Electronic Supplementary Information

for

Anti-inflammatory Activity and Enhanced COX-2 Selectivity of Nitric Oxide-Donating Zinc(II)-NSAID Complexes

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Experimental

All reagents and solvents were purchased from commercial sources and were used without further purification. Solvents were distilled and dried before use. 1,10-Phenanthrolinefuroxan (L^1) was prepared according to a literature procedure.[1]

Fourier transform infrared spectroscopy on KBr pellets was performed on a Shimadzu FT-IR 8400S instrument. Elemental analyses were performed on a Perkin Elmer 2400 series II CHN analyzer. Electro-spray ionization (ESI) mass spectra were recorded with a Waters QTOF Micro YA263 instrument. All room temperature NMR spectra were collected on a Bruker Avance 300 MHz spectrometer.

Synthesis of the Complexes:

$[(L^1)ZnCl_2]$ (1). A methanolic solution (5 mL) of zinc(II) chloride (0.025 g, 0.2 mmol) was added to a suspension of 1,10-phenanthrolinefuroxan (L^1) (0.048 g, 0.2 mmol) in methanol (10 mL). The reaction mixture was stirred for 12 h at room temperature to precipitate a white solid. The solid was isolated by filtration, washed thoroughly with methanol and dichloromethane, and dried under vacuum. Yield: 0.067 g (90%). Elemental analysis calcd (%) for **1**: C 38.49, H 1.61, N 14.96; Found: C 38.76, H 1.91, N 14.52. IR (KBr, cm^{-1}): 3456(br), 3056(m), 2923(m), 1631(s), 1589(m), 1510(s), 1411(s), 1087(m), 993(m), 853(m), 740(m), 676(m), 358(s).

$[(L^1)Zn(DCF)_2]$ (1a). A colorless methanolic solution (15 mL) of sodium diclofenac (NaDCF) (0.127 g, 0.4 mmol) was added to a white suspension of **1** (0.075 g, 0.2 mmol) in methanol (15 mL) and the resulting mixture was allowed to stir for 12 h. The solution was filtered and was kept for slow evaporation. A yellow solid was obtained from the concentrated solution. The solid was isolated by filtration, washed thoroughly with methanol and dried under vacuum. Yield: 0.12 g (70%). Elemental analysis calcd (%) for $C_{40}H_{26}Cl_4N_6O_6Zn$ (893.87 g/mol): C 53.75, H 2.93, N 9.40; Found: C 53.67, H 3.15, N 9.09. IR (KBr, cm^{-1}): 3265(br), 1629(s), 1587(s), 1605(s), 1508(s), 1450(s), 1409(s), 1305(m), 711(s), 746(s). ESI-MS (positive ion mode, DMSO/MeCN): *m/z* = 891.12 (35%, $[\mathbf{1a}+H]^+$), *m/z* = 260.98 (100%, $[L^1+Na]^+$). ^1H NMR (300 MHz, DMSO-d_6, 300K): δ (ppm) 3.48 (s, 4H, -CH_2 of DCF), 6.23 (d, 2H, *J* = 7.8 Hz, DCF), 6.76 (T, 2H, *J* = 7.0 Hz, DCF), 6.96 (t, 2H, *J* = 7.0 Hz, DCF), 7.08 (m, 4H), 7.42 (d, 4H, *J* = 8.1 Hz, DCF), 7.92 (m, 2H, phenfuroxan), 8.85 (s, 2H, -NH of DCF), 9.07 (m, 2H, phenfuroxan). ^{13}C NMR (125 MHz,

DMSO-d_6, 298K): δ (ppm) 41.8, 107.6, 115.9, 116.7, 119.3, 120.4, 124.2, 126.3, 126.7, 126.8, 127.0, 128.9, 130.3, 132.5, 134.1, 137.6, 142.9, 148.6, 150.4, 125.1, 176.3.

[(L^1)Zn(NPR)$_2$] (1b). An off-white complex was isolated following a similar method described for **1a** except that sodium naproxen was used instead of sodium diclofenac. Yield: 0.09 g (60%). Elemental analysis calcd (%) for $C_{40}H_{32}N_4O_8Zn$ (762.09 g/mol): C 63.04, H 4.23, N 7.35; Found: C 62.96, H 3.99, N 7.59. IR (KBr, cm^{-1}): 3431(br), 2970(w), 2935(w), 1631(s), 1600(s), 1568(m), 1508(w), 1411(s), 1390(m), 1267(s), 1230(m), 1029(m), 815(m), 734(m). ESI-MS (positive ion mode, DMSO/MeCN): *m/z* = 763.11 (40%, [**1b**+H]$^+$), *m/z* = 521.93 (100%, [**1b**-NPR+CH_3OH]$^+$). ^{1}H NMR (300 MHz, DMSO-d_6, 300K): δ (ppm) 1.33 (s, 6H, -CH_3 of NPR), 3.49 (s, 2H, -CH of NPR), 3.84 (s, 6H, -OCH_3 of NPR), 7.07 (d, 2H, *J* = 5.4 Hz, NPR), 7.22 (s, 2H, NPR), 7.42 (d, 2H, *J* = 6.9 Hz, NPR), 7.64 (m, 4H, NPR), 7.83 (m, 2H, NPR), 8.07 (s, 2H, phenfuroxan), 8.60 (m, 2H, phenfuroxan), 9.05 (s, 2H, phenfuroxan). ^{13}C NMR (125 MHz, DMSO-d_6, 298K): δ (ppm) 19.3, 46.6, 54.9, 105.3, 107.3, 116.3, 118.0, 124.8, 125.8, 126.7, 128.0, 128.7, 132.2, 132.5, 133.8, 138.6, 150.2, 151.2, 156.6, 179.3.

