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A bifunctional platinum(II) antitumor agent that forms DNA adducts with affinity for the estrogen receptor

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Abstract

A strategy is described for the re-design of DNA damaging platinum(II) complexes to afford elevated toxicity towards cancer cells expressing the estrogen receptor (ER). Two platinum-based toxicants are described in which a DNA damaging warhead, [Pt(en)Cl_2] (en, ethylenediamine), is tethered to either of two functional groups. The first agent, [6-(2-amino-ethylamino)-hexyl]-carbamic acid 2-[6-(7α-estra-1,3,5,(10)-triene)-hexylamino]-ethyl ester platinum(II) dichloride ((Est-en)$PtCl_2$), terminates in a ligand for the ER. The second agent is a control compound lacking the steroid; this compound, *N*-[6-(2-amino-ethylamino)-hexyl]-benzamide platinum(II) dichloride ((Bz-en)$PtCl_2$)), terminates in a benzamide moiety, which lacks affinity for the ER. Using a competitive binding assay, Est-en had 28% relative binding affinity (RBA) for the ER as compared to 17β-estradiol. After covalent binding to a synthetic DNA duplex 16-mer, the compound retained its affinity for the ER; specificity of the binding event was demonstrated by the ability of free 17β-estradiol as a competitor to disrupt the DNA adduct-ER complex. The (Est-en)$PtCl_2$ compound showed higher toxicity against the ER positive ovarian cancer cell line CAOV3 than did the control compound. (Est-en)$PtCl_2$ was also more toxic to the ER positive breast cancer line, MCF7, than to an ER negative line, MDA-MB231.

1. Introduction

Platinum compounds such as cisplatin, carboplatin and oxaliplatin are successful anticancer agents [1]. The former two agents are particularly effective in the treatment of testicular cancers, for which cure rates exceed 90% [2,3], whereas the third agent is used in treatment of colorectal cancer [4]. Platinum compounds are also used for treatment of other cancers, including those of the ovary, head and neck, and cervix [5]. However, as with most cancer therapeutics, clinical use of platinum antitumor agents is frequently limited by unwanted toxic side effects and acquired and/or intrinsic resistance against the drugs [1,6]. Efforts toward the development of next-generation platinum anticancer agents have been aimed at overcoming the aforementioned limitations.

The major mechanism of action of platinum compounds is through their ability to bind covalently to DNA [7]. Once DNA adducts are formed, cellular survival depends on the DNA-repair capabilities of the cells. Some cisplatin-resistant cell lines derived from tumors have increased rates of repair compared to cells from clinically responsive tumors [8]. Increased expression of the nucleotide excision repair (NER) endonuclease ERCC1 has been correlated with cisplatin resistance in ovarian cancer [9]. Cisplatin DNA-adducts also serve as recognition

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motifs for a variety of cellular proteins that are not involved in DNA repair [10]. Binding of these proteins also plays a role in modulating the cytotoxic effects of platinum compounds [11]. The relevance of these mechanisms to clinical responses is under continued investigation. Nevertheless, the discovery of proteins that bind tightly to platinum adducts provided the basis for a concept for the rational design of compounds that overcome resistance mechanisms and sensitize tumor cells to platinum compounds and other cytotoxic drugs.

Earlier work from our laboratory [12,13] resulted in the synthesis of compounds in which DNA-interactive nitrogen mustards were tethered to ligands for steroid receptors (either the androgen or the estrogen receptors). Some of these compounds have shown preferential toxicity against the relevant steroid receptor positive cells in culture and significant promise against animal xenograft models of human cancer. The objective of the current study was to explore the feasibility of using a steroid ligand, a derivative of estradiol, as a targeting agent that would enhance the toxicity of platinating agents in cells.

The compounds prepared in this work, and their intended mechanisms of action, are shown in Figure 1. The control compound, *N*-[6-(2-amino-ethylamino)-hexyl]-benzamide ((Bz-en)$PtCl_2$) (Fig. 1A) readily forms DNA adducts (*vide infra*) but does not interact with the ER owing to the absence of functional ligand. We propose that this compound forms adducts that can be readily repaired by the host cell nucleotide repair system. The compound may affect transcription but such effects would be assumed to be non-specific. By contrast, [6-(2-amino-ethylamino)-hexyl]-carbamic acid 2-[6-(7α-estra-1,3,5,(10)-triene)-hexylamino]-ethyl ester ((Est-en)$PtCl_2$) was designed to form adducts that interact with the ER (Fig. 1B). If such interactions occur in cells, the ER-adduct complex may camouflage the lesion from DNA repair systems that have the genome under surveillance in order to correct endogenously and exogenously generated genetic damage. If unrepaired, the adduct would possibly trigger a lethal event. As a second possible mechanism, the adducts of (Est-en)$PtCl_2$, if sufficiently abundant in the genome, could hijack a sufficient amount of the ER to prevent this transcription factor from performing its growth enhancing function in ER positive cancer cells. The third possibility is that the ER may form a complex with the free (Est-en)$PtCl_2$ compound and help deliver it to DNA. Before these models could be tested, it was necessary to synthesize a platinum warhead that could form DNA adducts capable of interacting with the ER in a biologically relevant manner. That issue is the subject of this initial paper.

2. Experimental

2.1 General Methods

All reagents and solvents were purchased from commercial sources and were of reagent quality unless otherwise stated. Moisture-sensitive reactions were handled under argon atmosphere using standard Schlenk techniques. Solvents used for synthesis were glass-distilled anhydrous or of the highest quality available, and were used without further purification. UV absorbance measurements were obtained with a Beckman DU®-65 spectrophotometer. ^{1}H NMR spectra were recorded on a Varian Mercury 300, Bruker Advance 400, Bruker Advance 401, or Varian INOVA 500 NMR spectrometers. ^{195}Pt NMR spectra were recorded on Varian INOVA 501 NMR spectrometer equipped with a tunable broad-band probe. The chemical shifts were reported as δ (ppm) values calibrated to solvent peaks for ^{1}H NMR spectra, whereas cisplatin (-2088 ppm in DMF) was used as an external reference for the ^{195}Pt NMR spectra. The splitting of proton resonances in the reported ^{1}H NMR spectra are defined as s = singlet, d = doublet, t = triplet, q = quartet, and m = multiplet. Quantification of radioactivity was performed on a Beckman LS 1801 Liquid Scintillation Counter or on a Molecular Dynamic Storm 840 PhosphorImager equipped with Molecular Dynamic ImageQuant software. High-resolution mass spectral analyses were carried out by the MIT Department of Chemistry Instrumentation

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Facility (DCIF). Elemental analyses were performed by Quantitative Technologies Inc. (QTI), Whitehouse, NJ.

