Design, Synthesis, DNA/HSA Binding, and Cytotoxic Activity of Half-Sandwich Ru(II)-Arene Complexes Containing Triarylamine–Thiosemicarbazone Hybrids

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Supporting Information

ABSTRACT: Organoruthenium complexes are potent alternatives for platinum-based complexes because of their superior anticancer activity. In this investigation, a series of new Ru(II)-arene complexes with triarylamine–thiosemicarbazone hybrid ligands with higher anticancer activity than cisplatin are reported. The molecular structure of the ligands and complexes was confirmed spectroscopically and supported by single-crystal X-ray crystallography. These complexes adopted a three-leg piano stool geometry. All the Ru(II)-arene complexes were systematically investigated for their in vitro cytotoxicity against human cervical (HeLa S3), lung (A549) cancer, and human normal lung (IMR-90) cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Interestingly, a pyrrolidine-attached Ru(II)-benzene complex exhibited superior activity against cancer cells with low IC50 values, and colony formation study showed complete inhibition at 5 and 10 μM concentration. Furthermore, morphological changes assessed by acridine orange and propidium iodide staining revealed that the cell death occurred by apoptosis. In addition, the interaction between synthesized Ru(II)-arene complexes and DNA/protein was explored by absorption and emission spectroscopy methods. These synthesized new organoruthenium complexes can be used for developing new metal-based anticancer drugs.

INTRODUCTION

The metal complexes are well exploited for their anticancer activity with an enormous impact on cancer chemotherapy. Because of their peculiar structure and better electronic, magnetic, thermodynamic, kinetic, and intrinsic properties, metal ions are widely used for the development of new highly potent anticancer agents, DNA probes, and cleaving agents. Cisplatin and related platinum-based chemotherapeutic drugs are active against various types of human cancers. The success of platinum-based drugs lagged behind because of their adverse effects on human health including kidney diseases, hemorrhage, and intrinsic and acquired resistance against various cancers. To overcome the aforementioned issues, researchers have diverted their attention from Pt-based drugs to design alternative biocompatible metal-based drugs. Ruthenium-based complexes are reported as target-specific and less toxic with high pharmacological effect. In particular, NAMI-A “imidazolium trans-\{tetrachlorido(DMSO)(1H-imidazole)-ruthenate(III)\}“ complexes exhibited remarkable activity against antimetastatic and solid tumors. At present, the formulation of KP1019 has been slightly modified because of solubility reasons and renamed as NKP-1339 (also IT-139). This formulation is currently being used in clinical trials and also got FDA orphan status in 2017, while the clinical trials for NAMI-A have been abandoned. Recently, Ru(II)-arene-based metallodrugs are projected as potential and effective candidates for cancer treatment. An impressive architecture was obtained by incorporating π-bonded arene moiety with biologically significant ligands, such as ethacrynic acid, anti-inflammatory drugs, quinolone, and thiosemicarbazone (TSC). The design and structure of arene moiety play a crucial role in establishing the pharmacological properties of the
complexes, viz. influence in cell uptake, binding to the active center, and inertness to other substituents. Another great advantage is the passive transport across the cell membrane because of the enhanced hydrophobicity. From literature, it has been evidently analyzed that the π-stacking of arene ligand enhances the interaction of metal complexes with DNA or protein which is the reason for their biological activity and various pharmacological properties. Arene moeity also stabilizes the oxidation state of Ru(II) and provides a hydrophobic face, enhances biomolecular recognition process, and improves the transport through cell membrane. The evaluation of Ru(II)-arene complexes with thepta (1,3,5-triaza-7-phosphaadamantane), \([\text{RuCl}_2(\eta^6-p\text{-cymene})(\text{pta})]\) (PAPTA-C), \([\text{RuCl}_2(\eta^6\text{-benzene})(\text{pta})]\) (PAPTA-B), and Ru(II)-arene-en (en-ethylenediamine) had been reported along with in vivo activity on the inhibition of metastasis growth with high selectivity and low toxicity (Figure 1).

![Figure 1. Ruthenium(II) complexes for cancer therapy (a) PAPTA-C, (b) PAPTA-B, and (c) Ru-arene-en.](image)

Thus, Ru(II)-arene with suitable ligand moeity proves to influence the cytotoxicity and efficiency. Recently, plenty of Ru(II)-arene-based complexes are being reported with high anticancer activity. These complexes unquestionably proved substantial in vitro cytotoxicity with apoptosis as the mechanism for cell death. TSC and triarylamide (TAA) molecules are significant constituents which are attracting the interdisciplinary field of research because of their notable geometry and chemical stability. TAAs possess impressive biological activity with enhanced fluorescence emission. The mechanism of inducing cell death upon photoactivation is associated with its unique style of interaction with the cell nucleus. TSCs are other vital constituents in the field of medicinal chemistry because of their coordination flexibility and biological versatility. A combination of TSC and TAA derivatives can result into a chief component for anticancer and other therapeutic drugs. Even though the latter is commonly used as a metal-ligating compound, the complexes with both TSCs and TAAs are not frequently reported. This paves a need for more discussion about the beneficial effects exhibited by this combination and by metal coordination. The valuable features of ruthenium as a metal with arene moeity and TSC/TAAs ligands inspired us to develop potent Ru(II)-arene complexes as a therapeutic agent.

In this work, we have synthesized a series of new half-sandwich Ru(II)-arene complexes containing TAAs and TSCs as chelating ligands. The unique combination of these ligands with Ru(II)-arene complexes has not been reported. The interaction of the new complexes with DNA and human serum albumin (HSA) is examined. In order to get an insight into the morphological changes, acridine orange (AO) and propidium iodide (PI) staining assays are also scrutinized. Cell proliferation behavior is analyzed by the colony formation method.

