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Maximizing Synergistic Activity When Combining RNAi and Platinum-based Anticancer Agents

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ABSTRACT: RNAi approaches have been widely combined with platinum-based anticancer agents to elucidate cellular responses and to target gene products that mediate acquired resistance. Recent work has demonstrated that platination of siRNA prior to transfection may negatively influence RNAi efficiency based on the position and sequence of its guanosine nucleosides. Here, we used detailed spectroscopic characterization to demonstrate rapid formation of Pt-guanosine adducts within 30 min after co-incubation of oxaliplatin [OxaPt(II)] or cisplatin [CisPt(II)] with either guanosine monophosphate or BCL-2 siRNA. After 3 h of exposure to these platinum(II) agents, >50% of BCL-2 siRNA transcripts were platinated and unable to effectively suppress mRNA levels. Platinum(IV) analogs [OxaPt(IV) or CisPt(IV)] did not form Pt-siRNA adducts but did display decreased *in vitro* uptake and reduced potency. To overcome these challenges, we utilized biodegradable methoxyl-poly(ethylene glycol)-block-poly-(ϵ -caprolactone)-block-poly(L-lysine) (mPEG-b-PCL-b-PLL) to generate self-assembled micelles that covalently conjugated OxaPt(IV) and/or electrostatically complexed siRNA. We then compared multiple strategies by which to combine BCL-2 siRNA with either OxaPt(II) or OxaPt(IV). Overall, we determined that the concentrations of siRNA (nM) and platinum(II)-based anticancer agents (μ M) that are typically used for *in vitro* experiments led to rapid Pt-siRNA adduct formation and ineffective RNAi. Co-incorporation of BCL-2 siRNA and platinum(IV) analogs in a single micelle enabled maximal suppression of BCL-2 mRNA levels (to < 10% of baseline), augmented the intracellular levels of platinum (by \sim 4x) and the numbers of resultant Pt-DNA adducts (by >5x), increased the cellular fractions that underwent apoptosis (by \sim 4x), and enhanced the *in vitro* anti-proliferative activity of the corresponding platinum(II) agent (by 10-100x depending on the cancer cell line). When combining RNAi and platinum-based anticancer agents, this generalizable strategy may be adopted to maximize synergy during screening or for therapeutic delivery.

INTRODUCTION

Since the late 1970's, platinum(II)-based anticancer agents, including cisplatin and oxaliplatin, have become the most common class of chemotherapies used worldwide.¹⁻² Whereas initial clinical response rates to platinum-containing regimens are high, the duration of activity in patients with metastatic cancers is generally short.³⁻⁴ The various mechanisms by which these tumors develop resistance and acquire

proliferative advantages are areas of very active investigation.⁵⁻¹¹ RNAi approaches have been used to screen for gene products that mediate these tumorigenic properties,¹²⁻¹⁶ to validate putative drug targets,¹⁷⁻¹⁹ and to develop therapeutic constructs.²⁰⁻²² Many of these experiments employ double-stranded small interfering RNAs (siRNAs) or microRNAs (miRNAs) that are synthesized with near-perfect complementarity to specific messenger RNA (mRNA) sequences. The siRNA (or

1 miRNA) is delivered into the cell via a lipid or polymer-based
2 transfection reagent, whereby it binds to the RNA-induced
3 silencing complex (RISC) and leads to cleavage of its target
4 mRNA.²³ The duration and level of mRNA knockdown are de-
5 pendent on the cell type and the concentration of the siRNA
6 that is delivered.²⁴ When combining siRNA and platinum(II)-
7 based anticancer agents, both species must be introduced
8 into the cellular environment within a narrow temporal win-
9 dow in order to capture synergistic treatment effects.¹²⁻¹⁹ The
10 success of such endeavors relies implicitly on the assumption
11 that the siRNA remains active, even for a few hours, when
12 utilized in combination with platinum-based therapies.

13 It has long been appreciated that platinum(II)-based an-
14 ticancer agents interact with DNA nucleobases, forming intra-
15 and interstrand cross-links through the generation of adducts
16 at the N7 positions of both guanine (G) and/or adenine (A).²⁵⁻
17 ²⁷ The most commonly formed cross-links include d(pGpG)
18 (i.e. the 1,2-guanosine-guanosine intrastrand cross-link) and
19 d(pApG) (i.e. the 1,2-adenosine-guanosine intrastrand cross-
20 link), which account for 47-50% and 23-28% of adducts, re-
21 spectively.^{25, 28} With increasing quantities of intracellular Pt-
22 DNA adducts, cell cycle arrest and eventually apoptosis en-
23 sue.²⁹⁻³² Given the base homology in RNA and DNA, recent
24 investigations have sought to determine the presence, as well
25 as to ascertain the biological consequences, of Pt-RNA ad-
26 ducts that may be similarly generated.

27 Cytoplasmic RNA is a major binding pool for platinum(II)-
28 based anticancer agents; and, the concentrations of Pt-RNA
29 adducts that are formed are ~4-20 times greater than those
30 of Pt-DNA adducts, following intracellular uptake of
31 cisplatin.³³⁻³⁵ Further, platinum(II)-containing agents readily
32 form bidentate GG, GA/AG, and GXG (X≠G) adducts with siR-
33 NA and miRNA.³⁶⁻³⁷ Whereas platination of neither the sense
34 strand nor the seed portions of the antisense strand seems to
35 interfere with silencing activity, Pt-RNA adducts that are
36 formed in non-seed regions of the antisense strand induce
37 thermal destabilization of the duplex of siRNA (or miRNA),
38 which leads to decreased silencing of the complementary
39 target mRNA.³⁶⁻³⁷ The results of these investigations raise
40 important questions as to whether the concentrations of siR-
41 NA and platinum(II)-based anticancer agents that are admin-
42 istered in combination treatments are sufficient to form Pt-
43 siRNA adducts in the cellular environment. siRNA inactivation
44 through Pt-siRNA adduct formation could have implications
45 for the results of numerous preclinical studies that have uti-
46 lized such approaches either for discovery or for therapeutic
47 intervention.¹²⁻²² Moreover, different strategies by which to
48 combine these two reactive species have not, hitherto, been
49 rigorously compared.

50 Here, we sought to determine a generalizable method by
51 which to combine RNAi and platinum-containing therapies in
52 order to prevent premature siRNA inactivation and to maxim-
53 ize synergistic activity. We utilized oxaliplatin [OxaPt(II)] and
54 cisplatin [CisPt(II)] as model platinum(II)-based anticancer
55 agents. To advance our design, we also synthesized chemical-
56 ly inert platinum(IV) derivatives, denoted by OxaPt(IV) and
57 CisPt(IV),³⁸⁻⁴⁰ respectively (**Schemes 1A, S1, and S2**). Plati-

num(IV) analogs are activated by reduction to their corre-
sponding platinum(II) species only after intracellular uptake
within cancer cells, which contain supraphysiologic levels of
glutathione and ascorbic acid (~100 to ~1000 fold excess over
normal cells).^{39, 41-45} Employing platinum(IV)-containing agents
offers an attractive means by which to overcome challenges
associated with combining RNAi and platinum(II)-based ther-
apies for discovery research and cancer treatment. The MCF-
7 breast cancer and the OVCAR4 ovarian cancer cell lines
were mainly employed as the *in vitro* model systems in our
studies; and, the anti-apoptosis protein BCL-2 was selected as
the target for RNAi. BCL-2 upregulation contributes to the
development of acquired CisPt(II) resistance in various solid
tumor malignancies.^{5, 46} Here, we used a BCL-2 siRNA that
contained multiple GG and GA regions but only a single GA
sequence outside of the seed portion of the antisense strand.
Platination at this latter site was expected to result in maxi-
mal thermal destabilization with decreased mRNA silencing³⁶⁻
37 (**Scheme 1A**). A micelle-based transfection reagent com-
prised of the triblock copolymer of methoxyl-poly(ethylene
glycol)-block-poly(ϵ -caprolactone)-block-poly(L-lysine)
(mPEG-b-PCL-b-PLL) was used to deliver OxaPt(IV) and/or
siRNA (**Scheme 1B**). This vehicle was also employed to vali-
date an optimal strategy for combining RNAi and platinum-
based anticancer agents (**Scheme 1C**).

EXPERIMENTAL SECTION

Materials. mPEG-b-PCL-b-PLL was synthesized as previ-
ously described.⁴⁷ Its structure was verified by ¹H-NMR spec-
troscopy and consisted of mPEG₁₁₄-b-PCL₂₅-b-PLL₂₅, where the
subscript denotes the degree of polymerization of each indi-
vidual monomer in a given block (**Figure S1**). This triblock
copolymer is hereafter abbreviated "P". N-
hydroxysuccinimide (NHS), 1-(3-dimethyl aminopropyl)-3-
ethylcarbodiimide hydrochloride (EDC·HCl), guanosine 5'-
monophosphate disodium salt (5'-GMP), and sodium ascor-
bate were purchased from Sigma-Aldrich. OxaPt(II) and
CisPt(II) were purchased from ChemiChem International De-
velopment Co., Ltd (Shenzhen, China). OxaPt(IV) analogs were
synthesized and characterized by nuclear magnetic resonance
(NMR) spectroscopy and mass spectrometry (MS), following
procedures described below and in our previous studies⁴⁸
(**Figures S2-S6**). The CisPt(IV) derivatives were similarly syn-
thesized and characterized as described.^{38, 40} All other chemi-
cals and solvents were used without further purification. Con-
trol (c-) siRNA that targeted the sequence 5'-
GGGUAAGUGUCCUACU-GAAGU-3', BCL-2 siRNA that targeted
the sequence 5'-UGUGGAUGACUGAGUACC UGA-3', and
Alexa488-labeled BCL-2 siRNA (Alexa488 siRNA) were pur-
chased from Integrated DNA Technologies (Coralville, Iowa);
the c-siRNA did not match any known sequence in the human
genome. Luciferase GL3 siRNA was purchased from
GenePharma Co. Ltd. (Shanghai, China).

