groups
BECPn	*bis*-estrogen-*cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(IV) compounds containing *n* methylene linker chain groups
BEEP	*bis*-17α-ethynylestradiol-*cis*-diamminedichloroplatinum(IV)
Bip	2,2′-bipiperidine
bp	base pair
BSO	buthionine sulfoxide
carboplatin	*cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II)
CBDCA	cyclobutanedicarboxylic acid
CCC	chirality-controlling-chelate
cis-DDP	*cis*- diamminedichloroplatinum(II)
cisplatin	*cis*-diamminedichloroplatinum(II)
CNC	chirality-neutral-chelate
CPG	carboxypetidase-G
CT	calf thymus
CTR	copper transporter
DACH	diaminocyclohexane
DCC	dicyclohexylcarbodiimide
DHD	dihydrodiol dehydrogenase
DIPC	diisopropylcarbodiimide
4-DMAP	4-dimethylaminopyridine
EDC	1-(3-dimethylaminopropyl)-3-ethylcarbodimmide hydrochloride
en	ethylene diamine
ER	estrogen receptor
ERCC1	excision repair cross complementation group 1
ERE	estrogen response element
ERα	estrogen receptor α
ERβ	estrogen receptor β
ER(+)	estrogen receptor-positive
ER(-)	estrogen receptor-negative
FACS	fluorescence-activated cell sorting
FACT	facilitates chromatin transcription
FBS	fetal bovine serum
FFC	first-to-first sphere communication
FITC	fluorescein isothiocyanate
FR	folate receptor
FSC	first-to-second sphere communication
G*	indicates a platinum bound guanine
GPI	glycosylphosphatidylinositol

GGR	global genome repair
HMG	high mobility group
HMGB1a	HMGB1 domain A
HMGB1b	HMGB1 domain B
HOBt	1, hydroxybenzotriazole-6-sulfonamidomethyl hydrochloride
HRP	horseradish peroxidase
IC_{50}	concentration required to kill 50% of treated cells
IC_{70}	concentration required to kill 70% of treated cells
MAPK	mitogen-activated protein kinase
Me_2ppz	N′,N′-dimethylpiperazine
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide]
NER	nucleotide excision repair
NF-κB	Nuclear factor -κB
NH_2Cba	cyclobutylamine
NH_2Cy	cyclohexylamine
PBS	phosphate buffered saline
2-pic	2-picoline
PMF	*mono*-folate-*cis*-diamminedichloroplatinum(IV) conjugate
Pol II LS	RNA polymerase II large subunit
PR+	progesterone receptor positive
Pt AAS	platinum atomic absorption spectroscopy
PMSF	phenylmethylsulfonylfluoride
PVDF	polyvinylidene fluoride
rcf	relative centrifugal force
rpm	revolutions per minute
RAGE	receptor for advance glycation end products
SRB	sulforhodamine B
TBP	TATA binding protein
TCR	transcription coupled repair
TFA	trifluoroacetic acid
TTBS	Tris buffered saline with 0.1% Tween 20
trans-DDP	*trans*-diamminedichloroplatinum(II)
tsHMG	testis specific HMG

Chapter 1
Cisplatin and Related Anticancer Drugs: Recent Advances and Insights.

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1. INTRODUCTION

Discovered serendipitously over thirty years ago[1], cisplatin (*cis*-diamminedichloroplatinum(II)) is used to treat breast, ovarian, bladder, lung, and head and neck carcinomas and affords greater than 90% cure rates in the case of testicular cancer.[2] Despite its success, the drug can cause severe side effects, is active against a limited range of cancers, and is often rendered inactive due to intrinsic or acquired resistance.[3] In order to develop more effective platinum analogs, there have been extensive studies of the mechanism of action of cisplatin. Over the past three decades, a great deal has been learned about the mechanism of cisplatin-induced cell death. As shown in Figure 1.1, cisplatin enters the cell both by passive diffusion and possibly also by an active transport mechanism.[4-6] Inside the cell, the chloride concentration is substantially lower, inducing cisplatin to undergo aquation.[7] The resulting cationic monoaqua species subsequently reacts with DNA, forming a variety of cross-links, with 1,2-intrastrand d(GpG) and d(ApG) cross-links accounting for 90% of all adducts.[7] A smaller percentage of the 1,3-intrastrand d(GNG) and interstrand cross-links also form.[7] The related drug carboplatin, or *cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II), forms a substantially greater number of 1,3-intrastrand cross-links.[8]

Cisplatin-DNA adducts inhibit DNA replication, block transcription by RNA polymerase II, and ultimately trigger programmed cell death, or apoptosis.[7,9] Furthermore, the 1,2-intrastrand cross-link induces a pronounced bend in the DNA helix.[10] The resulting wide and shallow minor groove opposite the platinum adduct serves as a recognition motif for a number of cellular proteins, including DNA repair proteins, histones, and HMG-domain proteins such as HMGB1.[5,7,10-12] The recognition of cisplatin-modified DNA by such proteins is an important cellular response to platinum damage because, among other functions, they signal for the repair

of cisplatin adducts, shield platinum-DNA cross-links from repair, and initiate apoptosis.[11] Understanding the role of such recognition proteins in mediating cisplatin cytotoxicity is of great interest.

This chapter is focused on the most recent developments in the cisplatin arena. In particular, we limit our discussion to papers that increase our understanding of the mechanism of action, reveal factors that influence the recognition and repair of platinum damage, and present new classes of platinum anticancer agents. We also treat exclusively mononuclear Pt(II) complexes with *cis* stereochemistry, since the chemistry and cytotoxicity of *trans*-platinum compounds, platinum(IV) complexes, and multinuclear platinum agents have been reviewed recently.[13] Several excellent reviews on various aspects of cisplatin cytotoxicity are also available.[4,5,7,14-18]

2. MECHANISM OF ACTION

2.1 Cellular uptake of cisplatin

It has long been accepted that cisplatin enters cells by passive diffusion. Evidence for such a pathway includes the following three observations: (1) the rate limiting factor for platinum uptake is drug concentration; (2) cisplatin accumulation is not inhibited by structural analogues; and (3) the uptake of cisplatin is not saturable.[19] Cisplatin accumulation also appears to be mediated by a membrane-associated protein transport system, as well as by additional agents including aldehydes, metabolic inhibitors and intracellular signaling mechanisms.[19] In some of these cases, the studies did not clearly delineate uptake from efflux processes. Cisplatin uptake has never been reported to be specifically inhibited by more than 50%, supporting the conclusion that passive diffusion must account for at least half of all drug influx, while protein-mediated

uptake may account for the remaining half.[19] The debate as to whether cisplatin can enter cells by facilitated diffusion is now more timely than ever because of a recently discovered relationship between cisplatin uptake and the copper transporter Ctr1.[6]

Direct evidence connecting copper transport and cellular cisplatin resistance was first obtained by the observation that cells transfected with *ATP7B*, a gene that encodes a copper transporting P-type ATPase, are resistant to both copper and cisplatin.[20] The gene product, ATP7B, is a 1465 amino acid, heavy-metal transporting protein that contains 6 Cu-binding motifs (GMTCXXC) at its N-terminus. The transfected cell line, KB/WD, was 8.9-fold more resistant to cisplatin than KB/CV cells (KB-3-1 cells transfected with an empty vector) and 2-fold more resistant to copper. KB/WD cells accumulated 60% less cisplatin than KB/CV cells and effluxed approximately 20% of the acquired platinum, which may account for the observed resistance.[20] A screen of yeast loss-of-function mutants for cisplatin resistance revealed that the strain defective in the MAC1 gene conferred the highest degree of resistance (2.5-fold).[21] The number of cells that survived a 2-h treatment of cisplatin was 1000 times greater in the mac1Δ mutant. All known Mac1p target genes (CTR1, FRE1, FRE7, CTA1 and CTT1) were individually deleted and only the CTR1 mutant, ctr1Δ, displayed a similar amount of cisplatin resistance. Additional cytotoxicity assays were carried out with mutants deficient in copper trafficking and utilization genes (ccc2Δ, fet3Δ, lys7Δ, sod1Δ, cox17Δ, and soc1Δ). None of these mutants showed comparative resistance with the ctr1Δ strain, indicating that defects in copper distribution or utilization did not lead to cisplatin resistance. Other copper transporters, Ctr3p, Ctr2p, and Fet4p, were also investigated; however, they did not lead to substantially increased levels of cisplatin resistance. Cisplatin accumulation in the ctr1Δ cell line was 59% of that in wild-type, suggesting that CTR1 mediates cisplatin uptake. Administration of copper decreases

cisplatin accumulation and increases cell survival by a factor of two. In addition, cisplatin diminishes copper uptake by 16% and mimics copper in its ability to trigger internalization and degradation of Ctr1p. Further experiments were performed with mouse Ctr1 knockout cells to investigate the role of mammalian Ctr1p in cisplatin resistance. Homozygous mutant cells were 8-fold more resistant and acquired 70% less cisplatin than wild-type cells.[21]

Further evidence linking copper transport with cisplatin uptake was provided in studies with yeast cells.[22] Accumulation of cisplatin and copper was determined in two isogenic *S. cerevisiae* strains, a parental line (BY4741) and a subline (*ctr1* BY4741-YPR124W) in which the CTR1 gene is deleted. Cellular copper was 16-fold reduced in the *ctr*1 line, and accumulation of cisplatin was 8-fold diminished. Addition of cisplatin to the culture medium also reduced the accumulation of copper in a concentration-dependent fashion. Moreover, *ctr1* cells were 1.9-fold more resistant to cisplatin and formed 2.3-fold fewer platinum-DNA adducts than wild type cells.[22] A connection between cisplatin and copper also occurs in human ovarian carcinoma cell lines (A2780/CP, 2008/C13*5.25, and IGROV-1/CP).[23] Cells selected for cisplatin resistance are also cross-resistant to copper, being between 5.7- and 8.1-fold more resistant to cisplatin and between 1.5- and 2.1-fold more resistant to $CuSO_4$. The cisplatin-resistant cells lines had reduced levels of platinum accumulation and 22-56% lower basal levels of copper. Interestingly, there was no clear correlation between whole cell uptake of cisplatin and DNA adduct formation. There is no known method to quantitate CTR1, but only one of the three resistant cell lines had reduced CTR1 mRNA relative to the parent line.[23]

The exact role that copper transport proteins play in mediating cisplatin resistance is unknown. Cisplatin uptake may be coupled to endocytosis and degradation of Ctr1p.[21] Alternatively, transporters and chaperones used to mediate copper homeostasis may import,

distribute, and export cisplatin in the same way as copper; however the kinetically more inert character of Pt(II) compared to Cu(I) or Cu(II) makes it difficult to understand such a parallel.[6,21-23] Although there is no known CTR cisplatin binding site, both yCtr1 and hCTR1 have three transmembrane segments including an extracellular methionine-rich N-terminal tail that could bind platinum (Figure 1.2).[24-29] It has been suggested that, because platinum binding affinity to methionine is sufficiently labile to afford transfer to histidine ligands, intracellular transport of cisplatin via binding to CTR is feasible.[22]

Investigation of the theory that protein-bound cisplatin serves as a platinum reservoir has provided much information about platinum-protein interactions.[15] Platinum compounds with chelating amines can migrate from methionine to histidine in peptides containing both.[15,30,31] There is not yet any evidence, however, that thioether-bound cisplatin will migrate to a histidine imidazole, much less to DNA.[30,32,33] The reaction of cisplatin with methionine- and histidine-containing peptides gives rise only to a variety of peptide chelates and the ammine ligand *trans* to the thioether is often dissociated.[30,32] Furthermore, a 1:1 complex between methionine and cisplatin does not react with CT DNA, even after 12 h.[34] Inside the cell, where glutathione concentrations can approach 10 mM, platinum thioether complexes are readily converted to platinum thiolates and subsequently exported from the cell.[33,35] These observations raise serious mechanistic questions about CTR mediated cisplatin transport. Further work is needed to elucidate the binding affinity of cisplatin to CTR and the fate of any transporter-bound platinum.

More recent studies have further questioned the role of CTR relating to cisplatin biological activity. The effect of hCTR1 expression on the activity of cisplatin was examined in human ovarian cancer cells.[36] Cisplatin treatment of cells that overexpress hCTR1 resulted in increased platinum accumulation and decreased cellular growth rates; however, increased

expression of hCTR1 had limited effect on cisplatin cytotoxicity and platinum-DNA adduct levels.[36] In addition, although cisplatin stimulates endocytosis and degradation of Ctr1 in yeast, the same is not observed in human embryonic kidney cells.[37] It appears that hCTR1 increases cellular platinum accumulation, but the platinum is sequestered into vesicles and is unavailable for DNA-binding.[36,37] Therefore, it is unlikely that hCTR1 contributes to the mechanism of action of cisplatin. Instead, copper-transport proteins may be more involved in resistance mechanisms of platinum-based anti-cancer drugs. For example, increased expression of ATP7A and ATP7B, copper-transporters located in the trans-Golgi apparatus, results in decreased cytotoxicity of cisplatin, carboplatin, and oxaliplatin but does not result in decreased cellular platinum levels.[38,39] Confocal microscopy studies reveal that cisplatin indeed co-localizes in vesicles with ATP7B and as a result cannot bind to DNA.[40]

2.2 Interaction of cisplatin with DNA

Cisplatin can bind to RNA, proteins or sulfur-containing biomolecules, but DNA is its primary biological target.[4,5,7] Understanding the factors that control cisplatin binding to DNA are crucial in elucidating its mechanism. Recent studies have examined the effects of flanking sequences on cross-link formation in order to understand the factors that might influence cisplatin sequence specificity. Cisplatin preferentially binds to poly-G-rich DNA sequences.[41] This apparent sequence specificity may be due to cisplatin targeting of such regions or may simply reflect the greater number of N7 platinum binding sites. Platination of M13mp18 viral DNA followed by examination by double-stranded replication mapping revealed that 89% of all GG sites blocked replication, indicating that cisplatin binds to most of the available poly-G sites.[41] Platination of a plasmid containing an 800 base-pair telomeric $(TTAGGG)_n$ repeat revealed the G-rich regions to

have more adducts than the random sequence segments; however, increased platination of the G-rich regions corresponded to an increase in platinum binding sites.[41] These data imply that cisplatin does not specifically target poly-dG sequences.[41]

The majority of cisplatin adducts are 1,2-intrastrand d(GpG) and d(ApG) cross-links, whereas the corresponding d(GpA) adduct does not form.[7] Furthermore, the 1,2-intrastrand d(GpG) cross-link is three times more prevalent than the d(ApG) cross-link.[42] It is of great interest to understand the factors that govern this cisplatin-adduct profile. With the use of hairpin-stabilized double-stranded oligonucleotides, the kinetics of platinum binding to GG and AG sequences were determined.[42] Reactions of *cis*-$[Pt(NH_3)_2Cl(OH_2)]^+$ and *cis*-$[Pt(NH_3)_2(OH_2)_2]^{+2}$ with d(TATGGTATT$_4$ATACCATA) (**I**) and d(TATAGTATT$_4$ATACTATA) (**II**) were monitored by HPLC. As expected based on charge and labile ligand availability, the monoaqua species reacts with these hairpin oligonucleotides between 20-150 times more slowly than the diaqua species. The diaqua species reacts 3 times more rapidly with **I** than with **II**, whereas the monoaqua species has similar reaction rates with both sequences. The relative platination rates of **I** and **II** suggest that *cis*-$[Pt(NH_3)_2Cl(OH_2)]^+$ should form similar amounts of GG and AG adducts, while *cis*-$[Pt(NH_3)_2(OH_2)_2]^{+2}$ should preferentially form GG adducts with cellular DNA.[42] In addition, platination rates of the hairpin oligonucleotides follow in the order *cis*-$[Pt(NH_3)_2(OH_2)_2]^{+2}$ > *cis*-$[Pt(NH_3)_2(OH_2)(OH)]^+$ > $[Pt(NH_3)_3Cl]^+$ >> *cis*-$[Pt(NH_3)_2Cl(OH_2)]^+$, which parallels the hydrogen bond donor ability of the complexes. Hydrogen bonding between the platinum ligands and the O6 of guanine may stabilize the transition state for Pt-nucleobase bond formation.[42] Such hydrogen bonding is not possible for adenine and may provide a rationale for the preference of GG over AG adducts. These observations suggest that the more significant DNA-binding species is *cis*-$[Pt(NH_3)_2(OH_2)_2]^{+2}$,

not the more widely accepted *cis*-$[Pt(NH_3)_2Cl(OH_2)]^+$.[42] Preassociation of the monoaqua species may drive the aquation equilibrium towards the diaqua form. Furthermore, polyanionic DNA repeals anions, such as Cl^-, and concentrates cations, like H_3O^+, which would favor the formation of the diaqua species. Thus, the second aquation reaction may occur while the monoaqua complex is associated with DNA before the first Pt-N7 bond is formed.[42]

Additional kinetic studies of cisplatin binding to three self-complementary oligonucleotides 5′-d(AATTAGTACTAATT)-3′ (-AG-), 5′-d(AATTGATATCAATT)-3′ (-GA-), and 5′-d(AATTGGTACCAATT)-3′ (-GG-) were carried out with the use of ^1H-^{15}N HSQC NMR spectroscopy.[43] Several kinetic models were used to evaluate the data and the most appropriate model was one that included a reversible aquation step and a single platinum binding site for the self-complementary duplex. The monoaqua species is first observed after 2 h, whereas the diaqua species appears only at 3.5 h. After 30 h at 298 K, 23% of the cisplatin remains and 65% has been converted to *cis*-$[Pt(NH_3)_2Cl(OH_2)]^+$. The presence of *cis*-$[Pt(NH_3)_2(OH_2)_2]^{+2}$ never accounts for more than 7% of total observable platinum. The rate of cisplatin aquation is slowed by 30-40% in the presence of DNA, with aquation in the presence of the -GA- oligomer being the slowest. The diminished rate of aquation may be explained by preassociation between cisplatin and DNA, leading to decreased solvent accessibility to the platinum center.[43] Different degrees of association between cisplatin and the -GG-, -AG-, and -GA- sequences may explain the different rates of aquation. The kinetics of cisplatin binding to -GG-, -AG-, and -GA- were also determined.[43] In general, the kinetics of monofunctional adduct formation correlate with the cisplatin adduct profile.[43]

The conclusions drawn in these two studies are clearly in conflict with one another.[16,42,43] Using their kinetic models, the latter investigators analyzed the hypothesis that *cis*-

$[Pt(NH_3)_2(OH_2)_2]^{+2}$ is the most important DNA-binding species. They concluded that reactions between DNA and the diaqua species only account for 0.9-1.2% of the platination products.[43] As discussed earlier, the diaquated species was first detected only after 3.5 h, at which time platination of DNA by the monoaqua form is already in progress. The chloride concentration in cancer cells (20-50 mM) probably further suppresses the formation of the diaqua species.[16,43]

2.2.1 Structural studies

Many platinum compounds are able to form 1,2-intrastrand cross-links, but their cytotoxicities do not approach that of cisplatin.[44] It is therefore of interest to characterize *cis*-PtA_2G_2 (G = guanine derivative, A = amine) adducts in order to assess whether subtle structural differences induced by the carrier ligand might influence the processing of platinum-DNA lesions and the attendant cytotoxicity. No two structural analyses of platinum-DNA adducts by NMR solution methods are the same, and the NMR results conflict with X-ray structures when analyzed in detail. Dynamic motion about Pt-N7 bonds greatly complicates the study of simple *cis*-PtA_2G_2 models by NMR methods.[45] Rotation about the Pt-N7 bond can give rise to either a head-to-head (HH) or head-to-tail (HT) orientation of the *cis* guanine bases.[46] There are two isomers possible for the HT orientation, ΔHT and ΛHT.[46] In addition, the phosphodiester backbone can adopt a normal (1) or opposite (2) backbone propagation direction. These possibilities give rise to four conformational classes: HH1, HH2, ΔHT1 and ΛHT2 (Figure 1.3).[46] The cisplatin 1,2-(GpG)-intrastrand cross-link adopts primarily the anti, anti head-to-head, or HH1, conformation, which minimizes steric clashes with the phophodiester linkage.[45] In order to study simple *cis*-PtA_2G_2 models and elucidate the effect of carrier ligands on adduct conformation, the dynamic motion about the Pt-N7 bond was slowed significantly by using bulky chelating amine ligands.[45] These

compounds are termed retro models and have utilized two classes of such ligands. The first class, known as chirality-controlling-chelate (CCC) ligands, have chiral carbon atoms near secondary amines so that the amine configurations are restricted to a particular chirality.[44,45,47-50] The platinum-bound guanine derivatives can assume a number of conformations in these retro model compounds because the CCC ligands lack bulk above and below the coordination plane. The control of Pt-N7 fluxionality arises from rigid bulk along the coordination plane.[45] The second ligand type, chirality-neutral chelate (CNC) ligands, have no chiral carbon atoms.[51-53] In addition, the CNC ligands have no axial or equatorial NH or alkyl groups, but effectively minimize rotation about the Pt-N7 bond.[51-53]

The study of retro model compounds by CD and NMR methods has provided valuable insights into the factors that influence conformer stability. In particular, the stability of isomers depends on several factors including dipole-dipole alignment (first-to-first sphere communication, or FFC), phosphate-*cis* G N1H interactions (second-sphere communication, or SSC), electrostatic repulsion, phosphate-carrier ligand NH interactions (first-to-second sphere communication, FSC), and N1H-G O6 hydrogen bonding (FFC).[44,47,49-51] The carrier ligand can significantly influence conformer stability in solution, which may be important in modulating cytotoxicty. In addition, the study of retro model compounds has led to the speculation that *cis*-[$Pt(NH_3)_2$(GpG)] may exist as a dynamic mixture.[48] Its ^{31}P NMR signal is more upfield shifted than expected for a purely anti, anti HH1 conformer, suggesting that a small population of anti, syn ΔHT1 conformer might be present. Furthermore, the downfield H8 proton signal is broad, which indicates a possible exchange between the anti, anti HH1 and anti, syn ΔHT1 conformers.[48]

The ability of the ammine ligands of cisplatin to form hydrogen bonds to the O6 atom of guanine has long been argued to be vital for cytotoxicity; however, it has recently been suggested that it is the small size of the NH_3 group, rather than its hydrogen-bonding ability, that is important.[53] In order to investigate the significance hydrogen bonding, the conformations of Pt(Me_2ppz)(GpG) and Pt(Bip)(GpG) were compared.[53] Pt(Me_2ppz)(GpG) lacks an NH group and therefore cannot hydrogen bond to guanine, whereas Pt(Bip)(GpG) is capable of hydrogen bonding. For the ΔHT1 conformers, hydrogen-bonding between the carrier ligand amine and the guanine O6 was weak and not important in stabilizing the interaction.[53] It is plausible that platinum withdraws electron density from the coordinated guanine base, weakening the hydrogen-bond accepting ability of the guanine O6.[53] Additional support for the lack of importance of hydrogen bonding in stabilizing the 1,2-intrastrand d(GpG) cross-link comes from examination of the cross-link structure itself. The base pair step adjacent to the platinum adduct, known as the Lippard base pair step, is unusually distorted.[53-55] As shown in Figure 1.4, the Lippard base pair step is characterized by a large positive slide and shift, caused by movement of the 5′-G* base pair relative to adjacent base pairs.[53-55] If the 5′-G* were hydrogen bonding to the platinum ammine ligand and retained normal base canting, a steric clash would result between the 5′-G* and the adjacent 5′-residue.[53] Therefore, the forces that favor formation of hydrogen bonds between the guanine O6 and platinum amine are dominated by the need to minimize steric clash.[53]

Recognition of the Lippard base pair step also helped to reconcile differences between the numerous NMR and X-ray structures of cisplatin-modified DNA mentioned above. Modeling cisplatin-modified DNA NMR data with the use of restrained molecular dynamics (RMD) afforded models with different base stacking, backbone conformation, base step rise, and the

extent and nature of PtG^*_2 geometry distortion.[56] Furthermore, NMR-derived solution structure positions differ by as much as 3.5 Å in the three-base-pair region 5′-XG*G*-3′ compared to a recently reported X-ray structure.[55,56] Marzilli proposes that NMR methods have previously failed to define the structure of the platinum-damaged duplex because of the unusual and unrecognized structure of this three-base-pair region.[56] The distortion significantly limits modeling by traditional RMD methods. Closer examination of the many reported NMR structures of cisplatin-damaged DNA revealed that all duplexes have the same structure in the three-base-pair region.[56] The NMR solution structure that models in the Lippard base pair step is quite similar to the X-ray structure of platinated DNA bound by the HMGB1a protein, suggesting that cisplatin-modified DNA requires little structural change in order to accommodate binding of this three-helix domain (see also 2.3.2 below).[55,56]

2.2.2. Effect of Chromatin Structure

The majority of studies characterizing cisplatin binding to DNA have been carried out using purified DNA. In the cell, DNA is condensed into a compact chromatin structure, which may affect cisplatin binding.[57] As a result, recent work has focused on elucidating the interactions between cisplatin and chromatin DNA. Early work with cisplatin binding to nucleosomal DNA revealed little effect of the core histones on DNA binding.[58] Nuclease digestion of chromatin to determine the location of platinum adducts indicated that DNA cross-links were favored over protein cross-links and that, at a low platinum concentration ($r_b < 0.05$), the linker DNA is targeted.[58-63] At high platinum concentrations ($r_b = 0.1$-0.2), however, there was no preference for linker DNA.[60] Current work with nucleosomal DNA utilizes reconstituted chromatin containing specifically designed DNA sequences. In reconstituted chromatin, cisplatin binds

preferentially to the nucleosomal linker regions.[64] Thus, it appears that the nucleosomal core region offers some protection against cisplatin-induced DNA damage.[64] Nucleosomal core particles lacking DNA linker regions form cisplatin interstrand cross-links in a similar manner as free DNA, suggesting that the major groove is still accessible to cisplatin attack.[65] In one study, the effect of chromatin structure was investigated in intact human cells, using the epsilon-globin gene promoter, which contains numerous transcription binding sites, as the target DNA sequence.[66] Platination was specifically enhanced at the CACC binding site where a transcription factor of the Sp1 family binds.[66] It is possible that the binding of transcription factors bends the DNA, therefore exposing it to cisplatin damage.[66]

More recently, the effect of chromatin structure on nucleotide excision repair has been investigated using a site-specifically platinated nucleosome containing either a d(GpG) or d(GpTpG) intrastrand cross-link.[67] The nucleosome inhibits excision of the d(GpG) platinum adduct to about 30% of the level observed with free DNA, whereas the d(GpTpG) platinum adduct is repaired at only 10% of the efficiency of free DNA. The excision efficiency of the platinum adducts was two-fold higher in native compared to recombinant nucleosomes, demonstrating that post-translational modification of the histones can effect the repair of damaged DNA.[67] This *in vitro* model provides a powerful tool for examination of the cellular processing of platinum adducts.

2.3 Role of cellular proteins

2.3.1 HMG-domain proteins bind to cisplatin-modified DNA

Since the initial discoveries over a decade ago,[68,69] numerous HMG-domain proteins have been found that specifically recognize and bind to cisplatin-modified DNA.[12] The HMG-domain is an

80 amino acid structure-specific DNA binding motif.[70,71] HMGB1 is an architectural protein important in maintaining chromatin structure. It increases transcription activation and functions as an extracellular signaling agent during inflammation, cell differentiation, cell migration and tumor metastasis.[72-74] Recent research has focused on understanding the factors that determine HMGB1 affinity to cisplatin-modified DNA. For example, acetylation of HMGB1 increases its affinity for cisplatin-modified DNA by 6-fold.[75] Furthermore, p53 interacts with HMGB1 and enhances binding to cisplatin-damaged DNA.[76] Discerning what governs DNA-protein interactions is essential for elucidating the roles of such proteins in mediating cisplatin cytotoxicity. The flanking sequence of cisplatin-modified DNA probes affects the binding affinity of HMGB1 and its individual domains, HMGB1a and HMGB1b. The affinity of HMGB1a for purine-rich probes is greatest when A·T base pairs flank the GG platination site.[77] The preference for A·T flanking sequences is most likely a consequence of the increased flexibility of the DNA.[78] Since the minor groove offers relatively few hydrogen-bonding atoms to assist in protein recognition and binding, it is likely that the intercalating residue serves as a DNA-recognition tool (Figure 1.5).[55,79] Thus platination of a flexible DNA sequence, like TGGA, significantly bends DNA and allows facile intercalation for minor-groove binding proteins.[79]

Auxiliary ligands on platinum also facilitate HMGB1 binding to platinated DNA.[80] The affinity of HMGB1a for the platinated DNA probe 5′-CCTCTCTGGATCTTC-3′ decreases as cisplatin > *cis*-$\{Pt(NH_3)(NH_2Cba)\}^{2+}$ > *cis*-$\{Pt(NH_3)(2\text{-pic})\}^{2+}$ ~ *cis*-$\{Pt(NH_3)(NH_2Cy)\}^{2+}$ > $\{Pt(en)\}^{2+}$ >> $\{Pt(dach)\}^{2+}$. When the flanking sequence is AGGC, HMGB1a affinity for the platinated probes is less discriminating. Overexpression of HMGB1 following steroid hormone treatment sensitizes cells to *cis*-$[Pt(NH_3)(NH_2Cy)Cl_2]$, but not to *cis*-$[Pt(dach)Cl_2]$ by the repair-

shielding mechanism (see below). This result reflects the lack of HMGB1 recognition of {Pt(dach)}$^{2+}$-DNA adducts.[80] Because HMGB1 is a minor-groove binding protein, it is unlikely that the amine ligands projecting into the major groove interact directly with the protein.[80] It is possible that bulky, rigid amine ligands restrict DNA flexibility, thereby reducing the affinity of HMGB1.[80]

The TATA-binding protein (TBP) also selectively binds to cisplatin-modified DNA. In fact, of all known platinated-DNA binding proteins, TBP has the highest specificity for platinum-damaged DNA.[80,81] TBP binds to the TATA box located 30 bp upstream from a transcription start site and recruits other vital transcription factors.[81] Inhibition of transcription by cisplatin can be restarted upon addition of TBP, suggesting that platinum-DNA adducts are able to 'hijack' TBP from its natural binding sites.[81] Platination of DNA containing a TATA-box significantly increases the binding affinity of TBP. The enhancement of TBP affinity is maximized when the platinum adduct is positioned near the intercalation site of the TBP phenylalanine.[81] The flanking sequence dependence of TBP is similar to that of HMGB1. In particular, having the flexible A·T bp flanking the platinum adduct site enhances TBP binding affinity.[80] TBP very slowly associates to and dissociates from both cisplatin-modified DNA and the TATA box.[82] Consequently, TBP is only able to shield cisplatin-adducts from nucleotide excision repair when allowed first to form the protein-DNA complex before exposure to the repair machinery.

A recently discovered transcription factor FACT (facilitates chromatin transcription), which is a heterodimer of SSRP1 and Spt16, preferentially binds to DNA damaged by cisplatin.[83] Surprisingly, the SSRP1 subunit of FACT does not bind to cisplatin-modified DNA; however, the isolated HMG-domain of SSRP1 does bind to the damaged DNA. The Spt16 subunit has no

independent affinity for the cisplatin-damaged DNA, but appears to alter the structure of SSRP1, rendering the HMG domain accessible for DNA binding.[83]

2.3.2 HMG-domain proteins mediate cisplatin cytotoxicity

HMGB1 and other cellular proteins that recognize platinum-DNA adducts may play a role in the mechanism of action of cisplatin cytotoxicity, as manifested by two main hypotheses that have evolved.[84] One hypothesis asserts that cisplatin-damaged DNA hijacks proteins away from their natural binding sites, leading to cellular stress and eventually cell death. The second hypothesis suggests that binding by cellular proteins shields cisplatin adducts from nucleotide excision repair (NER), allowing them to persist and drive apoptosis. These two hypotheses are not mutually exclusive. In support of the repair-shielding hypothesis, it has been demonstrated that HMG-domain proteins shield cisplatin adducts from repair in vitro and in cells.[85,86] Recently, it was discovered that exposing steroid receptor-positive cancer cells to either progesterone (for PR+ cells) or estrogen (for ER+ cells) induces upregulation of HMGB1.[87] Furthermore, this steroid-induced HMGB1 upregulation sensitizes ER+ or PR+ cells by a factor of 2-4 to cisplatin treatment, presumably by shielding the adducts from NER.[87] The timing of steroid hormone and cisplatin administration is critical for achieving the increased sensitivity. Only when cisplatin and steroid hormone are co-administered is the desired sensitization observed.[87] In order to achieve sensitization, the upregulation of HMGB1 must coincide with platinum adduct formation and attempted repair of the adduct. A clinical trial is currently under way to investigate the *in vivo* effect of estrogen and progesterone on tumor HMGB1 expression and growth inhibition. Expression of murine testis-specific HMG-domain (tsHMG) in HeLa cells enhanced transcription inhibition by cisplatin.[88] The effect of tsHMG on cisplatin-induced apoptosis was

less clear. Short incubation times with cisplatin showed that the presence of tsHMG increased the apoptotic response; however, for longer incubation times, tsHMG seemed to protect the cell from apoptosis.[88]

Although many studies have demonstrated that HMG-domain proteins potentiate cisplatin cytotoxicity, others reveal that HMG-domain proteins either have no effect or actually protect the cell from cisplatin damage. The cytotoxicity of cisplatin was investigated in two mouse embryonic fibroblast cell lines, $Hmgb1^{+/+}$ and $Hmgb1^{-/-}$.[89] Unexpectedly, the sensitivity of the two cell lines to cisplatin was equivalent. In addition, the levels of cisplatin-induced apoptosis were similar, suggesting that HMGB1 levels do not effect cisplatin cytotoxicity in mouse embryonic cells.[89] It is possible that HMGB1 is engaged in protein complexes and unavailable for repair shielding of cisplatin-DNA adducts. Alternatively, in the HMGB1 deficient $Hmgb1^{-/-}$ cells, other proteins such as HMGB2 may play a role in modulating cisplatin sensitivity.[89] Other work has indicated that HMGB1 is overexpressed in cisplatin-resistant cell lines and several yeast cell lines deficient in HMG-domain proteins are hypersensitive to cisplatin.[90,91] Clearly the effect of HMG-domain proteins in modulating the activity of cisplatin depends upon the cell type and/or context.

2.3.3 Other cisplatin-DNA recognition proteins

3-Methyladenine DNA glycosylase (AAG) was recently discovered to bind to cisplatin-damaged DNA.[92] AAG efficiently removes 3-methyladenine, 3-methylguanine, 7-methylguanine, and 1,N^6-ethenoadenine (εA) *in vivo*; however, in the presence of cisplatin-damaged DNA, the excision of εA by AAG is inhibited.[92] Although AAG recognizes and binds to platinum-DNA adducts, no evidence of attempted repair has been observed.[92] Interestingly, the highest mouse

levels of AAG mRNA are observed in the testis.[92] In addition, the tumor suppression protein p53 binds to cisplatin-modified DNA, but the presence of cisplatin-damage does not reverse its sequence-specific binding properties.[93,94] Moreover, platinum-DNA cross-links that form in the consensus DNA response element decrease p53 binding affinity.[94] Nonetheless, the ability of p53 to bind to cisplatin-modified DNA supports a possible role of p53 in potentiating cisplatin cytotoxicity.

3. CELLULAR DEFENSES AGAINST CISPLATIN

3.1 DNA repair mechanisms

Whether or not cisplatin-DNA adducts are removed by cellular repair mechanisms is a major determinant in drug toxicity. Cells deficient in DNA repair are hypersensitive to cisplatin, whereas overexpression of repair factors confers cisplatin resistance.[95] Understanding how cisplatin is repaired is thus vital for elucidating its mechanism of action and developing more effective platinum-based anticancer drugs. Cisplatin-DNA adducts are primarily removed by nucleotide excision repair (NER), which consists of recognition, incision, excision, repair synthesis and ligation.[7] Overexpression of NER factors may lead to cisplatin resistance. For example, ERCC1 (excision repair cross complementation group 1) expression at both the mRNA and protein levels is correlated to cisplatin response in human ovarian carcinomas.[96]

In general, NER can follow two pathways, transcription-coupled repair (TCR) or global genome repair (GGR).[97] TCR is responsible for repair of DNA damage on the transcribed strand of active genes. Repair of DNA damage on the non-transcribed strand of active genes or on the remainder of the nontranscribing genome is performed by GGR. A panel of human cell lines with various genetic deficiencies in NER were examined for cisplatin sensitivity.[97] TCR-

deficient cell lines were hypersensitive to cisplatin, regardless of GGR status.[97] Conversely, XP-C cells, defective in GGR and proficient in TCR, were not more sensitive towards cisplatin.[97] These results suggest that TCR, not GGR, is the primary repair response to cisplatin damage.[97] During transcription of a cisplatin-damaged gene, the large subunit of RNA polymerase II (Pol II LS) stalls upon reaching the cisplatin-adduct, thereby serving as a DNA-damage sensor.[9] Furthermore, cisplatin-induced transcription arrest signals for the ubiquitination and degradation of Pol II LS.[9,98] Ubiquitination of Pol II LS may recruit repair proteins and lead to subsequent removal of the cisplatin-damaged site. On the other hand, inhibition of ubiquitination could sensitize cells to cisplatin.[9]

Cisplatin can also induce caspase-mediated Pol II LS cleavage, which may signal that the cell has committed to apoptosis.[99] Ubiquitination is observed at early time points (0.25–1 h) after cisplatin damage, while caspase cleavage occurs at later times (16–24 h).[9,99] Thus, it appears that ubiquitination of Pol II LS is an initial defense mechanism against cisplatin cytotoxicity. Caspase cleavage of Pol II LS, and therefore apoptosis, may then result from failure to remove the stalled, ubiquitinated Pol II LS. The proteasome inhibitor N-acetyl leucyl-leucyl norlucinal (ALLnL) increases cisplatin cytotoxicity by 20–40% in human ovarian carcinomas.[100] In addition, ALLnL reportedly enhances platinum uptake, increases platinum adduct formation, and reduces repair of platinum adducts.[100] Proteasome inhibitors block the degradation of ubiquitinated proteins and promote deubiquitination of the histones H2A and H2B in nucleosomes.[101,102] The deubiquitination of H2A and H2B causes the nucleosome to adopt a higher-order, condensed structure that may shield cisplatin-adducts from repair.[101,102] ALLnL also suppresses cisplatin-induced ERCC1 mRNA overexpression, suggesting that proteasome inhibitors may sensitize cells to the drug by depleting NER machinery.[103] It is tempting to speculate that proteasome

inhibitors sensitize cells to cisplatin by preventing the removal of the stalled Pol II LS, therefore blocking repair of platinum damage and promoting apoptosis.

3.2 Cellular resistance to cisplatin

The mechanism of cisplatin resistance is extremely complex and mutlifactorial (reviewed recently, see refs. 104-107). Factors that appear to mediate cisplatin resistance include reduced uptake and increased efflux of platinum, increased levels of sulfhydryls (i.e., glutathione and metallothionein), increased DNA repair, and loss of mismatch repair.[106,107] As discussed earlier, proficiency in NER leads to the effective removal of cisplatin adducts and increased cell survival. Examination of a series of cisplatin-resistant ovarian cancer cell lines for expression of NER genes revealed only ERCC1 expression consistently to be correlated with resistance.[108] Recent research has focused on elucidating the genetic basis of cisplatin resistance. A number of genes have been directly linked to cisplatin resistance, including DNA mismatch repair, p53, and protein kinase A pathway genes.[104] cDNA microarrays were employed to determine what genes were differentially expressed in a cisplatin-resistant ovarian cancer cell line compared to the parent line.[109] Several genes were overexpressed in the resistant cell line, but only forced overexpression of dihydrodiol dehydrogenase (DHD) in the parent cell line resulted in a cisplatin-resistant cell line.[109] At present, it is not clear how DHD would potentiate cisplatin cytotoxicity.

The role of glutathione in conferring cisplatin resistance is a topic of debate. Although some studies have shown that increased levels of glutathione lead to cisplatin resistance, others have revealed no correlation between glutathione concentration and cell sensitivity. In a series of rat PC12 cell lines with various intracellular glutathione concentrations there was a strong

correlation between cisplatin cytotoxicity and glutathione concentration.[110] The parent PC12 cell line was 4-fold more sensitive to cisplatin than was the cell line with the highest intracellular glutathione concentration. The elevated glutathione concentration did not, however, lead to decreased cellular platinum content.[110] Depletion of glutathione by buthionine sulfoximine (BSO) increases DNA platination levels and sensitivity to cisplatin; however, previous work revealed that BSO has little to no effect on cisplatin cytotoxicity.[111,112] Inhibition of glutathione conjugate export pump and glutathione-S-transferase have no effect on cisplatin cytotoxicity.[111] γ-Glutamyl-transferase levels are increased in HeLa cells in response to cisplatin treatment, but overexpression of γ-glutamyl-transferase does not confer resistance.[113]

To date, no clear connection has been made between p53 status and cisplatin resistance. Recent clinical data indicate that ovarian cancer patients previously treated with platinum-based chemotherapeutics have tumors with increased glutathione levels, p53 positivity, and cisplatin resistance.[114] Ovarian cancer cells transfected with *HPV-16 E6* lose p53 function by way of E6 protein-mediated process.[115] The p53 deficient cells have similar platinum uptake as wild type cells, but are more sensitive to cisplatin.[115] The increased cisplatin cytotoxicity is due to loss of G_1/S checkpoint control and reduced repair of platinum adducts.[115] It has recently been suggested that p53 protects against cisplatin-induced apoptosis in TCR-proficient fibroblasts.[116]

4. NEW CISPLATIN ANALOGS AS ANTI-CANCER COMPOUNDS

Well over 3000 cisplatin analogs have been synthesized in pursuit of a platinum anticancer agent with broader tumor action, fewer side effects, and activity against cisplatin-resistant cancers.[3] Modifications include replacement of chloride ligands with various leaving groups, use of a plethora of amine and non-amine ligands, and compounds designed only to form monofunctional

DNA adducts.[3] Recent attention has also focused *trans*-platinum complexes, polyplatinum compounds, and platinum(IV) analogs (reviewed recently, see ref. 13). We summarize only briefly several new classes of platinum complexes, none closely related to cisplatin, and comment on the newest clinically approved platinum agent, oxaliplatin.

4.1 Overview of novel anticancer platinum compounds

Targeting platinum anticancer compounds to specific cell types may increase cytotoxic activity, reduce unwanted side effects, and diminish resistance due to limited uptake. As illustrated in Figure 1.6, one strategy for selective delivery of platinum anticancer agents has been to tether carbohydrate moieties to the platinum center.[117-123] Such compounds have primarily been designed to target liver cancer because of the galactose receptors on the surface of liver parenchymal cells.[120,122] Another strategy for targeting liver cancer is the use of bile acids, which are efficiently taken up by hepatoma cells via a Na^+-independent transport carriers.[124] A series of bile acid-platinum conjugates (Figure 1.6) have been synthesized and their cytotoxicity investigated both *in vitro* and in mouse models.[124-129] Bamet-UD2 exhibits enhanced uptake in hepatocytes, an ability similar to that of cisplatin to inhibit tumor growth, and a tendency to prolong survival time of mice implanted with tumors.[126,129]

Interest in platinum agents designed specifically to target DNA has been recently resurrected. Such use of DNA-targeting moieties might reduce platinum side reactions with other biomolecules, potentially reducing side effects.[130] The DNA intercalators employed in this work may also alter the kinetics and sequence specificity of DNA binding, possibly leading to a broadened spectrum of activity.[130] DNA-targeting agents such as phenazine-1-carboxamides, aminoacridines, and distamycin have been tethered to platinum amine ligands,[130-134] following

earlier design principles.[135,136] Such platinum complexes show increased cytotoxicity and are active *in vitro* against cisplatin-resistant cell lines. In addition, non-cisplatin-type platinum-acridinylthiourea conjugates have exhibited micromolar toxicitites in leukemia and ovarian cell lines.[137,138] Research has also focused on the synthesis of hydrophobic cisplatin analogues, cisplatin-loaded micelles for alternate methods of drug delivery, and oligonucleotide-tethered platinum complexes for targeting specific DNA sequences.[139-144]

4.2 High-throughput synthetic methodology

Statistically, 10,000 new compounds must be prepared and screened for activity before one clinically valuable compound is discovered.[145] To date, over 3,000 new platinum compounds have been prepared, and besides cisplatin only carboplatin and oxaliplatin have been approved for use in the United States.[145,146] It would therefore appear that, by significantly increasing the number of compounds synthesized and tested for cytotoxicity, the likelihood of obtaining an improved cisplatin analogue should increase. A high-throughput methodology has recently been developed that allows for the rapid synthesis and screening of platinum complexes.[147] Combinatorial synthesis was used to prepare over 3,600 platinum complexes, which were subsequently screened by using a transcription inhibition fluorescence-based assay.[147] Out of the 3,600 compounds prepared, four species showed promising activity, *cis*-[(isopropylamine)$_2$PtCl$_2$], *cis*-[ammine(cyclobutylamine)PtCl$_2$], *cis*-[(cyclobutylamine)$_2$PtCl$_2$], and *cis*-[ammine(2-amino-3-picoline)PtCl$_2$].[147] All four compounds had previously been identified as cytotoxic cisplatin analogues. Furthermore, the picoline-containing platinum compound is quite similar to ZD0473, currently being developed by AstraZeneca for use in the

clinic.[148] The high-throughput methodology could potentially serve as a new route for discovery of active cisplatin analogues.