### RESULTS AND DISCUSSION

The complexes were synthesized from thiosemicarbazide and aromatic aldehyde via condensation reaction (Scheme 1). The complexes \([(\eta^6-p\text{-cymene})\text{-Ru}^\text{II}(\text{L}1)\text{Cl}]\text{Cl} (1), [(\eta^6-p\text{-cymene})\text{-Ru}^\text{II}(\text{L}2)\text{Cl}]\text{Cl} (2), [(\eta^6-p\text{-cymene})\text{-Ru}^\text{II}(\text{L}3)\text{Cl}]\text{Cl} (3), and [(\eta^6-p\text{-cymene})\text{-Ru}^\text{II}(\text{L}4)\text{Cl}]\text{Cl} (4)] were obtained in good yield (82–87%) by using \([\text{RuCl}_2(\mu\text{-Cl})(\eta^6-p\text{-cymene})]\) and TSC ligands (Scheme 2). The TSC ligands (L1–L4) on treatment with Ru(II)-benzene dimer \([(\eta^6\text{-benzene})\text{-Ru}^\text{II}(\mu\text{-Cl})]\) gave ideal complexes \([(\eta^6\text{-benzene})\text{-Ru}^\text{II}(\text{L}1)\text{Cl}]\text{Cl} (5), [(\eta^6\text{-benzene})\text{-Ru}^\text{II}(\text{L}2)\text{Cl}]\text{Cl} (6), [(\eta^6\text{-benzene})\text{-Ru}^\text{II}(\text{L}3)\text{Cl}]\text{Cl} (7), and [(\eta^6\text{-benzene})\text{-Ru}^\text{II}(\text{L}4)\text{Cl}]\text{Cl} (8) (Scheme 3). All the TSC ligands and their corresponding Ru(II)-arene complexes were characterized by UV-visible, Fourier transform infrared (FT-IR), nuclear magnetic resonance (NMR), and mass spectrometry (MS) techniques.

The electronic spectral data of complexes 1–8 in methanol exhibits two absorption bands. The first band corresponds to the n → π* transition appeared around 268–295 nm. Subsequently, the second band in the region 339–365 nm was observed as a broad band owing to metal-to-ligand charge transfer (MLCT). The FT-IR spectra of TSC ligands exhibited very strong bands because of ν(N=H) in the region of 3453–3134 cm⁻¹.

Other characteristic bands were observed in the range of 1600–1584 and 1321–1244 cm⁻¹, which were assigned to ν(C=N) and ν(C=S), respectively. After the formation of complexes, there was a decrease in thiocarbonyl ν(C=S) and azomethine (C=N) stretching frequencies, which indicates the involvement of sulfur and nitrogen atoms in coordination with ruthenium ions.

Electrospray ionization (ESI)-MS spectra show molecular ion peaks of ligands L1–L4 (Figure S1) and complexes 1–8 (Figures S2 and S3). The ESI-MS spectra of ligand L1 showed [M + H]+ peaks and those of other ligands (L2, L3, and L4) exhibited [M – H]– peaks. The molecular ion peaks for complexes 1–8 were not observed, which may be due to possible fragmentation of the molecules. These complexes exhibited peaks due to [M – 2Cl – H]+, suggesting that the Cl group is labile.

In the 1H NMR spectra of ligands L1–L4 imine (=N–NH) and azomethine (HC=N), protons appeared at 9.12–8.76 and 7.69–7.54 ppm. The same protons in complexes 1–4 were deshielded and appeared at 13.79–14.53 ppm. The aromatic protons of the ligands and complexes resonate in the range of 7.52–7.01 ppm (Figure S4) and 8.27–7.09 ppm, respectively. The signals due to pyrroldine protons in L1, L2, L3, L4, 1, 2, 5, and 6 appeared in the range of 3.96–1.96 ppm. In the spectra of L3, L4, 3, 4, 7, and 8, signals of cyclohexyl protons...
are found in the regions 4.30−3.89 and 2.04−1.23 ppm. New signals were observed in the region 5.50−4.97 ppm, which is due to the presence of p-cymene complexes36 1–4 (Figure S6), whereas a signal at 5.56−5.54 ppm for complexes 5−8 (Figure S7) showed the presence of benzene ring.37 In the 13C spectra of ligands L1−L4 (Figure S5) and complexes 1−8, thiocarbonyl (C=S) and imine (C=N) carbon signals appeared around 176.1−175.7 and 174.8−171.2 ppm, respectively. All aromatic carbons in the ligands and complexes appeared in the range of 149.6−119.7 (ligands) and 150.6−119.2 ppm (complexes).13C NMR spectra of the complexes, a new signal at 103.9−82.1 ppm, confirmed the presence of p-cymene group in complexes 1−4 (Figure S8) and a new peak at 87.6−87.1 ppm validated the occurrence of benzene moiety in complexes 5−8 (Figure S9).

The molecular structures of L1, L2, L3, and L4 and 3 have been investigated by X-ray crystallography. Suitable crystals were obtained by slow diffusion of dichloromethane/methanol solution of samples and for L2 and L4 DMSO was used as an additive. Crystallographic data, description, and selected interatomic bond lengths and angles are given in Tables S1−S4. Thermal ellipsoid plots of compounds with the atomic labeling schemes are shown in Figures 2 and S10−S13. Our ligands are composed of three main parts: TAA group connected to pyrrolidine/cyclohexyl by TSC moiety. The structure revealed that the central nitrogen atom of TAA is in sp2 hybridization and the three benzene rings are twisted with respect to one other, existing in a propeller-like fashion. The molecule has E conformation with respect to the N2−N3; the bond length was 1.370, 1.385, 1.380, and 1.386 Å; and the dihedral angle was 177.0, 164.7, 173.3, and 173.2° for ligands. All molecules exhibited quasi-coplanarity in the plane of TSC and exists in thione (C=S) form, similar to other TSC systems.23 The molecular structure of complex 3 shows undoubtedly that the TSC ligand coordinates in a bidentate manner with Ru ions via thiocarbonyl sulfur (Sneutral) and azomethine nitrogen (Nneutral) along with one terminal chlorido and one arene moiety. This complex has adopted
the "piano stool" geometry, where \( \eta^6-p\)-cymene formed the top of the stool: the S, N (from TSC ligand) and chlorido atoms served as legs. Ru metal sharing with S and N atoms results in the formation of a five-membered chelate ring with bite angles 86.87° S(1)–Ru(1)–Cl(1) and 87.18° N(1)–Ru(1)–Cl(1). The twist in the bite angle has resulted in distorted octahedral geometry around the Ru center. The bond distances of Ru(1)–S(1), Ru(1)–N(1), and Ru(1)–Cl(1) are 2.3568, 2.1295, and 2.4171 Å, respectively. The \( \eta^6-p\)-cymene unit is strongly bonded to the Ru metal center with a typical Ru–C bond length of 2.2091 Å; moreover, the average C–C bond lengths are found to be 1.4168 Å. Upon complexation, the S(1)–C(S) and N(1)–C(1) bond characteristics (1.700 and 1.290 Å, respectively) remain almost unchanged, proving that the thione form coordinates with the Ru ions. All these observations are in agreement with other recent reports.38–40

