Supporting Information

Coordination-Triggered NO Release from a Dinitrosyl Iron Complex Leads to Anti-Inflammatory Activity

Kelsey M. Skodje, Min-Young Kwon, Su Wol Chung,* and Eunsuk Kim*

Contents **Page**

Materials and Methods

General. All non-biological syntheses and manipulations were performed under an inert atmosphere using an MBraun glovebox (< 0.1 ppm O_2, <0.1 ppm H_2O) or standard Schlenk line techniques unless stated otherwise. Solvents were purified by passing through alumina columns under an Ar atmosphere (MBraun solvent purification system) and stored over 4 Å molecular sieves prior to use. Silver hexafluorophosphate ($AgPF_6$), iron phthalocyanine (PcFe), and ferrocenium hexafluorophosphate ($FcPF_6$) were purchased from Sigma-Aldrich, St. Louis, MO and used as received. Nitric oxide (Matheson, 99%) was purified by following the literature method,[1] in which the NO gas stream was passed through an Ascarite column, and then distilled at –80ºC. Complexes $[Fe(TMEDA)(NO)_2]$ (**1**),[2] $[Fe(TMEDA)(NO)_2I]$ (**2**),[3] and $[Fe(TMEDA)Cl_2]_2$ (**3**)[4] were synthesized by following literature methods. Using the same procedures[2] as for the synthesis of $[Fe(TMEDA)(NO)_2]$ (**1**), ^{15}NO labeled **1** (~50 % ^{15}NO enrichment) was prepared using a 1:1 mixture of $Na^{15}NO_2$ (98% ^{15}N enriched, Aldrich) and $NaNO_2$ (Sigma-Aldrich, St. Louis, MO). Infrared spectra were collected with a Bruker Tensor 27 FT-IR spectrometer. X-band EPR spectra were recorded on a Bruker Elexsys II E500 spectrometer.

Reaction of $[Fe(TMEDA)(NO)_2]$ (1) with $FcPF_6$

Under a nitrogen atmosphere, 20 mg (85 mmol) of $[Fe(TMEDA)(NO)_2]$ (**1**) were dissolved in 2 mL MeCN in a 10 mL, 2-necked round-bottomed flask (rbf). One neck was sealed with a septum and the other was connected to a tubing adaptor with a 2-way stopcock. Rubber tubing was used to connect this flask to a 10 mL Schlenk flask, which was under negative pressure, and contained 48 mg iron(II) phthalocyanine (PcFe, 85 mmol, 1.0 eq.) in 3 mL MeCN. Then, a 1 mL MeCN solution containing 28 mg ferrocenium hexaflurophosphate ($FcPF_6$, 85 mmol, 1.0 eq.) was injected through the rubber septum of the rbf containing **1** and the stopcocks were opened so that the headspace gas could travel through the rubber tubing. Both flasks were stirred overnight. The next day, the solvent was removed from each by vacuum and infrared spectra of the solids were collected in KBr (Figure S1). For the ^{15}NO labeling experiment, the reaction

between **1** and $FcPF_6$ was prepared exactly as in the reaction described above, except that ^{15}NO enriched (50%) **1** was used (Figure S3).

Reaction of [Fe(TMEDA)$(NO)_2$I] (2) with silver hexafluorophosphate

The reaction between [Fe(TMEDA)$(NO)_2$I] (**2**) and silver hexafluorophosphate ($AgPF_6$) was set up in the same way as the reaction described previously with **1** and $FcPF_6$. In this case, 20 mg (55 mmol) of **2**, 32 mg (85 mmol, 1.0 eq.) of $FcPF_6$, and 32 mg of iron(II) phthalocyanine (55 mmol, 1.0 eq.) were used, in approximately the same volumes of MeCN. The course of the reaction was followed by IR spectroscopy (Figure S2).

Reaction of iron phthalocyanine (PcFe) with $NO_{(g)}$

In order to confirm the literature assignment of ν_{NO} in PcFe-NO,[5] a solution of 20 mg of PcFe in 3 mL MeCN was placed in a 25 mL flask under slight vacuum. Into the headspace of this flask, 2.4 mL $NO_{(g)}$ (110 mmol, 1.2 eq.) were injected and the solution was stirred overnight. The solvent was removed by vacuum the next day and an IR of the solid was taken in KBr (Figure S4).

