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Confocal and fluorescence lifetime imaging sheds light on the fate of a pyrene-tagged carbon monoxide-releasing Fischer carbene chromium complex

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Abstract

The synthesis of a new pyrene-containing Fischer carbene complex is described. The complex has a broad absorbance spectrum between 300 and 400 nm and, on excitation at 345 nm in CH_2Cl_2 solution, emission is observed at 395 and 415 nm. Emission is also observed in PBS buffer, but in this case the resulting spectra are much broader. Confocal and fluorescence lifetime imaging indicate that emission occurs on treating HeLa cells with the complex and co-localisation studies demonstrate that this is from the mitochondria and lipid-rich regions of the cell.

Introduction

Carbon monoxide has a well-defined physiological role and offers great potential in the therapeutic arena.[1] It is produced endogenously during the catabolism of heme, a process which is catalysed by the heme-oxygenase series of enzymes (HO-1 and HO-2) and CO acts as a gasotransmitter.[2] Remarkably, it has been shown that the administration of CO gas may have a beneficial effect: preventing organ rejection; as well as aiding the treatment of inflammation, hypertension, cerebral malaria, tumour growth and liver failure.[3] CO also shows interesting effects against certain types of bacteria. Carbon monoxide-releasing molecules (CO-RMs) have been employed as potential CO-prodrugs, which circumvent the requirement for non-selective administration of CO gas.[3] The fundamental concept is that a CO-RM will be able to deliver a well-defined therapeutic quantity of CO in a selective fashion.

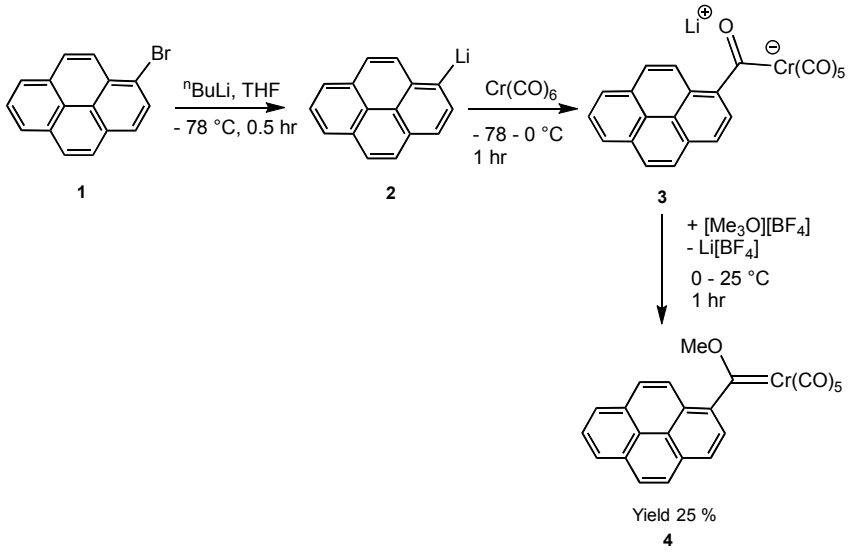
There have been a number of different structural types investigated as potential CO-RMs such as boronate esters.[4] The vast majority of CO-RMs, however, are metal carbonyls that may release CO with a thermal,[5] photochemical,[6] hydrolytic,[5e] or enzyme trigger.[7] In a number of important studies, it has been demonstrated that CO-RMs mimic the therapeutic ability of CO gas.[3] Indeed, CO-RMs have been shown to be effective anti-bacterial agents,[8] more so than CO gas alone. Given that one of the envisaged advantages of CO-RMs is their ability to selectively localise, it is somewhat surprising that there have only been a handful of studies focused on determining the cellular fate of a CO-RM. For example, a study using Raman spectromicroscopy has shown that $[Mn(tpm)(CO)_3]Cl$ (tpm = *tris*(1-pyrazolyl)methane) localises at the nuclear membrane and the nucleolus of HT29 cancer cells,[9] whereas IR spectromicroscopy has been used to study the localisation of a Mn-based CO-RM with a vitamin B12 ligand in 3T3 fibroblasts.[10] In addition, confocal fluorescence microscopy has been used to determine the fate of *fac*-$[Re(bpy)(CO)_3(thp)]OTf$ (bpy = 2,2′-bipyridine, thp = *tris*(hydroxymethyl)phosphine) in PCC-1 human prostatic carcinoma cells.[11]