[(L^1)Zn(MFN)$_2$] (1c). To a light yellow colored methanolic solution (10 mL) of sodium salt of mefenamic acid (NaMFN) (0.105 g, 0.4 mmol), a white suspension of **1** (0.074 g, 0.2 mmol) in methanol (10 mL) was added and the resulting mixture was allowed to stir for 12 h to precipitate an orange solid. The solid was isolated by filtration, washed thoroughly with methanol and dried under vacuum. Yield: 0.12 g (78%). Elemental analysis calcd (%) for $C_{42}H_{34}N_6O_6Zn$ (784.15 g/mol): C 64.33, H 4.37, N 10.72; Found: C 64.08, H 4.21, N 10.32. IR (KBr, cm^{-1}): 3471(br), 3265(m), 2925(m), 1635(m), 1614(s), 1679(s), 1498(s), 1409(s), 1384(s), 1284(s), 752(m), 676(m). X-ray quality single crystals were grown from a solvent mixture of dichloromethane and hexane at 273K. ^{1}H NMR (300 MHz, DMSO-d_6, 300K): δ (ppm) 2.01 (s, 6H, -CH_3 of MFN), 2.19 (s, 6H, -CH_3 of MFN), 6.59 (t, 2H, *J* = 7.6 Hz, MFN), 6.74 (d, 2H, *J* = 8.1 Hz, MFN), 6.86 (d, 2H, *J* = 6.9 Hz, MFN), 7.04 (m, 2H, MFN), 7.15 (t, 2H, *J* = 7.0 Hz, MFN), 7.88 (d, 2H, *J* = 6.6 Hz, MFN), 8.03 (m, 2H, phenfuroxan), 8.92 (d, 2H, *J* = 8.1 Hz phenfuroxan), 9.01 (d, 2H, *J* = 8.1 Hz, MFN), 9.19 (m, 2H, phenfuroxan), 10.14 (s, 2H, -NH of MFN). ^{13}C NMR (125 MHz, DMSO-d_6, 298K): δ (ppm) 13.5, 20.2, 108.0, 112.7, 115.9, 117.1, 119.9, 124.8, 125.6, 126.7, 129.7, 131.7, 132.4, 137.4, 139.6, 147.2, 148.9, 150.7, 152.5, 173.2.

[(L^1)Zn($IND)_2$] (1d). The complex was isolated following a similar method described for **1a** except that sodium salt of indomethacin was used instead of diclofenac. Yield: 0.14 g (72%). Elemental analysis calcd (%) for $C_{50}H_{36}Cl_2N_6O_{10}Zn$ (1017.15 g/mol): C 59.04, H 3.57, N 8.26; Found: C 57.52, H 3.67, N 7.30. IR (KBr, cm^{-1}): 2956(w),2929(w), 1676(s), 1623(s), 1593(s), 1475(s), 1406(m), 1321(s), 1226(s), 1089(w), 835(w). ESI-MS (positive ion mode, DMSO/MeCN): *m/z* = 1036.61 (20%, [**1d**+Na]$^+$), *m/z* = 657.78 (100%, [**1d-IND**]$^+$). ^{1}H NMR (300 MHz, DMSO-d_6, 300K): δ (ppm) 2.06 (s, 6H, -CH_3 of IND), 3.38 (s, 4H, -CH_2 of IND), 3.64 (s, 6H, -OCH_3 of IND), 6.57 (s, 2H, IND), 6.84 (m, 4H, IND), 7.62 (s, 8H, IND), 7.88 (s, 2H, phenfuroxan), 8.92 (m, 4H, phenfuroxan). ^{13}C NMR (125 MHz, DMSO-d_6, 298K): δ (ppm) 13.1, 31.8, 55.1, 78.9, 101.8, 107.4, 110.7, 114.0, 115.9, 116.4, 118.9, 126.3, 127.7, 128.9, 129.9, 130.9, 132.6, 133.8, 134.2, 137.4, 148.3, 150.1, 151.8, 155.1, 167.5, 175.3.

[(phendione)Zn($MFN)_2$] (2). A methanolic solution (5 mL) of zinc(II) chloride (0.025 g, 0.2 mmol) was added to a solution of 1,10-phenanthroline-5,6-dione (0.042 g, 0.2 mmol) in methanol (5 mL). The resulting mixture was allowed to stir for 5 min to produce a yellow solution. Then a colorless methanolic solution (10 mL) of sodium salt of mefenamic acid (NaMFN) (0.105 g, 0.4 mmol) was added and the resulting reaction mixture was stirred for about 12 h to obtain a deep green solid. The solid was isolated by filtration, washed thoroughly with methanol and dried under vacuum. Yield: 0.13 g (86%). Elemental analysis calcd (%) for $C_{42}H_{34}N_4O_6Zn$ (756.13 g/mol): C 66.71, H 4.53, N 7.41; Found: C 65.55, H 4.57, N 7.42. IR (KBr, cm^{-1}): 3421(br), 3263(m), 3068(w), 2941(w), 1691(s), 1614(s), 1579(s), 1508(s), 1472(m), 1431(m), 1388(s), 1286(s), 1155(m), 815(m), 752(m), 734(m). ESI-MS (positive ion mode, DMSO/MeCN): *m/z* = 756.2 (10%, [**2**+H]$^+$), 514.2 (40%, **[2-**MFN**]**$^+$). ^{1}H NMR (300 MHz, DMSO-d_6, 298K): δ (ppm) 2.03 (s, 6H, -CH_3 of MFN), 2.19 (s, 6H, -CH_3 of MFN), 6.59 (t, 2H, *J* = 7.3 Hz, MFN), 6.76 (d, 2H, *J* = 8.1 Hz, MFN), 6.87 (d, 2H, *J* = 6.9 Hz, MFN), 7.1 (m, 6H), 7.89 (d, 2H, *J* = 7.8 Hz, MFN), 7.94 (s, 2H, phendione), 8.64 (s, 2H, phendione), 9.09 (s, 2H, phendione), 10.22 (s, 2H, -NH of MFN). ^{13}C NMR (125 MHz, DMSO-d_6, 298K): δ (ppm) 13.6, 20.2, 111.3, 113.1, 116.2, 122.2, 125.2, 126.0, 129.0, 131.2, 131.7, 134.2, 135.7, 137.9, 138.4, 148.8, 152.3, 154.4, 170.2, 177.8.

Stability Studies: The UV-vis spectra of the compounds (0.1 mM) were recorded on an Agilent 8453 diode array spectrophotometer. Compounds were dissolved in DMSO and then diluted to

desired concentration with PBS buffer of pH 7.4, and collected the electronic spectra at different time interval.

