

# Recognizing and stabilizing miR-21 by chiral ruthenium(II) complexes

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## Research article

**Keywords:** Chiral ruthenium(II) complexes, MiR-21, RNA binding property, FRET

**Posted Date:** February 24th, 2020

**DOI:** <https://doi.org/10.21203/rs.2.24341/v1>

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**Version of Record:** A version of this preprint was published at BMC Chemistry on April 3rd, 2020. See the published version at <https://doi.org/10.1186/s13065-020-00672-8>.

# Abstract

MiR-21, a non-coding miRNA with 22 nucleotides, plays an important part in the proliferation, invasion, and metastasis of tumor cells. The present study demonstrates that isomers of chiral ruthenium(II) complexes with alkynes ( $\Lambda$ -1 and  $\Delta$ -1) were synthesized by Songogashira coupling reaction by using microwave-assisted synthetic technology. The isomers can recognize and stabilize miR-21, with the  $\Lambda$ -isomer showing a stronger binding capacity than the  $\Delta$ -isomer. Further studies showed that both isomers can be uptaken by MDA-MB-231 cells and enriched in the nucleus. Treatment with the  $\Lambda$ / $\Delta$ -isomer downregulated the expression of miR-21. In a word, the development of chiral ruthenium(II) complexes act as potential inhibitors against tumor cells by recognizing, stabilizing, and regulating the expression of miR-21.

## Introduction

Increasing attention has been focused on small-molecule targeting drugs over the last decades, and some of these drugs, including NAMI-A, KP1019, and CX-3543, have been undergone clinical trials [1–5]. However, with the rapid development of tumor targeting small molecules, the multidrug resistance and dependencies of cancer cells have become more serious [6–8]. Therefore, novel compounds that suppress the proliferation of tumor cells by regulating the expression of target genetic fragments must be designed [9]. MicroRNAs (miRNAs), a type of endogenous non-coding RNAs molecules with ~ 22 nucleotides that regulate the expression of protein by cleaving or repressing the translation of target mRNAs [10–14], provide the opportunities [15–16]. In the last decades, more than 1000 miRNAs have been found in human cells; some of these miRNAs are highly tissue-specific expression, whereas others are identified as house-keeping functional molecules [17–19]. MiR-21 (Fig. 1B), a miRNA overexpressed in almost all types of human malignancy, is involved in multifarious cancer-associated processes, including proliferation, invasion, and metastasis [20–22]. A growing number of evidence suggests that miR-21 as an “oncomir” in oncogenesis, which is up-regulation in most detected cancers, including breast, colorectal, pancreatic and glioblastoma cancer [23–25]. In recent years, miR-21 has been investigated as a potent target for small-molecule drugs [26–27]. For example, the gemcitabine sensitivity of cholangiocarcinoma cells increases by suppressing miR-21 [28]. In addition, curcumin could suppress tumor cells growth, invasion and metastasis by significantly inhibiting miR-21 expression [29]. A similar phenomenon was also observed in xenograft mouse models of gliomas, human hepatocytes, and breast cancer cells [30]. A recent study has found that miR-21 regulates cell apoptosis. Sasaki indicated that the apoptosis induction of tumor cells can be suppressed by upregulating the expression of miR-21 [31]. Further studies showed that overexpressing miR-21 can regulate the level of B-cell lymphoma-2 (Bcl-2) protein by downregulating Bcl-2 and upregulating B-cell lymphoma-associated x [32]. In addition, suppressing the expression of miR-21 activates the caspase signal pathway and downregulates the expression of caspase-3 [33]. Overexpressing miR-21 can inhibit the expression of the P53 gene, which exerts proapoptotic effect [34]. It can also repress the apoptosis of cells by regulating the programmed cell death 4 gene [35].

