Inhibition of TLR1/2 dimerization by enantiomers of metal complexes†

Li-Juan Liu,‡ Wanhe Wang,‡ Zhangfeng Zhong,§ Sheng Lin,§ Lihua Lu,§ Yi-Tao Wang,§ Dik-Lung Ma*§ and Chung-Hang Leung*§

a State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macao (China). E-mail: duncanleung@umac.mo

b Department of Chemistry, Hong Kong Baptist University, Kowloon Tong, Hong Kong (China). E-mail: edmondma@hkbu.edu.hk

‡ These authors contributed equally to this work.
Materials

Reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received unless specified. Deuterated solvents for NMR purposes were obtained from Cambridge Isotope Labs, iridium(III) chloride hydrate (IrCl$_3$·xH$_2$O) and rhodium(III) chloride hydrate (RhCl$_3$·xH$_2$O) were purchased from Precious Metals Online (Australia).

General experiment

High resolution mass spectrometry (HRMS) was carried out at the Mass Spectroscopy Unit in Hong Kong Baptist University, Hong Kong (China). $^1$H and $^{13}$C nuclear magnetic resonance (NMR) were recorded on a 400 MHz ($^1$H) and 100 MHz ($^{13}$C) Bruker instrument using chloroform-$d$, acetone-$d_3$, acetonitrile-$d_3$ or DMSO-$d_6$ as the solvent. $^1$H and $^{13}$C chemical shifts were referenced internally to solvent shift (CDCl$_3$-$d$: $^1$H δ1.94, $^{13}$C δ77.2; Acetonitrile-$d_3$: $^1$H, δ1.94, $^{13}$C δ118.7; Acetone-$d_6$: $^1$H δ2.05, $^{13}$C δ29.7; DMSO-$d_6$: $^1$H δ2.50, $^{13}$C δ39.52). Coupling constants are typically ± 0.1 Hz for $^1$H-$^1$H and ±0.5 Hz for $^1$H-$^{13}$C couplings. The following abbreviations are used for convenience in reporting the multiplicity of NMR resonances: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. All NMR data was acquired and processed using standard Bruker software (Topspin). The elemental analysis test of complexes was performed in Atlantic Microlab, Inc. (USA). Enantiomeric excesses were measured on a Shimadzu LC 20A QA&QC-HPLC-13 using Chiralpak® IF column.

Synthesis of iridium/rhodium complexes

General synthesis of [M$_2$(C^N)$_4$Cl$_2$] complexes

Cyclometalated dichloro-bridged dimers of the general formula [M$_2$(C^N)$_4$Cl$_2$], where M = Ir(III)/Rh(III), were synthesized according to a literature method.$^1$ In brief, MCl$_3$·xH$_2$O was heated to 130 ºC with 2.1 equivalents of cyclometallated C^N ligands in 3:1 methoxymethanol and deionized water under a nitrogen atmosphere overnight. The reaction was cooled to room temperature, and the product was filtered and washed with three portions of deionized water and then three portions of ether to yield the corresponding dimer.
General synthesis of $[\text{M}(\text{C}^\text{N})_2(\text{N}^\text{N})]\text{PF}_6$ complexes

These complexes were synthesized using a modified literature method.\(^1\) Briefly, a suspension of $[\text{M}_2(\text{C}^\text{N})_4\text{Cl}_2]$ (0.1 mmol) and corresponding N\(^\text{N}\) (0.21 mmol) ligands in a mixture of dichloromethane:methanol (1:1, 6 mL) was refluxed overnight under a nitrogen atmosphere. The resulting solution was allowed to cool to room temperature, and was filtered to remove unreacted cyclometallated dimer. To the filtrate, an aqueous solution of ammonium hexafluorophosphate (excess) was added and the filtrate was reduced in volume by rotary evaporation until precipitation of the crude product occurred. The precipitate was then filtered and washed with several portions of water (2 × 30 mL) followed by diethyl ether (2 × 30 mL). The solid was dissolved into acetone, and then the product was precipitated by adding diethyl ether, and filtered to yield.