4.3 Oxaliplatin

In the early 1970s, 1,2-diaminocyclohexane (DACH) platinum complexes became popular because they were non-cross-resistant with cisplatin.[149] Over the past decade, (1R, 2R-diaminocyclohexane)oxalatoplatinum(II), or oxaliplatin, has been regaining attention because it does not induce nephrotoxicity or myelosuppression and has a broader spectrum of activity than either cisplatin or carboplatin.[146,149-152] The first phase I clinical trial of oxaliplatin was stalled in the 1980s because of its unusual toxicity for peripheral sensory nerves and the GI tract; however, the side effects could be modulated by the Circadian time of administration.[149] Oxaliplatin was approved by France in 1996, China in 1998, and European Union in 1999.[149] In August of 2002, oxaliplatin (Eloxatin™) became the third platinum-based anticancer agent to be approved by the FDA. At present, oxaliplatin is only approved for treatment of metastatic colorectal cancer that has progressed or recurred within six months of completion of a first-line therapy.[153] Clinical studies with oxaliplatin have not demonstrated increased survival time or improvement of disease-related symptoms.[153] Instead oxaliplatin was approved by the FDA based on patient response rate and improved time-to-tumor progression.[153] Clinical trials are currently in progress to obtain approval of oxaliplatin as a first-line chemotherapeutic agent. Oxaliplatin has shown *in vivo* and *in vitro* activity against non-small cell lung cancer, germ cell malignancies, prostate cancer, breast cancer, non-Hodgkin's lymphoma, and malignant mesothelima.[152]

At present, it is unclear why oxaliplatin and cisplatin have different cytotoxicities and range of activity. Paradoxically, oxaliplatin, although more toxic than cisplatin in certain cell

lines, forms significantly fewer Pt-DNA adducts.[154] The adduct profile formed by oxaliplatin is similar to that of cisplatin with regard to the types and proportions of adducts formed; however, oxaliplatin induces more single strand breaks.[154] The difference in compound toxicity cannot be explained by differences in drug uptake or in ease of repair.[146,154] The recently determined X-ray structure of an oxaliplatin 1,2-d(GpG) intrastrand cross-link in a duplex DNA dodecamer has shed some light onto the differing activities of cisplatin and oxaliplatin.[155] The R,R-DACH ligand binding to the platinum center locks the C-N bond and fixes the position of the amine hydrogen atoms.[155] A hydrogen bond is then formed between the ammonia ligand *cis* to the 3′-guanine and the O6 atom of that nucleobase.[155] The inactive S,S,-DACH complex is incapable of forming such a hydrogen bond.[155] Differences in protein-recognition may explain the spectrum of activity of oxaliplatin as compared to cisplatin. Mismatch repair proteins (MMR) preferentially recognize cisplatin over oxaliplatin adducts and MMR deficient cells are resistant to cisplatin.[156] Loss of MMR does not confer resistance to oxaliplatin.[156] HMGB1 also has reduced affinity towards oxaliplatin-DNA adducts. In addition, cisplatin, but not oxaliplatin, activates c-Jun N-terminal and c-Abl kinases. The role of c-Jun N-terminal kinases in cisplatin toxicity is not clear.[146] Some studies suggest that c-Jun N-terminal kinases are required for cisplatin-induced apoptosis, while others assert that JNKs mediate cell survival.[157] Furthermore, oxaliplatin-DNA adducts are more easily bypassed by DNA polymerases β, ζ, γ, and η than cisplatin-adducts during replication.[158]

5. CONCLUSIONS AND FUTURE DIRECTIONS

Significant advances have been made to provide a better understanding of the mechanisms underlying the cytotoxicity of cisplatin. New methodologies are continually emerging that may

facilitate further insight and assist in the rational design of new platinum anticancer agents. Mass spectrometry is being used to study platinum-nucleotide interactions, atomic force microscopy reveals cisplatin changes the dynamics of DNA, and fluorescent tags may enable the visualization of cisplatin action *in vivo*.[159-163] Additional mechanistic insights may not only allow for the design of improved compounds, but may also improve upon combination therapies. For example, antisense oligonucleotides targeting against resistance genes increase the cytotoxicity of cisplatin in resistant cell lines.[164] The coming years will certainly continue to yield exciting developments in the arena of cisplatin chemistry in the cancer cell.

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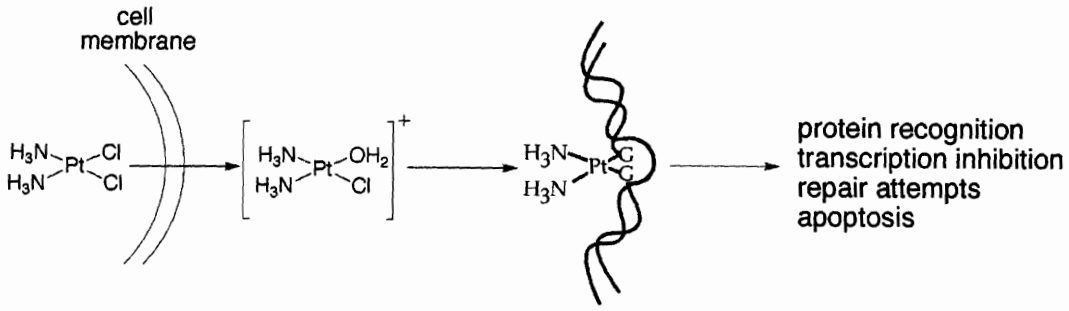

Figure 1.1. Schematic representation of components of the cisplatin mechanism of action.

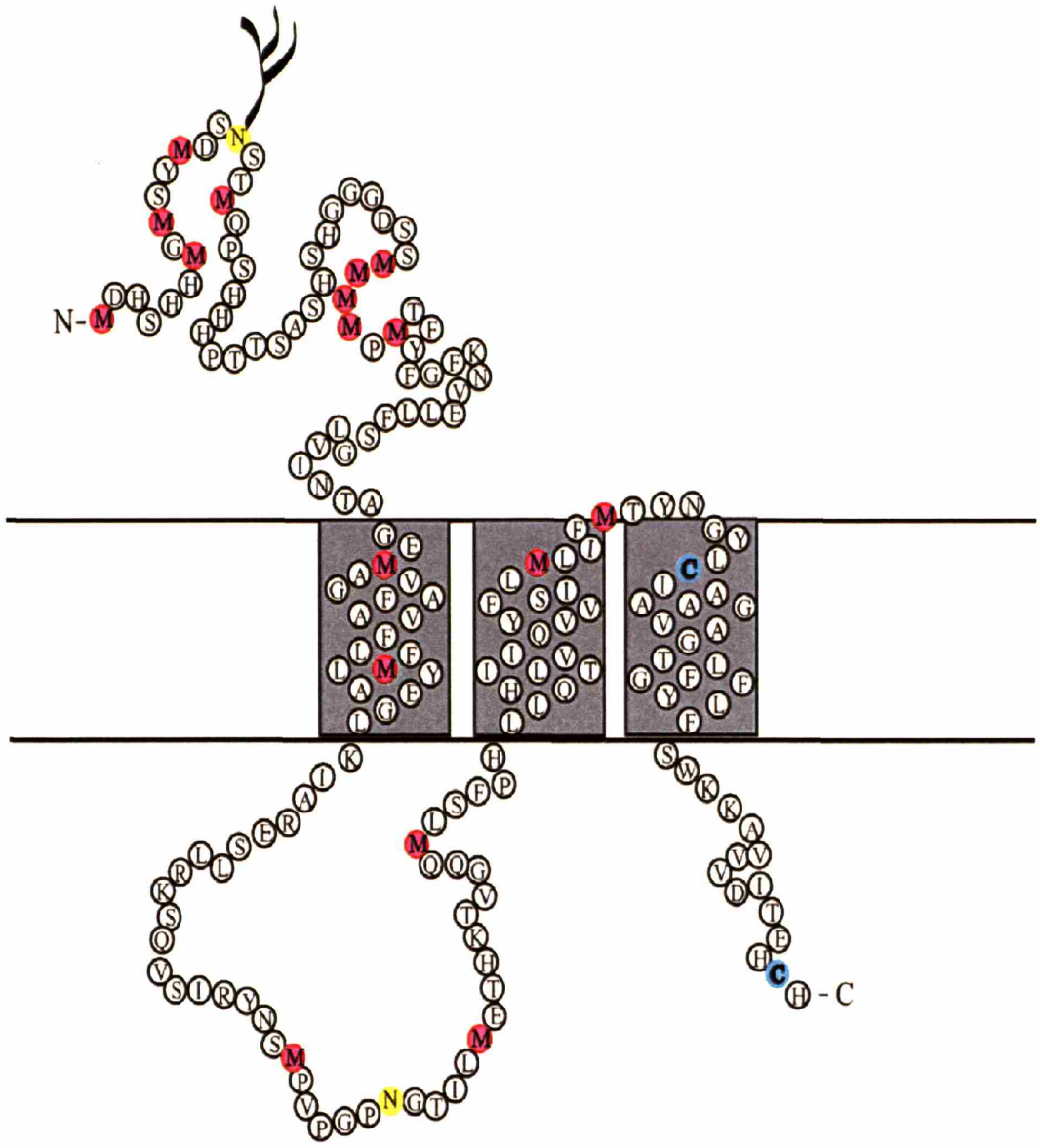

Figure 1.2. Model of hCTR1, the plasma membrane copper transporter in humans. The N-terminus of hCTR1 is extracellular and the C-terminus is an intracellular domain. Possible platinum methionine binding sites are indicated in red. (Reproduced by permission from Ref. 28)

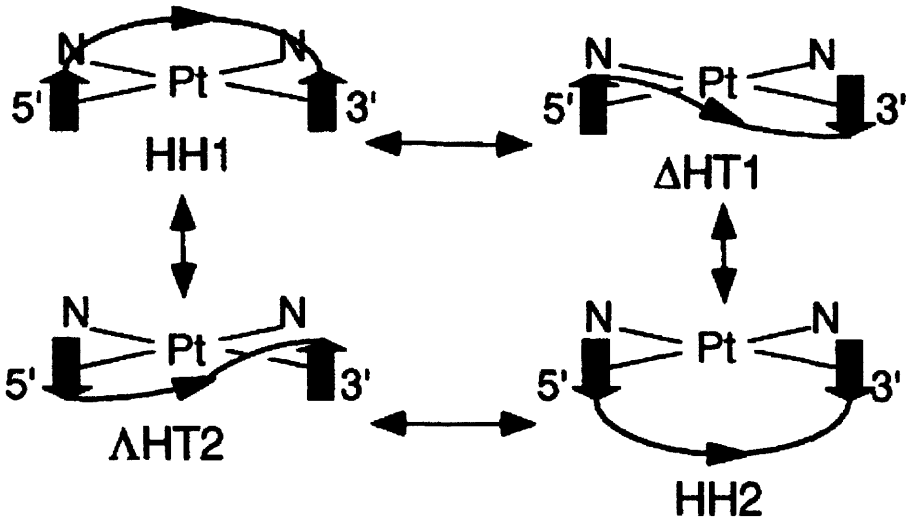

Figure 1.3. Platinum-guanine adduct conformers. The large arrow represents the guanine base with the H8 atom located at the arrow tip. The smaller arrow indicates the direction of phosphodiester linkage propagation. (Reproduced by permission from Ref. 46)

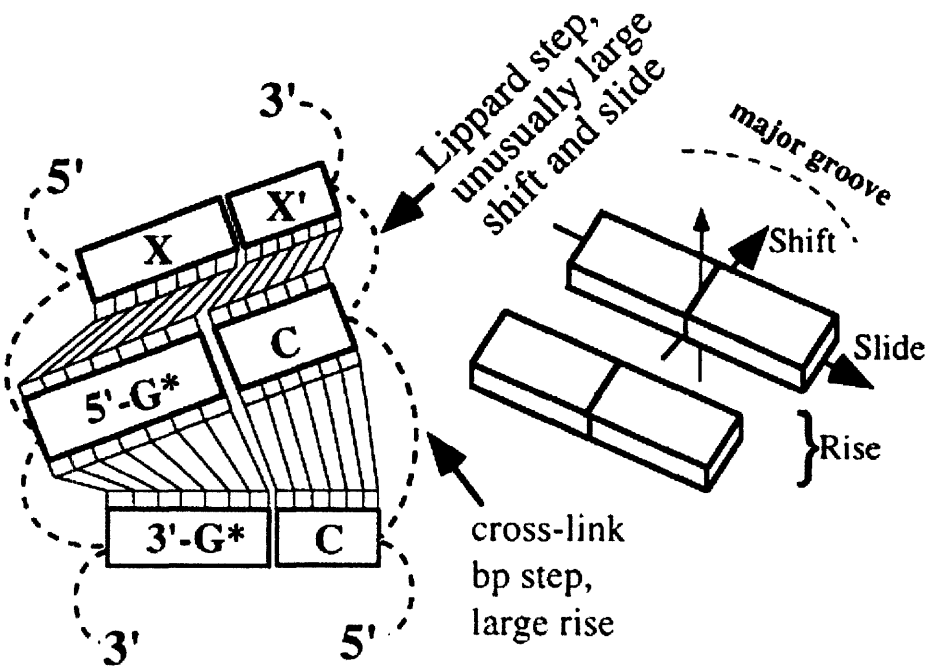

Figure 1.4. Model of DNA distorted by a Lippard base pair step. (Reproduced by permission from Ref. 54)

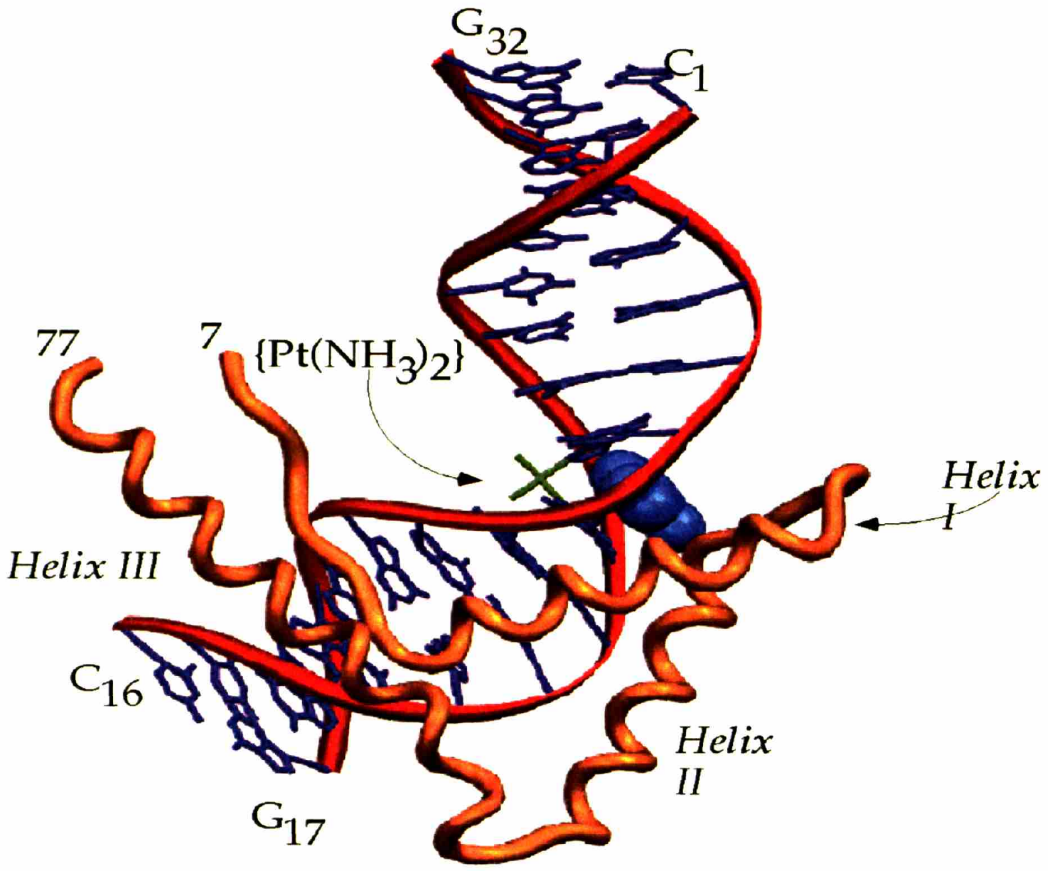

Figure 1.5. HMGB1a bound to cisplatin-modified DNA. Phe37 intercalates in the minor groove opposite the platinum adduct. (Reproduced by permission from Ref. 55)

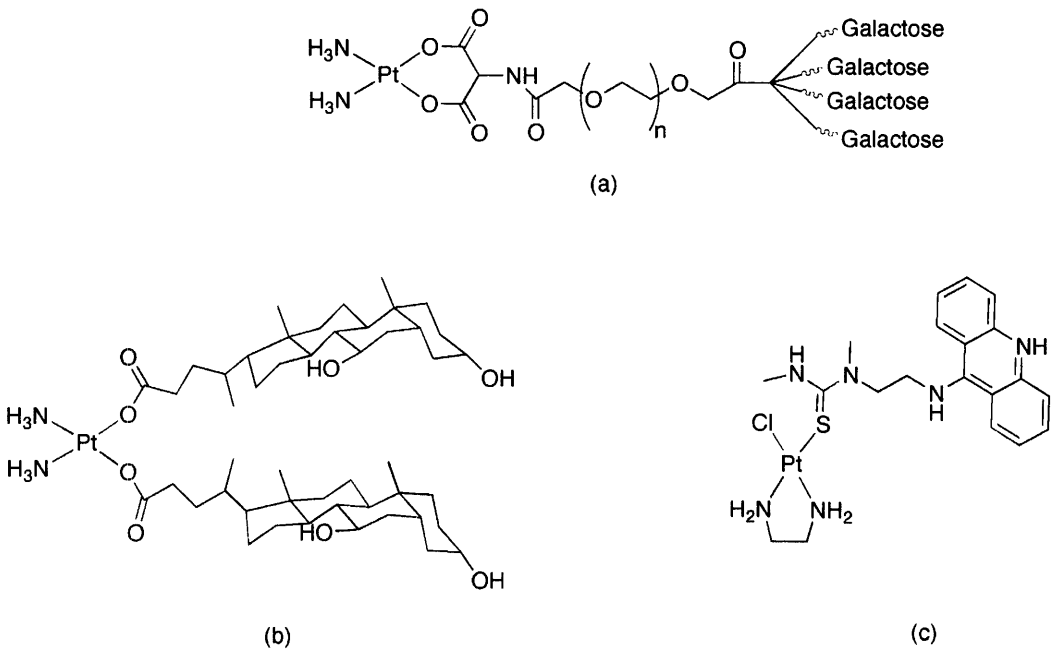

Figure 1.6. Several new classes of platinum anticancer agents: (a) Carbohydrate-platinum conjugate designed to target liver cells; (b) Bamet-UD2, bile acid-platinum derivative for targeting liver cancer; and (c) acridinylthiourea-tethered DNA-targeted platinum complex.

Chapter 2
Synthesis, Characterization, and Cytotoxicity of a Series of Estrogen-Tethered Platinum(IV) Complexes

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Introduction

The anticancer activity of cisplatin was discovered serendipitously over three decades ago.[1] Today cisplatin is used to treat testicular cancer, with greater than 90% success rates,[2,3] as well as breast, ovarian, bladder, lung, and head and neck carcinomas, either alone or in combination therapy.[4] Because cisplatin has adverse side effects, is limited to a narrow range of cancers and can be rendered inactive due to acquired resistance,[2] research has focused on elucidating its mechanism of action to guide the rational synthesis of improved platinum analogues.

Cisplatin can bind to RNA, proteins, and other sulfur-containing biomolecules, but DNA is its primary biological target.[5-7] Various adducts form upon cisplatin binding to DNA, with 1,2-intrastrand d(GpG) and d(ApG) cross-links accounting for ~90% of such interactions;[6,8] a smaller number of 1,3-intrastrand and interstrand cross-links also form. These adducts block transcription, inhibit replication, and ultimately induce apoptosis.[6] The DNA duplex is significantly distorted upon formation of cisplatin 1,2-intrastrand cross-links.[9] The resulting structure serves as a recognition motif for a variety of cellular proteins, including DNA repair components, histones, and HMG-domain proteins, such as HMGB1.[6,9,10] The binding of these proteins to platinum-DNA adducts modulates the cytotoxicity of cisplatin[11-13] and offers new strategies for improving the chemotherapeutic potential of platinum-based anticancer drugs.[14]

Recent work from our laboratory revealed that estrogen receptor-positive, ER(+), cells treated with estrogen are sensitized to cisplatin,[15] since estrogen induces overexpression of HMGB1, a protein that shields cisplatin-DNA adducts from nucleotide excision repair (NER).[11,15,16] This finding suggested to us that estrogen-tethered platinum(IV) complexes might prove valuable as novel anticancer drug candidates. The proposed mechanism is illustrated in Figure 2.1. Upon entering the reducing environment of the cell, the platinum(IV) complexes will

be reduced to platinum(II), simultaneously releasing one equivalent of cisplatin and two equivalents of a linker-modified estrogen. Subsequent binding of cisplatin to DNA and hydrolysis of the ester moieties should lead to HMGB1 upregulation, as described above, and sensitization of the cells due to repair-shielding of the cisplatin-DNA adducts.

The present chapter describes the application of this strategy through the design, synthesis, and characterization of a series of compounds **BEP1 – BEP5** (Scheme 2.2). In these molecules, estradiol is tethered to the terminal carboxylate groups of *cis, cis, trans*-diamminedichlorodisuccinatoplatinum(IV) through polymethylene chains of varying lengths. This variability was programmed into our study in order to allow for the best match between the kinetics of estrogen-mediated HMGB1 overproduction and cisplatin-DNA adduct formation in the cancer cell. The ability of the **BEPn** complexes to upregulate HMGB1, and their cytotoxic profile in both ER(+) and ER(-) cells, are reported and discussed.

Experimental Procedures

Materials and Methods

Potassium tetrachloroplatinate(II) was a gift from Engelhard. Cisplatin and *cis, cis, trans*-diamminedichlorodihydroxyplatinum(IV) were prepared as described in the literature.[17,18] The preparation of compounds **L2, L3, L4,** and **L5** were based on a previously reported methodology.[19] All chemicals and solvents were purchased from commercial sources unless specified otherwise. ^{1}H and ^{195}Pt NMR spectra were recorded on either a Varian 300 MHz or 500 MHz spectrometer at the MIT Department of Chemistry Instrumentation Facility (DCIF). High-resolution mass spectral analysis was carried out at the MIT DCIF.

Synthesis of *cis, cis, trans*-Diamminedichlorodisuccinatoplatinum(IV) (1). Succinic anhydride (4.1 g, 41 mmol) and *cis, cis, trans*-diamminedichlorodihydroxyplatinum(IV) (3.3 g, 10 mmol) were dissolved in 5 mL of DMSO. The solution was heated to 70 C for 15 h with constant stirring, cooled to room temperature, and filtered. The DMSO solvent was removed from the filtrate by lyophilization to yield a yellow solid. Recrystallization from acetone at -20 C afforded a pale yellow solid (3.7 g, 6.9 mmol, 69% yield). ^{1}H NMR (d_6-acetone, 300 MHz): δ 2.45 (m, 4H, CH_2), 2.53 (m, 4H, CH_2), 6.511 (broad singlet, 6H, NH_3). ^{195}Pt NMR (500 MHz): δ 1226.531. ERMS (ESI) calculated for $[M+H]^+$ 534.0004 amu, found 534.0001 amu.

Synthesis of 3-*tert*-Butoxycarbonylaminopropionic acid (L2). Triethylamine (6.2 mL, 45 mmol) was added to a solution of 3-aminopropionic acid (2.7 g, 30 mmol) in 50% aqueous dioxane (30 mL). BOC-ON (8.2 g, 33 mmol) was added and the reaction stirred for 3 h at room temperature. The reaction solution was diluted with H_2O (40 mL) and ethyl acetate (60 mL). The aqueous layer was isolated, washed with ethyl acetate, and acidified with a 5% citric acid solution. The aqueous layer was subsequently extracted with ethyl acetate. The organic fractions were combined and evaporated to yield **L2** as a cream solid (3.6 g, 19 mmol, 63% yield); mp=67-72 °C. ^{1}H NMR (d_6-DMSO, 300 MHz): δ 1.35 (s, 9H, CH_3), 2.33 (t, 2H, CH_2), 3.11 (q, 2H, CH_2), 6.67 (t, 1H, NH). HRMS (ESI) calculated for $[M-H]^-$ 188.0928 amu, found 188.0922 amu.

Synthesis of 4-*tert*-Butoxycarbonylaminobutyric acid (L3). This compound was prepared as described for **L2** from stirring 4-aminobutyric acid (3.9 g, 38 mmol) in 50% aqueous dioxane with triethylamine (8.0 mL, 57 mmol) and BOC-ON (10.4 g, 42 mmol). A yellow oil was

obtained that solidified upon addition of hexanes and cooling to –20 C to yield a cream solid (4.0 g, 19 mmol, 52% yield); mp=54-57 °C. ^{1}H NMR (d_6-DMSO, 300 MHz): δ 1.36 (s, 9H, CH_3), 1.57 (m, 2H, CH_2), 2.17 (t, 2H, CH_2), 2.90 (m, 2H, CH_2), 6.80 (t, 1H, NH). HRMS (ESI) calculated for $[M-H]^-$ 202.1085 amu, found 202.1075 amu.

Synthesis of 5-*tert*-Butoxycarbonylaminopentanoic acid (L4). This compound was prepared as described for **L2** from 5-aminopentanoic acid (3.4 g, 29 mmol), triethylamine (6.0 mL, 43 mmol), and BOC-ON (7.9 g, 32 mmol). A cream solid (3.1 g, 14 mmol) was isolated in 49% yield; mp=44-47 °C. ^{1}H NMR (d_6-DMSO, 300 MHz): δ 1.36 (s, 9H, CH_3), 1.43 (m, 4H, 2 CH_2), 2.17 (t, 2H, CH_2), 2.88 (m, 2H, CH_2), 6.73 (t, 1H, NH). HRMS (ESI) calculated for $[M-H]^-$ 216.1241 amu, found 216.1244 amu.

Synthesis of 6-*tert*-Butoxycarbonylaminohexanoic acid (L5). This compound was prepared as described for **L2** from 6-aminohexanoic acid (3.5 g, 27 mmol), triethylamine (5.5 mL, 40 mmol), BOC-ON (7.5 g, 30 mmol). A pale yellow oil (3.0 g, 13 mmol) was isolated in 48% yield. ^{1}H NMR (d_6-DMSO, 300 MHz): δ 1.20 (m, 2H, CH_2), 1.30 (m, 2H, CH_2), 1.36 (s, 9H, CH_3), 1.46 (m, 2H, CH_2), 2.16 (t, 2H, CH_2), 2.87 (m, 2H, CH_2), 6.74 (t, 1H, NH). HRMS (ESI) calculated for $[M-H]^-$ 230.1398 amu, found 230.1395 amu.

Synthesis of 17-(Aminoacetoxy)-estradiol-3-benzoate (EL1). Diisopropylcarbodiimide (3.7 mL, 24 mmol) was added to a solution of N-*tert*-butoxycarbonylglycine (4.2 g, 24 mmol) and 4-DMAP (3.0 g, 24 mmol) in THF (200 mL). The solution was stirred for 10 min before the addition of estradiol-3-benzoate (5.0 g, 13 mmol). After being stirred overnight, the solution was

filtered and the solvent was removed by rotary evaporation. The solid residue was dissolved in a 1M HCl/dioxane solution (265 mL) and stirred for an additional 6 h. Following evaporation of dioxane, the residue was suspended in 100 mL H_2O and the pH was adjusted to 10 with ammonium hydroxide. The solution was stirred for several hours and filtered to yield a white solid (4.3 g, 9.9 mmol, 75% yield); mp=125-129 °C. ^{1}H NMR (d_6-DMSO, 300 MHz): δ 8.12 (d, 2H, ArH), 7.74 (t, 1H, ArH), 7.59 (t, 2H, ArH), 7.34 (d, 1H, ArH), 7.01 (d, 1H, ArH), 6.96 (s, 1H, ArH), 4.66 (t, 1H, CH), 3.83 (d, 1H, CH), 2.83 (m, 2H, CH), 1.75 (bs, 2H, NH_2); 2.3-1.2 (m, 13H, CH_2), 0.796 (s, 3H, CH_3). HRMS (ESI) calculated for $[M+H]^+$ 434.2326 amu, found 434.2319 amu.

Synthesis of 17-(3-Aminopropionate)-estradiol-3-benzoate (EL2). A solution of **L2** (2.1 g, 11 mmol) and 4-DMAP (1.3 g, 11 mmol) was prepared in 100 mL of DMF. Diisopropylcarbodiimide (1.7 mL, 11 mmol) was added and the solution was stirred for 10 min. Estradiol-3-benzoate (2.6 g, 6.9 mmol) was added and the reaction was allowed to stir for 7 h. The solution was filtered, diluted with water, and extracted with ether. The organic fractions were combined and evaporated to dryness to yield a pink residue. The residue was dissolved in 800 mL of methylene chloride; 120 mL TFA was added and the solution was stirred for 2 h. The methylene chloride was evaporated to yield a pink oil, which was dissolved in H_2O and the pH adjusted to 10 with ammonium hydroxide. A white solid began to precipitate and the slurry was stirred for an additional hour. The solution was filtered to collect a white solid (~3 g of crude material); mp=156-160 °C. ^{1}H NMR (d_6-DMSO, 300 MHz): δ 8.08 (d, 2H, ArH), 7.73 (t, 1H, ArH), 7.58 (t, 2H, ArH), 7.32 (d, 1H, ArH), 7.00 (d, 1H, ArH), 6.94 (s, 1H, ArH), 4.67 (t, 1H, CH), 3.04 (t, 2H, CH_2), 2.83 (m, 2H, CH), 2.68 (m, 2H, CH_2), 2.30-1.20 (m, 13H, CH/CH_2), 1.70

(bs, 2H, NH_2), 0.814 (s, 3H, CH_3). HRMS (ESI) calculated for $[M+H]^+$ 448.2482 amu, found 448.2481 amu.

Synthesis of 17-(4-Aminobutanoate)-estradiol-3-benzoate (EL3). This compound was prepared as described for **EL2** using L3 (1.8 g, 8.9 mmol), 4-DMAP (1.1 g, 9.1 mmol), diisopropylcarbodiimide (1.4 mL, 8.9 mmol), and estradiol-3-benzoate (2.2 g, 5.7 mmol). The BOC-protecting group was removed by stirring in 700 mL methylene chloride and 100 mL TFA. A white solid was isolated (2.5 g crude) and used without purification; mp=110-115 °C. ^{1}H NMR (d_6-DMSO, 300 MHz): δ 8.09 (d, 2H, ArH), 7.73 (t, 1H, ArH), 7.59 (t, 2H, ArH), 7.33 (d, 1H, ArH), 7.00 (d, 1H, ArH), 6.95 (s, 1H, ArH), 4.65 (t, 1H, CH), 2.83 (m, 4H, CH_2), 2.42 (m, 2H, CH_2), 2.40-2.00 (m, 3H, CH_2), 1.81 (m, 4H, CH_2), 1.70 (bs, 2H, NH_2), 1.60-1.20 (m, 8H, CH), 0.813 (s, 3H, CH_3). HRMS (ESI) calculated for $[M+H]^+$ 462.2639 amu, found 462.2622 amu.

Synthesis of 17-(5-Aminopentanoate)-estradiol-3-benzoate (EL4). This compound was prepared as described for **EL2** using **L4** (1.3 g, 6.6 mmol), 4-DMAP (0.82 g, 6.7 mmol), diisopropylcarbodiimide (1.0 mL, 6.4 mmol), and estradiol-3-benzoate (1.6 g, 4.1 mmol). Deprotection of the amine was carried out by stirring in 480 mL of methylene chloride and 72 mL of TFA. A white solid was isolated (1.9 g crude); mp=107-113 °C. ^{1}H NMR (d_6-DMSO, 300 MHz): δ 8.09 (d, 2H, ArH), 7.73 (t, 1H, ArH), 7.59 (t, 2H, ArH), 7.32 (d, 1H, ArH), 7.00 (d, 1H, ArH), 6.95 (s, 1H, ArH), 4.65 (t, 1H, CH), 2.83 (m, 4H, CH_2), 2.36 (m, 2H, CH_2), 2.30-2.00 (m, 3H, CH), 1.90-1.72 (m, 4H, CH) 1.70 (bs, 2H, NH_2), 1.57 (m, 4H, CH_2), 1.42-1.20 (m, 6H, CH), 0.815 (s, 3H, CH_3). HRMS (ESI) calculated for $[M+H]^+$ 476.2795 amu, found 476.2800 amu.

Synthesis of 17-(6-Aminohexanoate)-estradiol-3-benzoate (EL5). This compound was prepared as described for **EL2** using **L5** (1.1 g, 4.6 mmol), 4-DMAP (0.58 g, 4.8 mmol), diisopropylcarbodiimide (730 μL, 4.7 mmol), and estradiol-3-benzoate (0.51 g, 1.4 mmol). Removal of the BOC-group was achieved by stirring in a solution of TFA (25 mL) in methylene chloride (240 mL). A white solid was isolated by filtration (0.60 g crude); mp=132-137 °C. ^{1}H NMR (d_6-DMSO, 300 MHz): δ 8.08 (d, 2H, ArH), 7.72 (t, 1H, ArH), 7.58 (t, 2H, ArH), 7.32 (d, 1H, ArH), 7.00 (d, 1H, ArH), 6.93 (s, 1H, ArH), 4.62 (t, 1H, CH), 2.82 (m, 2H, CH_2), 2.78-2.77 (m, 4H, CH_2), 2.31 (m, 2H, CH_2), 2.23-1.72 (m, 6H, CH), 1.70 (bs, 2H, NH_2), 1.57-1.20 (m, 10H, CH/CH_2), 0.798 (s, 3H, CH_3). HRMS (ESI) calculated for $[M+H]^+$ 490.2952 amu, found 490.2954 amu.

Synthesis of *cis, cis, trans*-Diamminedichloro-*bis*-(17-(N-carbonylmethylsuccinato)-estradiol-3-benzoate)platinum(IV) (BEP1). Diisopropylcarbodiimide (0.57 mL, 3.7 mmol) was added to a solution of **1** (0.81 g, 1.5 mmol) and 4-DMAP (0.48 g, 3.9 mmol) in DMF (100 mL). The solution was allowed to stir for 10 min at room temperature before the addition of **EL1** (1.6 g, 3.7 mmol). The solution was stirred for 15 h at room temperature, filtered, and the filtrate was diluted with 200 mL of ether. The resultant solution was cooled at –20 C for 10 h to facilitate precipitation. The crude product was filtered, triturated with ethanol (30 mL) and centrifuged (5 times). The tan solid was then triturated with boiling water (10 mL) five times and centrifuged to yield a pale yellow solid (0.72 g, 0.53 mmol, 35% yield). ^{1}H NMR (d_6-DMSO, 300 MHz): δ 8.33 (t, 2H, NH), 8.10 (d, 4H, ArH), 7.74 (t, 2H, ArH), 7.59 (t, 4H, ArH), 7.34 (d, 2H, ArH), 7.01 (d, 2H, ArH), 6.95 (s, 2H, ArH), 6.56 (bs, 6H, NH_3), 4.65 (t, 2H, CH), 3.80 (d,

4H, CH_2), 2.83 (m, 4H, CH_2), 2.60-1.20 (m, 34H, CH/CH_2), 0.785 (s, 6H, CH_3). HRMS calculated for $[M+H]^+$ 1364.4299 amu, found 1364.4253 amu.

Synthesis of *cis, cis, trans*-Diamminedichloro-*bis*-(17-(N-(2-carboxyethyl)-succinato)-estradiol-3-benzoate)platinum(IV) (BEP2). This compound was prepared as described for **BEP1** using **1** (0.37 g, 0.69 mmol), 4-DMAP (0.36 g, 2.9 mmol), diisopropylcarbodiimide (430 μL, 2.7 mmol), and **EL2** (1.2 g, 2.8 mmol). Crude **BEP2** was purified as described for **BEP1** to yield a pale yellow solid (0.13 g, 0.093 mmol, 14% yield). ^1H NMR (d_6-DMSO, 300 MHz): δ 8.10 (d, 4H, ArH), 7.95 (t, 2H, NH), 7.74 (t, 2H, ArH) 7.60 (t, 4H, ArH), 7.35 (d, 2H, ArH), 7.00 (d, 2H, ArH), 6.95 (s, 2H, ArH), 6.49 (bs, 6H, NH_3), 4.65 (t, 2H, CH), 3.23 (t, 4H, CH_2), 2.83 (m, 4H, CH), 2.43 (m, 4H, CH_2), 2.43-1.20 (m, 34H, CH/CH_2), 0.799 (s, 6H, CH_3). HRMS (ESI) calculated for $[M+H]^+$ 1391.4591 amu, found 1391.4562 amu.

Synthesis of *cis, cis, trans*-Diamminedichloro-*bis*-(17-(N-(3-carboxypropyl)-succinato)-estradiol-3-benzoate)platinum(IV) (BEP3). This compound was prepared as described for **BEP1** using **1** (0.65 g, 1.2 mmol), 4-DMAP (0.61 g, 5.0 mmol), diisopropylcarbodiimide (760 μL, 4.9 mmol), and **EL3** (2.3 g, 5.0 mmol). The crude material was purified as described for **BEP1** to yield a pale yellow solid (0.12 g, 0.084 mmol, 7.0% yield). ^1H NMR (d_6-DMSO, 300 MHz): δ 8.12 (d, 4H, ArH), 7.85 (t, 2H, NH), 7.74 (t, 2H, ArH) 7.59 (t, 4H, ArH), 7.35 (d, 2H, ArH), 7.00 (d, 2H, ArH), 6.96 (s, 2H, ArH), 6.50 (bs, 6H, NH_3), 4.69 (t, 2H, CH), 3.26 (t, 4H, CH_2), 3.04 (t, 4H, CH_2), 2.83 (m, 4H, CH_2), 2.47 (m, 4H, CH_2), 2.40-1.20 (m, 34H, CH_2), 0.805 (s, 6H, CH_3). HRMS (ESI) calculated for $[M+H]^+$ 1419.4904, found 1419.4890 amu.

Synthesis of *cis, cis, trans*-Diamminedichloro-*bis*-(17-(N-(4-carboxybutyl)-succinato)-estradiol-3-benzoate)platinum(IV) (BEP4). This compound was prepared as described for **BEP1** using **1** (0.22 g, 0.40 mmol), 4-DMAP (0.15 g, 1.2 mmol), diisopropylcarbodiimide (190 μL, 1.2 mmol), and **EL4** (0.62 g, 1.3 mmol). The crude material was purified as described for **BEP1** to yield a pale yellow solid (0.25 g, 0.17 mmol, 43% yield). ^{1}H NMR (d_6-DMSO, 300 MHz): δ 8.10 (d, 4H, ArH), 7.85 (t, 2H, NH), 7.74 (t, 2H, ArH) 7.60 (t, 4H, ArH), 7.35 (d, 2H, ArH), 7.00 (d, 2H, ArH), 6.97 (s, 2H, ArH), 6.51 (bs, 6H, NH_3), 4.64 (t, 2H, CH), 3.01 (t, 4H, CH_2), 2.84 (m, 4H, CH_2), 2.44 (m, 4H, CH_2), 2.30-2.21 (m, 8H, CH/CH_2), 2.20-1.20 (m, 34H, CH/CH_2), 0.804 (s, 6H, CH_3). HRMS (ESI) calculated for $[M+Na]^+$ 1469.5036, found 1469.5006 amu.

Synthesis of *cis, cis, trans*-Diamminedichloro-*bis*-(17-(N-(5-carboxypentyl)-succinato)-estradiol-3-benzoate)platinum(IV) (BEP5). This compound was prepared as described for **BEP1** using **1** (0.14 g, 0.26 mmol), 4-DMAP (0.10 g, 0.83 mmol), diisopropylcarbodiimide (130 μL, 0.81 mmol), and **EL5** (0.40 g, 0.82 mmol). The crude product was purified as described for **BEP1** to yield a pale yellow solid (0.085 g, 0.057 mmol, 22% yield). ^{1}H NMR (d_6-DMSO, 300 MHz): δ 8.09 (d, 4H, ArH), 7.81 (t, 2H, NH), 7.71 (t, 2H, ArH) 7.57 (t, 4H, ArH), 7.32 (d, 2H, ArH), 6.98 (d, 2H, ArH), 6.94 (s, 2H, ArH), 6.49 (bs, 6H, NH_3), 4.62 (t, 2H, CH), 2.98 (t, 4H, CH_2), 2.82 (m, 4H, CH_2), 2.40 (m, 4H, CH_2), 2.30-2.20 (m, 12H, CH/CH_2), 2.10-1.20 (m, 34H, CH/CH_2), 0.799 (s, 6H, CH_3). HRMS (ESI) calculated for $[M+H]^+$ 1475.5530 amu, found 1475.5551 amu.

Cell Culture Studies. MCF-7 cells were grown in DMEM (GIBCO/BRL) supplemented with 10% FBS, 1X antibiotic/antimycotic solution (GIBCO), and 2 mM L-glutamine. HCC-1937 cells were grown in RPMI-1640 media (ATCC) containing 10% FBS and 1X antibiotic/antimycotic solution. All cells were incubated at 37 C under a 5% CO_2 atmosphere.

Overexpression of HMGB1 Induced by BEPn Complexes. MCF-7 cells were grown to 70% confluence on 12-mm glass cover slips in 24-well plates. The cells were treated with either estradiol or **BEPn** and incubated for 4 h. The cells were then fixed and permeabilized with 25% acetic acid in methanol for 10 min at RT, washed with PBS, and incubated with a 1:100 dilution of anti-HMGB1 polyclonal antibody (PharMingen) for 1h at 37 C. The cells were subsequently incubated with a 1:200 dilution of goat anti-rabbit IgG conjugated to FITC (Biosource International) for 1 h at 37 C. The cover slips were then placed on microscope slides, fixed with gelvatol, and incubated at 4 C for 12 h. HMGB1 levels were then visualized under a fluorescent light microscope (Nikon) equipped with a digital camera (Diagnostic Instruments).

Cytotoxic Profile of BEPn. The cytotoxicities of the **BEPn** compounds were evaluated by using two methodologies, SRB and colony counting assays. For the SRB assay, MCF-7 and HCC-1937 cells were seeded onto 96-well plates at a density of 1000 cells per well and allowed to grow for 24 h. Cells were treated with compounds **BEP1** - **BEP5** at the following concentrations: 1, 2, 3, 4, 5, 6, 8, 10 μM. The 96-well plates were covered with Breathe-Easy gas permeable membranes (Diversified Biotech) and the cells incubated at 37 C for 96 h. After the incubation period, the cells were fixed by addition of 25 μL of 50% TCA and subsequent incubation at 4 C for 30 min. The viable cells were then stained by addition of 100 μL of sulforhodamine B in 0.1% acetic

acid followed by incubation for 30 min at RT. The cells were washed with 1% acetic acid and allowed to dry overnight. The SRB was solubilized by the addition of 100 μL of 10 mM Tris (pH 10.5) followed by shaking for 5 min. The number of viable cells was then quantified by measuring the absorbance at 492 nm.

For the colony counting assay, MCF-7 and HCC-1937 cells were seeded onto 6-well plates at a density of 1000 cells per well in 2 mL media and allowed to grow for 24 h at 37 C. Cells were then treated for 72 h with the **BEPn** complexes at the following concentrations: 0, 2, 4 6, 8, 10 μM. After 72 h, cells were washed with PBS, and fresh medium was added. After 7 days the colonies were counted by staining with a 1% methylene blue/50% ethanol (vol/vol) solution.

Results and Discussion

Synthesis and Characterization of BEP1 – BEP5

The preparation of several steroid-tethered platinum complexes has been reported.[20-24] None of these compounds enables the release of unmodified estrogen inside cells nor is any expected to effect the expression of HMGB1 because of their low affinity for the ER. All previously synthesized platinum-estrogen conjugates were designed to accumulate specifically in ER(+) cells by taking advantage of known steroid transport mechanisms.[20-22] The present study describes the first report of a hormone-conjugated platinum(IV) complex linked at the 17β-position. We chose this particular strategy for several reasons. Platinum(IV) complexes are relatively inert to ligand substitution, which allows for oral administration and differentiates their pharmacokinetic properties from those of cisplatin.[17,25] In addition, the designed platinum(IV) complexes assure simultaneous delivery of cisplatin and estrogen to the same population of cells.

Furthermore, release of cisplatin and unmodified estradiol may be achieved by use of the platinum(IV) platform. The concurrent delivery of cisplatin and estrogen allows for the selective targeting of ER(+) cells by achieving both DNA damage and HMGB1-induced repair shielding. Upon entering the cell, **BEPn** will be readily reduced by glutathione or other intracellular agents to afford cisplatin and two equivalents of the linker-modified estrogen.[25-28] The linkers were designed to be susceptible to hydrolysis by intracellular esterases, because the estrogen receptor does not recognize estrogens modified at the 17β-position.[29,30] Although the dose of the estradiol delivered to the cell cannot be optimized, the varying length of the estrogen-linkers permits control over the kinetics of estrogen hydrolysis.