With respect to the spectral data and X-ray diffraction (XRD) analysis, all the other seven complexes also possess similar geometrical features of [\( \eta^6-p\)-cymene]-Ru(II)(L3)Cl] (3).

**Interaction of the Complex with CT-DNA.** Electronic Absorption Studies. The binding mode of DNA with Ru(II)-arene complexes 1–8 was examined through electronic absorption spectroscopy studies. All complexes exhibited two absorption bands around 270–382 and 349–406 nm, which are assigned to ligand-to-metal charge transfer and MLCT, respectively. Upon successive addition of DNA to the fixed concentration of complexes, hypochromism was observed with 3–4 nm red shift: this indicates the intercalative mode of binding. The extent of shift and hypochromism can be related to the DNA binding affinity.41 The absorption spectra of the complexes in the presence and absence of CT-DNA is illustrated graphically in Figures 3 and S14. The binding constant of the complexes with CT-DNA (\( K_b \)) was obtained from the ratio of slope to intercept by plotting [DNA]/\( (\varepsilon - \varepsilon_i) \) versus [DNA] according to the equation

\[
\frac{[\text{DNA}]}{(\varepsilon - \varepsilon_i)} = \frac{[\text{DNA}]}{(\varepsilon_b - \varepsilon_i)} + 1/K_b (\varepsilon_b - \varepsilon_i),
\]

where [DNA] is the concentration of DNA in base pairs, \( \varepsilon_i \) is the apparent extinction coefficient value found by calculating \( A_{\text{observed}}/[\text{complex}] \), \( \varepsilon_i \) is the extinction coefficient of the free compound, and \( \varepsilon_b \) is the extinction coefficient of the compound in the fully bound form. Each set of data, when fitted into the above equation gave a straight line with a slope of 1/(\( \varepsilon_b - \varepsilon_i \)) and y-intercept of 1/\( K_b (\varepsilon_b - \varepsilon_i) \) (Figure S15).

The magnitude of intrinsic binding constants (\( K_b \)) complexes followed the order 8 > 7 > 6 > 2 > 3 > 1 > 5 > 6, and the values are presented in Table 1. The results revealed that the binding constants are in a similar range for the other reported complexes.33

**Fluorescence Spectroscopy Studies.** To further investigate the mode of binding of the metal complexes with CT-DNA, the fluorescence spectroscopy technique was employed. The fluorescence property was not observed for the complexes at room temperature in solution or in the presence of CT-DNA. Therefore, emission spectroscopy could not directly predict the binding of the complexes with DNA. Hence, a competitive binding study was carried out to comprehend the mode of DNA interaction with the complexes.34 Ethidium bromide (EB) emits intense fluorescence in the presence of CT-DNA because of the strong intercalation of the planar EB phenanthridine ring and the adjacent base pairs of the double helix. Therefore, EB can be considered as a typical indicator for intercalation. When a molecule that could bind more efficiently to DNA than when EB was added, the molecule will replace the bounded EB and there will be a quenching in the DNA-induced EB emission. The extent of quenching of CT-DNA–EB reflects the extent of interaction with the added molecule. The fluorescence intensity (607 nm) of CT-DNA pretreated EB system was observed to exhibit decreases (Figures 4 and S17) with successive addition of Ru(II) complexes (0–50 \( \mu M \)): quenching percentage with our complexes was calculated as 66, 61, 64, 58, 71, 60, 62, and 48% and resulted in hypochromic shift of 11, 8, 7, 10, 13, 15, 9, and 8 nm, respectively. The magnitude of interaction between complexes and DNA is quantitatively calculated by using Stern–Volmer equation,

\[
F'/F = 1 + K_q[Q],
\]

where \( F' \) and \( F \) are the fluorescence intensities in the absence and presence of the quencher, respectively, \( K_q \) is a linear Stern–Volmer quenching constant, and \([Q]\) is the concentration of complex. Figure S16,

![Figure 3. Absorption spectra of complexes (1 and 5) in Tris-HCl buffer upon addition of CT-DNA. [Complex] = 1.5 \times 10^{-5} \text{ M}, [DNA] = 0–40 \mu\text{M}. Arrow shows the decrease in absorption upon increasing DNA concentration.](image-url)
shows good Stern–Volmer plots, and the good linearity of the plots suggests a singular mode of quenching. The apparent DNA binding constant \( K_{\text{app}} \) was calculated by using the equation.\(^{46} \) \( K_{\text{EB}}[\text{EB}] = K_{\text{app}}[\text{complex}], \) where [complex] is the complex concentration at 50% reduction in the fluorescence intensity of EB, \( K_{\text{EB}} = 1.0 \times 10^{-7} \text{ M}^{-1} \) and [EB] = 5 \( \mu \)M. The study was carried out with an EB concentration of 10\(^{-3} \) M, DNA of 10\(^{-3} \) M, and metal complexes in 10\(^{-3} \) M with further dilution to 15 \( \mu \)M for EB and [DNA] and 5 \( \mu \)M for complexes. The probe was carried out with 10 equiv additions of metal complexes to the sample cuvette containing solution of buffer, EB, and DNA. The intrinsic fluorescence emission spectra of all the compounds were recorded separately before and after the addition of varying concentrations of complexes (0–50 \( \mu \)M) and the quenching constant \( K_q \) and \( K_{\text{app}} \) values follows order \( S > 6 > 1 > 2 > 7 > 3 > 4 > 8 \) and the values are listed in Table 1. From the absorption and emission spectra analysis, complex 5 is found to have better DNA binding nature than the other complexes.