Recently, Ruthenium(II) complexes have been extensively studied as potential anti-tumor agents [36–40]. Many studies showed that ruthenium(II) complexes containing planar aromatic rings can interact with DNA molecules through intercalative binding, groove-binding, and/or electrostatic interaction mode, and some entities with strong inhibitory effect have been researched [41–43]. Ruthenium complexes bearing different ligands could also bind and stabilize G-quadruplex DNA, including telomeric and proto-oncogene, which display great inhibition against tumor cells by regulating the expression of related proteins [44–48]. In our previous study found that polypyridyl ruthenium(II) complexes induced apoptosis through a mitochondria-mediated pathway rather than by direct-interaction DNA damage [49]. Further study suggested that chiral ruthenium(II) complexes coordinated by tFMPIP

(2-(trifluoromethylphenyl)-1H-imidazo[4,5-f][1, 10] phenanthroline) can induce the apoptosis of tumor cells by regulating the expression of Bcl-2 family proteins to activate the caspase signal pathway[50]. Chalikian et al. indicated that RNA can be recognized by small molecules [51]. However, whether ruthenium(II) complexes can bind to miR-21 remains unclear. In our previous study, we studied the interaction of the polypyridine ruthenium(II) complex with the total RNA of liver cells [52]. Moreover, we demonstrated that the effect of miR-21 on phosphatase and tensin homolog deleted on the chromosome ten/protein kinase B (AKT) signaling pathway is abrogated by the arene ruthenium complexes, and the miR-21 inhibitor could enhance the antitumor capability [53].

In the current study, the binding behavior of both chiral ruthenium(II) complexes ( $\Lambda$ -1 and  $\Delta$ -1, Fig. 1A) with miR-21 was first investigated. Results suggested that  $\Lambda$ -1 and  $\Delta$ -1 can bind and stabilize the hairpin structure of miR-21 molecules (Fig. 1B) with obvious enantiomer selectivity. Furthermore, the expression of miR-21 could be inhibited by both isomers after enriching in the nucleus of highly metastatic human breast cancer cells through real-time images and quantitative PCR experiments.

## Results And Discussion

### *Recognition of $\Lambda$ -1 and $\Delta$ -1 with miR-21.*

MiR-21, a miRNA overexpressed in almost all types of human malignancy, is involved in multiple cancer-associated processes, including proliferation, invasion and metastasis. Thus, the binding behaviors of  $\Lambda$ -1 and  $\Delta$ -1 with miR-21 were investigated to clarify the interaction of chiral ruthenium(II) complex on miR-21.

The recognition of miR-21 by  $\Lambda$ -1 and  $\Delta$ -1 was first demonstrated by electronic titration methods, which are commonly used to investigate the interaction of transition metal complexes with biological macromolecules [54]. The electronic spectra of  $\Lambda/\Delta$ -1 display the characterized metal-to-ligand charge transfer (MLCT) absorption with the peak at 465 nm and the characterized IL absorption with the peak at 286 nm (Figures 2A and 2B). Upon the addition of miR-21 solution, apparent hypochromism occurred for both isomers because of the electronic circumstance disturbed by miR-21 molecules [55]. At [miR-21]/[Ru]=0.083, the hypochromism values for  $\Lambda$ -1 and  $\Delta$ -1 were about 15.4% and 13.2%, with the binding

constant of  $\Lambda$ -1 and  $\Delta$ -1 are  $2.7 \times 10^5 \text{ M}^{-1}$  and  $1.14 \times 10^5 \text{ M}^{-1}$ , respectively. The characterized fluorescent for both isomers in the range of 500–700 nm underwent apparent increase in the presence of miR-21 (Figures 2C and 2D). At  $[\text{miR-21}]/[\text{Ru}] = 0.133$ , the emission intensities of  $\Lambda$ -1 and  $\Delta$ -1 increased to around 1.29 and 1.15 times than the original, with the intrinsic binding constant of  $\Lambda$ -1 and  $\Delta$ -1 are  $9.3 \times 10^4 \text{ M}^{-1}$  and  $1.23 \times 10^4 \text{ M}^{-1}$ , respectively. These data suggested that both isomers can bind to miR-21 in promising binding affinity, with the  $\Lambda$ -isomer having a stronger binding affinity than the  $\Delta$ -isomer. [56].