General measurements. NMR, MS, graphite furnace
atomic absorption spectroscopy (GFAAS), and UV-VIS spec-
troscopy were used to study the reactions of various platinum

species with either 5'-GMP or BCL-2 siRNA (see *Supplemental Materials and Methods* in the Supporting Information). In brief, ^1H , ^{13}C , and ^{195}Pt NMR spectra at RT were measured at 400, 100, and 86 MHz, respectively, using a Bruker NMR spectrometer (Bruker Corporation, Billerica, MA). MS measurements were performed on a Quattro Premier XE system equipped with an electrospray interface (ESI-MS; Waters, Milford, MA) as well as on a matrix-assisted laser-desorption ionization and time-of-flight MS (MALDI/TOF-MS) instrument (Bruker model MicroFlex). GFAAS and UV-Vis measurements were conducted using an AAnalyst 600 GFAAS instrument (Perkin Elmer, Waltham, MA) and an Infinite[®] M200 Pro microplate reader (Tecan Systems, Inc., CA, USA), respectively. GFAAS was also used to determine the Pt content in all micelle formulations. Inductively coupled plasma MS (ICP-MS; ICP-MS 7900, Agilent Technologies, CA, USA) was used for quantitative determination of trace levels of Pt in cancer cells and to quantify the numbers of intracellular Pt-DNA adducts that were formed after treatment with various experimental groups.

Cell Culture. MCF-7 (human breast cancer), LUC⁺ MCF-7 (luciferase expressing MCF7), OVCAR4 (human ovarian cancer), A2780 (CisPt(II)-sensitive), and A2780DDP (CisPt(II)-resistant) cell lines were obtained from ATCC and cultured at 37 °C, 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Carlsbad, CA; MCF-7) or RPMI 1640 (LUC⁺ MCF-7, OVCAR4, A2780, and A2780DDP) supplemented with 10% fetal bovine serum (FBS, Gibco).

Quantitative Reverse Transcription (RT-q)PCR. BCL-2 siRNA was first pre-incubated with platinum(II) or platinum(IV) species for various time periods and subsequently transfected into MCF-7 and OVCAR4 cells in 6-well plates, using an RNAiMAX Kit and by following the manufacturer's instructions (Invitrogen, Thermo-Fisher, USA). Briefly, 48 h after transfection, total RNA was isolated, using an RNeasy mini-kit (Qiagen, Germantown, MD), and quantified by NanoDrop[™] (NanoDrop 2000, Thermo Scientific). A total of 300 ng of mRNA was subsequently subjected to RT-qPCR analysis, utilizing primers that targeted BCL-2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the SYBR Premix Ex (Clontech, Mountain View, CA), and an Applied Biosystems StepOne Real-Time PCR System (Thermo Scientific). Relative gene expression values were determined by the $\Delta\Delta\text{CT}$ method using StepOne Software v2.1 (Applied Biosystems, Foster City, CA). Data are presented as fold differences in mRNA expression for BCL-2 normalized to the housekeeping gene GAPDH and were obtained by employing a standard curve; results are reported relative to untreated (control) cells. The sequence of the primers used for BCL-2 and GAPDH are as follows: BCL-2 forward – 5'-CTGCACCTGACGCCCTTACC-3'; BCL-2 reverse – 5'-CACATGACCCACCGAACTCAAAGA-3'; GAPDH forward – 5'-TTCACCACCATGGAGAAGGC-3'; and GAPDH reverse – 5'-GGCATGGACTGTGGTCA TGA-3' (Integrated DNA Technologies). The specificity of each set of primers was verified by melting curve analysis. Additional details may be found in *Supplemental Materials and Methods* in the Supporting Information.

Formation and Characterization of Polymeric Micelles. P-based micelles (M(P)) were prepared by an established nanoprecipitation method.⁴⁷ In brief, P (50 mg) was dissolved in DMF (10 mL); water (50 mL) was then added drop-wise into the flask under continuous agitation, forming M(P) in suspension. The suspension was then dialyzed against water to remove the organic solvent and subsequently freeze-dried for storage. The critical micelle concentrations (CMCs) of M(P) and other micellar formulations (*vide infra*) were measured as previously reported,⁴⁹ using Nile red as the fluorescent probe. Particle sizes and zeta potentials were measured using a Nano ZS90 Zetasizer equipped with a vertically polarized He-Ne laser (Malvern Instruments, Malvern, PA). Images of various micelle suspensions were obtained using a JEOL-1100 transmission electron microscope (JEOL USA, Peabody, MA).

Conjugation of OxaPt(IV) to M(P). EDC/NHS chemistry was employed to conjugate OxaPt(IV) to P via an amide bond. Briefly, EDC·HCl (0.191 g, 1 mmol) and NHS (0.115 g, 1 mmol) were dissolved in deionized H₂O under stirring. OxaPt(IV) (0.42 g, 0.8 mmol) was then added to the solution. After the suspension mixture became clear, P (0.5 g; containing 1.25 mmol NH₂ groups) was added to the reaction mixture (100 mL) and stirred at RT for 24 h; the suspension was then dialyzed against water for an additional 12 h and lyophilized to isolate OxaPt(IV)-conjugated micelles (M(OxaPt(IV))). The Pt content of the micelles was measured by GFAAS.

Complexation of siRNA with Polymeric Micelles. Suspensions of M(P) or M(OxaPt(IV)) were diluted with Opti-MEM media[®] (Invitrogen) to different concentrations, varying the final numbers of amino groups in solution. Equal volume solutions containing BCL-2 or c-siRNA were then added to the micelles, varying the initial ratios of free amines to phosphates (N/P) in suspension. siRNA-complexed micelles were then formed by gentle pipetting and allowed to equilibrate for 30 min at RT. For electrophoresis-retardation analyses, complexes formed from either M(P) or M(OxaPt(IV)) and siRNA at different initial N/P ratios, ranging from 0 to 4, were prepared for a fixed concentration of siRNA (1 mM). The samples were run on a 1% agarose gel in 0.5 mM tris-borate-EDTA buffer (TBE buffer; 89 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8.3) at 80 V and for 30 min. Bands containing free and micellar-bound siRNA were visualized using a UV illuminator (Tanon 3000, Biotanon, Shanghai, China) after ethidium bromide staining.

To determine the maximal loading of siRNA and the efficiency of micellar complexation, Alexa488-labeled BCL-2 siRNA was complexed with M(P) or M(OxaPt(IV)) at different initial N/P ratios, varying from 0 to 16, and subsequently quantified by both UV-VIS and fluorescence spectroscopy. To determine the amounts of micellar-complexed siRNA necessary for effective RNAi, Luciferase GL3 siRNA was complexed with M(P) or M(OxaPt(IV)) at an initial N/P ratio of 8; and, the percentage of initial luciferase mRNA expression (relative to controls) was quantified as a function of different concentrations of Luciferase GL3 siRNA that were used for transfection, ranging from 25-150 nM. Detailed information on micelle preparation and various siRNA-complexed formulations may

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be found in *Supplemental Materials and Methods* in the Supporting Information.

Intracellular Uptake of Fluorophore-labeled Micelles. For confocal microscopy experiments, MCF-7 and OVCAR4 cells were plated on coverslips in 6-well plates (4×10^5 cells/well) and cultured with RPMI 1640 supplemented with 10% FBS for 24 h. The cells were then incubated either for 30 min or for 4 h with micelles that were electrostatically complexed with Alexa488-labeled BCL-2 siRNA (50 nM) and that were covalently conjugated to Cy5.5 with and without OxaPt(IV), forming M(Cy5.5/Alexa488 siRNA) and M(Cy5.5/Alexa488 siRNA/OxaPt(IV)), respectively. After removal of the media, the cells were then washed twice with cold phosphate buffered saline (PBS, pH 7.4, 0.01 M) and fixed with 4% formaldehyde (Sigma-Aldrich, St. Louis, USA). To label the cell nuclei, samples were incubated with 1 μ L of DAPI (1 mg/mL; Sigma-Aldrich, St. Louis, USA) for 15 min in PBS and were subsequently rinsed extensively with PBS. Slides were mounted on coverslips and imaged, using a confocal laser scanning microscopy system (Olympus FV1000, Olympus Corporation, Bridgeport, CT).

For flow cytometry measurements, MCF-7 and OVAR4 cells were similarly cultured in 6-well plates (4×10^5 cells/well) prior to incubation with M(Cy5.5/Alexa488 siRNA) or M(Cy5.5/Alexa488 siRNA/OxaPt(IV)) either for 30 min or for 4 h. The cells were washed twice with cold PBS, lysed with trypsin-EDTA solution, collected by centrifugation (1500 rpm, 5 min), and, finally, analyzed for fluorescence content, using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Cells with fluorescence above the threshold intensity for untreated cells (i.e. blank) were quantified and compared. The percentage of these cells in the total population was considered as a measure of uptake efficiency. Data were analyzed using FlowJo software Version 7.6.2 (Tree Star, Ashland, OR).

Quantification of Intracellular Uptake of Platinum Species. MCF-7 cells were seeded in 12-well plates (1×10^5 cells/well) and incubated overnight in DMEM containing 10% FBS (1 mL). Various platinum-containing agents (OxaPt(II), OxaPt(II) + M(c-siRNA), OxaPt(II) + M(BCL-2), M(OxaPt(IV)/c-siRNA), or M(OxaPt(IV)/BCL-2)) were then added to each well (100 μ L aliquots and to final Pt concentration of 10 μ M). For siRNA-containing treatments, the final siRNA concentration in each well was 100 nM. After either 1 or 4 h of incubation, media were removed and the cells were thoroughly washed x5 with cold PBS. The cells were then lysed with nitric acid prior to ICP-MS measurements. For quantification of Pt-DNA adducts, cells were similarly incubated with each platinum-containing agent but for 24 h. The cells were then washed and the DNA was extracted using a Genome DNA extraction kit, following the manufacturer's instructions (Thermo Scientific, USA). DNA concentrations were determined by NanoDrop™; and, the quantity of DNA-bound Pt was determined by ICP-MS.

In vitro Cytotoxicity. MCF-7, OVCAR4, A2780, and A2780DDP cells were seeded in 96-well plates (5×10^3 cells/well). DMEM (MCF-7; 100 μ L) or RPMI-1640 (OVCAR4,

A2780, A2780DDP; 100 μ L) supplemented with 10% FBS was added to each well. The cells were then incubated at 37 °C for 24 h prior to the addition of OxaPt(II), OxaPt(IV), or M(OxaPt(IV)) with or without micellar-complexed BCL-2 or c-siRNA. For siRNA-containing treatments, an equivalent concentration of siRNA (100 nM) was added to each well. The cells were subsequently allowed to incubate at 37 °C for 72 h prior to evaluation of cellular viability, using the colorimetric MTT assay. In brief, MTT reagent in PBS buffer (5 mg/mL; 20 μ L) was added to each well and allowed to incubate for 4 h. DMSO (150 μ L) was then added to replace the MTT-containing media. After gentle agitation (5 min), the absorbance of each well at 570 nm was recorded on a Bio-Rad Plate Reader (Hercules, CA). ABS at 650 nm was set as background and used for subtraction. All experiments were conducted in triplicate.

