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Anticancer Activity for Targeting Telomeric G-Quadruplex and Antiangiogenesis of a Novel Ru(II)–Se Complex

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Abstract A new Ru(II)–Se complex, $Ru(bpy)_2L_2Cl_2$ (bpy=2,2′-bipyridine, L=1,10-phenanthrolineselenazole) (Ru-Se) has been synthesized and characterized. The G-quadruplex DNA-binding properties of the complex and its selenium ligand (Phen-Se) were evaluated by thermal denaturation study, polymerase chain reaction (PCR) stop assay, and telomerase repeat amplification protocol (TRAP). The results showed that the obtained complex could induce and stabilize G-quadruplex structure as well as exhibit potent inhibitory activity against telomerase. In vitro cytotoxicity studies showed that complex Ru-Se inhibited the cancer cell growth through apoptosis. However, the presence of the ligand Phen-Se did not appear to have a significant effect either on G-quadruplex binding or on biological activity. Furthermore, the cell migration assay and the tube formation assay also demonstrated that the complex Ru-Se significantly inhibited human umbilical vascular endothelial cell (HUVEC) proliferation, migration, and tube formation. These findings indicate that the Ru–Se complex may be a potential telomerase inhibitor and a viable drug candidate in antiangiogenesis for anticancer therapies.

Keywords Ru(II)–Se complex · Telomeric G-quadruplex · Anticancer activity · Apoptosis · Cell migration · Antiangiogenesis

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Abbreviations

Ru-Se	$Ru(bpy)_2L_2Cl_2$ (bpy=2,2′-bipyridine, L=1,10-phenanthrolineselenazole)
Phen-Se	1,10-phenanthrolineselenazole
CD	Circular dichroism
PCR	Polymerase chain reaction stop assay
TRAP	Telomerase repeat amplification protocol
MTT	3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

Introduction

Human telomeric DNA is characterized by short G-rich tandem repeat sequence $d[(TTAGGG)_n]$, which ends as the single strand [1]. This guanine-rich single-stranded DNA forms a novel structure named G-quadruplex in vitro [2–5], and it has been reported that the G-quadruplex may also exist in vivo [6, 7]. Small molecules that bind and stabilize this structure have been demonstrated to inhibit the enzyme telomerase, which has an elevated activity in 85–90 % of human cancer cells in comparison to normal somatic cells [8]. Thus, the inhibition of telomerase activity by inducing/stabilizing G-quadruplex formation and the detection of G-quadruplex DNA are expected to move into the forefront of research for new effective anticancer drugs [9–13]. Moreover, G-quadruplexes are finding wide implications in various biological phenomena including gene expression and cell proliferation [14, 15]. Stabilization of G-quadruplex structures within their genomic environments by the binding of small molecules can lead to various biological effects, and thus, makes it appealing for in vivo applications.

In recent years, metal complexes have been widely studied as new potential antitumor drugs since the established role of

cisplatin and carboplatin in cancer chemotherapy. Especially, ruthenium complexes have attracted much interest as a promising alternative to platinum, showing a remarkable antitumor activity. It is widely recognized that, besides exhibit low toxicity to normal cells, some Ru complexes are easily absorbed by tumor tissue and rapidly excreted from the body [16, 17]. Ru(II) complexes with polypyridyl ligands, which are typical octahedral metal complexes, have prominent DNA binding properties, and some of them have been investigated as nucleic acid probes, synthetic restriction enzymes, anticancer drugs, and DNA footprinting agents [18]. Over the last decade, ruthenium–polypyridyl complexes have attracted much attention due to their potential application as anticancer drugs [19, 20]. However, the molecular mechanisms of the apoptosis-inducing action of ruthenium polypyridyl complexes remain to be elucidated.