Initial attempts to prepare the estrogen-tethered complexes involved the synthesis of an ester-linked compound. A variety of coupling methodologies were tried to attach *cis, cis, trans*-diamminedichlorodisuccinatoplatinum(IV) directly to estradiol-3-benzoate; however, the desired compound could not be obtained. Next, 17-hemisuccinate-estradiol-3-benzoate was synthesized and numerous attempts were made to couple it to *cis, cis, trans*-diamminedichlorodihydroxyplatinum(IV), but without success.[31] Ultimately, the first member of the **BEPn** family of estrogen-tethered platinum(IV) complexes, **BEP1**, was obtained through coupling of a linker-modified estrogen with *cis, cis, trans*-diamminedichloro-disuccinatoplatinum(IV).

Cisplatin is readily oxidized by hydrogen peroxide to produce *cis, cis, trans*-diamminedichlorodihydroxyplatinum(IV).[17] As shown in Scheme 2.1, this *trans*-dihydroxyplatinum(IV) complex can be further modified by reaction with succinic anhydride to yield a *trans*-dicarboxylatoplatinum(IV) complex that is amenable to additional derivatization. The preparation of the *trans*-dicarboxylatoplatinum(IV) complex presented here is a variation of

a previously reported synthesis[32] that affords a similar yield, requires less stringent conditions, and leads to product in 15 h versus 24-48 h. The resulting *cis, cis, trans*-diamminedichloro-disuccinatoplatinum(IV) complex was characterized by ESI-MS, ^{1}H NMR, and ^{195}Pt NMR spectroscopy. The platinum ammine proton resonances appear as a broad singlet at 6.51 ppm, a value that is consistent with the ammine chemical shifts of other *trans*-dicarboxylatoplatinum(IV) complexes.[25] The succinato protons and ammine protons integrate in a 4:3 ratio, indicating the presence of two succinato ligands per platinum center. The ^{195}Pt NMR resonance at 1226 ppm is consistent with other known platinum(IV) carboxylates.[33,34]

The preparation of the polymethylene linkers (**Ln**) was based on previously published methodology.[19] Their ^{1}H NMR spectra agreed those with published previously (data not shown).[19] The series of linker-modified estrogens (**ELn**) were obtained by coupling estradiol-3-benzoate and BOC-protected aminoalkyl carboxylic acids (**Ln**) using diisopropylcarbodiimide, followed by removal of the BOC-protecting group. The free amine provides a suitable handle for coupling to the *trans*-disuccinatoplatinum(IV) complex. Because of their poor solubility, the **ELn** compounds were used without purification. Formation of the desired linker-modified estrogens was confirmed by ESI-MS and ^{1}H NMR spectroscopy (Table 2.1). A ^{1}H NMR spectrum of **EL1** is shown in Figure 2.2. A significant downfield shift of the H_{17} proton resonance is observed upon formation of the new ester linkage.

As shown in Scheme 2.2, the amine-modified estrogens are easily coupled to *trans*-dicarboxylatoplatinum(IV) utilizing diisopropylcarbodiimide, a common peptide coupling reagent. The ESI-MS and ^{1}H NMR spectroscopic data (Table 2.1) confirm the presence of the desired estrogen-tethered platinum(IV) complexes. A ^{1}H NMR spectrum of **BEP1** is shown in Figure 2.3. Coupling of the series of amino-modified estrogens to *cis, cis, trans*-

diamminedichlorodisuccinatoplatinum(IV) yielded the desired amide-linked compounds. Formation of the amide bond is evident from the appearance of a characteristic amide proton resonance at 7.80-8.33 ppm (H_{22}). The ^1H NMR data provide quantitative evidence for the presence of two estrogen moieties for every platinum center. The integrated intensity of the methyl protons (H_{18}) of the two estrogen groups match that of the platinum-ammine protons (H_{23}). In addition, there are an equal number of amide (H_{22}) and estrogen 17-α protons (H_{17}). The experimentally determined masses for **BEP1 - BEP5** are in excellent agreement with the calculated values (± 0.003%). The synthetic methodology presented provides a convenient route for preparing a variety of platinum(IV) compounds for testing as anticancer drugs and has the potential to target such complexes to specific tissue or cell types.

Overexpression of HMGB1 in MCF-7 Cells Following BEPn Treatment

The ability of compounds **BEP1 – BEP5** to upregulate HMGB1 levels was investigated by immunofluorescence microscopy. As shown in Figure 2.4, 4-hr incubation with 200 nM **BEP1 – BEP5** induces the overexpression of HMGB1 in MCF-7 cells. Treatment with **BEP1, BEP2, BEP3,** or **BEP4** increases HMGB1 expression to a similar degree as treatment with an equal amount of estradiol, whereas **BEP5** treatment induces considerably less protein expression. The ability of these estrogen-tethered platinum(IV) compounds to upregulate HMGB1 implies that all are taken into the cell and reduced to a Pt(II) species with concomitant release of the linker-modified estradiol. Moreover, hydrolysis of the linker-modified estradiol ester group must occur to allow for interaction of free-estradiol with the ER. Although hydrolysis of the estrogenic moiety alone could result in upregulation of HMGB1, reduction of Pt(IV) is required for DNA-

binding activity.[26] It is unlikely, however, that ester hydrolysis will precede platinum(IV) reduction because of the kinetics associated with these processes.[25,29]

Estrogen interaction with the hormone-binding domain induces the formation of ER homodimers, which then associate with the estrogen response element (ERE).[35] The exact mechanism by which estrogen binding to the ER induces overexpression of HMGB1 is not known; however, the presence of HMGB1 is required for ER interaction with the ERE.[36,37] HMGB1 may facilitate ER interaction with the ERE either by bending the element to provide a binding site for the ER or by stabilizing the distorted DNA of the ER-ERE complex.[35,37] The ability of the ER to bind to the ERE may also be affected by the intracellular levels of HMGB1 or by the number of free HMGB1 binding sites.[37] In addition, the transcriptional activity of the ER is increased by HMGB1.[38]

Selective Cytotoxic Behavior of BEP1 – BEP5 in ER(+) versus ER(-) Cells

The ability of **BEP1 – BEP5** to stimulate upregulation of HMGB1 suggests that ER(+) cells will be more sensitive than ER(-) cells towards these compounds. The cytotoxic behavior of **BEP1 – BEP5** and *cis, cis, trans*-[$Pt(NH_3)_2Cl_2(succinato)_2$] was evaluated in human breast cancer cell lines MCF-7 and HCC-1937, which are ER(+) and ER(-), respectively. Cell viability was evaluated using both the sulforhodamine B (SRB) and colony counting assays. The two methods provided comparable dose response curves, with the SRB assay resulting in less variance in the results. The colony counting method afforded more reliable data for **BEP5**, however. Since, in a control experiment (Figure 2.5), the sensitivities of the MCF-7 and HCC-1937 cells (IC_{50} = 4.0 μM) to *cis, cis, trans*-[$Pt(NH_3)_2Cl_2(succinato)_2$], the synthetic precursor of the **BEPn** family that carries no steroid appendage, were nearly identical, any difference in the ability of the

derivatized complexes to kill the two different cell lines can be attributed to the release of estrogen and subsequent upregulation of HMGB1. As illustrated in Figures 2.6 and 2.7, the induction of HMGB1 overexpression following treatment with the estrogen-tethered platinum(IV) complexes did not always translate to sensitization of MCF-7 cells. Instead, as shown in Table 2.2, the sensitivity of these cells to compounds **BEP1** – **BEP5** varies with linker-length. The cytotoxicities of **BEP1** and **BEP2** were quite comparable in MCF-7 and HCC-1937 cells, whereas **BEP5** was more cytotoxic to the HCC-1937 cells. One possible explanation for these results is that the elevated levels of HMGB1 produced by these complexes do not lead to a significant amount of platinum-DNA adduct repair-shielding. The cytotoxic profile of **BEP5** may be explained by the relatively low level of HMGB1 overexpression induced upon treatment of the MCF-7 cells. As shown in Figure 2.6, **BEP3** is significantly more cytotoxic in the MCF-7 cells. Based on IC_{50} values, MCF-7 cells were 1.8- and 1.3-fold more sensitive towards **BEP3** and **BEP4** treatment, respectively, than they were to HCC-1937 cells. The differential toxicity towards MCF-7 and HCC-1937 cells observed with **BEP3** and **BEP4** suggest that these compounds can upregulate HMGB1 in a manner that is kinetically competent to shield cisplatin-DNA adducts from repair and sensitize the cells. It is significant that **BEP3** is nearly two-fold more active in MCF-7 cells, because this degree of differential cytoxicity is similar to the enhancement achieved by cisplatin/estradiol co-treatment.[15]

The importance of the kinetics of HMGB1 upregulation in determining the toxicities of compounds **BEP1** – **BEP5** can be understood in the light of previous work from our laboratory demonstrating that estrogen-induced cisplatin sensitization was only achieved when the two compounds were co-administered.[15] In addition, sensitization to carboplatin was maximized when MCF-7 cells were pre-treated for 24 h with the platinum compound before administration

of estradiol. These observations suggest that the kinetics of HMGB1 overexpression and platinum-DNA adduct formation are critical for achieving an optimal level of repair shielding. The rate of ester hydrolysis depends upon the length of the substituent attached at the estrogen 17β-position.[30] The timing of hormone interaction with the ER and subsequent HMGB1 overexpression are therefore expected to be dependent upon the length of the ester linker chain. The inability of **BEP5** to induce significant HMGB1 upregulation after a 4 h treatment may be a consequence of the kinetics of ester hydrolysis. It is likely that compound **BEP3** stimulates HMGB1 overexpression at such a time that repair-shielding of the platinum DNA-adducts is maximized, whereas **BEP1** and **BEP2** induce HMGB1 overexpression, but the timing is insufficient to sensitize the cells to cisplatin-DNA lesions.

Conclusions

Despite the sensitization of ER(+) cells towards **BEP3** treatment, the degree of cytotoxicity achieved with cisplatin/estrogen co-treatment was not attained (IC_{50} = 1.0 μM). The diminished cytotoxicity of **BEP3** compared to cisplatin/estrogen co-administration may be a consequence of reduced uptake or of the different pharmacokinetics of the estrogen-tethered platinum(IV) complex. The concentration of estrogen delivered to the cell may also be a factor. When treating cells with cisplatin and estrogen alone, 200 nM concentrations of estradiol are used, whereas **BEPn** complexes deliver micromolar concentrations of the hormone. It is also possible that the estrogen dose administered by **BEPn** treatment itself induces a degree of cell proliferation. Alternatively, the micromolar concentration of estrogen could induce increased HMGB1 overexpression, which may actually inhibit cell death instead of leading to enhanced apoptosis.[39] Despite these caveats, the **BEPn** compounds provide a new model for the development of

platinum(IV) complexes designed to target and treat a variety of cancers in a specific manner, based on known cellular pathways. Animal studies are in progress to investigate further the potential utility of **BEP3** as an anticancer agent.

Acknowledgments

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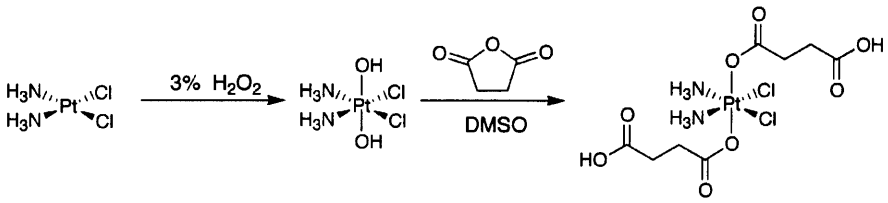
Table 2.1: ^{1}H NMR data (ppm) for estrogen ligands and platinum complexes in d_6-DMSO[a]

	H_1	H_2	H_4	H_6	H_{17}	H_{18}	H_{19}	H_{20}	H_{21}	H_{22}	H_{23}	H_a	H_b
EL1	7.34	7.01	6.96	2.83	4.66	0.796	8.12	7.59	7.74	1.75		3.83	
	d, 1H	d, 1H	s, 1H	m, 2H	t, 1H	s, 3H	d, 2H	t, 2H	t, 1H	bs, 2H		d, 2H	
EL2	7.32	7.00	6.94	2.83	4.67	0.814	8.08	7.58	7.73	1.75		3.04	2.68
	d, 1H	d, 1H	s, 1H	m, 2H	t, 1H	s, 3H	d, 2H	t, 2H	t, 1H	bs, 2H		t, 2H	m, 2H
EL3	7.33	7.00	6.95	2.83	4.65	0.813	8.09	7.59	7.73	1.70		2.83	2.42
	d, 1H	d, 1H	s, 1H	m, 2H	t, 1H	s, 3H	d, 2H	t, 2H	t, 1H	bs, 2H		m, 2H	m, 2H
EL4	7.32	7.00	6.95	2.83	4.65	0.815	8.09	7.59	7.73	1.70		2.83	2.36
	d, 1H	d, 1H	s, 1H	m, 2H	t, 1H	s, 3H	d, 2H	t, 2H	t, 1H	bs, 2H		m, 2H	m, 2H
EL5	7.32	7.00	6.93	2.82	4.62	0.798	8.08	7.58	7.72	1.70		2.78	2.31
	d, 1H	d, 1H	s, 1H	m, 2H	t, 1H	s, 3H	d, 2H	t, 2H	t, 1H	bs, 2H		m, 2H	m, 2H
BEP1	7.34	7.01	6.95	2.83	4.65	0.785	8.10	7.59	7.74	8.33	6.56	3.80	
	d, 2H	d, 2H	s, 2H	m, 4H	t, 2H	s, 6H	d, 4H	t, 4H	t, 2H	t, 2H	bs, 6H	d, 4H	
BEP2	7.35	7.00	6.95	2.83	4.65	0.799	8.10	7.60	7.74	7.95	6.49	3.23	2.43
	d, 2H	d, 2H	s, 2H	m, 4H	t, 2H	s, 6H	d, 4H	t, 4H	t, 2H	t, 2H	bs, 6H	t, 4H	m, 4H
BEP3	7.35	7.00	6.96	2.83	4.69	0.805	8.12	7.59	7.74	7.85	6.50	3.04	2.47
	d, 2H	d, 2H	s, 2H	m, 4H	t, 2H	s, 6H	d, 4H	t, 4H	t, 2H	t, 2H	bs, 6H	t, 4H	m, 4H
BEP4	7.35	7.00	6.97	2.84	4.64	0.804	8.10	7.60	7.74	7.85	6.51	3.01	2.44
	d, 2H	d, 2H	s, 2H	m, 4H	t, 2H	s, 6H	d, 4H	t, 4H	t, 2H	t, 2H	bs, 6H	t, 4H	m, 4H
BEP5	7.32	6.98	6.94	2.82	4.62	0.799	8.09	7.57	7.71	7.81	6.49	2.98	2.40
	d, 2H	d, 2H	s, 2H	m, 4H	t, 2H	s, 6H	d, 4H	t, 4H	t, 2H	t, 2H	bs, 6H	t, 4H	m, 4H

[a] See Scheme 2.2 for atom labeling diagram

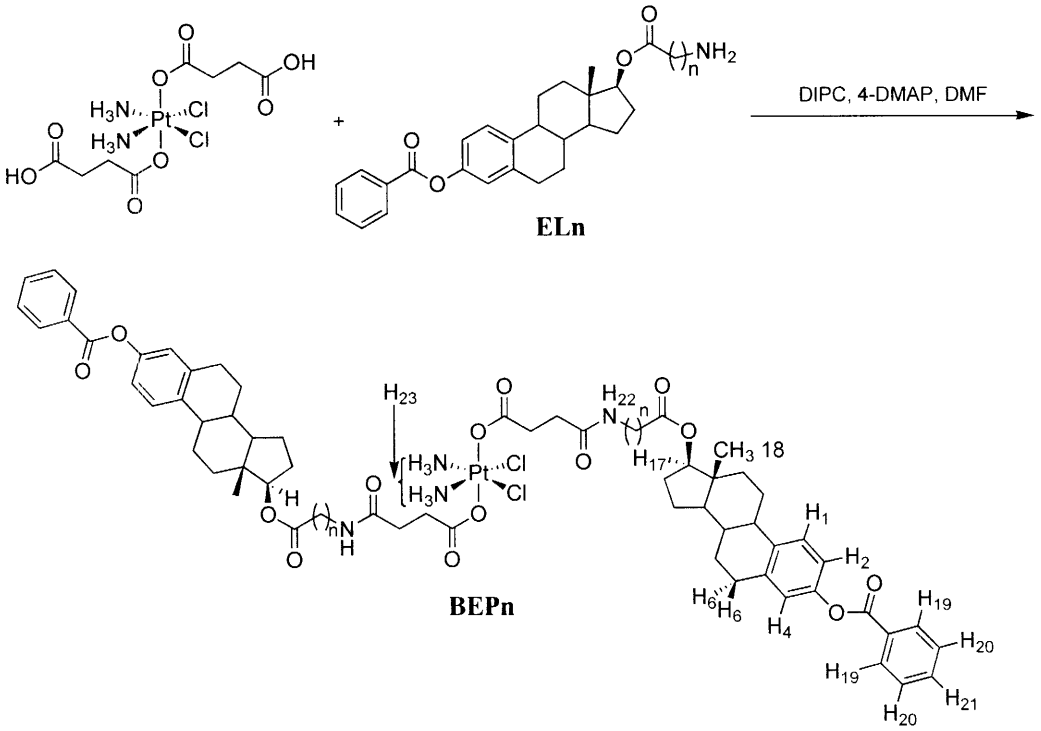
Table 2.2. IC_{50} values (μM) for **BEP1** – **BEP5**

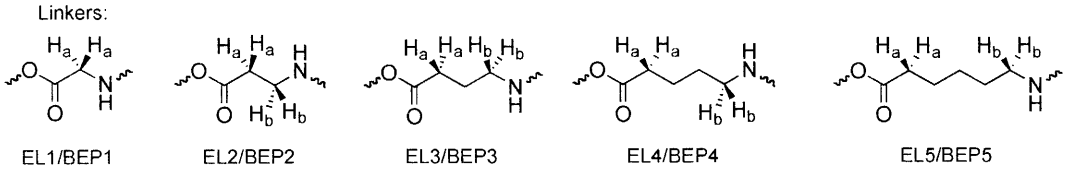
Compound	MCF-7	HCC-1937	Cytotoxicity Ratio (HCC-1937: MCF-7)
BEP1	3.2 ± 0.1	3.4 ± 0.4	1.1
BEP2	3.0 ± 0.2	3.6 ± 1.2	1.2
BEP3	2.1 ± 0.4	3.7 ± 0.9	1.8
BEP4	3.7 ± 0.5	4.8 ± 0.5	1.3
BEP5	5.5 ± 1.7	3.0 ± 0.2	0.55



1

Scheme 2.1.


DIPC, 4-DMAP, DMF
ELn
BEPn
CH3 18


Linkers:
EL1/BEP1
EL2/BEP2
EL3/BEP3
EL4/BEP4
EL5/BEP5

Scheme 2.2.

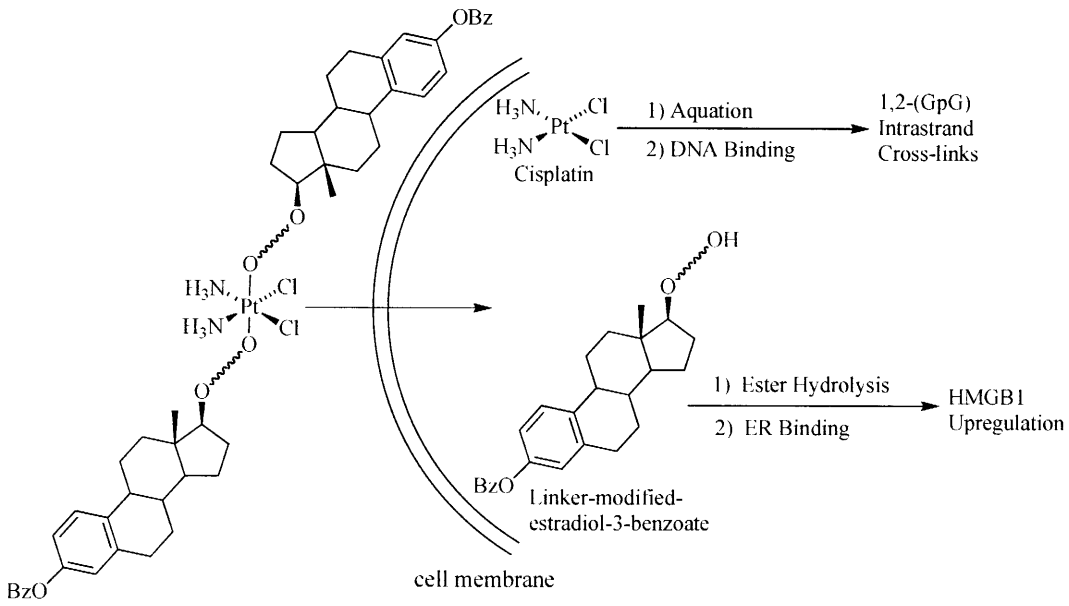


Figure 2.1. Proposed mechanism of action for estrogen-tethered platinum(IV) complexes. The reducing environment of the cell will convert platinum(IV) to platinum(II), releasing cisplatin and two equivalents of a modified estrogen. Upregulation of HMGB1 will shield cisplatin-DNA cross-links from repair, enhancing cell death.

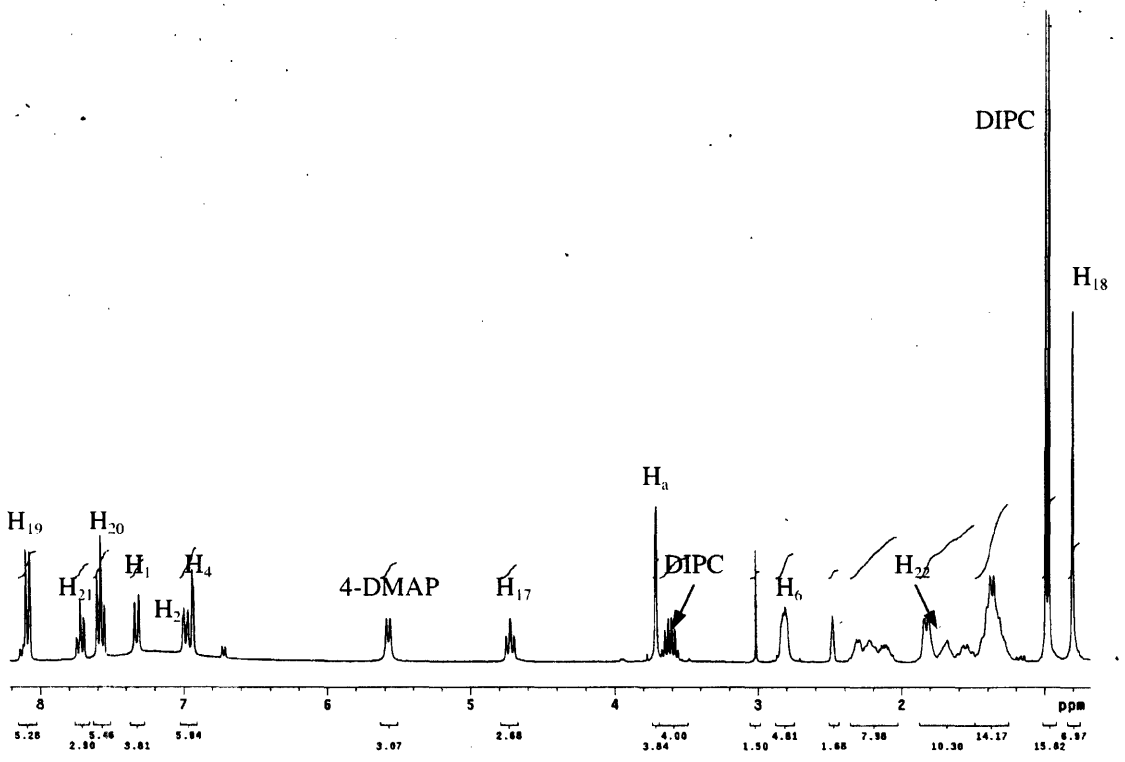


Figure 2.2. ^{1}H NMR spectrum of **EL1** in d_6-DMSO. See Scheme 2.2 for atom labeling diagram.

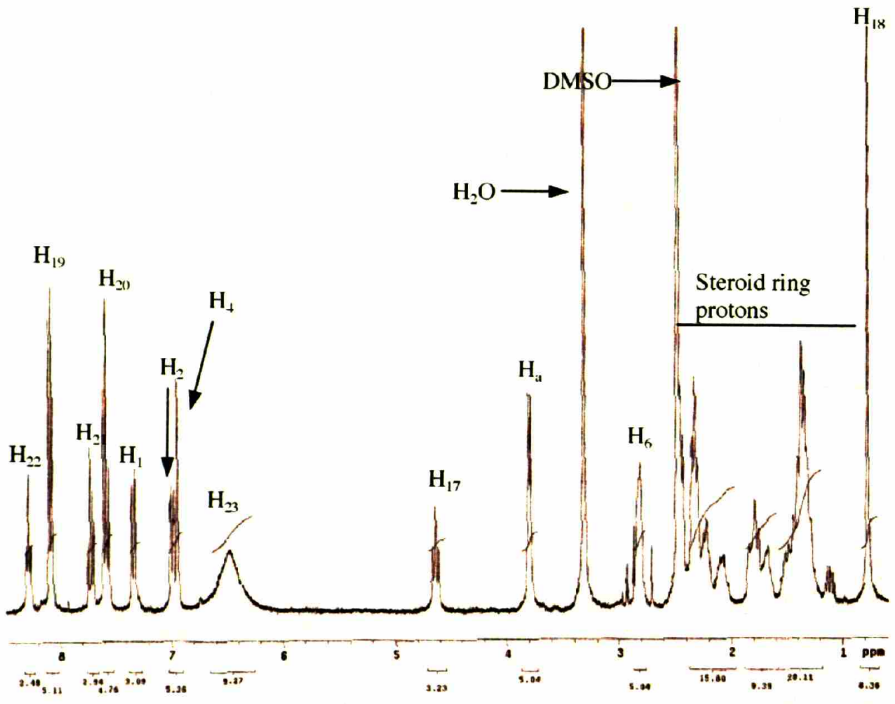


Figure 2.3. ^{1}H NMR spectrum of **BEP1** in d_6-DMSO. See Scheme 2.2 for atom labeling diagram.

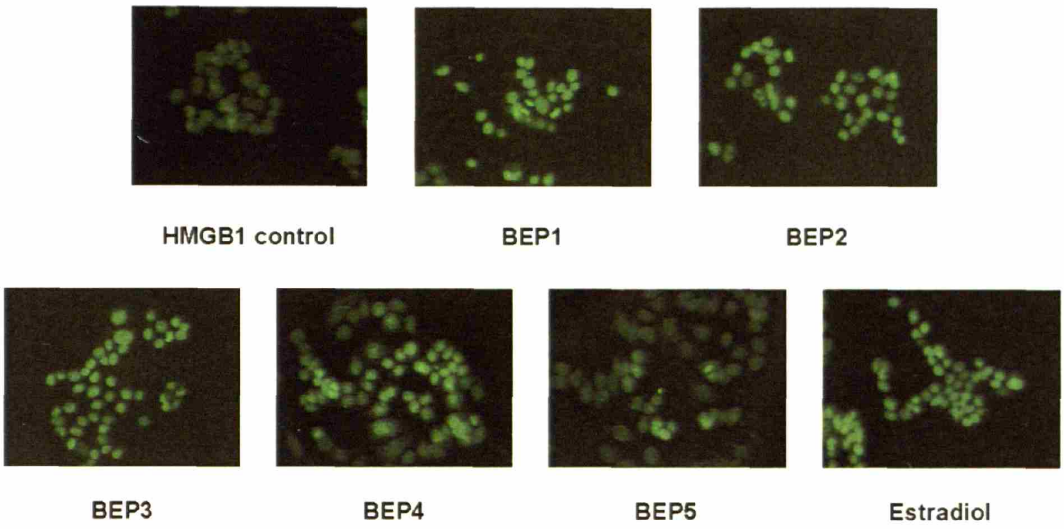


Figure 2.4. Expression of HMGB1 in MCF-7 cells as monitored by immunofluorescence microscopy. MCF-7 cells were grown on cover slips and subsequently incubated with 200 nM **BEPn** or estradiol for 4 h. The cell membrane was permeabilized and the cells were treated with anti-HMGB1 primary antibody followed by treatment with a FITC-conjugated secondary antibody to allow for HMGB1 visualization.

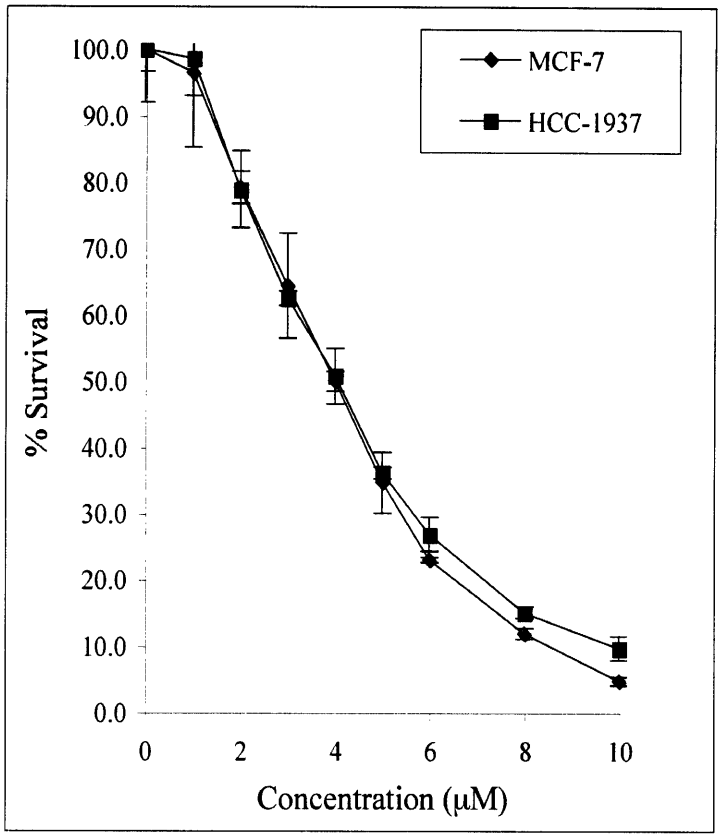


Figure 2.5. Cytotoxicity of *cis, cis, trans*-diamminedichlorodisuccinatoplatinum(IV) in MCF-7 and HCC-1937 cells. Cell survival is monitored by the SRB assay. Error bars represent one standard deviation.

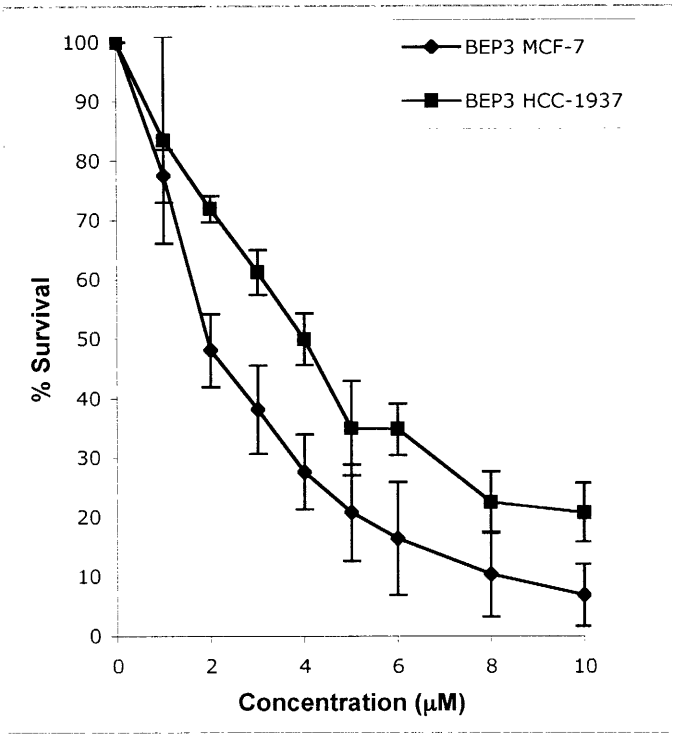


Figure 2.6. Effect of **BEP3** treatment on MCF-7 and HCC-1937 cell survival, as monitored by the SRB assay (± SD). Cells were treated continuously for 72 – 96 h. Experiments were carried out on at least three separate days with triplicate cultures for each drug concentration.

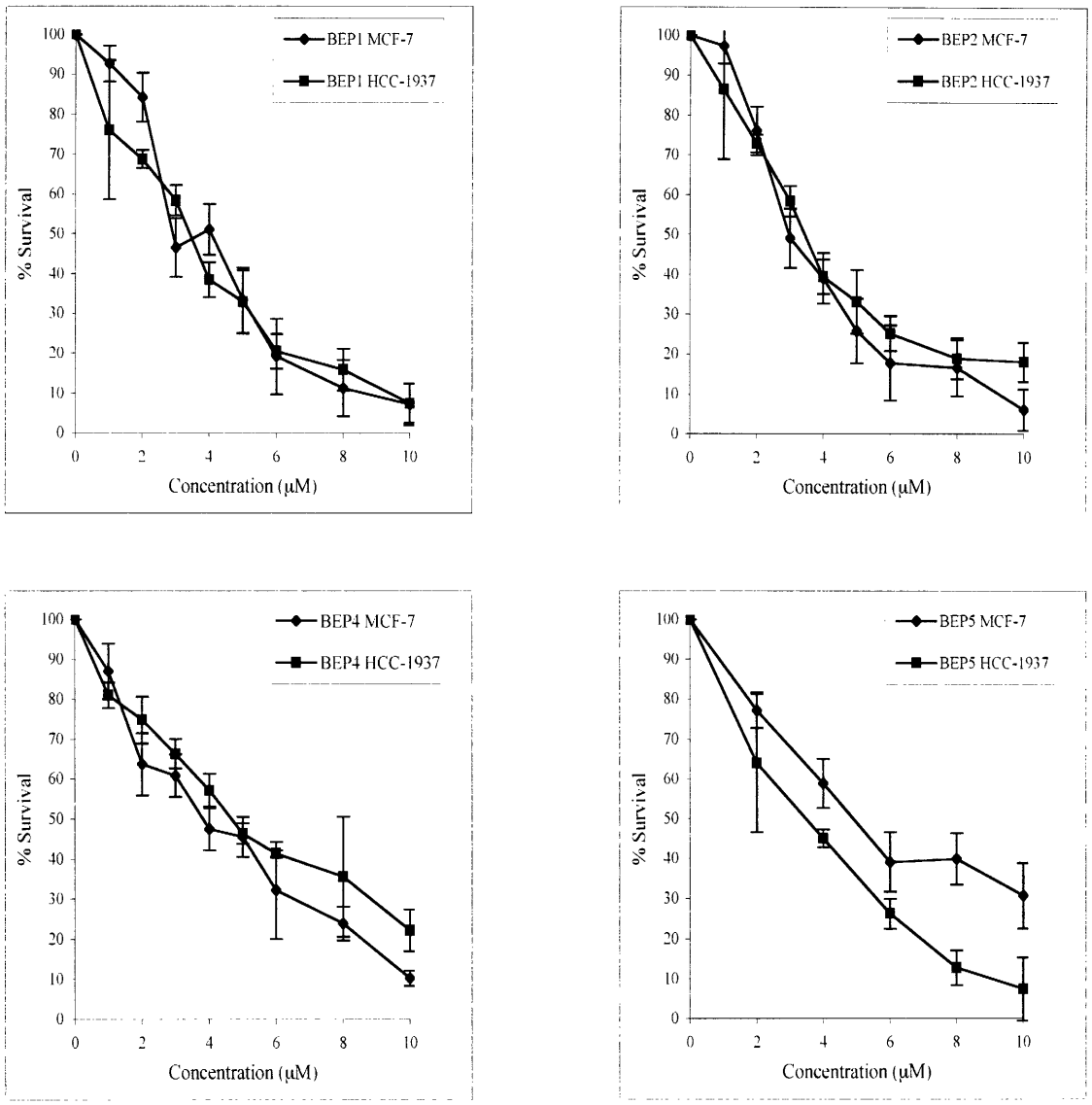


Figure 2.7. Cytotoxic investigation of **BEP1**, **BEP2**, **BEP4**, and **BEP5** in ER(+) MCF-7 and ER(-) HCC-1937 human breast cancer cells. Cell survival was determined by the SRB assay. Error bars represent one standard deviation.

Chapter 3
Synthesis and Biological Activity of a Series of Carboplatin-Based Estrogen-Tethered Platinum(IV) Compounds

Introduction

Carboplatin, or *cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II), is a widely used second-generation platinum anticancer drug. Developed by Bristol-Myers Squibb in collaboration with the Institute of Cancer Research and Johnson-Matthey, carboplatin was approved by the FDA in 1985 as a primary line of treatment for ovarian cancer.[1] Today, carboplatin is used alone or in combination therapy to treat a variety of malignancies, including head and neck, testicular, bladder, cervical, and small cell lung cancer.[1,2] Because of its lower toxicity, carboplatin can be administered at 8-15 fold higher doses than cisplatin and is effective in treating the same range of cancers.[1] Although cisplatin is generally regarded as the more potent therapeutic agent, carboplatin is often preferentially administered because of milder side effects associated with treatment.[3] Unfortunately, carboplatin is often rendered inactive by the same resistance mechanisms as cisplatin and is not effective against cisplatin-resistant tumors.[1]

The mechanism of carboplatin biological activity is generally similar to that of cisplatin. As with cisplatin, the primary biological target of carboplatin is DNA; however, the kinetics of nucleotide binding and adduct profile differ between the two compounds.[2,4-6] The chelating cyclobutanedicarboxylate (CBDCA) ligand is less labile than the cisplatin chloride ligands.[6] As a consequence, the DNA binding kinetics of carboplatin are considerably slower.[4] Upon reaction with DNA, carboplatin preferentially forms 1,2-intrastrand d(GpG) and d(ApG) cross-links, which account for approximately 50% of all adducts.[4,5] Interestingly, detailed analysis of DNA adduct profiles in CHO cells revealed that carboplatin forms more 1,3-intrastrand and interstrand cross-links (40% of total adducts) than cisplatin (9% of total adducts).[5] Formation of the 1,2-intrastrand d(GpG) cross-link distorts the DNA helix, giving rise to a wide and shallow minor groove that serves as a recognition site for a number of cellular proteins, including HMGB1.[7-9]

The interaction of HMGB1 with cisplatin-modified DNA prevents removal of the platinum damage by nucleotide excision repair (NER).[10,11]

In estrogen receptor-positive, or ER(+), MCF-7 human breast cancer cells, the cytotoxicity of cisplatin is enhanced 2-fold upon co-treatment with the steroid hormone estradiol.[12] Treatment of ER(+) cells with estradiol induces the overexpression of HMGB1 2-4 h after hormone treatment. The upregulation of HMGB1 is transient, with restoration to basal protein levels occurring 24 h after estrogen treatment. Estradiol treatment also enhanced carboplatin cytotoxicity by 2-fold when the hormone was administered 24 h after platinum treatment.[12] The different requirements for the timing of estradiol delivery reflect the different kinetics of carboplatin and cisplatin aquation and DNA-binding.[6,13] Furthermore, the influence of hormone treatment timing emphasizes the importance of optimizing the kinetics of DNA platination and HMGB1 upregulation. In order for shielding to occur, the expression of HMGB1 must be elevated at the time of platinum-DNA damage repair.[12]

The importance of optimizing the kinetics of HMGB1 upregulation and formation of Pt-DNA adducts was also manifest in studies of the biological effects of a series of estrogen-tethered platinum(IV) complexes (Figure 3.1).[14] Because of their ability to interact with the ER and subsequently induce HMGB1 upregulation, the **BEPn** compounds were expected to have enhanced cytotoxicity in ER(+) cells (*vide supra*). The series of compounds were designed to be reduced intracellularly resulting in the release of cisplatin and a linker-modified estrogen, which will be processed by intracellular esterases.[14] The rate of ester hydrolysis, and therefore the rate of interaction with the ER and subsequent HMGB1 overexpression, is influenced by the length and nature of the substituent tethered at the 17β-position.[15-17] Therefore, the length of the linker between the estrogen and the platinum center was varied from 1 to 5 methylene units in an

attempt to systematically alter the timing of HMGB1 upregulation. Compounds **BEP1-BEP4**, but not **BEP5**, were able to induce the overexpression of HMGB1 in MCF-7 cells after a 4 h incubation. In addition, the cytotoxic properties of **BEP1–BEP5** varied with linker length. Only **BEP3** displayed enhanced cytotoxicity in ER(+) MCF-7 cells compared to ER(-) HCC-1937 cells.[14]

The success of the **BEP3** compound inspired the synthesis of an analogous series of platinum(IV) compounds that would be reduced *in vivo* to yield carboplatin. The CBDCA leaving group may endow the series of compounds with greater stability and fewer side effects than the cisplatin analogues. In addition, this work represents the first example of a biologically relevant platinum(IV) compound derived from carboplatin. This chapter describes the synthesis, characterization, and biological activity of a series of carboplatin-based estrogen-tethered platinum(IV) compounds.

Experimental Procedures

Materials and Methods

Potassium tetrachloroplatinate(II) was a gift from Engelhard. Carboplatin was prepared as described in the literature.[18] The preparation of compounds **L2, L3, L4,** and **L5** were based on a previously reported methodology.[19] The syntheses of **EL1–EL5** were described elsewhere in this work (Chapter 2). *N*-Boc-*N*'-succinimidyl-4,7,10-trioxatri-decane-1,13-diamine, or **L17**, was prepared as described in the literature and its purity was confirmed by ^{1}H NMR spectroscopy.[20] All chemicals and solvents were purchased from commercial sources unless specified otherwise. ^{1}H and ^{195}Pt NMR spectra were recorded on either a Varian 300 MHz or 500 MHz spectrometer at the MIT Department of Chemistry Instrumentation Facility (DCIF). High-resolution mass

spectral analysis was carried out at the MIT DCIF. FT-IR spectra were recorded on a Thermo Nicolet Avatar 360 spectrometer with OMNIC software.

Synthesis of *cis, cis, trans*-Diamminecyclobutanedicarboxylatodihydroxoplatinum(IV) (1, Scheme 3.1). A solution of carboplatin (2.4 g, 6.4 mmol) in 15% aqueous H_2O_2 (22 mL) was allowed to stir in the dark for 2 h at 70 C and then for an additional 30 min at 60 C and subsequently cooled to RT. A white solid was isolated by centrifugation and dried *in vacuo* (0.74 g, 1.8 mmol, 28% yield); mp = decomposes above 230 C. ^{1}H NMR (d_6-DMSO, 300 MHz): δ 1.7 (m, 2H, CH_2), 2.5 (m, 4H, CH_2), 5.5 (bs, 6H, NH_3), 10.2 (s, 2H, OH). FT-IR (KBr, cm^{-1}): 3425 (s), 3296 (s), 3119 (s), 2961(m), 2313 (m), 2018 (w), 1650 (s), 1620 (s), 1344 (s), 1229 (w), 1095 (m), 909 (m), 775 (w), 548 (m), 467(w).

Synthesis of *cis, cis, trans*-Diamminecyclobutanedicarboxylatodisuccinatoplatinum(IV) (2). Succinic anhydride (1.7 g, 17 mmol) and **1** (1.7 g, 4.3 mmol) were dissolved in 25 mL of DMSO. The solution was heated to 70 C for 2.5 h with constant stirring, cooled to RT, and the DMSO solvent was removed by lyophilization to afford a sticky yellow solid. The crude product was rinsed with 20 mL of 1:1 acetone/ether, 30 mL of ether, and 30 mL of acetone to yield an off-white solid (1.6 g, 2.6 mmol, 60% yield). ^{1}H NMR (d_6-DMSO, 300 MHz): δ 1.8 (m, 2H, CH_2), 2.2-2.6 (m, 12H, CH_2), 6.4 (bt, J_{H-N} = 50.25 Hz, 6H, NH_3), 12.1 (s, 2H, COOH). ^{195}Pt NMR (H_2O, 500 MHz): δ 1893 (m, J_{Pt-N} = 250 Hz). ESI-MS calculated for $[M+Na]^+$ 628.3 amu, found 628.0 amu.