**Complex 1:** Reported\(^2\)

**Complex 2:** Reported\(^2\)

**Complex 3:** Yield: 60%. \(^1\)H NMR (400 MHz, Acetone-$d_6$) $\delta$ 9.81 (s, 2H), 9.33 (s, 2H), 8.97 (d, J = 8.3 Hz, 2H), 8.56 (d, J = 5.0 Hz, 2H), 8.41 (s, 2H), 8.09 – 8.06 (m, 2H), 7.86 (d, J = 8.1 Hz, 2H), 7.27 (t, J = 7.5 Hz, 2H), 7.06 (t, J = 7.5 Hz, 2H), 6.61 (d, J = 7.7 Hz, 2H), 1.55 (s, 6H); \(^{13}\)C NMR (100 MHz, Acetone-$d_6$) $\delta$ 183.53, 153.16, 151.79, 146.47, 141.82, 139.65, 134.35, 131.93, 131.51, 128.34, 127.75, 126.91, 125.09, 122.91, 116.99, 113.55, 10.30; MALDI-TOF-HRMS: Calcd. for C\(_{34}\)H\(_{26}\)N\(_6\)O\(_2\)Rh$[\text{M–PF}_6]^+$: 653.1668. Found: 653.1690. Anal.: (C\(_{34}\)H\(_{26}\)N\(_6\)O\(_2\)RhPF\(_6\)) C, H, N: calcd. 51.14, 3.28, 10.53; found 51.67, 3.38, 10.36.
Compounds Λ-(S)-S3 was synthesized using a modified literature method. A suspension of dimer S1 (120 mg, 141.5 μmol), (S)-4-tert-butyl-2-(2-hydroxyphenyl)-2-oxazoline (S)-S2 (41.2 mg, 188 μmol), silver trifluoromethanesulfonate (111 mg, 433 μmol), and triethylamine (78.5 μL, 566 μmol) in dichloromethane (30 mL) was stirred at room temperature overnight. The suspension was filtered through a short plug of silica with hexane/ethyl acetate (1:2). The crude product was collected, concentrated to dryness; the residue was purified by silica gel column chromatography. The diastereomers could be well separated using hexane/ethyl acetate/Et3N.
(100:50:1 to 100:100:1) as eluent. The resulting materials were dissolved in acetonitrile, washed five times with hexane, and concentrated to dryness to afford Λ-(S)-S3 as yellow solid (67.5 mg, 60%). We found that Λ-(S)-S3 decomposed during the purification and we did not obtain this intermediate. Because Λ-(S)-S3 was somewhat unstable during silica gel column chromatography, the pure $^1$H NMR and $^{13}$C NMR spectrum of Λ-(S)-S3 was not obtained. But HRMS indicated that it was the desired product. It could be used in the next step without further purification.

Λ-(S)-S3 (first eluted diastereomer): 1H NMR (400 MHz, CDCl$_3$): $\delta$ = 8.12 (dd, J = 9.2, 2.0 Hz, 2H), 8.00 (d, J = 2.8 Hz, 1H), 7.63 (dd, J = 8.0, 1.6 Hz, 1H), 7.47 (d, J = 2.4 Hz, 1H), 7.12-7.04 (m, 3H), 6.91-6.85 (m, 3H), 6.76-6.64 (m, 4H), 6.46 (t, J = 2.4 Hz, 1H), 6.30-6.22 (m, 2H), 6.01-5.98 (m, 1H), 4.51 (dd, J = 9.6, 2.4 Hz, 1H), 3.58 (dd, J = 8.4, 2.4 Hz, 1H), 0.31 (s, 9H) ppm. HRMS: calcd. for C$_{31}$H$_{30}$N$_5$O$_2$Rh M$^+$ 607.1449; found 607.1499.
Complex Λ-1: Complex Λ-1 were synthesized using a modified literature method.\(^3\) A suspension of Λ-(S)-S3 (67.5 mg, 111 μmol), 1,10-phenanthroline (40.3 mg, 203.5 μmol), and NH₄PF₆ (57.6 mg, 354 μmol) in acetonitrile (10 mL) was stirred at room temperature for overnight. The reaction mixture was concentrated to dryness and subjected to silica gel chromatography with dichloromethane/methanol (20:1). The combined product eluents were again concentrated to dryness, and the resulting material was dissolved in a minimum amount of dichloromethane. The product was precipitated by the addition of diethyl ether. The yellow precipitate was collected by centrifugation and dried under high vacuum to afford Λ-1 as its PF₆ salt (40.0 mg, 50%, 92.562% ee).