According to the result of epidemiological studies, selenium is thought to be associated with cancer prevention [21]. Besides, it is reported that selenocompounds with metal ions cause an increase in cytotoxic activity which are potential agents in cancer therapy [22]. In this report, efforts in our laboratory have been focused on the studies of antitumor properties of selenium–ruthenium containing complexes including their design, synthesis, structural modification, biological activity, and mechanisms [23–27]. In this context, a new Ru(II)–Se complex, $Ru(bpy)_2L_2Cl_2$ (bpy=2,2′-bipyridine, L=1,10-phenanthrolineselenazole) has been designed and synthesized (Fig. 1). Our objective was to study the interaction between the complex and G-quadruplex, as well as its biological properties including induction of apoptosis, inhibitory effects on cell migration, and tube formation.

Experimental

Reagents and Materials

Human telomeric DNA oligomer (HTG21) 5′-GGGTTAGG GTTAGGGTTAGG G-3′ (5′-G_3 $(T_2AG_3)_3$-3′) was purchased from Shanghai Sangon. Concentration of HTG21 was determined by measuring the absorbance at 260 nm after melting. Single-strand extinction coefficients were calculated from mononucleotide data using a nearest- neighbor approximation [28]. The formations of intramolecular G-quadruplexes were carried out as follows: the oligonucleotide samples were annealed in different buffers at 95 °C for 5 min, slowly cooled to room temperature, and then incubated at 4 °C overnight. Other reagents and solvents were purchased commercial sources unless otherwise specified. Doubly distilled water was used to prepare buffer solutions.

Physical Measurement

Electrospray ionization mass spectrometry (ES-MS) was recorded on a LQC system (Finnigan MAT, USA) by using CH_3CN as a mobile phase. Microanalysis (C, H, and N) was carried out with a Perkin–Elmer 240C elemental analyzer. Circular dichroism (CD) spectra were recorded on a Jasco J-810 spectropolarimeter.

Synthesis and Characteristics

The ligand 1,10-phenanthrolineselenazole (Phen-Se) was synthesized according to literature methods [29]. Ru-Se, a mixture of 1,10-phenanthrolineselenazole (1.71 g, 6 mmol), $RuCl_3 \cdot nH_2O$ (0.78 g, 3 mmol), and $LiCl \cdot H_2O$ (0.84 g, 14 mmol) were dissolved in DMF (10 ml). The mixture was refluxed at 140 °C for 8 h under an argon atmosphere. The solution was cooled to room temperature. After the solution was refrigerated overnight and upon addition of acetone (50 ml), a dark red precipitate was obtained. The precipitated complex was isolated by filtration and air-dried, then purified by chromatography over alumina. Yield 1.10 g (46 %); ES-MS (CH_3OH, m/z) 742.5 (M^+); Calcd for $RuC_{24}H_{12}N_8Se_2Cl_2 \cdot 3H_2O$ C 36.18, H 2.26, N 14.07; Found C 36.19, H 2.24, N 14.08.

Thermal Denaturation Study

Thermal denaturation study was carried out on a Jasco J-810 spectropolarimeter equipped with a Peltier temperature-controlling programmer PTP-6 using a quartz cell with an optical path length of 1 mm. With the use of the thermal melting program, the temperature of the cell containing the

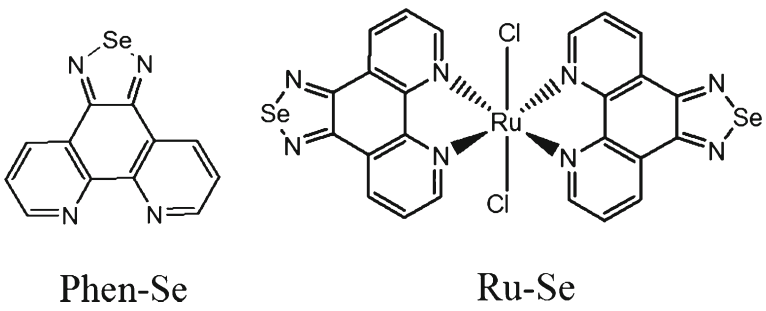

Fig. 1 Structures of complex Ru-Se and the ligand Phen-Se

cuvette was ramped from 30 to 100 °C. Thermal melting curves and ΔT_m calculation were performed in Tris–KCl buffer with 2 μM HTG21 DNA in the absence and presence of the complexes. The melting temperature T_m, which was defined as the temperature where half of the total base pairs was unbonded, was determined from the midpoint of the melting curves [30].