2.2 Synthesis of Pt compounds

(6-Hydroxyhexyl)-carbamic acid 2-(diphenylphosphinic-{6-[7α-estra-1,3,5,(10) triene-3,17β-bis-2-tetrahydropyranyl]-hexyl}-amino)-ethyl ester (2)—To a solution of 1 (460 mg, 0.485 mmol) in 30 mL of DMF, was added 6-aminohexanol (74 mg, 0.631 mmol) at room temperature. The reaction mixture was stirred for 12 h. After removing solvent under reduced pressure, the residue was re-dissolved in 50 mL of CH_2Cl_2 and partitioned between Na_2CO_3 saturated aqueous solution (50 mL) and CH_2Cl_2. The organic phase was dried over Na_2SO_4. The product was purified by column chromatography on silica gel (EtOAc/25% $CH_2Cl_2 \rightarrow CH_2Cl_2$/10% MeOH) to yield 2 as white solid (400 mg, 89 %). ^{1}H NMR ($CDCl_3$) δ 0.79, 0.81 (2s, 3H), 0.9–2.1 (42 H), 2.28 (m, 2H), 2.69 (2d, 1H), 2.85 (m, 1H), 2.95 (q, 2 H), 3.13 (q, 2H), 3.25 (m, 2H), 3.49 (m, 1H), 3.56–3.66 (m, 3H), 3.75 (t, 1H), 3.92 (m, 2H), 4.01 (t, 2H), 4.66 (m, 1H), 4.97 (t, 1H), 5.38 (2t, 1H), 6.76 (d, 1H), 6.84 (d, 1H), 7.18 (t, 1H), 7.40–7.5 (m, 6 H), 7.81 (m, 4H).

[6-(2-Amino-ethylamino)-hexyl]-carbamic acid 2-({6-[7α-estra-1,3,5,(10) triene-3,17β-bis-2-tetrahydropyranyl]-hexyl}-diphenylphosphinic-amino)-ethyl ester (3)—To a solution of 2 (400 mg, 0.43 mmol) in 10 mL of pyridine, was added methanesulfonyl chloride (67 μL, 0.86 mmol) via micro syringe at room temperature. The resulting mixture was stirred at room temperature for 30 minutes, after which 60 μL of water was added, followed by ethylendiamine (10 mL). After stirring at room temperature for 20 h, the volatile components of the reaction mixture were removed under the reduced pressure. The residue was dissolved in 10 mL of CH_2Cl_2, and partitioned between Na_2CO_3 saturated water and CH_2Cl_2. The organic phase was then dried over Na_2SO_4 and evaporated under reduced pressure. The residue was re-dissolved in 10 mL of acetonitrile and insoluble white precipitation was removed by filtration. The crude product in acetonitrile was purified by column chromatography on alumina gel (CH_2Cl_2/5% MeOH → CH_2Cl_2/5% MeOH/0.5% NH_4OH) to yield **3** (250 mg, 63 %). ^{1}H NMR ($CDCl_3$) δ 0.79, 0.81 (2s, 3H), 0.9–2.1 (42 H), 2.28 (m, 2H), 2.65–2.75 (m, 2H), 2.85- 2.99 (m, 6H), 3.10 (t, 2H), 3.25 (m, 2H), 3.48 (m, 1H), 3.59 (m, 1H), 3.73 (t, 1H), 3.91(m, 2H), 4.10 (m, 2H), 4.66 (m, 1H), 5.38 (2t, 1H), 6.75 (d, 1H), 6.84 (d, 1H), 7.18 (t, 1H), 7.40–7.45 (m, 6H), 7.78 (m, 4H)

[6-(2-Amino-ethylamino)-hexyl]-carbamic acid 2-[6-(7α-estra-1,3,5,(10)-triene)-hexylamino]-ethyl ester (4) (Est-en)—To a stirred solution of **3** (110 mg, 0.11 mmol) in 10 mL of methanol at room temperature, was added concentrated HCl (0.5 mL) drop wise. The resulting solution was stirred for 3–6 h with TLC monitoring. Solid Na_2CO_3 was added to the reaction mixture until all of HCl was neutralized. The excess Na_2CO_3 solid was removed by filtration and the solvent was evaporated under reduced pressure. The resulting products were re-dissolved in 20 mL of ethanol and insoluble material removed by filtration. Following removal of solvent the removed product was washed with CH_2Cl_2 (10 mL) several times to yield **4** (Est-en) (70 mg, 0.11 mmol, quantitative). ^{1}H NMR (CD_3OD) δ 0.73 (s, 3H), 0.92–1.77 (27 H), 1.87 (d, 1H), 1.98 (m, 1H), 2.25 (m, 2H), 2.64 (d, 1H), 2.77 (dd, 1H), 2.96 (t, 2H), 3.02–3.10 (m, 4H), 3.21 (m, 2H), 3.61 (t, 1H), 4.22 (m, 2H), 6.48 (d, 1H), 6.92 (dd, 1H), 7.03 (d, 1H). HRMS (ESI) calculated for [M+H] 601.4687, found 601.4684.

