Screening and Preliminary Biochemical and Biological Studies of [RuCl(p-cymene)(N,N-bis(diphenylphosphino)-isopropylamine)][BF₄] in Breast Cancer Models

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1. INTRODUCTION

Although most of the efforts in cancer drug development are based on the discovery of organic molecules, including those designed for specific molecular alterations, inorganic agents like platinum compounds are still the backbone for most therapies in the treatment of solid tumors. In this context, the identification of inorganic agents that can improve available therapies is a main goal. In fact, metallo-drugs in cancer therapy are being widely used and a variety of quite different metals with distinctive approaches are currently in research development. Ruthenium is a clear example. New metallo-drugs based on ruthenium have been tested against an important number of cancer cell lines. In this context, it is expected that ruthenium...
metallodrugs would offer much potential as cytostatic and cytotoxic drugs than platinum derivatives, as other mechanisms of action beyond the mere induction of DNA damage, can mediate their biological effect. Four Ru therapeutics have entered clinical trials during the last years (Figure 1). NAMI-A succeeded phase I clinical studies, but limited efficacy was obtained in phase II clinical studies, whereas the low solubility of KP1019 limited further development. The KP1339 is currently undergoing clinical studies. TLD1433 entered phase IIa clinical trials for nonmuscle-invasive bladder cancer. 

Because ruthenium(III) agents are pro-drugs and need activation by reduction, organometallic ruthenium(II) metallodrugs are being explored. Many of them, which are particularly attractive to the medicinal chemists because of their structural variability, have been reported as promising antitumoral agents. The most relevant candidates were reported by the group of Dyson. RAPTA-C derivatives are investigated as antimetastatic agents, whereas RAED-C displays potent cytotoxicity in vitro nonneoplastic cells.

In the field of organometallic ruthenium(II) metallodrugs, we recently reported aminophosphine and bis-aminophosphine compounds (from now on pnpRu) as potential chemotherapeutic agents (Figure 2). Some of the complexes displayed high in vitro antiproliferative activity in a series of cell lines, including the platinum-resistant A2780R. Given that a small library of such aminophosphine ligands could be rapidly obtained, this preliminary study highlighted the potential utility of this scaffold as an easily-tunable core in metallodrug discovery. Herein, we aimed to explore the potential of the lead pnpRu complexes as chemotherapeutic agents for breast cancer. We focused on this tumor type as there are limited therapeutic options mainly in the advanced stage. We report here on the screening and preliminary biochemical and biological studies of the lead pnpRu-14 compound in breast cancer therapy to provide solid support for further preclinical development of selected amino- and bisaminophosphine (pnp) Ru-based metallodrug candidates.

2. RESULTS AND DISCUSSION

2.1. Antiproliferative Studies in Vitro. The cationic ruthenium complexes pnpRu-14 and pnpRu-15 were chosen as lead compounds because of the high cytotoxicity they displayed against the tumor cells screened in the previous work. Specifically, pnpRu-14 was active against the Pt-resistant cell line A2780R with IC50 = 2.2 μM, while for the ferrocene containing pnpRu-15 (see Figure 2) this value was 3.1 μM. Moreover, these two compounds were somewhat less toxic toward noncancerous rat primary astrocytes and against HEK-293 cells.

The antitumoral properties of the lead pnp-Ru compounds pnpRu-14 and pnpRu-15 in comparison with the well-studied RAPTA-C analogue were assayed by monitoring the ability of these ruthenium complexes to inhibit cell growth using the MTT assay, which allows the determination of mitochondrial functionality in various types of breast malignancies, including HER2+ (SKBR3 and BT474 cancer cells) and HR+/HER2− (T47D and MCF7 cancer cells) breast cancer (Figure 3A).

While good levels of cytotoxicity (superior to those of RAPTA) were observed for the ferrocene-based pnpRu-15, pnpRu-14 was found to be particularly effective, reducing significantly the viability in all tumor cells screened. Importantly, inhibition of cell proliferation with pnpRu-14 took place in a concentration-dependent manner.

Among the currently used chemotherapeutics, platinum complexes are held in high regard. In several tumors like testicular cancer, nonsmall-cell lung cancer, or bladder cancer, the platinum therapy is well established. It is also used in combination with other chemotherapeutic agents in the treatment of some subtypes of breast cancer such as triple negative. Therefore, the cytotoxicity of the lead compound pnpRu-14 was compared, once again via the MTT assay, with the clinically used metallodrugs cisplatin and carboplatin.

Figure 1. Structures of the most relevant ruthenium metallodrugs in cancer therapy.

Figure 2. Structures of the pnpRu complexes previously reported. 