\(^1\)H NMR (400 MHz, CDCl₃): δ = 8.68 (dd, J = 8.4, 1.6 Hz, 2H), 8.44 (dd, J = 4.8, 1.2 Hz, 2H), 8.18 (s, 2H), 8.15 (d, J = 2.8, 2H), 7.83 (dd, J = 8.4, 5.2 Hz, 2H), 7.33 (dd, J = 7.6, 0.8 Hz, 2H), 7.17-7.13 (m, 2H), 7.00-6.96 (m, 2H), 6.78 (d, J = 2.0, 2H), 6.45 (t, J = 2.4, 2H), 6.39 (d, J = 7.6, 2H) ppm. \(^{13}\)C NMR (100 MHz, CDCl₃): δ = 150.6, 147.7, 145.9, 142.2, 139.1, 138.9, 134.1, 130.9, 128.3, 127.1, 126.4, 125.9, 124.4, 112.1, 108.9 ppm. HRMS: Calcd. for C\(_{30}\)H\(_{22}\)RhN\(_6\)[M–PF\(_6\)]\(^+\): 569.0956 Found: 569.0993. CD (MeCN): λ (Δε/ε−1cm−1) 328 (−5), 310 (+1).
Compounds $\Delta$-(R)-S$3$ was synthesized using a modified literature method.$^3$ A suspension of dimer S$1$ (120 mg, 141.5 $\mu$mol), (R)-4-tert-butyl-2-(2-hydroxyphenyl)-2-oxazoline (R)-S$2$ (41.2 mg, 188 $\mu$mol), silver trifluoromethanesulfonate (111 mg, 433 $\mu$mol), and triethylamine (78.5 $\mu$L, 566 $\mu$mol) in dichloromethane (30 mL) was stirred at room temperature overnight. The suspension was filtered through a short plug of silica with hexane/ethyl acetate (1:2). The crude product was collected, concentrated to dryness; the residue was purified by silica gel column
chromatography. The diastereomers could be well separated using hexane/ethyl acetate/Et₃N (100:50:1 to 100:100:1) as eluent. The resulting materials were dissolved in acetonitrile, washed five times with hexane, and concentrated to dryness to afford Δ-(R)-S₃ as yellow solid (33 mg, 30%). We found that Λ-(R)-S₃ decomposed during the purification and we did not obtain this intermediate. Because Δ-(R)-S₃ was somewhat unstable during silica gel column chromatography, the pure ¹H NMR and ¹³C NMR spectrum of Δ-(R)-S₃ was not obtained. But HRMS indicated that it was the desired product. It could be used in the next step without further purification.

Δ-(R)-S₃ (second eluted diastereomer): ¹H NMR (400 MHz, CDCl₃): δ = 8.13 (dd, J = 8.4, 2.0 Hz, 2H), 8.02 (d, J = 2.8 Hz, 1H), 7.63 (dd, J = 8.0, 2.0 Hz, 2H), 7.47 (d, J = 2.0 Hz, 1H), 7.13-7.06 (m, 3H), 6.91-6.86 (m, 2H), 6.76-6.65 (m, 4H), 6.48 (t, J = 2.4 Hz, 1H), 6.31-6.23 (m, 2H), 6.00 (d, J = 8.0 Hz, 1H), 4.52 (dd, J = 9.2, 2.4 Hz, 1H), 3.58 (dd, J = 8.4, 2.0 Hz, 1H), 0.31 (s, 9H) ppm. HRMS: calcd. for C₃₁H₃₀N₅O₂Rh M⁺ 607.1449; found 607.1448.
Complex Δ-1: Complex Δ-1 was prepared in a similar manner to Λ-1 from Λ-(S)-S3. After purification and precipitation, Δ-1 was obtained as its PF₆ salt (24 mg, 70%, 84.580% ee). ¹H NMR (400 MHz, CD₃CN): δ = 8.72 (dd, J = 8.0, 1.2 Hz, 2H), 8.45 (dd, J = 5.2, 1.2 Hz, 2H), 8.40 (d, J = 2.8, 2H), 8.21 (s, 2H), 7.85 (dd, J = 8.0, 4.8 Hz, 2H), 7.51 (dd, J = 8.0, 1.2 Hz, 2H), 7.19-7.15 (m, 2H), 7.00-6.96 (m, 2H), 6.88 (d, J = 2.0, 2H), 6.44 (t, J = 2.8, 2H), 6.38-6.36 (m, 2H) ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 150.8, 147.8, 147.5, 145.6, 142.4, 138.9, 138.4, 133.6, 130.6, 127.5, 127.3, 126.2, 125.7, 123.9, 117.0, 112.2, 108.2 ppm. HRMS: Calcd. for
$C_{30}H_{22}RhN_6[M–PF_6]^+$: 569.0956 Found: 569.0961. CD (MeCN): $\lambda$ ($\Delta\varepsilon/m$–1cm–1) 328 (+ 5), 310 (– 1).
Determination of the Enantiopurities by chiral HPLC

