Inteins, self-splicing protein elements, interrupt genes and proteins in many microbes, including the human pathogen Mycobacterium tuberculosis. Using conserved catalytic nucleophiles at their N- and C-terminal splice junctions, inteins are able to excise out of precursor polypeptides. The splicing of the intein in the mycobacterial recombinase RecA is specifically inhibited by the widely used cancer therapeutic cisplatin, cis-[Pt(NH₃)₂Cl₂], and this compound inhibits mycobacterial growth. Mass spectrometric and crystallographic studies of Pt(II) binding to the RecA intein revealed a complex in which two platinum atoms bind at N- and C-terminal catalytic cysteine residues. Kinetic analyses of NMR spectroscopic data support a two-step binding mechanism in which a Pt(II) first rapidly interacts reversibly at the N terminus followed by a slower, first order irreversible binding event involving both the N and C termini. Notably, the ligands of Pt(II) compounds that are required for chemotherapeutic efficacy and toxicity are no longer bound to the metal atom in the intein adduct. The lack of ammine ligands and need for phosphine represent a springboard for future design of platinum-based compounds targeting inteins. Because the intein splicing mechanism is conserved across a range of pathogenic microbes, developing these drugs could lead to novel, broad range antimicrobial agents.

Further studies reveal that the unique ability of an intein to break and form peptide linkages has led to their use in applications (1, 2) ranging from sensors of small molecules (3, 4) and environmental conditions (5) to single step protein purification platforms (6, 7). However, there are still underexplored reactions involving native inteins, notably their susceptibility as targets for antimicrobial drugs (8). In nature, inteins reside in key host proteins across several bacterial and fungal pathogens, including Mycobacterium tuberculosis, Mycobacterium leprae, and Cryptococcus neoformans (9).

These splicing elements divide the host protein into two parts, termed N- and C- inteins on the corresponding ends of the intein. In the precursor form, the host protein is usually non-functional because of the tendency of an intein to insert into highly conserved functional regions of the protein. In M. tuberculosis, inteins occur in three genes, recA, sufB, and dnaAB (9). The functionality of these proteins is key to the survival of the bacterium and relies upon the splicing of the intein from the respective host proteins. Because inteins do not occur in multicellular organisms, prevention of protein splicing provides a promising strategy for the development of novel antimicrobial therapeutics.

Canonical intein splicing occurs by a multistep process (10). First, a nucleophilic attack is initiated by the N-terminal cysteine residue (C1) of the intein on the preceding peptide bond, resulting in thioester formation (Fig. 1A, step 1). A second nucleophilic attack occurs from the first residue of the C-extein, usually a cysteine (C + 1), on the thioester bond, joining the flanking exteins in a branched intermediate (Fig. 1A, step 2). Finally, a C-terminal aspartagine of the intein undergoes cyclization, releasing the ligated exteins (Fig. 1A, step 3). Spontaneous N–S acyl rearrangement leads to the formation of a native peptide bond and a functional protein. The splicing process is mediated through a number of different residues that occur in conserved blocks across many inteins, such as the initiating cysteine in the A-block and the terminal G-block histidine and asparagine of the mycobacterial RecA intein (11). Residues sequentially far from the splice junctions but close spatially also mediate the splicing reaction at both the N and C termini of the intein, such as these F-block aspartate of RecA (Asp422) (12).

The M. tuberculosis RecA intein was previously minimized by removing an endonuclease domain not required for splicing (ΔAlh-SM) (13). This minimized intein is susceptible to inhibition by cisplatin with an IC₅₀ value in the low micromolar range. This result was mirrored in M. tuberculosis with cisplatin arresting mycobacterial growth by intein targeting (14). To devise a more rational approach to the design of intein-based therapeutics, we sought to provide structural and mechanistic insights into inhibitor binding. Additional platinum com-
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Compounds with safer toxicity profiles would be attractive for evaluation in the treatment of *M. tuberculosis*-related infections. Two newer platinum compounds, [Pt(tfbz)(NH$_3$)$_2$(NO$_3$)] (Ptffbz)$^3$ (15) and phenanthriplatin (16, 17) (Fig. 1B), both derived from cisplatin and assessed as tumor-inhibiting drug candidates, have been examined in the present study. In addition, we investigated Zeise’s salt, K[Pt(Cl$_3$(CH$_2$=CH$_2$)] (18) (Fig. 1B), a platinum-based organometallic compound, for its ability to inhibit intein splicing.

Here, we focus on characterizing the inhibition of the RecA intein by cisplatin, Ptffbz, and Zeise’s salt, which have an IC$_{50}$ in the same range and are >10-fold more effective than phenanthriplatin. We first developed an intein construct for metal binding (Fig. 1C) and then performed structural studies using a combination of mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, and x-ray crystallography. These results help inform the design of platinum-based drugs for splicing inhibition as the search for novel antimicrobials becomes pressing.

