Potential anticancer applications of the novel naringin-based ruthenium (II) complex

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Abstract

Ruthenium seems to be a promising alternative to platinum because of the wide range of oxidation states it has and its ability to form complexes with bioactive ligands. In this study, naringin, a naturally occurring flavonoid, was used to synthesize a novel ruthenium complex with potential anticancer activity. The characterization of the synthesized complex was done by UV–Vis spectroscopy, FTIR and NMR studies. In addition, the complex was tested against Human A549 cell lines to determine the anticancer effect, and against human dermal fibroblasts (HDFa) to find any underlying toxicity. Further, the morphological changes of the cancer cells can be determined by using bio-atomic force microscopy. Results showed that the synthesized complex was able to induce anticancer effects against A549 with minimal impact to HDFa. In this study, we investigated the anticancer properties of naringin-ruthenium (II) complex using live- and dead-cell staining assay, MTT, Trypan blue, and lactate dehydrogenase assay. Further, morphological changes were observed in the A549 cells using Bio-AFM. The Bio-AFM results have proven the better cytotoxic behavior of naringin-ruthenium (II) complex. The cell viability results also provided the anticancer efficacy of the complex.

Keywords Ruthenium-naringin complex · Flavonoid · Cancer A549 cell line · Human dermal fibroblasts (HDFa) · Bio-atomic force microscopy

Introduction

In cancer therapy, ruthenium (Ru) complexes are reflected as prospective alternatives for platinum-based drugs and have been established as encouraging anticancer drugs with high effectiveness and low side effects (Jayakumar et al. 2018). Ruthenium complexes affect different cancer types via the inhibition of metastasis, induction of apoptosis, or inhibition of growth and proliferation. Also, ruthenium exists in several oxidation states. Moreover, ruthenium complexes were found to bind the nucleic acids and proteins, interfering with replication and enzyme activity (Thangavel et al. 2017, 2018). This allows the activation of

ruthenium complexes only at the tumor site as a result of the local reducing environment (Roy et al. 2018). Furthermore, ruthenium can be complexed with bioactive ligands to enhance its activity (Zeng et al. 2017). Among the bioactive ligands, flavonoids, which are plant-derived bioactive compounds, can act as antiangiogenic, antiapoptotic, and antioxidant (Ijaz et al. 2018). Naringin, a flavonoid common in citrus fruits, shows promising antimetastatic and antiapoptotic activities by acting on different pathways as summarized in Fig. 1 (Alam et al. 2014). Interestingly, other flavonoid complexes such as quercetin–iron (II) complex demonstrated the cleavage activity on plasmid DNA (pbr322) by oxidative pathway in the physiological conditions (Raza et al. 2016). Ru(II)–DMSO-chalcone complexes exhibited effective binding capacity with DNA and significant topoisomerase II inhibition (Jovanovic et al. 2016). Chrysin complex with oxovanadyl (IV) inhibited the cell viability, and further affecting the morphology of the suppressed MG-63 tumor growth without toxic effects (León et al. 2016). In this study, a novel naringin-based ruthenium (II) complex was synthesized. The complex was characterized using UV–Vis, FTIR, and NMR

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Fig. 1 Schematic diagram of the anticancer pathways of naringin

spectroscopy. In addition, the effects of the synthesized complex were tested against A549 and HDFa cell lines using MTT assay, live and dead staining assay, and atomic force microscopy (AFM). To the best of our knowledge, chemotherapeutic activities of the ruthenium–naringin complex have not been investigated till date.