Cellular Apoptosis Assays. MCF-7 cells were cultured in 6-well plates (5×10^5 cells/well) and incubated with various treatments at a fixed Pt concentration (20 μ M). After 36 h, apoptotic cells were detected by flow cytometry, using the Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, San Jose, CA); the results were analyzed using FlowJo software Version 7.6.2.

RESULTS AND DISCUSSION

Monitoring the Reaction of siRNA with Various Platinum-based Species. OxaPt(II) and CisPt(II) bind preferentially to guanine and adenine bases in both DNA and RNA.^{25-27, 33-35} To compare the relative reactivity of OxaPt(IV) and CisPt(IV) to their platinum(II) counterparts, we first incubated each compound for different time intervals with 5'-GMP (i.e. 30 min, 1 h, 3 h, 6 h or 12 h) and in conditions that would mimic a typical *in vitro* RNAi experiment (i.e. co-incubation of the two species at 37 °C). Formation of Pt-(5'-GMP)₂ adducts was readily detectable by ESI-MS when 5'-GMP was incubated with OxaPt(II) but not with OxaPt(IV) (**Figures 1A, 1B and S7**). These findings were further confirmed by MALDI/TOF-MS measurements of the same suspensions, showing that Pt-(5'-GMP)₂ adducts were detected within 1 h in solutions containing OxaPt(II) or CisPt(II) but not OxaPt(IV) or CisPt(IV) (**Figures S8 and S9**). To further quantify differences in the kinetics of Pt-(5'-GMP)₂ adduct formation, ¹H NMR spectra were obtained for 5'-GMP solutions at various time intervals after the addition of different platinum(II) or platinum(IV) species and under identical conditions. The chemical shifts of the H8 proton on the guanosine base (8.5 ppm in the (diamino-1,2-cyclohexane)-Pt(5'-GMP)₂, DACHPt(5'-GMP)₂) adduct vs. 8.09 ppm in unreacted 5'-GMP) were monitored at several time points after co-incubation of 5'-GMP (10 mM) with either OxaPt(II) or OxaPt(IV) (5 mM) (**Figure S10**). By integrating the ratio of the two peaks at each time point, the relative amounts of Pt-bound 5'-GMP to free 5'-GMP were determined in solutions containing OxaPt(II) (green) or OxaPt(IV) (red) and plotted as a function of time (**Figure 1C**). The results showed that approximately 10% of the 5'-GMP pool consisted of DACHPt(5'-GMP)₂ adducts after 30 min of incubation with

OxaPt(II), that the number of adducts that were formed increased over the first 6 h, reaching 40% of the total pool, and that they continued to increase over time. Similar early formation and time dependent increases in the numbers of Pt(NH₃)₂(5'-GMP)₂ adducts were seen when 5'-GMP was incubated with CisPt(II) but not with CisPt(IV) (**Figure S11**). Taken together, these model reactions of 5'-GMP with various platinum species support a rationale for combining RNAi approaches with platinum(IV)- as opposed to platinum(II)-based anticancer agents, which would otherwise react with similar nucleosides in RNA macromolecules and which would lead to inactivation of the siRNA or miRNA species.

We next sought to ascertain the kinetics of Pt-siRNA adduct formation by using the anti-sense strand of BCL-2 siRNA. This single-stranded (ss-)siRNA (50 nM) was incubated with different platinum(II)- and platinum(IV)-based anticancer agents (500 nM), which were present in a 10-fold excess in order to account for the relative concentrations of RNA to platinum species that are typically employed in RNAi and cytotoxicity experiments, respectively.¹²⁻¹⁹ MALDI/TOF-MS spectra were taken at various time points after their co-incubation at 37 °C. These experiments demonstrated that Pt-siRNA adducts could be detected as early as 1 h after the incubation of ss-siRNA with OxaPt(II) (**Figures 1D** and **S12**). Three major peaks were detected and attributed to unreacted BCL-2 ss-siRNA (*m/z*=7227.888), BCL-2 ss-siRNA with one DACHPt²⁺ adduct (*m/z*=7535.592), and BCL-2 ss-siRNA with two DACHPt²⁺ adducts (*m/z*=7841.982). Note, the molecular weight of DACHPt was 309.08 and the Δ*m/z* of ss-siRNA upon formation of the siRNA-DACHPt²⁺ mono adduct was 307.704; ss-siRNA lost two H⁺ after binding with OxaPt(II) and added another H⁺, keeping it at the +1 charge state. The unmodified BCL-2 ss-siRNA had a molecular weight of 7224.401 and displayed a +1 charged mass peak at *m/z*=7226.122 (**Figure 1E**, inset). The margin of error for the MALDI/TOF-MS was +/- 0.1%; thus, for free ss-siRNA a variation of 0.722 Dalton units was within the range of precision for the MALDI/TOF-MS instrument. Compared to OxaPt(II), CisPt(II) is known to undergo more facile aquation of its planar ligands, decreasing its relative stability and increasing its reactivity towards ss-siRNA. When BCL-2 ss-siRNA was incubated with CisPt(II), numerous Pt-siRNA adducts were detected as early as 30 min after CisPt(II) addition (**Figure S13**). Notably, no Pt-siRNA adducts were detected in solutions of BCL-2 ss-siRNA containing either OxaPt(IV) or CisPt(IV) and even after 24 h of co-incubation under similar physiological conditions (pH 7.4; **Figure 1E**, **S12** and **S13**).

GFAAS measurements of ds-siRNA that had been isolated after co-incubation with various platinum species were also conducted to quantify the amounts of siRNA-bound Pt over time. These results indicate that a total of ~4 Pt atoms bound to ds-siRNA within 3-6 h after incubation with OxaPt(II) (**Figure S14**), which corresponded to platination of ~40% of the G nucleotides of the ds-siRNA (**Figure 1F**). Notably, no Pt was detected by GFAAS even in ds-siRNA solutions that had been incubated with OxaPt(IV) for up to 12 h. These results were further corroborated by UV-Vis measurements of the time-

dependent platination of ds-siRNA, which has been shown to convert it to ss-siRNA, via monitoring of the hyperchromic (i.e. Δ*A*-value at λ = 260 nm) and bathochromic shifts (from a maximum at λ ca. 258 nm to a maximum at λ ca. 260 nm) that follow nucleic acid duplex melting.^{37b} By first varying the initial concentrations of ds-siRNA (from 0.5-2 μM) for a fixed and excess (> 10-fold) amount of CisPt(II) (20 μM), we verified that ss-siRNA was generated via a pseudo-first order reaction and that the apparent second-order rate constant (*k*_{2,app}) could be derived from the linear relationship between the observed rate constant (*k*_{obs}) and the initial concentration of the platinum species (see **Figure S15** and **Table S1**):

$$[ss-siRNA]_t = [ds-siRNA]_o * (1 - e^{-(k_{obs} * t)})$$

$$k_{2,app} = k_{obs} / [\text{platinum species}]_o$$

UV-Vis spectroscopy was similarly used to observe the reactions of other platinum species with ds-siRNA, verifying that the *k*_{2,app} for OxaPt(II), CisPt(IV), and OxaPt(IV) were approximately 40%, 25% and 2% that of CisPt(II) (**Table 1**). Moreover, when ds-siRNA was incubated with CisPt(II) at concentrations that are typically employed for *in vitro* cytotoxicity experiments (i.e. > 10 μM), approximately 50% of the ds-siRNA transcripts were converted to ss-siRNA by 1-3 h. Notably, other investigators have previously measured higher reaction rate constants for CisPt(II) with ds-RNA, whereby similar results would be expected in less than 1 h of co-incubation (**Table 1** and ³³⁻³⁵). Given the decreased reactivity of OxaPt(II) when compared to CisPt(II), analogous levels of ds-siRNA deactivation (from conversion to ss-siRNA via platination under identical reaction conditions) could be expected between 3-6 h of co-incubation. Notably, as both CisPt(IV) and OxaPt(IV) were even less active than OxaPt(II), neither significant ds-siRNA platination nor deactivation were expected to proceed in their presence and under identical *in vitro* reaction conditions.

To determine the exact sites of platination on a nucleic acid transcript, we next conducted MALDI/TOF-MS experiments using a model ss-oligonucleotide of DNA that contained a single GG site, verifying the formation of mono- and bis-Pt-DNA adducts in the presence of OxaPt(II) but not OxaPt(IV) (**Figure S16**). MALDI/TOF-MS was then performed after enzymatic cleavage of the antisense strand of BCL-2 siRNA by RNase A, either before (**Figure 1G**) or after incubation with OxaPt(II). These experiments demonstrated two distinct sites on the ss-siRNA transcript that were platinated with either one or two DACHPt moieties, including the 5'-AGGU-3' segment in the seed portion and the 5'-AGU-3' site in the non-seed portion of the anti-sense strand (**Figures 1H** and **S17**).

Platination of the non-seed portion of the anti-sense strand was expected to affect RNAi efficacy.³⁶⁻³⁷ To test this hypothesis, we studied changes in the relative silencing activity of BCL-2 ds-siRNA that was pre-incubated with different platinum(II) and platinum(IV) agents prior to cellular exposure. Using a fixed concentration of siRNA (7.5 nM), different concentrations of platinum-based species (either 0.75 or 7.5 μM), and various incubation times (ranging from 30 min to 24

h), we effectively varied the numbers of Pt-siRNA adducts that were present on the siRNA transcript prior to transfection. The siRNA was subsequently diluted (to a final concentration of 1.5 nM) and combined with the commercially available transfection reagent Lipofectamine[®] (RNAiMAX) for delivery to both MCF-7 and OVCAR4 cells. RT-qPCR of BCL-2 mRNA isolated from these cells at 48 h after transfection showed ineffective BCL-2 silencing (i.e. greater than 30% of baseline mRNA expression) by siRNA (1.5 nM) that had been pre-incubated with a 1000-fold excess of OxaPt(II) for > 3 h (**Figure 2** and **S18**). CisPt(II) was more reactive than OxaPt(II) and ineffective BCL-2 mRNA suppression was seen even after 30 min of co-incubation of BCL-2 siRNA with CisPt(II) under identical conditions. The activity of the siRNA was unperturbed by > 24 h of exposure to similar concentrations of either OxaPt(IV) or CisPt(IV). Note, both the concentrations and the relative ratios of siRNA to platinum-based species that were used for these studies were typical of those employed for *in vitro* RNAi and cytotoxicity experiments, respectively.¹²⁻¹⁹ Taken together, these experiments support a generalizable phenomenon whereby siRNA rapidly reacts with platinum(II) species that are present at > 1 μ M concentrations, leading to irreversible Pt-siRNA adduct formation and ineffective RNAi within as little as 1-3 h of co-incubation. These results further highlight the value of combining RNAi approaches with chemically-inert platinum(IV) prodrugs to circumvent these limitations.

