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Tracking the cellular targets of platinum anticancer drugs: Current tools and emergent methods

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List of Abbreviations

Abbreviation	Definition
XR-Seq	Excision repair sequencing
TFIIH	Transcription Factor II H
HMGB1	High mobility group protein B1
rRNA	Ribosomal RNA
rDNA	Ribosomal DNA
RT	Reverse Transcriptase
CuAAC	Copper-catalyzed azide-alkyne cycloaddition
BODIPY	boron-dipyrromethene
SPAAC	Strain-promoted Azide - Alkyne Click
CuAAC	copper(I)-catalyzed azide alkyne cycloaddition
DIBO	dibenzocyclooctyne
LA-ICP-MS	Laser ablation-inductively coupled plasma-mass spectrometry
nHPLC-ESI-MS/MS	Nano high performance liquid chromatography-electrospray ionization-mass spectrometry-mass spectrometry
SEC-ICP-MS	Size exclusion chromatography-inductively coupled plasma-mass spectrometry
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
LC-MS/MS	Liquid chromatography-mass spectrometry-mass spectrometry
PDI	Protein disulfide isomerase

Abstract

Since its FDA approval just over 40 years ago, cisplatin and its more recently approved analogues, carboplatin and oxaliplatin, have been widely used to treat a spectrum of different cancers worldwide. Despite the overall success of this class of anticancer agents, identification of their precise cellular targets is still an area of intense study due to the side effects and resistance profiles of these drugs. As these compounds are small molecules with low specificity, target identification in the context of a complex cellular environment presents a significant challenge. In this review, we summarize established methods to identify RNA, DNA, and protein targets of platinum. We also review recent additions to the library of fluorescent-Pt derivatives as well as click-enabled platinum compounds that can be used for post-treatment labeling.

Keywords

Platinum anticancer drugs; click chemistry; proteomics; high-throughput sequencing; small molecule interactions

1. Introduction

Since the discovery and implementation of cisplatin as an anticancer compound, the mechanisms of action of platinum therapeutics (Figure 1) have been the topics of an immense body of research. Cellular pathways leading to antiproliferation, toxicity, side effects and resistance have all been subjects of intense study. Unraveling specific pathways is complicated by the pleiotropic responses induced by these relatively simple metal compounds. Identifying the range of targets of platinum compounds upon treatment in eukaryotic cells could aid in this goal, but represents a significant challenge. Unlike drugs with high specificity, these small metal compounds have many potential binding sites in cells including abundant potential primary ligands from amino acids and nucleobases as well as small molecules. In the context of a complex cellular environment, selectivity might be achieved by compartmentalization, accessibility, and other factors that are difficult to predict from *in vitro* work.

Recent advances in high-throughput and 'omics' methodologies provide new tools to approach complex questions such as comprehensive identification of small molecule targets in cells. In this review, we describe recent applications of these methods to identify cellular targets of Pt(II) anticancer compounds and their analogues. These include approaches for genome-wide detection of Pt-DNA and Pt-RNA adducts using high-throughput sequencing and various enrichment methods. Comprehensive proteomics-based methods to detect Pt-protein interactions in cells, and current results from those studies, are reviewed. We summarize synthetic tools, including recent literature on fluorescent probes used for platinum detection and click-enabled platinum derivatives for post-treatment labeling. It is our intent for the work described in this review to complement previously published reviews discussing molecular targets of Pt(II) anticancer compounds and methodologies to detect them [1–3].

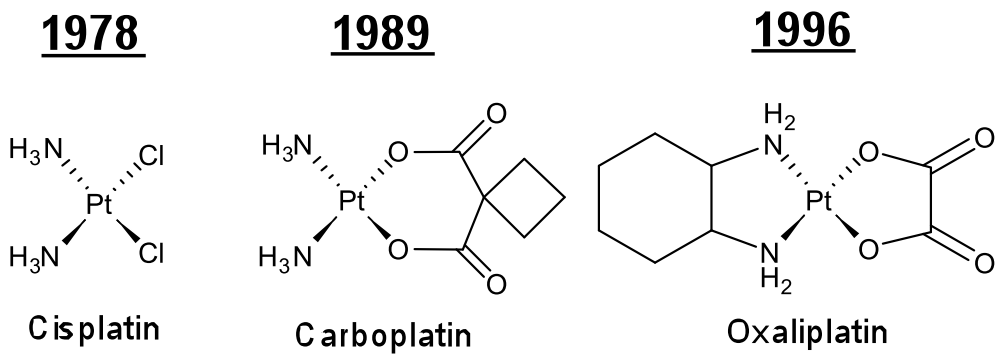


Figure 1. Pt(II) anticancer drugs currently approved for use in the United States. Included are years of FDA approval.

2. Pt(II) Interactions with nucleic acids

The most well-studied mechanism of action of Pt(II)-based chemotherapeutics is the formation of Pt(II) lesions on DNA, which can lead to inhibition of replication and ultimately cell death via DNA damage response (DDR) pathways [4]. Pt(II) lesions primarily form 1,2-intrastrand crosslinks on adjacent guanines, although they can also form 1,2-intrastrand crosslinks on AG dinucleotides, 1,3-intrastrand crosslinks on nonadjacent guanines, interstrand crosslinks, and monofunctional adducts [4]. This pattern has been observed both *in vitro* and *in vivo*, and has been described in detail in existing reviews [4–6]. Studies on the in-cell interactions between Pt(II) and nucleic acids that do not focus on the DDR are extremely limited. With recent evidence that oxaliplatin in particular causes cell death via ribosome biogenesis stress rather than DNA damage [7], the need for a better understanding of platinum's interactions with nucleic acids in cells has gained renewed interest.