The fluorescence resonance energy transfer (FRET) melting point assay was also carried out to clarify the stabilization of the structure of miR-21 in the presence of this chiral ruthenium(II) complex [57]. The melting point of miR-21 increased as the concentration of both isomers was increased. The melting point of miR-21 was about  $55.1 \text{ }^\circ\text{C}$  (Figure 3). When  $\Lambda$ -1 and  $\Delta$ -1 were added into the solution,  $T_m$  increased in a dose-dependent manner. At  $[\text{Ru}] = 3.0 \text{ }\mu\text{M}$ , the  $\Delta T_m$  values for  $\Lambda$ -1 and  $\Delta$ -1 were  $6.3 \text{ }^\circ\text{C}$  and  $4.0 \text{ }^\circ\text{C}$ , respectively.

Furthermore, the conformation changes of both isomers in the presence of miR-21 solution were further confirmed by using circular dichroism (CD) spectroscopy [58]. When miR-21 was added into the isomer solution, the ellipticity strength of the characterized CD signal for  $\Lambda$ -1 and  $\Delta$ -1 decreased obviously in a concentration-dependent manner. At  $[\text{miR-21}]/[\text{Ru}] = 0.183$ , the ellipticity strength for  $\Lambda$ -1 and  $\Delta$ -1 decreased to 34.9% and 30.7%, respectively (Figure 4A). These results indicated that the structure of both isomers can be disturbed by miR-21, and the change depends on the binding ability. Equilibrium dialysis is an important method to study the selective interaction of bio-macromolecules with small drug molecules. In general, the equilibrium dialysis was conducted at room temperature with 0.4 mL of miR-21 (25  $\mu\text{M}$ ) sealed in a dialysis bag and 10 mL of racemize  $[\text{Ru}(\text{bpy})_2(\text{p-TEPIP})](\text{ClO}_4)_2$  outside the bag. The CD signals of the dialyzate of racemized ruthenium(II) complex changed during dialysis. As shown in Fig. 4B, no detectable CD signal was observed before dialysis. After dialysis for 12 h, a distinct negative CD signal (300 nm) attributed to the signal of  $\Delta$ -1 peaked after 24 h. These results suggested that  $\Lambda$ -1 exhibited greater affinity to miR21 than  $\Delta$ -1.

### ***Expression of miR-21 regulated by $\Lambda$ -1 and $\Delta$ -1***

MiR-21 acts as a key regulator for cell apoptosis through a mitochondrial-dependent signal pathway. Thus, quantitative PCR (RT-qPCR) was evaluated to investigate the effect of the expression of miR-21, as shown in Figure 2B. The levels of miR-21 markers in MDA-MB-231 cells after treatment with both isomers were analyzed.

RT-qPCR showed that the expression levels of miR-21 were markedly suppressed with increasing amount of the isomers. At  $[\text{Ru}] = 50 \text{ }\mu\text{M}$ , 45.8% and 30.7% of miR-21 gene expression (mean expression of positive control=100) in MDA-MB-231 cells were smothered (Figure 5). The data indicated that the expression of miR-21 could be inhibited by this type of chiral ruthenium(II) complex.

# Materials And Methods

## *Chemicals.*

All materials and solvents were obtained commercially and used without further purification unless special statement. Mature miR-21 sequence, the complementary strand 5'-UAGCUUAUCAGACUGAUGUUGA-3', was purchased from Suzhou GenePharma Co., Ltd. F-miR-21 oligomers, the complementary strand 5'-FAM-UAGCUUAUCAGAC UGAUGUUGA-BHQ-1-3', was also purchased from Suzhou GenePharma Co., Ltd. Both isomers  $\Lambda/\Delta$ -[Ru(bpy)<sub>2</sub>(p-TEPIP)](ClO<sub>4</sub>)<sub>2</sub> ( $\Lambda/\Delta$ -1) were synthesized as previously described in the literature [59]. The detailed characterization data are listed in Supporting information (Figures S1–S4), and the purity of  $\Lambda/\Delta$ -1 was higher than 95% as tested by HPLC (Figure S5).