Nanoparticle Characterization. There are many examples of utilizing nanoparticles for the delivery of siRNA along with different anticancer agents.⁵⁰⁻⁵³ Whereas several studies have attempted nanoparticle incorporation of both siRNA and CisPt(II),⁵⁴⁻⁵⁶ the results presented here reveal the advantages of utilizing a platinum(IV) agent to prevent siRNA platination and premature inactivation. To date, however, there have been only a few examples that utilize this latter approach.⁵⁷⁻⁵⁹ Further investigations were, therefore, necessary to identify the optimal strategy by which to combine RNAi and platinum(IV) species to maximize their individual activities and to harness their synergy. Accordingly, we devised a transfection reagent capable of intracellular delivery of both platinum(IV) prodrugs and siRNA. For this work, we chose mPEG-b-PCL-b-PLL (P), which self-assembled into biodegradable micelles with a hydrophobic PCL core and a corona consisting of hydrophilic mPEG and positively-charged PLL segments (M(P), where "M" stands for micelles; **Scheme 1B**). OxaPt(IV) was coupled to the PLL polymer through formation of an amide bond between the carboxyl group of its auxiliary ligand and the free amino group of lysine, forming micelles that contained OxaPt(IV) (M(OxaPt(IV))). Similarly, siRNA was complexed with unreacted PLL chains through electrostatic interactions, forming micelles that contained BCL-2 (M(BCL-2)) or c-siRNA (M(c-siRNA)) (**Scheme S3**). Both OxaPt(IV) and siRNA species were also combined in a single micellar construct (M(OxaPt(IV)/BCL-2) or M(OxaPt(IV)/c-siRNA), respectively), consisting of a hydrophilic mPEG corona, a middle layer comprised of PLL/siRNA, and a hydrophobic core of PCL and PLL-OxaPt(IV) conjugates. When co-incorporated within a single

micelle, the siRNA was protected from interacting with the platinum species both by physical separation in different layers of the nanocomplex as well as by utilization of an unreactive platinum(IV) analog (e.g. OxaPt(IV)). Each of these micellar constructs was generated to examine its relative ability to protect siRNA from platination, thereby preserving the effectiveness of RNAi. When taken up into cancer cells, the Pt in M(OxaPt(IV)) rapidly reacted with intracellular reducing agents, including glutathione and ascorbic acid, liberating free OxaPt(II) that was capable of binding nuclear DNA (**Scheme 1C**).

M(P) had a mean diameter of 70.8 ± 0.5 nm, as determined by DLS, and a zeta potential of $+46.1 \pm 4.2$ mV (**Figure S19** and **Table S2**). To generate M(OxaPt(IV)), OxaPt(IV) was allowed to react with P at an initial molar ratio of 0.6:1 Pt to NH₂ groups (on the PLL block). Once coupled to PLL, the OxaPt(IV) conjugate became less water soluble, driving its segregation within the PCL core and leaving unreacted PLL chains in the outer layer of M(OxaPt(IV)). This assertion is supported by experimental evidence that demonstrated a decrease in the CMC from 37.2 to 24 μ g/mL (**Figure S20**), an increase in the mean particle diameter to 92.6 ± 2.6 nm (DLS), and a reduction in the zeta potential to $+34.8 \pm 1.9$ for M(OxaPt(IV)) as compared to M(P) (**Table S2**). Overall, M(OxaPt(IV)) was comprised of 10 wt% Pt (by GFAAS), corresponding to a final OxaPt(IV) content of 20.4 wt%. This degree of loading was expected to yield 72% unreacted amino groups on the PLL corona of M(OxaPt(IV)), which were available for additional complexation of siRNA through electrostatic interactions.

To prove that M(OxaPt(IV)) was able to complex with BCL-2 siRNA, agarose gel electrophoresis experiments were conducted on siRNA after co-incubation with either M(P) or M(OxaPt(IV)) (**Figure S21A** and **S21B**). The results showed that the mobility of the siRNA was completely inhibited when electrostatically complexed with M(P) above a ratio of nitrogen atoms in the PLL block of the polymer to phosphate units of the siRNA (N/P) that was greater than 2. Additionally, the mobility of the siRNA was reduced when complexed with M(OxaPt(IV)) above an N/P ratio of 4. By using Alexa488-labeled BCL-2 siRNA, the loading efficiency was determined to be approximately 99% and the final micelle composition consisted of 30% siRNA by weight (**Figure S21C-E**). Representative micelles, containing both BCL-2 siRNA and OxaPt(IV) formed at an N/P ratio of 8 (i.e. M(OxaPt(IV)/BCL-2)), were spherical with a mean particle diameter of 78.9 ± 10.4 nm and hydrodynamic size of 104.9 ± 2.1 nm, as determined by TEM and DLS, respectively (**Figure 3A** and **3B**). M(OxaPt(IV)/BCL-2) had a reduced zeta potential of $+26.6 \pm 1.5$ mV as compared to either M(P) or M(OxaPt(IV)) (**Table S2**), further supporting the shielding of free amines on the PLL corona of the micelles by siRNA. BCL-2 siRNA was also directly complexed to M(P) to generate M(BCL-2) with a mean particle diameter of 55.4 ± 0.7 nm (DLS) and a zeta potential of $+35.5 \pm 0.6$ mV (**Table S2**). M(OxaPt(IV)/BCL-2) rapidly released OxaPt(II) under high reducing conditions *in situ* (**Figure 3C**), modeling the intracellular environment of tumor cells. However, it exhibited a

1 slower and pH-dependent release of Alexa488-labeled siRNA
2 (**Figure 3D**). Together, these data support a mechanism of
3 rapid release of free OxaPt(II) followed by controlled release
4 of siRNA after the intracellular uptake of M(OxaPt(IV))/BCL-2.

5 *Cellular Uptake of Micelles.* In order to determine the opti-
6 mal conditions for siRNA delivery, we next used different
7 concentrations of Luciferase GL3 and BCL-2 siRNA to achieve
8 mRNA knockdown of their respective targets in a LUC⁺ MCF-7
9 cell line. For both M(P) and M(OxaPt(IV)), an N/P ratio of 8
10 was employed for siRNA complexation. For effective RNAi of
11 luciferase expression, 100 nM of Luciferase GL3 siRNA had to
12 be utilized with either micellar construct (**Figure S22**). Alt-
13 though this was a higher concentration value for siRNA than
14 was required with the commercially available RNAiMAX trans-
15 fection kit (typically 1-2 nM), it was within the range of siRNA
16 concentrations that have been utilized with other polymer-
17 based transfection reagents.⁶⁰⁻⁶² To better study the kinetics
18 and the mechanisms of micelle uptake and *in vitro* siRNA re-
19 lease, a Cy5.5-conjugated mPEG-b-PCL-b-PLL polymer was
20 synthesized (**Scheme S4**). Alexa488-labeled siRNA was then
21 complexed to micelles formed from this Cy5.5-conjugated
22 polymer, generating M(Cy5.5/Alexa488 siRNA). OxaPt(IV) was
23 also chemically bound to the Cy5.5-conjugated polymer to
24 yield dual fluorophore-labeled micelles (i.e. M(Cy5.5/Alexa488
25 siRNA/OxaPt(IV))), which enabled simulta-
26 neous but independent *in vitro* tracking of both polymer
27 and siRNA species (**Scheme S5**). MCF-7 cells were subse-
28 quently incubated with M(Cy5.5/Alexa488 siRNA/OxaPt(IV))
29 either for 30 min or for 4 h and were visualized by confocal
30 laser scanning microscopy (**Figure 3E**). After 30 min of incu-
31 bation, there was a co-localization of Cy5.5 (red) and Alexa488
32 fluorescence (green) in a punctate distribution within cells
33 (depicted in orange within the magnified overlay image), con-
34 sistent with the intracellular localization of intact micelles
35 within endosomes. After 4 h, however, Alexa488 emission
36 extended beyond the areas of corresponding Cy5.5 fluores-
37 cence and was apparent throughout the cytoplasm. Flow
38 cytometry was used to quantify the relative intracellular lev-
39 els of Cy5.5-labeled polymer and Alexa488-labeled siRNA
40 both in MCF-7 (**Figure 3F** and **3G**) and in OVCAR4 cells over
41 time (**Figure S23**). Notably, cells that were treated with either
42 M(Cy5.5/Alexa488 siRNA) or M(Cy5.5/Alexa488 siRNA/OxaPt(IV))
43 exhibited a 10-fold increase in Cy5.5-
44 conjugated polymer and 40-fold increase in Alexa488-labeled
45 siRNA after 4 h (**Figure 3F**), supporting active intracellular
46 uptake of each species. Utilization of different small molecule
47 inhibitors implicated multiple cellular uptake pathways but
48 with a predominance of clathrin-dependent endocytosis (**Fig-
49 ure S24**). Together these results support a mechanism of ac-
50 tive endocytosis of Cy5.5-conjugated micelles, rapid conver-
51 sion of M(OxaPt(IV)) to free OxaPt(II), and a slower cyto-
52 plasmic redistribution of Alexa488-labeled siRNA, which was
53 consistent with controlled release and endosomal escape.
54 This relative temporal and spatial separation between
55 OxaPt(II) and siRNA release was expected to limit the for-
56 mation of intracellular Pt-siRNA adducts and to promote the

individual and synergistic activities of these otherwise reac-
tive species.