Stability Studies of the Complexes under Cell Culture Conditions: *In vitro* stability of the complexes under cell culture conditions (10% DMSO-DMEM medium) was investigated based on a method reported in the literature.[2] Following this method, a zinc(II)-NSAID complex was dissolved in 500 µL DMSO-DMEM medium, and was transferred immediately to a dialysis bag with molecular weigµht cut off between 500 Da and 1000 Da. This choice of molecular weight cut off for dialysis bag was made because the small molecular weight NSAIDs (MW < 400 g/mol) could easily diffuse out of the dialysis bag if the corresponding complexes degraded in the culture medium. The bag was then placed in a beaker containing DMEM (80 mL). The outer phase was stirred continuously, and after specific intervals, 1.2 mL of sample was withdrawn from the outer phase. The outer phase was again replenished with 1.2 mL DMEM. The absorbance of the outer phase was monitored by UV-vis spectroscopy at different time interval over 72 h.

Nitric Oxide Release *in vitro*: Nitric oxide released from the complexes were measured according to the literature procedure.[3] A standard curve of nitrite concentration against absorbance was measured as reported in literature.[4] All the complexes were diluted to 150 µM with phosphate buffer solution (PBS) at pH 7.4 containing 5 mM L-cysteine and then Griess reagent was added to the solution to react with the nitrite at different time. The % of nitric oxide release was calculated from the absorbance of the peak at 540 nm.

Detection of Nitric Oxide in Living Cells: The release of nitric oxide from the complexes inside the cells was detected by the 'Nitric Oxide Flourometric Cell-Based Assay Kit' (10009419 Cayman Chemical). The assay was performed as per the manufacturer's protocol. According to the protocol, 1×10^5 RAW 264.7 cells were seeded in a 96 well plate and left to adhere overnight. Then the cells were treated with and without 100 µM of complex **1b.** After 4h the cell supernatants were removed and the wells were washed thoroughly with the assay buffer supplied with the Kit. Then the cells were incubated for 10 min with 100 µL of Hoechst dye solution. The wells were again washed with the assay buffer and examined under fluorescent microscope instantly. Nitric oxide distribution was detected using a filter at excitation and emission

wavelength of 485 and 535 nm, respectively. Hoechst dye staining cells were visualized with a UV filter.

Cytotoxicity Studies: Cytotoxicity of all the compounds (**1a-1d**), their parent compounds, and a physical mixture of 1,10-phenanthrolinefuroxan (L^1) and zinc(II) salt, against RAW 264.7 cells were determined by means of the colorimetric MTT [3 (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay.[5] In brief, 10^4 cells/well were seeded in a 96-well plate and left to attach overnight in a humidified incubator at 37˚C and 5% CO_2. The test complexes were prepared as a stock (2 mM) in DMSO and diluted with culture medium to desired concentration prior to addition to the cells. The culture medium was replaced with medium containing the compounds at various concentrations and incubated for 24 h, 48 h and 72 h at 37^{o}C in a 5% CO_2 incubator. The medium was then replaced with 100 µl MTT (1 mg/mL) stain per well, incubated for 3 h at 37˚C and subjected to DMSO (100 µL/well). Incubation was carried out for 10 min at 37˚C, and the absorbance was recorded at 570 nm using a plate reader (VARIOSKAN Flash). The yellow MTT, a tetrazole, gets oxidized by the cells with intact mitochondria and produce purple formazan compounds which gets dissolved in DMSO. Hence, only live cells can convert tetrazole to formazan giving a characteristic purple color. The percentage of cell viability in the treated wells was measured in comparison with the control wells. The values are expressed as the mean from three experiments and the bars in the figures indicate standard deviations. All the experiments were carried out in quintuplication.

Anti-Inflammatory Activity Assay: To check whether the complexes **1a-d** are able to act as anti-inflammatory agent or not, anti-inflammatory activity assay was performed by estimating the production of PGE_2 using standard literature protocol.[6] Briefly 1×10^6 RAW 264.7 cells were seeded in a 6-well culture plate and left to adhere overnight. The cells were treated with 1 µg/mL bacterial endotoxin lipopolysaccharide (LPS) and 100 ng/mL mouse cytokine interferon-gamma (IFN-γ) with and without test compounds for 24 h. The amount of secreted PGE_2 was determined in cell culture supernatants using a Prostaglandin E2 EIA kit (Cayman Chemicals, Ann Arbor, MI), according to the manufacturers protocol.

COX Inhibitor Screening Assay: In order to determine the pathway of the inhibition of PGE_2 production and the efficacy of the synthesized complexes **1a-d** in inhibiting COX enzyme *in vitro*, 'COX Fluorescent Inhibitor Screening Assay Kit' (700100; Cayman Chemical) was used.

The assay was performed as per the manufacturer's protocol and the assay plate was analyzed by ELISA plate reader (VARIOSKAN Flash) with an excitation wavelength of 540 nm and an emission wavelength of 595 nm. All the compounds were dissolved in DMSO and assayed in triplicate.

X-ray Crystallographic Data Collection, Refinement and Solution of the Structure: X-ray single-crystal data for **1c** were collected at 100 K using Mo-K_α (λ = 0.7107 Å) radiation on a SMART-APEX diffractometer equipped with CCD area detector. Data collection, data reduction, structure solution and refinement were carried out using the software package of APEX II.[7] The structure was solved by direct method and subsequent Fourier analyses and refined by the full-matrix least-squares method based on F^2 with all observed reflections.[8] The non-hydrogen atoms were treated anisotropically. CCDC 1430924 contains the supplementary crystallographic data for this paper. The data can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Crystal data of **1c**: MF = $C_{43}H_{34}Cl_2N_6O_6Zn$, MW = 867.03, Triclinic, space group-*P*-1, a = 11.3465(9), b = 12.7466(11), c = 15.1169(13)Å, V = 2073.0 Å^3, α = 105.715(3), β = 93.520(3), γ = 97.969(2)°, Z = 2, ρ = 1.389 mgm^{-3} F(000)= 892, μMo-Kα = 0.776 mm^{-1}, GOF = 1.028, A total of 28114 reflections were collected in the 1.41 ≤ θ ≤ 27.50 range of which 9174 were unique (R_{int} = 0.0360). R1(wR2) = 0.0839 (0.2731) for 527 parameters and 5905 reflections ($I > 2\sigma(I)$).