## *Quantitative reverse transcription-PCR assay for miR-21 expression.*

Quantitative PCR (Q-PCR) analysis of miR-21 transcript expression profiles of both isomers at various confluences were measured using the AB 7900HT Real-Time PCR system as previously described [60] with some modifications. All conditions of this experiment were similar to Bcl-2, except miR-21 was replaced. The primers were as follows: miR-21 Forward, 5'-ACACTCCAGCTGG GTAGCTTATCAGACTGA-3'; Reverse, 5'-GTGTCG TGGAGTCGGCAATTC-3'; U6 Forward, 5'-GTGCTC GCTTCGGCAGCACATATAC-3'; Reverse, 5' AAAAA TATGGAACGCTTCACGAATTTG-3'.

## *Electronic absorption measurements.*

Electronic absorption spectra of the ruthenium complexes (10  $\mu$ M) interacted with increasing concentration of miR21 (0, 0.0667, 0.1334, 0.2 ..... 0.667  $\mu$ M) were performed on a Shimadzu UV-2550 spectrophotometer at room temperature. [61]. The absorption spectrum was recorded after mixing the ruthenium(II) complex-miR-21 solution for 5 min. The addition of miR21 repeated some times until little changes were observed in the spectra, suggesting that the binding saturation was achieved.

## *Fluorescence measurements.*

Fluorescence experiments were carried out by increasing miR-21 solution to the ruthenium(II) complexes. The fluorescence of both ruthenium complexes were excited at 340 nm, and the emission curve was observed from 500-700 nm. The fluorescence spectrum was recorded after mixing the ruthenium(II) complex-miR-21 solution for 5 min. The addition of miR21 repeated some times until little changes were observed in the spectra, suggesting that the binding saturation was achieved. Due to the dilution after each titration experiment, the concentration of the ruthenium(II) complex only slightly changed.

## *Fluorescence resonance energy transfer melting point assay.*

The FRET melting point assay was performed to clarify the affinity of chiral ruthenium(II) complexes to bind miR-21. Fluorescence melting curves were measured by a Bio-Rad real-time PCR detection system

using a total reaction excitation at 470 nm constant temperature being maintained for 30 s prior to each reading to ensure a stable value [62].

### *Circular dichroism spectra measurements.*

Circular dichroism spectra were obtained using a Jasco J810 spectropolarimeter [63]. During the titration, a 2  $\mu$ L aliquot of buffered miR-21 solution was added to each cuvette of drugs, and the solutions were mixed by repeated inversion. After the solutions were mixed for  $\sim$ 5 min, the CD spectra were recorded. For each sample, CD experiments were measured at least three times at room temperature by using a quartz cell with a path length of 1 cm. The spectra were collected at wavelengths of 200–600 nm and with a scanning speed of 50 nm/min [64]. The instrument was flushed continuously with pure evaporated nitrogen throughout the experiment.

## Conclusion

The hairpin structure of miR-21 can be selectively recognized and stabilized by chiral ruthenium(II) complexes with alkynes ( $\Lambda$ -1 and  $\Delta$ -1), and the  $\Lambda$ -isomer exhibited stronger binding affinity than the  $\Delta$ -isomer. Furthermore, both isomers can be uptaken by MDA-MB-231 cells, and the expression of miR-21 was suppressed by both isomers. Thus, these chair ruthenium(II) complexes can act as potential inhibitors by recognizing, stabilizing, and downregulating the expression of miR-21. Investigation of the detailed mechanisms between their activity and downregulated miR-21 is now in progress.

## Abbreviations

**MiR-21:** MicroRNA-21 **MDA-MB-231:** Human breast cancer cells **Q-PCR:** Quantitative real time polymerase chain reaction **Bcl-2:** B-cell lymphoma-2 **Bax:** B-cell lymphoma-associated x **PDCD4:** programmed cell death 4 **PTEN:** phosphatase and tensin homolog deleted on chromosome ten **AKT:** protein kinase B **MLCT:** metal-to-ligand charge transfer **FRET:** Fluorescence resonance energy transfer **ITC:** Isothermal Titration Calorimetry **CD:** Circular dichroism **DEPC:** diethylpyrocarbonate.