[6-(2-Amino-ethylamino)-hexyl]-carbamic acid 2-[6-(7α-estra-1,3,5,(10)-triene)-hexylamino]-ethyl ester platinum(II) dichloride ((Est-en)$PtCl_2$)—To a stirred solution of **4** (Est-en) (85 mg, 0.141 mmol) in 5 mL of MeOH/H_2O (3:2 v/v), was added a solution of K_2PtCl_4 (70 mg, 0.17 mmol) in 4 mL of MeOH/H_2O (1:1 v/v). The resulting mixture was stirred for 24 h at room temperature in the dark. A 10% KCl aqueous solution was added

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and the reaction was stirred for additional 4 h in the dark. After letting the white precipitate settle to the bottom of the flask on ice for an hour, the supernatant was decanted. Any remaining suspended material was removed by centrifugation and combined with the original precipitate. The off-white solid was washed 4X with 1 ml of water by resuspension and centrifugation. Finally, the cream solid was dried under vacuum for 12 h to yield (Est-en)$PtCl_2$ (83 mg, 0.096 mmol, 68 %). *Note:* the reaction yield varied (25–70 %) depending on the scale of the reaction. The larger the reaction scale, the higher reaction yield. ^{1}H NMR (DMF-d_7) δ 0.79 (s, 3H), 0.9–1.80 (26 H), 1.85–2.0 (m, 3H), 2.25 (m, 2H), 2.60 (m, 2H), 3.05 (m, 3H), 3.75 (m, 1H), 4.02 (m, 2H), 4.58 (d, 1H), 5.22 (br, 2H), 6.10 (br, 1H), 6.49–6.70 (m, 2H), 7.00 (t, 1H), 7.13 (d, 1H), 9.20 (br, 1H). ^{195}Pt NMR (DMF-d_7) δ -2341. HRMS (ESI) calculated for [M+H] 867.3705, found 867.3681. Elemental analysis, calculated for $C_{35}H_{60}Cl_2N_4O_4Pt•2H_2O$: C 46.56; H 7.14; N 6.21. Found: C 46.89; H 7.01; N 6.14.

***N*-(6-Hydroxylhexyl)benzamide (5)—**A mixture of 6-aminohexanol (500 mg, 4.27 mmol) and benzoic anhydride (1 g, 4.42 mmol) was dissolved in 50 mL of DMF and stirred for 12 h at room temperature. After removing DMF, the products were partitioned between CH_2Cl_2 and aqueous Na_2CO_3 (sat.). The organic phase was dried over Na_2SO_4. The product was isolated by column chromatography on silica gel (EtOAc/33% hexanes) to yield **5** as white solid (870 mg, 92%). ^{1}H NMR (DMSO-d_6) δ 1.28–1.54 (m, 8 H, - C$\underline{H}_2$-), 3H), 3.25 (q, 2H, -C$\underline{H}_2$-NHC(O)-), 3.38 (q, 2H, -C$\underline{H}_2$-OH), 4.36 (t, 1H, -O$\underline{H}$), 7.41–7.51 (m, 3H, *meta & para* $C_6\underline{H}_5$-), 7.82 (m, 2H, *ortho* $C_6\underline{H}_5$-), 8.43 (t, 1H, -N$\underline{H}$C(O)-).

***N*-[6-(2-Amino-ethylamino)-hexyl]-benzamide (6) (Bz-en)—**To a stirred solution of **5** (2.5 g, 0.01 mol) in 50 mL of pyridine, was added methanesulfonyl chloride (1.7 mL, 0.02 mol) at room temperature. The resulting solution was stirred for 30 minutes, after which 2 mL of water was added. Excess ethylenediamine (10 mL) was then added to the reaction mixture which was further stirred for an additional 20 h. The volatile components of the reaction mixture were removed under the reduced pressure. The remaining residue was partitioned between aqueous Na_2CO_3 (sat.) and CH_2Cl_2. The organic phases were dried over Na_2SO_4. The product was isolated by column chromatography (alumina, 90% CH_2Cl_2/10% MeOH/1% NH_4OH) to yield **6** (Bz-en) as hygroscopic white solid (1.8 g, 68 %). ^{1}H NMR (D_2O) δ 1.22–1.30 (m, 4H, -C$\underline{H}_2$-), 1.34- 1.39 (m, 2H, -C$\underline{H}_2$-), 1.47–1.53 (m, 2H, -C$\underline{H}_2$-), 2.41 (t, 2H, -C$\underline{H}_2$- next to -$NH_{(2)}$), 2.49 (t, 2H, -C$\underline{H}_2$- next to -$NH_{(2)}$), 2.60 (t, 2H, -C$\underline{H}_2$- next to -$NH_{(2)}$), 3.26 (t, 2H, -C$\underline{H}_2$-NHC(O)), 7.42 (t, 2H, *meta* $C_6\underline{H}_5$-), 7.50 (t, 1H, *para* $C_6\underline{H}_5$-), 7.65 (d, 2H, *ortho* $C_6\underline{H}_5$-). HRMS (ESI) calculated for [M+H] 264.2070, found 264.2069.

***N*-[6-(2-Amino-ethylamino)-hexyl]-benzamide platinum(II) dichloride ((Bz-en) $PtCl_2$)—**To a stirred solution of **6** (Bz-en) (300 mg, 1.14 mmol) in water (3 mL) was added a solution of K_2PtCl_4 (473 mg, 1.14 mmol in 2 mL of H_2O). The resulting soultion was stirred for 25 h in dark. An aqueous solution of 5 % KCl (10 mL) was added to the reaction mixture which was stirred an additional 3 h. Suspended solids were then collected by centrifugation, triturated with water four times and finally the solid was dried under vacuum for 12 h to yield (Bz-en)$PtCl_2$ as a fine tan power (520 mg, 86 %). ^{1}H NMR (DMF-d_7) δ 1.31- 1.40 (m, 4H, -C$\underline{H}_2$-), 1.59- 1.65 (m, 3H, -C$\underline{H}_2$-), 1.91 (m, 1H, -C$\underline{H}_2$-), 2.55–2.82 (m, 4H, -C$\underline{H}_2$- next to -$NH_{(2)}$), 2.94 (m, 1H, -C$\underline{H}_2$- next to -$NH_{(2)}$), 3.08 (m, 1H, -C$\underline{H}_2$- next to -$NH_{(2)}$), 3.38 (t, 2H, -C$\underline{H}_2$-NHC(O)), 5.20 (s (br), 2H, -N$\underline{H}_2$), 6.05 (s (br), 1H, -N$\underline{H}$-), 7.46–7.50 (m, 2H, *para* $C_6\underline{H}_5$-), 7.95–7.98 (m, 2H, *ortho* $C_6\underline{H}_5$-), 8.47 (7.50 (t, 1H, *para* $C_6\underline{H}_5$-), 7.65 (d, 2H, *ortho* $C_6\underline{H}_5$-), 8.43 (t, 1H, -N$\underline{H}$C(O)-).). ^{195}Pt NMR (DMF-d_7) δ -2343. Elemental analysis, calculated for $C_{15}H_{25}Cl_2N_3OPt$: C 34.03; H 4.76; N 7.94. Found: C 34.32; H 4.74; N 7.85.