Synthesis of EL17 (Scheme 3.2). Diisopropylcarbodiimide (0.016 mL, 1.0 mmol) was added to a solution of **L17** (0.75 g, 1.8 mmol) and 4-DMAP (0.12 g, 1.0 mmol) in DMF (4 mL). The solution was allowed to stir for 15 min at RT before the addition of estradiol-3-benzoate (0.17 g, 0.45 mmol). The reaction was allowed to stir for 16 h, diluted with 15 mL of H_2O, and cooled to 4 C for an hour. The BOC-protected product was isolated by filtration and dried *in vacuo*. The white solid was dissolved in 10:1 dichloromethane/TFA (13.2 mL) and stirred for 2 h. Solvent was removed under reduced pressure. The resultant white solid was resuspended in water, the pH adjusted to 10 with ammonium hydroxide, and the suspension was stirred for 16 h. An oily white solid was isolated by centrifugation, dried *in vacuo*, and used without further purification (0.24 g, 0.35 mmol, 78% yield). ^{1}H NMR (d_6-DMSO, 300 MHz): δ 0.80 (s, 3H, CH_3), 1.2-2.0 (m, 12H, $CH/CH_2/NH_2$), 2.0-2.45 (m, 9H, CH/CH_2), 2.82 (m, 4H, CH_2), 3.0-3.6 (m, 16H, CH_2), 4.61 (t, 1H, CH), 6.95 (s, 1H, ArH), 7.0 (d, 1H, ArH), 7.35 (d, 1H, ArH), 7.60 (t, 2H, ArH), 7.75 (t, 1H, ArH), 7.85 (m, 1H, NH), 8.15 (d, 2H, ArH). ESI-HRMS calculated for $[M+H]^+$ 679.3953 amu, found 679.3965 amu.

Synthesis of *cis, cis, trans*-Diamminecyclobutanedicarboxylate-*bis*-(17(N-carbonylmethyl-succinate)-estradiol-3-benzoate)platinum(IV) (BECP1). Diisopropylcarbodiimide (0.10 mL, 0.63 mmol) was added to a solution of **2** (0.15 g, 0.25 mmol) and 4-DMAP (0.08g, 0.634 mmol) in DMF (5 mL). The solution was allowed to stir for 10 min at RT before the addition of **EL1** (0.22 g, 0.51 mmol). The solution was stirred for 15 h at room temperature, filtered, and the filtrate was diluted with 50 mL of ether. The resultant solution was cooled to –20 C for an hour to facilitate precipitation. The crude product was then triturated with ethanol (3x5 mL), boiling water (3x5 mL), and methanol (1x5 mL) and centrifuged for 3–5 min at 3000 rpm to yield a

white solid (0.17 g, 0.12 mmol, 48% yield); mp, decomposes above 197 C. ^{1}H NMR (d_6-DMSO, 500 MHz): δ 0.783 (s, 6H, CH_3), 1.2-1.6 (m, 4H, CH/CH_2), 1.60-1.95 (m, 10H, CH/CH_2), 2.0-2.7 (m, 14H, CH/CH_2), 2.83 (m, 4H, CH_2), 3.81 (d, 4H, CH_2), 4.65 (t, 2H, CH), 6.32 (bt, 6H, NH_3), 6.95 (s, 2H, ArH), 7.01 (d, 2H, ArH), 7.35 (d, 2H, ArH), 7.60 (t, 4H, ArH), 7.73 (t, 2H, ArH), 8.12 (d, 4H, ArH), 8.31 (t, 2H, NH). ESI-HRMS calculated for $[M+H]^+$ 1437.5211 amu. found 1437.5208 amu.

Synthesis of *cis, cis, trans*-Diamminecyclobutanedicarboxylate-*bis*-(17(N-(2-carboxyethyl)-succinate)-estradiol-3-benzoate)platinum(IV) (BECP2). This compound was prepared as described for **BECP1** using **2** (0.17 g, 0.28 mmol), 4-DMAP (0.09 g, 0.70 mmol), DIPC (0.11 mL, 0.70 mmol), and **EL2** (0.25 g, 0.56 mmol). Crude **BECP2** was triturated with ethanol (3x10 mL) and boiling water (3x10 mL) and centrifuged for 3-5 min at 3000 rpm to yield a white powder (0.24 g, 0.16 mmol, 57% yield); mp, decomposes above 191 C. ^{1}H NMR (d_6-DMSO, 500 MHz): δ 0.794 (s, 6H, CH_3), 1.2-2.6 (m, 44H, CH/CH_2), 2.84 (m, 4H, CH_2), 3.27 (t, 4H, CH_2), 4.64 (t, 2H, CH), 6.42 (bt, 6H, NH_3), 6.96 (s, 2H, ArH), 7.01 (d, 2H, ArH), 7.36 (d, 2H, ArH), 7.60 (t, 4H, ArH), 7.75 (t, 2H, ArH), 7.95 (t, 2H, NH), 8.11 (d, 4H, ArH). ESI-MS calculated for $[M+Na]^+$ 1487.5344 amu, found 1487.5308 amu.

Synthesis of *cis, cis, trans*-Diamminecyclobutanedicarboxylate-*bis*-(17-(N-(3-carboxypropyl)-succinate)-estradiol-3-benzoate)platinum(IV) (BECP3). This compound was prepared as described for **BECP1** using **2** (0.10 g, 0.17 mmol), 4-DMAP (0.05 g, 0.41 mmol), DIPC (0.065 mL, 0.41 mmol), and **EL3** (0.12 g, 0.26 mmol) in 3 mL DMF. Crude **BECP3** was purified as described for **BECP2** to yield a white powder (0.044 g, 0.030 mmol, 18% yield). ^{1}H

NMR (d_6-DMSO, 500 MHz): δ 0.801 (s, 6H, CH_3), 1.0-2.0 (m, 26H, CH/CH_2), 2.0-2.2 (m, 2H, CH), 2.2-2.6 (m, 16H, CH/CH_2), 2.83 (m, 4H, CH_2), 3.04 (m, 4H, CH_2), 3.26 (m, 4H, CH_2), 4.64 (t, 2H, CH), 6.36 (bt, 6H, NH_3), 6.96 (s, 2H, ArH), 7.01 (d, 2H, ArH), 7.47 (d, 2H, ArH), 7.60 (t, 4H, ArH), 7.74 (t, 2H, ArH), 7.85 (t, 2H, NH), 8.11 (d, 4H, ArH). ESI-HRMS calculated for $[M+H]^+$ 1493.5838 amu, found 1493.5856 amu.

Synthesis of *cis, cis, trans*-Diamminecyclobutanedicarboxylate-*bis*-(17-(N-(4-carboxy-butyl)-succinate)-estradiol-3-benzoate)platinum(IV) (BECP4). This compound was prepared as described for **BECP1** using **2** (0.17 g, 0.28 mmol), 4-DMAP (0.09 g, 0.70 mmol), DIPC (0.11 mL, 0.70 mmol), and **EL4** (0.27 g, 0.56 mmol). Crude **BECP4** was purified as described for **BECP2** to afford a white powder (0.20 g, 0.13 mmol, 46% yield); mp, decomposes above 185 C. ^{1}H NMR (d_6-DMSO, 500 mHz): δ 0.800 (s, 6H, CH_3), 1.2-2.6 (m, 52 H, CH/CH_2), 2.84 (m, 4H, CH_2), 3.03 (m, 4H, CH_2), 4.64 (t, 2H, CH), 6.34 (bt, 6H, NH_3), 6.96 (s, 2H, ArH), 7.01 (d, 2H, ArH), 7.34 (d, 2H, ArH), 7.61 (t, 4H, ArH), 7.74 (t, 2H, ArH), 7.84 (m, 2H, NH), 8.10 (d, 4H, ArH). ESI-HRMS calculated for $[M+Na]^+$ 1543.5970 amu, found 1543.5960 amu.

Synthesis of *cis, cis, trans*-Diamminecyclobutanedicarboxylate-*bis*-(17-(N-(5-carboxy-pentyl)-succinate)-estradiol-3-benzoate)platinum(IV) (BECP5). This compound was prepared in a similar manner as described for **BECP1** using **2** (0.17 g, 0.28 mmol), 4-DMAP (0.09 g, 0.70 mmol), DIPC (0.11 mL, 0.70 mmol), and **EL5** (0.27 g, 0.56 mmol). The solution was stirred for 15 h at RT and diluted with 45 mL water. The resultant solution was cooled to 4 C for 1 h to facilitate precipitation. Crude **BECP5** was purified as described for **BECP2** to afford a white powder (0.099 g, 0.064 mmol, 23% yield); mp, decomposes above 182 C. ^{1}H NMR (d_6-DMSO,

500 mHz): δ 0.801 (s, 6H, CH_3), 1.2-2.6 (m, 56H, CH/CH_2), 2.84 (m, 4H, CH_2), 3.00 (m, 4H, CH_2), 4.64 (t, 2H, CH), 6.33 (bt, 6H, NH_3), 6.96 (s, 2H, ArH), 7.01 (d, 2H, ArH), 7.35 (d, 2H, ArH), 7.61 (t, 4H, ArH), 7.75 (t, 2H, ArH), 7.81 (m, 2H, NH), 8.11 (d, 4H, ArH). ESI-MS calculated for $[M-H]^-$ 1547.7 amu, found 1547.0 amu.

Synthesis of *cis, cis, trans*-Diamminecylcobutanedicarboxylate-*bis*-17-(N-(trioxa-tridecanediamine-succinyl)-succinate)-estradiol-3-benzoate)platinum(IV) BECP17. This compound was prepared in a similar manner as described for **BECP1** using **2** (0.11 g, 0.18 mmol), 4-DMAP (0.06 g, 0.46 mmol), DIPC (0.07 mL, 0.460 mmol), and **EL17** (0.25 g, 0.37 mmol). The solution was stirred for 24 h at RT, diluted with 50 mL ether, and cooled to –20 C for 1.5 h. A white solid was collected by filtration, washed with boiling water, and dried *in vacuo* (0.15 g, 0.078 mmol, 43% yield); mp=105-110 C. ^1H NMR (d_6-DMSO, 300 MHz): δ 0.793 (s, 6H, CH_3), 1.2-2.0 (m, 24H, CH/CH_2), 2.0-2.45 (m, 18H, CH/CH_2), 2.45-2.50 (m, 12H, CH_2), 2.82 (m, 8H, CH_2), 3.0-3.6 (m, 32H, CH_2), 4.61 (t, 2H, CH), 6.3 (bs, 6H, NH_3), 6.97 (s, 2H, ArH), 7.00 (d, 2H, ArH), 7.35 (d, 2H, ArH), 7.62 (t, 4H, ArH), 7.75 (t, 2H, ArH), 7.86 (m, 4H, NH), 8.15 (d, 4H, ArH). ESI-HRMS calculated for $[M+Na]^-$ 1949.8444 amu, found 1949.9074 amu.

Cell Culture Studies. MCF-7 cells were grown in DMEM (GIBCO/BRL) supplemented with 10% FBS, 100 units/mL of penicillin (GIBCO), 100 μg/mL of streptomycin (GIBCO), and 2 mM L-glutamine. HCC-1937 cells were grown in RPMI-1640 media (ATCC) containing 10% FBS, 2.0 mM L-glutamine, 10 mM HEPES, 1.0 mM sodium pyruvate, 2 mM glucose, 100

units/mL of penicillin (GIBCO), and 100 μg/mL of streptomycin. All cells were incubated at 37 C under a 5% CO_2 atmosphere.

Overexpression of HMGB1 Induced by BECPn Complexes. MCF-7 cells were grown to 70% confluence on 12-mm glass cover slips in 24-well plates. Cells were treated with 200 nM **BECPn** for 4 h, rinsed with PBS, and further incubated for 20 h. The cells were fixed with 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at RT and washed in PBS for 30 min. Cells were permeabilized with 0.1% Triton-X100 in PBS for 10 min, rinsed with blocking buffer (PBS, 0.1% goat serum, 0.075% glycine), and incubated with a 1:100 dilution of anti-HMGB1 polyclonal antibody (Chemicon) for 1 h at 37 C. The cells were subsequently incubated with a 1:100 dilution of rabbit anti-mouse IgG conjugated to FITC (Chemicon) for 1 h at 37 C. The cover slips were then fixed on microscope slides with mounting medium (0.1% *p*-phenylenediamine in 75% glycerol) and incubated at 4 C for 12 h. HMGB1 levels were then visualized under a fluorescent light microscope (Nikon) equipped with a digital camera (Diagnostic Instruments).

Cytotoxic Profile of BECPn Compounds. Cell survival was analyzed by the MTT assay. MCF-7 or HCC-1937 cells were seeded into 96-well plates at a density of 1000 or 1500 cells per well, respectively. After 24 h, cells were treated with 0-30 μM **BECPn** and further incubated at 37 C. After incubating for 96-120 h, 20 μL of a 5 mg/mL MTT solution (PBS) was added to each well and incubated at 37 C for 5 h. The media was then removed from each well and the cells were redissolved in 100-200 μL of DMSO. The absorbance of each well was measured at 550 nm using a plate reader (SpectraMax 340pc, Molecular Devices). Percent cell survival was

calculated for each concentration by the ratio of the measured absorbance to the absorbance of the untreated wells.

Results and Discussion

Design, Synthesis, and Characterization of BECP1-5 and BECP17

The synthetic strategy in the present study mirrors that used in the preparation of the series of estrogen-tethered platinum(IV) complexes, **BEPn**, presented elsewhere in this work (Chapter 2).[14] As discussed earlier, the **BECPn** complexes will be reduced intracellularly, releasing carboplatin and a linker-modified estrogen. After **BECPn** reduction, carboplatin is available for DNA binding and the linker-modified estrogen will undergo esterase-facilitated hydrolysis to yield free estradiol. Since the kinetics of estradiol release are important, a longer ethylene glycol-based linker was prepared. To our knowledge, these compounds are the first example platinum(IV) complexes having the CBDCA ligand.

Carboplatin is readily oxidized by hydrogen peroxide to yield a *trans*-dihydroxyplatinum(IV) complex which was characterized by ^{1}H NMR and IR spectroscopy (Fig. 3.2). The platinum ammine protons are shifter significantly downfield as compared to their positions for carboplatin, which is consistent with the more positive formal oxidation state of platinum.[21] Oxidation of carboplatin to **1** is supported by the presence of the characteristic IR bands assigned to the OH stretch at 3425 cm^{-1} and the PtO stretch at 548 cm^{-1}.[21] The *trans*-dihydroxy complex was further characterized by X-ray crystallography, as described elsewhere.[22] As illustrated in Scheme 3.1, **1** reacts with succinic anhydride in DMSO to afford the *trans*-dicarboxylato complex **2,** which was characterized by ^{195}Pt and ^{1}H NMR spectroscopy. The ^{195}Pt NMR resonance at 1893 ppm (Fig. 3.3) is similar to other known platinum(IV)

carboxylates.[23] The platinum ammine proton resonances appear as a broad triplet at 6.4 ppm, a value that is in agreement with previously reported platinum(IV) complexes.[24,25] As shown in Figure 3.4, the ammine and succinato carboxylate protons integrate in a 3:1 ratio, indicating the presence of two succinato ligands per platinum center. The experimentally determined mass for **2** was in agreement with the calculated value to within 0.05%.

Synthesis of the ethylene glycol linker-modified estrogen **EL17** was achieved following the same protocol used to prepare **EL1-EL5**.[14] Formation of the ester linkage was confirmed by the significant downfield shift of the H_{17} proton resonance in the ^1H NMR spectrum of the product (Table 3.1). Isolation of the desired **EL17** compound was verified by ESI-MS; the value for the calculated mass was in excellent agreement with the experimentally determined mass within 0.00003%.

The synthesis of **BECPn** (n=1-5, 17) was achieved using the same methodology described for the preparation of the **BEPn** (n=1-5) compounds.[14] As illustrated in Scheme 3.2, **2** and the amine-modified estrogens are easily coupled using standard peptide coupling reagents to yield an amide-linked product. Purification of the **BECP** compounds is facile, with impurities being removed by washing the solid with water and ethanol. ESI-MS and ^1H NMR confirm the presence of the desired estrogen-tethered platinum(IV) compounds (see spectral analyses in Table 3.1). A ^1H NMR spectrum of **BECP1** is shown in Figure 3.5. Formation of the amide linkage between the estrogen and the platinum(IV) center is confirmed by the appearance of amide proton resonance H_{22} at 7.8-8.3 ppm. The ^1H NMR data also provide evidence for the presence of two estrogen substituents per carboplatin center. The platinum ammine protons (H_{23}) integrate with a 1:1 ratio to the 18-postion methyl group of the estrogen (H_{18}). Finally, the calculated masses for the **BECPn** compounds are in agreement with the experimentally

determined data to within 0.04–0.00002%. The synthetic methodology presented further extends previously presented strategies by providing a facile route for the preparation of carboplatin analogues suitable for targeting specific cell types.

Overexpression of HMGB1 in MCF-7 Cells Following BECPn (n=1-5, 17) Treatment

Immunofluorescence microscopy was used to evaluate whether or not the **BECPn** compounds induce overexpression of HMGB1 in MCF-7 cells. As discussed earlier, the kinetics of carboplatin binding to DNA is considerably slower than those of cisplatin. Therefore, the expression of HMGB1 was examined 24 h after addition of **BECPn**. As shown in Figure 3.6, a modest degree of HMGB1 upregulation occurs following 200 nM **BECPn** treatment. Qualitatively, the amount of HMGB1 overexpression achieved upon incubation with the **BECP** complexes does not equal the degree of protein upregulation observed with **BEPn** treatment.[14] The ability of the **BECP** compounds to induce overexpression of HMGB1 implies that the platinum(IV) centers are reduced inside the cell and that ester hydrolysis and subsequent release of free estradiol does occur. The modest degree of HMGB1 upregulation observed suggests that the kinetics of protein overexpression may not be optimized for achieving repair shielding.

Cytotoxicity Profiles of BECPn in ER(+) MCF-7 and ER(-) HCC-1937 Human Breast Cancer Cells

The cytotoxicity profiles of **BECPn** were evaluated in ER(+) MCF-7 and ER(-) HCC-1937 human breast cancer cells by the MTT assay. Unlike its cisplatin analogue, the *cis, cis, trans*-diamminecyclobutanedicarboxylatodisuccinatoplatinum(IV) complex has different cytotoxicities (see Figure 3.7) in MCF-7 (IC_{50} = 8.4±0.7 μM) and HCC-1937 (12.1±2.8 μM) cells. The

differential cytotoxicity of **2** is likely due to cell line differences in the uptake of the anionic compound. As shown in Figure 3.8, the cytotoxicities of the **BECP** compounds vary with linker length; however, the ER(+) MCF-7 cells are not more sensitive than the ER(-) HCC-1937 cells to any of the **BECP** compounds. This observation implies that no HMGB1 induced repair shielding of platinum-damaged DNA occurs in the ER(+) MCF-7 cells. The variation of compound cytotoxicity (see Table 3.2) may be due to the increased lipophilicity associated with longer linker length, favoring increased compound uptake. Interestingly, **BECP3** and **BECP17** were both more 1.5-fold more cytotoxic in the ER(-) HCC-1937 cell line. This observation may indicate that the concentration of hormone delivered to the cell may be sufficient to induces cell proliferation and reverses the trend seen for **BEP3** (Chapter 2).

Examination of the immunofluorescence data provides some insight into the lack of differential cytoxicity observed with the **BECP** compounds. The modest degree of HMGB1 upregulation observed after 24 h incubation may not be sufficient to affect the repair of platinum damaged DNA. As demonstrated in previous work, cisplatin-based compounds tethered to **EL1-EL4** induced significant upregulation of HMGB1 after 4 h.[14] Therefore, it is not unexpected that **BECP1–BECP4** would only moderately effect the expression of HMGB1 after 24 h. Because of the slower DNA-binding kinetics of carboplatin, it was predicted that longer linkers, which afford slower kinetics of HMGB1 upregulation, would be required to target ER(+) cells selectively. Unexpectedly, **BECP5** and **BECP17** did not improve upon the persistance of HMGB1 upregulation in MCF-7 cells (Figure 3.6, Table 3.2).

The DNA adduct profile of carboplatin provides another reason why the **BECP** compounds do not selectively kill ER(+) MCF-7 cells. As discussed earlier, carboplatin forms more 1,3-intrastrand and interstrand cross-links than cisplatin.[4,5] HMGB1 binds with specificity

to 1,2-intrastrand platinum-DNA cross-links, but the protein does not recognize either the 1,3-intrastrand or the interstrand cross-link with high affinity.[7] Therefore, HMGB1 may not be as effective in promoting the cytotoxicity of carboplatin compared to cisplatin. In support of this hypothesis, HeLa cells whose expression of HMGB1 was silenced by RNAi were 2.8-fold resistant to cisplatin, but only 1.6-fold less sensitive to carboplatin.[26]

Conclusions and Future Directions

A series of carboplatin-based estrogen-tethered platinum(IV) complexes, or **BECPn**, have been synthesized and characterized by ^{1}H NMR spectroscopy and ESI-MS. The synthetic methodology developed will allow for the facile preparation of other biomolecule-tethered carboplatin analogues. The **BECP** compounds induced a moderate degree of HMGB1 upregulation in ER(+) MCF-7 cells. The ability of **BECPn** to induce HMGB1 overexpression did not translate to increased cytotoxicity in ER(+) MCF-7 cells compared to ER(-) HCC-1937 cells.

Future work could involve the synthesis of additional linker-modified estrogens, such as polyethylene glycol-based compounds, that would induce HMGB1 overexpression 24 h after treatment. Detailed kinetic analysis of the rate of ester hydrolysis of substituents attached at the 17β-position could provide insight into the design of more appropriate linkers. Alternatively, synthetic estrogen agonists could be screened for their ability to act as long-lived steroid hormones. Additional work with tethering the steroid hormone progesterone may also provide useful carboplatin analogues.

Acknowledgements. Caroline Saouma was instrumental in performing all of the experiments described.

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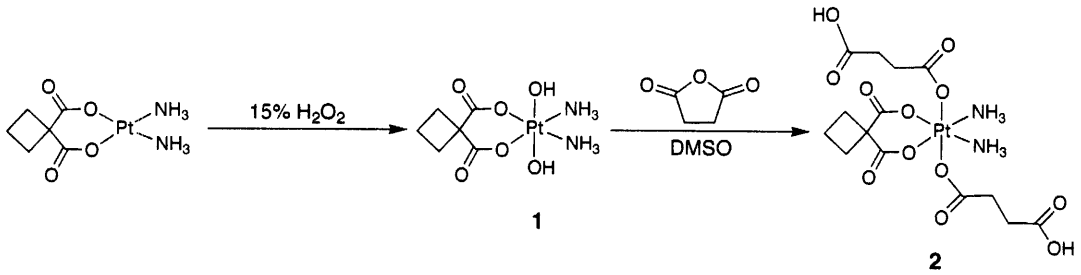
Table 3.1: ^{1}H NMR data (ppm) for estrogen ligands and platinum complexes in d_6-DMSO at 25 C[a]

	H_1	H_2	H_4	H_6	H_{17}	H_{18}	H_{19}	H_{20}	H_{21}	H_{22}	H_{23}	H_a
EL17	7.35	7.00	6.95	2.82	4.61	0.800	8.15	7.60	7.75	--	--	--
	d, 1H	d, 1H	s, 1H	m, 2H	t, 1H	s, 3H	d, 1H	t, 1H	t, 1H			
BECP1	7.35	7.01	6.95	2.84	4.65	0.783	8.12	7.60	7.73	8.31	6.32	3.81
	d, 2H	d, 2H	s, 2H	m, 4H	t, 2H	s, 6H	d, 4H	t, 4H	t, 2H	t, 2H	bt, 6H	d, 4H
BECP2	7.36	7.01	6.96	2.84	4.64	0.794	8.11	7.60	7.75	7.95	6.42	3.27
	d, 2H	d, 2H	s, 2H	m, 4H	t, 2H	s, 6H	d, 4H	t, 4H	t, 2H	t, 2H	bt, 6H	t, 4H
BECP3	7.47	7.01	6.96	2.83	4.64	0.801	8.11	7.60	7.74	7.85	6.36	3.26
	d, 2H	d, 2H	s, 2H	m, 4H	t, 2H	s, 6H	d, 4H	t, 4H	t, 2H	t, 2H	bt, 6H	m, 4H
BECP4	7.34	7.01	6.96	2.84	4.64	0.800	8.11	7.61	7.74	7.84	6.34	3.03
	d, 2H	d, 2H	s, 2H	m, 4H	t, 2H	s, 6H	d, 4H	t, 4H	t, 2H	t, 2H	bt, 6H	m, 4H
BECP5	7.35	7.01	6.96	2.84	4.64	0.801	8.11	7.61	7.75	7.81	6.33	3.00
	d, 2H	d, 2H	s, 2H	m, 4H	t, 2H	s, 6H	d, 4H	t, 4H	t, 2H	t, 2H	bt, 6H	m, 4H
BECP17	7.35	7.00	6.97	2.82	4.61	0.793	8.15	7.62	7.75	7.86	6.30	--
	d, 2H	d, 2H	s, 2H	m, 4H	t, 2H	s, 6H	d, 4H	t, 4H	t, 2H	m, 2H	bt, 6H	

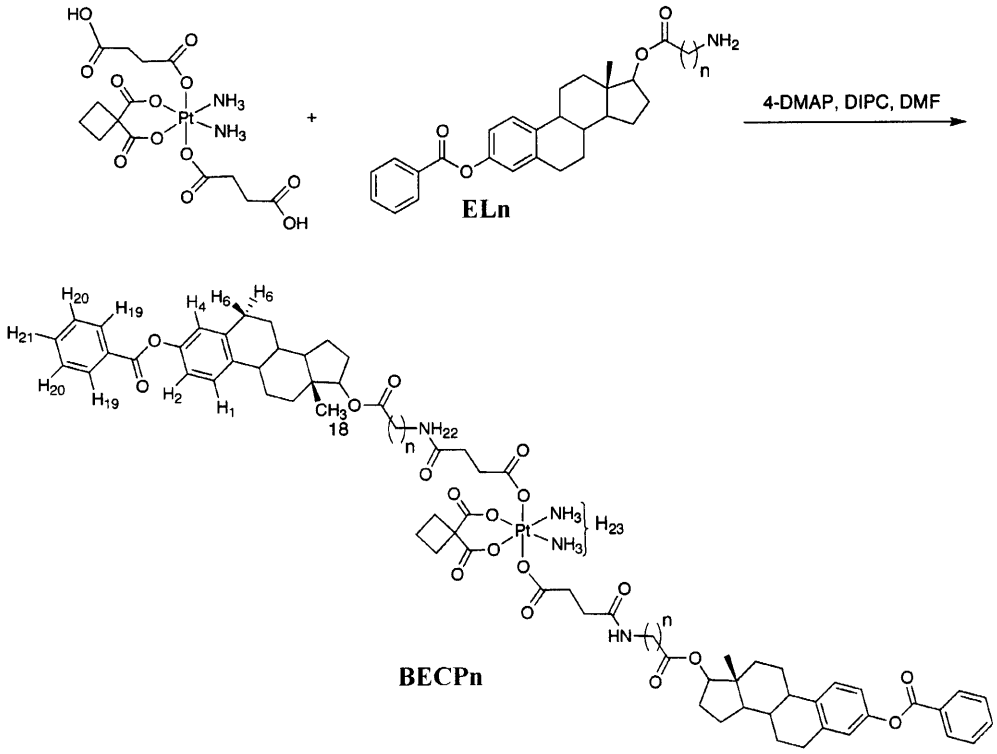
[a] See Scheme 3.2 for atom labeling diagram

Table 3.2. IC_{50} values (μM) for **BECP1–5** and **BECP17**

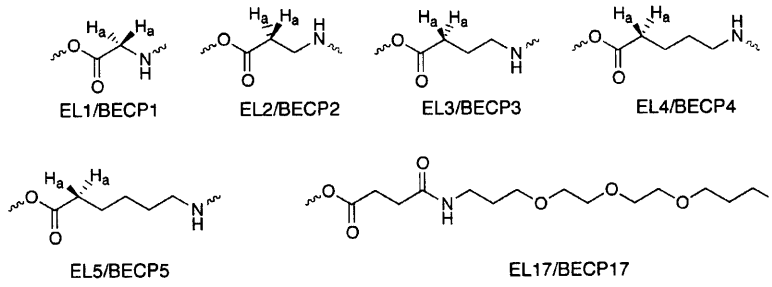
Compound	MCF-7	HCC-1937	Cytotoxicity Ratio (HCC-1937: MCF-7)
2	8.4 ± 0.7	12.1 ± 2.8	1.4
BECP1	9.5 ± 1.8	9.7 ± 1.1	1.0
BECP2	12 ± 1.4	11 ± 4.2	0.93
BECP3	18 ± 2.5	11 ± 3.0	0.65
BECP4	5.7 ± 1.0	5.5 ± 1.3	0.96
BECP5	5.6 ± 1.2	5.5 ± 0.9	0.97
BECP17	7.4 ± 1.5	4.8 ± 1.0	0.64



Scheme 3.1.



Linkers:



Scheme 3.2.

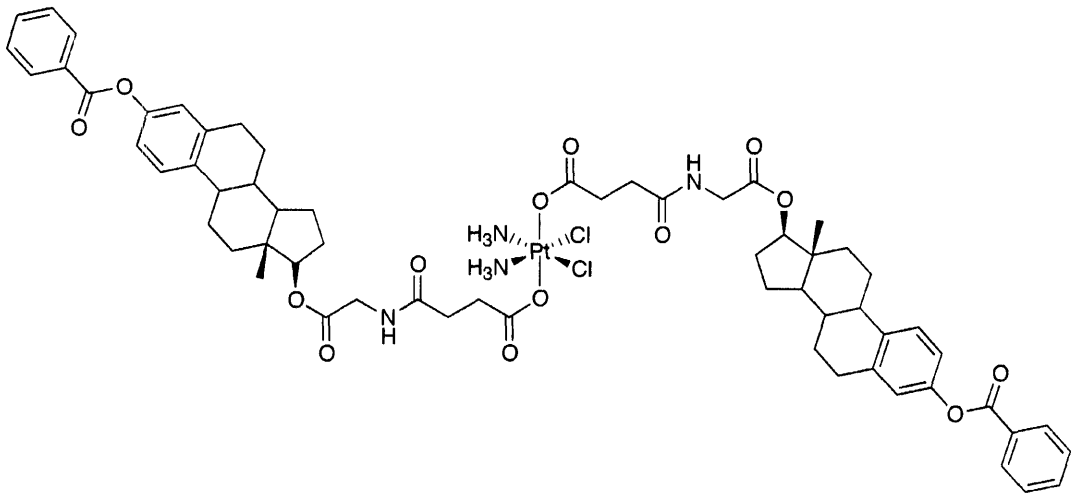


Figure 3.1. Schematic of a estrogen-tethered platinum(IV) complex, known as **BEP1**.

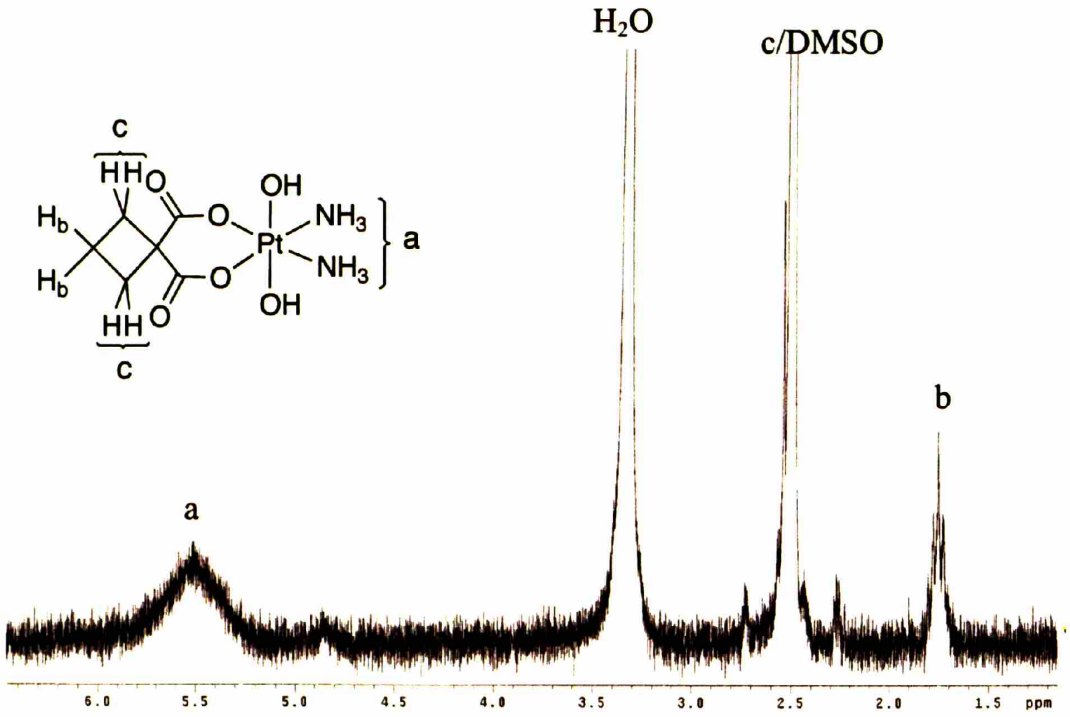


Figure 3.2. ^{1}H NMR spectrum of *cis, cis, trans*-diamminecyclobutanedicarboxylatodihydroxy-platinum(IV) in d_6-DMSO.

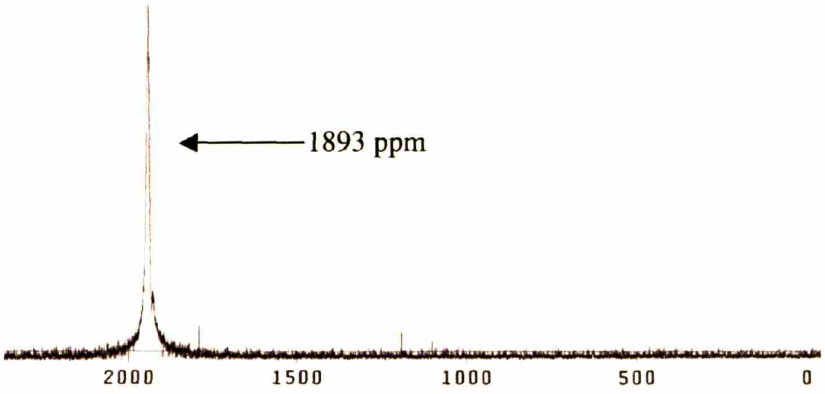


Figure 3.3. ^{195}Pt NMR spectrum of *cis, cis, trans*-diamminecyclobutanedicarboxylatodisuccinato-platinum(IV) in H_2O.

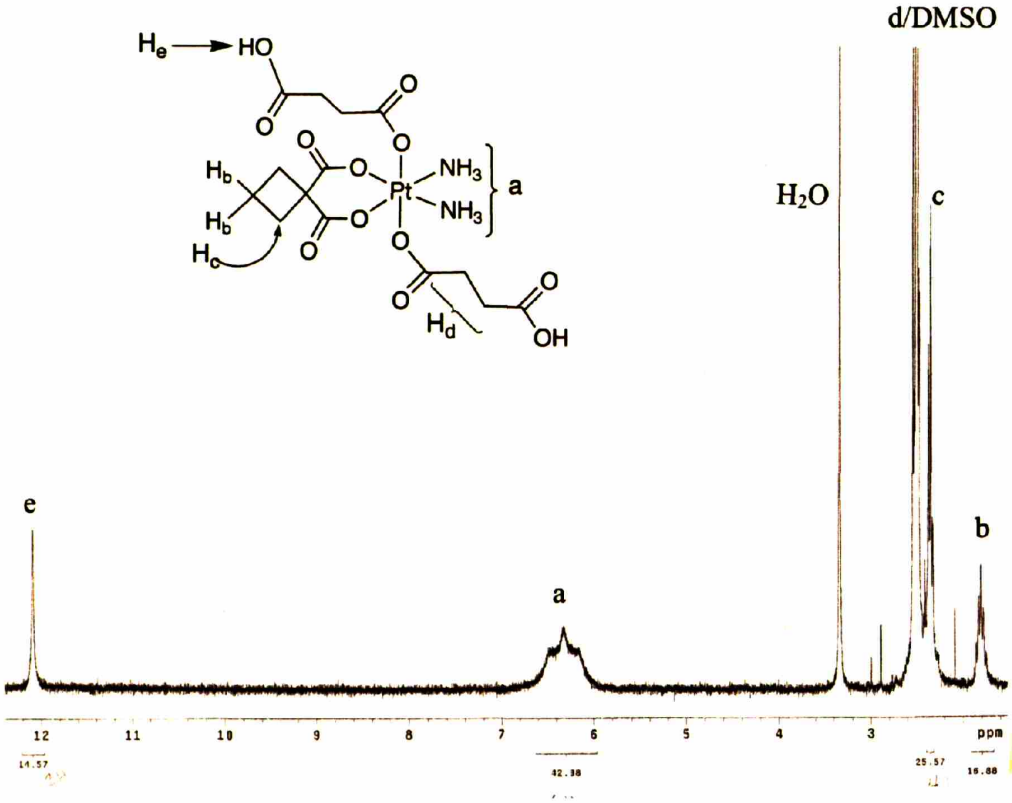


Figure 3.4. ^{1}H NMR of *cis, cis, trans*-diamminecyclobutanedicarboxylatodisuccinato-platinum(IV) in d_6-DMSO.

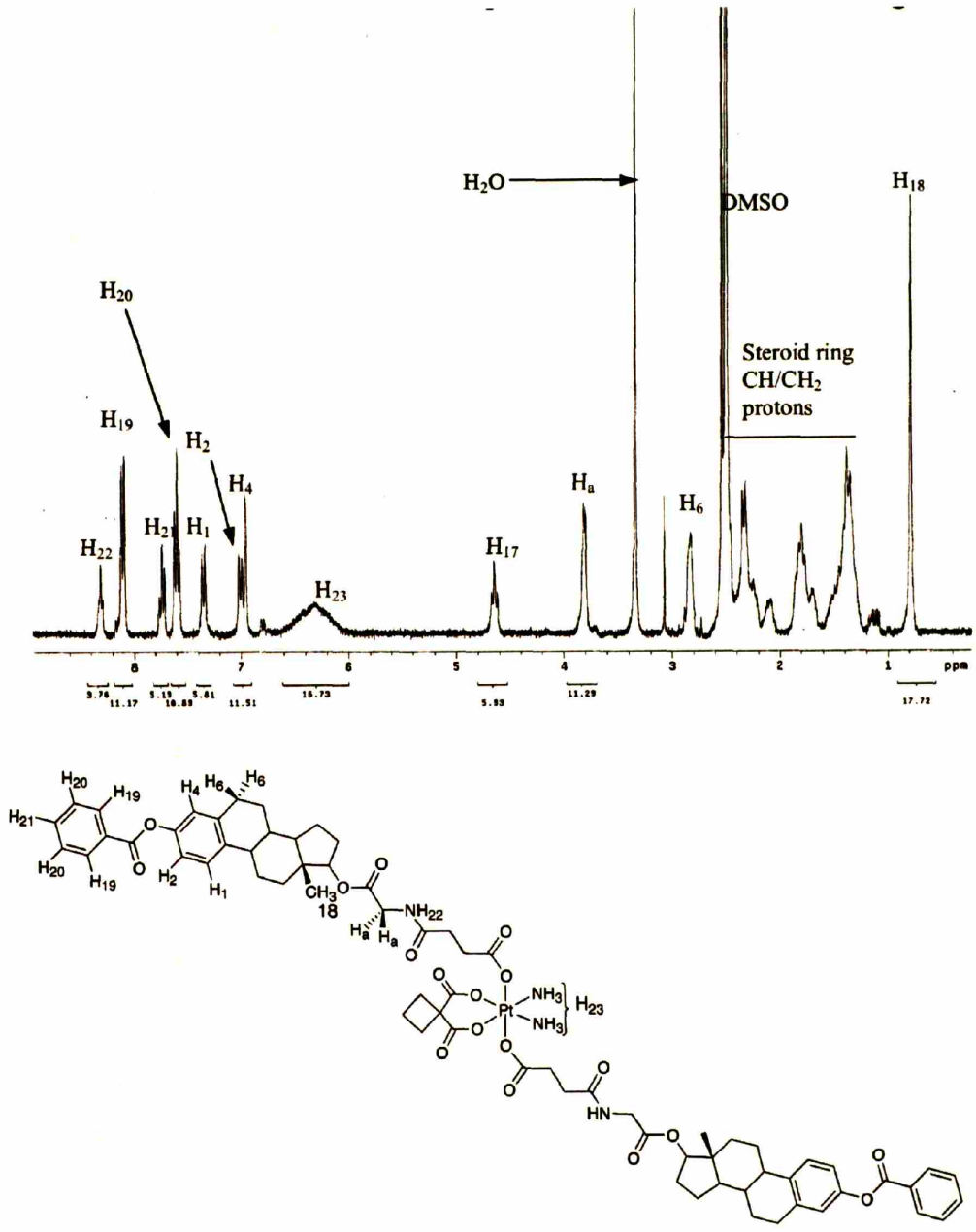


Figure 3.5. ^{1}H NMR spectrum of **BECP1** in d_6-DMSO.

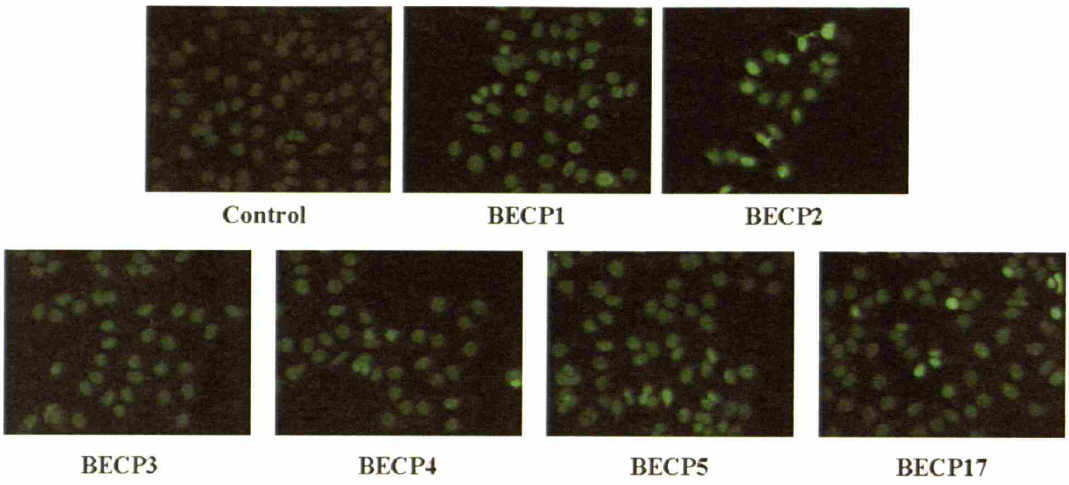


Figure 3.6. Expression of HMGB1 as visualized by immunofluorescence microscopy. Cells were treated with **BECP1-5** and **BECP17** for 4 h at 37 C and allowed to incubate for an additional 20 h before analyzing protein levels by immunofluorescence.

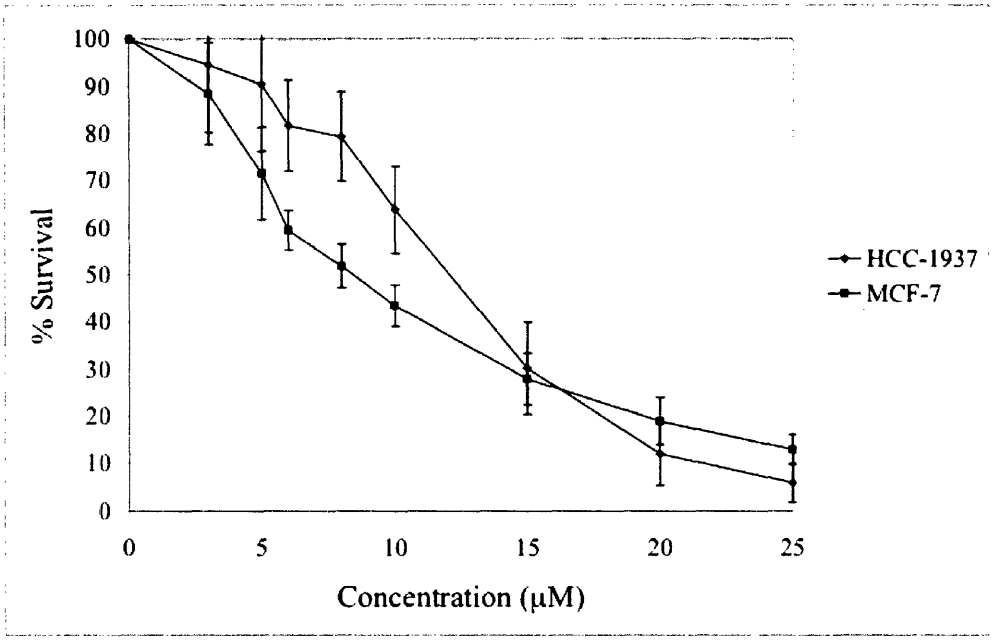


Figure 3.7. Cytotoxicity of **2** in ER(+) MCF-7 and ER(-) HCC-1937 human breast cancer cells. Cell survival was evaluated by the MTT assay. Error bars represent one standard deviation.