Polymerase Chain Reaction (PCR) Stop Assay

The test oligomers HTG21 (5′-$G_3(T_2AG_3)_3$-3′) and the corresponding complementary sequences HTG21rev (ATCG CT2CTCGTC3TA2C2) were used. The reactions were performed in 1×PCR buffer, containing 10 pmol of each oligonucleotide, 0.16 mM dNTP, 2.5 U *Taq* polymerase, and different concentrations of complexes. Reaction mixtures were incubated in a thermocycler with the following cycling conditions: 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. Amplified products were resolved on 15 % nondenaturing polyacrylamide gels in 1×TBE and silver stained.

Telomerase Repeat Amplification Protocol (TRAP) Assay

TRAP assay was performed by using a modification of the TRAP assay following previously published procedures [27, 31]. Telomerase extract was prepared from $HepG_2$ cell. PCR was performed in a final 50 μl reaction volume composed of reaction mix (45 μl) containing 20 mM Tris–HCl (pH 8.0), 50 μM dNTPs, 1.5 mM $MgCl_2$, 63 mM KCl, 1 mM EGTA, 0.005 % Tween-20, 20 μg/ml BSA, 3.5 pmol of primer HTG21 (5′-$G_3[T_2AG_3]_3$-3′), 18 pmol of primer TS (5′-AATC CG TCGAGC AGAGTT-3′), 22.5 pmol of primer CXext (5′-GTGCCCT TACCCTT ACCCTTA CCCTAA-3′), 7.5 pmol of primer NT (5′-ATCGCT TCTCGG CCTTTT-3′), 0.01 pmol of TSNT internal control (5′-ATTCCG TCGAGC AGAGTT AAAAGG CCGAGA AGCGAT-3′), 2.5 units of *Taq* DNA polymerase, and 100 ng of the extract. Compounds or distilled water were added under a volume of 5 μl. After telomerase elongation for 30 min at 30 °C, 30 cycles of PCR were performed (92 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s). Telomerase extension products were then analyzed on a nondenaturing 12 % polyacrylamide 1×TBE vertical gel and resolved at 200 V for 1 h.

Cytotoxicity Assay

The cytotoxicities of the complexes toward the indicated cell lines were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [32]. Briefly, cells were seeded on 96 well plates (1.0×10^3 per well) and exposed to various concentrations of complexes. The microplate was incubated for 48 h at 37 °C, 5 % CO_2, and 95 % air in a humidified incubator. After incubation, 10 ml of MTT reagent (5 mg ml^{-1}) was added to each well and further incubated for 2 h. The cells in each well were then treated with DMSO (150 ml for each well), and the optical density (OD) was recorded at 570 nm. The percentage of cell viability was calculated using the equation: (mean OD of treated cells/mean OD of control cells)×100 %. Cells treated with vehicle (1 % DMSO) were used as controls. Data were presented as averages of three independent experiments ± standard deviations.

Apoptosis Detection

Hela cells were supplemented by different concentration of drugs and incubated for 72 h, then washed once with ice-cold phosphate buffer solution (PBS) and fixed with 4 % paraformaldehyde for 30 min. After washed with ice-cold PBS, the cells were stained condensed chromatin of apoptotic cells more brightly than the looser chromatin of normal cells. Nuclei with chromatin condensed at the nuclear margin or in the center of the nucleus as well as fragmented nuclei were classified as apoptotic.

Inhibition of Cell Migration

The wound-healing assay was done as previously reported [33]. Approximately 2×10^5 human umbilical vascular endothelial cells (HUVECs) were seeded into 6 well plates and incubated for 24 h. Monolayer inactivated HUVECs were wounded by scratching with 1 ml pipette tip. Fresh medium was added with different concentrations of Ru-Se (5, 10, and 20 μM). Images were taken by Nikon digital camera after 24 h of incubation. The migrated cells were quantified by manual counting, and percentage inhibition was expressed using untreated wells at 100 %. At least three independent experiments were performed.