Results

Binding of Platinum Compounds to RecA Intein Depends on the Presence of a Reducing Agent—Three Pt(II) complexes were studied and compared with cisplatin for their ability to inhibit intein activity. The effectiveness of the compounds was tested with a fluorescence-based assay using a split GFP construct (29 kDa) containing a minimized RecA intein (15.3 kDa) as described previously (14, 19). Inhibition by the compounds was determined by fitting the data with a dose-response curve and calculating the inhibition constant, IC$_{50}$ (Fig. 2A and supplemental Fig. S1), defined as the concentration of inhibitor required for a 50% reduction in splicing activity. Cisplatin, Ptffbz, and Zeise’s salt have very similar IC$_{50}$ values (within one standard deviation of each other) of 1.67, 1.97, and 1.18 μM, respectively (Fig. 2A, CisPt, Ptffbz, and Zeise). By contrast, phenanthriplatin is not very active, having an IC$_{50}$ >20 μM (Fig. 2A, Phen), and was therefore not further analyzed.

The intein derivative ΔΔlh-SIC (structure inhibitor construct) was used to probe structural characteristics of cisplatin, Ptffbz, and Zeise’s salt binding. ΔΔlh-SIC is a derivative of ΔΔlh-SM with an N440A mutation of the terminal asparagine designed to retain the C-extein following N-terminal cleavage (Fig. 1C). The Cys$^+$1 residue, which is key to platinum coordination, was maintained with four additional C-extein residues. Native polyacrylamide gel electrophoresis was used to provide initial information about the binding of cisplatin, Ptffbz, and Zeise’s salt to the intein. Incubation of the intein in the presence of the reducing agent tris(2-carboxyethyl)phosphine (TCEP) led to the observation that TCEP is required for specific binding of cisplatin and Ptffbz to the intein. In the absence of TCEP, there is no visible shift with either cisplatin or Ptffbz (Fig. 2B, top, 1, 3 and 5). There is a substantial band shift corresponding to the Pt(II)-bound form of the intein in the presence of TCEP, which is suggestive of a structural rearrangement or change in the charge state (Fig. 2B, top, 2 and 4). TCEP immobilized on beads was also used as it allowed the intein to be reduced while not being freely available in solution, but no observable shift was seen upon addition of cisplatin to the reduced intein (supplemental Fig. S2). In contrast, Zeise’s salt bound in the absence of TCEP, yielding many reduced mobility intein bands that suggest a range of different binding events to the intein (Fig. 2B, bottom, 5). The band shifts for cisplatin, Ptffbz, and Zeise’s salt

$^3$The abbreviations used are: Ptffbz, [Pt(tfbz)(NH$_3$)$_2$(NO$_3$)] where tfbz is trifluorobenzoylacetonate; SIC, structure inhibitor construct; TCEP, tris(2-carboxyethyl)phosphine; r.m.s.d., root mean square deviation; HSQC, heteronuclear single quantum coherence; IPTG, isopropyl β-d-1-thiogalactopyranoside; DTT, dithiothreitol.

![FIGURE 1. Intein splicing and potential Pt(II) inhibitors. A, schematic of intein splicing and inhibition. Cys1 and Cys$^+$1 are the two catalytic nucleophiles in protein splicing (steps 1 and 2). C-terminal asparagine (shown in C; Asn440) cyclization releases the intein from the ligated exteins (step 3). Inhibition of the catalytic cysteines at the N- and C-terminal splice junctions prevents splicing (step 0). B, chemical structures of platinum-based compounds Ptffbz, cisplatin, Zeise’s salt, and phenanthriplatin. C, schematic of the RecA intein and the minimized derivatives used in this study. ΔΔlh-SIC is derived from ΔΔlh-SM through an N440A mutation, which inhibits C-terminal cleavage but permits cleavage at the intein N terminus. αα, amino acids.](https://example.com/figure1.png)
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in the presence of TCEP appear to be identical (Fig. 2B, bottom, 2 and 4), suggesting the same or a very similar intein-Pt(II) product formed. The complexes formed in the presence of these three platinum compounds were further investigated through mass spectrometry.

Mass Spectrometry Reveals Two Platinum Adducts to the Intein in the Presence of TCEP—MS was used to determine the mass of intein adducts. The ΔΔIhh-SIC construct was incubated with cisplatin, Pttfbz, or Zeise’s salt in the absence or presence of TCEP. A single peak was detected in the absence of the three platinum compounds, which corresponds to free intein (Mcal = 15885.11 Da, Mobss = 15884.57 Da) (Fig. 3A, 7). With cisplatin and Pttfbz alone in the absence of TCEP, only the free intein peak is observed (Fig. 3A, 2 and 4). Zeise’s salt shows a unique ability to bind to the intein in the absence of TCEP, resulting in a range of masses all larger than that of the intein (Fig. 3A, 6). Further investigation into Zeise’s salt binding in the absence of TCEP shows a concentration-dependent range of Pt(II) adducts to ΔΔIhh-SIC (supplemental Fig. S3). Although Zeise’s salt binding to ΔΔIhh-SIC in the absence of TCEP is interesting, the nonspecific nature of the association defied structural characterization.