EPR Spectroscopy

An X-band EPR spectrum of the product of the reaction between [Fe(TMEDA)$(NO)_2$] (**1**) and $FcPF_6$ (Figure S5) was recorded at 10 K in THF with a microwave power of 20 mW, frequency at 9.386 GHz, and modulation amplitude of 1.6 G at 100 kHz. The EPR sample was prepared by making a 1 mM THF solution of **1** and one equivalent of $FcPF_6$, mixing for 1-2 minutes, and then storing at 77 K until the spectrum was collected.

Cell culture and reagents. Lipopolysaccharide (LPS) from *Escherichia coli* was purchased from Sigma-Aldrich, St Louis, MO. The stock solutions of complexes **2** and **3** were freshly prepared for each experiment and were prepared in DMSO and serially diluted (in DMSO) to create 100 mM solutions. The prepared stock solutions were then

used to treat the cells with 10 to 500 μM. The final concentration of DMSO was maintained at 0.5 % throughout. Murine macrophages (RAW 264.7 cells) were cultured in T-75 flask with Dulbecco's modified Eagle's medium (Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% FBS (Invitrogen Life Technology, Carlsbad, CA) and 100 U⁄ml penicillin and streptomycin (Invitrogen Life Technology, Carlsbad, CA). During incubation, the culture medium was changed every 3 days. All cultures were maintained at 37°C under 5% CO_2 with 95% relative humidity. On the day prior to the treatment of **2** or **3**, the cells (2×10^6 cells/well) were plated into individual wells of 6-well plates and allowed to adhere overnight. On the following day, the cells were left treated with LPS (100 ng/mL), or treated with 10 to 500 μM of the test complexes (**2** or **3**) and LPS (100 ng/mL), or treated with 0.5 % DMSO as a vehicle for 0.5 to 12 hours.

Antibodies and Western blot analysis. Western imunoblotting was performed as previously described.[6] Briefly, the cells were harvested using RIPA buffer (Sigma-Aldrich, St Louis, MO) with protease inhibitors (Roche Applied Science, Mannheim, Germany). Protein concentrations of cell lysates were determined using Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL). The samples were resolved by 12% SDS-PAGE gels, and transferred to PVDF membranes (BIO-RAD, Hercules, CA) overnight (120 mA). The transferred membranes were hybridized with various antibodies (diluted 1:1000) overnight at 4°C, followed by HRP-conjugated IgG, and visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). HO-1 polyclonal antibody (StressGen Biotechnologies Corp., Victoria, Canada), anti-iNOS (Santa Cruz Biotechnology, Santa Cruz, CA), and GAPDH polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were used for Western blotting analysis.

RNA isolation and real-time reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from RAW 264.7 cells was isolated using TRIzol reagent (Invitrogen Life Technology, Carlsbad, CA), and reverse transcription was performed using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen Life Technology, Carlsbad, CA). The sequences of primers were as follows: mouse HO-1 forward primer, 5'- CGCCTTCCTGCTCAACAT T -3'; reverse primer, 5'-

TGTGTTCCTCTGTCAGCATCAC -3', mouse iNOS forward primer, 5'-AACGGAGAACGTTGGATTTG -3'; reverse primer, 5'- CAGCACAAGGGGTTT TCTTC -3', and β-actin forward primer, 5'- GATCTGGCACCACACCTTCT -3'; reverse primer, 5'- GGGGTGTTGAAGGTCTCAAA -3'. Amplification of cDNA started with 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 59°C. Real-time quantitative PCR for HO-1, iNOS, and β-actin was conducted using iQ^{TM} SYBR$^{®}$ Green Supermix (BIO-RAD, Hercules, CA).