In a recent study we have investigated the ability of a series of Group 6 metal carbonyl complexes to release CO.[12] In the family of Fischer carbene complexes $[M(CO)_5(=CRR')]$ (M = Cr, Mo, W; R = alkyl, aryl, alkynyl; R' = amino, alkoxy, thio) it was demonstrated that both the nature of the metal and the substituents on the carbene ligand affect the rate of release. For example, complexes based on chromium release CO rapidly; those based on molybdenum are slower; whereas the tungsten complexes are extremely slow. The nature of the π-donor substituent on the carbene also had a profound effect on the rate of CO-release with good donor such as amino-groups release CO at a slow rate ($t_{½}$ *ca.* 10^3-10^4 s), whereas those containing a sulfur substituent

released CO more rapidly ($t_{½}$ *ca.* 300 s). Given this level of control on the rate of CO-release and given that, in principle, up to a total of five molecules of CO may be liberated from each metal, we decided to explorer this series of compounds further. In particular, the incorporation of an emissive pyrene tag into the structure of the Fischer carbene could enable us to determine the site of cellular localisation for $[Cr(CO)_5(=CRR')]$ complexes.

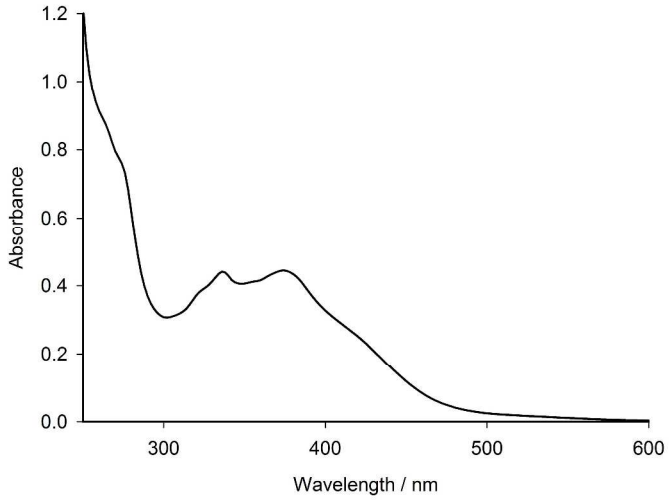
Results and discussion

The synthesis of the pyrene-substituted carbene complex, **4**, was undertaken using a modification of Fischer and Maasböl's original method to obtain $[Cr(CO)_5(=CPh\{OMe\})]$ (Scheme 1).[13] Lithiation of 1-bromopyrene, **1**, with ^{n}BuLi at –78 °C followed by addition of $Cr(CO)_6$ leads to the formation of the acyl anion **3**. This which was characterised *in situ* by IR spectroscopy (bands at 1908 and 2039 cm^{-1} in CH_2Cl_2 solution).[14] Methylation with $[Me_3O]BF_4$ resulted in the formation of **4** which could be isolated as orange crystals. The identity of **4** was confirmed by NMR and IR spectroscopic analysis. For example, in the $^{13}C\{^{1}H\}$ NMR spectrum a resonance was observed at δ 358.9 for the carbene carbon. Resonances at 216.4 (CO ligands *cis* to the carbene) and 225.3 (CO ligand *trans* to the carbene), along with infrared bands at 2063 and 1948 cm^{-1} in the IR spectrum confirmed the presence of the $Cr(CO)_5$ group.

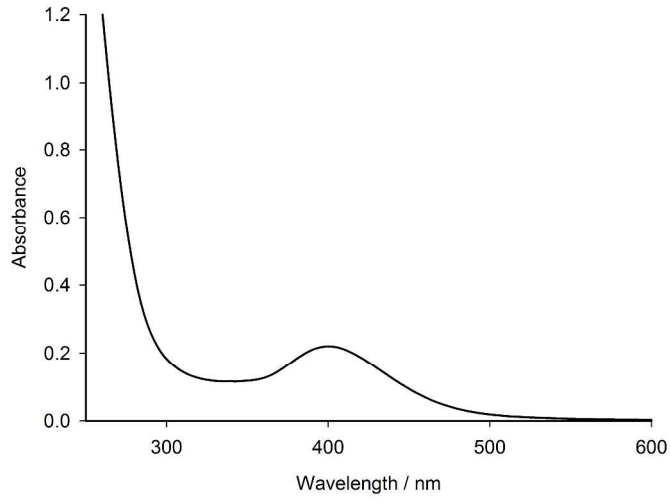


Scheme 1 Preparation of pyrene-substituted carbene complex **4** using a three step synthetic sequence.