Rather than focusing on the *in vitro* interactions of Pt(II) with nucleic acids and the propensity of these compounds to form particular types of adducts, this review will describe more recent methods employed to isolate and identify DNA and RNA targets of these drugs in a cellular context. The studies reviewed have used a combination of enzymatic, immunological, and chemical methods to approximate the location of these lesions following treatment *in vivo* or *in cellulo* (Table 1). Enzymatic methods leverage the features of platinum lesions which block

polymerases [8,9] or protect regions of oligonucleotides from digestion [10]. Primer extension assays use polymerases to extend primers on platinated template strands of DNA or RNA until they are blocked by platinum adducts, revealing lesions at polymerase stop sites. These methods can be coupled with a variety of downstream methods including traditional sequencing gels, PCR, high throughput sequencing, and mass spectrometry. Recently, click-enabled Pt compounds have promised a more direct method of locating these lesions on DNA and RNA.

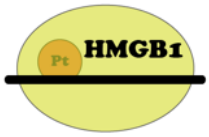
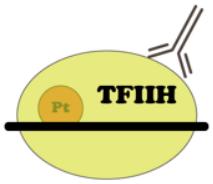
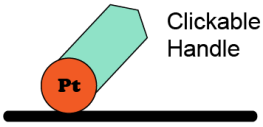
Method	Uses	References
a. Primer extension 	Sequencing gels to ID rRNA targets in yeast and bacteria	[12, 13, 14]
	High throughput ID of rRNA targets - yeast	[16]
	High throughput ID of DNA lesions - human cell lines	[19-23]
b. Pulldown - HMGB1 enrichment 	Enrichment of Pt sites prior to high throughput primer extension	[23]
c. Pulldown – IP of DNA damage repair proteins 	Enrichment prior to high throughput sequencing of repair sites	[19, 20]
d. Pulldown – IP of GG adducts formed by cisplatin 	Pulldown of platinated DNA for microarray	[25]
	Enrichment prior to high throughput primer extension	[19, 20]
e. Enzymatic and chemical probing 	Mapping sites on bacterial rRNA	[10]
f. Post treatment labeling - click chemistry 	In-gel fluorescent detection of Pt on yeast tRNA and rRNA	[13]
	Fluorescent microscopy of Pt adducts in tissue culture	[27, 34, 41, 43-44]

Table 1. Methods used to detect Pt(II) binding sites on nucleic acids

2.1 Pt(II) Interactions with RNA

There is abundant evidence for platinum accumulation on cellular RNA, and several *in vitro* studies of Pt-RNA interactions have shown propensity for GG and AG adducts as well as Pt-induced crosslinking in structured RNAs [1,11]. Studies from our lab and others have utilized reverse transcriptase (RT) primer extension and sequencing gels (Table 1a) to probe for lesions on RNA isolated from platinum-treated bacteria and yeast [12–15]. On yeast ribosomal RNA (rRNA), cisplatin-induced Pt binding sites were identified on the 18S helix [12], while both cisplatin and oxaliplatin lesions were described on the sarcin-ricin loop of rRNA [13]. Recently, this RT primer extension has been applied in a high throughput context to identify cisplatin adducts formed *in vivo* on yeast rRNA [16], with several new sites being identified. These sites - located on helices 23, 24, 58, 80, and 89 of the yeast ribosome - were confirmed by traditional gel-based primer extension analysis.

In bacteria, RT primer extension analysis has been used to assess the interaction of cisplatin and amino acid-modified platinum compounds with the 16S bacterial rRNA [14]. Adjacent to these polymerase-based enzymatic mapping methods, RNase and chemical probing techniques (Table 1e) have been used to reveal platination sites on bacterial rRNA hairpins [10]. A 2016 study identified platinum binding sites on bacterial ribosomes following *in vitro* cisplatin treatment using X-ray crystallography [17], but the relevance of these findings to the formation of adducts *in vivo* is unknown. The DeRose lab has utilized click chemistry to identify *in vitro* Pt(II) binding to *S. cerevisiae* rRNA and tRNA [13], described in more detail in section 4 of this review.

2.2 Pulldown studies to identify Pt(II) Interactions with DNA

Recent studies have used pulldown techniques to enrich for platinum-bound genomic DNA prior to analysis with other methods [18]. Hu et al. utilized a high-throughput sequencing

assay called “Damage-seq” which relies on immunoprecipitation enrichment of Pt-bound DNA (Table 1d) with an anti-Pt-DNA antibody followed by analysis of polymerase stop sites (Table 1a) [19]. This assay was performed alongside Excision Repair Sequencing (XR-seq), which selects sequences from damaged fragments excised in Nucleotide Excision Repair (NER) using an antibody that recognizes Transcription Factor II H (anti-TFIIH, Table 1c). For Damage-seq, enrichment of Pt-bound DNA fragments was conducted via immunoprecipitation with a commercially available antibody raised against cisplatin-induced Pt-GG adducts. Following this enrichment, amplification of isolated DNA was performed such that fragments with a platinum lesion resulted in a truncated amplification product (Table 1a). These samples are then subjected to high-throughput sequencing and analyzed. This Damage-seq method was conducted on human lymphocyte GM12878 cells that had been treated with 200 μM cisplatin for 1.5 hours or 200 μM oxaliplatin for 3 hours. Results from cisplatin-treated samples revealed uniform distribution of lesions on the genome correlating with GG dinucleotide frequency. The exception was a very slight enrichment at nucleosome centers, which the authors suggest protect lesions from repair. In contrast, XR-seq revealed a heterogeneous pattern of repair on the genome, with NER excision products disproportionally originating from sequences known to be located in active chromatin sites. Similar results were found from samples treated with oxaliplatin. In sum, the authors describe homogeneously distributed genome-wide platinum binding as measured by Damage-seq and heterogenous patterns of genome repair as measured by XR-seq. From this, they conclude that the observed sites of platinum damage on the genome is mostly dictated by repair efficiency, rather than lesion formation. The same group later developed “high-sensitivity-Damage-seq” [20], or HS-Damage-seq, which is designed to deplete undamaged DNA fragments from the extracted genomic DNA prior to high-throughput sequencing. This technology was used to demonstrate that Pt adduct formation is generally reduced around certain transcription factor binding sites. The authors suggest that this is

caused by occlusion of these sites by the transcription factors occupying them, effectively blocking cisplatin from accessing the sites. Damage-seq and XR-seq have been used in mouse models to generate cisplatin damage and repair maps in different mouse organs [21], and XR-seq has been used to map excision repair sites in mouse tissues after cisplatin treatment at different stages of the circadian cycle in mice [22].