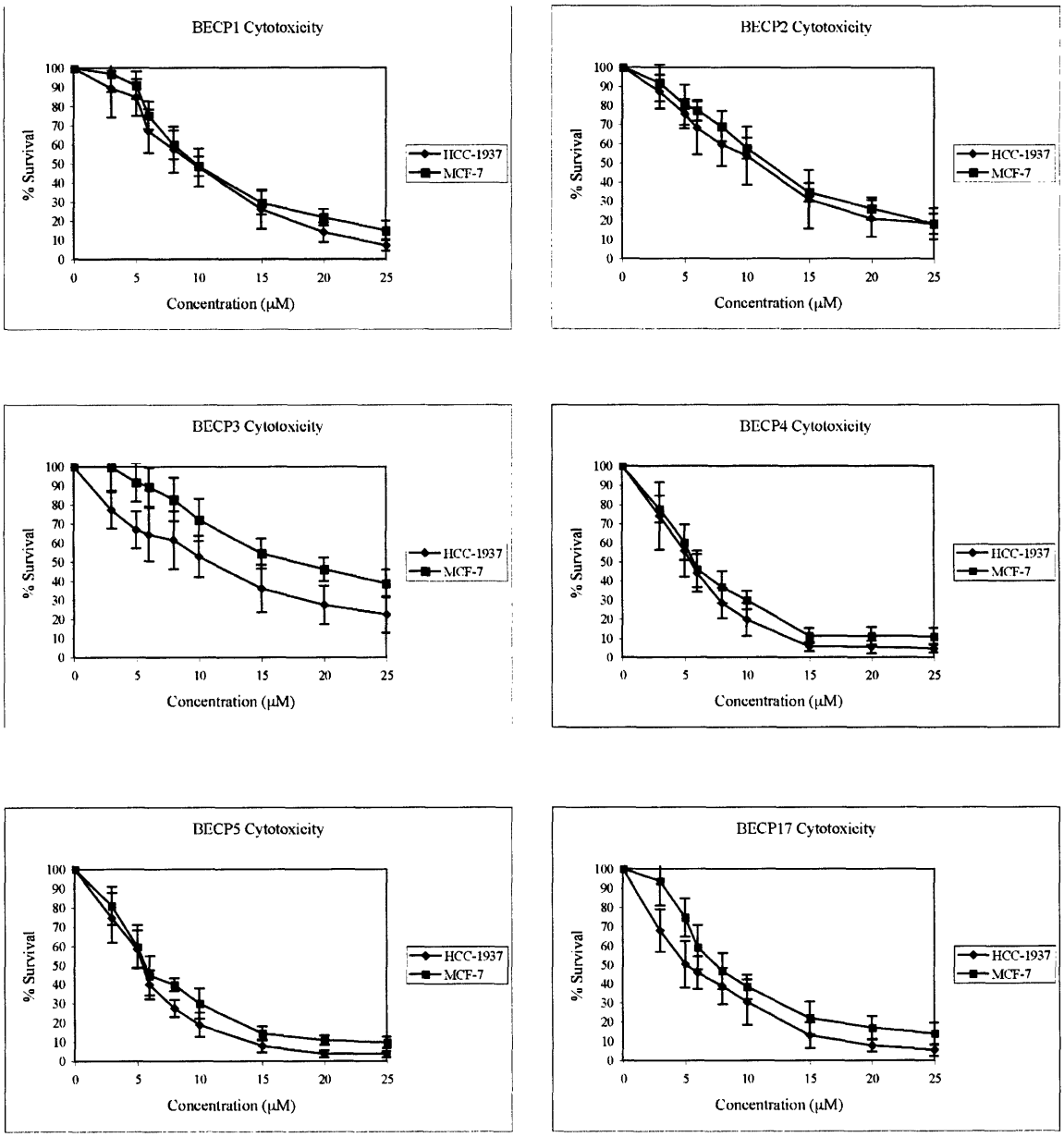


Figure 3.8. Cytotoxicity of **BECP1-5** and **BECP17** in ER(+) MCF-7 and ER(-) HCC-1937 human breast cancer cells. Cell survival was evaluated by the MTT assay after incubation with the cytotoxic agents for 96 h. Error bars represent one standard deviation.

Chapter 4
Design, Synthesis, and Biological Evaluation of an 17α-Ethynylestradiol-Tethered Platinum(IV) Complex Designed to Target Estrogen Receptor-Positive Cells

Introduction

Receptor-mediated uptake is a promising strategy in the quest for development of cancer-cell specific drug delivery. A prominent difference between cancer and healthy cells is the variable expression of different receptors, including the estrogen receptor (ER).[1-3] Both ERα and ERβ are expressed in human breast cancer; but each receptor is differentially expressed in specific disease types.[4-6] For example, ERβ is expressed in both normal mammary gland and in benign breast tumors, whereas the expression of ERα is limited in normal mammary tissue.[4] ERα appears to be involved in the initiation of malignancy and is overexpressed in ductal carcinoma, but not in medullary breast cancer. On the other hand, the expression of ERβ is upregulated in both ductal and medullary breast cancer. [4] Because ERα and ERβ have different functions, many of which have yet to be elucidated, the binding affinity for both receptors of compounds that target them should be evaluated.

A number of hormone-tethered compounds that bind the ER have been prepared for evaluation as breast cancer imaging agents or as components of chemotherapeutic drug candidates (Figure 4.1).[7-12] Structure-activity relationships have revealed that ER-binding affinity is maintained when estradiol is modified at the 7α-, 11β-, or 17α-position.[10] A series of 7α-modified estrogens have demonstrated high relative binding affinities (RBA) for the ER.[9,10,13] For example, enhanced cytotoxicity in ER(+) cells was observed for a 7α-estradiol-tethered mustard (Figure 4.1A).[9,14] In addition, selective uptake into ER(+) cells has been demonstrated with a porphyrin-estradiol conjugate linked through the 11β-position (Figure 4.1B).[8,11] Modification of estradiol at the 17α-position has also yielded potentially useful compounds with high RBAs.[7,12,15] For instance, a 17α-alkylamide-tethered platinum(II) estradiol conjugate (Figure 4.1C) is twice as cytotoxic in ER(+) cells as compared to ER(-) cells.[16]

The differential cytotoxicity of the aforementioned compounds in ER(+) cell lines is often attributed to increased uptake in the target cell line or, alternatively, to other ER-dependent responses.[9,15] Although increased uptake of estradiol occurs in ER(+) tissue, the mechanism is unknown.[1] In the blood, estrogens are bound to the plasma proteins albumin and sex hormone-binding globulin and therefore have low clearance rates, which could affect tumor uptake. Studies with radioactive steroids confirm that estradiol is selectively taken up and concentrated in the breast. In addition, malignant breast tissue accumulates more estradiol than the healthy breast. Binding of estradiol to the ER in the cytoplasm results in translocation of the complex to the nucleus.[1]

In addition to enhanced uptake into ER(+) cell lines, compounds with affinity for the ER may also elicit their cell-type dependent action by induction or down-regulation of estrogen-dependent genes.[17,18] In the absence of estradiol, the ER is bound by heat shock proteins (HSP) and can only undergo ligand-independent activation controlled by growth factor receptor signalling.[6] Binding of estradiol to either ERα or ERβ initiates signal transduction cascades through induction of conformational changes that result in release of HSP and subsequent receptor dimerization.[1,5] Zinc-finger DNA-binding domains mediate interaction of the ER dimer with estrogen responsive elements of target genes.[6] Estrogen stimulation in MCF-7 cells regulates the expression of 105 genes including (Table 4.1) genes responsible for the control of DNA replication and repair, transcription, cell cycle, cellular signaling, extracellular matrix, protein degradation and synthesis, and apoptosis.[17] A number of these genes (e.g., DNA polymerase, HMGB1, XRCC5, caspase 7, and metallothionein) have been associated with cisplatin activity or resistance.[19-24] Previous work from our laboratory demonstrated that estrogen treatment induces the overexpression of HMGB1 in ER(+) MCF-7 cells and, in turn, sensitizes

the cell to cisplatin treatment.[25] HMGB1 binds to cisplatin 1,2-intrastrand d(GpG) cross-links and shields the Pt-DNA adducts from repair.[26] Therefore, a platinum complex with affinity for the ER could be selectively target ER(+) cells by either of two mechanisms: increased uptake or the regulation of estrogen-dependent genes, such as HMGB1, that influence cell survival. This chapter describes the synthesis and characterization of one such complex, 17α-ethynylestradiol-tethered platinum(IV), and its effect on ER(+) breast cancer cells.

Experimental

General Considerations. The synthesis of *cis, cis, trans*-diamminedichlorodisuccinato-platinum(IV) is described elsewhere in this work (Chapter 2). 17α-(Benzylamine)-ethynyl-estradiol was prepared as described in the literature and characterized by ESI-MS and ^{1}H NMR spectroscopy.[27] ^{1}H NMR spectra were recorded on a Varian 300 MHz spectrometer at the MIT Department of Chemistry Instrumentation Facility (DCIF). High-resolution mass spectral analysis was carried out at the MIT DCIF. Platinum atomic absorption spectral analysis (Pt AAS) were performed on a Perkin Elmer AAnalyst spectrometer equipped with a HGA-800 graphite furnance system and a AS-72 autosampler.

Synthesis of *bis*-Ethynylestradiol-*cis*-diamminedichloroplatinum(IV), BEEP. A solution of *cis, cis, trans*-diamminedichlorodisuccinatoplatinum(IV) (0.299 g, 0.560 mmol), EDC (0.539 g, 2.81 mmol), and HOBt (0.382 g, 2.83 mmol) was prepared in 5 mL of DMF. After 10 min, a solution of 17α-(benzylamine)-ethynylestradiol (0.670 g, 1.67 mmol) in DMF (5 mL) was added and the reaction was allowed to stir at RT for 16 h. The reaction solution was diluted with 40 mL of diethyl ether and cooled to –20 C to facilitate precipitation. The crude product was isolated

by centrifugation and washed with water (5x20 mL). The tan solid was then triturated with 75% ethanol in water and centrifuged to yield a pale tan solid (270 mg, 0.21 mmol, 37% yield); mp, decomposes above 165 C. ^{1}H NMR (d_6-DMSO, 300 MHz): δ 8.97 (bs, 2H, OH), 8.37 (t, 2H, NH), 7.26 (m, 8H, ArH), 7.05 (d, 2H, ArH), 6.45 (bs, 6H, NH_3), 6.48 (d, 2H, ArH), 6.41 (s, 2H, ArH), 5.46 (s, 2H, OH), 4.22 (d, 4H, CH_2), 2.68 (m, 4H, CH_2), 2.4-1.6 (m, 25H, CH/CH_2), 1.4-1.2 (m, 9H, CH/CH_2), 0.791 (s, 6H, CH_3). HRMS calculated for $[M+Na]^+$ 1323.4323 amu, found 1323.4194 amu.

Cell Culture. MCF-7 cells were grown in DMEM (GIBCO/BRL) supplemented with 10% FBS (GIBCO/BRL), 1 mM sodium pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin. HCC-1937 cells were grown in RPMI-1640 media (ATCC) containing 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin. All cells were incubated at 37 C under a 5% CO_2 atmosphere.

Overexpression of HMGB1 by Treatment of Cells with Estrogen-Containing Compounds. MCF-7 cells were grown to 70% confluence on 12-mm glass coverslips (Fischer Scientific) in 24-well plates. The cells were treated with either 200 nM β-estradiol, 17α-ethynylestradiol or **BEEP** and incubated for 4 h. The cells were washed with PBS (3x), fixed in 2% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) at RT for 1 h, and rinsed again in PBS (3x). The cells were then permeabilized with 0.1% Triton-X100 in PBS for 10 min at RT, washed in blocking buffer (PBS, 0.1% goat serum, 0.075% glycine), and incubated with a 1:100 dilution of anti-HMGB1 polyclonal antibody (PharMingen) for 1 h at 37 C. The cells were subsequently washed in blocking buffer and incubated with a 1:200 dilution of goat anti-rabbit

IgG conjugated to FITC (Biosource International) for 1 h at 37 C. The coverslips were then placed on microscope slides, fixed with 0.1% *p*-phenylenediamine in glycerol, and stored O/N at 4 C. HMGB1 levels were visualized under a fluorescent light Nikon Eclipse TS100 microscope using a FITC-HYQ filter cube (excitation 460-500, bandpass 510-560 nm). Images were captured with an RT Diagnostics camera operated with Spot Advanced software.

Estrogen-Receptor Binding Affinity. Human recombinant ERα or ERβ (0.50 μM, Calbiochem) was incubated with 5.0 μM BEEP at 4 C in TE buffer (10 mM Tris, 1.5 mM EDTA, pH 7.4) for 18 h. The solution was diluted to 3 mL total volume and free and ER-bound BEEP were separated by dialysis using a MWCO 8000 dialysis cassette (Pierce) against PBS for 4 h at 4 C. The concentration of ER-bound BEEP was determined by using Pt AAS.

Uptake of BEEP into ER(+) MCF-7 and ER(-) HCC-1937 Cells. MCF-7 and HCC-1937 cells were seeded onto 10-cm plates and grown until 80-90% confluent. Cells were incubated with 10 μM **BEEP** for 4 h at 37 C, harvested, and washed extensively with PBS. The total protein content was determined by the bicinchoninic acid protein assay and the platinum concentration by Pt AAS. The degree of **BEEP** uptake was expressed as a ratio of platinum concentration (mg/L) to the total protein concentration (mg/L) of the isolated cell pellet.

Cytotoxicity Studies. In a 96-well plate, MCF-7 cells were seeded at a density of 1000 cells per well in 100 μL of DMEM. After 24 h, the cells were either treated with 0–20 μM cisplatin alone or co-treated with 200 nM 17α-ethynylestradiol and 0–20 μM **BEEP**. The cells were incubated for 96 h at 37 C and evaluated for survival by the MTT assay.[28] Briefly, cells were treated with 20 μL of 5 mg/mL MTT (PBS, pH 7.4) and incubated for 5 h at 37 C. The media was removed

and the cells resuspended in 100-200 μL of DMSO. The absorbance of each well was measured at 550 nm by a plate reader (SpectraMax 340 pc, Molecular Devices). Percent cell survival was calculated for each concentration by the ratio of the measured absorbance to the absorbance of the untreated wells.

Results and Discussion

Effect of 17α-Ethynylestradiol on the Cytotoxicity of Cisplatin. For our strategy to be effective, it was important to establish that 17α-ethynylestradiol upregulates HMGB1 and sensitizes ER(+) cells to cisplatin treatment to the same degree as β-estradiol.[25] Figure 4.2 demonstrates that incubation of the cells with 200 nM 17α-ethynylestradiol for 4 h induces the overexpression of HMGB1 to a similar degree as estradiol alone. In addition, co-treatment of ER(+) MCF-7 breast cancer cells with cisplatin and 17α-ethynylestradiol sensitizes cells to platinum treatment by nearly a factor of three (Figure 4.3). In addition to maintaining affinity for the ER, 17α-ethynylestradiol also recapitulates the activity of β-estradiol in its ability to modulate the expression of HMGB1.

Synthesis of a 17α-Ethynylestradiol-Tethered Platinum(IV) Complex. The synthesis, characterization, and biological activity of a series of 17β-estradiol-tethered platinum(IV) complexes, known as **BEPn**, designed to target ER(+) cells were described in Chapter 2. Like these compounds, the 17α-ethynylestradiol-tethered platinum(IV) complex (**BEEP**) will be reduced intracellularly to yield cisplatin and a linker-modified estrogen.[29] The **BEP** compounds differ from **BEEP** mainly in the nature of the steroid tether. As discussed earlier, compounds with affinity for the ER are selectively taken into ER(+) breast cancer cells. Because the **BEP**

compounds are linked at the 17β-position of the steroid ring, they cannot bind the ER. Interaction with the ER will only occur after reduction of **BEP** and subsequent hydrolysis of the linker-modified estrogen. The resultant free estradiol can then bind the ER and cause HMGB1 upregulation. **BEEP** was designed to have binding affinity for the ER prior to these processing steps in order to take advantage of both ER(+)-dependent mechanisms, selective uptake and HMGB1 upregulation. Since the linker-modified estrogen released upon **BEEP** reduction contains a non-hydrolyzable component, it is critical that the estrogen-tethered complex itself binds to the ER.

The synthesis of *bis*-17α-ethynylestradiol-*cis*-diamminedichloroplatinum(IV), **BEEP**, was achieved by coupling two readily available intermediates: 17α-(benzylamine)-ethynylestradiol and *cis, cis, trans*-diamminedichlorodisuccinatoplatinum(IV). The preparation of 17α-(benzylamine)-ethynylestradiol was based on a previously reported methodology.[27] 3-Bromobenzylamine was first BOC protected and subsequently allowed to react with sodium iodide to yield the more reactive 3-iodobenzyl-carbamic acid tert-butyl ester. Sonogashira coupling of the BOC-protected benzylamine and 17α-ethynylestradiol, followed by deprotection of the amine group, afforded 17α-(benzylamine)-ethynylestradiol in 67% overall yield. The experimentally determined mass for the desired benzylamine-modified estradiol ($[M+H]^+$= 402.2 amu) is in exact agreement with the calculated value ($[M+H]^+$=402.2 amu).

Scheme 4.1 summarizes the synthesis of **BEEP**. The amine-modified 17α-ethynylestradiol is coupled to *cis, cis, trans*-diamminedichlorodisuccinatoplatinum(IV) using common peptide coupling reagents, EDC and HOBt. Isolation of the desired platinum-estradiol conjugate was confirmed by ^{1}H NMR spectroscopy (see Figure 4.4) and ESI-MS. Formation of the amide linkage is supported by a new proton resonance at 8.37 ppm, which is consistent with

known platinum(IV) bioconjugates.[30] The amide proton (H_b) integrates in a 1:1 ratio with the estradiol –OH group (H_3). Finally, the experimentally determined mass was in excellent agreement with the calculated value (± 0.0009%).

Biological Studies of BEEP

*Effect of **BEEP** Treatment on the Expression of HMGB1.* The ability of **BEEP** to upregulate HMGB1 was investigated by immunofluorescence microscopy. As shown in Figure 4.2, treatment with 200 nM **BEEP** for 4 h does not induce overexpression of HMGB1 in MCF-7 cells. The failure of **BEEP** to induce HMGB1 upregulation suggests that the compound may have no affinity for the ER.

*Binding Affinity of **BEEP** for ERα and ERβ.* The binding affinity of **BEEP** for ERα and ERβ was approximated by incubation of the isolated receptors with **BEEP** and subsequently separating free from bound platinum by dialysis. The results (Table 4.2) indicate that **BEEP** has very low affinity for ERα (K_d ~ 970 nM) and none for ERβ.

Because of the high number of ER molecules expressed per cell (10^3-10^4), a relative binding affinity (RBA) of ~1% would be sufficient for providing some degree of ER(+)-cell targeting.[31] The K_d value for estradiol binding to the ER is 0.05-0.10 nM and therefore the RBA of **BEEP** is 0.005-0.01%. This very low RBA of **BEEP** explains the failure of **BEEP** to induce HMGB1 regulation in MCF-7 cells, and implies that **BEEP** will not achieve any ER(+)-selective targeting of the compound.

Whole Cell Uptake of BEEP into ER(+) MCF-7 and ER(-) HCC-1937 Cells. In spite of the low ER binding affinity of **BEEP**, its uptake was evaluated in MCF-7 and HCC-1937 cells. Cells were incubated with 10 μM **BEEP** for 4 h at 37 C, washed with PBS, and harvested. The

whole-cell platinum content was determined by AAS and compared to the total protein content of the isolated cell pellet. For ER(+) MCF-7 cells the concentration of platinum to total protein content ratio was 0.004 ± 0.002, and for ER(-) HCC-1937 cells the ratio was 0.02 ± 0.01. Therefore, it is clear that **BEEP** is not selectively taken up by ER(+) cells, a result is that reflects the low ER binding affinity observed for **BEEP**.

*Cytotoxic Profile of **BEEP** in ER(+) MCF-7 and ER(-) HCC-1937 Cells.* The cytotoxicity of **BEEP** was evaluated in ER(+) MCF-7 and ER(-) HCC-1937 human breast cancer cell lines by the MTT assay. As shown in Figure 4.5, the cytotoxicity of **BEEP** is nearly identical in MCF-7 (IC_{50}=1.8 ± 0.1 μM) and HCC-1937 (IC_{50}=2.2 ± 0.1 μM) cells. This observation is consistent with the inability of **BEEP** to induce HMGB1 overexpression and to bind to the ER.

A number of platinum-estrogen conjugates have been prepared to obtain a compound suitable for targeting ER(+) breast cancer.[32-40] Although some compounds maintained affinity for the estrogen receptor and are cytotoxic in ER(+) cell lines, evidence for estrogen-mediated selective-targeting of platinum compounds is scarce. With the exception of the 17α-alkylamide-tethered estrogen-platinum(II) conjugate discussed earlier (Figure 4.1C), most compounds have similar cytotoxicities in ER(+) and ER(-) cell lines.[16] Two 17α-ethynylestradiol platinum(II) conjugates designed for selective uptake into breast cancer cells were recently reported.[41] The complexes had poor affinity for the ER (RBA <1%) as a result of their hydrophilic character and no cytotoxicity data were presented. As with the present compounds, it is possible that the hydrophilic nature of the succinate linker hinders binding to the ER.[41]

Conclusions and Future Prospects

A 17α-ethynylestradiol platinum(IV) complex designed to specifically target ER(+) cells was synthesized and characterized by ^{1}H NMR and ESI-MS. **BEEP** had low affinity for the ER and, therefore, could not induce overexpression of HMGB1 in ER(+) human breast cancer MCF-7 cells. In addition, no selective uptake of **BEEP** into ER(+) MCF-7 cells occurred. **BEEP** is moderately cytotoxic in MCF-7 cells, but has no differential toxicity compared to ER(-) HCC-1937 human breast cancer cells.

Future work could target the synthesis of a 7α- or 11β-estradiol-tethered platinum complex. We attempted to prepare a 7α-estradiol-tethered platinum complex, but the ligand synthesis proved problematic because of low product yields. An alternative would be to obtain a more hydrophobic linker for attachement at the 17α-position. Modification at this site is more readily achieved, so a variety of linkers could easily be screened for ER binding affinity. However, in light of the general failure of platinum-estrogen conjugates to target ER(+) cell lines, attention should be focused on other targeting moieties such as peptides, carbohydrates, or folic acid.

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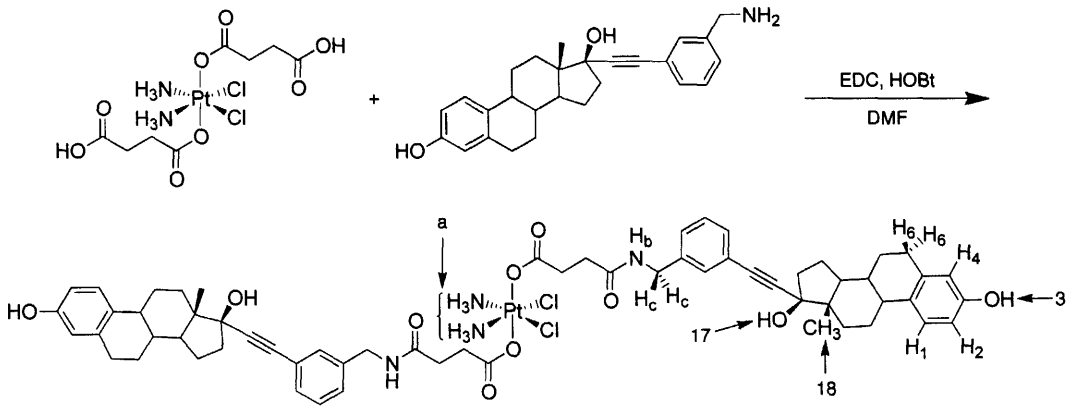
Table 4.1. Selected estrogen-regulated genes in MCF-7 human breast cancer cells.[a] Adapted from Lobenhofer, E.K., Bennett, L.l Cable, L., Li, L., Bushel, P.R., and Afshari C.A., *Mol. Endocrinology* (2002), **16**: 1215-1229.

Function/Protein Affected	Effect on Expression
DNA Replication/Repair	
DNA polymerase ε	+
DNA polymerase Δ	+
Uracil-DNA glycosylase	+
Dihydrofolate reductase	+
XRCC5	-
Transcription/Chromatin Structure	
Sp3 transcription factor	+
HMGB1	+
H2B	-
JUN B	-
STAT1	-
Cell Cycle	
Cdc28 protein kinase 2	+
Cyclin D1	+
Cyclin A2	+
Tubulin	+
Cyclin-dependent kinase	-
Cellular Signaling	
Protein kinase inhibitor β	+
Tyrosine kinase receptor C	+
Ephrin-A1	-
TGF-β2	-
Extracellular matrix/cell structure	
Filamin B	+
Laminin 3	+
Vitronectin	+
Protein degradation/Synthesis/Targeting	
Karyopherin α2	+
Heat shock 10-kDa protein 1	+
Mannose-6-phosphate receptor	-
Apoptosis	
Caspase 7	+
NF-κB	-
Amyloid β precursor protein	-
Miscellaneous	
Sterol Δ-7-reductase	+
Metallothionein	-

[a] Increased expression is indicated by + and decreased expression is denoted by -.

Table 4.2. Binding affinity of **BEEP** for ERα and ERβ. **BEEP** was incubated with ER and ER at 4 °C for 18 h. Free receptor-bound **BEEP** were separated by dialysis against PBS and the concentration of the latter was determined by Pt AAS.

Sample	[**BEEP**] (nM)	[ER] (nM)	[**BEEP**·ER] (nM)	~K_d (nM)
ERα	830	83	71	970
ERβ	830	83	0	--



BEEP

Scheme 4.1

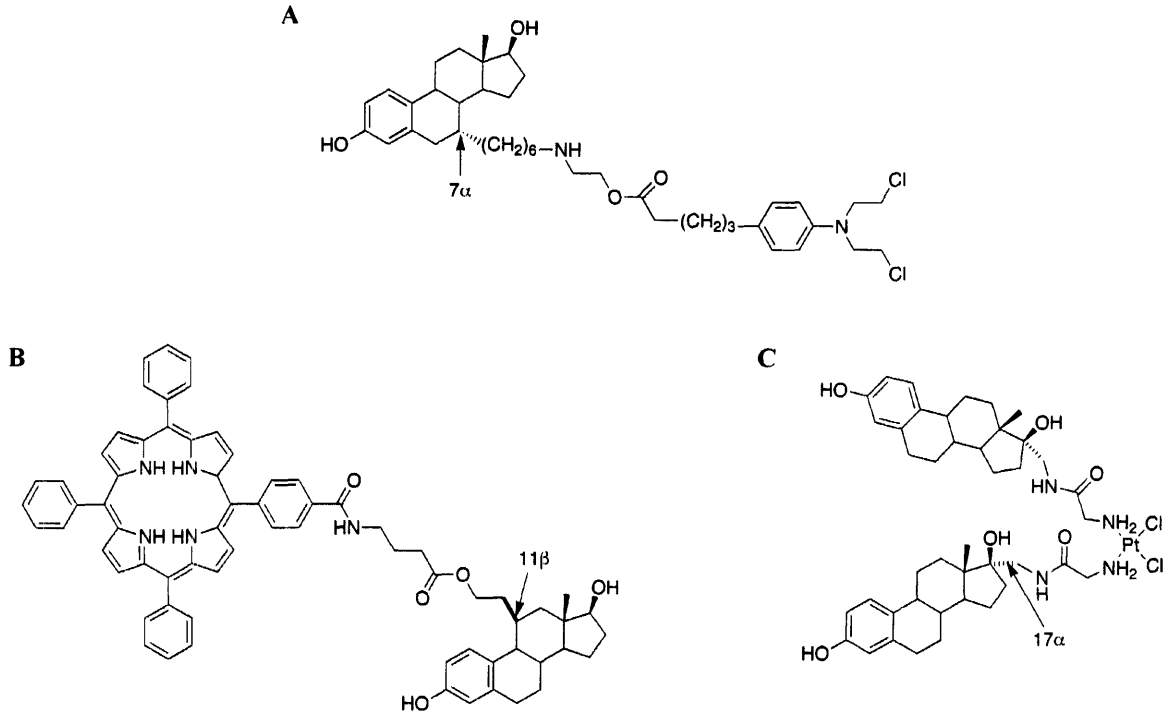


Figure 4.1. Compounds designed to target ER(+) cells: (A) 7α-Estradiol-tethered mustard[14]; (B) Porphyrin-estradiol conjugate linked through the 11β-position[11]; (C) 17α-alkylamide-tethered platinum(II) estradiol conjugate.[16]

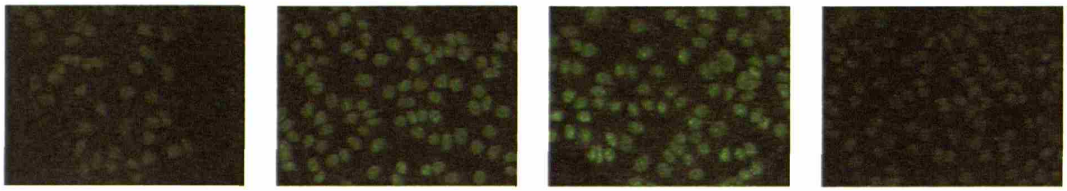


Figure 4.2. Expression of HMGB1 as visualized by fluorescence microscopy. MCF-7 cells were grown on glass coverslips and incubated with 200 nM β-estradiol, 17α-ethynylestradiol, or **BEEP** for 4 h at 37 C. The cells were fixed, permeabilized, and treated with a 1:100 dilution of anti-HMGB1 antibody followed by a 1:100 dilution of anti-IgG antibody conjugated to FITC.

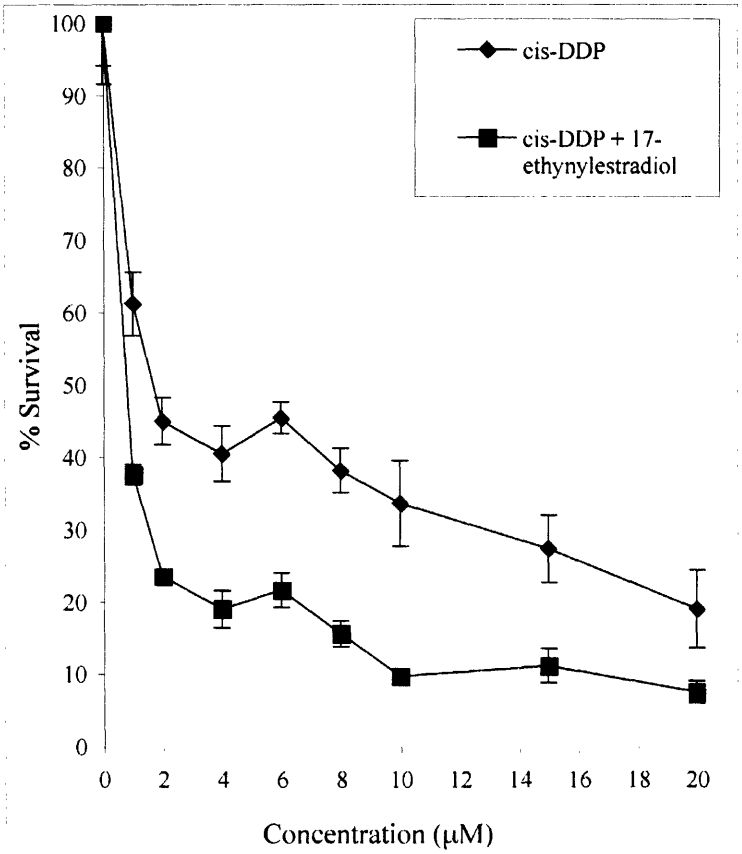


Figure 4.3. Cytotoxicity of cisplatin with or without 17α-ethynylestradiol co-treatment in MCF-7 cells. Cell survival was evaluated by the MTT assay. Error bars represent one standard deviation.

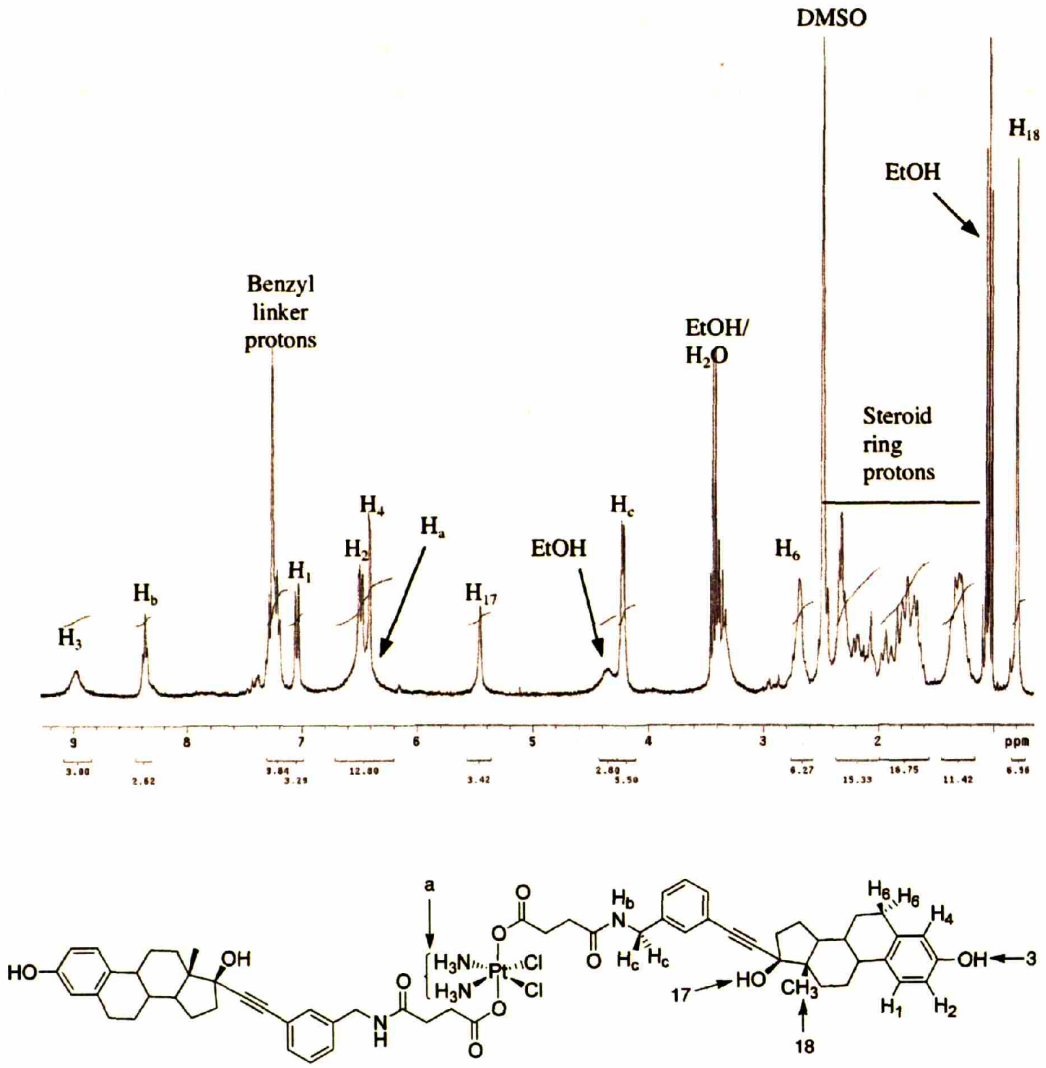


Figure 4.4. ^{1}H NMR spectrum of **BEEP** in d_6-DMSO.

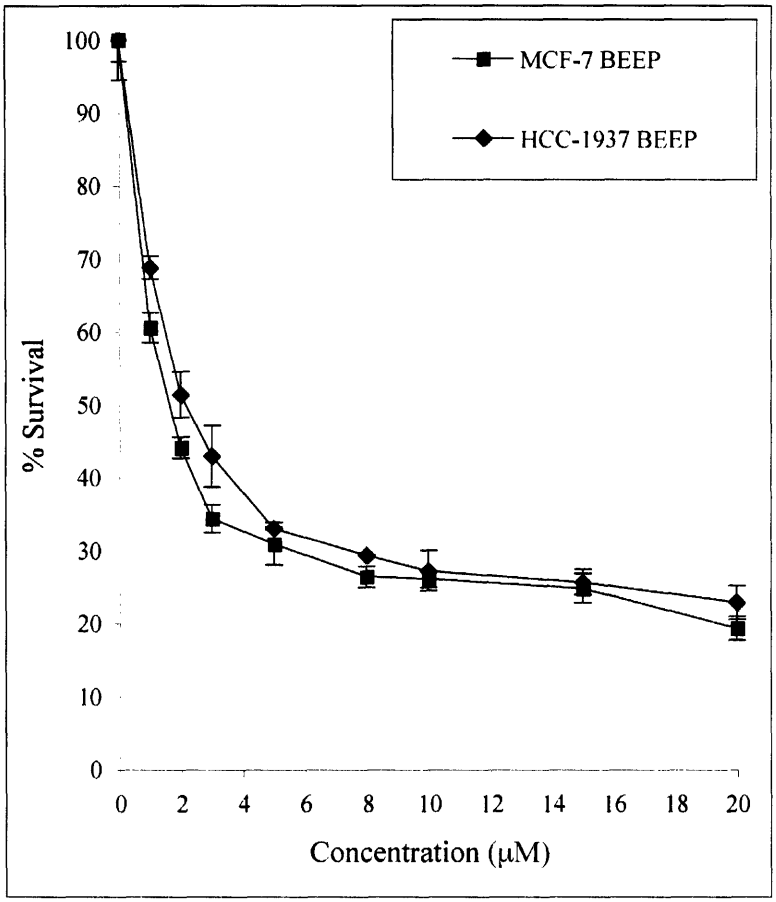


Figure 4.5. Survival of ER(+) MCF-7 and ER(-) HCC-1937 human breast cancer cells treated with 0-20 μM **BEEP** as monitored by the MTT assay. Error bars represent one standard deviation.

Chapter 5
Targeting Platinum(IV) Complexes to Cancer Cells Through Folate Receptor-Mediated Uptake

Introduction

The vitamin folic acid is essential for one-carbon transfer reactions and is required for nucleotide base biosynthesis and cell growth.[1,2] Mammalian cells cannot synthesize folic acid, therefore efficient uptake mechanisms exist to ensure an adequate supply of the nutrient.[2] The folate receptor (FR) is responsible for the major folic acid uptake pathway. The mechanism for receptor-mediated uptake of folic acid may involve via clathrin-coated pits, caveolae, or submicron domains, but the exact process in unclear at present.[3-7] As shown in Figure 5.1, once folic acid is internalized the pH of the encapsulating endosome drops to between 4.3-6.9, releasing a portion of bound folate[1,8] The remainder is recycled back to the extracellular environment.[1] Although the efficiency of folic acid internalization is only 15-25%, folate-receptor mediated endocytosis can import ~$2x10^5$ molecules per cell per hour.[1]

There are three isoforms of the folate receptor: FRα, FRβ, and FRγ.[9] Both FRα and FRβ are anchored to the cell membrane through a glycosylphosphatidylinositol (GPI) linkage, whereas the less abundant FRγ lacks the GPI moiety and is soluble.[1,9] The α- and β-isoforms differ with respect to their affinities for folic acid and are differentially expressed. FRα, considered the high affinity isoform (K_d~0.1 nM), is moderately expressed in the kidney, placenta, and choroid plexus. The expression of the low affinity FRβ isoform (K_d~1 nM) is limited to hematopoietic and non-epithelial cells (i.e., spleen, thymus).[1] In addition to expression in normal tissue, both FRα and FRβ are overexpressed in a variety of malignancies.[1,7,10,11] As shown in Table 5.1, the distribution of FR isoforms varies considerably and FRα is significantly upregulated in a number of cancers including ovarian, uterus, endometrium, brain, kidney, head and neck, and mesothelium.[12] In fact, overexpression of FRα is a marker for ovarian cancer.[13] The overexpression of FRβ is mostly limited to myelogenous leukemia and sarcomas.[1]

Because the expression of the FR is upregulated in such a wide variety of cancers, targeting anti-cancer drugs through FR-mediated uptake is an attractive proposition. The relatively low expression of the FR in normal tissue suggests that folate-tethered anti-cancer compounds would be selectively taken up in malignant tissue, which would allow for higher dosing with fewer side effects.[1,12] In addition, the expression of the FR in healthy tissue is limited to the apical membrane surface, meaning that the receptor is directed towards the body cavity. Therefore, access to the FR is prohibited through the blood stream and primarily occurs through the urine and airways. In malignant tissue epithelial cells lose their polarity and, as a result, the FR is accessible to folate-tethered compounds in the blood.[1] *In vivo* studies with radionuclide folate conjugates have confirmed high levels of uptake in tumor masses.[7] Unfortunately, significant uptake of the radiolabeled folic acid also occurs in the kidneys, which could result in unwanted nephrotoxicity. However, the high clearance rates of such compounds make kidney toxicity less likely.[7]

The use of folic acid conjugation has provided many potentially useful compounds with the ability to target cancer cells selectively *in vitro* and *in vivo*.[7,14-18] A cisplatin folic acid conjugate may be particularly powerful for treating a variety of cancers. Previous work has afforded a carboplatin folate conjugate; however, the compound failed with respect to enhanced uptake and selective cytotoxicity.[19] One shortcoming of the compound was that the carboplatin and folic acid moieties were connected through a polyethylene glycol (PEG) linker. Although the use of PEG linkers have been successful in some cases, the bulky nature of the linker can result in poor release from internalized endosomes.[14] The stability of the carboplatin derivative in the acidic environment of the endosome may also have contributed to the poor biological activity of the compound. Therefore, our strategy in the present study was to synthesize a folic acid

conjugate based on a platinum(IV) platform with a shorter linker. Use of platinum(IV) affords a more stable compound with the potential to release unmodified cisplatin upon intracellular reduction.[20] The shorter tether also alleviates problems associated with poor release of PEG-conjugates from endosomes. This chapter describes the synthesis and biological activity of the target folate-tethered platinum(IV) complex.

Experimental

Materials. Potassium tetrachloroplatinate(II) was a gift from Engelhard. *Cis, cis, trans*-diamminedichlorodisuccinatoplatinum(IV) was prepared as described previously.[20] *N*-Boc-4, 7, 10-trioxatridecane-1, 13-diamine (**BOC-TDA**) was prepared as described in the literature and its purity was confirmed by ^{1}H NMR spectroscopy.[21]

Synthesis of Trioxatridecanediamine-folate (TDA-folate, Scheme 5.1). DCC (0.52 g, 2.5 mmol) and pyridine (190 μL, 2.4 mmol) were added to a stirred solution of folic acid (1.2 g, 2.7 mmol) in 100 mL of DMSO. After 15 min, **BOC-TDA** (1.7 g, 5.3 mmol) was added and the solution was allowed to stir in the dark for 24 h at RT. The pyridine was removed under reduced pressue and the DMSO by lyophilization to yield a sticky orange solid, which was washed with 30% acetone in ether. The BOC protecting group was removed by stirring in 10% TFA in dichloromethane (100 mL) and the solvent under reduced pressure to afford an orange oil. The crude product was resuspended in an aqueous solution of ammonium hydroxide (pH 9) for 2 h, isolation by centrifugation, and dried *in vacuo* to give a yellow solid (0.75 g, 1.2 mmol, 48% yield); mp, decomposes above 150 C. ^{1}H NMR (d_6-DMSO, 300 MHz): δ 8.63 (s, 1H, ArH), 7.85 (m, 1H, NH), 7.60 (m, 3H, ArH), 7.00 (bt, 1H, NH), 6.63 (d, 2H, ArH), 4.49 (d, 2H, CH_2),

4.20 (m, 1H, CH), 3.33 (m, 18H, CH_2), 2.81 (t, 2H, CH_2), 1.5-2.0 (m, 4H, CH_2). HRMS (ESI) calculated for $[M+H]^+$ 644.3151 amu, found 644.3165 amu.

Synthesis of a Monofolate-Tethered Platinum(IV) Complex (PMF, Scheme 5.2). A solution of EDC (0.085 g, 0.44 mmol), NHS (0.051 g, 0.44 mmol), and *cis, cis, trans*-diamminedichloro-disuccinatoplatinum(IV) (0.096 g, 0.22 mmol) was prepared in 10 mL of H_2O and allowed to stir for 10 min. A solution of **TDA-folate** (0.11 g, 0.17 mmol) in water (10 mL) was added and the solution was allowed to stir for 15 h at RT in the dark. The reaction solution was dialyzed against 5 mM sodium bicarbonate buffer (pH 9.0) to remove unreacted starting material (2-4x) and subsequently desalted by dialysis against water (2x). A hydrated yellow powder (0.18 g, 0.15 mmol, 90% yield) was isolated upon lyophilization; mp, decomposes at 222 C. ^1H NMR (D_2O, 500 MHz): 8.73 (s, 1H, ArH), 7.67 (d, 2H, ArH), 6.81 (d, 2H, ArH), 4.65 (s, 2H, CH_2), 4.27 (m, 1H, CH), 2.63 (t, 4H, CH_2), 2.40 (t, 4H, CH_2). HRMS (ESI) calculated for $[M+H]^+$ 1160.2970 amu, found 1160.2954 amu.