In contrast, incubation of the intein with the three platinum compounds in the presence of TCEP yielded an identical complex of heavier mass. In each case, the complex corresponds to the intein associated with two Pt(II) atoms, one TCEP, and three water molecules (Mcal = 16579.50 Da, Mobss = 16580.90 Da) (Fig. 3A, 1, 3, and 5). A number of small, lower molecular mass peaks can be seen, reflecting incorporation of lysine residues instead of arginine (leading to shifts differing by 28 Da each) (20) and small peaks of masses that are larger than the major peak, possibly corresponding to salt adducts to the protein. The identical products formed by cisplatin, Pttfbz, and Zeise’s salt in the presence of TCEP may suggest a similar reaction mechanism for the three compounds in which the TCEP mediates a common Pt(II) intermediate in binding to the intein.

To determine the relationship between the two Pt(II) binding events, a titration was performed with cisplatin (Fig. 3B, S:1, 2:1, and 1:1). Interestingly, only higher molar ratios of cisplatin to ΔΔIhh-SIC intein led to formation of the intein-Pt(II)3 complex. At substoichiometric concentrations of cisplatin to intein (≤1:1), no binding was observed. The ΔΔIhh-SIC-Pt(II)2·TCEP complex seems to be stable only when two platinum ions are bound to the intein, highlighting the importance of both binding sites in stabilizing the complex.

Crystal Structure of Platinum Adducts Reveals a Quaternary Complex—A ΔΔIhh-SIC-Pt(II)2·TCEP crystal was crystallized. The crystal structure, solved at 1.50-Å resolution (supplemental Table 1), confirmed binding of two Pt(II) atoms and one TCEP unit. The Pt(II) atoms are bound at the active sites in stabilizing the complex.

FIGURE 2. Intein inhibition by platinum-based compounds. A, IC50 of Pttfbz, cisplatin (CisPt), Zeise’s salt, and phenanthriplatin (Phen) in the context of a ΔΔIhh-SM split GFP construct. IC50 was calculated using a normalized fluorescence output, fitted to a dose-response curve, and averaged among four technical repeats. Error bars of 1 S.D. are shown (see also supplemental Fig. S1). B, native band shifts on 15% polyacrylamide gels. Binding of cisplatin and Pttfbz to the ΔΔIhh-SIC intein occurs only in the presence of TCEP (top, 2–5), whereas binding of Zeise’s salt (Zeise) also occurs in the absence of TCEP (bottom, 5). The TCEP-bound products appear identical in all three cases. The lower free intein band is likely a conformer that is formed in the 15% polyacrylamide non-denaturing gel. Gel shift assays were replicated, and representative gel images are shown.
thiolate sulfur atom and the N-terminal amino nitrogen of Cys1. However, the crystal structure does not show any ligands in the expected trans coordination with crystals derived from independent protein preparations (Fig. 4B and supplemental Fig. S4A). Crystal structures lacking expected ligands are not unprecedented (21, 22). Furthermore, a Zeise’s salt co-crystal with similar coordination geometry shows two aqua ligands in the trans positions (supplemental Fig. S5A and Table 2A). The Pt(II) at the C-terminal splice junction is coordinated to Cys/H11001 and His439 through the thiolate and imine nitrogen of the imidazole, respectively (Fig. 4C and supplemental Fig. S4B). His439 is a highly conserved penultimate intein residue in the G-block involved in chemistry at the C-terminal splice junction. The TCEP molecule was shown to be coordinating the square planar Pt(II) ion, cis to a coordinated water molecule or hydroxide ion. The plane of best fit through these atoms was calculated, and the atoms exhibited a r.m.s.d. of 0.11 Å from this plane (supplemental Table 2B). The terminal carboxylates of TCEP are in hydrogen bonding distance to the amines from Lys36 and Lys74. Nearly identical coordination geometry at the C terminus was also observed in the Zeise’s salt co-crystal (supplemental Fig. S5B).

Comparison of the crystal structure of the complex with that of another minimized RecA intein construct, ΔΔIhh-TR, which differs from ΔΔIhh-SIC by a T70A mutation that affects cleavage at the N terminus but is identical at the C terminus (23), showed that Pt(II) binding resulted only in localized conformational changes at the C-terminal binding site. The r.m.s.d. of the free ΔΔIhh-TR and ΔΔIhh-SIC-Pt(II)2TCEP complex is 0.18 Å. The two most prominent structural changes are indicated in Fig. 4D. A shift of 3.3 Å occurs upon coordination of the Cys/H11001 residue with the Pt(II) atom (Fig. 4D). A twist of the His439 imidazole ring orients the Ne2 atom for Pt(II) binding with Cys/H11001, TCEP, and a water molecule forming a square planar geometry (Fig. 4C). Two other weakly associated water molecules are also present in coordination with the C-terminal Pt(II). The distance between the two Pt(II) ions is 11.3 Å, and their presence leads to Cys1 and Cys/H11001 being moved farther apart from each other from 9.4 to 12.7 Å. This new location for Cys/H11001 is significantly greater than the average distance (8–9 Å) required for an intein to function (24, 25).