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against a panel of breast cancer cell lines (Figure 3B). Gratifyingly, the \textit{pnpRu-14} was 8-fold more efficient at inhibiting the growth of SKBR3 and BT474 cells, and 4-fold more efficient at T47D and MCF7, than cisplatin or carboplatin. Additionally, \textit{pnpRu-14} showed a concentration-dependent profile in T47D and MCF7 in contrast to carboplatin and cisplatin (Figure 3B). The outstanding potency of \textit{pnpRu-14}, particularly in the MCF7 and the BT474 cell lines, suggested that this compound may constitute a promising drug lead candidate suitable for further testing. In addition, the administration of the compound increased the reduction of the % MTT transformed of the anti-HER2 antibody Herceptin in SKBR3, particularly at higher doses (Figure 3C).

2.2. \textit{PnpRu-14} Induces Cell Cycle Arrest at G1 on Cell Cycle. Aiming to shed light on the suppression of cancer cell proliferation by \textit{pnpRu-14}, the mechanism implied was further investigated (Figure 4A). For this purpose, the two hormone receptor positive cell lines, MCF7, T47D and the two HER2+ cell lines, and SKBR3 and BT474, were treated with \textit{pnpRu-14} for 24 h and DNA staining with propidium iodide/RNAse was performed. Addition of \textit{pnpRu-14} to four cell lines increased the percentage of cells in G0/G1, although for T47D did not reach a statistical significant level (Figure 4A). A concomitant decrease of cells in the G2/M phase was also observed. The biochemical analyses demonstrated a reduction of Cyclin E and D in T47D, BT474, and SKBR3, indicative of an arrest in G1, thus confirming the results observed with flow cytometry (see Figure 4B and statistical analysis in Figure S3 in Supporting Information).

In addition to the effects of \textit{pnpRu-14} on the cell cycle, we also explored the possibility that the compound can be responsible for cell death (Figures 5 and S1 in Supporting Information). The treatment with 1 \textmu M of \textit{pnpRu-14}-augmented Annexin V staining of MCF7, BT474 and SKBR3 cells, and did not modify AV+ staining (Figure 5A). Clear indications of apoptosis were observed in three cell lines, MCF7, BT474 and SKBR3. Degradation of PARP was only identified in BT474 cell lines in line with an increase of caspase 3 demonstrating that in this particular cell line, cell death was
mediated by activation of caspases (Figure 5B,C). A similar effect, but to a less extent was observed in MCF7 and SKBR3. Finally, phosphorylation of H2AX was observed in SKBR3, indicative of an induction of DNA damage. Taken together, these results demonstrate that pnpRu-14 inhibits proliferation by more than one target, including via cell cycle arrest and the induction of apoptosis, and this effect is cell-dependent.

2.3. Reactivity with Biomolecules. The mechanisms by which ruthenium-based drugs exert their toxicity effects remain to be fully elucidated. In order to understand the molecular bases, several computational physicochemical for the lead compound pnpRu-14 were evaluated by DFT calculations (Table S1 in Supporting Information). Some of these parameters are frequently used in quantitative structure-activity relationship methods (Table 1).13 As was expected, the lipophilicity of pnpRu-14 based on the calculated \( \alpha \) and SASA values was lower than that of cisplatin. For cisplatin, the limiting step in the mechanism of the cytotoxicity induction is generally attributed to the hydrolysis of the Pt–Cl bonds.14 DFT studies indicated a more favorable solvation (\( \Delta G_{\text{solv}} \)) of pnpRu-14 than cisplatin, which may lead to a more favorable subsequent hydrolysis. This fact may be consistent with the high cytotoxicity of pnpRu-14 in case DNA would be the main target. Hence, as has already been observed with other Ru-based metallo drugs, it might point to a differential mode of action of pnpRu-14 with respect to that of cisplatin.

It is very well-known the high affinity to DNA of most platinum metallo drugs.15 However, NAMI-A is known to have weaker interactions with DNA than cisplatin, whereas KP1019 undergoes interactions similar to cisplatin. RAPTA derivatives exhibit pH-dependent DNA damage. Although DNA is known as a major target for metallo drugs, the environment of the nucleic acids, especially the protein regions of chromatin also appears to be an important target.16 Histone H1 is a lysine-rich

Table 1. Calculated Physicochemical Parameters for pnpRu-14

| parameter                     | pnpRu-14 (eV) | cisplatin |
|-------------------------------|---------------|-----------|
| \( E_{\text{HOMO}} \)         | −8.26         | −6.30     |
| \( E_{\text{LUMO}} \)         | −4.51         | −1.96     |
| \( \mu \) (D)                 | 5.09          | 11.3      |
| \( \alpha \) (Bohr\(^2\))     | 475           | 87.0      |
| SASA (Å\(^2\))               | 778           | 174       |
| \( V_{\text{m}} \) (cm\(^3\) mol\(^−1\)) | 453          | 97.0      |
| \( \Delta G_{\text{solv}} \) (kcal mol\(^−1\)) | −36.1        | −30.8     |