Interaction of the Complex with HSA. Fluorescence Titration Studies. HSA plays a vital role in the transportation of drugs and compounds such as fatty acids which bind reversibly to HSA. Binding of the drugs to this extracellular protein can alter its metabolism and distribution as well as concentration. Reports show that the forces of interaction between HSA and complexes consist of hydrogen bond formation, van der Waals forces, electrostatic forces, and hydrophobic interactions. Thus, it is necessary to study the interaction of the drugs with HSA. Fluorescence spectrophotometry is a common technique to study molecular interaction with proteins. Tyrosine, tryptophan, and phenylalanine are the three residues responsible for the autofluorescence activity of HSA. HSA comprises 585 amino acid residues with a single tryptophan residue (Trp 214) obligation for the intrinsic fluorescence of HSA.\(^{47} \) The fluctuations in the graph thus obtained after the addition of the metal complexes suggested that binding occurs on the protein. Here, we have performed the emission-quenching experiments by increasing the amount of the complex solution to a fixed quantity of HSA ([Figure S18]), and fluorescence intensity at 345 nm decreases up to 69, 59, 64, 66, 78, 68, 62, and 70% for complexes 1–8, with a hypochromic shift of 8, 7, 11, 10, 13, 12, 11, and 9 nm for complexes 1–8, respectively. The complexes interact hydrophobically with proteins, which is evident from the observed hypochromism. The fluorescence quenching is described by the Stern–Volmer\(^{48} \) relation given as \( F_0/F = 1 + K_q[Q], \) where \( F_0 \) and \( F \) are the fluorescence intensities in the absence and presence of quencher, respectively. \( K_q \) is a linear Stern–Volmer quenching constant and \( [Q] \) is the quencher concentration. A linear plot is obtained from the graph of \( F_0/F \) versus \( [Q] \) plot ([Figure S19]). Further, the equilibrium between free and bound molecules is represented by the Scatchard equation: \( \log([F_0 − F]/F) = \log K_q + n \log[Q], \) where \( K_q \) is the binding constant of the complex with HSA and \( n \) is the number of binding sites (Table S1),\(^{49} \) from the plot of \( \log ([F_0 − F]/F) \) versus \( \log[Q] \) ([Figure S20]).

Anticancer Activity. All the synthesized Ru(II)-arene complexes (1–8) were tested against A549 and HeLa S3 cancer cell lines. The graph of percentage (%) of cell viability versus concentration is presented in Figure S23, and the IC\(_{50}\) (half minimum inhibition concentration) value of the complexes is shown in Table 2. Among all the complexes, complex 5 showed promising cytotoxic activity against both HeLa S3 and A549 cancer cells. Complex 5 showed 50% of inhibition at 5.3 ± 3.8 \( \mu \)M in HeLa S3 cells and 7.24 ± 5.4 \( \mu \)M in A549 cells. In addition, complexes 1, 3, and 6 showed moderate cytotoxicity activity in HeLa S3 cells with an IC\(_{50}\) value of 69.5 ± 4.5, 77.6 ± 3.5, and 85.6 ± 4.2 \( \mu \)M, respectively. However, complex 6 also showed moderate cytotoxic activity in A549 cells with an IC\(_{50}\) value of 18.9 ± 5.8 \( \mu \)M (Table 2). The cytotoxic effects of complexes 1, 3, 5, and 6 against human normal lung IMR-90 cell line ([Figure S24]) showed less toxicity compared with cancer cells with an IC\(_{50}\) value of 81.5 ± 1.6, >100 ± 1.8, 39 ± 4.7, and >100 ± 5.9 \( \mu \)M. It is remarkable to mention that complex 5 is more active compared with cisplatin.\(^{50-51} \) The present reported complexes, particularly 5 and 6, displayed low IC\(_{50}\) values compared with

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**Table 2. IC\(_{50}\) Values of Synthesized Complexes against HeLa S3, A549, and IMR-90 Cells**

| complexes | HeLa S3 (IC\(_{50}\)–\( \mu \)M) | A549 (IC\(_{50}\)–\( \mu \)M) | IMR-90 (IC\(_{50}\)–\( \mu \)M) |
|-----------|-------------------------------|-------------------------------|-------------------------------|
| 1         | 69.5 ± 4.5                    | 413.2 ± 2.1                  | 81.5 ± 1.6                   |
| 2         | >100 ± 7.1                    | >100 ± 1.5                  | NT                           |
| 3         | 77.6 ± 3.5                    | 71.1 ± 3.9                  | >100 ± 1.8                  |
| 4         | >100 ± 1.9                    | >100 ± 7.8                  | NT                           |
| 5         | 5.3 ± 3.8                     | 7.24 ± 4.5                  | 39 ± 4.7                     |
| 6         | 85.6 ± 4.2                    | 18.9 ± 5.8                  | >100 ± 5.9                  |
| 7         | >100 ± 4.4                    | 96.4 ± 5.6                  | NT                           |
| 8         | >100 ± 1.2                    | >100 ± 3.7                  | NT                           |
| cisplatin | 18 ± 3.1                      | NT                           | NT                           |

\(^{NT} = \text{not tested.}\)
the previously reported Ru-\textit{p}-cymene and Ru-benzene complexes against A549 and HeLa S3 cancer cells.\textsuperscript{34,52} The activity of complexes 2 and 3 was also comparable with reported ruthenium-arene complexes against HeLa S3 and A549 cell lines.\textsuperscript{53} It is evident from the comparison that our complexes showed good cytotoxic property with the earlier reported ruthenium-arene complexes (Figure 5). Cytotoxicity results indicate higher activity of complexes because of the nature of the chelating TSC/TAA ligand and arene moiety.