NMR Kinetic Studies of Platinum Binding to the Intein Reveals a Two-step Reaction—We assigned 95% of the backbone amide proton and nitrogen chemical shifts of the ΔΔIhh-SIC to ΔΔIhh-SIC. Compounds form an identical product in the presence of TCEP (1, 3, and 5). Zeise’s salt shows a range of adducts in the absence of TCEP (6). In the absence of compound (7) or absence of TCEP for cisplatin (2) and Pt(tfbz) (4), only free intein peak is seen. B, cisplatin titration. Increasing molar ratios of cisplatin show a mixture of free ΔΔIhh-SIC (15,884.57 Da) and ΔΔIhh-SIC-Pt(II)2TCEP species (16,580.90 Da). Average mass error of measurements was calculated at 41 ppm. Plots are representative of at least two biological replicates.

### Figure 3

**Binding of platinum compounds as monitored by positive electrospray ionization-MS (m/z = 7).**

**A.** Binding of cisplatin (CisPt), Pt(tfbz), and Zeise’s salt (Zeise) to ΔΔIhh-SIC. Compounds form an identical product in the presence of TCEP (1, 3, and 5). Zeise’s salt shows a range of adducts in the absence of TCEP (6). In the absence of compound (7) or absence of TCEP for cisplatin (2) and Pt(tfbz) (4), only free intein peak is seen. B, cisplatin titration. Increasing molar ratios of cisplatin show a mixture of free ΔΔIhh-SIC (15,884.57 Da) and ΔΔIhh-SIC-Pt(II)2TCEP species (16,580.90 Da). Average mass error of measurements was calculated at 41 ppm. Plots are representative of at least two biological replicates.

| Compound | TCEP |
|----------|------|
| 1        | CisPt |
| 2        | CisPt |
| 3        | Pt(tfbz) |
| 4        | Pt(tfbz) |
| 5        | Zeise |
| 6        | Zeise |
| 7        | -    | +    |

Pt : Intein

5:1
2:1
1:1
CisPt only
TCEP only
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SIC-Pt(II)₂-TCEP complex (supplemental Fig. S6A) based on the previously assigned M. tuberculosis minimized RecA intein constructs (23, 26). The incompleteness of the assignments can be traced to low signal-to-noise ratios due to amide proton exchange processes in the loop and β-sheet structures and the presence of five prolines that interrupted sequential connectivities. Despite these gaps in backbone assignments, the majority of backbone resonances assignments are complete for the ΔΔIhh-SIC-Pt(II)₂-TCEP complex, providing site-specific reporters for binding events upon complex formation.

Because chemical shifts are exquisitely sensitive to changes in the chemical environment of nuclei, we used 15N heteronuclear single quantum coherence (HSQC) NMR spectroscopic experiments to monitor temporal changes in the backbone amide proton and nitrogen chemical shifts of ΔΔIhh-SIC caused by addition of a molar excess of cisplatin and TCEP. NMR spectra were collected every 2 h for a period of 50 h. Comparison of the NMR spectrum of the untreated sample with that of ΔΔIhh-SIC 20 min following addition of cisplatin and TCEP shows chemical shift changes in five residues: Lys27, Thr70, Asp72, Ala84, and Phe421 (Fig. 5A, left). Mapping these residues onto the solved crystal structure revealed that, with the exception of Lys27, they cluster proximal to the N-terminal Pt(II) binding site (Fig. 5A, right). This result suggests that there is a rapid binding to the N-terminal site first. Because no single adducts of ΔΔIhh-SIC-Pt(II) were found by MS, we assumed that this binding is reversible and a covalent ΔΔIhh-SIC-Pt(II) complex was not formed.