Table S1 *Selected bond distances (Å) and angles (°) for complex [(L^1)Zn(MFN)$_2$] (**1c**).*

Zn1-O5	1.942(4)	Zn1-O3	1.964(3)
Zn1-N4	2.094(4)	Zn1-N3	2.099(4)
O4-C27	1.255(5)	O3-C27	1.276(5)
O6-C42	1.258(5)	C1-N1	1.327(6)
O5-C42	1.298(5)	N1-O1	1.406(6)
N2-C2	1.301(6)	N2-O2	1.150(6)
N2-O1	1.426(6)		
O5-Zn1-N3	109.03(13)	C27-O3-Zn1	102.1(3)
O3-Zn1-N3	125.35(14)	O5-Zn1-O3	111.00(14)
N4-Zn1-N3	79.12(14)	O5-Zn1-N4	122.85(14)
C42-O5-Zn1	104.7(3)	O3-Zn1-N4	107.31(13)

Table S2 *IC_{50} values of complexes* ***1a-d*** *on RAW 264.7 cell line.*

Complex	IC_{50} (μM)
1a	16.66 ± 1.24
1b	14.08 ± 0.78
1c	17.10 ± 0.58
1d	17.16 ± 1.04

Table S3 *Nitric oxide release by the complexes in the presence of excess L-cysteine.*

Compound	% of NO release (24 h)	% of NO release (72 h)
L^1	-	52
1a	16	25
1b	36	42
1c	27	33
1d	33	35

Table S4 *Release of nitric oxide (NO) from complex* ***1b*** *at different pH for 24h and 72h.*

24h			72h		
pH 7.4	**pH 6.5**	**pH 5.5**	**pH 7.4**	**pH 6.5**	**pH 5.5**
36	34	33	42	40	38

Table S5 *Production of PGE_2 in RAW 264.7 cell induced by LPS and IFN-γ in the absence/presence of test compounds at 10 μM concentration. Values in parentheses denote the standard deviation.*

Compound	% of PGE_2 production	Compound	% of PGE_2 production
Control	0.06	L+ γ	100
L+ γ+L^1	90.35 (± 8.10)	L+ γ+Zn	80.62 (± 19.41)
L+ γ+NaDCF	0.97 (± 0.07)	L+ γ+**1a**	1.12 (± 0.80)
L+ γ+NaNPR	3.20 (± 1.76)	L+ γ+**1b**	2.41 (± 0.10)
L+ γ+MFN	0.63 (± 0.10)	L+ γ+**1c**	1.00 (± 0.08)
L+ γ+IND	1.02 (± 0.07)	L+ γ+**1d**	1.06 (± 0.11)

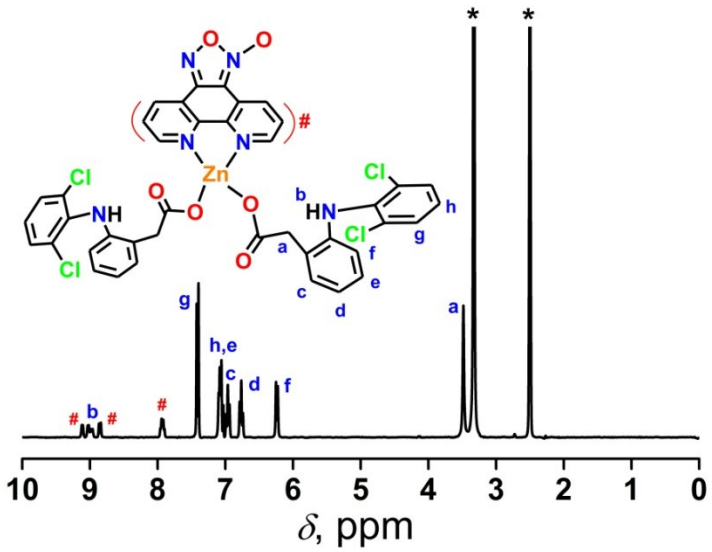

Fig. S1 *^{1}H NMR spectrum (300 MHz, 300 K, DMSO-d_6) of complex **1a**. Peaks marked with * are derived from residual solvents, and the peaks marked with # are from 1,10-phenanthrolinefuroxan ligand.*

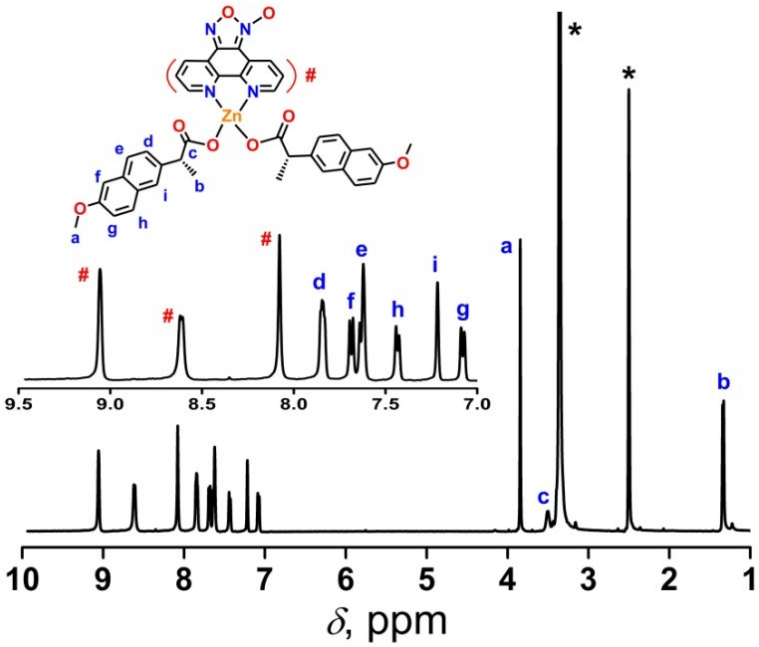

Fig. S2 *^{1}H NMR spectrum (300 MHz, 300 K, DMSO-d_6) of complex **1b**. Peaks marked with * are derived from residual solvents, and the peaks marked with # are from 1,10-phenanthrolinefuroxan ligand.*