Shu et al. have recently developed a technology called “cisplatin-seq” [23]. This is another high-throughput sequencing assay relying on the ability of Pt-DNA adducts to block polymerases. In this study, Pt-bound DNA was first enriched with a protein construct (Table 1b) derived from a high mobility group (HMG) box protein known to bind Pt-DNA lesions formed by cisplatin, HMGB1 [24]. The construct was demonstrated via dot blotting to have a high affinity for both GG and AG crosslinks , in contrast to the commercially available cisplatin antibody which was biased towards GG crosslinks (Table 1d). HMGB-enriched fragments were amplified with DNA polymerase and analyzed via high throughput sequencing. Cisplatin sites were identified if a stop was measured in sequencing that was also within a sequence that was enriched via the HMGB1 pulldown. This cisplatin-seq method was applied to HeLa cells that had been treated for 3, 12, or 24 hours with 50 μM cisplatin. An analysis of Pt(II) accumulation by chromosome distribution revealed an enrichment of platinum crosslinking sites on mitochondrial DNA relative to nuclear chromosomes. Within nuclear DNA, the researchers found relative enrichment in promoter regions and transcription termination sites, but determined that this enrichment could be attributed to a higher frequency of GG dinucleotides in those regions. Similarly, chromosomes found to have a relative enrichment of platinum lesions had a higher GG frequency. Interestingly, strong nucleosome signals were associated with higher crosslink frequency regardless of GG density. Higher crosslink frequency was also observed on sequences associated with non-histone DNA binding proteins. The authors suggest from this

correlation that these proteins protect crosslinking sites from NER, and that shielding from repair affects the accumulative pattern of lesions on the genome.

Powell et al. performed a microarray-based pulldown study using the anti-cisplatin antibody (Table 1d) [25]. The antibody was used to enrich for both cisplatin- and oxaliplatin-damaged DNA in yeast and normal human dermal fibroblast cell culture, with treatment concentrations up to 1 mM. Enriched samples were analyzed via microarray and compared to an unenriched input sample. The human microarrays covered a randomly selected 10 Mbp section of chromosome 17, while the yeast microarray probed the whole genome. These results were examined in association with a ChIP-on-chip microarray dataset from yeast to correlate DNA damage maps with histone H3 acetylation changes, which are expected to be more abundant in regions of DNA damage. A low resolution map of DNA damage was generated and revealed heterogeneous distribution of Pt-induced DNA damage, with some correlation to expected damage sites based on GG and AG sequence distributions. H3K9 acetylation patterns were found to be the same regardless of the type of damage.

Taken together, these high-throughput studies have confirmed an expected preference for platinum lesion formation on GG dinucleotides and more generally regions of high guanine frequency on the genome. They have also suggested that nucleosomes and DNA binding proteins may shield Pt(II) lesions from DNA repair machinery. A relationship between nucleosome positioning and the formation of Pt(II)-DNA lesions is supported by studies that have demonstrated either an overall increase [26] or redistribution [27] of Pt(II)-DNA lesions upon induction of a euchromatic state in cells using histone deacetylase inhibitors.

Beyond these general conclusions, these studies have revealed mixed results regarding the initial formation and accumulative patterns of Pt adducts. Several limitations have permeated these studies, with one major challenge being the enrichment of platinated fragments prior to sequencing. Some rely on the commercially available anti-cisplatin antibody

for enrichment (ab103261, Abcam) [19,25]. While this antibody recognizes the GG lesions formed by cisplatin and carboplatin, it has little to no immunoreactivity to AG adducts [23] and has not been demonstrated to recognize the adducts formed by oxaliplatin. Similarly, the HMG protein used by Shu et al. to enrich for platinated DNA has been shown to have a higher affinity for lesions formed by cisplatin than those formed by oxaliplatin [5,28]. Additionally, these methods were all developed using high concentrations of Pt compounds that are above clinical relevance. All rely on one or more "secondary properties" of the lesions, such as their ability to block polymerases, or the affinity of specific proteins or antibodies to Pt-damaged DNA. More direct methods such as those utilizing click chemistry to recognize and enrich for the Pt compounds themselves regardless of cargo have the potential to further our understanding of Pt accumulation on DNA genome-wide and nucleic acids more generally in the cell.

3. Detection of Platinum Using Pre-tethered Fluorophores

Methods of tracking platinum compounds in cells are important tools for understanding molecular targets. Atom-based methods such as installation of a radioactive platinum atom, X-ray fluorescence, electron microscopy, NMR, and mass spectrometry can provide information on platinum location and binding partners and have been reviewed previously [3]. These methods, while useful for *in vitro* and non-cellular work, are difficult to apply to live cells. One approach for potential live-cell imaging is the tethering of a fluorophore directly onto the platinum compound.

Cisplatin, oxaliplatin, and carboplatin are small, low or no- carbon molecules (Figure 1) in contrast to most fluorophores. The attachment of a fluorophore to platinum compounds can give rise to differences in charge, lipophilicity, solubility, polarity, or reactivity from the parent platinum compounds, as noted previously by Wexselblatt et al. [2]. For platinum compounds, small changes can result in large differences to toxicity. For example, the addition of a methyl or

ethyl to the cyclohexane ring changes the toxicity of oxaliplatin derivatives [29]. Another example demonstrated by Rijal et al. showed altered binding specificities of different amino-acid modified platinum compounds towards 16S rRNA [14]. If such small changes can make large impacts in these examples, it would stand to reason that larger deviations, such as an added fluorophore, could significantly affect activity. Also of concern may be the assumption that the fluorescent ligand remains tethered to the platinum in the cell. Fluorescence detection only gives information on the location and presence of the fluorophore and not the platinum itself. Fluorophores tethered through a monodentate ligand could be susceptible to trans-labilization [2].