After 50-h incubation, changes in chemical shifts greater than 1.0 ppm were observed for Leu2, Asp422, Glu424, Val425, Leu428, Val432, Ala433, and Ala440 (Fig. 5B, top). These changes, which affected both N- and C-terminal residues of ΔΔIhh-SIC, can be rationalized based on the crystal structure of the complex. Leu2 and Asp422 are within 3 Å from the N-terminal Pt(II) (Fig. 4F), and Ala440 is greatly affected by the significant conformational changes of His439 and Cys+1 coordinating the C-terminal Pt(II) (Fig. 4D). A conformational change in Cys+1 upon Pt(II) binding resulted in a new hydrogen bond between the backbone carbonyl of Cys+1 and amide of Val425, which would also lead to shifts in Glu424. Leu428 is probably affected by the 3.0-Å proximity of the carboxylate of the TCEP ligand following Pt(II) binding (Fig. 5B, bottom, left). Val432 and Ala433 could potentially have undergone an allosteric effect similar to that associated with the previously discovered mutation V76L (13), also in the central portion of the intein (Fig. 5B, bottom, right). The major shifts support the binding of Pt(II) in two distinct locations, one at the N-terminal splice junction and the other at the C-terminal splice junction, with the shifts in the C-terminal junction being more extensive due to the presence of TCEP.

To characterize the kinetics of the Pt(II) binding to the ΔΔIhh-SIC intein, we plotted the time course changes in the NMR peak intensities of well resolved N-terminal residues Leu2 and Asp422 and two representative C-terminal residues. Because of clustering of peaks, we tracked Val75 and Val438 (supplemental Fig. S6B), two residues that are well resolved and close to the C terminus (supplemental Fig. S7), over the period from 20 min up to 50 h (Fig. 5C). To simplify our analysis, we chose residues that were not affected by the rapid initial binding of Pt(II) to ΔΔIhh-SIC. Within error, the time course curves for all four residues were in agreement.

To obtain an estimate of rates, we assumed that the binding process can be described by three reactions (supplemental Equations 1, 2, and 3) where a Pt(II) complex can first bind the N-terminal site, which we assume occurs quickly and reversibly. The subsequent binding of an activated Pt(II) species to the C-terminal site leads to a global shift involving irreversible binding of Pt(II) at both termini. We determined a rate constant for the irreversible binding of a Pt(II) species to both termini of the intein (supplemental Equations 2 and 3) under the conditions of a molar excess of cisplatin + TCEP. Although the exact composition of the prebinding species has not been determined, we suspect that it contains an activating TCEP ligand not only because TCEP is present in the final complex but also because of the relative kinetic stability of the Pt–P bond and the strong trans effect exhibited by the phosphate. A mean irreversible forward rate constant, $k_f$, of 0.012 ± 0.0004 h⁻¹ was obtained from a fit to the experimental data for both binding sites, which suggests a global change in the structure of the intein. The quality of the fit, judging by $R^2 = 0.989$ and 0.987 for N-terminal and C-terminal fits, respectively, was good. The simple model suggests that, after 50-h incubation, Pt(II) binds irreversibly to both termini of the intein. The changes in the
chemical shifts after 20 min and the ability of a first order irreversible rate equation to adequately describe the slower reaction are in accord with our proposed reaction scheme. Reversible binding of Pt(II) at the N terminus occurs rapidly for $t < 20$ min followed by a slower irreversible association of a Pt(II) at the N-terminal Cys1 and the addition of a second Pt(II) species at the C-terminal Cys+1, leading to the complex observed by MS and in the co-crystal.
Discussion

Tuberculosis is a major health concern with high global incidence rates of >9.6 million in 2014 (27). Appearance of multi- and extensively drug-resistant strains of M. tuberculosis highlights the need for novel treatment options targeting different cellular processes (28, 29). It was shown previously that the chemotherapeutic agent cisplatin is a potent intein inhibitor that arrests the growth of M. tuberculosis in a targeted fashion (14). Here, through testing of more Pt(II) compounds, we discovered that Pttfbz and Zeise’s salt are of equivalent potency in vitro with IC₅₀ values in the 2 μM range. Intein-Pt(II) binding interactions were characterized, leading to the observation of a ΔΔIhh-SIC-Pt(II)₂-TCEP complex. The association between cisplatin and the ΔΔIhh-SIC intein, established at 1.50Å resolution, provides mechanistic insights and suggestions for further drug design.

The Pt(II) ligands in the crystal structure are of particular interest in the context of protein splicing because cisplatin targets key catalytic residues at each step of the pathway (Fig. 4). Cys1 and His439 are highly conserved throughout inteins (11), and Cys +1 is often found as the first residue of the C-extein. Cys1 is the initiating catalytic residue in the first step of splicing, serving as the attacking nucleophile to form the first thioester intermediate (Fig. 1A). The next step in the pathway is mediated by the Cys +1 residue to form a branched intermediate. The Cys +1 residue is trapped by the C-terminal Pt(II) and is prevented from functioning. The third step, cyclization of the terminal asparagine, is mediated by a conserved G-block histidine (His439), which forms a charged relay system with water and the terminal asparagine (30). His439 is rotated to bind effectively to the Pt(II) (Fig. 4D), thus displacing any potential interactions with water. Despite the absence of Asn440 in ΔΔIhh-SIC, comparison with a previously crystallized minimized RecA intein shows the Asn440 is oriented away from the binding position of the Pt(II) (31), suggesting it would not be involved in platinum binding as expected from a knowledge of simple coordination chemistry.