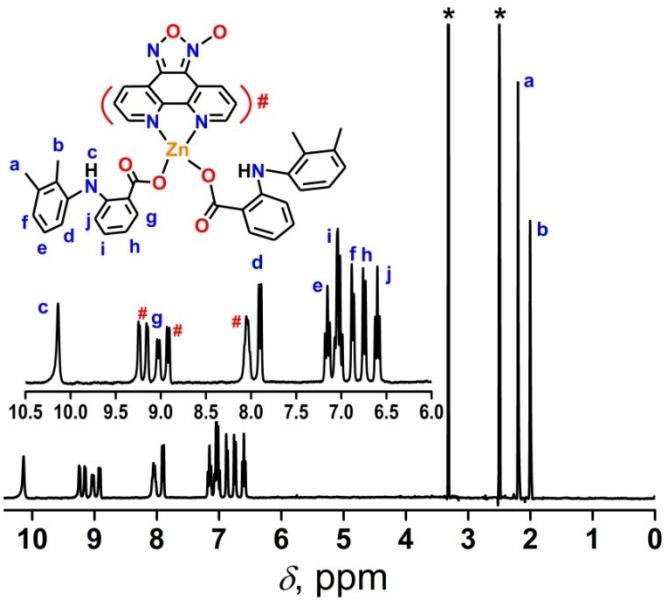

Fig. S3 *^{1}H NMR spectrum (300 MHz, 300 K, DMSO-d_6) of complex **1c**. Peaks marked with * are derived from residual solvents, and the peaks marked with # are from 1,10-phenanthrolinefuroxan ligand.*

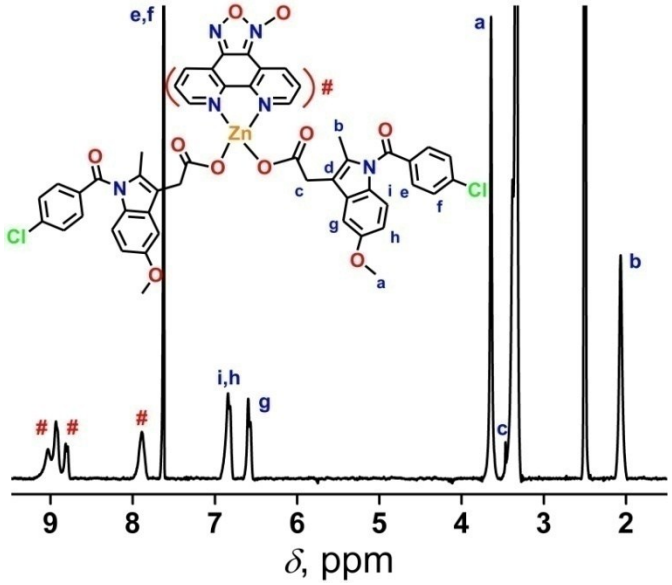

Fig. S4 *^{1}H NMR spectrum (300 MHz, 300 K, DMSO-d_6) of complex **1d**. Peaks marked with * are derived from residual solvents, and the peaks marked with # are from 1,10-phenanthrolinefuroxan ligand.*

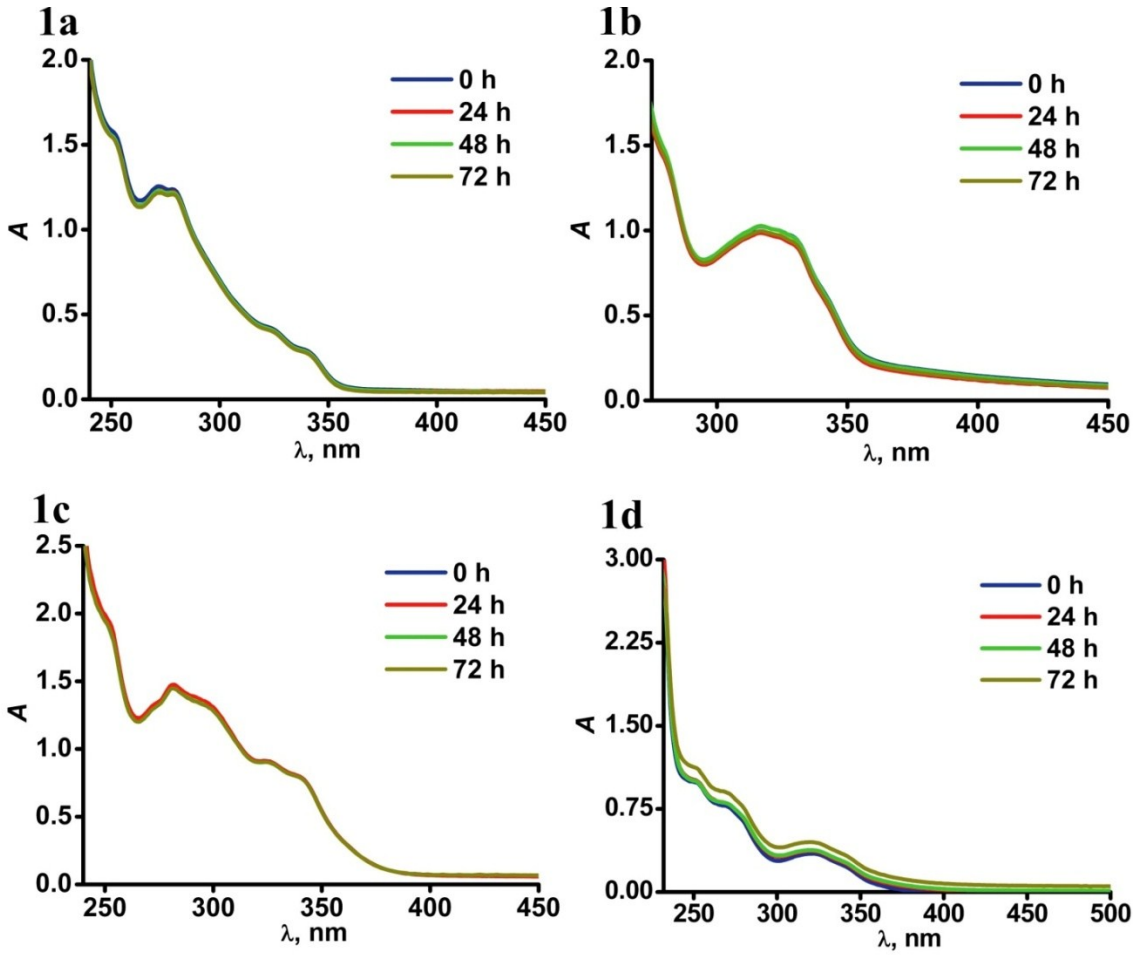

Fig. S5 *UV-Vis spectra of the complexes* ***1a-d*** *(0.1 mM) in 2% DMSO-PBS buffer solution recorded at different time interval over 72h of incubation at 310 K.*