Enzyme-linked immunosorbent assay (**ELISA).** The mouse TNF-α, IL-6, and IL-10 released into the culture medium was measured using an ELISA kit (mouse TNF-α and IL-6) from R&D Systems, Inc. (Minneapolis, MN), following the manufacturer's instructions. In brief, the ELISA plates (BD Biosciences, San Jose, CA) were coated with a monoclonal anti-mouse TNF-α, IL-6, and IL-10 antibody (2 mg/mL) in coating buffer (1% BSA in PBS, pH 7.2~7.4) for overnight at room temperature. Then, the plates were blocked with coating buffer for 2 hours at room temperature, and incubated with wither recombinant mouse TNF-α, IL-6, and IL-10 standards or the samples collected in quadruplicate (100 ml/well) for another 2 hours. The plates were then incubated with a biotinylated mouse TNF-α, IL-6, and IL-10 antibody (150 ng/mL) for 2 hours, and freshly diluted streptavidin-HRP for 20 minutes subsequently in dark. After each step, the plates were washed three times with the washing buffer. A chromogen substrate tetramethylbenzidine (100 ml/well) (eBioscience, Inc., San Diego, CA) was added and incubated for 5 minutes in dark. The reaction was stopped by adding 2 N H_2SO_4 (50 ml/well), and the plates were read at 450 nm with an automatic ELISA reader (MERK SensIdent Scan, Helsinki, Finland).

Cell Viability Assay. Cells were seeded at 1×10^4 cells per well in 96-well plates and were treated for 24 hours with vehicle (0.5 % DMSO), **2** or **3** at serial concentrations (0, 10, 100, 500 μM) in the presence of LPS (100 ng/ml). Cell viability was determined based on mitochondrial dehydrogenase activity assays using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI) according to the manufacturer's instructions. The CellTiter 96® AQueous One Solution reagent contains a

tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium] and an electron coupling reagent (phenazine ethosulfate). Briefly, after 24 hours treatment, 20 µL of CellTiter 96® AQueous One Solution was added to each well containing 100 µl of culture medium. Plates were incubated for an additional 3 hours at 37°C, and absorbance at 490 nm of the plates was measured using a SpectraMax M2 microplate reader (Molecular Devices, USA) to calculate the cell survival percentages.

Statistical analysis. Data are represented as mean ± SD. For comparisons between two groups, we used Student's two-tailed unpaired *t* test. For comparisons of timed series experiments, we performed Student's paired *t* tests. Statistically significant differences were accepted at $p<0.05$.

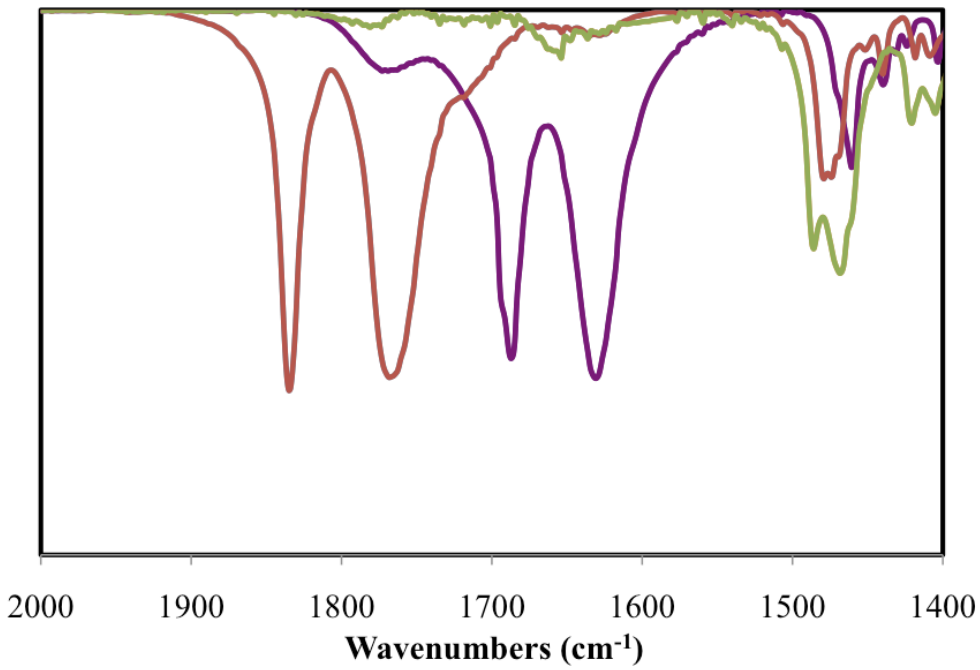

Figure S1. IR spectrum (KBr) of [Fe(TMEDA)$(NO)_2$] (**1**) before (purple line, ν_{NO} = 1629, 1690 cm^{-1}) and after mixing with ferrocenium hexafluorophosphate ($FcPF_6$) for 5 min. to form meta-stable intermediate (red line, ν_{NO} = 1769, 1835 cm^{-1}), and after several hours (green line)