Materials and methods

Materials

Tetrakis (dimethyl sulfoxide) dichlororuthenium (II) [RuCl₂(DMSO)₄], naringin, trypsin-EDTA, penicillin–streptomycin and LDH assay kit was purchased from Sigma-Aldrich, USA. Solvents used in the study were purchased from Daejung chemicals. HDFa cells, human dermal fibroblast growth medium, and supplements were purchased from CEFO Ltd in Seoul, South Korea. Fetal bovine serum (FBS) was purchased from Young in Frontier, Seoul, Korea. Cell culture flasks were purchased from SPL Life Sciences Co., Ltd., South Korea. DMEM (Dulbecco modified eagle medium) and Live/Dead™ Viability/Cytotoxicity Kit for mammalian cells were purchased from Thermo Fisher Scientific, Massachusetts, USA. MTT Cell Proliferation Assay Kit was purchased from Bio Vision, USA. All other reagents were of pharmaceutical grade and purchased from Sigma-Aldrich, St. Louis, USA. Mill-Q water (18.2 MΩ) was used for all experiments.

Synthesis of naringin–ruthenium complex

The synthesis was performed in accordance with the method of Shao et al. with modifications (Shao et al. 2016). A methanolic solution of naringin (1 mmol) was prepared, added with 0.4 mmol of citric acid, and refluxed for 30 min. Then, a methanolic solution of RuCl₂(DMSO)₄ (1 mmol) was added dropwise to the naringin solution and the reaction mixture was refluxed for 10 h. Subsequently, the mixture was cooled and filtered. The resulting crystals were recrystallized using ethanol prior to characterization and cell study. The amber-colored crystals were stable and very soluble in water.

Characterization of the complex

The synthesized complex was characterized using UV–Vis, FTIR, and NMR spectroscopy. In UV–Vis spectroscopy, small amounts of naringin, RuCl₂(DMSO)₄, and the complex were dissolved in dimethyl sulfoxide. UV–Vis spectra of the samples were obtained using Optizen POP UV/VIS spectrophotometer. For FTIR, dried samples of naringin, RuCl₂(DMSO)₄, and the complex were ground, together with KBr and placed in the spectrophotometer. FTIR spectra of the samples were acquired using JASCO FTIR-4600 spectrophotometer in ATR mode. Lastly, ¹H and ¹³C NMR of naringin and the complex were obtained using Bruker Avance III 500 MHz NMR spectrometer. Naringin was dissolved in DMSO-d₆ while the complex was dissolved in deuterium oxide.

Cell culture

The A549 cells were acquired from ATCC, and grown in DMEM, supplemented with 10% FBS and 1% penicillin–streptomycin. Human dermal fibroblast cells (HDFa) were obtained from CEFO Ltd (Seoul, South Korea) and cultured using their respective growth medium. The cells were incubated at 37 °C, 5% CO₂, followed by subculture upon reaching 70–90% confluency.

Cell viability study (MTT assay)

A549 and HDFa cells were seeded on 96-well plate and incubated at 37 °C, 5% CO₂ until 50% confluency was achieved. A549 cells were treated with naringin, RuCl₂(DMSO)₄, and the complex at different concentrations (0, 10, 20, 30, 40, 50, 60 and 70 µg/mL) and HDFa cells with (0, 20, 40, 60, 80, 100, and 120 µg/mL) concentrations for 24 h. After the treatment period, MTT assay was performed as per the manufacturer's protocol.

Live and dead staining assay

The cell viability of A549 cells was further examined using live and dead staining assay obtained from Thermo Fisher Scientific, Massachusetts, USA at different time intervals. Treated cells were stained, and incubated for 30 min, at room temperature, in the dark. Stained samples were imaged using fluorescent microscopy. Images of the control and treated cells were taken using Nikon live cell capture software. Live cells were indicated by green fluorescence while the dead cells were stained red (Thangavel et al. 2018).

LDH release assay

The LDH assay was assessed by employing LDH detection assay kit by Sigma-Aldrich Inc., USA. A549 and HDFa cells were cultured in 96-well plates about 1×10^6 cells per well. Then the medium was replaced with newly prepared medium consisting of the complex at different concentrations (0, 5, 10, 50, 100, and 150 µg/mL) and incubated at for 37 °C, 5% CO₂ for 4 h. Cytotoxicity of the complex was measured by recording the absorbance at 490 nm.

