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A ruthenium-platinum metal complex that binds to Sarcin Ricin Loop RNA and lowers mRNA expression

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IT127 is a dinuclear transition metal complex that contains a Pt(II) and a Ru(III) metal center. We have shown that IT127 is significantly more effective in binding the 29-base Sarcin Ricin Loop (SRL) RNA in comparison to Cisplatin, a hallmark anticancer agent. Binding site analysis shows that IT127 prefers purine bases and the GAGA tetraloop region of SRL RNA. Our results with a dihydrofolate reductase (DHFR) model system reveal that IT127 binding to mRNA reduces translation of DHFR enzyme and that the Ru(III) and Pt(II) centers in IT127 appear to work in a synergistic manner.

RNA molecules have the remarkable ability to form complex, three-dimensional folded structures. This rich structural diversity of RNA and its role in a variety of cellular functions have led to its emergence as an important biological target.[1,2] Small molecule interactions with RNA have the potential of disrupting important cellular processes. Folded RNA structures present base-paired stems and tertiary structure elements like hairpins, loops, and embedded regions that can serve as relevant sites of action for chemotherapeutic agents. Hence investigations of small molecule interactions with RNA are an appropriate and timely endeavour.

Transition metal compounds such as Cisplatin (shown in Chart 1) have long been used to treat cancer, inhibit tumour growth and trigger apoptosis by forming covalent DNA crosslinks in cells.[3-6] Treatment with Cisplatin, like other platinum-containing compounds such as Oxaliplatin and Carboplatin, leads to varying degrees of cytotoxicity and drug resistance.[7] Furthermore, these compounds are not very effective in metastatic tissues.[8] Because of these shortcomings, chemotherapeutic compounds containing a host of other transition metals such as gold, iron, gallium, silver, rhenium, titanium, and ruthenium have been the subject of recent investigations.[9-15] In particular, ruthenium(III) compounds,

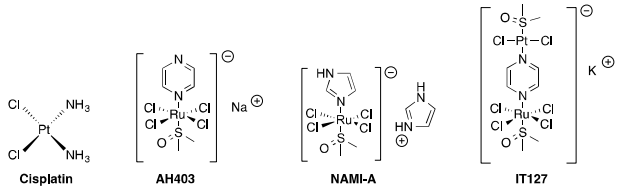

Chart 1: Chemical structures of metal complexes. From left: Cisplatin, AH403, NAMI-A, IT127.

such as NAMI-A (Chart 1), have gained attention as potential anticancer agents having different modes of action and improved capacity against metastatic tissues than platinum-based drugs.[16-17]

In this work, we have investigated the interactions of IT127 (a transition metal complex containing two metal centers) with RNA model systems. We hypothesize that simultaneous incorporation of multiple metal centers into a single compound could yield a potential therapeutic agent with improved properties and mechanistic action compared to a compound with a single metal center. Compounds containing multiple platinum centers have previously been shown to be more effective in circumventing resistance in tumour cells.[18] Mixed-metal compounds containing ruthenium/platinum, gold/iron, and gadolinium/platinum centers have also been synthesized previously by others.[19-21] In 2016, the Zhu group showed that dinuclear Pt(IV)/Ru(II) compounds called ruthplatins demonstrated enhanced cell death in numerous Cisplatin-resistant cancer cell lines.[22] However, most of the previous work has determined that DNA and proteins are the molecular targets of these compounds. There are only a limited number of studies that have explored the binding of transition metal complexes such as platinum to RNA.[23] And to the best of our knowledge, investigations of RNA targeting by multinuclear metal complexes have not been carried out. Towards this end, ruthenium(III)-platinum(II) complexes such as IT127 have been recently designed to potentially deliver both the antimetastatic activity of NAMI-A and antineoplastic payload of Cisplatin (chemical information for IT127 is provided in ESI, Table S1).[24]

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Sarcin Ricin Loop (SRL) RNA is a universally conserved 29-base sequence found in 28S ribosomal RNA (Figure 2a).[25] It plays an important role in peptide elongation during protein translation and it has been the subject of extensive inquiry.[26] The short sequence of SRL RNA and the availability of high-resolution structures make it a good system for exploration of metal complex binding. The gel shift assay results in Figure 1 show that IT127 has a significantly greater effect on electrophoretic mobility of SRL RNA than both Cisplatin and AH403 (Chart 1) under identical experimental conditions. AH403 is the Ru(III) containing complex that is an intermediate during synthesis of IT127. Together with Cisplatin, AH403 is a good choice for comparison with IT127. A cocktail of Cisplatin + AH403 shows minimal gel shift compared to IT127 (ESI, Figure S1).