The complexes were analyzed with a Daicel CHIRALPAK® IF (0.46 cm I.D. × 25 cm L × 5 μm) HPLC column on Shimadzu LC 20A QA&QC-HPLC-13 System. The flow rate was 0.7 mL/min, the column temperature 25 °C, and UV-absorption was measured at 254 nm. Solvent A = 0.1% triethylamine a.q. (pH 3.0, adjust with H₃PO₄), solvent B = acetonitrile, with a linear gradient of 30% to 70% B in 40 min. Sample was prepared as 1.0 mg/ml in ACN/H₂O = 50/50 (v/v), injection volume is 1 μl.

Materials and reagents. All chemicals were purchased from Sigma-Aldrich and were used as acquired. Pam₃CSK₄ (N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteiny-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine·3HCl) were purchased from InvivoGen. TurboFect
transfection reagent was purchased from Thermo Fisher Scientific. Antibodies were purchased from Santa Cruz Biotechnology.

**Stability Analysis.** The complexes were stored in 1% Fetal Bovine Serum (FBS) Dulbecco's Modified Eagle Medium (DMEM) at 298 K for 0 h and 6 h. Absorption spectra were recorded on Cary UV-100 Spectrophotometer.4

**Cell culture.** RAW 264.7 cells were cultivated in DMEM supplemented with 10% FBS. The cells were maintained at a density of 1 × 10^7 cells in a T-25 cm² flask and cultured at 37 °C, 5% CO₂, 95% air in a humidified incubator. The cells were pre-treated with complexes (2 µM) for 6 h and then stimulated with Pam₃CSK₄ (200 ng/mL) for 1 h for the following experiments.

**In vitro TNF-α ELISA.** RAW 264.7 cells were placed in 24-well plates at a density of 20,000 cells per well and grown overnight. After 24 h, cells were treated with vehicle, CU-CPT22 and complexes in 1% FBS DMEM for 6 h, and were subsequently co-treated with Pam₃CSK₄ (200 ng/mL) for an additional 1 h. The conditioned medium was collected and the levels of secreted TNF-α were determined by using immunoassay kits (Neobioscience) according to the manufacturer’s protocol. Briefly, 50 μL of Assay Diluent was added to each well, followed by adding 50 μL of the conditioned medium in to the wells. The plate was incubated at 37 °C for 1.5 h to allow the secreted TNF-α to bind to the immobilized antibodies specific for TNF-α. The wells were then washed with wash buffer 5 times and incubated with 100 μL of TNF-α Conjugate at 37 °C for further 1 h. The plate was washed 5 times before the addition of 100 μL of substrate solution to each well. After 30 min incubation at 25 °C in the dark, 100 μL of stop solution was added to each well. The color intensity was measured by using SpectraMax M5 microplate reader (Molecular Devices, California, USA) with excitation at 450 nm and emission at 570 nm.

**NF-κB translocation assay.** The drug-treated RAW 264.7 cells were fixed with 4% formaldehyde diluted in warm PBS for 15 min at 25 °C. The cells were then washed with PBS for three times and blocked in blocking buffer (1× PBS/5% normal serum/0.3% Triton™ X-100) for 1 h. Washed as before, the cells were subsequently incubated in diluted primary antibody overnight at 4 °C. The cells was washed, followed by probing with Alexa Fluor® 594 dye diluted in antibody dilution buffer for 1 h at 25 °C in the dark. After incubation, DAPI was added
to the cells for 15 min. The cells were then imaged using Leica TCS SP8 confocal microscope under 20× magnification.