Trypan blue dye assay

To investigate the in vitro cytotoxicity of complex on A549 and HDFa cells, Trypan blue exclusion assay was employed. Cells were cultured and different concentrations (0, 5, 10, 50, 100, and 150 µg/mL) of the samples were added to the cells and incubated at 37 °C, 5% CO₂ for 30 min. 100 µL of Trypan blue dye was added and the number of live and dead cells was counted using haemocytometer by compound

microscope. The percentage of cytotoxicity was calculated by using the formula.

$$\% \text{ Dead cells} = \frac{\text{Number of dead cells}}{\text{Sum of dead cells and living cell}} \times 100$$

Atomic force microscopy sample preparation

Cells were grown overnight at a cell density of 5×10^5 on a six-well plate with glass cover-slips pretreated with 0.2% gelatin. Next, A549 cells were treated with the complex at different time intervals (0, 12, and 24 h). Afterwards, cells were washed with PBS and fixed with 4% paraformaldehyde solution for 30 min. Subsequently, the cells were washed with PBS, air-dried, and imaged using bio-AFM. Images of the cells were obtained and processed through JPEK software.

Statistical analysis

All the data shown in this paper are the mean ($\pm$ standard deviation, SD) of three independent experiments.

Results

Characterization of the complex

The UV–Vis absorption spectra of the samples are shown in Fig. 2. The sharp absorption peak of naringin at λ_{max} 270 nm can be attributed to the $n \rightarrow \pi^*$ transition of the carbonyl group or the phenol group of naringin. The broad absorption peak of RuCl₂(DMSO)₄ at 290 nm can be attributed to the DMSO region of the compound. Moreover, the complex demonstrated the characteristic peak of naringin at 270 nm. However, the complex showed an altered secondary peak when compared to naringin.

The IR spectra of the samples shown in Fig. 2 reveals that both naringin and the complex displayed vibrational frequencies at 3650–2950/cm region and 1700–1650/cm region, emphasizing the presence of hydroxyl and carbonyl groups respectively. The difference in peak intensity was attributed to the possible coordination of a ruthenium atom to the aromatic hydroxyl and carbonyl of naringin. Moreover, RuCl₂(DMSO)₄ also displayed peaks for DMSO ligands at 3014 and 2924/cm. These peaks were found to be retained in the complex.

The ¹H and ¹³C NMR of the naringin and complex were recorded, and the results are presented in Fig. S1–S4. ¹³C NMR spectra of naringin and the complex, both showed the bands for carbonyl (190+ ppm), aromatic (110–170 ppm) and aliphatic carbons (30–105 ppm), indicating the persistence of naringin in the structure of the complex. In ¹H NMR, the

Fig. 2 **a** UV–Vis absorption spectra of naringin, $\text{RuCl}_2(\text{DMSO})_4$ and complex **b** FTIR spectra of naringin, $\text{RuCl}_2(\text{DMSO})_4$, and complex

disappearance of signals at 5.35 and 5.44 ppm, pertaining to phenolic hydrogen (4.6–7.0 ppm), in the complex were marked as a sign that the aromatic hydroxyl bonded with a ruthenium atom (Figs. S1–S4).

Anti-proliferative activity naringin–ruthenium complex

MTT cell proliferation assay was used to evaluate the anticancer effects of naringin, $\text{RuCl}_2(\text{DMSO})_4$ and complex against A549 cells. Based on the results, the complex demonstrated higher anticancer effects against A549 than its parent materials (Fig. 3a). At 40 $\mu\text{g/mL}$ of complex, only 50% of the cells were viable. For comparison, HDFa cells were treated with the samples. No samples induced significant changes in the viability of HDFa cells (Fig. 3b).

The toxic effects of the complex by live- and dead-cell staining assay

The viability of A549 cells treated with complex was further evaluated using live- and dead-cell staining assay. Live cells were indicated by green fluorescence whereas dead cells emit red fluorescence. Figure 4 shows that the number of dead A549 cells increased with longer exposure to the complex. The complex had demonstrated a time-dependent effect against A549. Remarkably, HDFa cells did not show signs of cell death. Thus, the toxicity of the complex was only apparent to the A549 cells. The percentage of the cell viability observed in the HDFa and A549 cells, when treated the complex is shown in the Fig. S5.