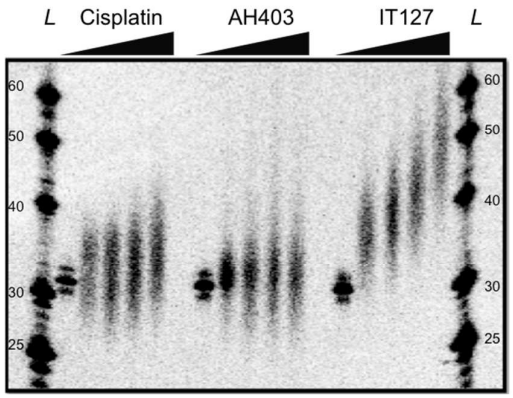

Figure 1: Electrophoretic mobility shift assay of metal complexes bound to the 29-base SRL RNA. Lanes marked as L represent a single-stranded RNA ladder with the size (in bases) indicated next to the band. RNA (40 μM) was incubated for 2 hours at 37°C with metal complexes at 0, 50, 100, 250, 500 μM concentration prior to gel electrophoresis.

The improved ability of IT127 to inhibit RNA mobility may be due to: (i) the greater molecular weight of IT127 which causes an upward shift of the RNA gel band to a greater extent than smaller metal complexes, and (ii) conformational changes brought about because of RNA binding to multiple metal centers. The marked enhancement of the dinuclear species' mobility shift relative to AH403 (the mononuclear ruthenium species) implies that the Pt(II) center plays a crucial role in IT127 binding to RNA. In recent work, platinum-containing compounds have shown a greater affinity for RNA in comparison to DNA.[27] Furthermore, the strength of binding of dinuclear complexes like IT127 is likely due to a chelating effect where the binding of platinum center positions the ruthenium for additional interactions with neighbouring nucleotides.[24] Therefore, it is not surprising to observe that IT127 binds to SRL RNA with greater efficacy than Cisplatin and AH403. Gel mobility shift was accompanied by a concentration-dependent tendency for the RNA bands to migrate as diffuse streaks. Intramolecular cross-linking of RNA loop structures by metal complexes can induce multiple conformations in RNA leading to a collection of adducts with only marginally different gel mobility. Similar protracted bands have been observed following incubation of metal complexes with *Tetrahymena thermophila* ribozyme RNA.[28] Nonetheless, this mobility shift assay shows that IT127 is significantly more effective in reducing RNA gel mobility than Cisplatin and AH403. These results also indicate that the presence of multiple metal centers in IT127 maybe playing a synergistic role in binding to RNA and its subsequent gel shift.

Sarcin Ricin Loop RNA (Figure 2a) was also used as a template for DNA primer extension in order to analyze RNA:metal complex binding sites. Our results in Figure 2b show that, in the absence of metal complex, a short DNA primer is fully extended (denoted by * in Figure 2b) by the reverse transcriptase (RT) enzyme. As the concentration of IT127 increases, a noticeable decline in the intensity of the full-length product band is observed. However, the decline in the amount of full-length product with Cisplatin is marginal, which indicates that IT127 is more effective at inhibiting reverse transcription. More importantly, there is a significant increase in the intensity of several shorter DNA fragments, which represents partial extension of the DNA primer by reverse transcriptase enzyme. These fragment bands are boxed in Figure 2b and the integrated band intensity values are reported in Table 1.

Inhibition of reverse transcriptase activity has been used to precisely indicate cross-linked nucleic acid adducts (small molecules covalently bound to nucleotides).[29] The partial extension of a DNA fragment by reverse transcriptase denotes a potential RNA binding site for the metal complex. An increase in the concentration of metal complex likely leads to an increase in the concentration of metal complex bound to RNA at a specific position. This yields a reverse transcription stop at or near that site (*vide infra*). This stoppage manifests as an increase in the concentration of the reverse transcribed DNA fragment of a particular length. Therefore, metal complex binding will only change the band intensities at those particular RNA sites within the sequence. It is also clear from Figure 2b that there are several other bands in SRL RNA that do not change in intensity as the concentration of IT127 or Cisplatin increases. This indicates that these sites are unaffected by the presence of the metal complex.

Figure 2b and Table 1 show five primer extension bands that increase in intensity as IT127 concentration increases. These bands correspond to binding sites in the purine rich regions of SRL RNA: G19, G18, A17, U11, and G10 (Figure 2a). Cisplatin has three purine rich binding sites (G3, G14, A15). In case of Cisplatin, binding interactions occur in the purine rich tetraloop (G14, A15) as well as the stem region (G3) of SRL RNA. It has long been established that platinum complexes like Cisplatin have a high degree of preference for purines over pyrimidines.[27,30] In case of IT127, the first cluster, as shown in Figure 2b, is G19-G18-A17 and the second cluster is U11-G10. In addition to these regions being purine rich, computational analysis has previously shown that the GAGA tetraloop (yellow) and the GUA base triple (green) in SRL RNA are significantly open and accessible to solvent.[26,27] The high degree of three-dimensional accessibility is likely required for IT127, which has a heterodinuclear structure that is chemically