The UV-vis spectrum of **4** recorded in CH_2Cl_2 solution exhibited two peaks in the visible region 376 nm and 334 nm (Figure 1a). The former having a shoulder and being somewhat similar to the lowest energy band in $[Cr(CO)_5(=CPh\{OMe\})]$ (Figure 1b) whereas the peak at 334 nm occurredat a similar energy to those observed in the hydroxyl-substituted pyrene $C_{16}H_9(CH_2)_4OH$ (Figure 1c). TD-DFT calculations (see E.S.I for details) indicate that for $[Cr(CO)_5(=CPh\{OMe\})]$ there are three transitions at $\lambda >$ 300 nm, the most intense (and highest energy in this region) is predicted to be at 372 nm. These correspond to MLCT transitions from filled metal-based orbitals to the LUMO, which is located on the carbene ligand. In the case of **4**, the calculations indicate a total of seven transitions between 316 and 466 nm, consistent with the presence of the extra bands in this region in the experimental spectrum (Figure 1a) when compared to $[Cr(CO)_5(=CPh\{OMe\})]$ (Figure 1b). In this case, the dominant contributions to the transitions are from filled orbitals based on both the metal and the pyrene fragment to unoccupied orbitals with significant localisation on the pyrene and carbene carbon.



(a)



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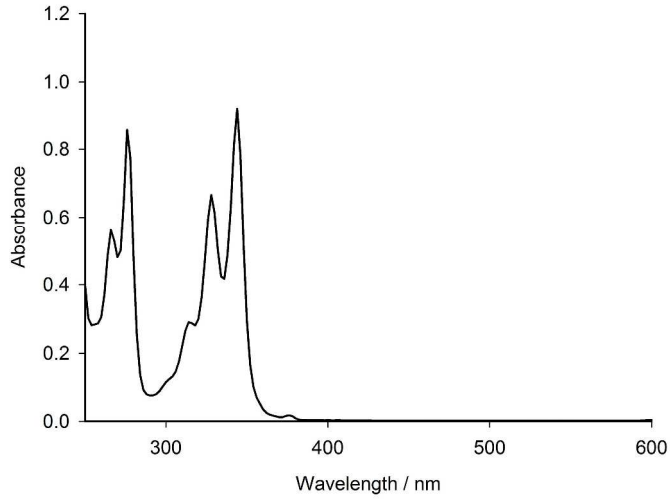
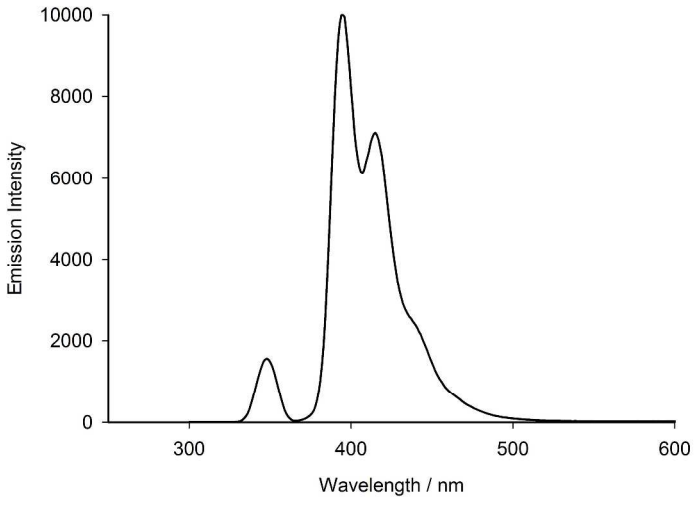


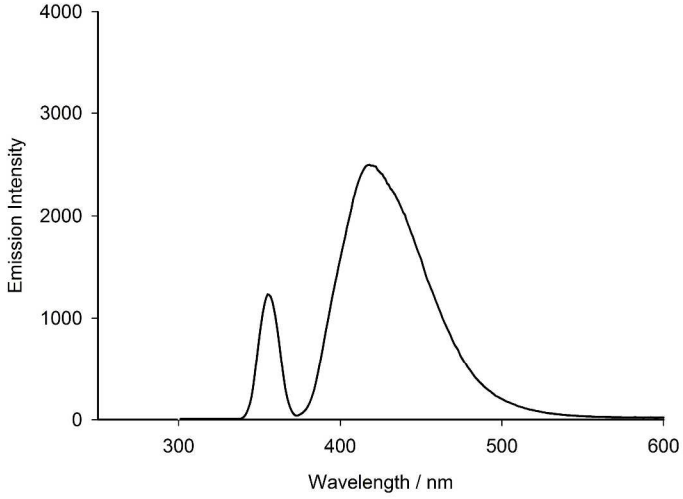
(c)