Cisplatin derivatives have provided a number of promising leads for use in cancer treatment (32). The potential for these drugs to also treat tuberculosis by targeting inteins is explored here in vitro. A potential antitumor candidate, Pttfbz, which is synthesized through direct replacement of the chloro ligands of cisplatin with a diketonate ligand, is also an effective intein inhibitor. The presence of the [Pt(NH₃)₂Cl₂]⁺ moiety in both cisplatin and Pttfbz provides an explanation for a similar efficacy in intein inhibition for the two compounds. Phenanthriplatin, a second cisplatin derivative investigated in this study, has been shown previously to react only slowly with thiols, which is favorable in its intended use as a chemotherapeutic (16) but makes the compound a less effective inhibitor of intein splicing. Unlike Pttfbz and phenanthriplatin, Zeise’s salt is not a derivative of cisplatin, which may account for its different binding to the intein.

We found that binding of cisplatin and Pttfbz to the RecA intein is mediated by the reducing agent TCEP. In the crystal structure, the TCEP associated with the C-terminal Pt(II) is stabilized by hydrogen bonds with the side chain amines of Lys36 and Lys74. The presence of TCEP as a ligand to Pt(II) binding to an active site cysteine has been documented previously (33). Furthermore, the TCEP phosphine has been shown to increase the binding of cisplatin to a Sp1 zinc finger protein (34). We speculate that the phosphine facilitates substitution of the ammine ligand in the trans position (35) and binding of Pt(II) to ΔΔIhh-SIC (supplemental Fig. S4). The soft acid/base properties of cysteine thiolates favor the formation of a Pt(II)-thiolate complex. The structure of Pt(II) at the C-terminal splice junction supports the proposed mechanism with direct evidence of platinum ligands in a square planar geometry (supplemental Table 2A). The thiolate residue of the intein binds trans to the phosphine, whereas the remaining two ligands are replaced by both an ammine, donated by His439, and a water molecule. Proximity of His439 to Cys +1 and the highly active nature of His439 promote coordination of imidazole to the C-terminal Pt(II). Given that cisplatin is able to effectively inhibit splicing in M. tuberculosis, there is likely a native compound that exerts a similar effect in activating cisplatin in vivo.

The intein needs both the N- and C-terminal platinum binding sites to be filled for the complex to be stable. Tracking of cisplatin binding 20 min after addition shows that the Pt(II)-TCEP complex binds rapidly to the N-terminal binding site (Fig. 5A). As there is a distinct absence of singly bound Pt(II) in the titration of substoichiometric concentrations of cisplatin (Fig. 3B), this binding of Pt(II) at the N-terminal splice site must be reversible. Previous testing of ΔΔIhh-TR, a construct that does not contain a free thiol at the N-terminal Cys1 position (23), showed a lack of binding by cisplatin. The inability of cisplatin to bind in the absence of a free Cys1 thiol supports the importance of requiring both N- and C-terminal Pt(II) association for irreversible binding. Through tracking by NMR, it was determined that after initial reversible binding of Pt(II) to the N terminus (<20 min) further binding to both termini was first order, irreversible, and of equal rate.

Zeise’s salt, which contains an ethylene ligand that has a strong trans effect (36) and lacks ammine ligands, does not require TCEP to facilitate interaction with the intein in sharp contrast to cisplatin and Pttfbz. The binding behavior exhibited by Zeise’s salt is likely due to the strong trans effect of the ethylene ligand, which allows the formation of a more reactive intermediate that is able to bind to the intein. The intein products formed upon incubation with low concentrations of Zeise’s salt contain a species that is stable with a single Pt(II) adduct, indicating that an ammine ligand is not required for binding. At
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High concentrations of Zeise’s salt, a range of Pt(II) adducts form, although in the presence of TCEP, only the ΔΔIhh-SIC-Pt(II)₂₃TCEP complex is identified, identical to that with cisplatin and Pt(II) in presence of TCEP. The TCEP molecule thus uniformly targets the platinum compounds specifically to the two cysteines at the N- and C-terminal splice junctions and prevents off-target interactions. The unusual trans location of the phosphine is possibly due to hydrogen bonding of the TCEP carboxyether groups with the protein residues Lys34 and Lys76.

Strikingly, none of the original cisplatin or Zeise’s salt ligands appear in the bound crystal structure. This result is noteworthy because the ammines associated with cisplatin are required for potent function involving DNA binding (37, 38). This study sharpens the type of platinum compounds that might serve as candidates for initiating a broader platinum-based drug design program for combattng M. tuberculosis. In particular, less toxic versions of the platinum drugs used in the study can be conceived and could lead to novel, non-toxic treatments.