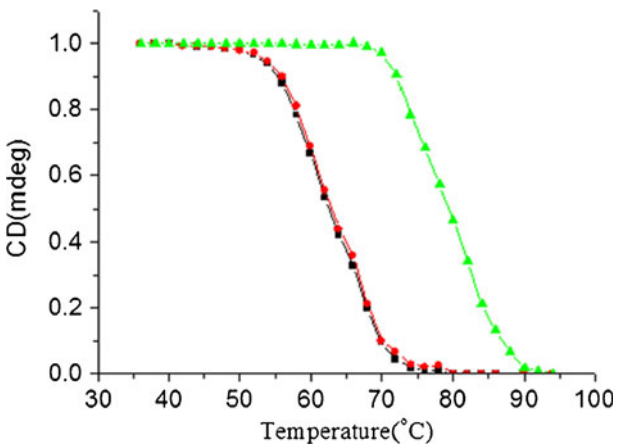

Fig. 2 CD melting curves for G-quadruplex DNA (*square*), G-quadruplex DNA+Phen-Se (*circle*), G-quadruplex DNA+Ru-Se (*triangle*), in Tris–KCl buffer (pH 7.0). [DNA]=2 μM, [complex]=20 μM. The stability of G-quadruplexes DNA was assessed by CD at 295 nm

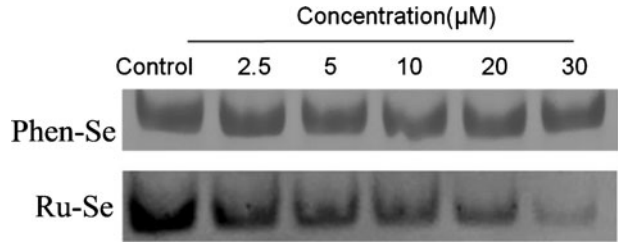

Fig. 3 Reactions of Ru-Se and Phen-Se to HTG21 in PCR stop assay

Table 1 Growth inhibition of complex Ru-Se and Phen-Se on selected cancer cells

Complex	Cytotoxicity (IC_{50}, μM)				
	Hela	A549	CNE	HepG2	MDA-MB-231
Phen-se	33.6±3.0	53.6±2.2	42.0±2.5	59.2±1.8	71.1±3.4
Ru-se	12.7±0.8	26.5±1.9	21.6±2.8	32.1±2.6	33.3±4.1
Cisplatin	13.2±2.0	18.4±2.5	12.3±0.97	26.6±1.8	19.7±1.0

Tube Formation Assay

In order to further evaluate the ability of Ru-Se to inhibit the formation of blood vessels. Tube formation assay was performed, Matrigel was dissolved at 4 °C for overnight, and each well of 96 well plates was coated with 30 μl Matrigel and incubated at 37 °C for 45 min. HUVECs (3×10^4) were added in 200 μl fresh medium with various concentration of complex Ru-Se (5 μM, 10 μM, 20 μM). After 24 h of incubation at 37 °C, 5 % CO_2, HUVECs cell tube formation were subsequently captured by an inverted microscope (Axio Observer Z1, Carl Zeiss, Germany). Tubular structures were quantified by manual counting of low-power fields, and percent inhibition was expressed using untreated wells as 100 %.

Results and Discussion

Thermal Denaturation Study

The stabilization of the complex Ru-Se to the G-quadruplex structure was primarily determined by the change of the melting temperature of the G-quadruplex DNA and by using CD spectroscopy in the presence of K^+. The ligand Phen-Se was utilized as the control here. According to the previous studies [34], 295 nm was chosen to study the influence of the complexes on the stability of G-quadruplex DNA. The CD intensity of HTG21 with the complexes in a Tris–HCl buffer at 295 nm versus the temperature was shown in Fig. 2. The transition temperature of the G-quadruplex increased from 61.5 to 79 °C when induced by complex Ru-Se, and an increase in the melting temperature of the quadruplex indicated a stabilizing effect, therefore complex Ru-Se could stabilize the quadruplex. However, even when the ratio of Phen-Se to HTG21 DNA was 10, the increase in the melting temperature (ΔT_m) of the G-quadruplex did not increase. That is to say, complex Ru-Se showed more stabilization effect on G-quadruplex than that of Phen-Se. The different behaviors between the complex and the ligand inspired us to further study their different DNA-binding properties by variety of research methods.