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2.3 Reaction of Pt compounds with DNA

Platinated compounds (4 nmol) were allowed to react with a 5′[^{32}P]-labeled self-complementary oligonucleotide 5′-d(ATTATTGGCCAATAAT) (0.8 nmol) dissolved in 40 μL phosphate buffer (50 mM, pH=7.4) for 12 h at 37°C. The DNA was recovered by ethanol precipitation and analyzed by separation on a denaturing 20% polyacrylamide gel. Quantification of radioactivity was performed on a Molecular Dynamic Storm 840 PhosphorImager equipped with Molecular Dynamic ImageQuant software. The level of modification was estimated by the ratio of the amount of unmodified DNA to the amount to DNA with lower mobility in each lane on the gel. Analysis of DNA modification by (Bz-en) $PtCl_2$ and (Est-en)$PtCl_2$ was performed twice, whereas the analysis of DNAs treated DMSO or cisplatin was performed once.

2.4 Affinity of (Est-en)$PtCl_2$ for the estrogen receptor

The relative affinity of (Est-en)$PtCl_2$ for the estrogen receptor was determined by a competitive binding assay using the human ER ligand binding domain (hER-LBD) (Invitrogen, Carlsbad, CA). [^{3}H]-17β-Estradiol (50 nM) was combined with unlabeled competitors (0.1 nM to 100 μM) and 2.7 nM hER-LBD in 150 μL of 10 mM Tris-Cl pH 7.5, 10% glycerol, 2 mM dithiothreitol, 1 ml/ml BSA. The solution was incubated at 4°C for 12 h. Bound and free ligands were separated by absorbtion of the hER-LBD to hydroxyapatite (HAP). After incubation, 100 μL of a 50% slurry of HAP in binding solution was added and allowed to absorb the hER-LBD for 15 min at 4°C. The HAP was washed three times with binding buffer. HAP pellets were resuspended in 2 ml ethanol, scintillation fluid added and radioactivity measured. The relative binding affinity (RBA) was calculated as the percent ratio of 17β-estradiol divided by the test compound required to reduce bound radioactivity by 50%. The experiment was performed in duplicate.

2.5 Affinity of (Est-en)$PtCl_2$-DNA adducts for hER-LBD

The [^{32}P]-labeled self-complementary 16-mer deoxyoligonucleotide was allowed to react with platinum compounds as described above. Unreacted (Est-en)$PtCl_2$ was removed using a Micro Bio-Spin 6 column (BioRad, Hercules, CA). DNA containing adducts formed by (Est-en) $PtCl_2$ or unreacted DNA (7.6 pmol) was combined with hER-LBD (3.9 pmol) in 10% glycerol, 2 mM DTT and 10 mM Tris pH 7.5 (20 μL final volume). 17β-Estradiol (25 fmol – 25 pmol) was added as competitor to the solutions where indicated. After 1 h at room temperature complexes were separated on a 5% polyacrylamide gel containing 0.5xTBE and run at 4°C. The data shown represent the results of three independent experiments.

2.6 Cell culture

The cell lines used in this study were obtained from the American Type Culture Collection (ATCC, Rockville, MD). HeLa, MDA-MB231 and MCF-7 cell lines were maintained in Minimal Essential Media (MEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 2 mM glutamine, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids. The GM04312 cell line (i.e., Xeroderma Pigmentosum Complementation Group A; XPA) was maintained in MEM supplemented with 10% FBS and 2 mM glutamine. CAOV-3 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% FBS. Cells were grown in a humidified 5% CO_2/air atmosphere at 37 °C.

2.7 Growth inhibition and clonal survival assays

For the growth inhibition assay cells were seeded at 7×10^4 cells/well in 6-well plates and allowed to adhere to the surface for 24 h. Cells were then exposed to the platinated test compounds dissolved in DMSO, as well as DMSO alone in growth medium with a reduced

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serum content (0.5% FBS) for 48 h. At the end of the incubation, cells were trypsinized and cell numbers determined using a Coulter Counter. The percent growth inhibition is the ratio of cell number in compound-treated and DMSO-treated wells multiplied by 100. The results represent the averages of three independent experiments each of which was performed in duplicate or triplicate for each concentration of test compound. The LD_{50} values were estimated from the growth inhibition curves.

Clonal survival assays with HeLa or XPA (GM04312) cells were performed by seeding 6-well dishes at 1000 cells/well. Cells were allowed to adhere to the surface for 24 h. Cells were then exposed for 24 h to test compounds in growth media with a reduced serum content (0.5% FBS). Fresh growth media (containing 10% FBS) was then added cells were grown for 5–7 days. Colonies were fixed with acetic acid/methanol, stained with crystal violet and counted. The assay was repeated twice with triplicates for each concentration of test compound.

3. Results

A number of platinum complexes linked to steroids have been designed to take advantage of steroid transport mechanisms to increase the intracellular accumulation of drug [14,15,16,17, 18,19]. In addition to affecting toxicant transport, our study was aimed at the construction of a molecule that would disrupt the processes of DNA repair and transcription. We chose the estrogen receptor as the cancer cell-expressed target for several reasons. The ER is expressed in most ovarian cancers which, although initially responsive to platinum chemotherapeutics, frequently develop resistance through increased DNA repair abilities [9]. Moreover, the ER, when up regulated, drives genes for growth of cancer cells. Our goal in this work was to determine, in part, if our repair-blocking or transcriptional disruption strategies (Fig. 1) might be able to enhance the selective toxicity of platinating agents against cancer cells expressing the ER. For this study, we used a linker we had shown in earlier work permits the juxtaposition of the ER with DNA damage sites in the genome; this linker is attached to the steroid residue at the 7α position [13].