Figure 1 UV-vis spectra of **4** (a), [Cr(=CPh{OCH_3})$(CO)_5$] (b) and $C_{16}H_9(CH_2)_4OH$ recorded in CH_2Cl_2 solution.

The effect of the incorporation of the chromium carbene on the emission spectra of the pyrene was then investigated. Excitation of a CH_2Cl_2 solution of **4** resulted in emission at 395 and 415 nm (Figure 2a). Emission was also observed in PBS solution (Figure 2b), although the bands were somewhat broader and the intensity reduced. Only weak emission was observed from $[Cr(=C\{OCH_3\}Ph)(CO)_5]$ under identical conditions, indicating the incorporation of the pyrene groups was vital to this effect.



(a)



(b)

Figure 2 Emission spectra of **4** recorded in (a) CH_2Cl_2 solution at 25 °C, excitation wavelength 345 nm. (b) PBS solution at 25 °C, excitation wavelength 345 nm.

The ability of **4** to release CO was estimated *via* an assay based on the capture of liberated CO by deoxy-myoglobin.[15] This process results in the formation of carboxymyoglobin, Mb-CO, and the change was monitored spectrophotometrically. In this case a correction was applied to the data to account for the absorbance of **4** in the region of the proteins Q-bands: the resulting data are shown in Figure 3.[15] CO-release is typically quantified by the half-life, $t_{½}$, (defined as the amount of time required for an *X* μM solution of CO-RM to generate a solution of Mb-CO with concentration *X*/2 μM) which provides a mechanism-independent measure of CO-release. For complex **4** the $t_{½}$ values were 2773 s, 2765 s and 1764 s for solutions of concentration of 60 μM, 40 μM and 20 μM respectively, which is somewhat slower than $[Cr(=C\{OCH_3\}Ph)(CO)_5]$ ($t_{½}$ 332 s for a 60 μM solution) and more similar to the amino-substituted carbenes $[Cr(=C\{NR^1{}_2\}R^2)(CO)_5]$.[12]

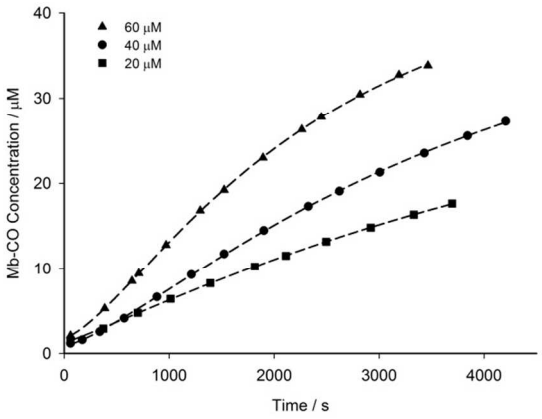


Figure 3 CO-release profiles for assays performed on 60 μM, 40 μM and 20 μM solutions of **4**.