Fig. 3 Cell viability of **a** A549 and **b** HDFa cell lines treated with naringin, $\text{RuCl}_2(\text{DMSO})_4$, and complex for 24 h

Fig. 4 Fluorescent microscopy images of **a** A549 and **b** HDFa cells treated with the complex for 6, 12, and 24 h

LDH leakage assay

To assess the activity of complex on the A549 and HDFa cells, lactate dehydrogenase assay (LDH) was carried out. Fig. S6 shows the percentage of LDH leakage in A549 cells (red curve) with dose-dependent manner.

As the concentration of the complex was increased, cell death was also increased, which suggests that the complex has significant cytotoxicity towards A549 cells. On the other hand, the percentage of LDH leakage in HDFa cells (black curve) does not show much effect using the complex.

Trypan blue exclusion method

The cytotoxicity of complex towards the A549, HDFa cells is estimated using Trypan blue assay method. Fig. S7 demonstrates that as the concentration of the complex was increased, percentage in the number of dead cells was also increased in the case of A549 cells (black curve) whereas in the case of HDFa cells (red curve) less significant effects are observed.

Morphological evaluation of A549 cell line using Bio-AFM

Bio-AFM was utilized to study any morphological changes induced by the complex. High-resolution 2D and 3D images were acquired and analyzed. A549 cells treated with the complex for 0, 12, and 24 h are described in Fig. 5. No cell damage was observed at the start of the experiment. At 12 h, the cells started to show leakage of cytoplasmic fluid out of the cell. Further treatment at 24 h resulted in a compromised cell membrane. Cytoplasm and other cellular structures seemed to have excluded out of the A549 cell. Here, a time-dependent cytotoxicity was observed for the complex against A549. Longer exposure to the complex leads to reduced cell-membrane integrity.

Discussion

Recently, much interest has gained in the study of ruthenium (Ru) complexes using different kinds of ligands for the manufacturing of cancer drugs (Popolin et al. 2017). Naringin, a flavanone glycoside mostly available in the citrus fruits with many biological benefits such as antioxidant, anti-inflammatory, antimicrobial, antiviral, and anticancer etc., (Camargo et al. 2012). Potential benefits of naringin have already proved that apoptosis of HeLa cells done by means of caspase pathways (Zeng et al. 2014). Recent studies have already explained the effectiveness and capacity of naringin drug in different kinds of cancers such as cervical, liver, breast, bladder, stomach, and colon cancers. However, our study focuses on the effects of naringin–ruthenium complexes on the lung cancer cells (Li et al. 2013). As ruthenium compounds possess very minimal side effects and low cytotoxicity regarding cancer cells when compared to the platinum compounds, they are emerging as the most interesting anticancer agents (Yang et al. 2012). Naringin exerts significant characteristics including apoptosis, morphological changes, internucleosomal DNA fragmentation, and reduced mitochondrial transmembrane potential using mitochondrial pathways (Chen et al. 2016).

Fig. 5 Bio-AFM images of A549 cells treated with the complex at 12 and 24 h

Conclusion

In this study, a novel ruthenium complex was synthesized using a flavanoid, naringin and the complex was formed as a result of potential binding between the ruthenium atom and the phenolic and carbonyl groups of naringin. The anticancer activity of the synthesized complex was tested against A549. Cell viability of A549 cells treated with 40 µg/mL of the complex for 24 h decreased up to 50%. Moreover, no significant change was observed in HDFa cells. Thus, indicating a selective action of the complex on the A549 cells. Lastly, exposure of A549 cells to the complex for 24 h resulted in cytotoxic activity, indicated by the compromised cell membrane. Our findings from the in vitro study support the continued investigation of ruthenium–naringin complex possessing a potential chemotherapeutic activity against non-small cell lung cancer lines.

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Compliance with ethical standards

Conflict of interest The authors declare no competing financial interests.

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