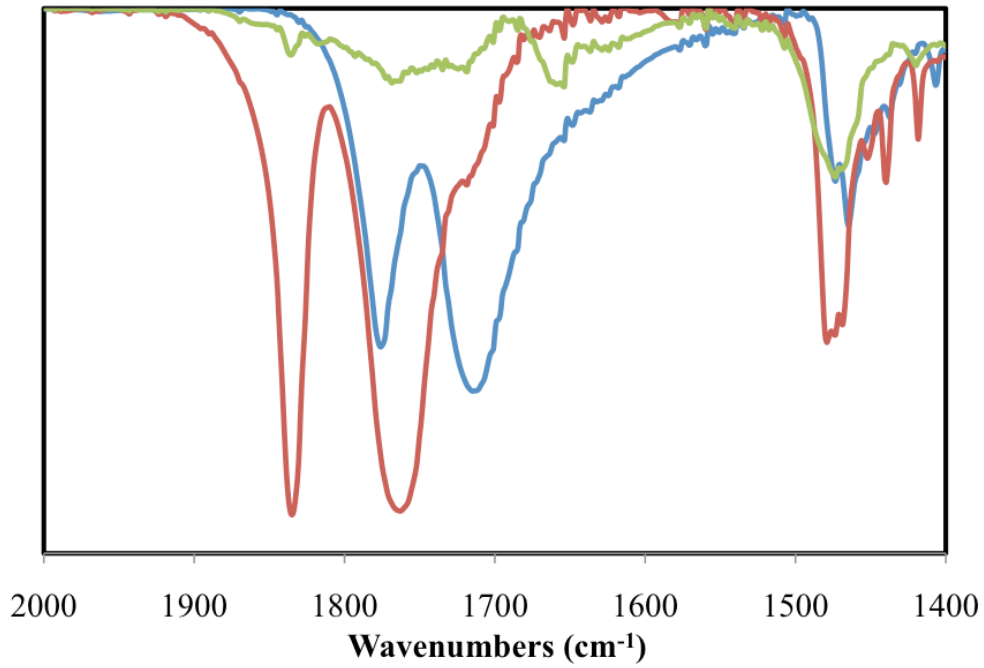

Figure S2. IR spectrum (KBr) of [Fe(TMEDA)$(NO)_2$I] (**2**) before (blue line, ν_{NO} = 1719, 1777 cm^{-1}) and after mixing with silver hexafluorophosphate ($AgPF_6$) for 5 min. to form meta-stable intermediate (red line, ν_{NO} = 1769, 1835 cm^{-1}), and after several hours (green line).