The preparation of the estradiol-platinum compound (Est-en)$PtCl_2$ (Scheme 1) was based in part on previously published methods. The key precursor, the estradiol-linked carbonate (**1**) was prepared by methods described in an earlier publication by our research group [13]. Carbonate (**1**) was then allowed to react with aminohexanol to form the carbamate (**2**). The terminal alcohol group of (**2**) was activated and coupled to ethylenediamine to yield, after acid deprotection, (**4**) Est-en. The platinum complex was prepared by addition of an aqueous solution of K_2PtCl_4 to a solution of the Est-en ligand and the product isolated directly from the reaction mixture. Using similar methods, we synthesized a *N*-(6-hexyl)benzamide derivative of ethylenediamminePt(II), (Bz-en)$PtCl_2$ (Scheme 2). This molecule was designed as a control to investigate that role played by the steroid moiety in the estradiol-linked Pt compound (Est-en)$PtCl_2$. The coordination of the Pt to the diamine in each complex was confirmed by NMR. The ^{195}Pt chemical shift values of (Est-en)$PtCl_2$ (-2341) and (Bz-en) $PtCl_2$ (-2343) are characteristic of platinum(II) complexes with a diamine accompanied by two chloride atoms [20,21].

We used gel electrophoresis to assess the ability of the platinum compounds to react with DNA and form covalent adducts. Upon overnight incubation with a radio-labeled self complementary 16-mer, 5′[^{32}P]-d(AATATTGGCCAATATT), containing the preferred platinum binding site d(GpG) [22] (Figure 2 inset, indicated by arrows), cisplatin as well as the (Est-en)$PtCl_2$ and (Bz-en)$PtCl_2$ compounds displayed their ability to modify DNA by the appearance of slower migrating bands (Figure 2; bands in boxes). While only a trace amount of DNA remained unmodified by cisplatin, greater amounts of unmodified DNA are present after reaction with

the (Est-en)$PtCl_2$ and (Bz-en)$PtCl_2$ compounds indicating the order of reactivity as cisplatin > (Bz-en)$PtCl_2$ > (Est-en)$PtCl_2$.

We next examined the ability of free (Est-en)$PtCl_2$ to bind to the ER using a competitive ligand binding assay. In the presence of cell extracts containing the human ER ligand binding domain (hER-LBD) and [^{3}H]-17β estradiol, increasing concentrations of (Est-en)$PtCl_2$ resulted in deceased radioligand associated with the ER (Figure 3a). In comparison to estradiol, (Est-en)$PtCl_2$ had a relative binding affinity (RBA) of 28% (i.e., the RBA of 17β-estradiol = 100).

We next used an electrophoretic mobility shift assay (EMSA) to determine whether the ER can bind to the DNA adducts of (Est-en)$PtCl_2$. The self-complementary 16-mer was allowed to react with (Est-en)$PtCl_2$ as described above and the product was incubated with purified hER-LBD at room temperature for 45 min. Analysis on a 5% non-denaturing polyacrylamide gel revealed that DNA containing (Est-en)$PtCl_2$ adducts produced slowly migrating bands in the presence of the ER-LBD (Figure 3b, lane 3). The presence of two complexes with slightly different mobilities likely results from different orientations of Pt bound in the oligonucleotide DNA. Similar observations of isomeric DNA binding with asymmetric platinum complexes have been previously reported [23]. It is also possible that the two bands represent complexes formed by the ER binding to inter- and intra-strand crosslinked DNA. The slowly migrating complexes disappeared when increasing amounts of 17β-estradiol were added to the reactions (lanes 4–8) (Figure 3b). The ability of the natural ligand to dissociate the ER from modified DNA suggests that complex formation results from a specific association between (Est-en)$PtCl_2$-DNA adducts and the ER.

The cytotoxic effects of (Est-en)$PtCl_2$ and (Bz-en)$PtCl_2$ were compared in the human ovarian cancer cell line CAOV3, which expresses the ERα [24]. Toxicity was evaluated by comparing the number of cells in wells after 48 h treatment with either compound to the number of cells in wells treated with vehicle only. The results in Figure 4A show that (Est-en)$PtCl_2$, with an LD50 of 25 μM, is much more potently cytotoxic than (Bz-en)$PtCl_2$ against CAOV3 cells. This enhanced toxicity is possibly owed to (i) diminished repair, (ii) enhanced uptake or (iii) disrupted transcription, as indicated in Figure 1. It is noteworthy that the high differential toxicity of (Est-en)$PtCl_2$ over (Bz-en)$PtCl_2$ is achieved despite its lower inherent ability to damage DNA (Fig. 2).

We next examined whether the ability to perform nucleotide excision repair (NER) was capable of modulating the cytotoxic effects of (Est-en)$PtCl_2$. The NER pathway is one of the major cellular defenses against DNA damage caused by cisplatin [6]. The cytotoxicity of (Est-en)$PtCl_2$ towards an XPA (NER-negative) cell line was compared with its cytotoxic effects towards the NER repair proficient HeLa cell line. Using a clonal survival assay, we found repair-deficient XPA cells suffered greater toxicity from (Est-en)$PtCl_2$ than did repair-proficient HeLa cells (Figure 4B). This result supports the notion that (Est-en)$PtCl_2$ DNA lesions are substrates for NER and helps to identify DNA as an important cellular target of this drug candidate.

Finally, we tested the role of the ER in mediating the cytotoxicity of (Est-en)$PtCl_2$ in vitro by determining its antiproliferative effects on the breast cancer cell lines MDA-MB231 (ER-) and MCF-7 (ER+). Figure 4C shows that at concentrations < 20 μM (Est-en)$PtCl_2$ inhibited growth of the MCF-7 cell line to a greater extent than it did the MDA-MB231 cell line. These results along with those shown in Figure 4A indicate that both the presence of the estradiol moiety in (Est-en)$PtCl_2$ and expression of the ER in target cells lead in enhanced toxicity.