Inhibition of Amplification in HTG21

In order to further evaluate the ability of the complex to stabilize G-quadruplex DNA, PCR stop assay was carried out. The sequences of HTG21 and its corresponding complementary sequence (HTG21 rev) can hybridize a final double-stranded DNA–PCR product when used with *Taq* DNA polymerase as the catalyst. However, in the presence of some G-quadruplex stabilizers, the template sequence HTG21 was induced into a G-quadruplex structure that blocked the hybridization and the detection of the final PCR product. As shown in Fig. 3, in the presence of Phen-Se, the final double-stranded DNA–PCR product could still be detected, indicating that the ligand Phen-Se could not stabilize the G-quadruplex

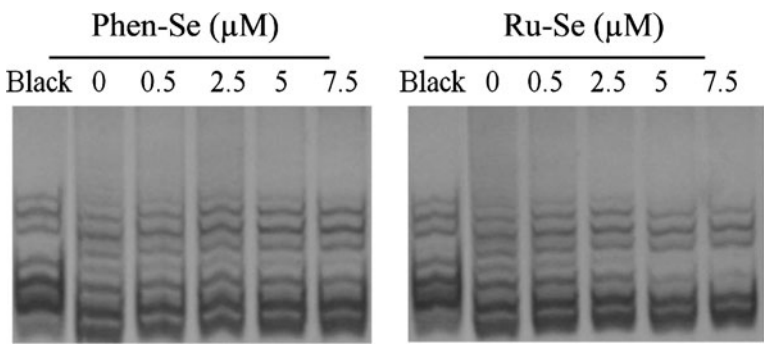

Fig. 4 Effects of Ru-Se and Phen-Se on telomere activity of $HepG_2$ cells. Increasing concentrations of drugs (0–7.5 μM) and solutions were added to the telomerase extract. The elongated products were amplified using PCR, which was followed by polyacrylamidegel electrophoresis. The *Black line* is a negative control which was run under identical conditions but omitting the telomerase extract to ensure absence of PCR artifacts. Lane 0 is a positive control, in which the buffer did not contain dimethyl formamide (DMF)

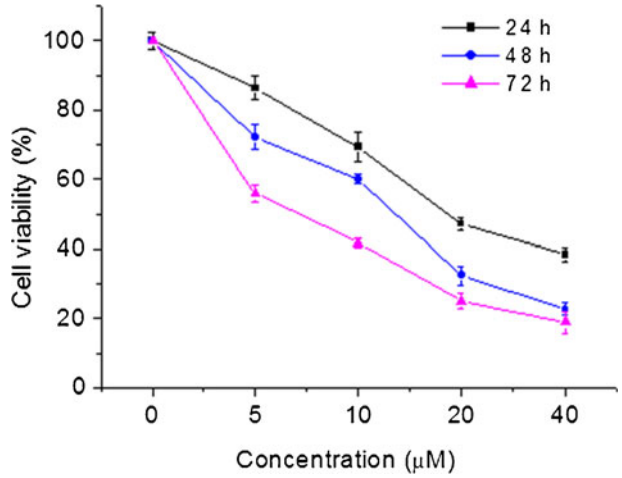

Fig. 5 Antiproliferative activities of complex Ru-Se on Hela cells. Cells were treated with different concentrations of the complex for 24 h, 48 h, and 72 h, respectively

in HTG21. However, in the presence of Ru-Se, 5′ to 3′ primer extension by DNA *Taq* polymerase was arrested and the final double-stranded DNA–PCR product could not be detected at 30 μM. Moreover, the concentration of Ru-Se that inhibited amplification by 50 % (IC_{50}) was found to be 15.35 (±2.13) μM. These indicated that complex Ru-Se, which has Ru metal group, exhibited stronger inhibitory effect compared to Phen-Se. This result is consistent with the thermal denaturation results, further implying that complex Ru-Se possesses a higher stabilizing ability than Phen-Se.