Despite these limitations, platinum-tethered fluorophores have proved invaluable to platinum research and continue to be synthesized. There have been many new compounds that incorporate a fluorophore created within the last few years. Kitteringham et al. have created a carboplatin mimic with an incorporated BODIPY fluorophore and have imaged it in both cisplatin sensitive and resistant cells [30]. Kalayda et al. created an oxaliplatin mimic which has been functionalized with a fluorescein and used to study oxaliplatin resistance [31]. A glucose and BODIPY conjugate has also been synthesized that shows enhanced uptake into cells [32]. Xue et al. created a BODIPY-incorporated platinum compound modified with a photosensitizer to induce reactive oxygen species [33]. Yao et al. have also produced a cysteine-directed derivative using their click enabled, acridine-tethered compound **7** (Table 2) [34]. Additionally, platinum(IV) has been derivatized to release fluorophores from the axial positions to investigate payload release upon reduction. Some recent Pt(IV) derivatives utilize aggregation-induced emission for visualization [35] and an EGFR-targeted platinum compound tethered to a fluorescein [36].

Fluorescent platinum probes have been valuable to the field and continue to be used to further study platinum accumulation and targets; however, the chemical concerns of the derivatives

compared to the parent compounds have gone largely unaddressed. A more direct method, such as selectively tethering fluorophores to platinum compounds after native activity has already occurred, is highly desirable.

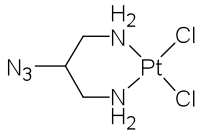
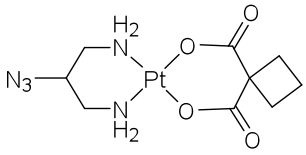
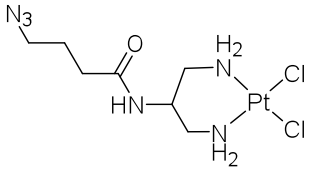
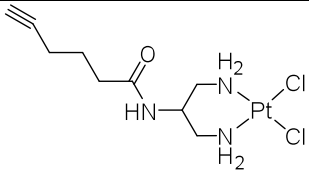
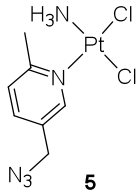
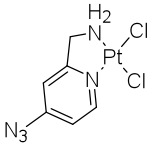
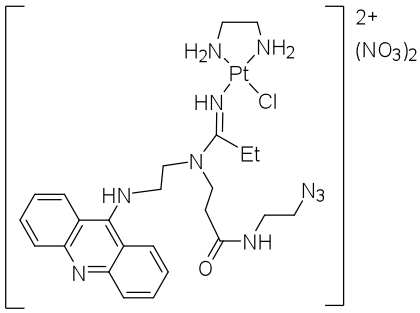
Structure	Use	Reference
 **1**	Library synthesis of carboplatin derivatives	[38]
	Fluorescent labeling of DNA and ribosome	[39]
	Protein target identification	[40]
 **2**	Synthesis of carboplatin derivatives	[38]
	Toxicity	[30]
 **3**	Protein target identification	[40]
	DNA pulldown	[42]
	Cellular imaging	[41]
	Fluorescent labeling of DNA	[41]
 **4**	Cellular Imaging	[41]
 **5**	Fluorescent labeling of RNA, ribosomal RNA and tRNA	[13]
 **6**	Cellular imaging, gene expression, DNA binding	[27]
 **7**	Synthesis of acridine derivatives	[34]
	Cellular imaging	[44]
	DNA labeling	[44]
	Investigation of DNA and RNA synthesis, Cell cycle investigation	[43]

Table 2. Click-enabled Platinum(II) Derivatives

4. Click chemistry for post-treatment tethering to platinum compounds

Click chemistry refers to reactions that are distinguished as being modular and high yielding [37]. A widely used bioorthogonal click reaction is azide-alkyne cycloaddition, either Cu-catalyzed (CuAAC) or strain-promoted (SPAAC). An azide group is easy to incorporate via a substitution reaction into many molecules of interest. The small size, biological inertness, and overall neutral charge have made it an enticing option for incorporation into platinum ligands. The installation of a small azide may have less influence on the localization or activity of the platinum compound than the installation of a large fluorophore. The selectivity of the CuAAC and SPAAC reactions for post-treatment labeling provide direct detection of the platinum compound.

There are currently a handful of available click-capable platinum compounds (Table 2). All of the compounds have been used for cellular work and for a variety of other applications. Kitteringham et al. determined the IC_{50} of the azide-modified compound **2**, which had similar toxicity to the parent carboplatin and much higher toxicity than the pre-tethered BODIPY conjugate mentioned above [30,38]. Compound **1** originally synthesized by Urankar et al. has been used to label DNA as well as characterize cellular RNA targets in *S. cerevisiae* by Moghaddam et al. [38,39]. It was also used for *in vitro* fluorescent protein labeling and an enzymatic assay [40]. Compound **3** has been used to enrich for Pt-bound proteins for target identification in *S. cerevisiae* [40], in addition to its application to enrich for and fluorescently label Pt-bound DNA *in vitro* [41,42]. For a more comprehensive look at Pt binding in a cellular context, compound **3** and compound **4**, its alkyne derivative, were used in cellular imaging studies [41]. Compound **5**, another click chemistry enabled compound developed in the DeRose lab, was used to fluorescently label ribosomal RNA and tRNA from treated *S. cerevisiae* [13]. Zacharioudakis et al. synthesized compound **6** in order to determine localization of Pt-DNA

adducts upon treatment with a histone deacetylase inhibitor [27] and to investigate the effect of platinum on gene expression. Lastly, compound **7** was used by Qiao et al. to determine the cellular distribution of a Pt-acridine compound [43]. It has since been used to synthesize new derivatives that incorporate cysteine-directed moieties as described above. Compound **7** has been used to label proteins, investigate DNA and RNA synthesis after platinum introduction, and to fluorescently label DNA [34,43,44].

The click chemistry-enabled Pt compounds described above have been used to further understanding of Pt interactions in cells through the direct detection of the Pt reagent post-treatment, allowing for Pt localization that is theoretically unbiased from the influence of fluorophore properties. However, these compounds have all been restricted to the CuAAC reaction which requires a cytotoxic Cu(I) catalyst, or to strain-promoted reactions that require a non-cell permeable DIBO [45]. Further work into diversifying the click-capable platinum compounds to other types of bioorthogonal reactions may allow expansion into live-cell imaging and other types of investigations based on post-treatment derivatization.