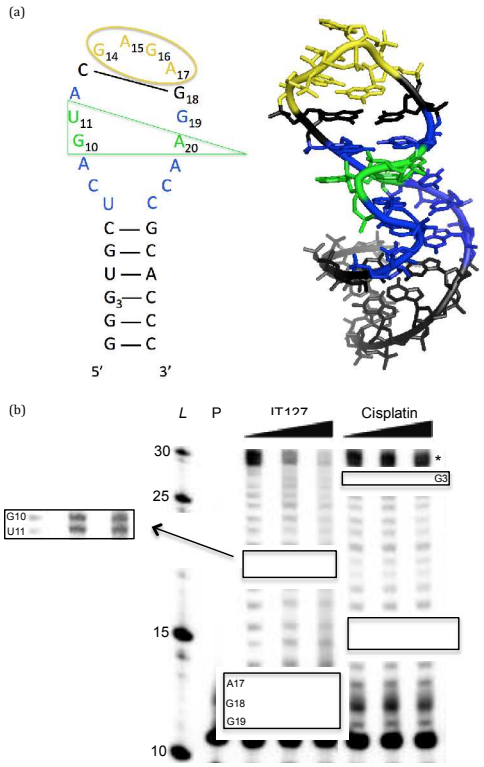

Figure 2: a) Secondary (left) and tertiary (right) structures of SRL RNA. The two structures are color-coded identically with the GAGA tetraloop (yellow oval) and GUA base triple (green triangle) highlighted. b) DNA primer extension by reverse transcriptase enzyme was carried out in the presence of 0, 50, and 100 μM IT127 and Cisplatin. Lane labeled as L represents a single-stranded DNA ladder with the size (in bases) indicated next to the band. Primer DNA alone was loaded in the lane labeled as P. Band indicated with an asterisk (*) represents the fully extended cDNA product. Boxed bands indicate potential SRL binding sites for the metal complexes.

Table 1. Intensity counts indicating metal complex binding to specific sites of SRL RNA.

SRL Base	Complex	0 μM	50 μM	100 μM
G19	IT127	14.4	19.5	20.8
G18	IT127	56.9	72.9	82.4
A17	IT127	10.5	17.3	20.9
U11	IT127	1.0	2.8	2.9
G10	IT127	1.7	3.8	3.8
A15	Cisplatin	2.0	4.8	7.1
G14	Cisplatin	3.4	6.4	8.6
G3	Cisplatin	2.6	6.6	8.7

Table 1. Boxed gel bands in Figure 2b were integrated to determine intensity counts. An increase in intensity as a function of metal complex concentration indicates an RNA binding site. Counts were normalized to the U11 band at 0 μM.

larger and more complex than Cisplatin. Studies using psoralen have shown that reverse transcriptase enzyme extends up to the base where psoralen binds to RNA.[29] And while psoralen does not have a metal center, other studies with metal complexes have shown that primer extension by reverse transcriptase does not stop until it reaches the bound nucleotide or one nucleotide prior to the binding site.[27,30] Therefore, we have strong evidence to support the aforementioned SRL binding regions for Cisplatin and IT127.

We were further interested in examining whether metal complex binding to RNA inhibits vital cellular processes. For example, mRNA occlusion can result in problems associated with protein synthesis and even induce apoptosis, which is considered an attractive target for different modes of anticancer activity.[31] We developed an *in vitro* dihydrofolate reductase (DHFR) model system to test whether the binding of metal complexes to mRNA interferes with protein translation. DHFR enzyme, essential for de novo synthesis of purines, catalyzes the reduction of dihydrofolate to tetrahydrofolate using NADPH as a co-factor (Figure 3a). Inhibition of DHFR enzyme activity has long been a target for anticancer drug discovery since this pathway is known to cause apoptosis.[32-34] In this study, the activity of the translated DHFR enzyme was determined by measuring the change in absorbance at 340 nm because NADPH oxidation, which occurs upon dihydrofolate reduction, leads to a decline in absorbance at 340 nm. The graph in Figure 3b illustrates the results from our positive control sample (labeled Control), which utilizes purified and unbound mRNA as a template for DHFR translation.