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4. Discussion

Acquired or intrinsic resistance limits the clinical use of platinum-based therapeutics to a relatively narrow range of tumor types. One strategy described in the literature to overcome resistance mechanisms and expand the range of treatable tumors has been to link platinum (II)-coordination complexes to amino acids, steroids, DNA intercalators or other ligands that alter transport or chemical reaction properties [21,25,26]. These manipulations have been intended to achieve selective transport and accumulation of platinum compounds in the DNA of tumor cells. The notion that estradiol-linked platinum complexes might be selectively enriched in ER + tumor cells led to the design and evaluation of platinum compounds with affinity for the ER [19,18,15,27].

We were not solely interested, however, in improving the transport properties of the platinum-warhead complex into cells, although that mechanism would certainly be beneficial. The key feature of the designed genotoxicant, (Est-en)$PtCl_2$, is the linker that adjoins the warhead and ligand portions of the molecule. This linker was designed to permit the steroid portion to interact with the ER even after the platinum warhead has reacted with DNA (Figure 3). Thus the molecule was designed to combine some features of molecules designed by others that provide enhanced bioavailability, and new features that incorporate different mechanisms of toxicity, namely DNA repair inhibition and quenching of transcriptional responses.

At present, three non-mutually exclusive models are proposed to explain the enhanced toxicity of the platinum-steroid conjugate: (i) preferential uptake and sequestration of (Est-en)$PtCl_2$ into ER positive cells, (ii) shielding of DNA adducts of (Est-en)$PtCl_2$ from DNA repair enzymes by the ER, and (iii) hijacking of the growth promoting transcriptional activity of the ER by (Est-en)$PtCl_2$-DNA adducts.

Regardless of mechanism, the impact of the steroid ligand as a structural feature to target the toxicity of (Est-en)$PtCl_2$ is very significant, as indicated in Figure 4A. This compound is less reactive with DNA *in vitro* than the ligand-less control, (Bz-en)$PtCl_2$, yet it shows strikingly enhanced toxicity against an ER positive ovarian cancer line. Likewise, increased toxicity of (Est-en)$PtCl_2$ towards the ER+ breast cancer cell line MCF-7 (compared to ER- MDA-MB231; Figure 4C) suggests that one aspect of its mechanism of action involves the ability to bind to the ER. The fact that we observe no differences at >20 uM could represent a threshold at which the number of DNA adducts overwhelms the repair capacity in either cell line – in which case diminished repair in the ER+ line would not lead to greater toxicity. While detailed mechanistic studies have not been done on (Est-en)$PtCl_2$, the data in Figure 4B provide insight into the cellular target responsible for toxicity. The greater toxicity of (Est-en)$PtCl_2$ against DNA repair deficient cells than against wild type cells indicates that the compound is indeed reaching its intended target, DNA. Future studies will investigate the relative contributions of the hypothetical mechanisms of DNA repair shielding and steroid receptor transcriptional hijacking along with cellular uptake to the overall toxicity of (Est-en)$PtCl_2$ in steroid receptor positive cancer cells.

Acknowledgments

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Abbreviations

DMF	dimethylformamide

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DTT	dithiothreitol
ER	estrogen receptor
ESI	electron spray ionization
FBS	fetal bovine serum
HRMS	high resolution mass spectrometry
LBD	ligand binding domain
NER	nucleotide excision repair
RBA	relative binding affinity
TBE	tris-borate-EDTA

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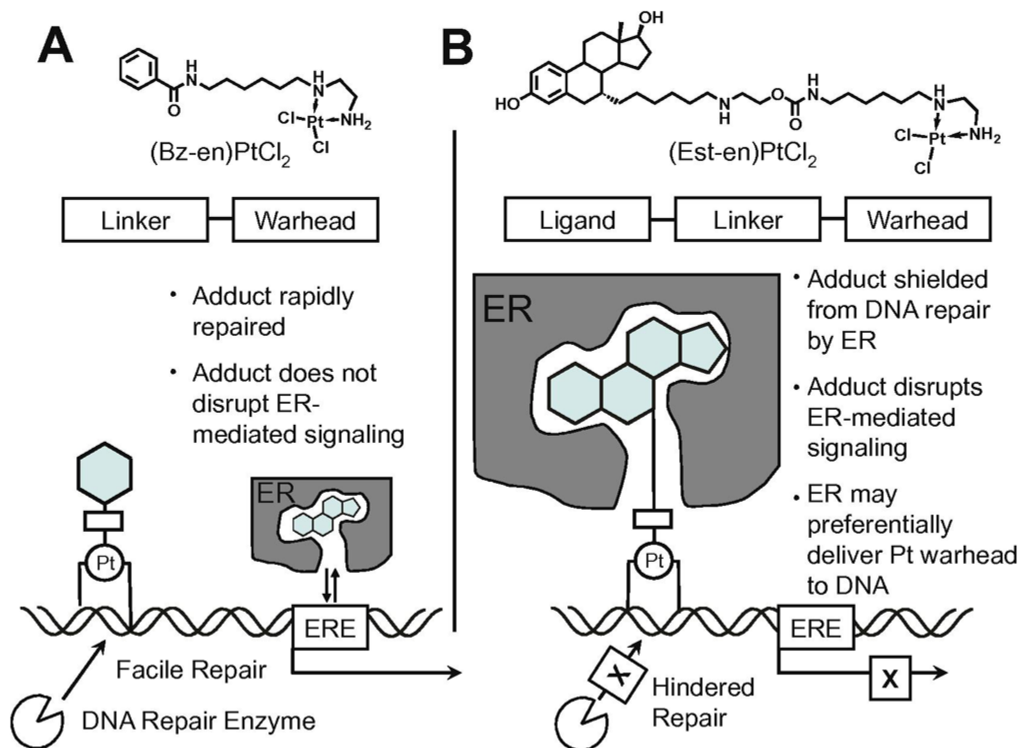

Figure 1.
Structures and proposed mechanisms of toxicity of Pt(II) complexes. A. The (Bz-en)$PtCl_2$ compound, which does not bind to the ER, forms DNA adducts that can be repaired and does not antagonize ER function. B. The (Est-en)$PtCl_2$ compound contains a ligand that enables the ER to bind to DNA adducts and prevent repair. Association of the ER with adducts also disrupts transcription of ER-regulated genes.