5. Pt interactions with proteins

As a soft Lewis acid, Pt(II) is reactive towards soft nucleophiles, and has a preference for coordinating sulfur- and nitrogen-containing molecules. Thus, proteins, especially those that are rich in Cys, Met, and His residues, are an attractive target for Pt(II) [46]. Pt-protein interactions are an understudied aspect of Pt(II) drug treatment, as most work is done investigating Pt(II) interactions with DNA. Because of their hypothesized role in Pt(II) toxicity and resistance mechanisms, investigations into Pt-protein interactions *in vitro* have expanded in the past decade. Various model proteins have been employed to study Pt(II) binding to proteins, and these studies have been carefully reviewed previously [47–51]. Overall, *in vitro* investigations have shown that contrary to expectations, Cys residues are not necessarily the

main or only binding site available on proteins. Met and His residues are highly targeted, as well as some oxygen-containing residues such as Asp, Glu, Tyr, and Ser. These studies have revealed interesting conformational and functional changes to Pt(II)-bound proteins [52–55]. However, the differences in treatment conditions and analysis technologies have not provided much consensus on specific binding sites. Most studies require the use of high treatment concentrations or long treatment times to generate enough signal to be detected among the "noise" of unplatinated proteins. These conditions are not analogous to cellular conditions, and observed binding sites may not represent those occurring during treatment. A handful of investigations have attempted to address in-cell reactivity using methods ranging from shotgun proteomics without enrichment, enrichment using sequential hyphenated techniques, enrichment and identification by pre-tethered Pt(II) compounds, and more recently post-treatment enrichment by click chemistry (Figure 2). A summary of Pt-bound proteins following in-cell treatment that have been detected by multiple methods is provided in Table 3.

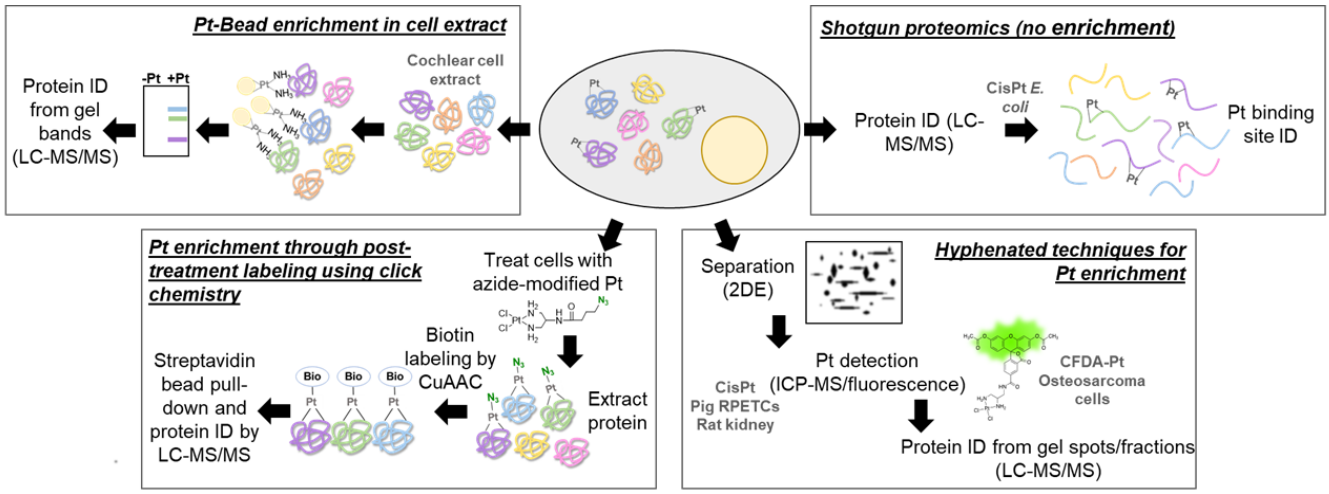


Figure 2. Proteomics strategies for identification of Pt-bound proteins in treated cells. Shotgun proteomics with no prior enrichment for Pt-proteins has been performed on cisplatin-treated *E. coli* with successful identification of Pt-bound peptides. A variety of studies have employed hyphenated techniques such as 2D electrophoresis and ICP-MS upstream of LC-MS/MS to enrich for Pt-proteins in either cisplatin or CFDA-Pt treated cells. Pt(II)-conjugated agarose beads have been used to identify Pt-interacting proteins from cochlear cell extract. Pt-proteins in treated yeast have been enriched for through a post-treatment biotin labeling method using click chemistry.

5.1 Pt-protein identification with no prior enrichment

Will et al. identified specific Pt binding sites on proteins in cisplatin-treated *E. coli* using multidimensional liquid chromatography and tandem mass spectrometry (MudPIT) with no prior enrichment [56]. They were able to identify Pt(II) binding sites on a total of 31 proteins, including DNA mismatch repair protein MutS, DNA helicase II (UvrD), ATP-dependent RNA helicase DbpA, and abundant enzymes like malate dehydrogenase (Mdh), enolase (Eno) and glyceraldehyde-3-phosphate dehydrogenase (G3p1). This report used high concentrations of cisplatin for treatment, at 1 mM for 3 hours. To identify targeted proteins at a more therapeutically relevant concentration, Stephanopoulou et al. used the same methodology to identify Pt-bound proteins in *E. coli* treated with 50 μM cisplatin for 3 hours [57]. In this study, 18 total proteins were found to have Pt binding sites. In accordance with Will et al., they identified modification on Mdh, Eno, and elongation factor Tu (Tuf1), however different residues were predicted as Pt binding highlighting the relevance of treatment concentration. New targets were also found, including Hsp70 family protein DnaK, universal stress protein (UspA), and multiple 30S ribosomal proteins (RpsA, RpsF, RpsK). While sulfur- and nitrogen-containing residues are predicted to be preferred binding sites of Pt in proteins, both studies observed significant modification at oxygen-containing Ser, Thr, Tyr, Glu and Asp residues. Although His, Cys, and Met residues are thermodynamically preferred by Pt, the kinetic preference of oxygen-containing residues appears to be captured at short treatment times of 3h. The authors of both these studies specifically note that this methodology likely favors identification of abundant proteins, and is not able to provide information on less abundant proteins. Additionally, low signal to noise prevents easy identification of Pt binding residues on targeted peptides.