Inhibition of Telomerase Activity in Cell-Free System

Further analysis was performed by using the modified TRAP assay [35]. Although the limitation of TRAP was reported recently [36], the data from this experiment could be used to make the comparison between complex and the ligand (Fig. 4). In this experiment, solution of the complex or the ligand was added to the telomerase reaction mixture containing an extract from cracked Hela cell lines. At the concentrations between 0.5 and 7.5 μM, only the complex Ru-Se revealed a dose-dependent inhibition of the telomere, and the number of bands clearly decreased with respect to the control. By contrast, no complete inhibition was observed in the presence of Phen-Se, even at 7.5 μM. This result not only agreed to the experimental data from the above studies but also indicated that complex Ru-Se might be a potential human telomerase inhibitor.

In Vitro Cytotoxicity

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay was then performed to preliminarily evaluate the cytotoxicity of the complexes on several human cancer cell lines including Hela (human epithelial carcinoma), A549 (human lung carcinoma), CNE (human nasopharygeal carcinoma), HepG2 (human hepatocellular liver carcinoma), and MDA-MB-231 (human breast cancer). Table 1 demonstrated the IC_{50} values of Ru-Se and Phen-Se after 48 h of treatment. Cisplatin was used as control. The results showed that the ligand had no cytotoxicity against selected cell lines, but the five tested cancer cell lines were all susceptible to the complex Ru-Se. The IC_{50} values were 12.7, 26.5, 21.6, 32.1, 33.3 μM for Hela, A549, CNE, HepG2, and MDA-MB-231 cells, respectively, indicating a high cytotoxic effect of the complex Ru-Se. Moreover, it is noteworthy that, among the tested cancer cells, the complex Ru-Se is more toxic than cisplatin against Hela cells. Thus, Hela cells were used for

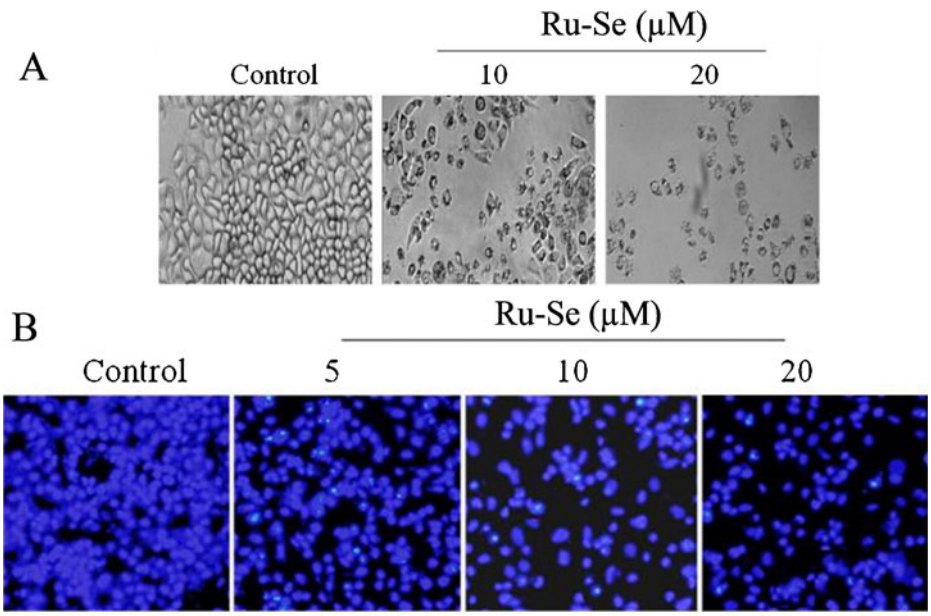

Fig. 6 Cell morphological changes induced by complex Ru-Se in Hela cells. **a** Cells incubated with 10 and 20 μM drug were visualized using light microscopy. **b** Fluorescence microscope images of Hela cells that incubated with complex Ru-Se at different concentrations for 24 h. Cell nuclei were stained with Hoechest 33342 prior to observation