HPLC Analysis of CPG-Mediated Cleavage of TDA-folate. Carboxypeptidase-G (CPG) digestion was carried out following a previously published methodology.[22] Briefly, 500 μL of a 155 μM **TDA-folate** solution in Tris buffer (150 mM, pH 7.3) was incubated with 0.5 units of CPG in 250 μL of Tris buffer for 24 h at 30 C. The cleavage of glutamic acid was followed by RP HPLC (100% H_2O to 50% acetonitrile over 30 min), monitoring absorbance at both 254 and 280 nm.

FITC Conjugation of TDA-folate. A solution of **TDA-folate** (33 mg, 0.051 mmol) and FITC (20 mg, 0.051 mmol) in 5 mL DMF was allowed to stir for 24 h at RT in the dark. An additional 75 mg (0.12 mmol) of **TDA-folate** was then added to ensure that no free FITC remained. The FITC-conjugated **TDA-folate** was isolated by lyophilization and used without further purification. The ratio of [folate]:[FITC] was found to be 3.65 as determined by UV/vis spectrophotometry (folate ε_{282}=22,600 $M^{-1}cm^{-1}$; FITC ε_{494}=68,000 $M^{-1}cm^{-1}$).

Cell Culture. Human breast cancer MCF-7 and cervical cancer HeLa cells were purchased from the American Type Culture Collection. BG-1 ovarian carcinoma cells were a gift from J. Barrett (NIH, MD). MCF-7, HeLa, and BG-1 cells were grown in DMEM (GIBCO/BRL) supplemented with 10% FBS (Hyclone), 1 mM sodium pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin. All cells were incubated at 37 C under a 5% CO_2 atmosphere.

Uptake of FITC-Conjugated TDA-folate. Cells were grown to 80% confluence in 6-well plates and incubated with 2.5 μM of FITC-conjugated **TDA-folate** at 37 C for 0-6 h. Following incubation, the cells were harvested, and washed extensively with PBS. For each sample, 10^4 cells were analyzed by fluorescence-activated cell sorting (FACScan, Beckton Dickinson) and the resultant data were analyzed with CELL QUEST. Non-treated cells were referenced as the control, with less than 5% of the cells exhibiting native fluorescence.

Whole Cell Uptake of Cisplatin and PMF. Cells were seeded onto 10-cm plates and grown until 80-90% confluent. Cells were incubated with 10 μM **PMF** for 4 h at 37 C, harvested, and washed extensively with PBS. The total protein content was determined by the bicinchoninic

acid protein assay and platinum concentration by AAS. The degree of **PMF** uptake was expressed as a ratio of platinum concentration (mg/L) to the total protein concentration (mg/L) of the isolated cell pellet.

Cytotoxicity Studies. In a 96-well plate, cells were seeded at a density of 1000 cells per well in 100 μL of DMEM. After 24 h, the cells were either treated with 0–10 μM cisplatin or 0-3 μM **PMF**. The cells were incubated for 96 h at 37 C and evaluated for survival by the MTT assay. Briefly, cells were treated with 20 μL of 5 mg/mL MTT (PBS) and incubated for 5 h at 37 C. The media was removed and the cells resuspended in 100-200 μL of DMSO after which the absorbance of each well was measured at 550 nm by a plate reader (SpectraMax 340 pc, Molecular Devices). Percent cell survival was calculated for each concentration as the ratio of the measured absorbance to that of the untreated wells.

Results and Discussion

Synthesis and Characterization of PMF

Initial attempts to prepare a platinum(IV) folate conjugate involved the use of an ethylenediamine linker; however, the linker-modified folic acid compound had poor solubility in most aqueous and organic solvents. Therefore, a longer, more hydrophilic linker was chosen. As shown in Scheme 5.1, the linker-modified folate, **TDA-folate**, was readily obtained from the DCC-mediated coupling of folic acid and **BOC-TDA**. HRMS confirmed the presence of the desired linker-modified folic acid; the experimentally determined mass was in excellent agreement with the calculated value (±0.0002%).

The reaction of **BOC-TDA** with folic acid yields two isomers: α- and γ-linked conjugates. In general, modification of the γ-carboxyl group occurs more readily; however, because the folate receptor binds γ-modified folic acid more readily than α-modified conjugates, it is necessary to quantify the ratio of the two isomers.[22] LC-MS analysis of the **TDA-folate** compound reveals the presence of two species of equal mass (m/z 644.3) with retention times of 11.8 (65%) and 13.4 (35%) min, indicating that both α- and γ-isomers are present. The ratio of α- to γ-isomers was determined by a CPG digestion assay; the enzyme cleaves only γ-modified folic acid.[22] As illustrated in Figure 5.2, upon incubation with CPG, the peak with a retention time of 11.8 min disappears, while the species with a retention time of 13.4 min remains unchanged. Therefore, it is clear that the **TDA-folate** compound is 65% γ-modified, which is consistent with literature values for other folic acid synthetic conjugates.[19]

Cis, cis, trans-Diamminedichlorodisuccinatoplatinum(IV) and **TDA-folate** were coupled to afford the desired folic acid conjugate using standard peptide coupling reagents (Scheme 5.2). Purification of the conjugate is facile and achieved by dialysis against sodium bicarbonate buffer (pH 9) using molecular weight 1000 cut-off dialysis tubing. When EDC and NHS were used to prepare the folic acid conjugate, greater than 90% of the mono-substituted platinum(IV) complex (**PMF**) was isolated before purification. The presence of **PMF** was confirmed by ^{1}H NMR spectroscopy and ESI-HRMS. The **PMF** compound is only moderately soluble in D_2O, so a high quality NMR spectrum was not obtained; however, the results provide quantitative evidence for the presence of one folate moiety for each platinum center. As shown in Figure 5.3, the integration of the succinate protons (H_6 and H_7) is twice that of the integration of the *p*-aminobenzoic acid protons (H_3 and H_4). Finally, the experimentally determined mass was in excellent agreement with the calculated value (±0.0001%). The purity of **PMF** as evaluated by

HPLC is shown in Figure 5.4. When subjected to HPLC, significant decomposition of **PMF** occurs. Product decomposition was proven by comparing the chromatographs of the original **PMF** sample (Fig. 5.4, left trace) to that of a HPLC-purified sample (Fig. 5.4, right trace). Both traces contain extraneous peaks with similar retention times.

Cellular Uptake of FITC-Conjugated TDA-Folate

The expression of the FR was evaluated by monitoring the uptake of FITC-conjugated **TDA-folate** in BG-1, MCF-7, and HeLa cells by FACS. In general, the expression of the folate receptor correlates with the uptake of folic acid.[12,19,22,23] Cells were incubated with 2.5 μM FITC-conjugated **TDA-folate** for 0-6 h at 37 C and the cellular fluorescence was subsequently determined. As illustrated in Figure 5.5, the uptake of the FITC-labeled **TDA-folate** is moderately cell-line dependent. The level of folate uptake increases as MCF-7<HeLa<BG-1. After a 6 h incubation 67% of BG-1 have internalized the modified folic acid, compared to only 57% of MCF-7 cells.

Cytotoxicity of Cisplatin and PMF Toward HeLa, MCF-7, and BG-1 Human Cancer Cells

The cytotoxicity of **PMF** was determined in HeLa human cervical cancer, MCF-7 human breast cancer, and BG-1 human ovarian cancer cells and compared to the biological activity of cisplatin. These three cell lines were chosen because of known overexpression of the folate receptor in cervical, breast, and ovarian cancers. Figures 5.6, 5.7, and 5.8 show cell survival plots for cisplatin and **PMF** in BG-1, HeLa, and MCF-7 cells, respectively. It is evident that **PMF** is significantly more cytotoxic than cisplatin in each cell line. In addition, the **PMF** compound is 100-fold more cytotoxic than the previously reported folate-tethered carboplatin analogue.[19]

Examination of the ratio of IC_{50} (μM) values for cisplatin and **PMF** reveals a cell line-dependent trend (see Table 5.2). Furthermore, the cytotoxicity data correlate with the uptake of the FITC-conjugated **TDA-folate**. In BG-1 cells, **PMF** is nearly 10 times more cytotoxic than cisplatin, whereas, **PMF** is only 6 times as active as cisplatin in MCF-7 cells. This observation supports the conclusion that **PMF** activity is a consequence of folate-receptor mediated uptake. It is noteworthy that **PMF** is particularly active against the BG-1 human ovarian cancer cell line. As mentioned earlier, overexpression of the folate receptor is a marker for ovarian cancer. A compound that could selectively target ovarian cancer cells would be extremely valuable given the fact that ovarian cancer causes more deaths than any other cancer of the female reproductive system.[24] In 2005, it is expected that over 20,000 new cases of ovarian cancer will be diagnosed and more than 40,000 woman will succumb to the disease.[24]

Mechanism of Action

The cytotoxicity of **PMF** can be attributed to folate receptor-mediated uptake of the cytotoxic agent, and not related to any additional biological activity conferred by folic acid. To test this hypothesis, the effect of folic acid on the cytotoxicity of cisplatin was independently examined. Cotreatment of HeLa, BG-1, and MCF-7 cells with folic acid and cisplatin did not alter the cytotoxicity of the platinum compound (data not shown). Therefore, the cytotoxicity of **PMF** is probably not affected by release of the modified folic acid. However, amide hydrolysis of **TDA-folate** to yield folic acid inside the cell is expected to be slow; so additional control experiments with cotreatment of cells with **TDA-folate** are planned.

The cellular uptake of **PMF** in HeLa, BG-1, and MCF-7 was evaluated and compared to that of cisplatin. Cells were incubated with 10 μM **PMF**, 10 μM **PMF** + 200 μM folic acid, or

10 μM cisplatin for 4 h at 37 C, washed with PBS, and harvested. The whole-cell platinum content was determined by platinum AAS and compared to total protein in the isolated cell pellet, estimated by the BCA assay. The results are summarized in Table 5.3. In MCF-7 and HeLa cells, uptake of **PMF** is essentially equivalent to the uptake of cisplatin. In addition, the uptake of **PMF** is unaffected by the addition of free folic acid. In BG-1 cells, the amount of **PMF** internalized is twice that of cisplatin; however, the uptake of **PMF** is only slightly affected by competition with free folic acid. It is not surprising that **PMF** uptake is greatest in BG-1 cells, the most sensitive cell line. Previous studies of cisplatin passage into in an ovarian cell line revealed that the rate of passive diffusion was ~$5x10^7$ molecules per cell per hour, which is over 200 times faster than folate receptor-mediated endocytosis.[25] Therefore, comparison of **PMF** and cisplatin uptake levels may not allow for differentiating passive diffusion from an active transport mechanism. It seems unlikely, however, that **PMF** uptake would occur by passive diffusion because the molecule has a molecular weight over 1000 and is a dianion at pH 7. Similar uptake experiments with the anionic **PMF** precursor *cis, cis, trans*-diamminedichlorodisuccinatoplatinum(IV) did not reveal any detectable internalization of the compound, supporting an active transport mechanism for **PMF**.

Based on their similar uptake levels, it is unclear why **PMF** is more cytotoxic than cisplatin. One possibility is a difference in stability in culture media between the two compounds. Over time, platinum(II) compounds are inactivated in culture media through binding to components of fetal bovine serum, such as albumin.[26] On the other hand, platinum(IV) compounds are exceedingly stable in culture media.[26] Therefore, during long incubation times cells will continue to internalize biologically active **PMF**, while cisplatin will be protein-bound and unable to bind to DNA. It is possible that the enhanced cytotoxicity of **PMF** is due both to

increased stability of the platinum(IV) compound and to folate receptor-mediated uptake. Another possibility is that cisplatin is not internalized as efficiently as **PMF** and binds to the cellular membrane. In order to evaluate this possibility, experiments to determine nuclear platinum levels after cisplatin and **PMF** treatment are planned.

Conclusions and Future Directions

The folic acid-tethered platinum(IV), **PMF**, was synthesized and characterized by HRMS and HPLC. Its cytotoxicity was evaluated in MCF-7, HeLa, and BG-1 human cancer cell lines and compared to the biological activity of cisplatin. **PMF** is significantly more cytotoxic than cisplatin in each cell line. Furthermore, the ratio of cisplatin to **PMF** cytotoxicity correlates with the ability of each cell line to internalize FITC-conjugated folic acid. **PMF** had enhanced cellular uptake as compared to *cis, cis, trans*-diamminedichlorodisuccinatoplatinum(IV), but had uptake levels similar to those of as cisplatin. The mechanism of **PMF** cytotoxicity remains to be determined.

The *in vitro* cytotoxicity of **PMF** may be enhanced by purification of the γ-conjugated isomer. As discussed earlier, the α- and γ-conjugated **TDA-folate** compound can be separated by HPLC and subsequently allowed to react with *cis, cis, trans*-diamminedichlorodisuccinato-platinum(IV). Additional *in vitro* studies should also be carried out in cell lines that express little to no folate receptor. If the mechanism of action of **PMF** involves enhanced uptake, then cell lines without the folate receptor should be less sensitive to **PMF**.

The ability of **PMF** to selectively target cancer cells can be further tested by animal studies. One concern with **PMF** is whether it is stable enough in the blood to allow for selective targeting of tumors. Biotransformation studies with *cis, trans, cis*-

$[PtCl_2(OAc)_2NH_3(c\text{-}C_6H_{11}NH_2)]$, or JM216, revealed that the compound is reduced to a variety of platinum(II) metabolites in blood within 15 min.[27] On the other hand, two *trans*-dihydroxy platinum(IV) compounds were stable in the blood serum and reduced intracellularly to yield the active platinum(II) metabolites.[27] In order to achieve selective targeting *in vivo* the platinum folic acid conjugate must remain intact until it is internalized by folate receptor-mediated endocytosis. Therefore, a compound with a lower reduction potential may be valuable to test in animal studies. As shown in Figure 5.9, two other folate-tethered platinum complexes have been designed to obtain a compound with greater stability in blood serum. The first compound, **PMFII**, is based on **PMF** with one axial succinate ligand being replaced with a hydroxyl group. This modification should afford the **PMFII** complex with a lower reduction potential than that of **PMF**. The second compound, **PMFIII**, is based on the previously reported carboplatin folic acid conjugate; however, **PMFIII** contains the smaller trioxatridecanediamine linker instead of the PEG-based linker used in the previously described compound.[19] Furthermore, the platinum(II) folate complex will be oxidized with hydrogen peroxide yielding a stable *trans*-dihydroxy platinum(IV) compound. The resultant platinum(IV) folic acid conjugates (**PMF-PMFIII**) should afford valuable a series of compounds for *in vivo* evaluation.

Acknowledgement

Caroline Saouma synthesized and characterized the **PMF** complex.

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Table 5.1. Expression of FRα and FRβ is normal and malignant tissue. The levels of FRα and FRβ are given in arbitrary units that reflect the receptor mRNA levels with respect to an internal standard. Adapted from Ross, J.F., Chaudhuri, P.K., and Ratnam, M., *Cancer* (1994), **73**, 2432-2443.

Tissue	FRα	FRβ	[^{3}H] Folic acid bound (pmol/mg protein)
Normal ovary	0.3	2.2	0.6
Ovarian carcinoma	30.9	4.9	28.4
Normal uterus	<0.2	3.5	<0.2
Uterine carcinoma	58.5	2.5	72.1
Normal colon	0.9	3.2	1.3
Colon carcinoma	1.1	0.8	0.6
Normal kidney (case 1)	7.2	0.8	ND[a]
Normal kidney (case 2)	2.6	2.3	ND
Normal kidney (case 3)	<0.2	0.8	ND
Kidney carcinoma (case 1)	23.3	1.5	ND
Kidney carcinoma (case 2)	3.5	1.7	ND
Normal lung	7.1	0.8	ND
Lung carcinoma (case 1)	92.0	10.5	ND
Lung carcinoma (case 2)	0.6	1.1	ND
Normal brain	0.7	3.5	0.4
Meningioma (brain tumor)	3.2	45.0	21.0
Osteosarcoma	<0.2	45.3	16.0
Uterine sarcoma	<0.2	24.9	10.6
Lymphoma (case 1)	0.3	10.7	4.2
Lymphoma (case 2)	13.8	8.7	23.0

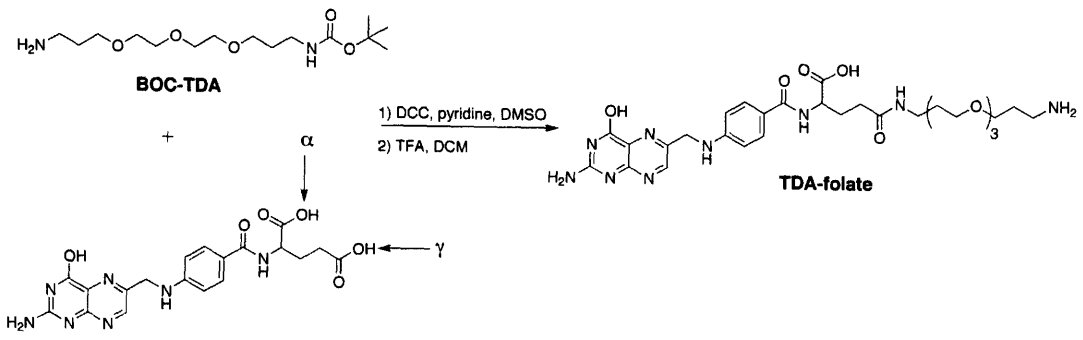
[a] ND: Not determined

Table 5.2. IC_{50} (μM) values for cisplatin and **PMF** in BG-1, MCF-7, and HeLa cells

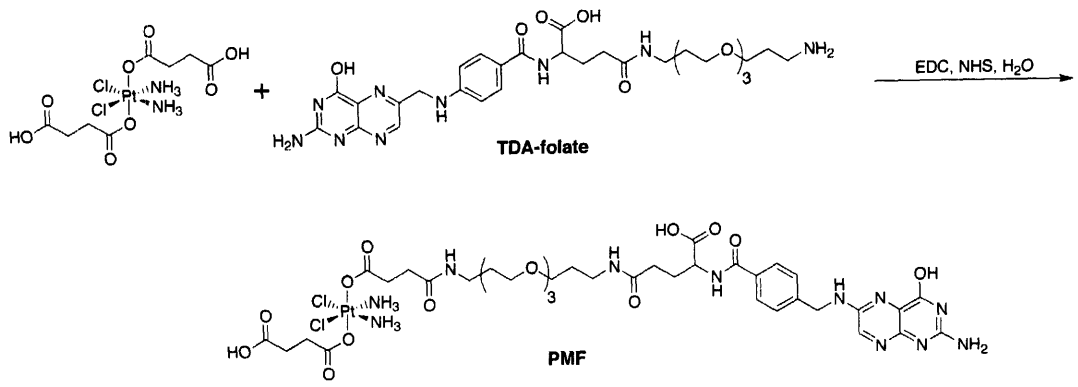
Cell line	Cisplatin	**PMF**	Cytotoxicity Ratio (Cisplatin:**PMF**)
BG-1	2.4±0.4	0.25±0.04	9.6
MCF-7	1.4±0.01	0.22±0.04	6.4
HeLa	1.6±0.2	0.22±0.04	7.3

Table 5.3 Uptake of cisplatin and **PMF** in MCF-7, HeLa, and BG-1 cells. Uptake levels are expressed as the ratio of platinum (mg/L) to protein concentration (mg/L).

Sample	[platinum]/[protein] x 10^5
MCF-7 Cells	
Cisplatin	2.0
PMF	1.7
PMF + folic acid	2.0
HeLa Cells	
Cisplatin	1.9
PMF	1.6
PMF + folic acid	2.5
BG-1 Cells	
Cisplatin	1.3
PMF	3.0
PMF + folic acid	2.8



Scheme 5.1.



Scheme 5.2.

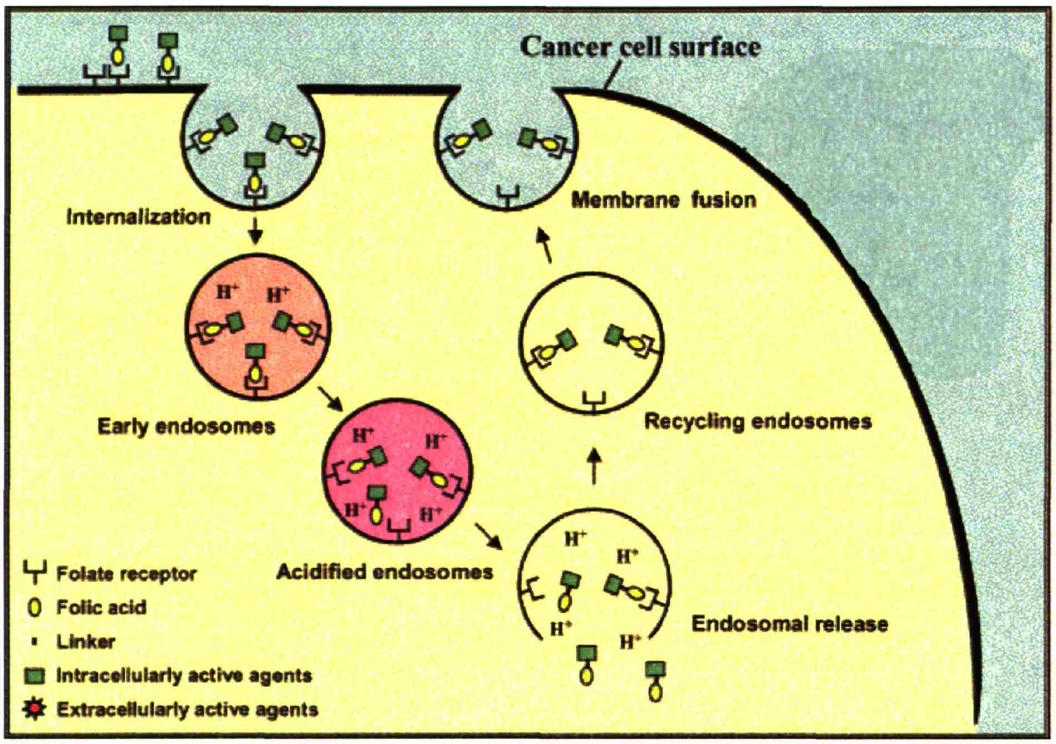


Figure 5.1. Folate receptor-mediated uptake of folic acid conjugates. Folic acid binds the folate receptor and is subsequently internalized by an endocytotic pathway. The pH of the endosome drops, thereby releasing folic acid from the receptor and then the endosome. Folic acid that remains receptor-bound is recycled back the the extracellular environment. Adapted from Yingjuan, L., Low, P.S., *Advanced Drug Delivery Reviews* (2002), **54**, 675-693.

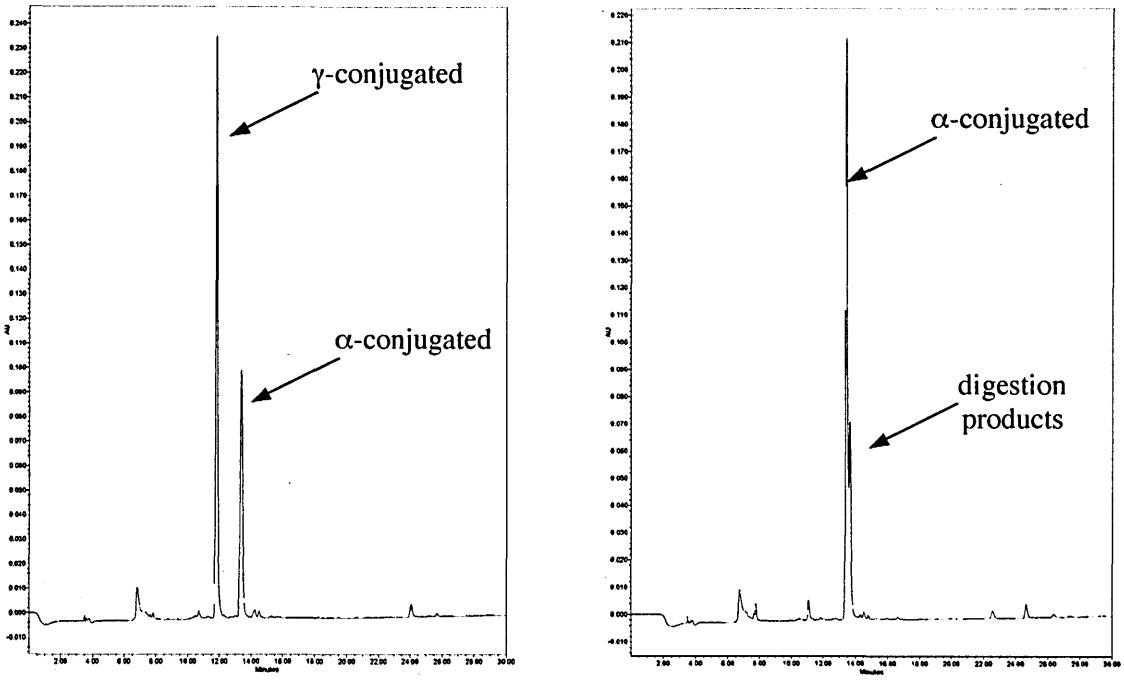


Figure 5.2. Analysis of the CPG digestion of **TDA-folate** by RP HPLC (100% H_2O to 50% acetonitrile over 30 min). The left panel shows the HPLC trace before digestion and clearly reveals the presence of two species. Mass spectrometry confirmed that the two peaks were of the two isomers. The right panel shows the HPLC chromatogram following CPG digestion. The γ-conjugated isomer is completely digested, while the α-isomer remains unchanged. The distribution of isomers is 35% α- and 65% γ-conjugated.

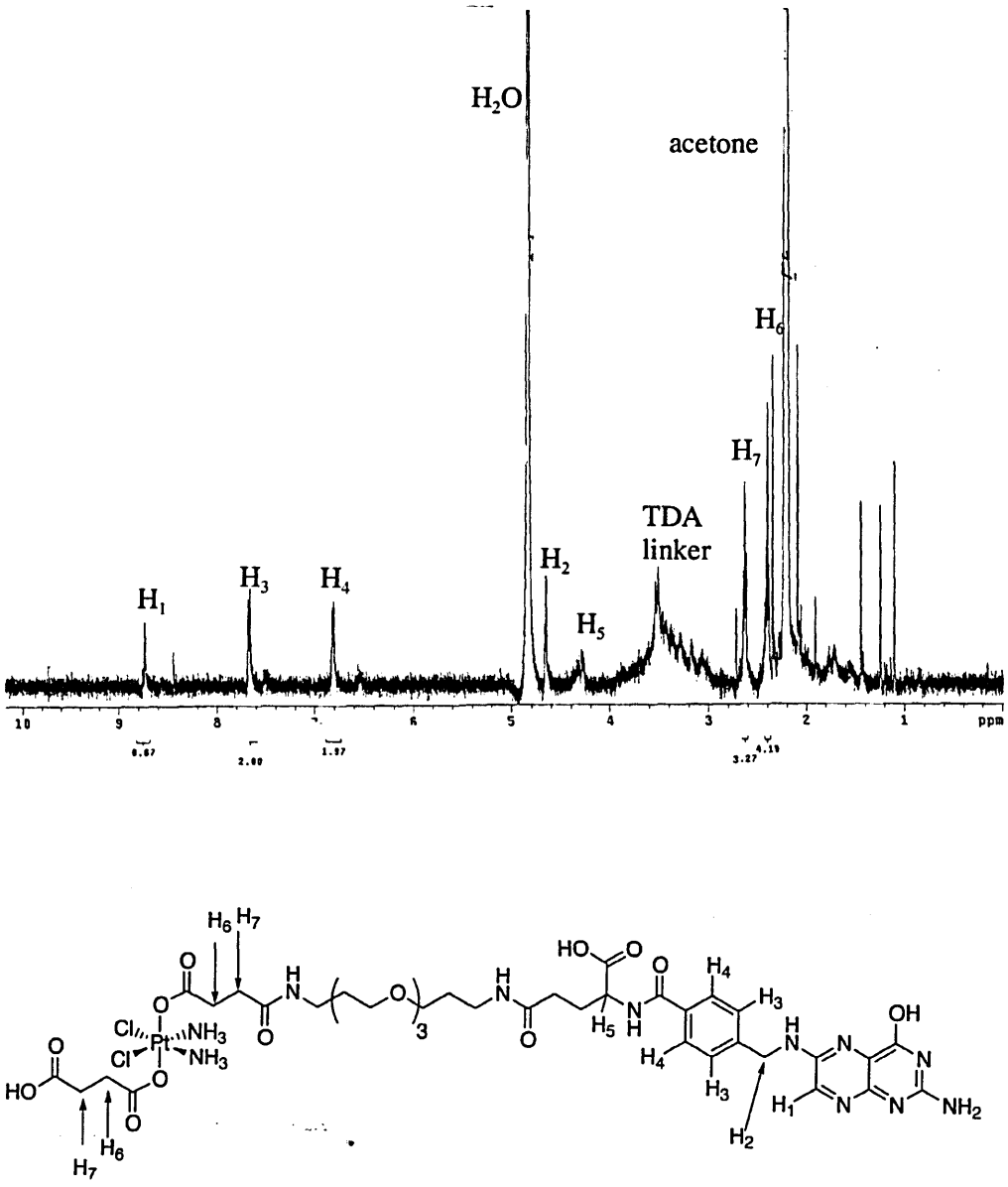


Figure 5.3. ^{1}H NMR (500 MHz) of **PMF** in D_2O.

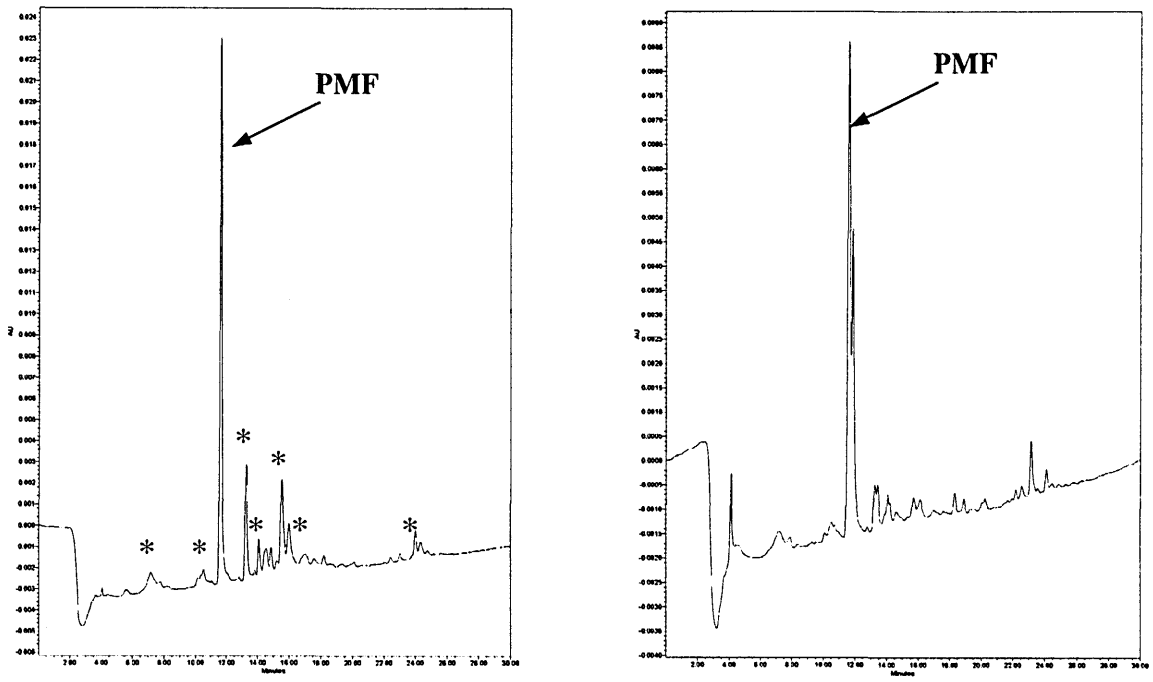


Figure 5.4. Purity analysis of **PMF** by HPLC. The left chromatogram shows the trace of **PMF** purified by dialysis, while the right trace is of **PMF** further purified by HPLC. Peaks labeled with an asterisk on the left trace are attributed to product decomposition on the HPLC column because they also appear on the trace of the HPLC-purified sample (right).

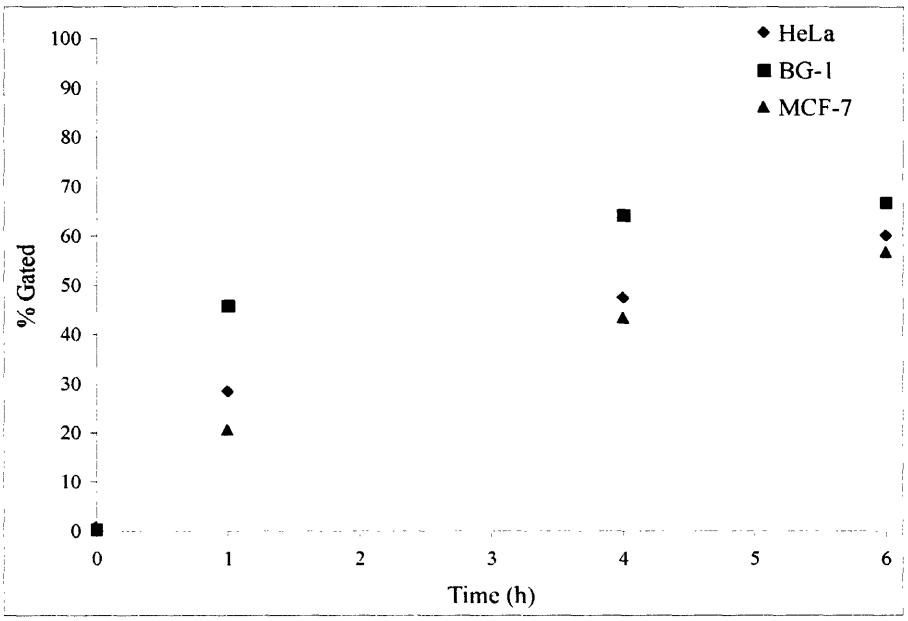


Figure 5.5. Uptake of FITC-conjugated folic acid as monitored by flow cytometry. Cells were incubated with 2.5 μM FA-FITC at 37 C for 0-6 h, harvested, and analyzed by FACS.

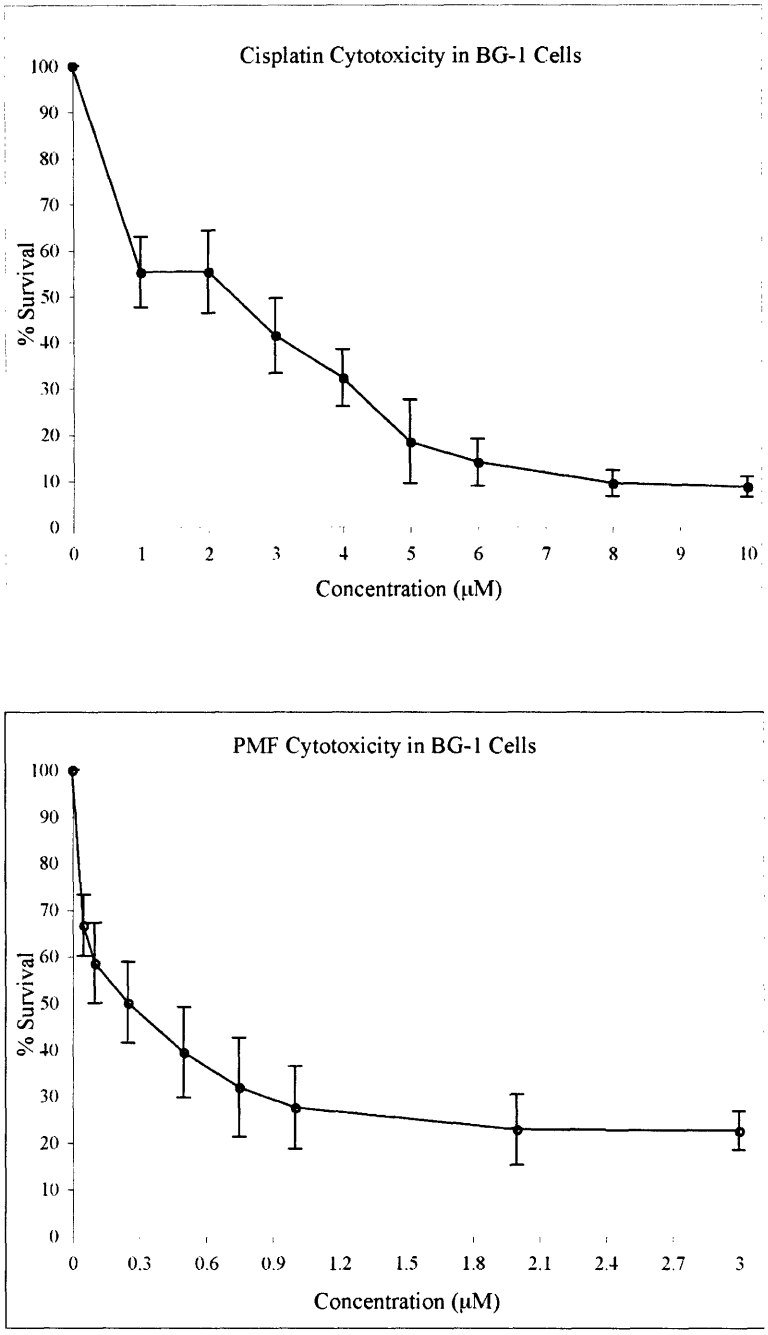


Figure 5.6. Cytotoxicity of cisplatin and **PMF** in BG-1 human ovarian cancer cells as determined by the MTT assay.

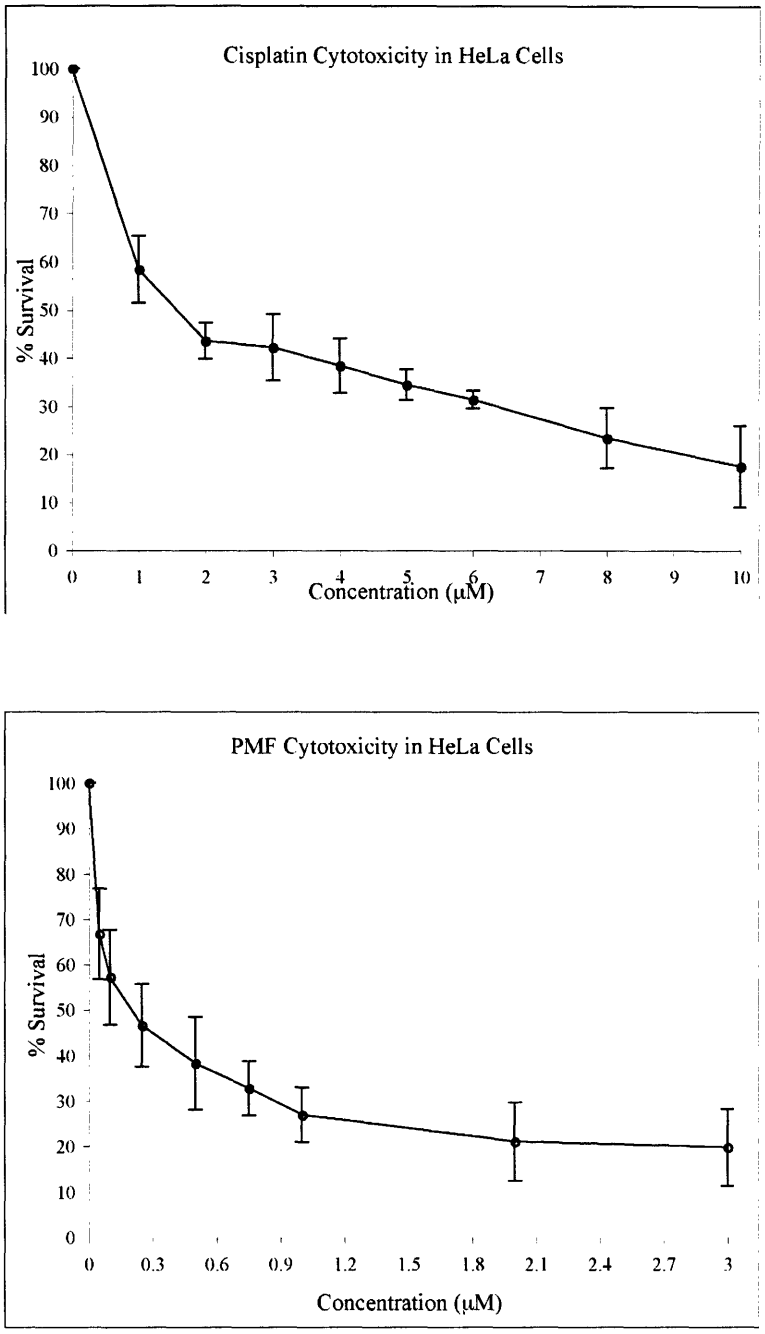


Figure 5.7. Cytotoxicity of cisplatin and **PMF** in HeLa human cervical cancer cells. Percent cell survival determined by the MTT assay.

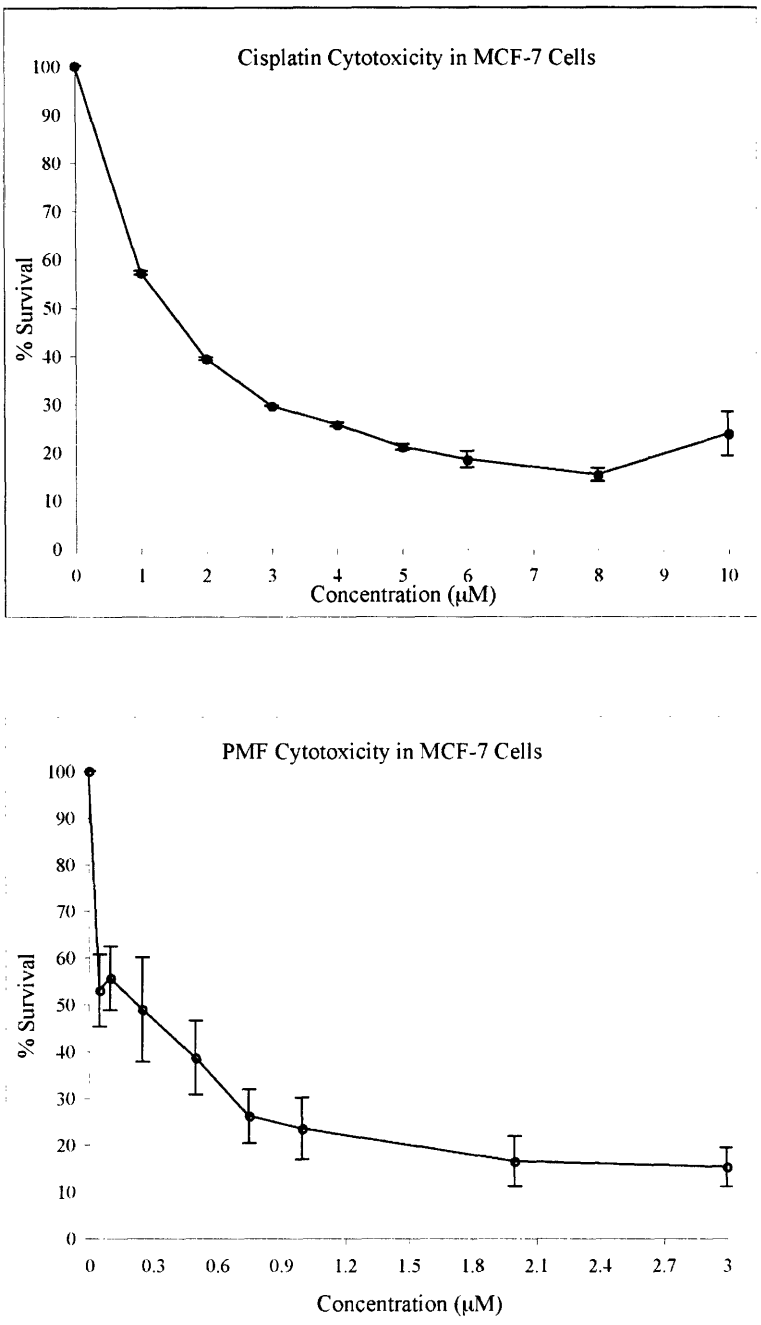
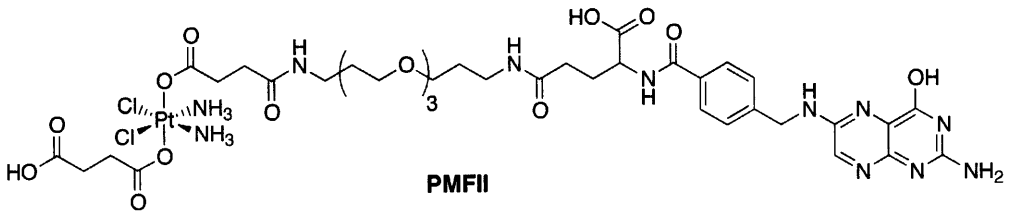
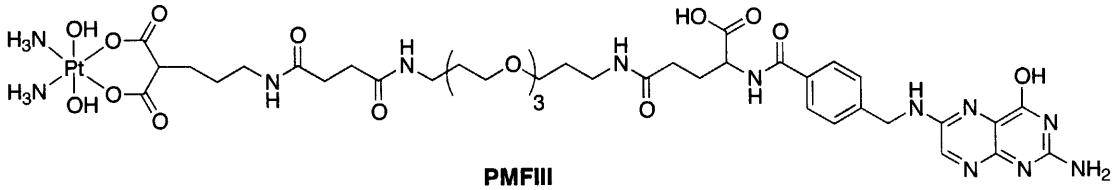


Figure 5.8. Cytotoxicity of cisplatin and **PMF** in MCF-7 human breast cancer cells as determined by the MTT assay.