Experimental Procedures

Split GFP Assay—BL21(ADE3) cells were transformed via electroporation with pHGmU, containing GFP split by a minimized RecA intein, or pHGsp, containing uninterrupted GFP (19). Cells were grown to an OD of 0.6 following subculture into LB medium (BD Biosciences) and induced with 0.4 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 3 h at 37 °C. Cultures were pelleted and stored at −80 °C until use. Split GFP was expressed as inclusion bodies, and cells were lysed using bacterial protein extraction reagent (Thermo Scientific). The inclusion bodies were washed once with 20 mM sodium phosphate, pH 8.0, 500 mM NaCl, 1 mM EDTA, and 2% Triton X-100 (USB Corp., Cleveland, OH) and an additional three times in the same buffer without EDTA or Triton X-100. Inclusion bodies were solubilized in 8 M urea, spun down to remove any additional insoluble portions, and stored at 4 °C. Refolding was carried out by dilution of protein to a concentration of 35 μM in buffer containing 20 mM sodium phosphate, pH 9, 0.5 M NaCl, and 0.5 M arginine.

Platinum solutions were prepared fresh in the dark immediately prior to each experiment by dissolving each compound in 20 mM sodium phosphate buffer, pH 7, 0.5 M NaCl, and 0.5 M arginine to a final concentration of 300 μM using sonication and vortexing. Independently weighed platinum solutions were used for each inhibition assay repeat.

In black, flat bottom 96-well plates (Costar, Kennebunk, ME), 100-μl wells were prepared to contain 20 mM sodium phosphate, pH 7, 0.5 M NaCl, 0.5 M arginine, 200 μM TCEP, 200 μM EDTA, and platinum concentrations varying from 0 to 80 μM. Refolded protein was added to a final concentration of 200 nM, and an initial fluorescence reading was immediately taken using a Synergy H1 plate reader (BioTek, Winooski, VT) at 485-nm excitation and 528-nm emission. Samples were allowed to react for 18 h at 25 °C in the dark prior to a second fluorescence reading.

Construction of pET45b ΔΔIhh-SIC Plasmid—The ΔΔIhh-SIC protein used for MS, NMR spectroscopic, and X-ray crystallographic experiments was based on the ΔΔIhh-SM construct (Fig. 1C) (13, 23). ΔΔIhh-SM, with a minimized RecA intein and five N-terminal and five C-extein residues, was cloned into pET30b using NdeI and HindIII (New England Biolabs, Ipswich, MA). An N440A mutation was made to the ΔΔIhh-SM construct using the primer 5’-AAAGCTTCA-CGCGGCGGAACAGGCGTGCAGCAGAACCCCTTCCGCGACGAGC-3’ to prevent C-terminal cleavage. The resulting ΔΔIhh-SIC was subcloned into pET45b from pET30b using XbaI and HindIII. Ligated constructs were transformed into DH5α, and isolated plasmids were sequenced to confirm muta-tion of the intein. The ΔΔIhh-SIC is active for N-terminal cleavage and was used in the purification through addition of an N-terminal His-tag, which is cleaved from the intein and C-extein following purification. The five C-extein residues are maintained, including Cys + 1, which is thought to be important for the metal ion coordination.

ΔΔIhh-SIC Expression and Purification—Expression of ΔΔIhh-SIC was carried out in an oxidizing background, Origami B(ADE3) cells, to promote disulfide bond formation between Cys1 and Cys + 1 of the construct and prevent premature splicing. Cells were grown to an OD of ~0.6 in LB medium and induced with 1 mM IPTG for 16 h at 16 °C. Harvested cells were stored at −80 °C until use. Protein was purified over a nickel-nitrilotriacetic acid column (GE Healthcare) using a column buffer of 20 mM sodium phosphate and 500 mM NaCl, pH 7.0, with 500 mM imidazole in the column buffer for elution. Following elution, 100 mM DTT was added to fractions containing target protein and incubated at 25 °C for ~18 h to induce N-terminal cleavage of the intein from the His-tag and linker region. The buffer was exchanged through a desalting column into buffer containing 75 mM imidazole and loaded onto a nickel-nitrilotriacetic acid column to remove His-tag cleavage product and any uncleaved protein.

Native Polyacrylamide Gel Electrophoresis Assay—The gel shift reactions were performed by incubating 8 μM purified ΔΔIhh-SIC protein in binding buffer (50 mM sodium phosphate and 100 mM NaCl, pH 7.0) with 100 μM platinum compound (cisplatin, Pt(II), or Zeise’s salt) in the presence or absence of 200 μM TCEP at 25 °C for 18 h in the dark. Cisplatin and Zeise’s salt were dissolved in binding buffer, and Pt(II) was made in water due to poor solubility in binding buffer. Binding interactions were analyzed using native polyacrylamide gel electrophoresis to retain the protein in a folded state. Unboiled samples were loaded onto 15% native Tris-glycine gels with a 29:1 acrylamide:biacrylamide ratio in running buffer (250 mM Tris and 1.92 M glycine). Running buffer lacked SDS to maintain non-denaturing conditions. A 1:3 ratio of non-denaturing loading dye (10% v/v glycerol, 12.5% v/v stacking buffer, and 0.1% w/v bromphenol blue without SDS and β-mercaptoethanol) to sample was used. Gels were run under a constant voltage of 150 V for a minimum of 2 h to obtain optimal separation. Bands were visualized using Coomassie Brilliant Blue staining.