\( \Delta G_{\text{r}} \) energy: \( E_{\text{HOMO}} \) \( E_{\text{LUMO}} \) \( \mu \); polarizability: \( \alpha \); solvent-accessible surface area: SASA; molar volume: \( V_{\text{m}} \); Gibbs free energy of solvation: \( \Delta G_{\text{solv}} \); hydrolysis: \( \Delta G_{\text{r}} \).
histone fraction of chromatin and its role as DNA linker might modulate the binding of any drug to DNA. On the other hand, human serum albumin (HSA) can bind most therapeutic drugs. It has been demonstrated that the distribution and the metabolism of drugs can be significantly altered because of their binding to this protein. Regarding the binding studies to HSA, higher affinity by certain rhenium antitumoral agents has been observed compared to those of cisplatin.17

To get further insights into the pharmacological properties at the molecular level, information about the affinity of pnpRu-14 to DNA, HSA, and H1 histone were obtained by UV–vis absorption and fluorescence spectroscopy studies. In a first step of the study, DNA-binding studies to UV–vis spectroscopy with DNA were conducted for pnpRu-14 and RAPTA-C (the study with cisplatin was previously reported by our laboratory).18 The complex pnpRu-14 presented one intense absorption band centered at 300 nm corresponding to the charge transfer between Ru and the cymene moiety throughout the P atoms. The assignments of the transitions were guided by the results from time-dependent TD-DFT calculations (see Figure S2 in Supporting Information).

Figure 6A shows the plots of $A_0/(A_0 - A)$ versus [compound]/[DNA] of pnpRu-14, RAPTA-C and cisplatin in the presence of DNA (eq 1). The binding constant, $K_a$ values, were determined by linear regression, and values are shown in Table 2. Additionally, the interaction of pnpRu-14, RAPTA-C, and cisplatin with HSA and H1 histones was examined in fluorescence quenching experiments (see Experimental Section). The binding constant, $K_a$ values, were determined by linear regression of a plot of $(F_0 - F)/F$ versus log[Q] (eq 4), as shown in Figure 6B,C. Table 2 summarizes the binding constants of pnpRu-14, RAPTA-C, and cisplatin to DNA, HSA, and H1 histone.

The compounds interacted with DNA with binding constants, $K_a$, within 103–104 M$^{-1}$, a magnitude comparable with those of metal complexes binding DNA through noncovalent interactions.19 Based on the molecular structure and positive charge of pnpRu-14, electrostatic interactions with DNA could be expected with the negatively charged phosphate groups of DNA. However, considering that the $K_a$ value of the neutral complexes, RAPTA-C and cisplatin were of similar magnitude, a different action mechanism for its cytotoxicity could be involved. Examination of the binding constants to the HSA and H1 proteins indicated a strong interaction of the cationic pnpRu-14 with both proteins, whereas the neutral compounds cisplatin and RAPTA-C show a significant lower protein affinity. Remarkably, the binding affinity of pnpRu-14 toward H1 histones is two order of magnitude higher than RAPTA-C, while the corresponding binding cisplatin and H1 is practically negligible. This behavior is also appreciable when compared with the number of H1 binding sites (n), 0.48 for cisplatin versus ≈1.18 for both Ru derivatives. This set of results supports preceding studies in which histones and HSA proteins are good candidates for organometallics rhenium(II) complexes action.16,18 It seems that pnpRu-14 recognizes the chromatin structure with higher affinity than free DNA and this fact could strongly influence in its mechanism of action.

### 2.4. Pharmacokinetic and Biodistribution Study

Accumulation and biodistribution are important factors in drug development. The pharmacokinetic and biodistribution for the drug candidate pnpRu-14 were achieved by determining the content of Ru in 19 nude mice after intraperitoneal (i.p.) administration of a single concentration of 5 mg kg$^{-1}$. The mice were sacrificed 0.1, 1.5, 4, 12, 36, and 48 h after injection. Serum, liver, spleen, kidneys, heart, lungs, brain, prostate, ovary, and reproductive organs were collected from each mouse and the Ru content was determined by inductively coupled plasma–mass spectrometry (ICP–MS). The pharmacokinetic parameters of pnpRu-14 calculated in the 19 mice used for the in vivo study are displayed in Table 3 and the pharmacokinetic graphic in Figure 7A (see procedure in Experimental Section). pnpRu-14 was quickly absorbed into serum ($t_{1/2abs} = 0.5$ h) and, after approximately 1.5 h, the peak serum concentration was reached. Then, pnpRu-14 was progressively eliminated from the blood compartment with an elimination half-life greater than 12 h (Table 3). The $t_{max}$ calculated is faster than the $t_{max}$ of 2.5 h found for the biodistribution study on KP-1339/IT139, although it should