PMFII



PMFIII

Figure 5.9. Schematic diagram of two proposed platinum folic acid conjugates designed to have greater *in vivo* stability.

Chapter 6
Effect of Exogenous HMGB1 on the Sensitivity of HeLa and MCF-7 Cells to Cisplatin and *trans*-DDP

Introduction

HMGB1 is a ubiquitous and abundant (10^6 copies per cell) nuclear protein that contains two tandem homologous DNA-binding domains and a glutamate- and aspartate-rich C terminus.[1] HMGB1 binds primarily to the minor groove of DNA with little or no sequence specificity, but preferentially recognizes distorted structures including 4-way junctions and cisplatin-damaged DNA. Although the exact function of HMGB1 in the nucleus is unknown, its interaction with transcription factors such as Hox and Pou proteins, p53, TBP, and steroid hormone receptors increases their binding affinity with their respective target DNA sequences.[2] HMGB1 has long been thought to act only as an intracellular architectural transcription factor; however, recent research has revealed its role as an extracellular cytokine, uncovering an unexpected new function of the protein.[1]

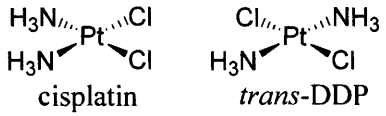
HMGB1 is loosely associated with chromatin and readily leaks into culture medium when a cell is permeabilized or undergoes necrosis.[3] Conversely, HMGB1 remains tightly bound to chromatin during apoptosis.[3] Monocytes and macrophages actively secrete HMGB1 by hyper-acetylating the protein in order to direct it for export.[4] Once secreted from the cell, binding of HMGB1 to RAGE, the receptor for advanced glycation end products, signals for a variety of cellular events that ultimately effect cell motility, metastatic pathways, activation of NF-κB, and the production of other cytokines.[1,2,5-7] The extracellular presence of HMGB1 has been connected to a variety of disease states, such as inflammation, sepsis and arthritis.[8-10] Both RAGE and HMGB1 are overexpressed in a variety of tumors, including colorectal, breast, prostate, and gastrointestinal cancers, and are suggested to be important in cancer progression.[6,7,11] Furthermore, inhibition of RAGE/HMGB1 signaling cascades slows tumor

growth and metastasis in human tumor xenograft bearing mice.[6] As a cytokine, HMGB1 appears to be a mediator of inflammation, sepsis, and stem cell recruitment.[10,12,13]

HMGB1 has been connected to the biological activity of cisplatin in a number of studies.[14,15] Formation of cisplatin 1,2-d(GpG)-intrastrand cross-links distorts the DNA duplex, giving rise to a wide and shallow minor groove that serves as a recognition site for HMG domain binding.[16] Interaction of HMGB1 with cisplatin-damaged DNA prevents nucleotide excision repair (NER) of the platinum adducts *in vitro* and therefore is proposed to drive apoptosis *in vivo*.[17] NER of DNA damaged by the clinically inactive isomer *trans*-DDP is not affected by the presence of HMGB1.[17] In support of such a repair-shielding hypothesis, steroid hormone-induced overexpression of HMGB1 sensitizes cells by a factor of two to cisplatin, but not to *trans*-DDP treatment.[18] Furthermore, silencing of HMGB1 expression by RNA interference in HeLa human cervical cancer cells decreases cisplatin sensitivity by 2.8-fold.[19] On the other hand, overexpression of HMGB1 has been linked to cisplatin-resistance and inhibition of apoptosis in mammalian cells, suggesting dual roles or cell-type specific action of the protein.[20-22]

The ability of HMGB1 to act as a cytokine leads us to propose a mechanism by which the extracellular protein might mediate the cisplatin sensitivity of solid tumors. As shown in Figure 6.1, the vasculature of malignant growths cannot support the entire tumor and, as a result, the core is starved of nutrients and oxygen and dies by a necrotic pathway. The necrotic core of the tumor passively releases HMGB1, which could enter surrounding cells and enhance the cytotoxicity of cisplatin, either by preventing NER of platinum-DNA adducts or otherwise interfering with tumor cell biology. HMGB1 has been demonstrated to be a non-viral gene delivery agent, suggesting that the protein can indeed permeate cells.[23] To evaluate our hypothesis, we investigated in the present study the consequences of exogenous HMGB1 on

HeLa human cervical and MCF-7 human breast cancer cells by flow cytometry and western blot analysis. In addition, the ability of extracellular HMGB1 to sensitize cells to cisplatin and the clinically ineffective isomer *trans*-DDP was examined.



Experimental

Materials. Recombinant rat HMGB1 was expressed and purified from *Escherichia coli*.[24] The expression of HMGB1 was silenced in HeLa cells by constitutively expressing short hairpin RNA from a plasmid vector.[19] MCF-7 and HeLa cells were purchased from ATCC. Cisplatin and *trans*-DDP were prepared according to literature procedures from potassium tetrachloroplatinate(II), a gift from Engelhard.[25,26]

Cell Culture. MCF-7 and HeLa cells were grown in DMEM (GIBCO/BRL) containing 10% FBS (GIBCO/BRL), 4 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. HMGB1-silenced HeLa cells were grown in DMEM (GIBCO/BRL) supplemented with 10% FBS, 100 μg/mL zeocin (Invitrogen), 4 mM glutamine, 1 mM sodium pyruvate, and 100 units/mL penicillin, and 100 μg/mL streptomycin. All cells were incubated at 37 C under a 5% CO_2 atmosphere.

Monitoring Expression Levels of HMGB1 by Immunofluorescence Microscopy. HeLa cells were grown to 70% confluence on 12-mm glass cover slips in 24-well plates. The cells were then treated with 2-4 nM HMGB1 and incubated for 0 to 48 h. The cells were then permeabilized with 25% acetic acid in methanol for 10 min at RT, washed with PBS, and incubated with a 1:100

dilution of anti-HMGB1 polyclonal antibody (PharMingen) for 1 h at 37 C. The cells were subsequently treated with a 1:100 dilution of goat anti-rabbit IgG conjugated to FITC (Biosource International) for 1 h at 37 C. The coverslips were then placed on microscope slides, fixed with glycerol containing 0.1% *p*-phenylenediamine, and incubated at 4 C for 12 h. HMGB1 levels were then visualized under a fluorescent light Nikon Eclipse TS100 microscope using a FITC-HYQ filter cube (excitation 460-500, bandpass 510-560 nm). Images were captured using an RT Diagnostics camera operated with Spot Advanced software.

Fluorescent Microscopy of Cells Treated with FITC-conjugated HMGB1. Recombinant HMGB1 was fluorescently labeled using FluoReporter FITC protein labeling kit (Molecular Probes). Briefly, 200 μL of 2.5 mg/mL HMGB1 were mixed with 20 μL of 1 M sodium bicarbonate buffer (pH 9). Reactive dye solution in DMSO (37 μL) was added to the stirring protein solution and the reaction was allowed to stir for an additional 1 h at RT in the dark. The FITC-conjugated protein was purified by extensive dialysis and analyzed by UV-visible spectroscopy.

HeLa cells were grown to 70% confluence on 12-mm glass cover slips in 24-well plates. The cells were treated with 2 nM to 1 μM portions of HMGB1 and incubated for 4-48 h at 37 C. The cells were then washed with PBS and H_2O, mounted on microscope slides using glycerol containing 0.1% *p*-phenylenediamine, and stored at 4 C for 12 h. Cells were imaged as described above.

Uptake of Extracellular HMGB1 by Flow Cytometry. MCF-7 and HeLa cells were incubated with 100 nM FITC-conjugated HMGB1 at 37 C for 1-48 h, harvested by trypsinization, and

washed copiously with PBS. For each sample, 10,000 cells were analyzed by fluorescence-activated cell sorting (FACScan, Beckton Dickinson) and the resultant data were manipulated by using CELL QUEST. Non-treated cells were gated as the control, with approximately 1% of the cells exhibiting native fluorescence.

Uptake of Extracellular HMGB1 by Western Blot Analysis.

Preparation of Nuclear and Cytosolic Extracts. HMGB1-silenced HeLa cells were grown to 70% confluence in 10-cm dishes and then for an additional 48 h in the presence or absence of 10-500 nM extracellular HMGB1. The cells were harvested, collected by centrifugation for 5 min at 450 x g, and washed twice with PBS. The cells were resuspended in 100 μL of lysis buffer (10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, and 1 mM PMSF) and incubated on ice for 15 min. The cell suspension was centrifuged for 5 min at 420 x g. The supernatant was discarded and the cell pellet was resuspended in 20 μL of lysis buffer. The cell pellet was subsequently disrupted with 10 strokes from a narrow-gauge needle and centrifuged at 10,000 x g for 20 min at 4 C. The supernatant (cytosolic extract) was transferred to a new tube, snap frozen, and stored at –80 C until use. The resultant crude nuclear pellet was resuspended in 10 μL of extract buffer (25% glycerol, 0.2 mM EDTA, 420 mM NaCl, 1.5 mM $MgCl_2$, 20 mM Hepes pH 7.9, 1 mM DTT, and 1 mM PMSF) and disrupted as described above. The suspension was shaken at 4 C for 30 min, centrifuged at 20,000 x g for 5 min, and the supernatant (nuclear extract) was transferred to a fresh tube, snap-frozen, and stored at –80 C until use.

Western Blot Protocol. For examination of HMGB1 levels, cytosolic and nuclear extracts were thawed and allowed to come to 4 C before analysis. For analysis of RAGE expression, whole cell pellets were lysed in SDS-PAGE sample buffer (60 mM Tris·HCl pH 6.8, 10% glycerol, 2%

SDS, 0.025% bromophenol blue, and 0.7 M 2-mercaptoethanol) at 95 C for 15 min. Proteins were separated by SDS-PAGE for 90 min at 150 V on a BioRad 4-20% Tris·HCl Ready-Gel. The separated proteins were then transferred to a PVDF membrane at 100 V for 60 min, blocked with TTBS + 5% milk for 90 min, and probed with a 1:2,000 dilution of anti-HMGB1 polyclonal antibody (Upstate), a 1:20,000 dilution of anti-actin polyclonal antibody (Upstate), or a 1:1000 dilution of anti-RAGE polyclonal antibody (Chemicon) O/N at 4 C. The PVDF membrane was washed with TTBS + 5% milk and incubated with a 1:3,000 dilution of anti-rabbit-HRP (HMGB1 and RAGE, Amersham Biosciences) or anti-mouse-HRP (actin, Bio-Rad) secondary antibody for 1 h at RT. The PVDF membrane was then incubated with ECL western blotting detection solution (Amersham Biosciences) for 90 sec and blotted dry. Proteins were then detected by exposing the PVDF membrane to BioMax MR autoradiography film (Eastman Kodak Co.).

Cytotoxicity Assays. Cell survival was analyzed by the MTT assay.[27] Cells were seeded into 96-well plates at a density of 250-500 cells per well. After 24 h, cells were treated with 2 nM-1 μM of HMGB1 or were left untreated and further incubated at 37 C for 48 h. Cells were then treated with either 0-20 μM cisplatin or 0-500 μM *trans*-DDP. After incubation for 96 h, 20 μL of a 5 mg/mL MTT (Sigma) solution in PBS was added to each well and incubated at 37 C for 5 h. The media was then removed from each well and the cells redissolved in 100-200 μL of DMSO. The absorbance of each well was measured at 550 nm by a plate reader (SpectraMax 340pc, Molecular Devices). Percent cell survival was calculated for each concentration by the ratio of the measured absorbance to the absorbance of the untreated cells.

Effect of MAPK Inhibitors on the Cytotoxicity of Cisplatin and *trans*-DDP. Cytotoxicity assays were setup as described in the previous section. After incubation of MCF-7 or HeLa cells with 10 nM or 1 μM of HMGB1 for 44 h, the cells were further treated with either 1μM of SKF-86002 (Calbiochem), 40 nM of JNK Inhibitor II (Calbiochem), or 2.5 μM of ERK Activation Inhibitor Peptide I (Calbiochem) for 4 h at 37 C before treatment with cisplatin or *trans*-DDP. Cell survival was determined by the MTT assay.

Effect of Necrotic Media on the Cytotoxicity of Cisplatin and *trans*-DDP. MCF-7 or HeLa cells (75 cm^2 flask, ~10^7 cells) were frozen at –80 C and then rapidly thawed at 37 C. This cycle was repeated 4 to 6 times, after which the cells were incubated at 37 C for an additional 1 h. The media bathing the necrotic cells was isolated by centrifugation of the cellular suspension at 1,500 rpm for 10 min at 4 C and filtering the supernatant through a 0.2 μm Millipore filter. The media was used immediately for cytotoxicity assays. Cells were seeded into 96-well plates at a density of 250–500 cells per well in 100 μL regular or necrotic media. After 48 h, cells were treated with 0-20 μM cisplatin and allowed to incubate for an additional 96 h. Cell viability was assayed by the MTT assay.

Results and Discussion

The chromasomal protein HMGB1 is actively secreted as a pro-inflammatory cytokine from macrophages and monocytes and passively released from necrotic cells.[3,4] We have examined the ability of extracellular HMGB1 to be taken up in cultured cells and to mediate the cytotoxicity of cisplatin and *trans*-DDP. The effect of extracellular HMGB1 on the cytotoxicity of platinum compounds depends on the cell line and the mechanism by which HMGB1 alters the cytotoxicity of cisplatin and *trans*-DDP is platinum-compound specific.

Cellular Uptake of Exogenous HMGB1

The levels of exogenously administered HMGB1 were examined in HeLa cells by immunofluorescence microscopy. HeLa cells were incubated with 2-4 nM HMGB1 for 0.5 h-48 h. As shown in Figure 6.2, the fluorescence signal from the antibody-labeled HMGB1 increases after 41.5 h and maximizes at 48 h. Because of the high background associated with immunofluorescence techniques, HMGB1 was labeled with a fluorescent tag to facilitate the imaging of its uptake. As shown in Figure 6.3A, micromolar concentrations of the FITC-labeled HMGB1 were required for imaging. Time-course experiments showed that intracellular HMGB1 levels begin to increase after as little as 4 h. Figure 6.3B illustrates that uptake of FITC-HMGB1 increases with longer incubation times and appears to maximize at ~50 h. The FITC-labeled HMGB1 appears to accumulate in both the nucleus and the cytoplasm. Wheras the immunofluorescence and fluorescent microscopic data provide evidence for HMGB1 uptake, the kinetics seemed slow and assignment of the cellular localization of the labeled protein was tenuous, at best. Therefore, the HMGB1 uptake was further investigated by flow cytometry and western blot analysis.

The kinetics of HMGB1 uptake in MCF-7 and HeLa cells were analyzed by flow cytometry. In HeLa cells, HMGB1 internalization maximizes after 8-10 h incubation with the FITC-labeled protein. More than 80% of HeLa cells incorporate FITC-labeled HMGB1 after 8 h, and the levels of HMGB1 remain constant for 48 h. As shown in Figure 6.4, the kinetics of HMGB1 uptake are considerably slower in MCF-7 cells. Maximum uptake of FITC-HMGB1 occurs after incubation for 18 h at 37 C. In addition, the number of MCF-7 cells that take up FITC-HMGB1 is much lower than for HeLa cells. After an 18 h incubation period with FITC-

HMGB1, only 40% of the MCF-7 cells internalize the extracellular protein. The level of HMGB1 uptake remains constant after incubation for 48 h.

Uptake of extracellular HMGB1 was also confirmed by western blot analysis. HeLa-HMGB1 silenced cells were incubated with either 0 nM, 20 nM, 100 nM or 500 nM of HMGB1 for 48 h. Accumulation of HMGB1 was readily observed in whole cell extracts of HMGB1-silenced HeLa cells (see Figure 6.5A). The distribution of HMGB1 was further examined by preparation of nuclear and cytosolic extracts from HMGB1-silenced HeLa cells treated with 500 nM of HMGB1 (see Figure 6.5B). The western blot of cell extracts from HMGB1-silenced HeLa cells show the presence of protein in both the cytoplasm and the nucleus of the protein-treated cells.

The utility of HMGB1 as a non-viral gene delivery agent was previously explored.[23,28] It is therefore not unexpected that HMGB1 would be cell permeable. DNA packaged with HMGB1 was efficiently transfected into several mammalian cell types and was observed to accumulate in the nucleus of the transfected cells. In mouse Ltk^- cells, approximately 40% of transfected cells accumulated the HMGB1/DNA complex in the nucleus, which is similar to the degree of uptake observed in MCF-7 cells in the present study.[28] The variable degree of HMGB1 uptake demonstrates a potentially critical difference between MCF-7 and HeLa cells. Endocytosis has been proposed as the mode of HMGB1 uptake; however, both the mechanism of HMGB1 facilitated transfection and uptake have yet to be elucidated.[23,28]

HMGB1 is known to bind RAGE, but it is not clear whether RAGE mediates the uptake of the extracellular protein or is only involved in mediating signal transduction pathways.[29,30] The expression of RAGE was examined and compared to actin in MCF-7 and HeLa cells (see Figure 6.5C). RAGE is present primarily as a transmembrane receptor, but also occurs as several

truncated soluble forms.[11] These species account for the presence of the three bands observed by western blot analysis. The expression levels of RAGE in MCF-7 and HeLa cells do not parallel the uptake of HMGB1 in the two lines. Although MCF-7 and HeLa cells express equivalent levels of RAGE, internalization of HMGB1 is twice as efficient in HeLa cells. Therefore, it seems unlikely that RAGE is responsible for mediating the uptake of HMGB1. Other candidates for receptor-mediated HMGB1 uptake are the Toll-like receptors. HMGB1 has recently been established as a ligand for TLR2 and TLR4; however, to our knowledge, neither receptor has been connected to uptake of extracellular molecules.[31] As with RAGE, TLR2 and TLR4 facilitate signal transduction pathways induced by extracellular cytokines.[31]

Effect of Extracellular HMGB1 on the Cytotoxicity of Cisplatin and *trans*-DDP

The ability of exogenous HMGB1 to be taken up by HeLa and MCF-7 cells suggested that its presence in the extracellular medium may alter their sensitivity to cisplatin. In HeLa cells, control experiments with HMGB1 showed no protein-induced toxicity at concentrations up to 1 μM. Cotreatment of HeLa cells with 10 nM–1 μM HMGB1 and cisplatin did not effect the cytotoxicity of the latter over the 0-10 μM concentration range. Pretreatment of HeLa cells for 48 h with 1 μM HMGB1, however, led to increased sensitivity toward the drug. As shown in Figure 6.6A, 48 h pretreatment of HeLa cells with 1 μM HMGB1 enhances cisplatin activity by 1.8-fold at the IC_{50} value and by more than 5-fold at IC_{70}.

Modulation of cisplatin cytotoxicity by extracellular HMGB1 was also studied in MCF-7 cells. Control experiments with HMGB1 revealed that the protein is not cytotoxic in these cells at low concentrations (less than 10 nM); however, treatment with 100 nM or 1 μM HMGB1 reduces cell viability by 30%. Pretreatment with 2 nM HMGB1 does not effect cisplatin

cytotoxicity, whereas incubation with 5–10 nM HMGB1 for 48 h sensitizes MCF-7 cells towards cisplatin exposure (Figure 6.7A). As summarized in Table 6.1, a 3.0-fold enhancement in cisplatin cytotoxicity at IC_{50} was achieved upon pretreatment with 10 nM HMGB1.

The mechanism of HMGB1-induced sensitization of cells to cisplatin is cell-line dependent. One possibility is that the exogenous HMGB1 in the nucleus recognizes and binds to the cisplatin-DNA 1,2-intrastrand cross-links, shielding them from NER. Evidence for the repair-shielding hypothesis has been previously demonstrated in both cell lines. HeLa cells with HMGB1 expression silenced by RNAi are nearly 3-fold resistant to cisplatin compared to wild-type cells.[19] In MCF-7 cells, steroid hormone-induced overexpression of HMGB1 enhances the cytotoxicity of cisplatin treatment by two-fold.[18] Although HeLa cells accumulate more HMGB1 than MCF-7 cells, it seems that the protein is not as available for DNA binding in the former cells. A large amount of protein (1 μM) is required to sensitize HeLa cells to cisplatin, whereas as little as 5 nM exogenously administered HMGB1 significantly enhances cisplatin cytotoxicity in MCF-7 cells. It is possible that, in HeLa cells, HMGB1 must be post-translationally modified in order to interact with cisplatin-modified DNA. In fact, acetylated-HMGB1 binds cisplatin-damaged DNA with greater affinity than the unmodified protein.[32]

Co-treatment of MCF-7 or HeLa cells with HMGB1 and cisplatin does not sensitize them to the drug. This observation suggests that uptake of HMGB1 is a prerequisite for achieving enhanced cisplatin cytotoxicity. Previous studies demonstrated the importance of the kinetic factors for HMGB1 upregulation as a determinant of cisplatin-induced cytotoxicity.[18] In order for repair-shielding to occur, the levels of HMGB1 must be sufficiently elevated at the time of platinum-damage formation so that HMGB1 binding can occur prior to repair of the platinum-damaged DNA. Therefore the need to pre-treat the MCF-7 and HeLa cells with HMGB1 in order

to achieve maximally enhanced cisplatin cytotoxicity can be explained by the relatively slow (8-18h) uptake of the protein. Thus repair shielding is a viable model to explain these results.

In order to evaluate further the repair-shielding hypothesis, the effect of extracellular HMGB1 on the cytotoxicity of *trans*-DDP was determined. Unlike cisplatin, *trans*-DDP is unable to form 1,2-(GpG)-intrastrand DNA cross-links. Therefore, *trans*-DDP DNA adducts are not recognized by HMGB1.[24] In the present study, we discovered that HMGB1 sensitizes HeLa but not MCF-7 cells to *trans*-DDP. Pretreatment with 1 μM HMGB1 enhanced the cytotoxicity of *trans*-DDP by nearly an order of magnitude (Figure 6.6B and Table 6.1). Even co-treatment with 1 μM HMGB1 and *trans*-DDP sensitized HeLa cells by 4-fold.

The observation that the cytotoxicity of *trans*-DDP is not modulated by exogenous HMGB1 in MCF-7 cells supports the hypothesis that addition of the protein to the extracellular media affects the repair of cisplatin-DNA adducts by the shielding mechanism described above. Conversely, the results for HMGB1 and *trans*-DDP treatment of HeLa cells suggest that a different mechanism may be involved.[a] Repair-shielding of a *trans*-DDP DNA adduct seems unlikely especially since the degree of HMGB1-induced sensitization is significantly greater than for cisplatin in HeLa cells. Furthermore, as mentioned above no evidence exists to support a interaction between HMGB1 and *trans*-DDP damaged DNA. Since co-treatment with 1 μM HMGB1 in HeLa cells affects *trans*-DDP, but not cisplatin, cytotoxicity, we conclude that HMGB1 sensitizes HeLa cells to the two diamminedichloroplatinum(II) isomers platinum compounds by different mechanisms.

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[a] Because of the need for high levels of HMGB1, repair-shielding of DNA damaged by *trans*-DDP may occur in HeLa cells. HMGB1 binds with modest affinity to cisplatin interstrand cross-links and to interstrand cross-links on DNA damaged by a polynuclear *trans*-platinum complex.[33,34] Moreover, HMGB1 is thought to modulate the repair of psoralen interstrand cross-links through formation of a ternary complex with replication protein A (RPA).[35]

Effect of MAPK Inhibitors on HMGB1-induced Enhancement of *trans*-DDP Cytotoxicity

From the foregoing results we wondered whether HMGB1 might sensitizes cells to *trans*-DDP by functioning as a cytokine. HMGB1 binding to the RAGE receptor induces several signal transduction pathways including translocation of NF-κB to the nucleus and phosphorylation of the MAP kinases ERK, JNK, and p38.[1,29] Activation of NF-κB is typically associated with the prevention of apoptosis, but a number of genes that it regulates are pro-apoptotic.[36] The balance of pro-survival and pro-apoptotic signals induced by the HMGB1/RAGE interaction may be modified by platinum treatment. The ability of HMGB1 treatment alone to induce cell death in MCF-7 cells suggests that the protein may induce pro-apoptotic signal transduction pathways. In support of this supposition, exposure to HMGB1 induces apoptosis in macrophages via a proposed JNK pathway.[37]

We therefore investigated the ability of p38, JNK, and ERK inhibitors to modulate the ability of exogenous HMGB1 to render *trans*-DDP more cytotoxic toward HeLa cells. The ERK inhibitor was inherently cytotoxic and treatment with the p38 inhibitor did not effect HMGB1-induced sensitization to *trans*-DDP. As illustrated in Figure 6.8, however, the JNK inhibitor dramatically reversed the sensitization of HeLa cells to *trans*-DDP following HMGB1 pretreatment. Conversely, pretreatment of the cells with the JNK inhibitor had no effect on the ability of HMGB1 to sensitize HeLa cells to cisplatin (data not shown).

Several studies have previously connected JNK activation to *trans*-DDP cytotoxicity.[38-40] Treatment of mouse keratinocytes with *trans*-DDP induces transient phosphorylation of JNK, a phenomenon typically associated with a pro-survival cellular response.[39,40] Prolonging JNK activation by treatment with sodium orthovanadate, a tyrosine phosphatase inhibitor, increases mouse keratinocyte sensitivity to *trans*-DDP.[39] Perhaps in HeLa cells, exogenous HMGB1

prolongs JNK activation and thereby sensitizes cells to *trans*-DDP in an analogous fashion. The fact that treatment with a JNK inhibitor reduces the sensitivity of HMGB1-treated HeLa cells to *trans*-DDP is consistent with this proposal.

Effect of Necrotic Media on the Cytotoxicity of Cisplatin

Induction of Necrosis. As discussed earlier, HMGB1 is passively released from necrotic cells. It was therefore of interest to examine the effect of exposing cells to media harvested from necrotic cell cultures. Necrosis can be induced by subjecting cells to 4-6 rapid freeze/thaw (-80 C/37 C) cycles followed by incubation at 37 C for 1 h.[41] In a typical experiment, ~10^7 cells grown in 12 mL of media were made necrotic and the surrounding media were harvested and used for cytotoxicity assays. Assuming that the majority of the 10^7 cells undergo necrosis and release their entire supply of HMGB1 (10^6 molecules/cell), the media will contain approximately $8x10^{11}$ HMGB1 molecules per mL of media, or 1.4 nM.

Effect of Media from Necrotic Cell Cultures on Cisplatin Sensitivity. The computed concentration of HMGB1 in the necrotic media is considerably lower than the amount of the exogenous protein required to sensitize HeLa and MCF-7 cells to cisplatin. However, as shown in Table 6.2, cells grown in media bathing necrotic cell cultures were more sensitive to cisplatin treatment. HeLa cells were sensitized by a factor of two to cisplatin, and the cytotoxicity of cisplatin toward MCF-7 cells was enhanced 3-fold.

Mechanism of Cisplatin Sensitization. It remains to be proved whether HMGB1 alone is responsible for the cellular sensitization to cisplatin following exposure to necrotic cell media, but preliminary observations support this premise. The cytotoxicity of *trans*-DDP toward MCF-7 cells remained unchanged when they were grown in necrotic cell media. Necrotic media was also

prepared from HMGB1-silenced HeLa cells, which should contain little or no released HMGB1. HeLa cells grown in such media were actually resistant to cisplatin (IC_{50} = 5.2±0.2 μM) compared to cells grown in regular media (IC_{50} = 2.6±0.3 μM). This result supports the hypothesis that it is the HMGB1 released from necrotic cells that sensitizes HeLa cells to cisplatin, but it also underscores the complexity of the components released from necrotic cells. As mentioned earlier, the maximum concentration of HMGB1 released into 12 mL of media would be 1.4 nM, which is significantly lower than the concentration of recombinant protein used to sensitize MCF-7 and HMGB1 cells to cisplatin. This result might be a consequence of other released necrotic factors that sensitize cells to cisplatin. Alternatively, HMGB1 released from necrotic cells may differ from the recombinant protein added extracellularly. HMGB1 actively secreted from monocytes is hyperacetylated in order to direct the protein for export, but HMGB1 passively released from necrotic cells is not.[4] However, HMGB1 exists in several acetylated forms in vivo.[3] Therefore, it is possible that such post-translational modifications of HMGB1 enhance uptake and activity of the protein.

Conclusions and Future Directions

The ability of extracellular HMGB1 to mediate the cytotoxicity of platinum compounds was evaluated in MCF-7 human breast cells and HeLa human cervical cancer cells. Externally added HMGB1 permeates the cell membrane and localizes in both the nucleus and cytoplasm of HeLa cells. Extracellular HMGB1 sensitizes cells to platinum compounds in a cell line-dependent fashion. In HeLa cells, 1 μM of HMGB1 is required to enhance the cytotoxicity of cisplatin. Pretreatment with HMGB1 also sensitizes HeLa cells to *trans*-DDP through a JNK-dependent mechanism. Pretreatment with 10 nM HMGB1 sensitizes MCF-7 cells to cisplatin, but not to

trans-DDP. These observations support the hypothesis that HMGB1 mediates the cytotoxicity of cisplatin by a repair-shielding mechanism. The ability of HMGB1 to sensitize HeLa cells to *trans*-DDP is proposed to occur via a signal transduction mechanism involving JNK.

Media from necrotic cell cultures sensitizes MCF-7 and HeLa cells to cisplatin treatment, presumably through the release of HMGB1. In support of this hypothesis, media that bathed necrotic HMGB1-silenced HeLa cells actually rendered HeLa cells less sensitize to cisplatin. These observations raise many interesting questions concerning the mechanism of cisplatin cytotoxicity enhancement by necrotic media. Future work could involve immunoprecipitation of HMGB1 from necrotic media or examination of the role of HMGB2. These experiments also suggest that post-translational modifications of HMGB1 may be important in achieving cisplatin sensitization. Future experiments should use native HMGB1 instead of recombinant protein in order to evaluate this possibility. In addition, HMGB1 released from necrotic cells could be characterized by mass spectrometry.

The question of whether HMGB1 sensitizes cells to platinum treatment by a repair-shielding or signal transduction pathway could be explored by using truncates of the protein. Previous work showed that the cytokine function of HMGB1 was localized to the B box, and more specifically required amino acids 89-108.[42] Different protein truncates should be screened and evaluated for their ability to bind platinum-damaged DNA and to sensitize cells to platinum treatment.

More evidence for HMGB1 sensitizing cells to cisplatin treatment via a repair-shielding mechanism could be obtained by examination of Pt-DNA adduct levels. Numerous attempts to acquire such data through quantifying platinum levels by Pt AAS have failed; however, it may be

possible to use monoclonal antibodies raised against cisplatin-modified DNA to examine repair of platinum damage.

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Table 6.1. IC_{50} values (μM) for cisplatin and *trans*-DDP in HMGB1-pretreated HeLa and MCF-7 cells.

HMGB1 Concentration	No Protein	10 nM	100 nM	1 μM
HeLa cells				
cisplatin	2.6 ± 0.3	2.0 ± 0.6	2.1 ± 0.3	1.4 ± 0.4
trans-DDP	90 ± 13	80 ± 10	50 ± 8	10 ± 2
HMGB1 Concentration	No Protein	2 nM	5 nM	10 nM
MCF-7 cells				
cisplatin	5.8 ± 0.8	6.5 ± 0.1	3.4 ± 0.2	1.8 ± 0.2
trans-DDP	55 ± 15	78 ± 6	68 ± 10	67 ± 10

Table 6.2. Effect of necrotic media on the cytotoxicity of cisplatin in MCF-7 and HeLa cells as monitored by IC_{50} (μM) values.

Media Type	Regular	Necrotic
HeLa cells		
cisplatin	2.6±0.3	1.2±0.2
MCF-7 cells		
cisplatin	5.8±0.8	1.8±0.3

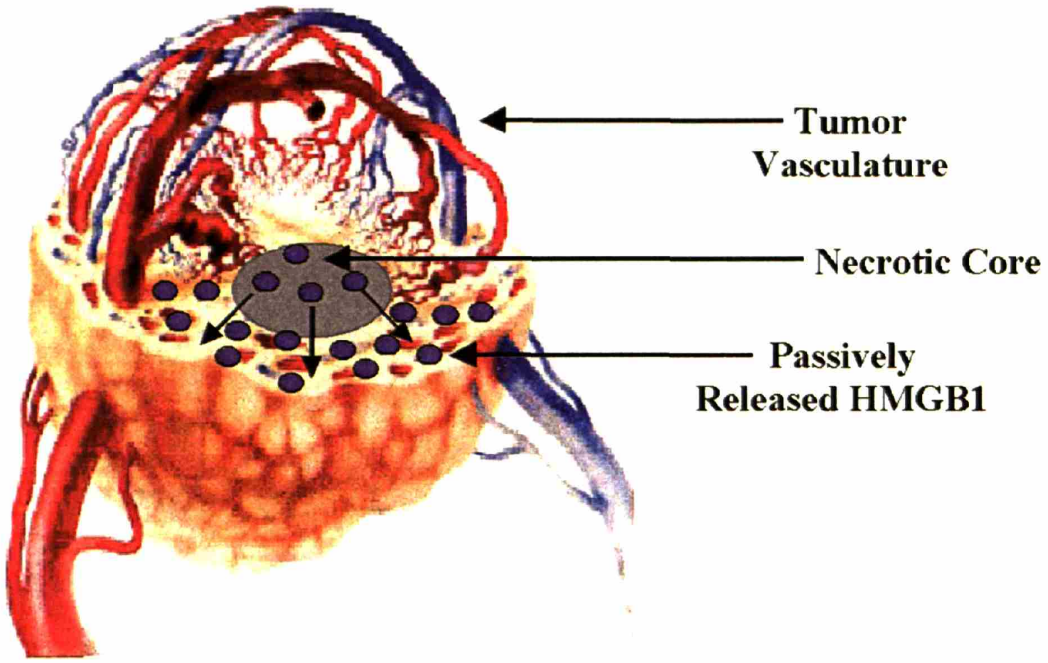

Figure 6.1. Schematic representation of a solid tumor with a necrotic core. HMGB1 is passively released from necrotic cells and interacts with surrounding viable tumor tissue. Figure modified from www.lillie.inserm.fr/mordon/tumor01.jpg.

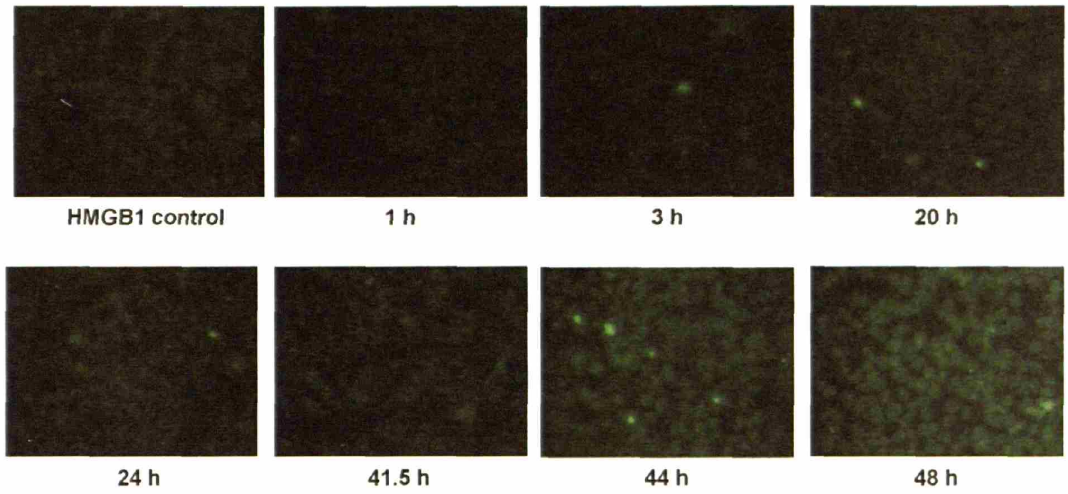

Figure 6.2. Cellular levels of HMGB1 as detected by immunofluorescence (40X magnification). HeLa cells were treated with 2 nM HMGB1 and incubated at 37 C for 0–48 h. Fixed cells were treated with an anti-HMGB1 primary antibody followed by treatment with a FITC-conjugated secondary antibody to allow for HMGB1 visualization.

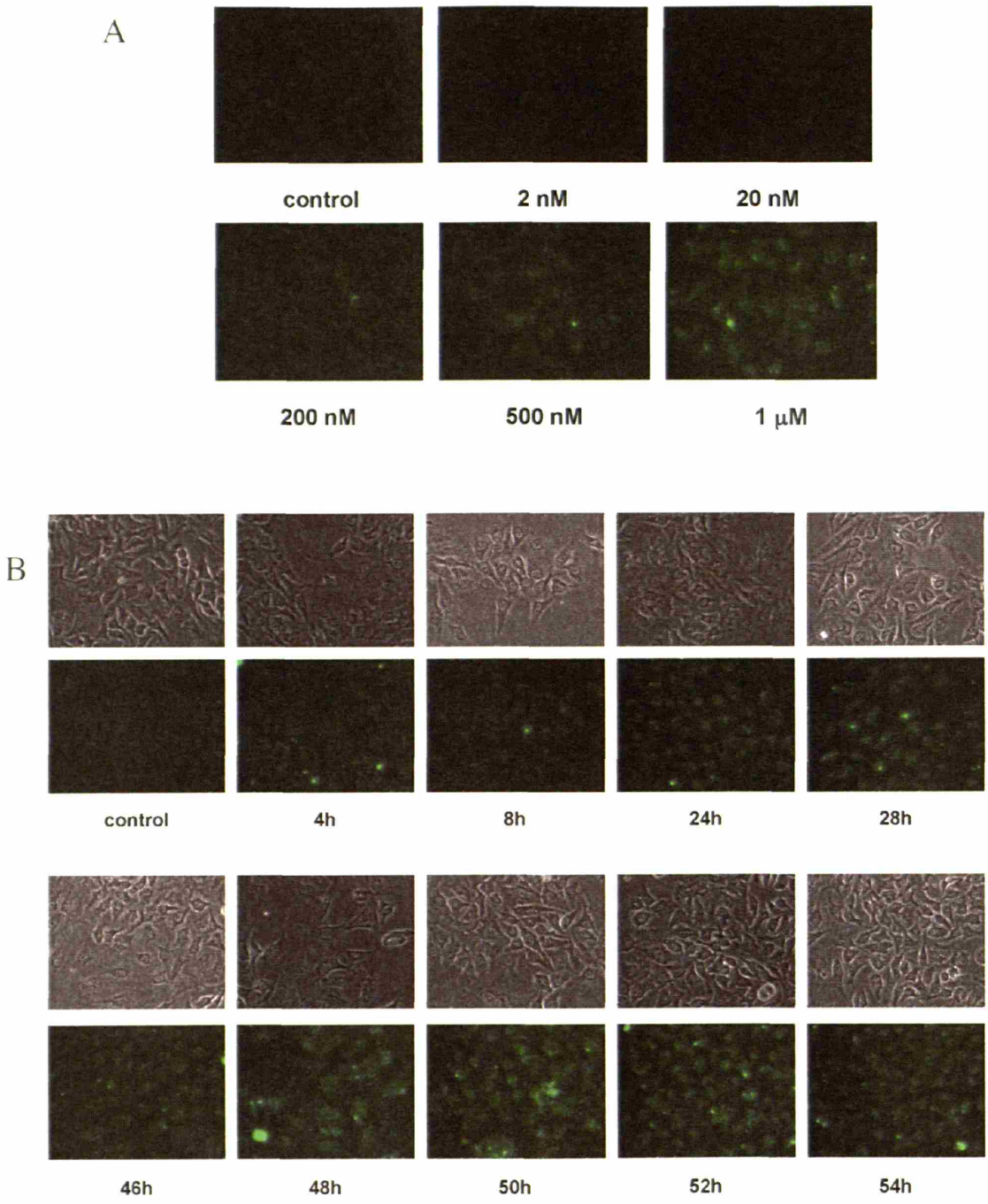

Figure 6.3. Fluorescence microscopy images of cells incubated with FITC-HMGB1 (40X magnification). (A) Determination of minimal FITC-HMGB1 concentration required for HMGB1 detection. HeLa cells were treated with 0-1 μM HMGB1 for 48 h at 37 C. (B) Kinetics of FITC-HMGB1 uptake in HeLa cells. Cell were treated with 500 nM HMGB1 and incubated at 37 C for 0–54 h. Both phase contrast and fluorescent images are shown.

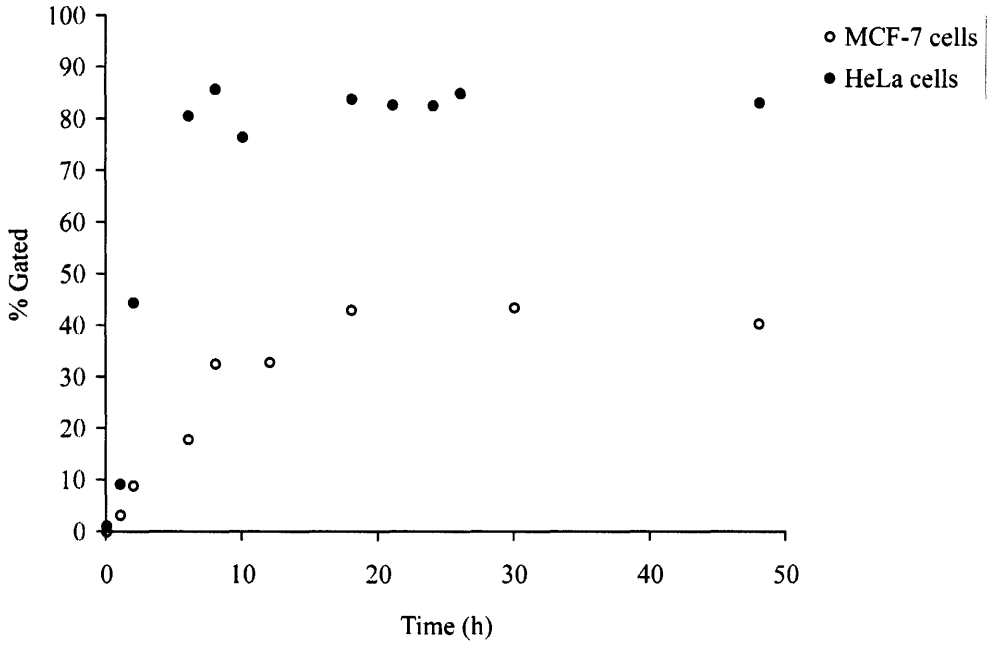

Figure 6.4. Uptake of FITC-labeled HMGB1 as monitored by flow cytometry. HeLa and MCF-7 cells were incubated with 100 nM FITC-HMGB1 at 37 C for 0–48 h, harvested, and analyzed by FACS.

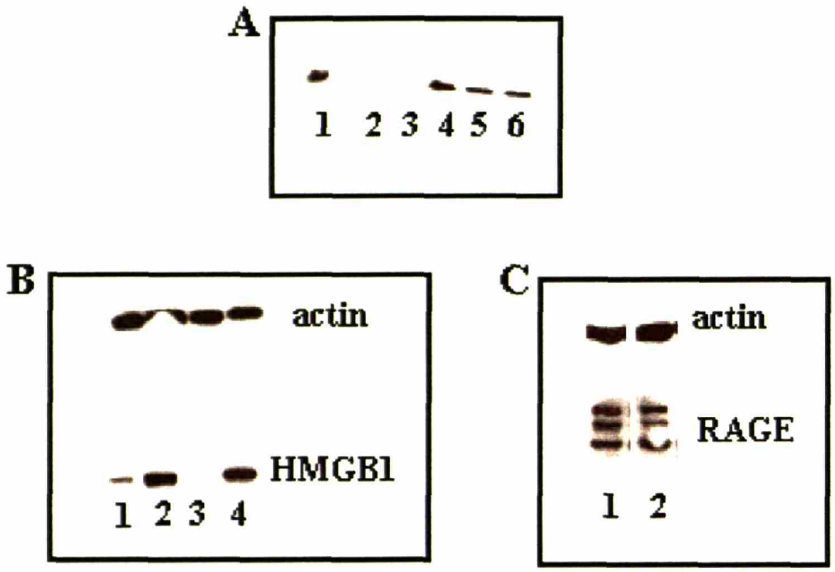

Figure 6.5. (A) Western blot analysis of HMGB1 levels in whole cell extracts of HMGB1-silenced HeLa cells treated with 0 (lane 2), 2 (lane 3), 20 (lane 4), 100 (lane 5), or 500 (lane 6) nM of exogenous recombinant HMGB1. Lane 1 is recombinant HMGB1 alone as a positive control. (B) Western blot of HMGB1 expression levels in HMGB1-silenced HeLa cells. Lanes 1 and 3 are the cytosolic and nuclear extracts of HMGB1-silenced HeLa cells, respectively. Lanes 2 and 4 are the cytosolic and nuclear extracts of HMGB1-silenced HeLa cells allowed to incubate with 500 nM HMGB1 for 48 h at 37 C, respectively. (C) Expression of RAGE in HeLa (lane 1) and MCF-7 (lane 2) cells. Actin was used as the loading control.