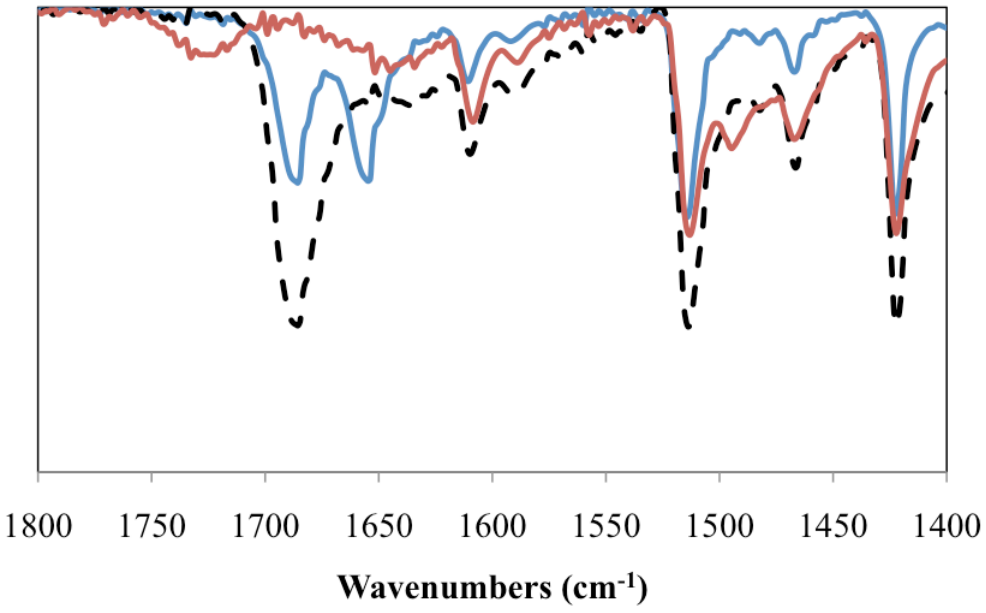

Figure S3. IR spectra (KBr) of iron(II) phthalocyanine (PcFe) before (red line) and after (black dashed line) exposure to headspace of [Fe(TMEDA)$(NO)_2$] (**1**)/$FcPF_6$ reaction (ν_{NO} = 1686 cm^{-1}), and that after exposure to headspace of 50% enriched [Fe(TMEDA)$(^{15}NO)_2$] (**1**)/$FcPF_6$ reaction (blue line, ν^{15}_{NO} = 1654 cm^{-1}, $\Delta\nu$ ($^{15}NO/^{14}NO$) = 32 cm^{-1}).

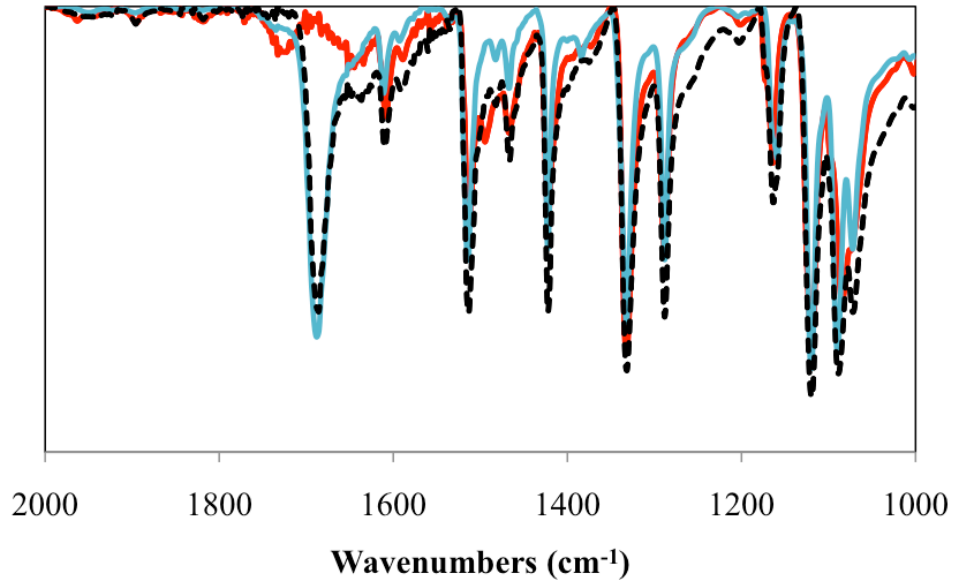

Figure S4. IR spectrum (KBr) of PcFe before (red line) exposure to $NO_{(g)}$, after exposure to $NO_{(g)}$ (light blue line, ν_{NO} = 1686 cm^{-1}), and after (black dashed line) exposure to headspace from [Fe(TMEDA)$(NO)_2$] (**2**)/$AgPF_6$ reaction (ν_{NO} = 1686 cm^{-1}).