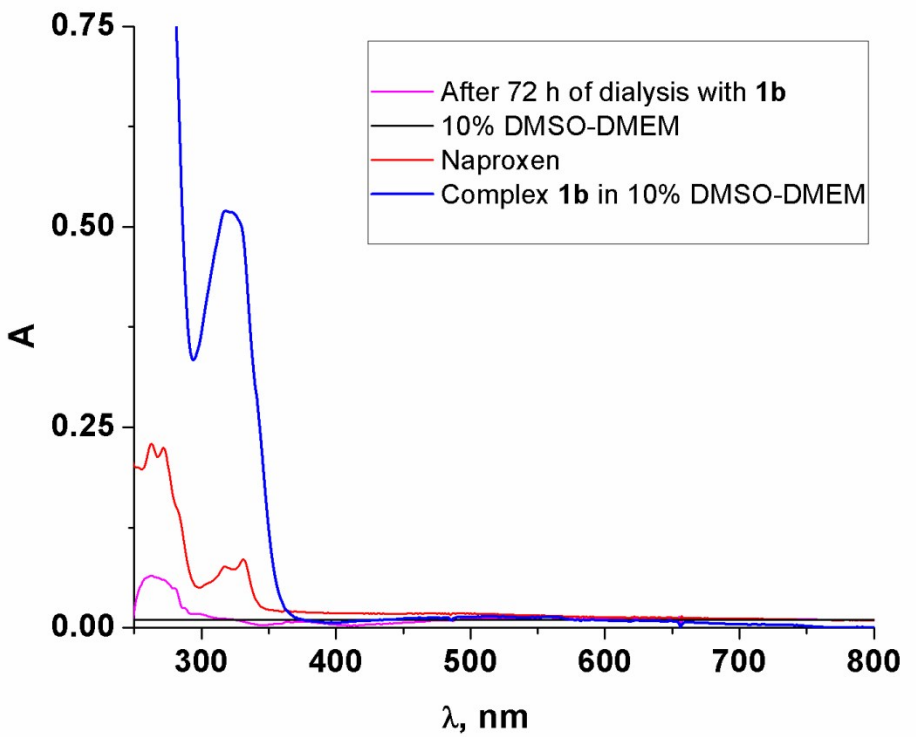

Fig. S6 *UV-Vis spectra of complex* ***1b*** *(0.1 mM in 10% DMSO-DMEM medium), naproxen and the solution after dialysis of* ***1b*** *for 72 h.*

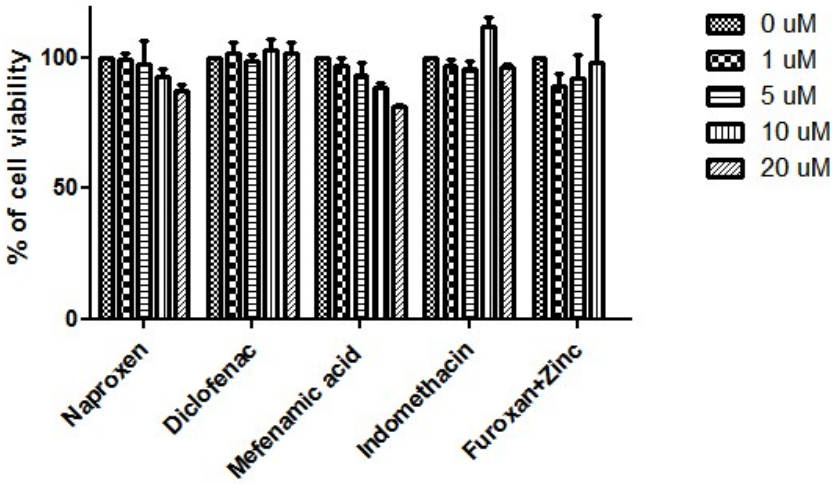

Fig. S7 *MTT assay of the free NSAIDs, and a physical mixture of of 1,10-phenanthrolinefuroxan (L^1) and zinc(II) salt on mouse macrophage RAW 267.4 cell line for 72h.*