![Figure 6](image-url)

**Figure 6**. (A) Plot of $A_0/(A_0 - A)$ vs 1/[DNA] for the titration of pnpRu-14 and RAPTA-C with DNA. (B) Plot of log[(F0 − F)/F] vs log[Q] for the titration of HSA with pnpRu-14, cisplatin and RAPTA-C. (C) Plot of log[(F0 − F)/F] vs log[Q] for the titration of H1 with pnpRu-14, cisplatin, and RAPTA-C.

### Table 2. Binding Parameters ($\pm 2\sigma$) Obtained for the Interaction of pnpRu-14, RAPTA-C, and Cisplatin with DNA, HSA, and H1 Biomolecules

| comp. | biomol. | $K_a \times 10^{-3}$ | $n$ |
|-------|---------|----------------------|----|
| pnpRu-14 | DNA | 3.91 ± 1.91 | |
| | HSA | 3.23 ± 1.04 | 162 ± 59.6 | 1.11 ± 0.21 |
| | H1 | 5.24 ± 1.34 | 140 ± 14.7 | 1.17 ± 0.02 |
| RAPTA-C | DNA | 47.1 ± 10.6 | |
| | HSA | 7.06 ± 1.37 | 3.28 ± 0.59 | 0.87 ± 0.04 |
| | H1 | 1.85 ± 0.21 | 10.9 ± 2.78 | 1.06 ± 0.03 |
| cisplatin | DNA | 26.4 ± 0.73 | |
| | HSA | 0.58 ± 0.14 | 39.8 ± 10.8 | 1.18 ± 0.21 |
| | H1 | 2.28 ± 0.59 | 0.02 ± 0.01 | 0.48 ± 0.14 |
Table 3. Non-Compartmental Pharmacokinetics Analysis Determined in Mice after Intraperitoneal Administration of a 5 mg kg⁻¹ Dose of pnpRu-14

| Pharmacokinetic Parameters | pnpRu14 | RAPTA-C²⁰ |
|-----------------------------|---------|-----------|
| Kₚ (h⁻¹)                   | 0.055   |           |
| t₁/₂α (h)                  | 12.65   | 11.47     |
| t₁/₂β (h)                  | 0.5     |           |
| tₚmax (h)                  | 1.5     |           |
| Cmax (μg/L)                | 53.87   |           |
| AUCtotal (μg h L⁻¹)        | 791.98  | 891       |
| Vd (L)                     | 1.914   | 0.153     |
| CLapp (L h⁻¹)              | 0.105   | 0.009     |

![Figure 7](image-url)

Figure 7. Ruthenium concentration in serum after the administration of a single dose of 5 mg kg⁻¹ pnpRu-14 (A); and Ru content in liver, kidneys, spleen, lung, heart, ovary, prostate, and the reproductive system 0.5, 1.5, 4, 12, 36, and 48 h after intraperitoneal administration of pnpRu-14 (B). The values indicated are the average of data collected from samples taken from three animals at each time-point (n = 2 for 48 h postdose).

be noted that in the latter study, a concentration of 40 mg kg⁻¹ was used. The elimination half-life of pnpRu-14 is similar to compounds RAPTA-C,⁹ NAMI-A,¹¹ and KP1019,²² previously reported. When the total area under the concentration–time curve (AUCtotal) was analyzed, 7.91% of the AUC was extrapolated from the last timepoint at 48 h, thus enhancing the confidence on the data calculated for AUC, volume of distribution (Vd), and apparent clearance (CLapp).

Comparison with RAPTA-C studies indicate a higher Vd which could be attributed to a higher solubility of pnpRu-14 or a higher affinity to plasma proteins. Serum concentration at 12 h after the injection was 28.4 ± 10.7 ng mL⁻¹, which is lower than the Cmax, thus suggesting a low tissue accumulation of pnpRu-14. Similar results were reported for water-soluble iminophosphorane ruthenium(II) compounds. The results could support the binding studies reported above, in which pnpRu-14 showed a higher affinity to HSA than RAPTA-C and cisplatin.