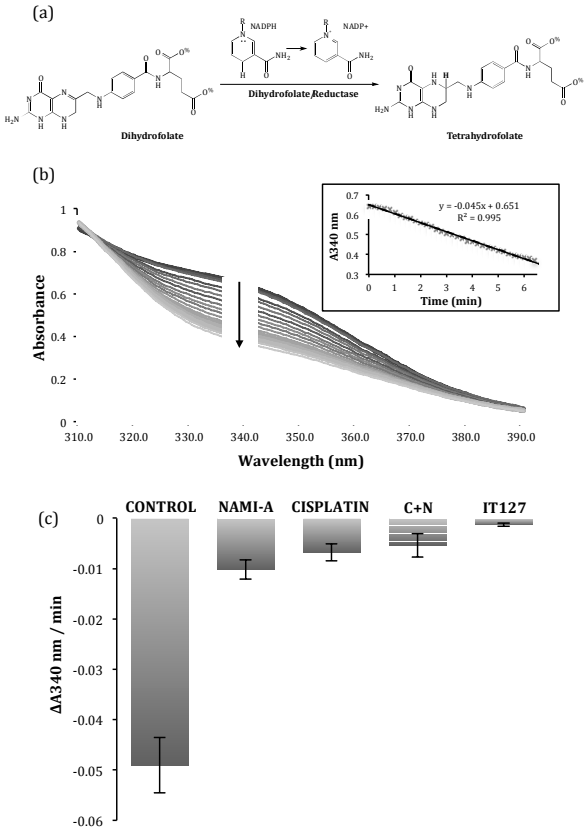

Figure 3. a) Reaction scheme illustrating reduction of dihydrofolate to tetrahydrofolate catalyzed by DHFR enzyme using NADPH as a cofactor b) Change in Abs at 340 nm illustrates NADPH oxidation (indicated by the black arrow). Inset: Abs at 340 nm was plotted against time to determine rate. c) Bar graph illustrating NADPH oxidation rate (as shown in inset plot) for different metal complexes. Prior to translation, Cisplatin, NAMI-A, IT127 and a 1:1 mixture of Cisplatin and NAMI-A (C+N) were incubated with mRNA (3000 μM RNA in base and 500 μM metal complex). The control sample is the unbound, purified mRNA template.

The translation inhibition capacity of IT127 was compared with Cisplatin, NAMI-A, and a cocktail containing Cisplatin and NAMI-A (C+N). The 1:1 cocktail mixture of Cisplatin and NAMI-A as a combination of individual Pt(II) and Ru(III) drugs was tested in comparison with the dinuclear Pt(II)-Ru(III) containing IT127. Our data in Figure 3c illustrates that all samples successfully produced active DHFR enzyme. In comparison with Cisplatin, NAMI-A and the cocktail combination, IT127 sample demonstrated the lowest rate of NADPH oxidation and thus demonstrated the greatest translation inhibition capacity. Direct incubation of IT127 with purified DHFR enzyme only leads to a minimal loss in enzyme activity (ESI, Figure S2).

The key step in protein translation is the formation of the translation initiation complex, which has proven to be an attractive mechanistic target for cancer drugs. Cisplatin has been shown to block *in vitro* protein synthesis by preventing the 60S subunit binding to the 48S pre-initiation subunit, which disrupts the peptide chain elongation process.[35] The binding of IT127 to mRNA might inhibit the translational machinery in a similar manner. Specifically, IT127 binding to DHFR mRNA may prevent mRNA binding to the ternary complex, which can inhibit binding of the 60S ribosomal subunit to the pre-initiation complex for chain elongation. Compared to Cisplatin, the dinuclear complex IT127 interferes much more significantly with the activity of ribosomes and the synthesis of DHFR enzyme, and thus the amount of functional enzyme produced was much less in the case of IT127 than Cisplatin (ESI, Figure S3). This leads us to believe that IT127 binding to mRNA maybe causing disruption of the translation initiation process. Zhu et al. have recently shown that Ruthplatin 1, a multinuclear Pt(IV) – Ru(II) compound is more cytotoxic than the mixture of Cisplatin and the ruthenium drug PPA-Ru(II).[22] Our results in Figure 3c also suggest that multinuclear complexes such as IT127 with two distinct metal centers can exhibit a synergistic effect thereby yielding greater biological inhibition than mononuclear metal complexes like Cisplatin and NAMI-A. The presence of both ruthenium and platinum in a single complex was also previously suggested to facilitate cross-linking of unique proteins with DNA.[36] Thus, the presence of two linked metal centers in IT127 might enable binding interactions with biomolecules that are likely unrealized by complexes with a single metal center.

In conclusion, we have carried out proof of principle experiments to show that heteromultinuclear transition metal complexes like IT127 have a binding preference for specific RNA nucleotides. Furthermore, the reduction in RNA gel mobility and mRNA expression realized by IT127 is far greater than anticancer drug benchmarks like Cisplatin. Future studies with cancer cell lines aim to explore the effectiveness of metal complexes like IT127 in slowing tumour metastasis. Finally, this work opens a potential novel area for researchers looking for newer targets for chemotherapeutic agents.

Conflicts of interest

There are no conflicts of interest to declare.

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