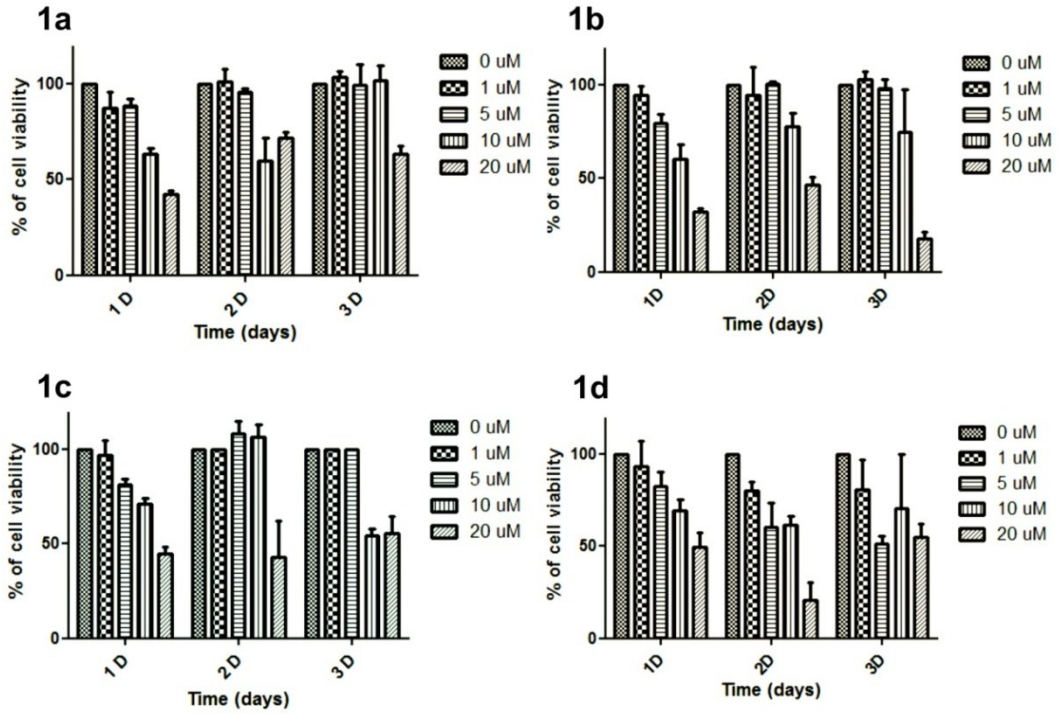

Fig. S8 *MTT assay of the complexes on mouse macrophage RAW 267.4 cell line; 1D, 2D, and 3D stand for 24, 48, and 72 h, respectively.*

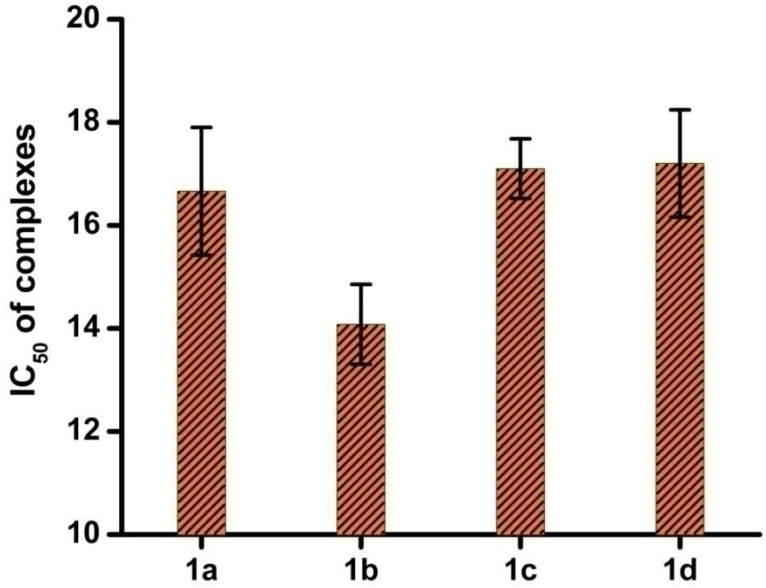

Fig. S9 *IC_{50} values of the complexes **1a-d** on mouse macrophage cell line RAW 264.7 after 24h.*

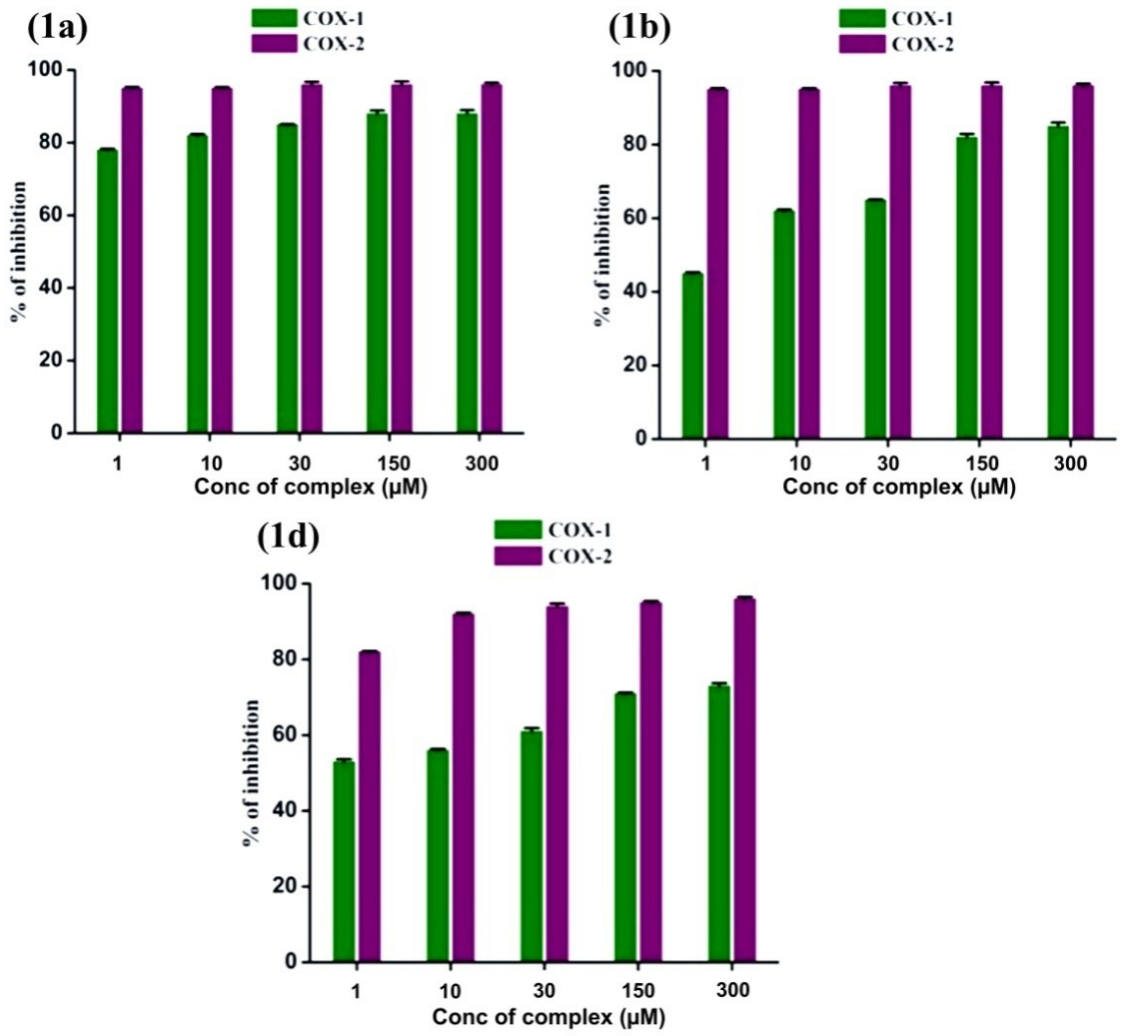

Fig. S10 *COX inhibition assay of complexes* ***1a****,* ***1b*** *and* ***1d****.*