*Optimum Strategy for Combining RNAi and Platinum-
based Anticancer Agents.* After having validated effective
intracellular delivery with our transfection reagent, we next
compared various strategies for combining BCL-2 siRNA and
platinum-based anticancer agents in order to achieve maxi-
mal synergistic activity. For all siRNA-containing groups, the
concentration of siRNA (100 nM) was kept constant and was
transfected using various polymeric micelles formed at an
N/P ratio of 8. These micelles had no inherent effects on cel-
lular viability in the absence of the platinum-based species
(**Figure S25**). We next conducted a series of experiments in
which MCF-7 cells were treated with increasing concentra-
tions of different platinum formulations (i.e. free OxaPt(II),
free OxaPt(IV), or M(OxaPt(IV))), either individually or in
combination with M(BCL-2) or M(c-siRNA). Cells were also
treated with siRNA and platinum-based species that were co-
delivered via a single micellar construct (i.e. M(OxaPt(IV))/BCL-
2) or M(OxaPt(IV))/c-siRNA). In all cases, BCL-2 mRNA levels
were measured by RT-qPCR, intracellular Pt concentrations
and the numbers of Pt-DNA adducts were quantified by ICP-
MS, and cellular viability was determined by the colorimetric
MTT assay after each treatment.

Decreased amounts of intracellular Pt were detected af-
ter both 1 and 4 h of exposure to free OxaPt(IV) as compared
to free OxaPt(II) (**Figure 4A** and **Table S3**). Intracellular Pt
levels were markedly enhanced (by ~3-8 fold) when treated
with M(OxaPt(IV)) as opposed to various free drug formula-
tions of either OxaPt(II) or OxaPt(IV) that were administered
at identical Pt concentrations. The intracellular levels of Pt
also increased 2-fold between 1 and 4 h after administration
of M(OxaPt(IV)), which, again, supported a time-dependent
mechanism of micelle uptake via active endocytosis.

Co-administration of M(OxaPt(IV)) and a separate micel-
lar construct containing only siRNA (e.g. M(BCL-2)), however,
resulted in a relative 2-fold decrease in the intracellular con-
tent of Pt as compared to cells that were treated with either
M(OxaPt(IV)) alone or M(OxaPt(IV))/BCL-2 at equivalent Pt
concentrations. These results indicate that micelle uptake via
endocytosis was not only time-dependent but also saturable.
These observations were further supported by analogous
measurements of the numbers of intracellular Pt-DNA ad-
ducts that could be detected after 24 h of treatment (**Figure
4B** and **Table S3**). A markedly enhanced number of Pt-DNA
adducts (~10 fold increase) were measured after treatment
with M(OxaPt(IV)) as opposed to either free OxaPt(II) or free
OxaPt(IV) administered at equivalent Pt concentrations and
for similar incubation periods. Co-administration of two sepa-
rate micellar constructs of M(OxaPt(IV)) and M(BCL-2) result-
ed in the generation of half the numbers of Pt-DNA adducts
as compared to treatment with M(OxaPt(IV)) alone or with
M(OxaPt(IV))/BCL-2, which, again, supported a saturable limit
for micelle uptake by MCF-7 cells. To promote the highest
intracellular levels of Pt and the maximal numbers of Pt-DNA
adducts, combined delivery of OxaPt(IV) and BCL-2 siRNA via
a single micellar construct was found to be optimal.

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Thereafter, we examined the relative levels of BCL-2 mRNA within cells after treatment with each experimental group (Figure 4C and S26). Whereas free OxaPt(II) decreased BCL-2 mRNA levels to 65% that of untreated (control) cells, consistent with its known pro-apoptotic activity,⁶³ effective mRNA suppression was only achieved with BCL-2 siRNA treatment (i.e. suppression to less than 30% of baseline mRNA levels). Co-administration of free OxaPt(II), free OxaPt(IV), or M(OxaPt(IV)) together with M(BCL-2) did not further inhibit mRNA levels beyond that which was obtained with M(BCL-2) treatment alone. These results indicate that when siRNA was electrostatically complexed with polymeric micelles prior to platinum(II) exposure, it was effectively protected from platination. Notably, co-incorporation of BCL-2 siRNA and OxaPt(IV) in the same micellar construct (i.e. M(OxaPt(IV)/BCL-2)) did achieve a statistically significant improvement in BCL-2 mRNA suppression (to less than 10% of baseline levels). As such, delivery of BCL-2 siRNA and OxaPt(IV) with a single micelle not only ensured siRNA transcript stability but also promoted maximal suppression of mRNA levels beyond that which was achievable with either agent only or when they were combined by alternative means.

Ultimately, the goals of combining RNAi with platinum-based therapies are to explore changes in treatment responses and biological activities that are mediated by the suppression of specific gene products. We, therefore, interrogated changes in the *in vitro* viability of MCF-7 cells that were treated with various experimental combinations of platinum-based species and BCL-2 siRNA. We measured changes in cellular viability as a function of increasing quantities of free OxaPt(II), free OxaPt(IV), or M(OxaPt(IV)), either as single treatments or in combinations with either M(BCL-2) or M(c-siRNA) (Figure 4D and S27). Cells were also treated with M(OxaPt(IV)/BCL-2) or M(OxaPt(IV)/c-siRNA) at equivalent Pt concentrations for comparative purposes. Synergistic treatment responses from any combination of platinum-based species and siRNA were defined as a decrease in the IC₅₀ value as compared to cells that were exposed to the same platinum-based therapy alone. Although the combination of free OxaPt(IV) with M(BCL-2) did show a synergistic treatment effect, which was not obvious in the combination of free OxaPt(II) and M(BCL-2), free OxaPt(IV)-containing treatments were approximately 10-fold less potent than free OxaPt(II) at inducing cellular toxicity (Figure 4E). By comparison, M(OxaPt(IV)) was 100x more potent than free OxaPt(IV) and displayed 10-fold greater cytotoxicity than free OxaPt(II). Whereas the combination of M(BCL-2) and M(OxaPt(IV)) did result in a statistically significant improvement in anti-proliferative activity, M(OxaPt(IV)/BCL-2) displayed the highest *in vitro* potency, yielding an IC₅₀ value of 60 nM as compared to 23 +/-0.25 μM for free OxaPt(II) alone. This dramatically enhanced efficacy for M(OxaPt(IV)/BCL-2) was further corroborated by testing each of the various treatment combinations on additional cell lines (i.e. OVCAR4, A2780 and A2780DDP; Table 2). To investigate the biological mechanisms underlying this improved activity, we performed flow

cytometry to quantify the populations of MCF-7 cells that were apoptotic after 36 h of exposure to each experimental group (Figure 4F and S28). When compared to treatment with free OxaPt(II) (at equivalent Pt concentrations and for identical durations of time), treatment with M(OxaPt(IV)/BCL-2) resulted in a 4-fold increase in the total number of cells that had undergone apoptosis. Notably, this level of pro-apoptotic activity was twice that of M(OxaPt(IV)) alone and 1.5-fold greater than that of the combination of M(OxaPt(IV)) and M(BCL-2). These experiments confirmed that the delivery of BCL-2 siRNA and OxaPt(IV) via a single micellar construct resulted in the highest numbers of apoptotic events as compared to all other strategies for combining BCL-2 siRNA with different platinum-based species.

CONCLUSIONS

RNAi approaches are extensively used to study molecular responses that mediate acquired cellular resistance to platinum-containing regimens. The success of these endeavors relies on the preserved activity of the exogenous RNAi transcript when combined with platinum-based anticancer agents. Here, we demonstrated rapid and extensive Pt-siRNA adduct formation when siRNA was incubated with platinum(II)-based agents at concentrations and for time intervals that are typically used for *in vitro* RNAi experiments. Notably, siRNA was protected from inactivation by combining it with a platinum(IV) analog, but this combination resulted in reduced cytotoxicity as compared to using the active platinum(II) agent alone. In order to promote effective RNAi and to maximize the individual and synergistic activities of siRNA and platinum-based anticancer agents, we found that both species had to be co-incorporated within a single micelle delivery vehicle. A generalized adoption of this strategy may lead to the development of novel therapeutics and may be used to identify drug targets that would otherwise be missed in conventional screens that typically combine RNAi with platinum(II)-based anticancer agents.

ASSOCIATED CONTENT

Supporting Information

"Supporting Information.PDF" is available free of charge via the Internet at <http://pubs.acs.org> and contains the *Supplemental Materials and Methods, Schemes S1* thru *S5, Figures S1* thru *S28, and Tables S1* thru *S3*.