Mass Spectrometry—Binding experiments for MS were performed in binding buffer (50 mM sodium phosphate and 100 mM NaCl, pH 7.0). Incubation was carried out for ~18 h in the dark. Samples were buffer-exchanged into 150 mM ammonium acetate through desalting columns and concentrated using 3-kDa Amicon Ultra centrifugal filter units (EMD Millipore, Billerica, MA). Mass spectrometry analysis of the protein was
carried out on an LTQ Orbitrap Velos instrument (Thermo Scientific) in the positive ion mode.

**NMR Spectroscopic Experiments**—Expression of uniformly labeled [U-13C, 15N]ΔNlh-SIC was conducted as described (39). Origami B(ΔADE3) containing the pET45b ΔNlh-SIC plasmid was grown in LB medium with 100 μg/ml ampicillin to an OD of ~0.8. Cells were washed with 1× M9 salts and suspended in a quarter of the volume of M9 medium containing 2% U-13C-labeled glucose as the sole carbon source and U-15N-labeled ammonium acetate. The culture was allowed to recover for 1.5 h at 37 °C with shaking at 250 rpm prior to induction with 1 mM IPTG. Purification was performed as described above. Samples were prepared in 50 mM sodium phosphate and 100 mM NaCl, pH 7, in 90% H2O and 10% D2O at 25 °C. Cisplatin and TCEP were added from 4 and 200 mM solutions, respectively. The sample was then concentrated from a larger volume to 450 μl using 3-kDa molecular mass cutoff centrifugal filter units to a final concentration of 250 μM ΔNlh-SIC, 5 mM TCEP, and 1.5 mM cisplatin for collection of spectra. This concentration step leads to a 20-min delay between adding cisplatin and TCEP to the intein sample and initiating data acquisition. Avance 700-MHz and Avance II 500-MHz spectrometers (Bruker, Billerica, MA) equipped with TXI and TCI cryoprobes, respectively, were used. NMR spectra were acquired at 25 °C, processed with TopSpin 2.1 software (Bruker), and analyzed with CARA (40). 15N HMQC experiments were used to monitor the kinetics of cisplatin binding to the intein. Standard triple resonance HNCA, CBCACONH, and HNCACB experiments were used for the chemical shift assignment of the ΔNlh-SIC-Pt(II)2TCEP complex (41).

**X-ray Crystallography**—Following initial purification as described above, 23 mg of ΔNlh-SIC in 7 ml was further purified by gel filtration through a 16/60 Superdex 75 column (GE Healthcare). Intein-containing fractions were pooled and concentrated to 8.3 mg/ml in 20 mM Tris, pH 8.0, 100 mM NaCl, and 2 mM TCEP. Initial crystallization conditions were obtained by screening the Hampton Research Index HT crystallization screen and existing crystallization conditions for RecA intein variants (12) using the hanging drop vapor diffusion method. Upon optimization, large intein-Pt(II) complex crystals were grown by mixing 1 μl of intein and 1 μl of reservoir solution containing 2 mM cisplatin (or 2 mM Ziese’s salt), 54–59% ammonium sulfate, 0.1 mM Tris, pH 8.5, and 2 mM TCEP. The intein-Pt(II) complex crystallizes in space group P2_12_1 and has one intein molecule per asymmetric unit. Prior to data collection, all crystals were transferred to a cryoprotectant solution containing crystallization buffer supplemented with 25% glycerol. The crystals were flash cooled directly in liquid nitrogen. Diffraction data for the co-crystals were collected at 100 K using a MAR325 CCD detector at the BL14-1 beamline of the Stanford Synchrotron Radiation Laboratory. Data were processed, scaled, and reduced using the programs HKL2000 (42) and PHENIX suite (43). The structure of the intein was determined by molecular replacement with Protein Data Bank code 2IN0 as a search model using the PHASER program suite (44). Structure refinement was carried out using the PHENIX program suite (supplemental Table 1).

**Author Contributions**—H. C., C. S. P., and C. M. G. performed biochemical assays and expression of protein for structural studies. H. C. and C. M. G. performed and analyzed mass spectrometry experiments. Z. L., J. Z., and H. L. performed and analyzed crystallographic experiments. H. C. and A. S. performed and analyzed NMR experiments. H. C., C. S. P., and G. B. analyzed kinetic data. M. B. designed and provided supervision of the overall project. All authors were involved in discussion of results and editing of the manuscript.

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