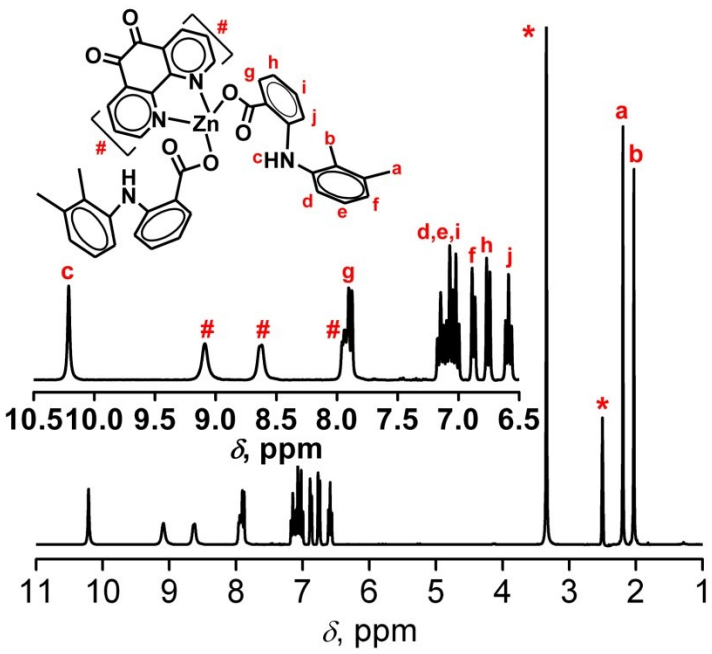

Fig. S11 *^{1}H NMR spectrum (300 MHz, DMSO-d_6, 300 K) of [(phendione)$Zn^{II}(MFN)_2$] (**2**). The peaks marked with * are from residual solvents, # is from phendione unit and the other peaks are from the mefenamic acid unit.*

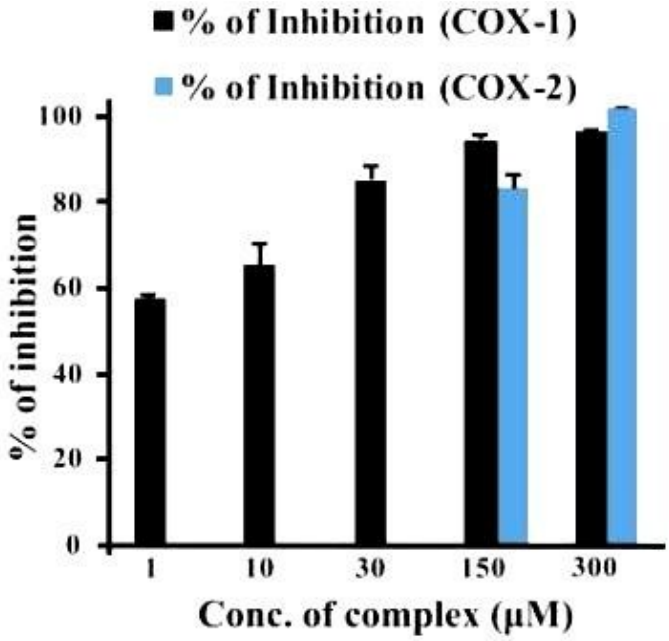

Fig. S12 *COX inhibition assay of complex **2**.*

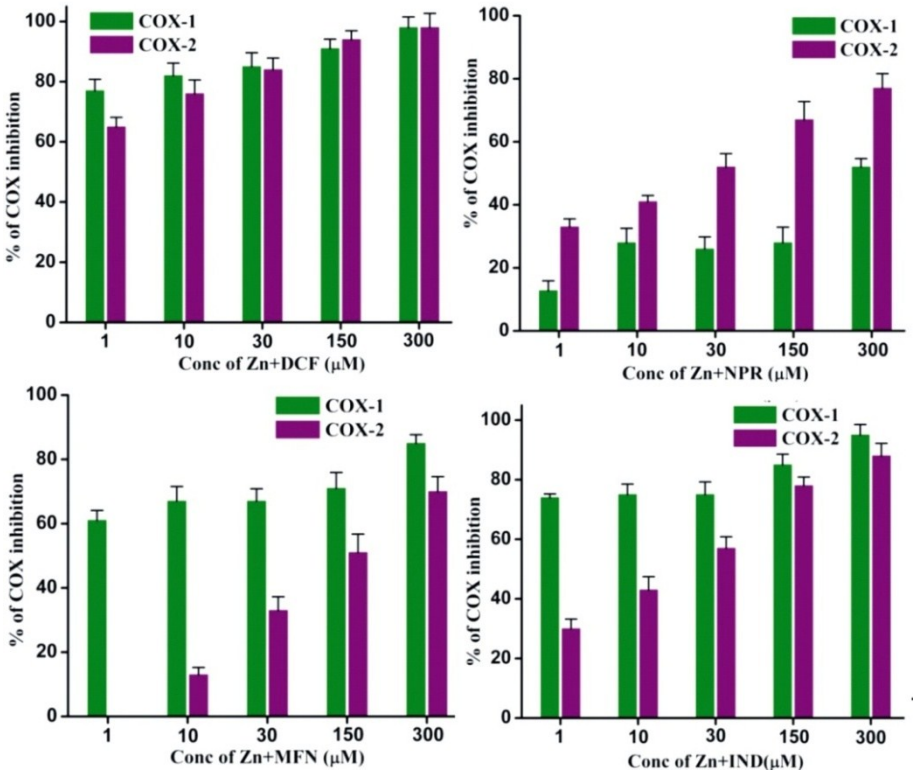

Fig. S13 *COX inhibition assay of the physical mixtures of zinc(II) salt and free NSAIDs.*

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