5.2 Hyphenated techniques for identification of Pt-protein interactions

A few studies have utilized hyphenated techniques to enrich for platinated populations of proteins. Moreno-Gordaliza et al. identified Pt-bound proteins from pig renal proximal tubule epithelial cells treated with 1 mM cisplatin for 6 hours [58]. To accomplish this, proteins were first separated by 2D electrophoresis, then Pt-containing spots in the gel were identified by LA-ICP-MS. A total of 26 Pt-containing spots were excised and proteins identified by nHPLC-ESI-MS/MS. In agreement with studies performed in *E. coli*, they also identified malate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, enolase, and elongation factor Tu in excised gel spots. Additional proteins found in spots with high Pt signal include cytoskeletal protein vimentin, ER chaperone GRP94, nucleolar protein nucleolin, and core histones (H2A, H2B, H3, and H4). Pt modified peptides were not observed, which the authors note is likely due to the significantly lower abundance of Pt bound peptides in comparison to unmodified peptides.

Moraleja et al. also employed a combination of techniques to enrich for Pt-bound proteins in kidney tissue from a rat treated with cisplatin [59] (16 mg/m^2 for 3 days). To do this, proteins were first separated by molecular weight and Pt content monitored using SEC-ICP-MS. They then selected a Pt-rich fraction for further separation by isoelectric focusing. Pt content was again determined using ICP-MS for each of the collected fractions after IEF, and 4 of the most Pt rich fractions were analyzed by nLC-MS/MS. From these fractions, albumin, cytochrome C, thioredoxin, and copper transporter ATOX1 were identified. No Pt-modified peptides could be identified in this study either, which was again reported to likely be due to low abundance of Pt-peptides.

5.3 Pre-tethered Pt(II) compounds for enrichment of Pt(II)-bound proteins

While the use of multiple protein separation and Pt detection technologies prior to mass spectrometry has provided important insight into Pt-protein interactions in the cell, these methods are time intensive and not suitable for high-throughput studies. Karasawa et al. used a different approach for Pt-peptide enrichment, synthesizing Pt(II)-conjugated agarose beads [60]. As they were specifically interested in Pt protein interactions through the ammine ligands of cisplatin, the beads contained conjugated $[Pt(NH_3)_2]^{2+}$. Lysates from mouse cochlear cells were incubated with these beads for 2h, and bound proteins separated by SDS-PAGE. Bands with high intensity were then chosen for LC-MS/MS analysis and a total of 17 proteins were identified. Quite a few are related to function of the endoplasmic reticulum, including GRP94, the ER ATP-ase VCP, Ca^{2+} storage protein calreticulin, and chaperones GRP78 and protein disulfide isomerase. As a selection of high intensity bands from pulldown experiments were used for mass spectrometry, the authors note that the identified proteins might represent more abundant proteins, excluding identification of less abundant proteins. Again, no specific Pt binding sites on these proteins are identified.

More recently, Kotz et al. [61] used a fluorescently labeled cisplatin analogue, carboxy-fluoresceindiacetate covalently linked to $Pt(en)Cl_2$ (CFDA-Pt) [62], to identify Pt-bound proteins prior to analysis. This compound was initially synthesized to track the subcellular localization of Pt(II) in human osteocarcinoma cells [62]. Human ovarian carcinoma cells (A2780) were treated with 25 μM CFDA-Pt for 2 hours and proteins separated by 2-D electrophoresis [61]. The fluorescent moiety of CFDA-Pt allowed for visualization of Pt-bound proteins in-gel using fluorescence scanning. Fluorescent spots were then excised from the gel and proteins identified by ESI-MS/MS. Spots that were analyzed were determined to be GRP78, PDIA1, PDIA6, PDIA3, β-actin, and vimentin. Low abundance of Pt-bound peptides is again hypothesized to be responsible for the lack of identification of specific platination sites, with the need for pre-concentration steps highlighted by the authors.

5.4 Post-treatment labeling of Pt(II)-bound proteins using click chemistry

These studies establish that there is still a need for increased specificity and easily implemented enrichment methods for Pt-bound proteins in cellular systems. Our lab addresses this need through a click chemistry-based approach, using azide and alkyne-containing Pt(II) compounds that are suited for post-treatment labeling and enrichment steps. In a recent report, *S. cerevisiae* were treated for 6 hours with 75 μM of the azide-modified Pt(II) compound azidoplatin (AzPt, **3**, Table 2), and Pt-bound proteins were labeled with biotin by the CuAAC click reaction [40]. Pt-bound and biotin labeled proteins were then enriched for using streptavidin coated beads and bound proteins identified by LC-MS/MS. In total, we identified 152 proteins that were significantly enriched in AzPt treated *S. cerevisiae*. Enriched proteins are distributed in predicted cellular abundance, and a number of low abundance proteins are included. Of note were a number of highly enriched proteins involved in cellular stress pathways (Table 3) such as ER stress, metal homeostasis, cell redox homeostasis, and the DNA damage response. In addition to the many novel proteins found to be enriched in Pt-treated cells, proteins previously identified as Pt(II) binding were also identified in this study, providing further evidence of their targeting and validating our click chemistry-based method of enrichment (Table 4).

Metal-binding proteins have been identified as Pt(II) targets across multiple *in vitro* and *in cellulo* studies. ATOX1 is a copper transporter chaperone that is responsible for the trafficking of Cu(I) from copper transporter 1 (CTR1) to ATPase copper transporters that deliver Cu(I) to the golgi apparatus or secretory vesicles [63]. ATOX1 and the yeast homologue ATX1 contain the metal-binding domain CXXC, which has been shown to bind Pt(II) following cisplatin treatment *in vitro* [51,52,64,65]. ATOX1 was identified in Pt-enriched fractions from cisplatin treated rat kidney [59], and ATX1 was enriched in AzPt-treated yeast samples. One way in

which cisplatin and other Pt(II)-based drugs enter the cell is by active transport through CTR1 [66]. Identification of copper binding proteins in cellular studies further lends to the evidence that this is one mechanism through which Pt(II) influx and efflux is mediated.