## Declarations

### Authors' contributions

YF and JS performed experiments including RT-qPCR and FRET melting point UV titration and Fluorescence titration. LZY and YTL wrote and revised this paper together. LBY provide help to perform the experiments including CD spectra and Equilibrium dialysis. WJM and YD provided the funds, guided the researches and the results analysis, wrote and revised the manuscript. All authors read and approved the final manuscript.

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## Acknowledgements

All authors thank long-term help and support Prof. Xicheng Wang and Prof. Weijun Tan.

## Competing interests

The authors declare that they have no competing interests.

## Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

## Supporting information

Real-time fluorescence images, the movie for the dynamic imaging process, Quantitative Reverse Transcription-PCR and Isothermal Titration Calorimetry experiments. This material is available free of charge *via* the Internet at <https://bmcchem.biomedcentral.com/>

## Funding

This research was funded by the National Natural Science Foundation of China (Grant 81572926), the National Science Foundation for Young Scientists of China (Grant 81703349), the Provincial Major Scientific Research Projects in Universities of Guangdong Province (Grant 2014KZDXM053), the Innovation Team Projects in Universities of Guangdong Province (Grant 2016KCXTD018), the Science and Technology Project of Guangdong Province (Grant 2014A020212312), and the Innovation Projects in Universities of Guangdong Province (Grant 2015cxqx151). All funding bodies played no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

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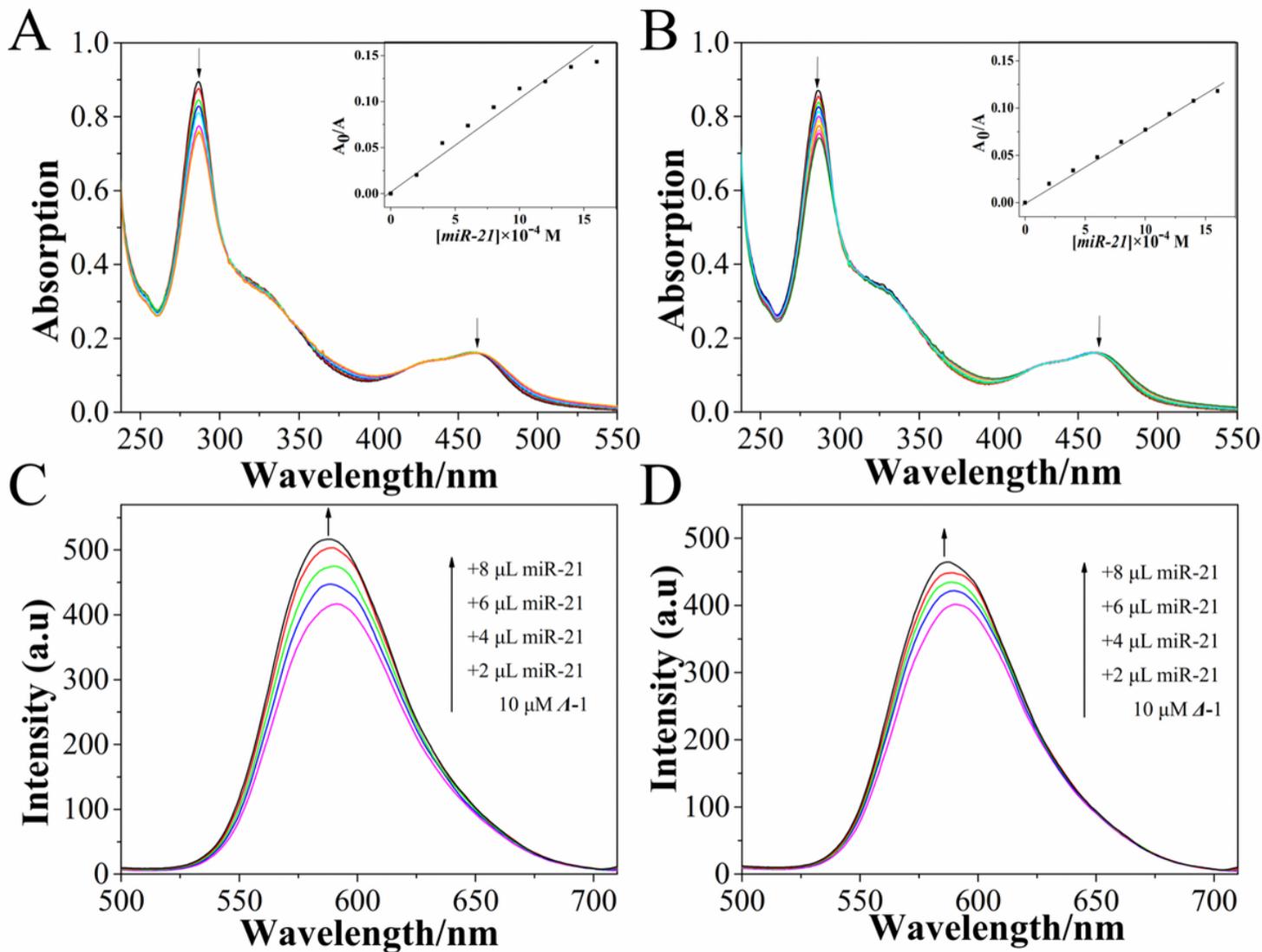
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**Figure 2**