It is noteworthy that ruthenium was found to specifically accumulate not only in liver and kidneys, the organs related to biotransformation, and elimination of xenogenous compounds such as pnpRu-14 but also relatively high concentrations were detected in reproductive organs such as prostate, ovary, fallopian tubes as well as in the afferent and efferent ducts (Figure 7B). Nevertheless, the number of female subjects analyzed is reduced to establish a hypothesis related to a potential activity toward these tissues, also considering the remarkable occurrence of interindividual variability.

On the contrary, the lowest levels of the compound were detected in lungs and heart. As expected, the organ samples taken from the animals sacrificed within 90 min after administration contained the highest levels of Ru, except for ovary and lungs, and slightly higher levels (approx. 3-fold) were observed in kidneys and liver only 1.5 h after injection. As in similar works, the concentration of pnpRu-14 in kidneys was remarkably higher than in the spleen,¹⁰ whereas RAED-C pharmacokinetic studies revealed the highest levels of the compound associated with the liver and kidneys,²⁴,²⁵ a phenomenon also observed for pnpRu-14. This phenomenon, together with the moderate clearance and relative short half-life calculated would indicate a considerably high rate of elimination of pnpRu-14 through liver metabolism and/or renal clearance. Moreover, the decrease of pnpRu-14 levels in kidney between 1.5 and 12 h after dosing will indicate that a significant amount of pnpRu-14 is being excreted by glomerular filtration, while a second peak is observed in kidneys 12 h after the injection, possibly because of a second elimination step of pnpRu-14 previously accumulated in several organs, such as the reproductive system or the prostate.

Over time, the Ru concentration was detected in all tissues analyzed, paralleling the total Ru levels in serum, except for the brain, in which levels below the limit of quantification of the ICP-MS methodology or no Ru was detected for any of the timepoints analyzed. This observation might be related to the low potential of this hydrophilic and charged drug compound to cross the blood–brain barrier. Nevertheless, the rapid elimination of pnpRu-14 from the organs analyzed and the bloodstream, partly because of its charged nature, would indicate the potential absence of serious side effects.

3. CONCLUSIONS

Chemotherapy is one of the most potent strategies to treat cancer. However, many limitations do exist including the identification and selection of a specific subgroup of responsive patients. This work is focused on the validation of an organometallic ruthenium(II) complex, pnpRu-14, as a novel anticancer drug for breast cancer therapy. Overall, pnpRu-14 exhibits a marked antitumoral activity in vitro against HER2+ and RH+/HER2− breast cancer, associated with low toxicity and favorable clearance properties. Initial mechanistic studies indicate a caspase-dependent apoptosis with cycle arrest in the G0/G1 phase cells. The interaction of pnpRu-14 with DNA is weak and electrostatic in nature and strong with HSA and H1 histones in comparison with the reference metallo-drugs, RAPTA-C, and cisplatin. Pharmacokinetic studies show a quick absorption in plasma with an elimination half-life similar.
to that reported for other ruthenium derivatives. Importantly, pnpRu-14 is not significantly accumulated in any tested organs. The simple accessible synthesis of this compound and its preliminary biological activity in vitro makes pnpRu-14 a good candidate for further evaluation in vivo as a potential chemotherapeutic agent against breast cancer.

4. EXPERIMENTAL SECTION

4.1. Synthesis of Compounds. Compounds RAPTA-C, pnpRu-14, and pnpRu-15 were prepared as previously described.1 Cisplatin was obtained from Sigma Chemical Co.

4.2. Cell Culture pnpRu-14. The positive hormonal receptor cell lines T47D and MCF7 and the HER2+ cell line BT474 and SKBR3 were obtained from the American Type Culture Collection (ATCC; CCL-227) (Manassas, VA). All lines were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 5 mM L-glutamine, and grown in incubators at 37 °C and 5% CO2. Cell culture mediums and supplements were obtained from Sigma-Aldrich (St. Louis, MO). Carboplatin, cisplatin, and herceptin were purchased from Accord Healthcare (United Kingdom, MA).

4.3. MTT Metabolization Assays. For concentration response and synergy studies, T47D, MCF7, BT474, and SKBR3 were plated at a density of 10,000 cells per well in 48-multiwell plates. The following day, cells were treated with different doses of the drugs (pnpRu-14, RAPTA-C, pnpRu-15, carboplatin, and cisplatin), alone or in combination (RU14 and herceptin), for three days (72 h).