Endoplasmic reticulum (ER) stress has been suggested to be an important cytoplasmic mediator of apoptosis upon cisplatin treatment [67–69]. Interestingly, proteins involved in ER stress have been identified as targets of Pt(II) in most cellular studies investigating Pt-protein binding, including our own. One of the most frequently identified proteins targeted by Pt(II) in cells is the protein folding chaperone protein disulfide isomerase (PDI), which has been proposed to play an important role in the cellular response to cisplatin treatment [70,71]. Pt(II) treatment of PDI *in vitro* has been shown to inhibit PDI activity [40]. The active site of PDI contains a conserved thioredoxin-like domain, CXXC [72], which we hypothesize is targeted by Pt(II) to inhibit PDI activity. Additional work is needed to confirm that this inhibition happens in a cellular context, and to determine which specific residues of PDI are bound by Pt(II). This identification of Pt(II)-binding at the amino acid level *in cellulo* has proven difficult, as evidenced by the lack of information regarding bound residues gained from previous cellular studies. Application of enrichment methods such as post-treatment labeling by click chemistry may improve detection of platinated peptides.

Protein	Description	Accession	Binding site	Organism	Reference	Pt(II) compound
DbpA	ATP-dependent RNA helicase	P21693	D399	*E. coli*	[56]	Cisplatin
DBP2 + DED1	ATP-dependent RNA helicase	P06634 + P24783	No ID	*S. cerevisiae*	[40]	Azidoplatin
HisI	Histidine biosynthesis bifunctional protein	P06989	M37 (Y36)	*E. coli*	[56]	Cisplatin
HIS4	Histidine biosynthesis trifunctional protein	P00815	No ID	*S. cerevisiae*	[40]	Azidoplatin
Tuf1	Elongation factor Tu 1	A7ZSL4	M369 (M359, H365, T362)	*E. coli*	[56]	Cisplatin
Tuf1	Elongation factor Tu 1	A7ZSL4	M113	*E. coli*	[57]	Cisplatin
TEF1	Elongation factor 1-alpha	P02994	No ID	*S. cerevisiae*	[40]	Azidoplatin
TUF1	Elongation factor Tu, mitochondrial	P02992	No ID	*S. cerevisiae*	[40]	Azidoplatin
Mdh	Malate dehydrogenase	P61889	T298 (D297)	*E. coli*	[56]	Cisplatin
Mdh	Malate dehydrogenase	P61889	T283, S285	*E. coli*	[57]	Cisplatin
MDH3	Malate dehydrogenase, peroxisomal	P32419	No ID	*S. cerevisiae*	[40]	Azidoplatin
TPM2	Beta-tropomyosin	A1X899	No ID	Renal proximal tubule epithelial cells (*Sus scrofa*)	[58]	Cisplatin
TPM1	Tropomyosin-1	P17536	No ID	*S. cerevisiae*	[40]	Azidoplatin
TUBA1A	Tubulin alpha-1A chain	P02550	No ID	Renal proximal tubule epithelial cells (*Sus scrofa*)	[58]	Cisplatin
TUB2	Tubulin beta chain	P02557	No ID	*S. cerevisiae*	[40]	Azidoplatin
ALDH2	Aldehyde dehydrogenase, mitochondrial	Q2XQV4	No ID	Renal proximal tubule epithelial cells (*Sus scrofa*)	[58]	Cisplatin
ALD4 + ALD5	Aldehyde dehydrogenase	P46367 + P40047	No ID	*S. cerevisiae*	[40]	Azidoplatin
TrxA	Thioredoxin 1	P0AA25	H7	*E. coli*	[56]	Cisplatin
TXN	Thioredoxin	P11232	No ID	Kidney tissue (*Rattus norvegicus*)	[59]	Cisplatin
TRX1 + TRX2	Thioredoxin-1 and Thioredoxin-2	P22217 + P22803	No ID	*S. cerevisiae*	[40]	Azidoplatin
CTSD	Cathepsin D (aspartyl protease)	P24268	No ID	Kidney tissue (*Rattus norvegicus*)	[59]	Cisplatin
PEP4	Saccharopepsin (aspartyl protease)	P07267	No ID	*S. cerevisiae*	[40]	Azidoplatin
PABPC1	Polyadenylate-binding protein 1	Q9EPH8	No ID	Kidney tissue *(Rattus norvegicus*)	[59]	Cisplatin
PAB1	Polyadenylate-binding protein	P04147	No ID	*S. cerevisiae*	[40]	Azidoplatin
EIF5A	Eukaryotic translation initiation factor 5A-1	Q3T1J1	No ID	Kidney tissue (*Rattus norvegicus*)	[59]	Cisplatin
HYP2	Eukaryotic translation initiation factor 5A-1	P23301	No ID	*S. cerevisiae*	[40]	Azidoplatin
ATOX1	Copper transport protein	Q9WUC4	No ID	Kidney tissue (*Rattus norvegicus*)	[59]	Cisplatin
ATX1	Metal homeostasis factor	P38636	No ID	*S. cerevisiae*	[40]	Azidoplatin
VCP	Transitional endoplasmic reticulum ATPase	P55072	No ID	HEI-OC1 cell line (*Mus musculus*)	[60]	$2NH_3$Pt-agarose
CDC48	Cell division control protein 48	P25694	No ID	*S. cerevisiae*	[40]	Azidoplatin
PDIA4	Protein disulfide isomerase A4	P13667	No ID	HEI-OC1 cell line (*Mus musculus*)	[60]	$2NH_3$Pt-agarose
PDIA4	Protein disulfide-isomerase A4-like	F1SAD9	No ID	Renal proximal tubule epithelial cells (*Sus scrofa*)	[58]	Cisplatin
PDI-P5	Protein disulfide isomerase P5	E1CAJ6	No ID	Renal proximal tubule epithelial cells (*Sus scrofa*)	[58]	Cisplatin
PDIA3	Protein disulfide-isomerase A3	P11598	No ID	Kidney tissue (*Rattus norvegicus*)	[59]	Cisplatin
P4HB	Protein disulfide isomerase A1	P07237	No ID	A2780 cell line (*Homo sapiens*)	[61]	CFDA-Pt
PDIA6	Protein disulfide isomerase A6	Q15084	No ID	A2780 cell line (*Homo sapiens*)	[61]	CFDA-Pt
PDIA3	Protein disulfide isomerase A3	P30101	No ID	A2780 cell line (*Homo sapiens*)	[61]	CFDA-Pt
PDI1	Protein disulfide isomerase	P17967	No ID	*S. cerevisiae*	[40]	Azidoplatin