\textbf{AO/PI Staining.} The morphological change associated with apoptotic cell death was confirmed by fluorescence images using AO/PI. AO is an indication of live cells and PI is an indication of dead cells. After 24 h treatment of complex 5 (10 \( \mu \text{M} \)), dramatic morphological changes, nuclear condensation, and cell shrinkage were observed in HeLa S3 and A549 cancer cells (Figures 6 and 7).

\textbf{Colony Formation Studies.} Cell proliferation was confirmed by colony formation study. Complex 5 was treated with HeLa S3 and A549 cancer cells to assess the colony formation ability (Figure 8). The results clearly indicated that complex 5 exhibited complete inhibition of colony formation at 5 and 10 \( \mu \text{M} \) in HeLa S3 cells and 10 and 20 \( \mu \text{M} \) in A549\textsuperscript{54,55} cells. Therefore, complex 5 not only shows cytotoxic effects but also induces morphological changes and inhibits colony formation in HeLa S3 and A549 cells.

\section*{CONCLUSIONS}
In this work, we have designed and synthesized eight new Ru(II)-arene complexes bearing bidentate S\textsubscript{(neutral)} and N\textsubscript{(neutral)} chelating TSC ligands. The molecular structure of the ligands and complex 3 was established by single-crystal XRD studies. On the basis of crystallographic data, the typical piano stool geometry of complex was projected. The binding affinity of the complexes with DNA/protein was assessed using spectrophotometric methods. The spectroscopic values have interpreted that the complexes exhibit good binding affinity toward DNA.
with the appreciable binding constant. The complexes are bounded to DNA via intercalation. The complex with a five-membered pyrrolidine substituent shows the higher binding ability. In vitro studies show that complexes 5 and 6 have very low toxicity in HeLa S3 and A549 cancer cell lines. IC50 values indicated that complex 5 is appreciably superior to the prominent anticancer drug cisplatin. Nucleus staining was confirmed by fluorescence images using AO/PI. After 24 h treatment of complex 5 (10 μM), dramatic morphological changes, nuclear condensation, and cell shrinkage were observed in HeLa S3 and A549 cancer cells. Furthermore, cell proliferation was identified using colony formation ability. Studies. The results clearly indicated that complex 5 exhibited complete inhibition in HeLa S3 and A549 cancer cells at lower concentrations. The toxicity of complexes (1, 3, 5, and 6) was also studied against human normal lung IMR90 cell line. Our present investigation demonstrated that introduction of TAA–TSC ligand to the Ru(II)-arene complexes can be a key for developing potent anticancer drugs.

**EXPERIMENTAL SECTION**

**Materials and Methods.** RuCl3·3H2O was purchased from Sigma-Aldrich. All the other reagents and solvents were received from various suppliers and used without further purification. FT-IR spectra in the range of 4000–500 cm−1 were obtained as attenuated total reflection (ATR) pellets using a PerkinElmer Frontier FT-IR/far-infrared spectrometer. Both 1H and 13C NMR spectra were recorded in CDC13 solvent by using tetramethylsilane as an internal standard in a Bruker spectrometer (400/500 and 100/125 MHz, respectively). Electronic absorption spectra were recorded by a Jasco V-670 spectrophotometer. The melting points (triplicate measurements) were determined in open capillary tubes on a Lab India instrument and uncorrected. The ESI-MS spectra of ligands and complexes were recorded on a Thermo Lab India instrument and uncorrected. The ESI-MS spectra of complexes were recorded on a Thermo ExactivePlus mass spectrometer in positive mode (L1 and L–8) and negative mode (L2, L3, and L4). The elemental analyses were performed using a Vario EL-III CHNS analyzer.

**Synthesis of TSC Ligands.** The new TSC ligands L1–L4 were synthesized by direct condensation of corresponding thiosemicarbazide with modified TAA's. Both reactants were suspended in methanol (30 mL) containing a few drops of glacial acetic acid. The mixture was refluxed at 60–70 °C for 6 h and allowed to attain room temperature. The product was obtained as a yellow solid; it was then filtered, washed with cold methanol, and dried in vacuo. Methanol and dichloromethane (1:1) mixture was used to obtain crystals of the compounds.

**N-(4-(Di phenylamino)benzylidene)pyrrolidine-1-carbothiohydrazides (L1).** Pyrrolidine-1-carbothiohydrazide (0.1452 g, 1 mmol) and 4-(diphenylamino)benzaldehyde (0.2733 g, 1 mmol) were used. ESI-MS calcd for C24H25N4S: C, 71.97; H, 6.04; N, 13.99; S, 8.01. Found: C, 71.92; H, 5.92; N, 13.92; S, 7.92. UV-vis (CH3OH): λmax nm (ε, dm3 mol−1 cm−1) 210 (48126), 294 (23146), 368 (43746). FT-IR (ATR, cm−1): υ(N–H); 3134 (s), υ(C–H); 2954 (s), υ(C=N); 1587 (s), υ(C=S); 1244 (m), 756 (m). 1H NMR (500 MHz, CDC13): δ ppm 8.76 (s, 1H, J = 4.0 Hz, aromatic-H), 7.45 (d, 4H, aromatic-H), 7.06–7.12 (m, 6H, aromatic-H), 7.01 (d, J = 5.0 Hz, 2H), 3.96–1.95 (m, 8H, pyrrolidine-H). 13C NMR (125 MHz, CDCl3): δ ppm 176.2 (C = NH), 147.0 (C=S), 149.5, 141.5, 129.5, 125.2, 123.8, 122.1 (aromatic C), 75.6 (m) (aliphatic C).