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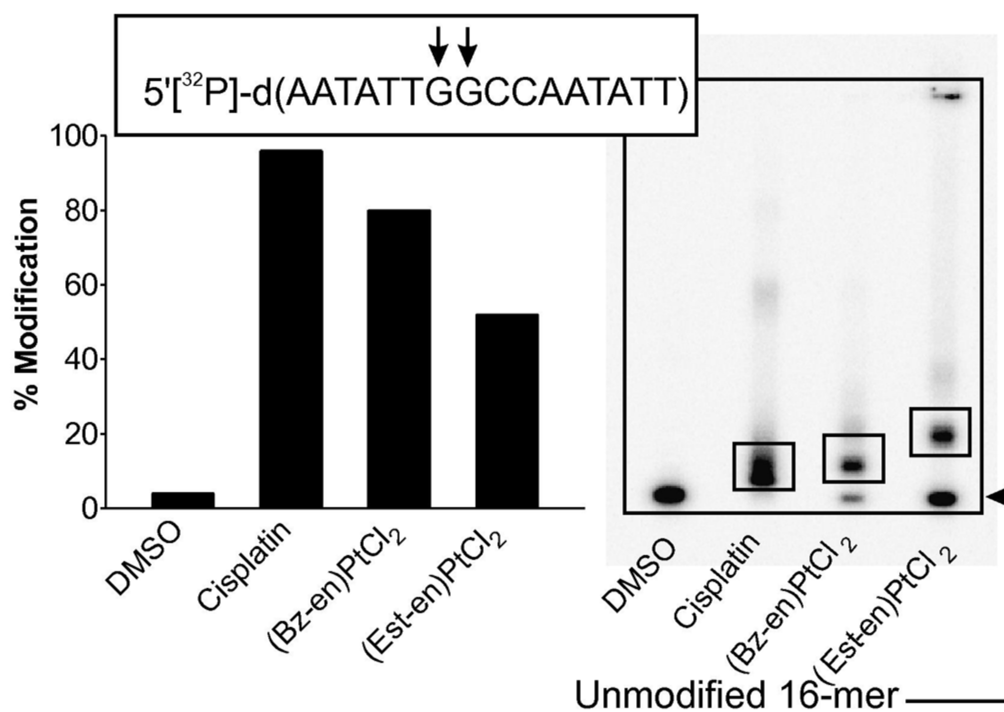

Figure 2.

Covalent modification of a 16-mer deoxyoligonucleotide by platinum compounds: (Right Panel) Separation of modified and unmodified DNA by gel electrophoresis; (Left Panel) Percentage of DNA modified by each compound as determined by PhosphorImager analysis. (Representative of two independent experiments.)

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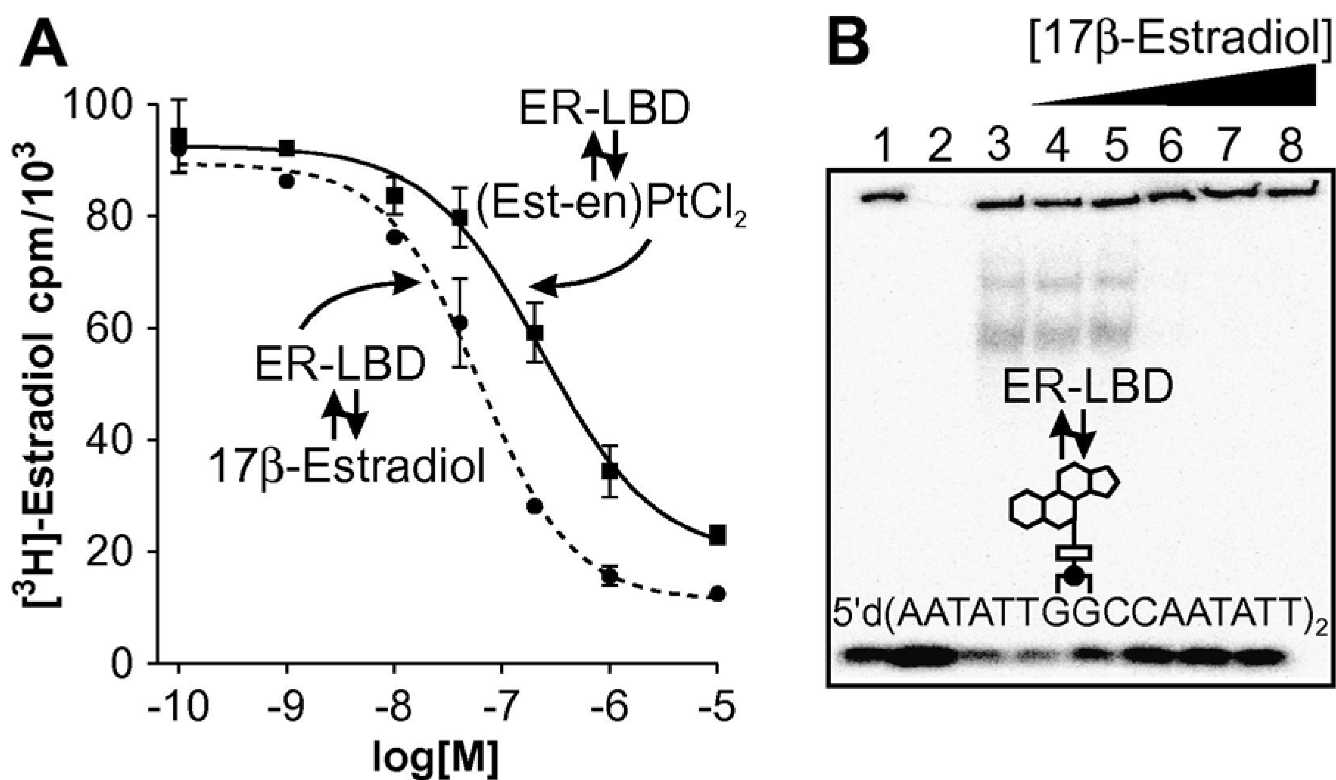

Figure 3.
Ability of (Est-en)$PtCl_2$ and (Est-en)$PtCl_2$-DNA adducts to bind to the hER-LBD: A. Competitive binding of 17β-estradiol (-•-) or (Est-en)$PtCl_2$ (-■-) with [^{3}H]-17β-estradiol for the hER-LBD (Points, mean ± S.D.). B. Gel mobility shift assay: Lane 1, (Est-en)$PtCl_2$-modified DNA; Lane 2, unmodified DNA + hER-LBD; Lane 3, (Est-en)$PtCl_2$-modified DNA + hER-LBD; Lanes 4–8 (Est-en)$PtCl_2$-modified DNA + hER-LBD + 17β-estradiol (0.13 – 13 uM) (Representative of three independent experiments).