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Notes

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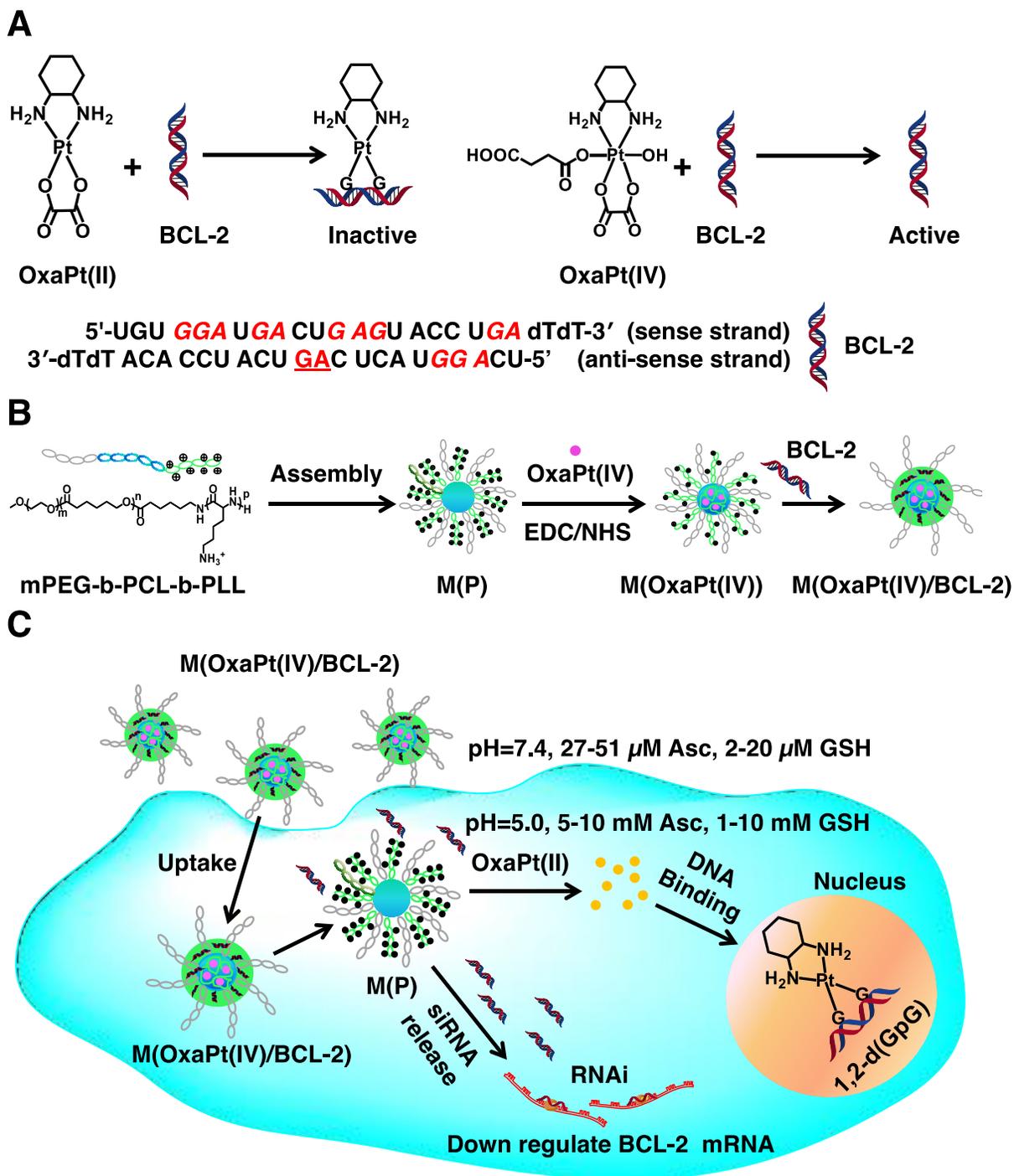
ABBREVIATIONS

BCL-2, B-cell lymphoma 2; CisPt(II), cisplatin; CisPt(IV), cisplatin(IV); mRNA, messenger ribonucleic acid; miRNA, micro-ribonucleic acid; OxaPt(II), oxaliplatin; OxaPt(IV), oxaliplatin(IV); Pt-RNA, platinum-ribonucleic acid adduct; siRNA, small interfering ribonucleic acid.

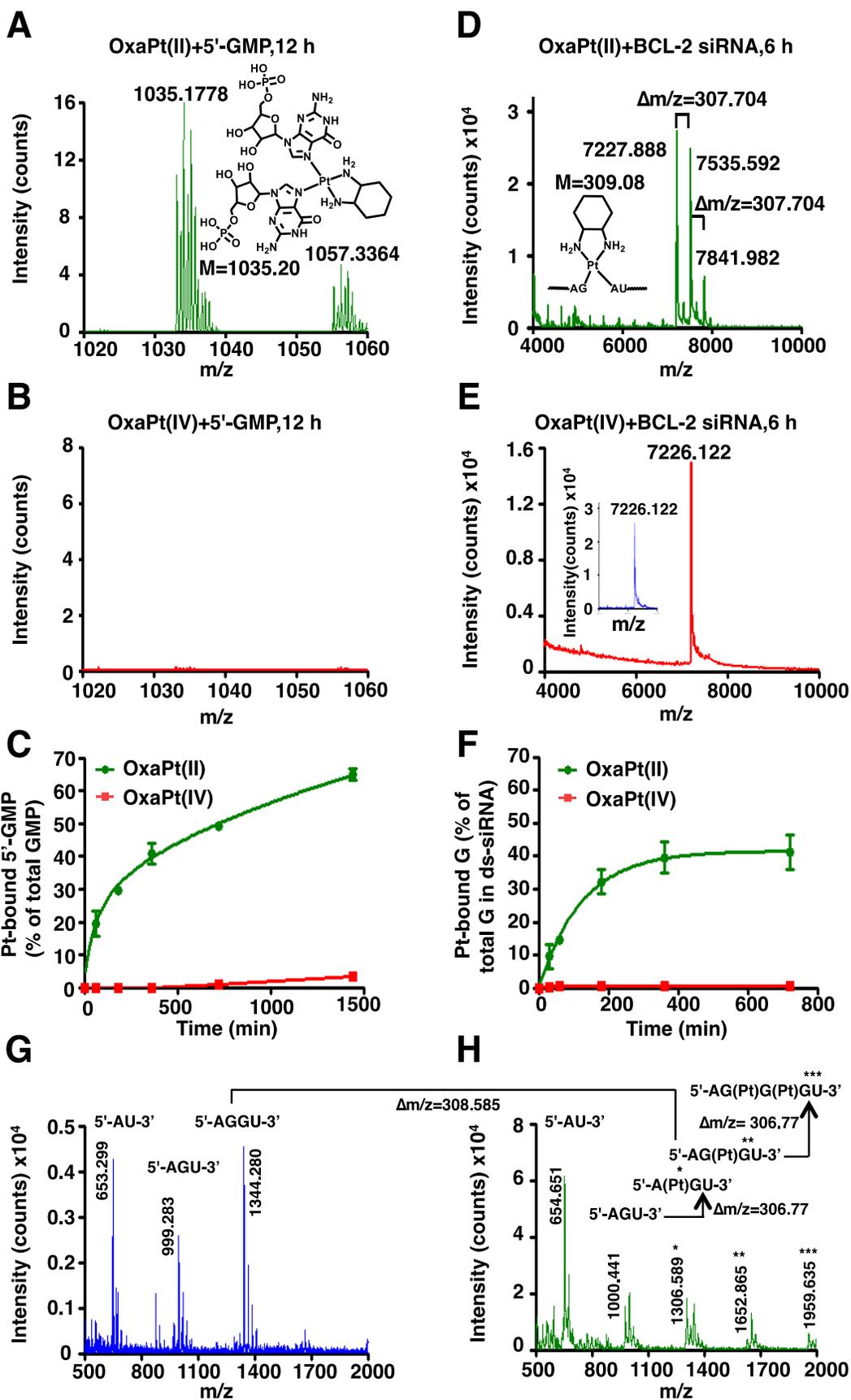
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1 **Scheme 1. OxaPt(II) but not OxaPt(IV) deactivates siRNA and a strategy for combining RNAi with an OxaPt(IV) prodrug.** A) Planar
2 OxaPt(II) rapidly forms Pt-RNA adducts with BCL-2 siRNA, resulting in transcript inactivation; kinetically inert octahedral OxaPt(IV) spe-
3 cies, however, do not. As predicted from the sequence of the BCL-2 siRNA, platination of a single GA region in the non-seed portion of
4 the anti-sense strand (red/underlined) would be expected to affect silencing efficacy; the sense and anti-sense strands also contain other
5 potential sites for further platination (red/italics). B) Illustration of mPEG-b-PCL-b-PLL micelles (M(P)), incorporating OxaPt(IV)
6 (M(OxaPt(IV)) or both OxaPt(IV) and BCL-2 siRNA in a single construct (M(OxaPt(IV)/BCL-2)); note, OxaPt(IV) is linked through an amide
7 bond while BCL-2 siRNA is bound through electrostatic complexation to amino groups on the PLL chains of the micelles. C) Intracellular
8 uptake and the ultimate fate of M(OxaPt(IV)/BCL-2). The micelles are taken up by endocytosis, where high intracellular concentrations of
9 ascorbic acid (Asc) and glutathione (GSH) rapidly convert OxaPt(IV) to OxaPt(II); free OxaPt(II) subsequently binds genomic DNA, resulting
10 in Pt-DNA adduct formation. BCL-2 siRNA is liberated more slowly from the micelles, escaping from the endosome to down regulate BCL-
11 2 mRNA expression in the cytoplasm.
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1 **Figure 1: OxaPt(II) rapidly binds 5'-GMP as well as siRNA, forming Pt-RNA adducts, while OxaPt(IV) does not.** Representative ESI-MS
2 spectra of solutions of 5'-GMP (10 mM) at 12 h after incubation with either A) OxaPt(II) (5 mM) or B) OxaPt(IV) (5 mM) at 37 °C. The
3 presence of the DACHPt(5'-GMP)₂ adduct was monitored over time by measuring changes in the relative height of the peak at
4 $m/z=1035.1778$; the structure and molecular weight of bis-adducts are shown in A, inset. Note: other peaks in the ESI spectra correspond
5 to adducts formed with Na⁺ and K⁺; see also **Figure S7** (Supporting Information) for spectra at other time points. C) Kinetics of the binding
6 of various Pt-based agents to 5'-GMP; results are depicted as the molar percentage of the total 5'-GMP pool in solution and were deter-
7 mined by ¹H NMR spectroscopy. Representative MALDI/TOF-MS spectra of the products formed after 6 h of incubation of BCL-2 ss-siRNA
8 (50 μM) with either D) OxaPt(II) (500 μM) or E) OxaPt(IV) (500 μM) at 37 °C. The spectrum of unreacted ss-siRNA is shown in E, inset. F)
9 Binding kinetics of OxaPt(II) and OxaPt(IV) (50 μM) to BCL-2 ds-siRNA (5 μM) as measured by GFAAS. Data are expressed as the percent-
10 age of platinum-bound G nucleotides within the ds-siRNA suspension. MALDI/TOF-MS was also conducted after enzymatic cleavage of
11 the antisense strand of BCL-2 siRNA (50 μM) by RNase A either G) before or H) after incubation with OxaPt(II) (500 μM) to demonstrate
12 sites of platination. 5'-AC-3', 5'-AGU-3', and 5'-AGGU-3' are the cleavage products of the native BCL-2 ss-siRNA. Platination of the G
13 and/or A sites in the BCL-2 ss-siRNA resulted in the addition of one or more DACHPt moieties, yielding 5'-A(Pt)GU-3'(*), 5'-A(Pt)GGU-
14 3'(**), and 5'-A(Pt)G(Pt)GU-3'(**); note, here Pt denotes DACHPt.