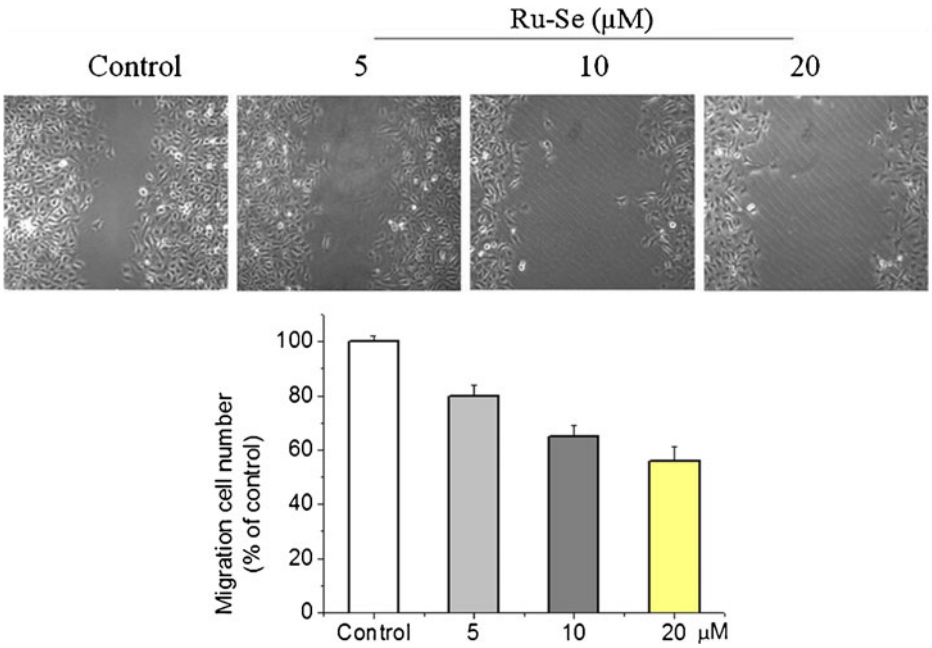

Fig. 7 Effects of complex Ru-Se on HUVEC migration in wound migration assays. Cells were wounded with pipette in the presence or absence of 5, 10, and 20 μM of drug, respectively. After incubation, the migrated cells were quantified by manual counting. Data are presented as the percentages of the control group, which was set at 100 %

further studies. The cell viability (%) obtained with continuous exposure for 72 h are depicted in Fig. 5. The toxicity of the complex Ru-Se was found to be concentration dependent, i.e., the cell viability decreased when the concentration of the complex increased.

Induction of Apoptosis

Next, the morphological changes of Hela cells upon exposure to complex Ru-Se (10 μM and 20 μM, respectively) for 48 h were evaluated by light microscope. As illustrated in Fig. 6a, besides losing their normal morphology, apoptotic features—in particular membrane blebbing, cell shrinkage, and rounding—appeared in most of the cells. Moreover, the morphological changes of the cells were dose-dependent and significant apoptotic bodies were observed at the high concentration of 20 μM. To evaluate the nuclear morphological features of Hela cells on exposure to the complex, cells staining with Hoechst were analyzed by fluorescence microscopy. As Fig. 6b indicated, the control cells exhibited homogeneous nuclear staining, but the complex-treated cells displayed typical apoptotic changes (e.g., staining bright, condensed chromatin, and fragmented nuclei [37, 38]. These results suggested that cell death induced by the complex is mainly due to apoptosis.