Electronic spectra of  $\Lambda$ -1 (A) and  $\Delta$ -1 (B) with and without increasing amounts of miR-21.  $[Ru]=10 \mu M$ ; Fluorescence emission spectra of  $\Lambda$ -1 (C) and  $\Delta$ -1 (D) with and without increasing amounts of miR-21.  $[Ru]=10 \mu M$ .

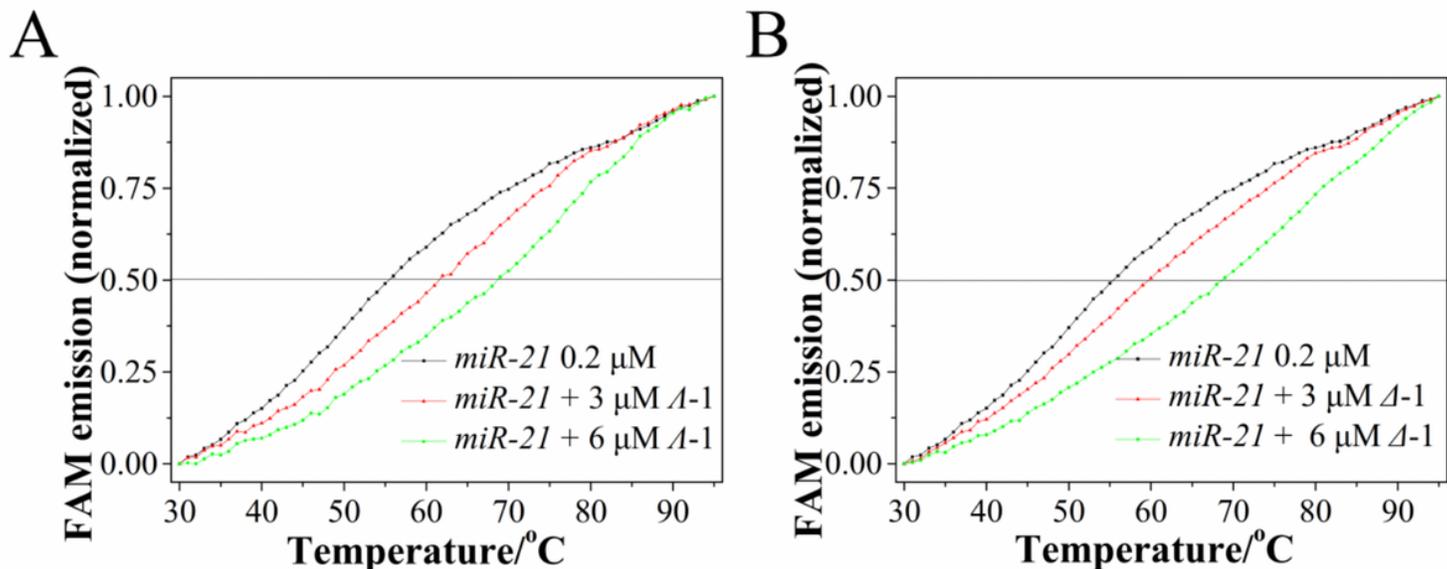


Figure 3

Fluorescence resonance energy transfer (FRET) melting profiles of miR-21 in the absence and presence of  $\Delta$ -1 (A) /  $\Delta$ -1 (B). [miR-21]=0.2  $\mu$ M.