4.4. Cell Cycle and Apoptosis Assay. For cell cycle analyses, T47D, MCF7, BT474, and SKBR3 were plated at 100,000 cells per well in 6-multiwell plates. The following day, cells were treated with pnpRu-14 (1 μM) during 24 h. Then, cells were trypsinized, centrifuged at 800 g, and supernatant was discarded. Cells were fixed and permeabilized with 300 μL of cold-ice ethanol (70% in phosphate-buffered saline (PBS)) for 30 min; then, 700 μL of a BSA solution (2% BSA in PBS, Sigma-Aldrich, St. Louis, MO) was used to wash cells and avoid aggregate formation. Then, cell pellets were incubated with propidium iodide/RNase staining solution (Immunostep S.L., Salamanca, Spain). Cell cycle was analyzed in a FACSContigo II flow cytometer (BD Biosciences).

For apoptosis analyses, 100,000 cells were treated with pnpRu-14 (1 μM) for 48 h. Then, cells and supernatants were collected and centrifuged at 900 rpm for 5 min. Pellets were resuspended in 1X Annexin V binding buffer (Immunostep S.L., Salamanca, Spain) and incubated for 1 h in the dark with Annexin V and PI staining solution (2.5 μL Annexin V-DE-634, 8 μL of PI [2 mg/mL final concentration], 300 μL binding buffer, Immunostep S.L.). Percentage of apoptotic cells was determined using a FACSContigo II flow cytometer (BD Biosciences). Both early (Annexin V-positive, PI-negative) and late apoptotic (Annexin V-positive and PI-positive) cells were included in cell death analyses.

4.5. Western Blot. T47D, MCF7, BT474, and SKBR3 cells were treated with pnpRu-14 (1 μM) for 24 and 48 h. Then, cells were washed with PBS and lysed with ice-cold lysis buffer (20 mM Tris-HCl [pH 7.0], 140 mM NaCl, 50 mM EDTA, 10% glycerol, 1% Nonidet P-40, 1 μM pepstatin, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mM phenylmethyl sulfonyl fluoride, and 1 mM sodium orthovanadate). Lysates were centrifuged at 10,000g at 4 °C for 10 min and the clarified cell extracts were quantified with BCA (Bicinchoninic Acid Kit for Protein Determination, BCA1-1KT Sigma-Aldrich) Protein extracts (80 μg) loaded in on 6–15% SDS-PAGE gels, depending on the molecular weight of the proteins to be analyzed. After electrophoresis, proteins in gels were transferred to PVDF membranes (Millipore Corporation). Membranes were blocked in Tris-buffered saline containing Tween (TBST) (100 mM Tris [pH 7.5], 150 mM NaCl, and 0.05% Tween 20) containing 1% of bovine serum albumin for 2 h and, then, incubated with the corresponding antibody in TBST for 2–16 h. After washing with TBST, membranes were incubated with HRP-conjugated anti-mouse or anti-rabbit secondary antibodies (1:10,000 dilution) for 30 min and bands were visualized by using ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, United Kingdom).

Antibodies against cyclin E, cyclin D1, p27, and p21 were from Cell Signaling Technologies (Beverly, MA, USA). Antibodies against PARP and cyclin B were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The rabbit polyclonal anti-calnexin was obtained from Stressgen Bioreagents (Victoria, BC, Canada), anti-Rabbit from Bio-Rad and anti-Mouse is from GE Healthcare.

4.6. Caspase 3 Assay. Fifty micrograms of protein extracts from pnpRu-14 48 h-treated cells were placed in 96-well plates in triplicate. The final volume of the lysates was taken to 100 μL by 1X Caspase buffer (25 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 10% sucrose). One hundred μL of 2X caspase reaction buffer (50 mM HEPES pH 7.4, 300 mM NaCl, 2 mM EDTA, 0.2% CHAPS, 20% sucrose, 20 mM DTT, and 10 μM fluorescently labelled caspase 3 substrate Ac-DEVD-AFC) was added to each well containing cell lysates. The plate was shaken to mix the solution and incubated at 37 °C for 1 h from light. Signals were measured at 400/505 nm in a fluorescent reader (BioTek).

4.7. In Vivo Studies. To study the pharmacokinetics and biodistribution of pnpRu-14 in vivo, BALB/c/cAnNΔ1-Rf1 Fox1 nu/Fox1 nude mice (male and female, 10 weeks old, body weight 16–27 g) were utilized. A 2:1 ratio between female and male animals was established at each experimental group, except for the control and 48 h group. Animal care complied with the Principles of Laboratory Animal Care and Guide for the Care and Use of Laboratory Animals. The selected dose (5 mg/kg) of pnpRu-14 was intraperitoneally administered into the animals, which were divided into seven groups: (i) vehicle control group (*n = 2), (ii) 30 min post-treatment (*n = 3), (iii) 90 min (*n = 3), (iv) 4 h (*n = 3), (v) 12 h (*n = 3), (vi) 36 h (*n = 3), and (vii) 48 h post-treatment (*n = 2), respectively. First, blood was drawn from each animal in different tubes containing EDTA, and then centrifuged at 1500g by 10 min to obtain the serum aliquots required for the pharmacokinetic analysis. These samples were then stored at −80 °C until ICP–MS analysis. Furthermore, liver, kidneys, spleen, lungs, heart, brain, and reproductive organs were collected at the indicated times to determine the biodistribution of pnpRu-14.