Table 3. Pt-bound proteins identified in multiple studies. Reported proteins are closest matched homologues. See reference for details.

Protein	Description	Enrich	P-Value	GO biological process
CDC48	*Cell division control protein 48*	∞	1.02E-05	Response to stress/ ER associated misfolded protein catabolic process
PDI1	*Protein disulfide isomerase*	6.2	7.90E-03	Response to stress/Protein folding
PRE9	*Proteasome subunit alpha type-3*	∞	8.21E-04	Proteasomal protein catabolic process
PUP2	*Proteasome subunit alpha type-5*	3.8	2.74E-02	Proteasomal protein catabolic process
RPN10	*26S proteasome regulatory subunit RPN10*	∞	5.55E-03	Proteasomal protein catabolic process
RPN2	*26S proteasome regulatory subunit RPN2*	∞	3.40E-04	Proteasomal protein catabolic process
RSP5	*E3 ubiquitin-protein ligase RSP5*	∞	6.17E-03	Proteasomal protein catabolic process
ATX1	*Metal homeostasis factor ATX1*	∞	1.01E-04	Response to stress/Copper chaperone activity
CCS1	*Superoxide dismutase 1 copper chaperone*	4.6	3.18E-02	Response to stress/Intracellular copper ion transport
CUP1-1	*Copper metallothionein 1-1*	6.3	4.48E-03	Response to stress/Detoxification of copper ion
TRX1	*Thioredoxin-1*	2.1	2.36E-02	Response to stress/Cell redox homeostasis
TRX2	*Thioredoxin-2*	3.7	2.71E-02	Response to stress/Cell redox homeostasis
NHP6A	*Non-histone chromosomal protein 6A*	∞	4.96E-03	Response to stress/DNA replication-independent nucleosome organization
POL30	*Proliferating cell nuclear antigen*	∞	3.84E-03	Response to stress/Nucleotide excision repair

Table 4. Subset of proteins involved in cellular stress responses identified by click chemistry in AzPt-treated *S. cerevisiae*. Enrichment value is reported as fold enrichment in AzPt treated samples following normalization in all samples (∞, protein was not identified in control samples). Listed proteins are grouped by response: ER stress, metal homeostasis, cell redox homeostasis, and DNA damage response. Reprinted with permission from Cunningham R.M. and DeRose V.J. *ACS Chem. Biol.* 2017, 12, pp 2737-2745. Copyright 2017 American Chemical Society.

5.5 Identification of Pt(II) mediated protein-DNA crosslinks

In addition to binding cytoplasmic proteins, there is much interest in the interactions of Pt(II) with serum proteins. As the most abundant protein in serum, significant work has been done investigating Pt(II) binding to serum albumin [73]. Recently, we used our azide-modified Pt(II) compounds to conjugate Pt-bound biomolecules to a hexanyl-modified DNA for DNA hybridization-based pulldown [42]. Unexpectedly, when we applied this methodology to label Pt-bound bovine serum albumin (BSA) we found that platinated BSA readily crosslinked DNA in the absence of the click reaction. Protein-DNA crosslinking was also observed with cisplatin-treated BSA, indicating that a Pt-BSA adduct may also crosslink to nucleic acids presumably through additional ligand exchange at Pt. We also observed some transfer of Pt from cisplatin treated BSA to free DNA. These observations indicate that Pt that has bound to proteins--in particular serum albumin--may still be reactive to DNA. Interestingly, while free DNA is readily degraded by serum nucleases, DNA crosslinked to BSA by cisplatin was nearly completely protected from degradation for up to 24 hours in serum [42].

Pt-facilitated protein-DNA crosslinks have been previously reported in *in vitro* studies [74–76]. More recently, Ming et al. used proteomics to identify proteins involved in Pt-mediated protein-DNA crosslinks in human fibrosarcoma cells treated with 100 μM cisplatin [77]. Of the 256 proteins that they identified, approximately half are classified as nuclear proteins, and unsurprisingly, a majority are DNA and RNA-binding proteins. The authors also note that many of the identified proteins are involved in important cellular processes such as DNA damage repair, RNA processing, regulation of transcription, and cell cycle regulation. These protein-DNA crosslinks have been shown to inhibit DNA replication and DNA damage repair processes *in vitro* [74], suggesting that protein-DNA crosslinks may be a particularly potent consequence of Pt(II) binding.

6. Conclusion

Upon entering a cell, platinum anticancer agents encounter an array of biomolecules. Despite the ubiquitous use of these drugs and their diverse clinical applications, the specific targets of cisplatin and its analogues in a cellular context remain elusive. Historically, attempts to understand the targeting profiles of these drugs have either relied on the examination of their extensive biological and clinical effects, or on highly specific *in vitro* structural studies. A growing body of research attempts to reconcile these disparate realms of study using unbiased, comprehensive high-throughput approaches towards target identification. In this review, we have described recent work which has begun to outline the patterns of platinum targeting and accumulation in the cell, as well as factors that determine in-cell binding specificity. We preview emergent methods and technologies that enable high-throughput identification of platinum targets. Further use and development of these methods will generate a more comprehensive understanding of the molecular mechanisms of action of platinum anticancer drugs in a cellular context.

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