**4-(Bis(4-(thiophen-3-yl)phenylamino)benzylidene)pyrrolidine-1-carbothiohydrazide (L2).** Pyrrolidine-1-carbothiohydrazide (0.1452 g, 1 mmol) and 4-(4-(thiophen-3-yl)benzaldehyde) (0.4375 g, 1 mmol) were used. ESI-MS calcd for C32H27N4S3: C, 72.12; H, 6.02; N, 13.92; S, 8.01. Found: C, 72.12; H, 6.02; N, 13.92; S, 8.01. UV-vis (CH3OH): λmax nm (ε, dm3 mol−1 cm−1) 212 (69809), 306 (28900), 385 (54 S23). FT-IR (ATR, cm−1): υ(N–H); 3189 (s), υ(C–H); 2947, υ(C=N); 1602 (s), υ(C=S); 1326 (s), 784 (w). 1H NMR (400 MHz, CDC13): δ ppm 8.88 (s, 1H, J = 8.0 Hz, 4H, thiophene), 7.58 (s, 1H, J = 8.0 Hz, 4H, aromatic-H), 7.28 (t, J = 8.0 Hz, 4H, aromatic-H), 7.06–7.12 (m, 6H, aromatic-H), 7.01 (d, J = 5.0 Hz, 2H), 3.96–1.95 (m, 8H, pyrrolidine-H). 13C NMR (125 MHz, CDCl3): δ ppm 176.2 (C=S), 147.0 (C=N), 149.5, 141.5, 129.5, 125.2, 123.8, 121.2 (aromatic C), 46.2 (aliphatic C).
2H, aromatic-H), 3.96–1.96 (m, 8H, pyrrolidine-H). 13C NMR (100 MHz, CDCl3): δ ppm 176.0 (C=S), 145.9 (C=N), 149.0, 141.6, 131.4, 128.1, 127.46, 126.9, 126.3, 126.1, 125.2, 122.6, 119.7 (aromatic C), 54.9, 24.1 (aliphatic C).

N-Cyclohexyl-2-(4-(diphenylamino)benzylidene)-hydrazinecarbothioamid (L3). N-(4-Cyclohexylthiosemicarbazide (0.1732 g, 1 mmol) and 4-(diphenylamino)benzaldehyde) (0.2259 g, 0.4 mmol) were used. ESI-MS calc for C26H28N4S2 [M - Cl - H], 427.1956; found, 427.1966. Yellow solid. Yield: 94%. mp 219 °C.

[(η⁴-p-Cymene)-RuIICl(η⁴-p-Cymene)Cl]Cl (L2). L2 (0.2259 g, 0.4 mmol) was used. ESI-MS calc for C₃₄H₃₂N₄S₂RuCl [M - 2Cl - H], 799.1537; found, 799.1506. Orange solid. Yield: 84%. mp 225 °C. Anal. Calcd for C₃₄H₃₂N₄S₂RuCl: C, 57.78; H, 6.04; N, 7.62; Cl, 10.15; S, 13.89. Found: C, 57.97; H, 4.88; N, 6.43; Cl, 11.04.

Synthesis of [(η⁴-p-Cymene)-RuIICl(η⁴-p-Cymene)Cl]Cl complexes. [(η⁴-p-Cymene)-RuIICl(μ-p-Cymene)Cl] was prepared using a previously reported method. The [η⁴-p-Cymene]-Ru(η⁴-p-Cymene)Cl complexes were obtained by reacting the dimer [RuCl(μ-p-Cymene)]Cl with TSC ligands. To a warm solution (34 °C) of TSC in a CHCl₃/CH₂OH mixture (20 mL v/v: 3:1), one portion of [RuCl(μ-p-Cymene)]Cl in CHCl₃ (4 mL) was added and stirred for 12-14 h at room temperature. The dark red color solution was concentrated to ~2 mL under reduced pressure, and addition of hexane (20 mL) gave a colored solid. The product was collected by filtration, washed with petroleum ether, and dried in vacuo.

[(η⁴-p-Cymene)-Ru(η⁴-p-Cymene)Cl]Cl (L1). L1 (0.1602 g, 0.4 mmol) was used. ESI-MS calc for C₃₄H₳₂N₄S₂RuCl [M - 2Cl - H], 635.1782; found, 635.1799. Brown dark solid. Yield: 87%. mp 236 °C. Anal. Calcd for C₃₄H₳₂N₄S₂RuCl: C, 72.86; H, 6.58; N, 13.07; S, 7.48. Found: C, 72.94; H, 6.43; N, 12.87; S, 7.18. UV-vis (CH₂OH): λmax nm (ε, dm³ mol⁻¹ cm⁻¹) 295 (44850), 209 (72195), 373 (91420). FT-IR (ATR, cm⁻¹): λ max, nm (ε, dm³ mol⁻¹ cm⁻¹) 3156, 2934, 2854, 2050, 3452, 3012, 2925, 2916, 1740, 1496, 1420, 1379, 1328, 1327, 1291, 1282, 1277, 1265, 1261, 1202 (aromatic carbons of TAA ligand), 103.8–82.1 (aromatic carbons p-cym), 52.1, 30.6, 26.5, 23.2, 21.5, 21.4, 18.6 (aliphatic carbons of pyrrolidine and p-cym).
228 °C. Anal. Calcd for C_{14}H_{14}Cl:N,RuS_{2}: C, 58.78; H, 5.16; N, 6.23; S, 10.70. Found: C, 58.81; H, 5.23; N, 6.11; S, 10.35. UV-vis (CH_{3}OH): λ_{max} nm (ε, dm^{3} mol^{-1} cm^{-1}) 268 (5853), 345 (3424). FT-IR (ATR, cm^{-1}): ν (N−H): 3343 (m), 3124 (w), ν (C−H): 2923 (s), ν (C=NH): 1590 (s), ν (C=S): 1280 (m), 772 (w). 1H NMR (400 MHz, CDCl_{3}): δ ppm 14.53 (1H, 1H−cym−H), 8.73 (s, 1H, HC═N), 8.09 (d, J = 8.0 Hz, 2H, aromatic-H), 7.39−7.09 (m, 16H, aromatic-H), 5.47 (d, J = 4.0 Hz, 1H, p-cym-H), 5.01 (d, J = 4.0 Hz, 1H, p-cym-H), 4.95 (d, J = 4.0 Hz, 1H, p-cym−H), 3.88−3.90 (m, 1H, 1H p-cym CH(CH_{3})_{2}), 2.12 (s, 3H, p-cym C(CH_{3})_{2}), 2.04−1.36 (m, 10H, 1H. 11.9 (d, J = 8.0 Hz, 6H, p-cym C(CH_{3})_{2}). 13C NMR (100 MHz, CDCl_{3}): δ ppm 174.86 (C═S), 146.03 (C=N), 151.3, 132.4, 129.8, 129.1, 128.2, 126.2, 125.2, 123.7, 119.3 (aromatic carbons of TAA ligand), 103.6−82.4 (aromatic carbons p-cymene), 34.7, 32.2, 30.7, 25.2, 24.3, 23.0, 21.4, 18.5 (aliphatic carbons of cyclohexyl and p-cymene).