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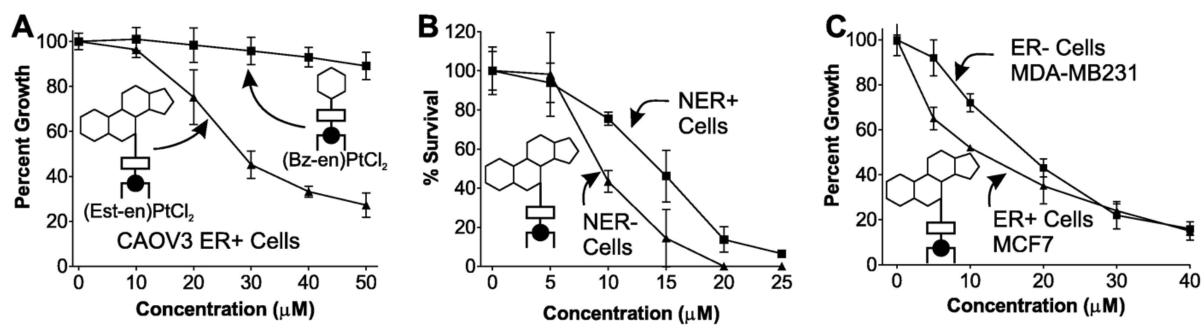

Figure 4.

Effect of platinum compounds on cell survival: A. Cytotoxicity of (Est-en)$PtCl_2$ (-▲-) and (Bz-en)$PtCl_2$ (-■-) towards CAOV-3 cells as measured by growthinhibition. B. Cytotoxicity of (Est-en)$PtCl_2$ towards NER-deficient XPA cells (-•-) and NER-proficient HeLa cells (-◆-) as measured by clonal survival. C. Cytoxicity of (Est-en)$PtCl_2$ towards ER-, MDA-MB231 (-■-) and ER+ MCF-7 (-▲-) breast cancer cells as measured by growth inhibition. Points, mean of two independent experiments performed in triplicate ± S.D.

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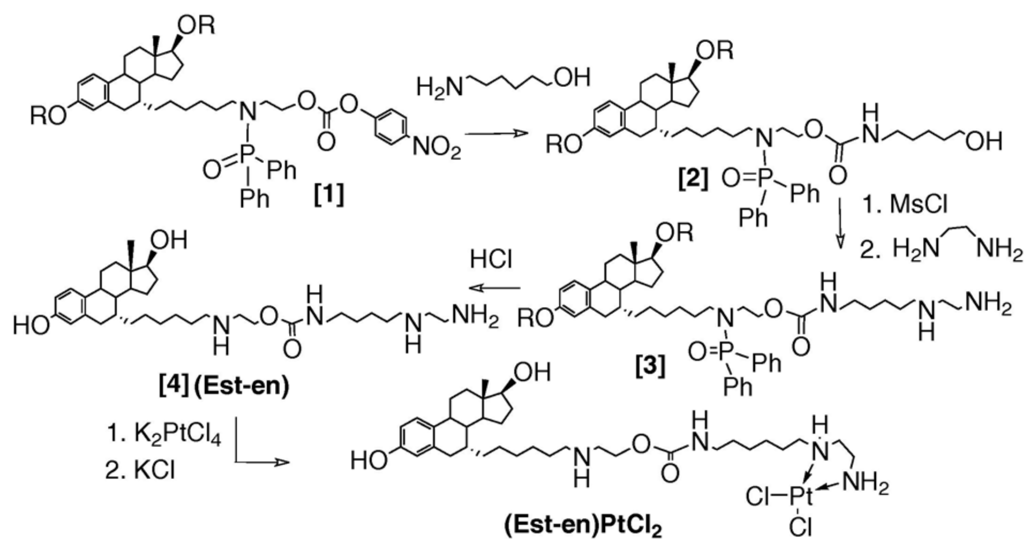

Scheme 1.
Strategy for the preparation of (Est-en)$PtCl_2$

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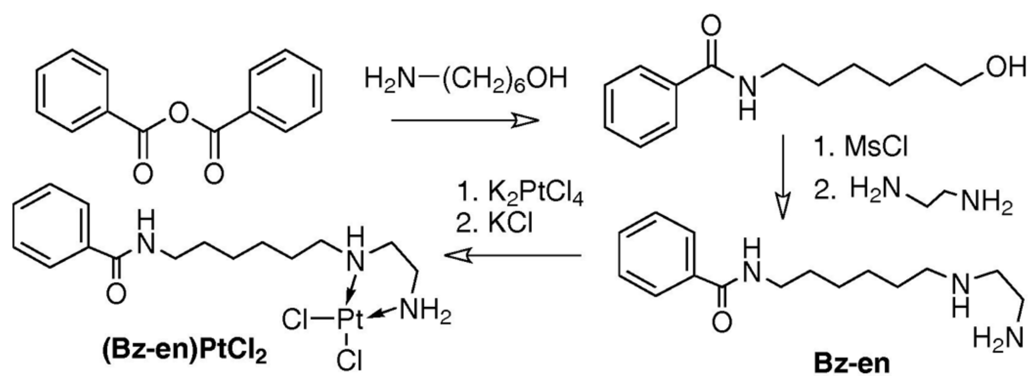

Scheme 2.

Strategy for the preparation of (Bz-en)$PtCl_2$