**Cell morphology imaging.** The living cells after drug treatment were then imaged to capture the changes of cell morphology using Leica TCS SP8 confocal microscope with a highly sensitive photomultiplier (PMT) transmission detector under 63× magnification.

**Pull down assay.** RAW 264.7 cells were pre-treated with vehicle, CU-CPT22 or compound 1 in 1% FBS DMEM for 6 h and then treated with Pam3CSK4 (200 ng/mL) in a 6-well plate for 1 h. The immunoprecipitation assay was performed according to the instructions from the manufacturer. 200 µg of the cell lysates were mixed with 1 µg of anti-TLR2 for overnight. The mixture were then added to Sera-Mag SpeedBeads protein A/G magnetic particles and incubated for additional 1 h of incubation at 25 °C under constant rotation. The particles were collected by a magnetic stand, and the supernatant was discarded. The particles were washed with washing buffer for three times to remove non-specific binding protein. The particles with bound proteins were eluted by boiling in 2× SDS loading buffer at 95 °C for 5 min. The eluted proteins were subjected to electrophoresis SDS-PAGE. The signals of bands were detected by probing with anti-TLR1 and anti-TLR2 antibodies.

**Cell viability assay.** RAW 264.7 cells were seeded at 4,000 cells per well in 96-well plates and incubated overnight at 37 °C. Serial dilutions of complex 1 and its enantiomers ranging from 0–8 µM were added to each well and the plates were incubated at 37 °C in a humidified CO2 incubator for further 72 h. 100 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-tetrazolium bromide) reagent (1 mg/mL) was added to each well. After 4 h incubation, the medium was replaced with 100 µL of DMSO and the plates were incubated at 25 °C for 10 min with shaking. Color intensity was measured at 570 nm using a microplate reader. IC50 values of complex 1 and its enantiomers were determined by the dose-dependence of the surviving cells after exposure 6 and 24 h.

**Data analysis.** All data were presented as the means of three separate experiments. Group comparisons between the control group and various drug treatment groups were done by a one-way ANOVA using GraphPad Prism software (Prism 6.0). Significance was considered if a P-value of <0.05 was reached. Densitometry of western blots was performed by scanning of the exposed film and using Image Lab (Bio-Rad).
Figure S1. UV/Vis absorption of complexes at a concentration of 50 μM in 1% FBS DMEM at 298 K at 0 h and 6 h.
Figure S2. Effect of ligands on TNF-α secretion in Pam₃CSK₄-stimulated RAW 264.7 cells as determined by TNF-α ELISA. (A) Chemical structures of ligand a and b. (B) Pre-treated with ligand a or b at 2 µM for 6 h, then stimulated with Pam₃CSK₄ at 200 ng/mL for 1 h. Culture supernatants were transferred to microtitre wells containing immobilized human TNF-α monoclonal antibody. TNF-α binding was detected using TNF-α primary antibody and HRP-conjugated secondary antibody. Error bars represent the standard deviation of triplicate results.
**Figure S3.** HPLC trace for complex A-1. Integration of peak areas: 92.290: 7.710 e.r., enantiomeric excess is 84.580%.

**Figure S4.** HPLC trace for complex A-1. Integration of peak areas: 96.281: 3.719 e.r., enantiomeric excess is 92.562%.
Figure S5. HPLC trace for complex rac-1.
Figure S6. Cell morphology imaging of the RAW 264.7 cells treated with or without complex 1 and its enantiomers. Cells were pre-treated with complex 1 and its enantiomers at 2 µM for 6 h, then stimulated with or without Pam₃CSK₄ at 200 ng/mL for 1 h. The morphology of the living cells were imaged under 63× magnification with a highly sensitive photomultiplier transmission detector using a Leica TCS SP8 confocal microscope.
Figure S7. Dose-response effect of complex 1 and its enantiomers on RAW 264.7 cell viability as determined by an MTT assay. The cells were treated different concentrations of complex 1 and its enantiomers for 6 h (A) and 24 h (B). IC\textsubscript{50} values of complex 1 and its enantiomers (Δ-1 and Λ-1) after 24 h: 3.1 ± 1.0 μM, 3.1 ± 1.5 μM and 4.7 ± 1.3 μM, respectively. Error bars represent the standard deviation of triplicate results.
References