Table 1: Summary of different ds-RNA macromolecules, various platinum-containing anticancer agents, their reaction rate constants ($k_{2,app}$), and the percentages of ds-siRNA that are expected to be deactivated due to platination as a function of reaction time and initial concentration of the platinum-containing species.

| REF | RNA | Length | # Gs | Species | $k_{2,app}$ ($M^{-1}s^{-1}$) | Std Dev | % ds-siRNA deactivated due to platination (i.e. % ss-siRNA) | | | | | | | | | |
|------------------|-------------|--------|------|-----------|-----------------------------------|------------|---|---------------|--------------|---------------|--------------|---------------|--------------|---------------|--------------|---------------|
| | | | | | | | 30 min | | 1 h | | 3 h | | 6 h | | 24 h | |
| | | | | | | | 1 μM | 10 μM | 1 μM | 10 μM | 1 μM | 10 μM | 1 μM | 10 μM | 1 μM | 10 μM |
| 37a | RNA-1 | 15 | 6 | CisPt(II) | 22.1 | 1.7 | 3.9 | 33 | 7.6 | 55 | 21 | 91 | 38 | 99 | 85 | 100 |
| | RNA-2 | 15 | 6 | CisPt(II) | 20.1 | 1.1 | 3.6 | 30 | 7.0 | 52 | 20 | 89 | 35 | 99 | 82 | 100 |
| | miR-146a | 21 | 9 | CisPt(II) | 20.4 | 0.6 | 3.6 | 31 | 7.1 | 52 | 20 | 89 | 36 | 99 | 83 | 100 |
| 37b | RNA-1 | 15 | 6 | CisPt(II) | 7.7 | 0.5 | 1.4 | 13 | 2.7 | 24 | 8.0 | 57 | 15 | 81 | 49 | 100 |
| | RNA-1-1-S | 13 | 5 | CisPt(II) | 10.5 | 0.6 | 1.9 | 17 | 3.7 | 32 | 11 | 68 | 20 | 90 | 60 | 100 |
| | RNA-1-2 | 13 | 6 | CisPt(II) | 23.6 | 1.0 | 4.2 | 35 | 8.1 | 57 | 23 | 92 | 40 | 99 | 87 | 100 |
| | RNA-1-3 | 15/16 | 6 | CisPt(II) | 29.7 | 1.1 | 5.2 | 41 | 10 | 65 | 27 | 96 | 47 | 100 | 92 | 100 |
| | RNA-1-4 | 17 | 6 | CisPt(II) | >30.0 | N/A | 5.3 | 42 | 10 | 66 | 27 | 96 | 48 | 100 | 93 | 100 |
| This work | BCL-2 siRNA | 20 | 10 | CisPt(II) | 12.7 | 1.2 | 2.3 | 20 | 4.5 | 37 | 13 | 75 | 24 | 94 | 67 | 100 |
| | | | | OxaPt(II) | 5.3 | 2.3 | 0.9 | 9 | 1.9 | 17 | 5.6 | 44 | 11 | 68 | 37 | 99 |
| | | | | CisPt(IV) | 3.0 | 1.2 | 0.5 | 5 | 1.1 | 10 | 3.2 | 28 | 6.2 | 47 | 23 | 92 |
| | | | | OxaPt(IV) | 0.2 | N/A | 0.0 | 0.4 | 0.1 | 0.7 | 0.2 | 2.2 | 0.4 | 4.3 | 1.7 | 16 |

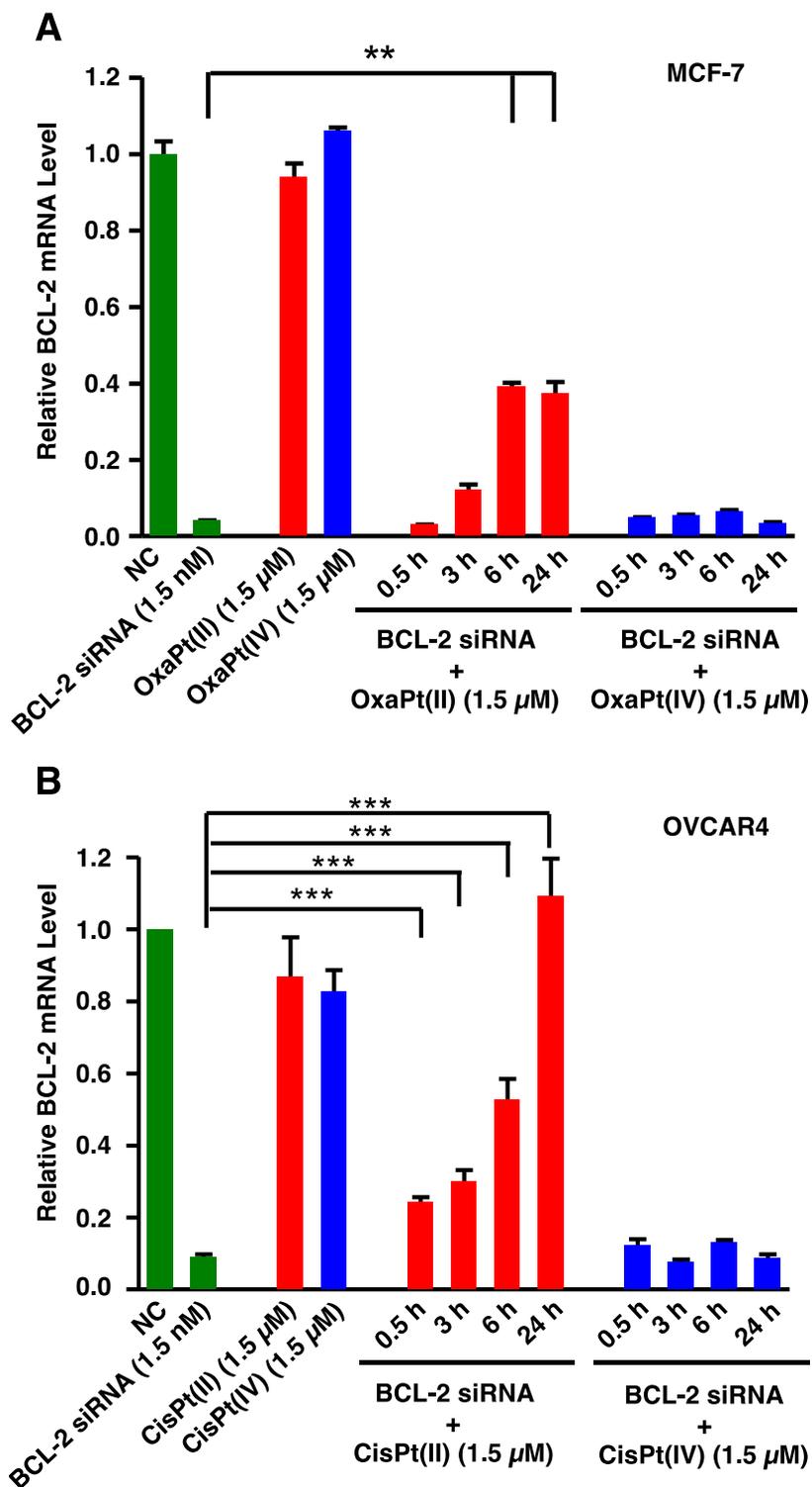


Figure 2: Incubation of siRNA with OxaPt(II) or CisPt(II) results in a time-dependent decrease in RNAi efficacy. Relative BCL-2 mRNA levels in A) MCF-7 breast cancer cells and B) OVCAR4 ovarian cancer cells at 37 °C and at 48 h after Lipofectamine[®]-based transfection of BCL-2 siRNA. BCL-2 siRNA (1.5 nM) was utilized directly or was first pre-incubated with either different platinum(II)-based anticancer agents (i.e. OxaPt(II) or CisPt(II)) (1.5 μ M) or with their platinum(IV) analogs (i.e. OxaPt(IV) or CisPt(IV)) (1.5 μ M) for different time periods, ranging from 0.5 to 24 h prior to transfection. BCL-2 mRNA levels were quantified by NanoDrop[™] and the data are presented as relative to that of untreated cells, which served as the negative control (NC) treatment. Data are depicted as mean values \pm SEM with $n = 3$ samples per condition; note, 3 experimental replicates were similarly conducted for each cell line and treatment, yielding analogous results. Significance is defined as ** $p < 0.01$; *** $p < 0.001$.