In this case, these samples were lyophilized to homogenize them and for their preservation.

4.8. Statistical Analysis. To determine significant and fully understand group differences, to equal variances Tukey’s test was used with ANOVA, respectively, and to compare the media of results, Student t test was used. The values for the statistical analyses are: *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001.
4.9. Sample Preparation. Ruthenium content in explanted organs (i.e., liver, kidneys, spleen, lungs, heart, brain, prostate, and reproductive system) from mice treated with pnpRu-14 or PBS solution were evaluated by ICP–MS. Additionally, the serum content of this metal was measured at fractions collected 0.5, 1.5, 4, 12, 36, and 48 h after administering the dose of pnpRu-14.

Optimized digestion of samples was carried out in a microwave digester (Ethos Plus Microwave Labstation, Milestone Inc., Monroe, CT, USA) by placing 50 μL of serum, or a fragment of each organ specimen previously weighed, in a Teflon digestion vessel where 2 mL of 20% HNO₃ (diluted from 69% purity HNO₃) was added. The vessels were closed, and the samples digested with 125 W of power per vessel using a four-stage program (stage 1 heating to 220 °C for 5 min; stage 2 holding at 200 °C for 5 min; stage 3 heating to 220 °C for 5 min; and a final step of 5 min at 220 °C). Then, the vessels were allowed to cool to room temperature for 15 min prior to opening the vessels. Digested solutions were then adjusted with ultrapure water to obtain a 10 mL solution with the correct acid concentration to perform the analysis. All the solutions were prepared using ultrapure water obtained from a Millipore Milli-Q system.

4.10. Sample Analysis. The quantitative analysis of ruthenium in the processed samples was carried out by ICP–MS using a 7900 Series (G8403A) Agilent ICP–MS instrument (Agilent Technologies, CA, USA) equipped with a MicroMist glass concentric nebulizer, a quartz Scott-type double pass-cooled spray chamber, and SPS 4 autosampler. The metal content was determined by flow injection analysis under the following operating conditions: power 1550 W, carrier gas 0.99 L min⁻¹, make-up gas 0.00 L min⁻¹, sample depth 10 mm, nebulizer pump 0.1 rps and spray chamber temperature 2 °C. The ICP–MS instrument was equipped in helium collision mode for unsurpassed interference removal. A MicroMist glass concentric nebulizer, a quartz Scott-type double pass-cooled spray chamber, and SPS 4 autosampler. The metal content was determined by flow injection analysis under the following operating conditions: power 1550 W, carrier gas 0.99 L min⁻¹, make-up gas 0.00 L min⁻¹, sample depth 10 mm, nebulizer pump 0.1 rps and spray chamber temperature 2 °C. The ICP–MS instrument was equipped in helium collision mode for unsurpassed interference removal. A typical performance test in He mode is as follows: He flux 5 mL/min, m/z 7 (327 cps), m/z 89 (2542 cps), m/z 205 (481 cps). Every sample contained 0.5 ng mL⁻¹ erbium as internal standard for ICP–MS measurements and all determinations were conducted by monitoring the ¹⁰¹Ru signal. Serum from control mice was spiked with the test compound to determine the extraction efficiency.

The ICP–MS instrument was tuned using a solution containing 1 μg mL⁻¹ each of Ce, Co, Li, Mg, Tl and Y (Agilent), and calibration curves were obtained using aqueous standard solutions in identical matrix to the samples (with regard to internal standard and 2% nitric acid) with appropriate stock standards dilutions (Sigma-Aldrich, Switzerland).

4.11. Affinity Studies. 4.11.1. Materials and General Procedure. Salmon sperm DNA was obtained from Sigma-Aldrich in 10 g L⁻¹ aliquot. A stock solution of 1300 μM was prepared by dissolving the sample in 0.02 M Bis-Tris buffer solutions at pH 7.4 containing 0.1 M NaCl. Bis-Tris (Sigma) and NaCl (Panreac) had a purity of no <99.0%. Water was purified in a Milli-RO System (Millipore) and stored at −10 °C. The nucleotide DNA concentration was determined spectrophotometrically. The ratio A₂₆₀/A₂₈₀ for this solution was >1.8.