**Synthesis of [η^6-Benzene]-Ru^II-(TSC)Cl\(_2\) Complexes.**

[η^6-Benzene]-Ru^II-(TSC)Cl\(_2\) (μ-Cl) was synthesized as per the reported method. The prepared Ru(II)-benzene dimer (0.100 g, 0.2 mmol) and ligand (L) were combined in 20 mL of CH\(_2\)Cl\(_2\)/CH\(_3\)OH and the resultant mixture was stirred for 17−20 h at room temperature. The color of the reaction mixture changed to dark red. The dark red color solution was concentrated to ~2 mL under reduced pressure, and addition of hexane (20 mL) gave a colored solid. The product was collected by filtration, washed with hexane, and dried in vacuo.

[(η^6-Benzene)-Ru^II(μ-L)Cl(Cl)] (5). L 1 (0.1602 g, 0.4 mol) was used. ESI-MS calced for C\(_{38}\)H\(_{34}\)Cl\(_2\)N\(_4\)RuS\(_3\) [M − 2Cl − H\(^+\)], 579.1156; found, 579.1079. Light orange solid. Yield: 85%. mp 213 °C. Anal. Calcd for C\(_{38}\)H\(_{34}\)Cl\(_2\)N\(_4\)RuS\(_3\): C, 55.38; H, 4.65; N, 8.61; S, 4.93. Found: C, 56.51; H, 4.69; N, 8.56; S, 4.81. UV-vis (CH\(_3\)OH): λ_{max} nm (ε, dm^{3} mol^{-1} cm^{-1}) 290 (2749), 352 (4242). FT-IR (ATR, cm^{-1}): ν (N−H): 3064 (w), ν (C−H): 2912 (s), ν (C=NH): 1569 (s), ν (C=S): 1191 (m), 752 (m). 1H NMR (400 MHz, CDCl\(_3\)): δ ppm 9.61 (s, 1H, 1H−cym−H), 8.21 (s, 1H, HC═N), 7.36 (t, J = 4.0 Hz, 4H, aromatic-H), 7.20 (d, J = 8.0 Hz, 8H, aromatic-H), 7.11 (d, J = 8.0 Hz, 2H, aromatic-H), 5.56 (s, 6H, benzene-H), 3.79−2.01 (m, 8H, pyrrolidine-H). 13C NMR (100 MHz, CDCl\(_3\)): δ ppm 171.2 (C═S), 146.1 (C=N), 151.4, 133.2, 129.8, 126.2, 125.1, 124.1, 119.3 (aromatic carbons), 87.5 (aromatic carbons of benzene), 52.2, 26.6, 25.1 (pyrrolidine carbons).

[(η^6-Benzene)-Ru^II(μ-L)Cl(Cl)] (6). L 2 (0.2259 g, 0.4 mmol) was used. ESI-MS calced for C\(_{40}\)H\(_{38}\)Cl\(_2\)N\(_4\)RuS\(_3\) [M − 2Cl − H\(^+\)], 670.1469; found, 670.1389. Orange solid. Yield: 87%. mp 217 °C. Anal. Calcd for C\(_{40}\)H\(_{38}\)Cl\(_2\)N\(_4\)RuS\(_3\): C, 56.63; H, 5.05; N, 8.26; S, 4.72. Found: C, 56.72; H, 5.11; N, 8.19; S, 4.68. UV-vis (CH\(_3\)OH): λ_{max} nm (ε, dm^{3} mol^{-1} cm^{-1}) 289 (4920), 351 (6016). FT-IR (ATR, cm^{-1}): ν (N−H): 3184 (s), 3022 (s), ν (C=NH): 2923, 1568 (s), ν (C=S): 1181 (m), 747 (m). 1H NMR (400 MHz, CDCl\(_3\)): δ ppm 9.81 (s, 1H, 1H−cym−H), 8.73 (s, 1H, HC═N), 8.15 (s, J = 4.0 Hz, 2H, aromatic-H), 7.38 (t, J = 8.0 Hz, 4H, aromatic-H), 7.20 (d, J = 4.0 Hz, 6H, aromatic-H), 7.09 (d, J = 4.0 Hz, 2H, aromatic-H), 5.52 (s, 6H, benzene-H), 3.88−3.90 (m, 1H, cyclohexyl-H), 2.01−1.30 (m, 10H, cyclohexyl-H). 13C NMR (100 MHz, CDCl\(_3\)): δ ppm 174.3 (C═S), 145.9 (C=N), 151.5, 132.7, 129.9, 128.3, 126.3, 125.3, 123.5, 119.1 (aromatic carbons), 87.1 (aromatic carbons of benzene), 54.9, 32.3, 25.1, 24.5 (cyclohexyl carbons).