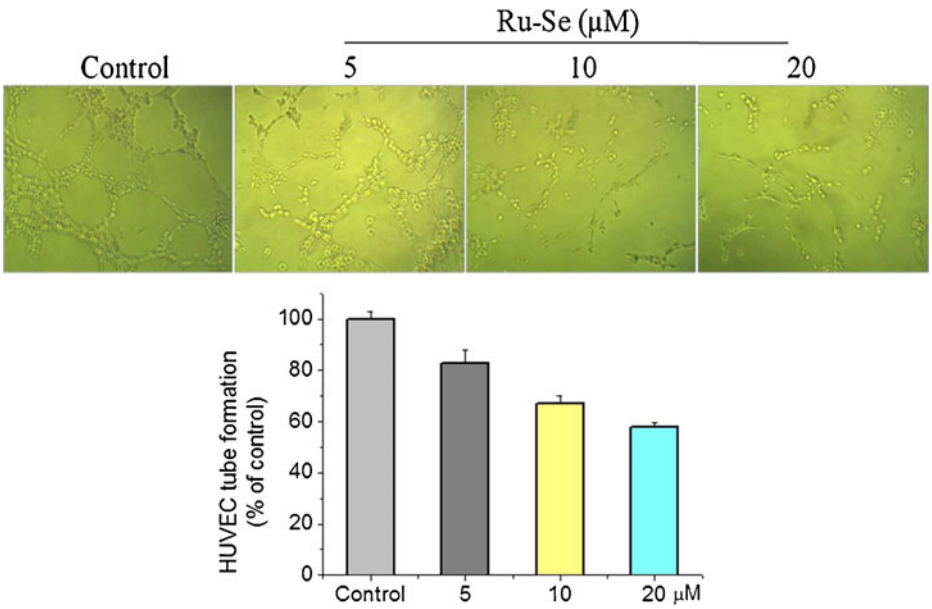

Fig. 8 Effects of complex Ru-Se on HUVEC tube formation. After 24 h of incubation, HUVEC tube-like formation was assessed with an inverted photomicroscope. These experiments were performed thrice with similar results, and significant differences from control group were observed ($p<0.05$). Data are presented as the percentages of the control group, which was set at 100 %

HUVEC Migration and Tube Formation Studies

Effect of drug treatment on cell motility on endothelial cells is further assessed by wound-healing assay, because cell migration is a key step for endothelial cell to form blood vessels in angiogenesis. As indicated in Fig. 7, the control cells moving across the gap was clearly observed in 24 h. However, the complex Ru-Se significantly inhibited HUVEC migration at 10 μM in scratching cell assays. The results demonstrated that the complex Ru-Se had potent inhibitory effects on HUVEC cell migration. Hence, we guessed that Ru-Se might inhibit tumor blood vessels growth.

As we have known, matrigel assay can be used to examine the potential effects of drugs on the tube formation of HUVEC. As shown in Fig. 8, we observed that 10 μM Ru-Se inhibited 50 % tube formation of HUVEC on matrigel, and 20 μM Ru-Se can completely inhibit the formation of tubular structures. Network formation, as judged by total tube length, was significantly lower ($P<0.001$) when treated with the complex compared to that of the control. These results showed that Ru-Se had potential effects on blocking in vitro angiogenesis by inhibiting cell migration and tube formation.

Conclusions

In conclusion, we found that a new Ru(II) complex with ligand Phen-Se was capable of stabilizing G-quadruplex structure by CD melting curves and PCR stop assay. The binding modes of the complex with G-quadruplex would thanks to two interaction mechanism. On the one hand, the selenium ligand of Ru(II)–Se complex with the ability to display strong π–π interactions with HTG21 DNA. Others, the electron withdrawing properties of the Ru(II) induce Ru(II)–Se combined to the G-quadruplex DNA. TRAP assay showed that the Ru(II)–Se complex exhibited potent inhibitory activity against telomerase. The G-quadruplex DNA-binding properties of the complex were studied, but the ligand showed no effect on G-quadruplex DNA. We speculated that the bioactivity of Ru(II)-Se is better than that of Phen-Se, thanks to its more stabilization effect on G-quadruplex DNA than that of Phen-Se, for DNA with the ability to control the cellular life activities. MTT and morphological studies revealed that the complex Ru-Se exhibited quite potent antitumor activities through induction of apoptosis. Furthermore, the complex Ru-Se exhibited higher inhibitory effects on cell metastasis and showed the potential and significant role in preventing tube formation. Taken together, our results demonstrated that the synthesized complex Ru-Se could be a potential telomerase inhibitor and a promising candidate for further evaluation as chemotherapeutic agent for human cancers.

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Conflict of Interest There are no conflicts of interest to declare.

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