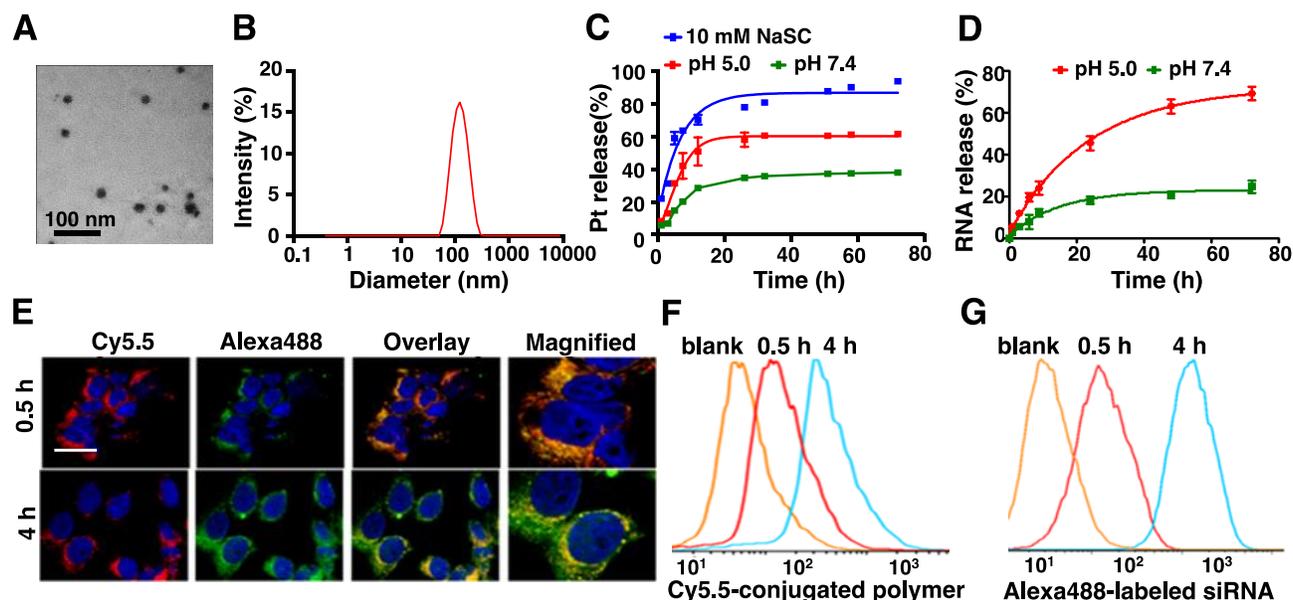
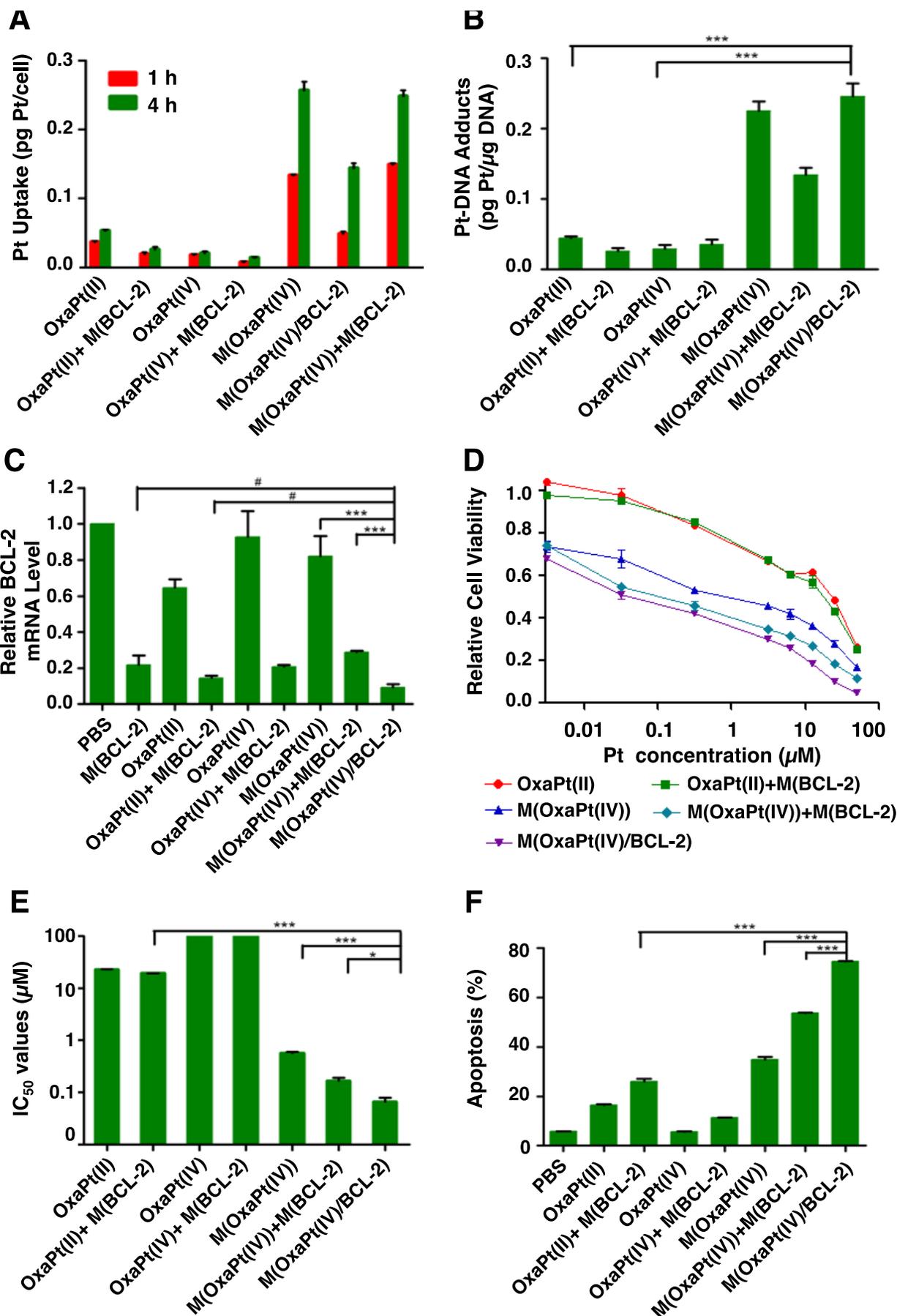


Figure 3: Micelles that covalently conjugate OxaPt(IV) and that are electrostatically complexed with BCL-2 siRNA demonstrate controlled release of both species. A) TEM and B) DLS results depicting the average size distributions for M(OxaPt(IV))/BCL-2) that were formed at an amine (per monomer unit, N) to phosphate (per nucleic acid, P) ratio (N/P ratio) of 8. The resultant micelles had a mean particle diameter of 78.9 +/- 10.4 nm by TEM and 104.9 +/- 2.1 nm by DLS. C) Rapid *in situ* release of free OxaPt(II) was observed in high reducing environments (10 mM sodium ascorbate, NaSC) as compared to under physiologic (pH 7.4) or acidic (pH 5.0) *in situ* conditions. Individual data points are expressed as mean values \pm SD for an n = 3 technical replicates per condition. A statistically significant separation in the curves was observed at all time points after 12 h ($p < 0.05$ via ANOVA). D) The release of Alexa488-labeled siRNA from M(OxaPt(IV))/Alexa488 siRNA) was enhanced in low pH environments (pH 5.0 vs. pH 7.4). Individual data points are again expressed as mean values \pm SD for an n = 3 technical replicates per condition. A statistically significant separation in the curves was observed at all time points after 1 h ($p < 0.05$ via two-tailed t-test). E) Confocal laser scanning microscopy images were taken to visualize cellular uptake and cytoplasmic delivery of M(Cy5.5/Alexa488 siRNA/OxaPt(IV)). Images were obtained at 30 min (upper panel) and at 4 h (lower panel) after the addition of micelles to the MCF-7 cells. Note, cell nuclei were stained with DAPI (blue); Cy5.5-conjugated mPEG-b-PCL-b-PLL polymer (red) and Alexa488-labeled BCL-2 siRNA (green) were utilized to simultaneously and independently track the polymer and RNAi species, respectively (scale bar = 100 μ m). Flow cytometry was employed to quantify the relative uptake of F) Cy5.5-conjugated polymer and G) Alexa488-labeled BCL-2 siRNA at various time points after the addition of M(Cy5.5/Alexa488 siRNA/OxaPt(IV)) to MCF-7 cells. Signals generated from the untreated (blank) cells are included for reference.



1 **Figure 4: Co-encapsulation of OxaPt(IV) and BCL-2 siRNA within a single micellar transfection reagent (i.e. M(OxaPt(IV)/BCL-2)) quanti-**
2 **tatively maximizes the intracellular uptake of Pt, the numbers of Pt-DNA adducts that are formed, the suppression of BCL-2 mRNA**
3 **levels, and the cytotoxicity to MCF-7 cells through augmentation of cellular apoptosis.** A) Pt uptake into MCF-7 cells after 1 h (red) and
4 4 h (green) of cellular exposure to various treatments as determined by ICP-MS. Equal concentrations of Pt (10 μ M) and/or BCL-2 siRNA
5 (100 nM) were used in each group and were representative of the concentrations of each species that are typically employed for *in vitro*
6 cytotoxicity and RNAi experiments, respectively. B) Total numbers of Pt-DNA adducts as assessed after 24 h of incubation with each ex-
7 perimental group. DNA was isolated after cellular lysis and the content of DNA-bound Pt was determined by ICP-MS. C) Relative BCL-2
8 mRNA levels as determined by RT-qPCR conducted at 48 h after treatment with each experimental group. D) Dose dependent cell viabil-
9 ity plots and E) IC₅₀ values for cellular toxicity as determined by the MTT cellular viability assay, which was performed at 72 h after incu-
10 bation with each experimental group. Experiments in A-D were performed in triplicate. IC₅₀ values were derived from three independent
11 experiments that each had three technical replicates per condition. Data are displayed as the mean values \pm the standard deviation of
12 the mean. F) Total percentages of MCF-7 cells that underwent apoptosis as determined by flow cytometry at 36 h after treatment with
13 various experimental groups. Equivalent concentrations of Pt (10 μ M) +/- BCL-2 siRNA (100 nM) were used for each condition. Data are
14 depicted as mean values \pm SEM with n = 3 samples per condition. In all cases, significance is defined as *p<0.05, **p<0.01, and ***
15 p<0.001. # denotes p>0.05.
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Table 2: Summary of IC₅₀ values (μM) for MCF-7, OVCAR4, A2780 and A2780DDP cells treated with various combinations of platinum-containing anticancer agents and/or siRNA species.

| Treatment groups | MCF-7 | OVCAR4 | A2780 | A2780DDP |
|-------------------------|--------------|--------------|--------------|--------------|
| OxaPt(II) | 23.05 ± 0.25 | 15.45 ± 0.50 | 2.90 ± 0.28 | 18.80 ± 0.99 |
| OxaPt(II)+M(c-siRNA) | 28.51 ± 0.34 | 21.60 ± 0.71 | 3.75 ± 0.91 | 22.25 ± 1.63 |
| OxaPt(II)+M(BCL-2) | 19.20 ± 0.42 | 9.24 ± 1.50 | 1.60 ± 0.14 | 10.55 ± 1.34 |
| OxaPt(IV) | >100 | >100 | 15.85 ± 2.76 | >100 |
| OxaPt(IV)+M(c-siRNA) | >100 | >100 | 12.65 ± 1.20 | >100 |
| OxaPt(IV)+M(BCL-2) | >100 | >100 | 8.60 ± 1.13 | >100 |
| M(OxaPt(IV)) | 0.54 ± 0.03 | 1.79 ± 0.10 | 0.87 ± 0.13 | 1.45 ± 0.07 |
| M(OxaPt(IV))+M(c-siRNA) | 0.43 ± 0.01 | 1.37 ± 0.12 | 0.72 ± 0.09 | 1.06 ± 0.20 |
| M(OxaPt(IV))+M(BCL-2) | 0.17 ± 0.02 | 0.43 ± 0.01 | 0.51 ± 0.03 | 0.60 ± 0.08 |
| M(OxaPt(IV))/c-siRNA | 0.43 ± 0.02 | 1.80 ± 0.25 | 0.59 ± 0.06 | 0.72 ± 0.05 |
| M(OxaPt(IV))/BCL-2 | 0.06 ± 0.01 | 0.12 ± 0.01 | 0.39 ± 0.02 | 0.42 ± 0.08 |

Table of contents (TOC) graphic