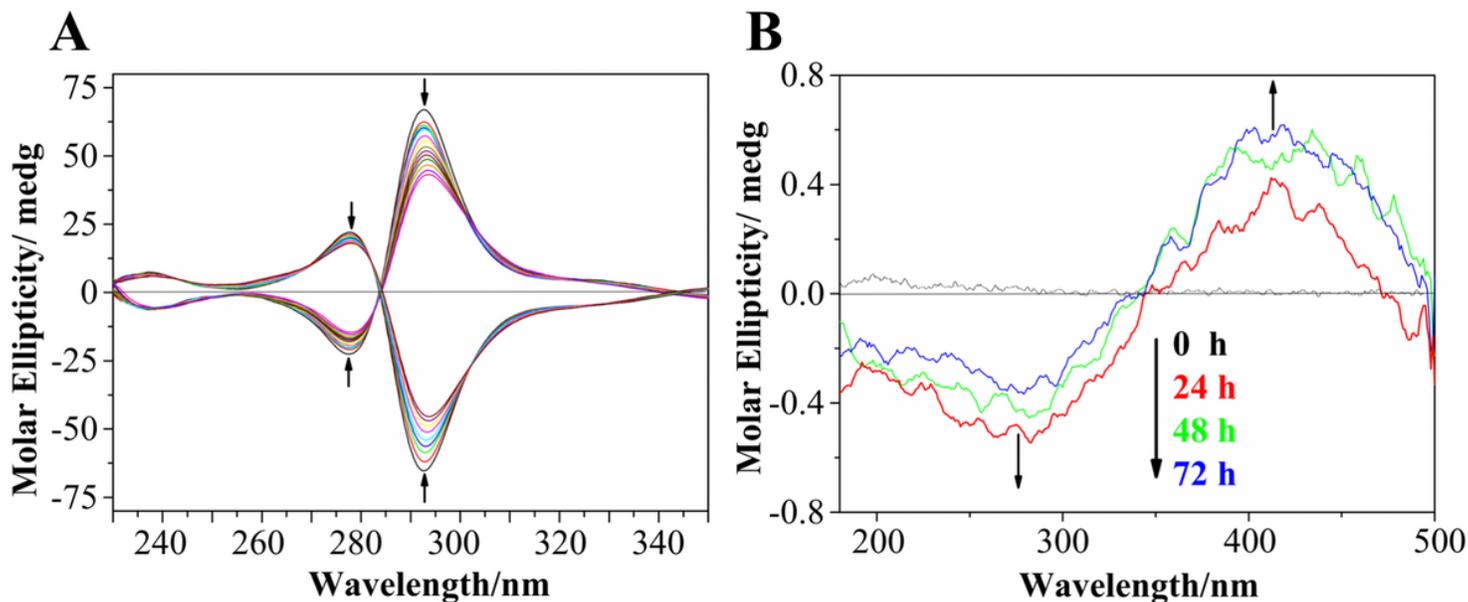


Figure 4

A) CD spectra of  $\Delta$ -1 and  $\Delta$ -1 in the absence and presence of miR-21. [Ru]=10  $\mu$ M. B) CD spectra of racemized ruthenium(II) complex dialyzed against miR-21 at t=0, 24, 48, and 72 h. ([Ru]=5  $\mu$ M; [miR-21]=2.5  $\mu$ M).