HSA (≥99%) and H1 histone (%) were supplied by Sigma-Aldrich. For HSA and H1 binding experiments, working solutions of proteins (5 μM) were daily prepared in buffer solution. Stock solutions of pnpRu-14 and RAPTA-C of 180 and 216 μM, respectively, were prepared by dissolving the sample in methanol while cisplatin stock solution (330 μM) was obtained by dissolving the compound in DMSO.

4.11.2. DNA-Binding Studies by UV–Vis Spectroscopy. Absorbance spectra were recorded on an ETCR-762 (Jasco) spectrophotometer in a 10 × 10 mm quartz cuvette operating at 1 nm resolution. Titration experiments were performed at constant concentration of metallo-drugs in the sample cell. Stock DNA was added in increasing amounts (0–200 μM) to the sample cell containing 3 mL of a solution 10 μM of drugs in buffer and 3 mL of the solution of buffer in the reference cell. The decrease in the absorption at λ_max was measured at each addition. The binding constants were then determined according to the Benesi–Hildebrand equation.27

\[
\frac{A_0 - A}{A_0} = \frac{\epsilon_G}{\epsilon_G - \epsilon_{HG}} + \frac{\epsilon_G}{\epsilon_G - \epsilon_{HG}} K[DNA]
\]

where K is the binding constant, \(\epsilon_G\) and \(\epsilon_{HG}\) are the absorption coefficients of the coordination compound and the coordination compound/DNA complex, respectively. Three experiments were carried out for each compound (Table 2).

4.11.3. Protein Binding Studies by Steady-State Fluorescence Spectroscopy. Fluorescence spectra of the samples were recorded by means of a FSS (Edinburgh Instruments) spectrophotometer equipped with a time-correlated single photon counting detector. As a light source, an Xe lamp of 150 W was employed to record the steady-state fluorescence spectra. In the protein (HAS, H1)/metallo-drug binding experiments, 3 mL protein solutions (5 μM) were titrated in cuvette by successive addition of a metallo-drug stock solutions. The excitation wavelengths were 295 and 283 nm for H1 and HAS, respectively, whereas the emission fluorescence intensities were collected at 330 and 372 nm, respectively. The excitation and emission slits were fixed at 1 and 5 nm, respectively. The step and dwell times were 1 nm and 0.1 s, respectively. For the inner filter effect fluorescence was corrected through

\[
F_{corr} = F_{obs}10^{(ΔA_{as}+ΔA_{sm})/2}
\]

where \(F_{corr}\) and \(F_{obs}\) are the corrected and observed fluorescence intensities, and \(ΔA_{as}\) and \(ΔA_{sm}\) are the absorbance of the system at excitation and emission wavelengths. Temperature was controlled at 300 K by a temperature-controlled cuvette holder, TC 125 (Quantum Northwest) and the experiment was repeated three times.

The Stern–Volmer equation was then used to evaluate the quenching constant KSV of the studied drug–protein systems

\[
\frac{F_0}{F} = 1 + K_{SV}[Q]\]

where \(F_0\) and \(F\) are the emission intensities in the absence and the presence of the quencher, respectively, \([Q]\) is the concentration of the quencher (metallo-drug), and KSV is the Stern–Volmer constant.28

For static quenching, the relationship between fluorescence intensity and the quencher concentration, \([Q]\), is described by the following equation

\[
\log \left( \frac{F_0}{F} \right) = \log K_s + n \log [Q]
\]
where $F_0$ corresponds to the fluorescence intensity of the protein in the absence of quencher, $n$ and $K_q$ are the number of binding sites and binding constant, respectively.\textsuperscript{29--31}

### 4.11.4. Computational Methodology

The Gaussian09 (Rev. C.01) software package was used to carry out the theoretical calculations,\textsuperscript{32} and the hybrid density functional B3LYP method employed to generate geometries. The effective core potential LANL2DZ was used to describe Ru atoms and the 6-31+G(d,p) basis set was employed for the other atoms.\textsuperscript{33,34} The solvation model based on density was employed to include solvent effects.\textsuperscript{35}

HOMO and LUMO energies, dipole moments, and polarizabilities were calculated at the B3LYP level in the gas phase. Solvent-accessible surface areas and molecular volumes were extracted from calculations in aqueous solution. Solvation free energies of the chloride compound were obtained as the difference of their free energies in water solution and in the gas phase. Electronic transitions were analyzed at the time-dependent (TD)-B3LYP level for the solvated molecules.

### ASSOCIATED CONTENT

5 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b00296.

DFT-optimized geometry of pnpRu-14 in Cartesian coordinate system and calculated electronic transitions and HOMO and LUMO orbitals for pnpRu-14 (PDF)

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Notes

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