XRD Studies. A Leica MZ 75 microscope was used to identify faces with dimensions of representative sample crystal. The selected crystal was fixed in nitrogen stream (Oxford) at 100 K with the help of a nylon loop. All reflection data were acquired on a Bruker APEX2 X-ray diffractometer. Program APEX2 was employed to obtain the data-integrated intensity of each reflection, which was possible by data frame reduction. Three-dimensional profiling algorithmic integration was used and the collected data were corrected with respect to crystal decay effects and polarization factors. The absorption effect data were collected from SADABS. A solution was produced by SHEXLTL (XS). The H atoms were geometrically fixed and set riding on the corresponding parent atoms. The final data representation and structure plots were acquired by Olex.

DNA Binding Studies. DNA binding experiments were performed by UV−vis and fluorescence spectroscopy methods. The required concentration of the compounds was prepared by dissolving the complexes in 5% dimethylformamide/Tris-HCl/NaCl. CT-DNA was dissolved in 50 mM NaCl/5 mM Tris-HCl (pH 7.2) and stored at 4 °C. The DNA solution was diluted to get an absorbance corresponding to 6600 M⁻¹ cm⁻¹ at 260 nm. The 15 μM concentration of the complex was titrated against CT-DNA (0−40 μM). The spectra were recorded after equilibration for 3 min, allowing the compounds to bind to the CT-DNA.

The competitive binding of the complexes were investigated with EB by fluorescence technique. EB solution was prepared using Tris-HCl/NaCl buffer (pH 7.2). The test solution was added in aliquots of 5 μM concentration to DNA−EB and the change in fluorescence intensities at 596 nm (450 nm excitation) was recorded.
HSA Binding Studies. The binding of Ru(II)-arene complexes (1–8) with HSA was studied using fluorescence spectra, recorded at a fixed excitation wavelength corresponding to HSA at 280 nm and monitoring the emission at 335 nm. The excitation and emission slit widths and scan rates were maintained for all the experiments. Stock solution of HSA was prepared in Tris-buffer (50 mM NaCl/5 mM Tris-HCl, pH 7.2) and stored in the dark at 4 °C for further use. Concentrated stock solutions of each test compounds were prepared by dissolving them in Tris-buffer and diluted with Tris-buffer to get required concentrations. HSA (2.5 mL) solution was titrated by successive additions of 10−6 M stock solution of complexes using a micropipette.32 For synchronous fluorescence spectra measurements, the same concentration of HSA and the complexes were used and the spectra were measured at two different Δλ (difference between the excitation and emission wavelengths of HSA) values of 15 and 60 nm.

Cytotoxicity. Cytotoxic study was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. AS49, HeLa S3, and IMR-90 cells were purchased from ATCC, USA. AS49 and HeLa S3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (10% fetal bovine serum and 2 mL L-glutamine, along with antibiotics) at 37 °C with 5% CO2. Cells/well (2 × 103) were seeded in 96-well plates and incubated at a humidified condition. The Ru(II)-arene complexes were dissolved in dimethyl sulfoxide (DMSO, 0.5%). The cells were treated with different concentrations of Ru(II)-arene complexes (100−3.125 μM) and incubated for 24 h. After treatment, cells were washed with phosphate-buffered saline (PBS) and incubated with fresh DMEM containing MTT (5 mg/mL). The plates were incubated for 3−4 h at dark condition and 100 μL of DMSO was added to each well. The cytotoxicity data were measured at 570 nm. The percentage of cytotoxicity was calculated by the following formula, inhibition (%) = A − B/A × 100 (A = control group and B = treated group).

Cell Death Analysis Using Fluorescence Probes. HeLa S3 and AS49 (3 × 103) cancer cells were seeded in 30 mm dishes and incubated overnight at a humidified condition (37 °C with 5% CO2). HeLa S3 and AS49 cancer cells were treated with complex 5 for 24 h. After treatment, cells were incubated with AO/PI (10 μM) solution for 15 min at dark condition. The images were captured under a fluorescence microscope (Biorevo, BZ-9000, Keyence, 20×).

Colony Formation Studies. HeLa S3 and AS49 cancer cells (1 × 103) were seeded in a 24-well plate and incubated overnight. The HeLa S3 and AS49 cells were treated with complex 5 for 24 h. After 24 treatments, the cells were washed with PBS and incubated with fresh DMEM for 10 days. The cells were stained with crystal violet solution and the data were calculated by ImageJ software.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b01022.

HSA binding constant, quenching constant, and number of binding sites; crystal data and structure refinement for L1, L2, L3, and L4; selected bond lengths and angles of L1, L2, L3, and L4; crystal data and structure refinement for complex; selected bond lengths and angles; cytotoxic effects of complexes 1–8 against HeLa S3 and AS49 cells; cytotoxic activity of complexes 1, 3, 5, and 6; graphs of binding studies for complexes, and NMR and mass spectra of all the ligands and complexes (PDF). Crystallographic data of L1 (CIF)

Crystallographic data of L2 (CIF)

Crystallographic data of L3 (CIF)

Crystallographic data of L4 (CIF)

Crystallographic data of 3 (CIF)

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Notes

The authors declare no competing financial interest. The crystallographic data for the structures reported in this paper have been deposited with the Cambridge Crystallographic Data Centre (CCDC) as supplementary publication numbers CCDC 1884650, 1884651, 1884652, 1884653, and 1884654. Copies of the data can be obtained free of charge from the CCDC (12 Union Road, Cambridge CB2 1EZ, UK; Tel.: +44-1223-336408; Fax: +44-1223-336003; e-mail: deposit@ccdc.cam.ac.uk; web site: http://www.ccdc.cam.ac.uk).

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