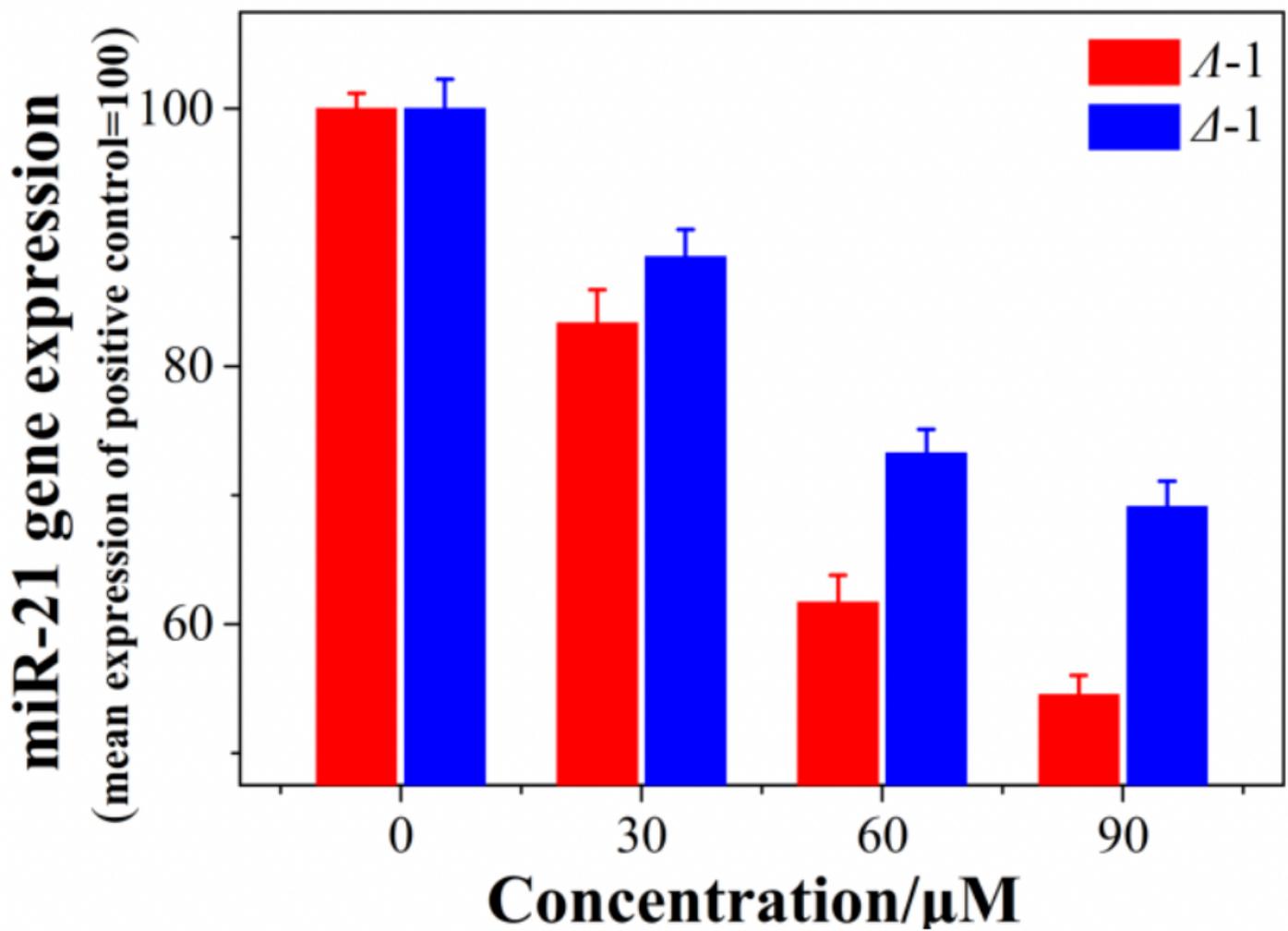


Figure 5

Downregulation of miR-21 after treatment with different concentration complexes in cell level tested by quantitative PCR (RT-qPCR). The amplification plot and dissociation curve of RT-qPCR of miR-21 transcript expression profiles of both isomers at various confluences are shown in Figure S6.

## Supplementary Files

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