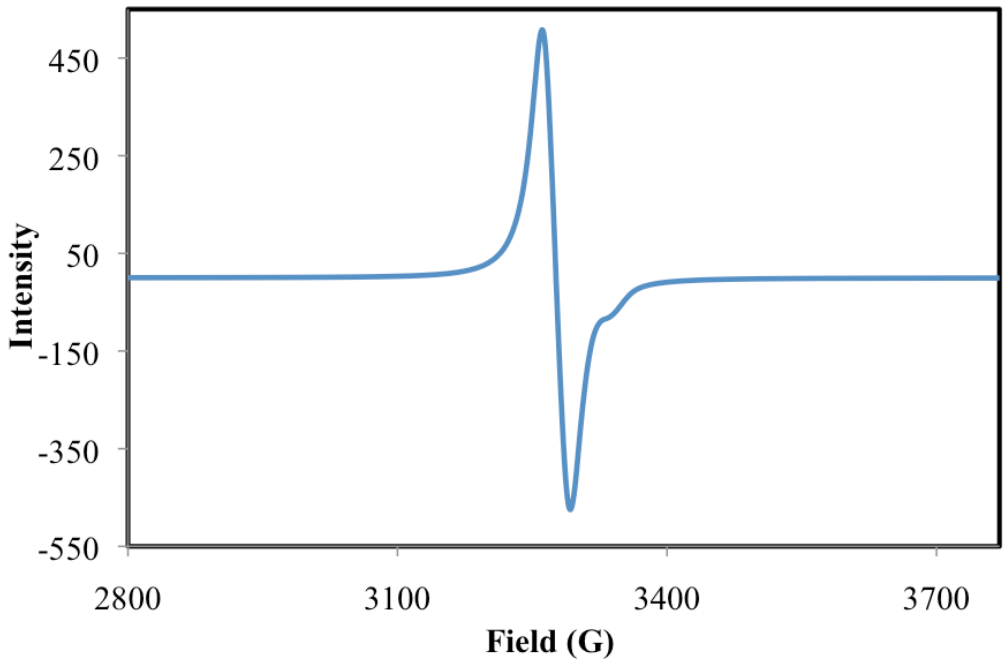

Figure S5. X-band EPR spectrum of a meta-stable reaction intermediate generated from [Fe(TMEDA)$(NO)_2$] (**1**) and ferrocenium hexafluorophosphate ($FcPF_6$) taken at 10 K in THF. (g = 2.04).

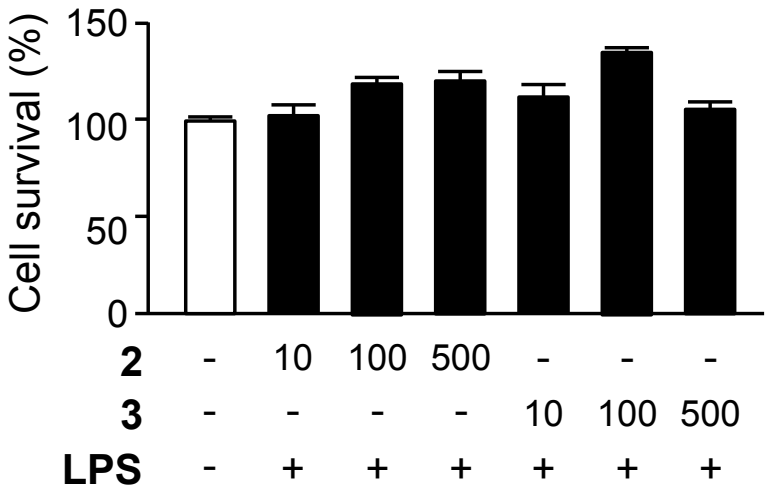

Figure S6. The effects of [Fe(TMEDA)$(NO)_2$I] (**2**) and [Fe(TMEDA)$Cl_2]_2$ (**3**) on cell survival. RAW 264.7 cells were treated with vehicle (DMSO), **2** (10, 100, 500 μM), or **3** (10, 100, 500 μM) plus LPS (100 ng/mL), and MTS solution was added 24 hours after treatment. After 3 hours incubation with MTS solution, absorbance was measured at 490 nm to calculate the cell survival. Values are mean ± SD, n=8. Results are representative for three independent experiments.

References

1. Schopfer, M. P.; Mondal, B.; Lee, D.-H.; Sarjeant, A. A. N.; Karlin, K. D., *J. Am. Chem. Soc.* **2009**, *131*, 11304-11305.
2. Hung, M.-C.; Tsai, M.-C.; Lee, G.-H.; Liaw, W.-F., *Inorg. Chem.* **2006**, *45*, 6041-6047.
3. Chen, C.-H.; Ho, Y.-C.; Lee, G.-H., *J. Organomet. Chem.* **2009**, *694*, 3395-3400.
4. Davies, S. C.; Hughes, D. L.; Leigh, G. J.; Sanders, J. R.; De, S. J. S, *J. Chem. Soc., Dalton Trans.* **1997**, 1981-1988.
5. Ercolani, C.; Neri, C., Phthalocyanines of metals of the first transition series. I. Nitrosyl derivatives. Synthesis and properties. *J. Chem. Soc. A* **1967**, 1715-18.
6. Chung, S.W.; Kwon, M.Y.; Kang, Y.H.; Chung, H.T.; Lee, S.J.; Kim, H.P.; Perrella, M.A., *J. Cell. Physiol.* **2012**, *227*, 351-60.