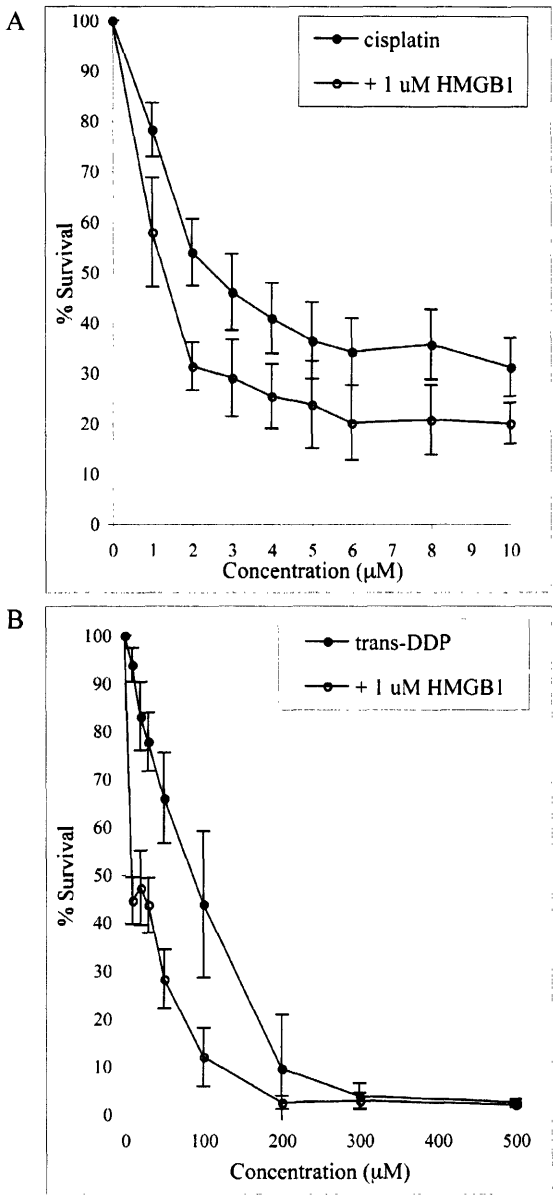

Figure 6.6. Cytotoxicity of cisplatin (A) and *trans*-DDP (B) in HeLa cells as monitored by the MTT assay. Cells were incubated with 1 μM HMGB1 for 48 h before platinum treatment. Error bars represent one standard deviation.

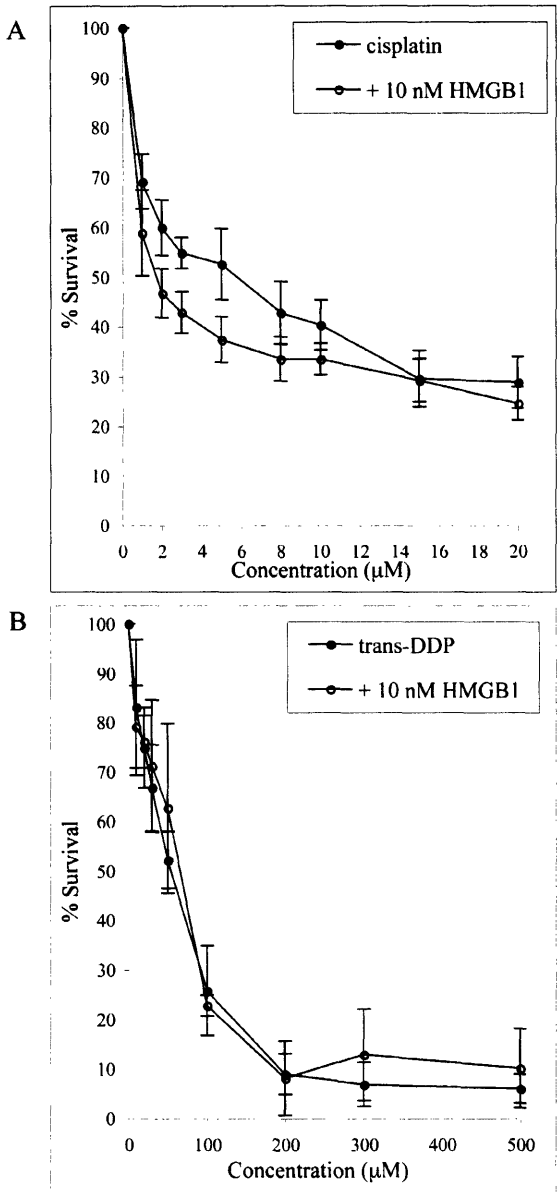

Figure 6.7. Cytotoxicity of cisplatin (A) and *trans*-DDP (B) in MCF-7 cells as monitored by the MTT assay. Cells were incubated with 10 nM HMGB1 for 48 h before platinum treatment. Error bars represent one standard deviation.

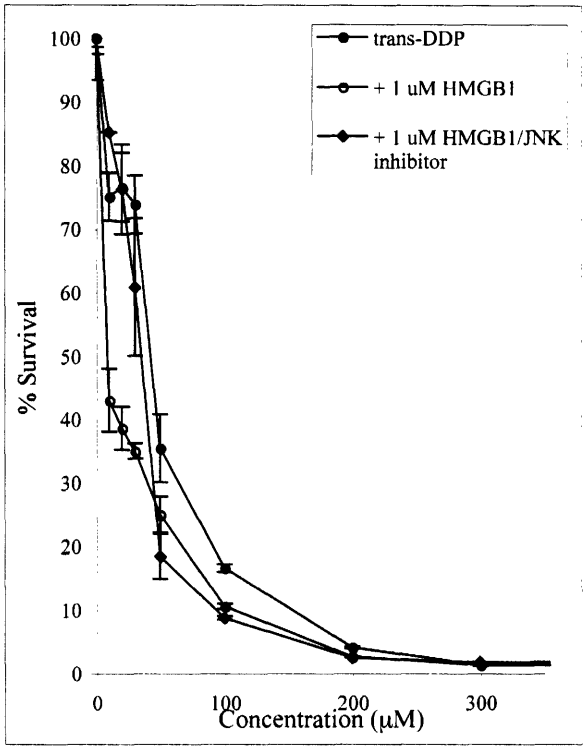

Figure 6.8. Effect of MAPK inhibitors on the cytotoxicity of *trans*-DDP in HMGB1-pretreated HeLa cells. Cells were incubated for 44 h with 1 μM HMGB1 and for 4 h with MAPK inhibitors before platinum treatment. Cell survival was determined by the MTT assay and error bars represent one standard deviation.

Appendix A
Post-Translational Modifications of Histone H4 Following Platinum Treatment

Introduction

Cells treated with cisplatin, *cis*-diamminedichloroplatinum(II), respond to the resultant DNA damage in a variety of ways, and many of which remain to be established.[1] In addition to initiating DNA-damage recognition and repair mechanisms, cisplatin treatment activates signal transduction cascades, including mitogen-activated protein kinase (MAPK) pathways.[1] In HeLa cells, exposure to cisplatin results in the phosphorylation of histone H3 at Ser-10 and Ser-28 and acetylation of H4, the former being mediated by the p38 MAPK pathway.[2] Histone modifications alter chromatin structure, which increases the accessibility of nucleosomal-packaged DNA to proteins that process the genome.[2] Elucidating cisplatin-induced post-translational modifications of histones is important for understanding how cells respond to and repair platinum-damaged DNA.

Experimental Procedures

General Considerations. The isolation of histones from harvested nuclei and their mass spectrometric analysis were carried out by Mike Boyne and Professor Neil Kelleher at the University of Illinois at Urbana-Champaign (UIUC), in the manner described previously.[3]

Cell Culture. Human MCF-7 and HCC-1937 breast cancer, NTeraII testicular carcinoma, and HeLa cervical cancer cells were purchased from the American Type Culture Collection. MCF-7 and HeLa cells were grown in DMEM (GIBCO/BRL) supplemented with 10% FBS (Hyclone), 1 mM sodium pyruvate, 100 units/mL of penicillin, and 100 μg/mL streptomycin. NTeraII and HCC-1937 cells were grown in RPMI-1640 media (ATCC) containing 10% FBS, 2 mM L-

glutamine, 1 mM sodium pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin. All cells were incubated at 37 C under a 5% CO_2 atmosphere.

Treatment of Cells. Cells were grown to 90% confluence in 175 cm^2 flasks (~$2x10^7$ cells) and treated with either 100 μM sodium butyrate, 5-20 μM cisplatin, 100 μM *trans*-DDP, or 10 μM *cis*-[$PtCl_2$(DACH)] for 16 h at 37 C. Cells were then washed with cold PBS, harvested, snap frozen, and stored at –80 C until the isolation of their nuclei.

Preparation of Nuclei. NIB-250 buffer was prepared with the following components: 15 mM Tris·HCl pH 7.5, 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 250 mM sucrose, 1 mM DTT, 10 mM sodium butyrate and 0.5 mM PMSF. Frozen cell pellets were thawed and allowed to reach 4 C. Cells were suspended in NIB-250 buffer supplemented with 0.3% NP-40 ($2x10^7$ cells/mL of buffer) and incubated on ice for 5 min. The nuclei were pelleted by centrifugation at 600 rcf for 5 min at 4 C and the supernatant was discarded. The nuclear pellet was resuspended in NIB-250 buffer without NP-40 ($2x10^7$ cells/mL of buffer) and centrifuged at 600 rcf at 4 C for 5 min. The supernatant was discarded and the cells were again resuspended in NIB-250 buffer ($2x10^7$ cells/mL of buffer). Samples (1 mL) were apportioned into 1.5 mL microcentrifuge tubes and centrifuged at 600 rcf at 4 C for 5 min. Finally, the supernatant was discarded and the nuclei were resuspended in an equal volume of NIB-250 supplemented with 20% glycerol, snap frozen, and stored at –80 C. Samples were sent to UIUC on dry ice.

Results and Their Significance

Nuclei were isolated from HeLa, MCF-7, NTeraII, and HMGB1-silenced HeLa cells treated with either cisplatin or sodium butyrate (see Table A.1 for treatment conditions) and post-translational modifications of histone H4 were monitored by mass spectrometry.[3] Sodium butyrate is a histone deacetylase inhibitor, so cells treated with this compound served as a positive control. As shown in Figures A.1 and A.2, cells treated with sodium butyrate contain several acetylated forms of histone H4 that are not observed in untreated cells. Conversely, cells treated with cisplatin, carboplatin, or *trans*-DDP have essentially the identical histone H4 patterns as untreated cells. In addition, the ratio of acetylated to unmodified histones is unchanged upon platinum treatment. Therefore, it appears that treatment with cisplatin does not effect the post-translational modification of histone H4. Alternatively, any differences in post-translational modifications may be below the detection limit of the current mass spectrometric analyses. The detection limit of mass spectrometric techniques is dependent upon sample preparation and instrument sensitivity. For analysis of histone modifications, the detection limit is between 50:1 and 300:1.[4]

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Table A.1. Treatment conditions for samples analyzed by mass spectrometry.

Cell Line	Treatment Conditions
HeLa	Untreated
	100 μM sodium butyrate, 16 h
	5 μM cisplatin, 16 h
	10 μM cisplatin, 16 h
	20 μM cisplatin, 16 h
	100 μM carboplatin, 48 h
	100 μM trans-DDP, 16 h
	10 μM cis-[$PtCl_2$(DACH)], 16 h
MCF-7	Untreated
	100 μM sodium butyrate, 16 h
	5 μM cisplatin, 16 h
	10 μM cisplatin, 16 h
	20 μM cisplatin, 16 h
NTeraII	Untreated
	100 μM sodium butyrate, 16 h
	5 μM cisplatin, 16 h
	10 μM cisplatin, 16 h
	20 μM cisplatin, 16 h
HeLa-HMGB1	Untreated
silenced and	100 μM sodium butyrate, 16 h
–pcDNA control	10 μM cisplatin, 16 h

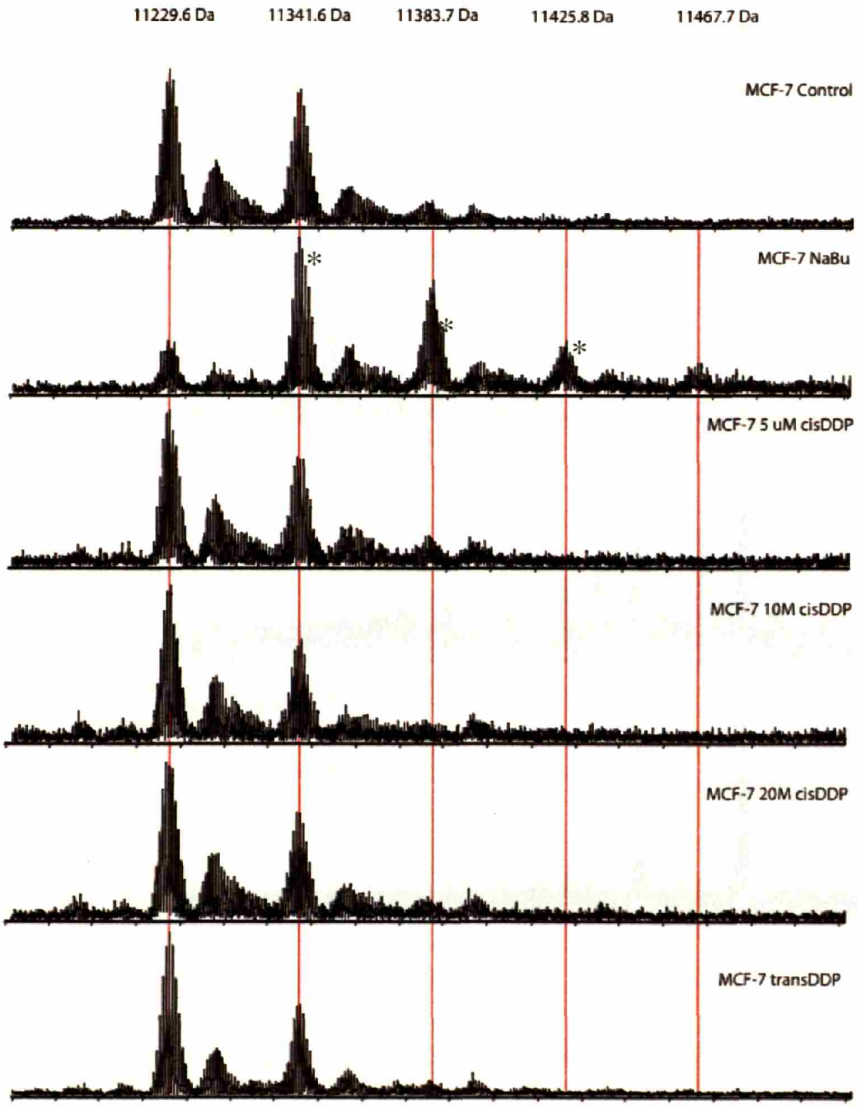

Figure A.1 Mass spectrometry of histone H4 from cisplatin-treated MCF-7 nuclei. Acetylated histone H4 isoforms of interest are indicated by an asterisk.

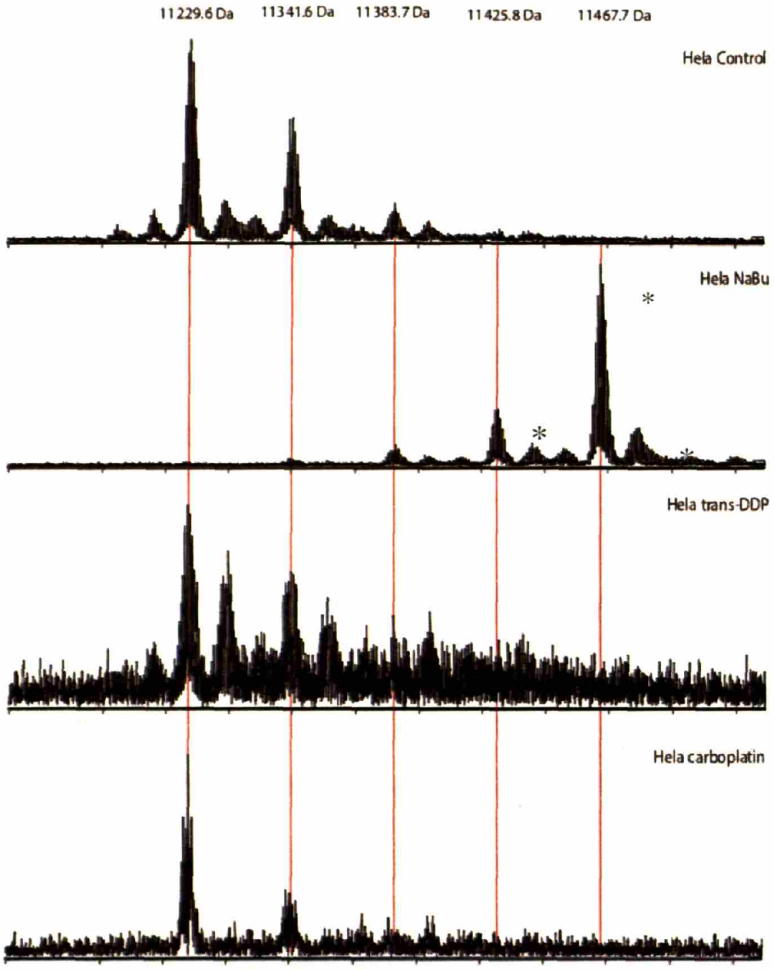

Figure A.2. Mass spectrometry of histone H4 isolated from platinum-treated HeLa cells. Acetylated histone H4 isoforms of interest are indicated by an asterisk.

Appendix B
Expression of HMGB1 in Paraffin-Embedded Seminoma Tissue from Human Cancer Patients

Introduction

Formerly defined vaguely as an architectural protein, HMGB1 has recently been shown to have an extracellular role as a cytokine.[1] The protein is actively exported from the nuclei of macrophages and monocytes and is passively leaked from necrotic cells to induce a variety of signal transduction cascades.[2-4] This newly discovered signaling role of HMGB1 inspired the hypothesis that the extracellular protein may mediate the cytotoxicity of cisplatin in solid tumors (Chapter 6). Externally added HMGB1 is taken up by HeLa and MCF-7 human cancer cells grown in culture (Chapter 6). Furthermore, pretreatment with the extracellular protein sensitizes HeLa and MCF-7 cells to cisplatin (Chapter 6).

In order to assess the potential clinical relevance of our hypothesis, the expression level of HMGB1 was evaluated in paraffin-embedded human tumor tissue. More than 90% of patients with testicular cancer are cured with cisplatin treatment. We therefore chose to examine the expression of HMGB1 in testicular seminomas. This appendix describes the histological evaluation of testicular seminoma tissue performed by our collaborators Dr. Robert Colvin and Dr. J.P. Bissonnette at Massachusetts General Hospital. In addition, determination of HMGB1 expression in paraffin-embedded tumor tissue by western blot analysis is discussed.

Experimental Procedures

Materials. Microscope slides with thin slices (~5 μM) of paraffin-embedded formalin-fixed seminoma tissue were provided by the Pathology Department at Massachusetts General Hospital (10 slides per case). Histology staining with haematoxylin and eosin characterized the samples as either predominately viable or necrotic. HMGB1 levels were visualized using standard immunohistochemical techniques. Table B.1 summarizes sample information.

Preparation of Extracts from Paraffin-Embedded Formalin-Fixed Tissue. The preparation of protein extracts was based on a previously reported methodology.[5] Tissue slices were washed with xylene to remove the paraffin and then hydrated by washing in ethanol and water. This procedure was repeated 2-3 times and the tissue was allowed to dry in the air. The tissue from each case was scraped from the microscope slide using a razor blade and resuspended in 200 μL of RIPA buffer (1 mM NaH_2PO_4, 10 mM Na_2HPO_4, 154 mM NaCl, 1% triton x-100, 12 mM sodium deoxycholate, 2% SDS, 0.2% NaN_3, 0.95 mM NaF, 2 mM PMSF, 5 μg/mL aprotinin, 5 μg/L leupeptin). RIPA buffer can be prepared without the protease inhibitors (PMSF, aproptinin, and leupeptin) and stored at 4 C, these protease inhibitors should be added immediately before use. The tissue suspension was heated at 100 C for 20 min, and allowed to incubate at 60 C for 2 h. The tissue lysates were centrifuged at 13500 rpm at 4 C for 15 min and the supernatant was collected and analyzed for protein content by the bicinchoninic acid assay. The extracts were then concentrated to ~50 μL, snap frozen, and stored at –80 C until further analysis.

Western Blot Analysis. For examination of HMGB1 levels, the tissue extracts were thawed and allowed to come to 4 C before analysis. Proteins were separated by SDS-PAGE for 90 min at 150 V on a BioRad 4-20% Tris·HCl Ready-Gel. The separated proteins were transferred to a PVDF membrane at 100 V for 60 min, blocked with TTBS + 5% milk for 90 min, and probed with a 1:2,000 dilution of anti-HMGB1 polyclonal antibody (Upstate) or a 1:20,000 dilution of anti-actin polyclonal antibody (Upstate) O/N at 4 C. The PVDF membrane was washed with TTBS + 5% milk and incubated with a 1:3000 dilution of anti-rabbit-HRP (HMGB1, Amersham Biosciences) or anti-mouse-HRP (actin, Bio-Rad) secondary antibody for 1 h at RT. The PVDF membrane was then incubated with ECL western blotting detection solution (Amersham

Biosciences) for 90 sec and blotted dry. Proteins were then detected by exposing the PVDF membrane to BioMax MR autoradiography film (Eastman Kodak Co.). The expression level of HMGB1 was compared to that of the loading control standard actin for each sample. The ratio of HMGB1 to actin expression was determined by measuring the number of pixels in each protein band from the immunoblot using Quantity Analysis Software (BioRad, version 4.5.0).

Results and Discussion

The expression levels of HMGB1 were evaluated in testicular seminoma tissue by histological techniques and by western blot analysis. Clinical samples were chosen based on their distribution of viable and necrotic tissue. As summarized in Table B.1, the seminoma tissue was characterized as either predominately viable or necrotic.

Histological Evaluation

Two clinical cases were examined by haematoxylin and eosin staining and by immunohistochemistry in order to determine the levels of viable/necrotic tissue and HMGB1 expression, respectively. As shown in Figure B.1, Case MS-04-X41943 is a testicular seminoma characterized by patchy areas of necrosis surrounded by viable tumor tissue. The distribution of HMGB1 correlates with the histology of the tumor, with the necrotic tissue having less HMGB1 expression than the viable region. This observation is consistent with a report that necrotic cells passively release HMGB1.[6] There was no direct evidence for selective uptake of HMGB1 released from necrotic cells by the viable tissue, however. Cells farthest away from and adjacent to the necrotic region appear to contain equivalent levels of HMGB1.

Case MS-04-D60785 is a testicular seminoma with large areas of necrotic and viable tissue. This case is particularly interesting because the necrotic and viable regions are separated

by a fibroinflammatory rind (Figure B.2). The necrotic tissue shows only patchy expression of HMGB1, wheras the fibroinflammatory rind is intensely stained for HMGB1. It is possible that HMGB1 release from the necrotic tissue acts to recruit inflammatory cells to respond to the region of damaged cells.[6,7] The viable cells adjacent to the fibroinflammatory rind also stain positive for HMGB1 expression. Again, there was no evidence for selective HMGB1 uptake exists in this case; however, a clinical pattern of significant levels of HMGB1 in testicular seminomas was demonstrated.

Western Blot Analysis

In an attempt to obtain evidence for cellular uptake of extracellular HMGB1, the level of the protein was compared in predominantly viable versus necrotic seminoma tissue. HMGB1 concentrations were normalized to the amount of actin in each sample. As shown in Figure B.3, all nine of the seminoma samples contain HMGB1 regardless of the tissue histology. Figure B.4 summarizes the ratio of HMGB1 to actin in the seminoma samples. In general, there is no correlation between HMGB1level and the identity of the tissue as necrotic or viable. Case MS-04-D60785 is particularly valuable since the tumor contains adjacent necrotic and viable tissue (Figure B.2). The viable region of the seminoma sample MS-04-D60785 does contain slightly more HMGB1 than the necrotic region of the tumor; however, it is unclear whether this difference is significant.

Conclusions and Future Directions

Both histological and immunoblotting techniques demonstrated that HMGB1 is present in testicular seminomas; however, direct evidence for internalization of extracellular HMGB1 was not obtained. Visualization of HMGB1 by western blot analysis is somewhat problematic given

the large amount of tissue required for analysis. For example, tissue from 10 microscope slides was harvested for each case. In addition, true quantification of protein levels from clinical samples is difficult. Nevertheless, this study provides an impetus for further investigation of HMGB1 expression levels in seminomas. Since HMGB1 overexpression has been associated with cisplatin resistance and anti-apoptotic signals, it is possible that a tissue-type dependence exists for this phenomenon.[8,9] It is tempting to speculate that the regions of necrosis in testicular seminomas sensitize the surrounding viable tissue to cisplatin through the release of HMGB1.

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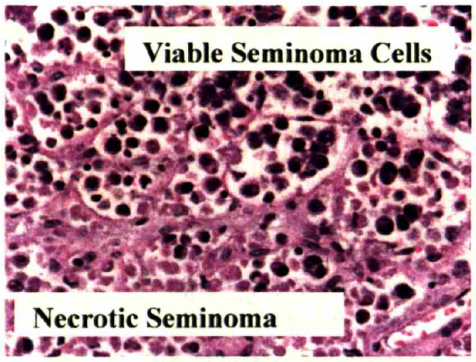
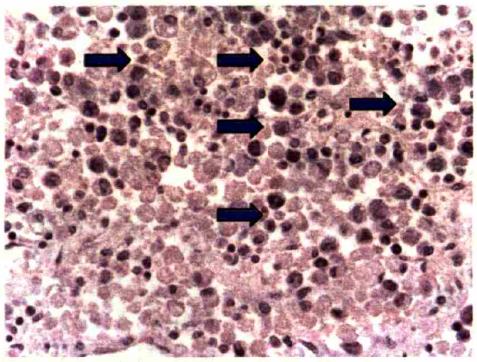
Table B.1. List of clinical testicular seminoma samples examined in this study. Samples were provided as paraffin-embedded formalin-fixed tissue attached to microscope slides. Histology was determined at Massachusetts General Hospital Pathology Department.

Sample/Lane Number	Case Number	Histology
1	MS-04-D39239, block A3	Predominately viable
2	MS-04-X41943, block A5	Predominately viable
3	MS-04-D60785, block A9	Predominately viable
4	MS-05-D7074, block A13	Predominately viable
5	MS-04-X59021, block A1	Predominately necrotic
6	MS-04-X59021, block A4	Predominately necrotic
7	MS-04-D60785, block A3	Predominately necrotic
8	MS-04-D60785, block A4	Predominately necrotic
9	S02W39192, block P	Markedly necrotic



H&E Stain



Immunohistochemistry for HMGB1

Figure B.1 Histochemistry for Case MS-04-X41943, testicular seminoma with patchy areas of necrosis surrounded by viable tissue (40X magnification). The left panel is tissue stained with haematoxylin and eosin (H&E) for differentiating viable (black) and necrotic (pink) tissue. The right panel shows HMGB1 expression levels as visualized by immunohistochemistry. The blue arrows indicate select areas positive for HMGB1 expression (brown nuclei).

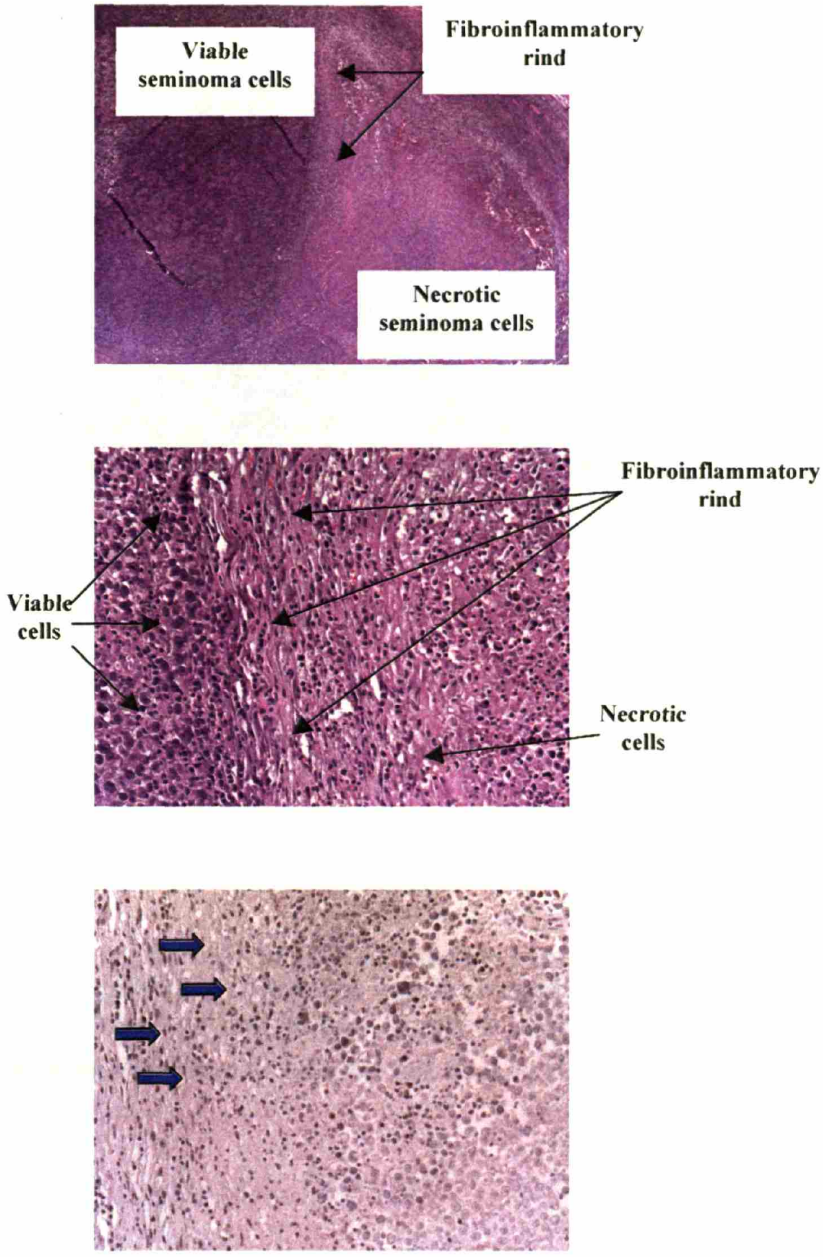


Figure B.2. Histochemistry for Case MS-04-D60785, a testicular seminoma with a large area of necrosis that is separated from viable tumor tissue by a fibroinflammatory rind (top panel, 2X magnification). The center panel is tissue (10X magnification) stained with haematoxylin and eosin (H&E) for differentiating viable (black) and necrotic (pink) tissue. The bottom panel (10X magnification) shows HMGB1 expression levels as visualized by immunohistochemistry. The blue arrows indicate select areas positive for HMGB1 expression (brown nuclei). The necrotic area has patchy, diffuse HMGB1 expression, wheras the fibroinflammatory rind is mostly positive for HMGB1 expression.

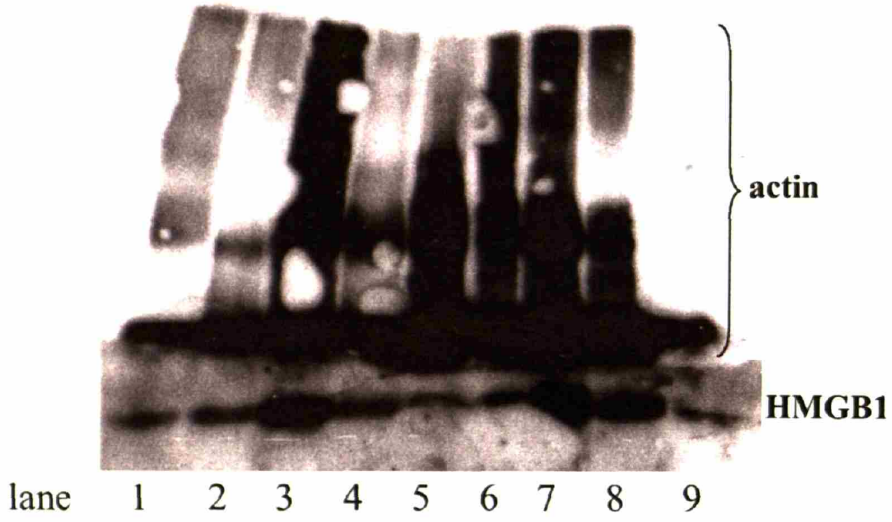


Figure B.3. Expression levels of actin and HMGB1 in testicular seminoma samples. See Table B.1 for lane assignments.

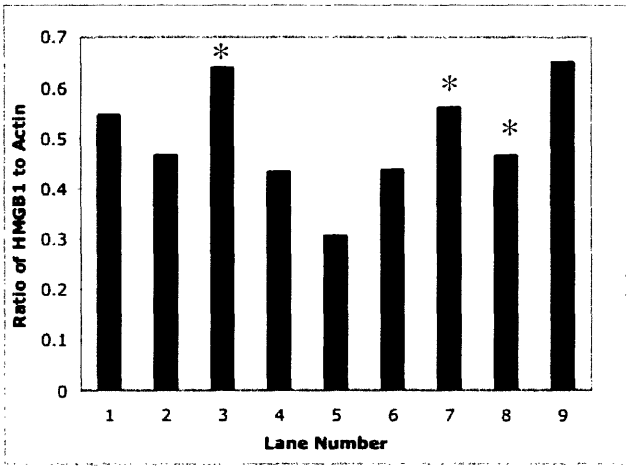


Figure B.4. A bar chart showing the ratio of HMGB1 to actin expression levels in testicular seminoma samples with variant histology. The ratios were determined by western blot analysis. See Table B.1 for lane assignments. Bars marked with an asterisk (*) are from the same patient.

Appendix C
Solid Phase Synthesis of a Platinum(IV)-RGD Peptide Conjugate

Introduction

Angiogenic tumor blood vessels express $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, which specifically recognize RGD and NGR tripeptide motifs.[1] Recent work from our laboratory afforded a series of platinum(IV) peptide conjugates prepared to target angiogenic endothelial cells through interaction with such integrin receptors.[2] The platinum(IV) peptide conjugate containing the RGD peptide motif was particularly active against bovine capillary endothelial cells. Although the Pt(IV)-RGD conjugate was not as cytotoxic as cisplatin, it was more active than related platinum(IV) compounds tethered to non-specific peptides.[2] In order to evaluate the targeting potential of the Pt(IV)-RGD conjugate, animal studies are planned. Unfortunately, current synthetic methodologies only yield 1-5 mg quantities of the Pt(IV)-RGD conjugate and several hundred milligrams of material are required for animal studies. This appendix describes an improved solid-phase synthesis of the Pt(IV)-RGD conjugate that yields 20 mg of pure material.

Experimental Procedures

Materials and Methods. *Cis, cis, trans*-Diamminedichlorodisuccinatoplatinum(IV) was synthesized as described previously.[3] Rink amide resin and all peptides were purchased from NovaBiochem.

Solid-Phase Synthesis of a Mono-Substituted Pt(IV)-RGD Conjugate.

Synthesis of RGD. Rink amide resin (0.17 g, 0.61 mmol/g resin, 0.10 mmol) was washed with DMF and soaked in 20% piperidine/DMF for 5 min to remove the Fmoc protecting group (deblocking step). For the first coupling reaction, Fmoc-Asp-(OtBu)-OH (0.44 g, 1.1 mmol) was dissolved in a solution of DIPC (140 μL, 0.89 mmol) and HOBt (0.13 g, 0.99 mmol) in DMF (5 mL) and allowed to stir for 5 min before addition to the resin. After 90 min, the resin was rinsed

with DMF and the deblocking procedure was repeated. The coupling reaction was repeated with Fmoc-Gly-OH (0.31 g, 1.1 mmol), DIPC (140 μL, 0.89 mmol) and HOBt (0.13 g, 0.99 mmol) in DMF (5 mL) and the Fmoc protecting group was removed with 20% piperidine/DMF. The last peptide coupling reaction was carried out with Fmoc-Arg-(Pbf)-OH (0.67 g, 1.0 mmol), DIPC (140 μL, 0.89 mmol), and HOBt (0.13 g, 0.99 mmol) and the deblocking procedure was repeated. Finally, the resin-linked RGD peptide was washed with DMF.

Platinum(IV) Conjugation. Cis, cis, trans-Diamminedichlorodisuccinatoplatinum(IV) (1.1 g, 2.0 mmol) was activated with DIPC (270 μL, 1.7 mmol) and HOBt (0.24 g, 1.8 mmol) in DMF (5 mL) for 5 min at RT. The activated platinum(IV) compound was added to the resin-linked RGD peptide and the reaction was allowed to stir at RT in the dark. After 14 h, the resin was rinsed with DMF, methanol, and methylene chloride. The product was cleaved from the resin by stirring in 10 mL 95% TFA in water for 1.5 h at RT in the dark. Crude product was isolated by ether precipitation, resuspended in H_2O, and lyophilized to dryness. The mono-substituted Pt(IV)RGD conjugate was purified by reverse-phase HPLC (C18 semi-preparative column, 40 mm x 250 mm, linear gradient from 100% H_2O to 15% acetonitrile over 30 min) and isolated as a pale yellow powder (21 mg, 0.025 mmol, 24% yield). As shown in Figure C.1, comparison of the HPLC traces shows that the solid phase methodology yields a much cleaner reaction mixture. Isolation of the desired Pt(IV)-RGD conjugate was confirmed by LC-MS.

Significance

The manual solid phase synthesis of the mono-substituted RGD-conjugated diamminedichloro-platinum(IV) compound represents a significant improvement over the previous procedure for the preparation of peptide-tethered platinum(IV) compounds. The previous methodology

involved automated solid-phase synthesis and subsequent purification of peptide. The peptide was then allowed to react with the platinum complex to afford product in 11% yield (4.3 mg) after purification. The manual solid phase synthesis (see Scheme C.1) affords the pure RGD-conjugated platinum(IV) compound in 24% yield (21 mg) and only requires one purification step. The ability to isolate larger quantities of the Pt(IV)-RGD conjugate is essential for *in vivo* evaluation of its cytotoxicity. Further improvements could involve purification of the Pt(IV)-RGD conjugate by dialysis instead of HPLC.

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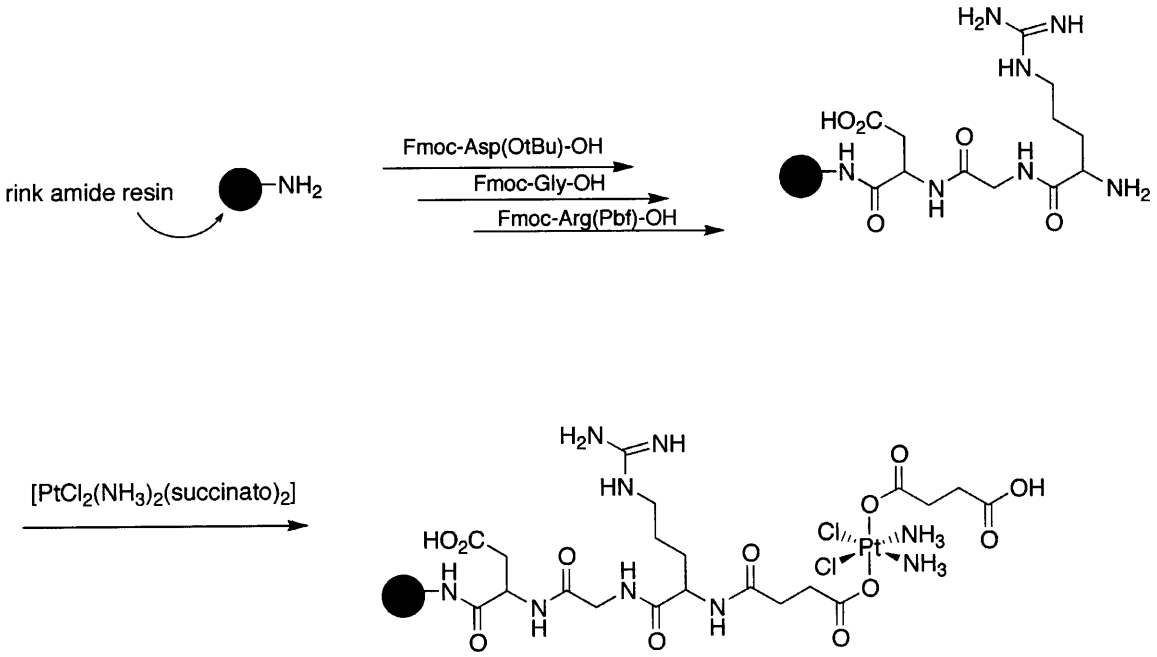
rink amide resin
NH2
Fmoc-Asp(OtBu)-OH
Fmoc-Gly-OH
Fmoc-Arg(Pbf)-OH
[PtCl2(NH3)2(succinato)2]
H2N
NH
HN
HO2C
H
N
O
N
H
NH2
OH
Cl
Pt
NH3

Scheme C.1.

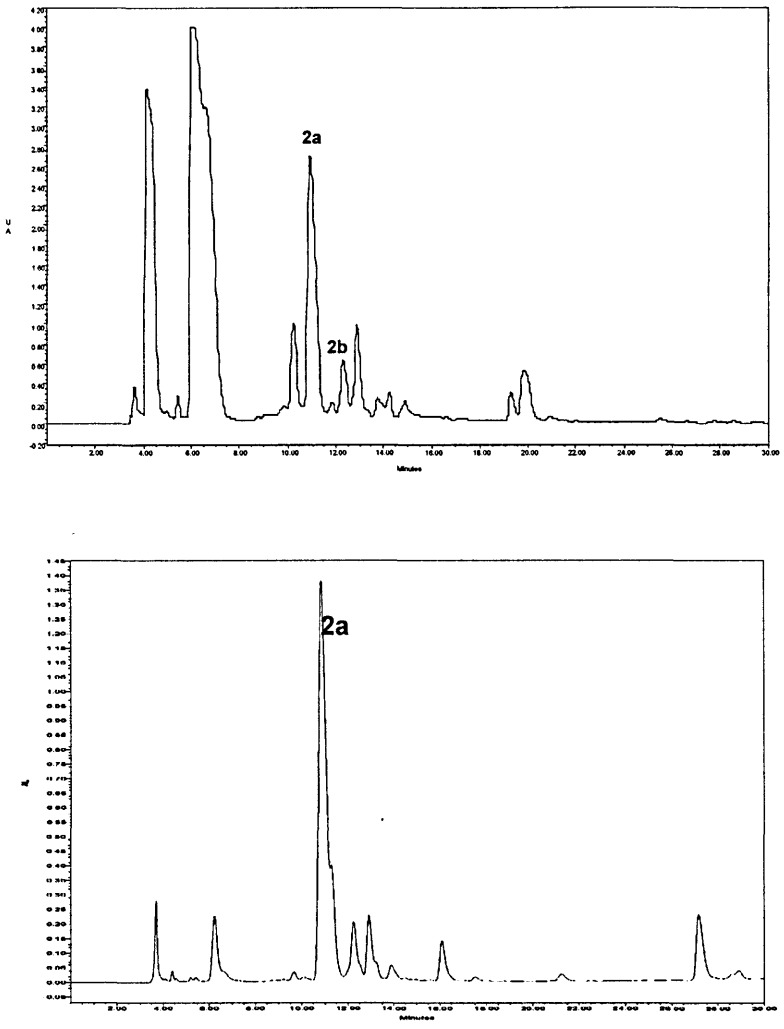

Figure C.1. HPLC chromatographs of crude RGD-conjugated Pt(IV) complex. The mono-substituted conjugate is indicated as **2a**, and the *bis*-substituted product is labeled as **2b**. The top panel is the HPLC trace from a previous reaction[2] and the bottom panel is the trace from the current work.

Biographical Note

The author was born Katie Elizabeth Robinson on April 12, 1978, to Michael and Sally Robinson, in Charleston, WV. At the age of four she moved to Washington, PA, just outside of Pittsburgh, where she grew up with two brothers, Dan and Matthew. While in high school, her interest in chemistry was spawned under the guidance of Mr. Dan Flynn. The author graduated Washington High School with honors in 1996. She then moved to Raleigh, NC where she followed in the footsteps of her uncles and grandfather and attended North Carolina State University. Under the direction of Dr. Anton Schreiner, she majored in chemistry and graduated in the Fall of 1999 as valedictorian of her class. While at NC State, she performed research in the laboratories of Dr. Anton Schreiner, Dr. Russell Linderman, and Dr. Stefan Franzen. Also at NC State, she met Neil Randolph Barnes whom she married in May of 2000. With Randy by her side, the two moved to Boston, MA during the summer of 2000. In September 2000, the author began graduate studies in inorganic chemistry at MIT and joined the lab of Professor Stephen J. Lippard. Her dissertation research has focused on the rational design of platinum-based anticancer compounds. She also mentored the laboratory studies of an undergraduate student, Caroline Sauoma. Following graduation, she plans to join industry in the Boston area.

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