Having established that **4** was a viable CO-releasing molecule which was emissive in both organic media and aqueous solution, we elected to investigate if the localisation of this complex with cancer cells could be observed by confocal microscopy. Compound **4** and 1-bromopyrene, **1**, (used as a ligand model) were incubated with HeLa cells as described in the Experimental and imaged using laser scanning confocal microscopy and fluorescence lifetime imaging microscopy (FLIM). The free ligand precursor showed negligible uptake, whereas addition of the chromium complex **4** resulted in notable fluorescence emission when excited at 405 nm, with insignificant emission observed when exciting at 488 nm and 543 nm (see E.S.I.). It was therefore appropriate to carry out colocalisation experiments with dyes such as Mitotracker Red® and Nile Red, which displayed strong corroboration with complex **4** (Figures 4 and 5), indicating that this compound localises both in mitochondria and lipid-rich regions of the cell. Furthermore, blebbing (which sometimes accompanies cell death)[16] was observed upon continuous illumination for 10 mins with lasers of 488 nm (see E.S.I.). Currently there is no technique that provides a full understanding of complex stability in cells, however based upon our previous studies, the FLIM is capable of providing information on the chemical environment of a molecule, hence speciation.[17] FLIM experiments were therefore carried out to better understand how complex **4** interacts with cells. First, excited state lifetime experiments in solution, were carried out in DMSO using time-correlated single photon counting (TCSPC). The ligand precursor required fitting to two exponential decay components, with a short first component, τ_1 of 0.6 ns and a much longer second component τ_2 of 2.6 ns ($\chi^2 = 1.00$). In contrast complex **4** possessed a single component, which was 4.3 ns ($\chi^2 = 1.25$). Interestingly, in HeLa cells complex **4** localised in both punctate and cytoplasmic regions and displayed two components, a short component of 0.3 ns ± 0.2 ns and a longer component of 6.5 ns ± 2.8 ns. Furthermore, in the punctuated regions the longer component lifetime was significantly higher and was in excess of 10 ns. The proligand did not enter cells, therefore this indicates that either dissociation occurred after entering the cell or that the presence of the ligand was evident (by the lifetime component). Furthermore, the increase of the excited state for the second component is probably an indication of the stability of the complex. The cytotoxicity of **4** over a 10 - 100 μM concentration range was evaluated by Almar Blue and LDH assays against RAW264.7 Murine Macrophages (see E.S.I.). The resulting data demonstrated a small loss of cell viability at concentrations of 10 μM of **4** over a 4 hour (Alamar Blue) and 15 min (LDH) time period, whereas concentrations of 100 μM resulted in a *ca.* 90 % loss of viability in both cases. Therefore, it appears that **4** is cytotoxic to both the HeLa and macrophages at high concentration (as is the case for many organic compounds). The mechanism for the toxicity is unknown, although the results we have obtained through the imaging measurements have allowed for the visualisation of this process.

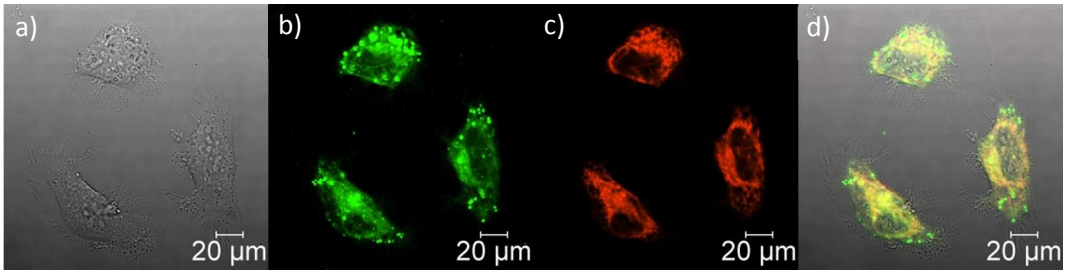


Figure 4. Laser scanning confocal microscopy of complex **4** incubated with HeLa cells for 15 minutes at 50 µM (2% ethanol), co-stained with Mitotracker Red® at 200 nM a) DIC micrograph, b) green channel with λ_{ex} = 405 nm, red channel with λ_{ex} = 543 nm and d) is an overlay of the previous three images. Where green represents fluorescence emission from compound **4**, red is emission from Mitotracker Red® and yellow represents colocalisation of both dyes.

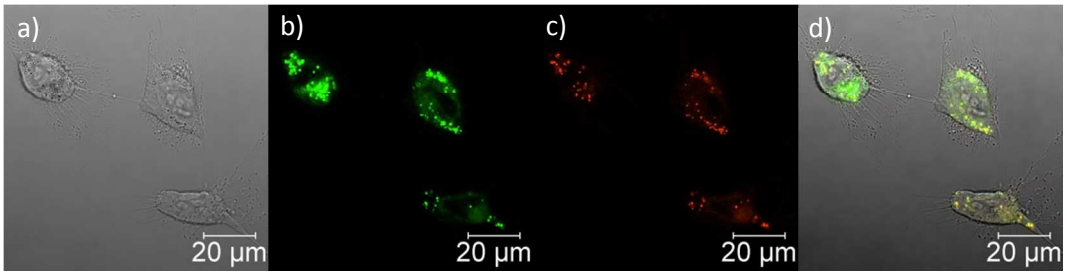


Figure 5. Laser scanning confocal microscopy of complex **4** incubated in HeLa cells for 15 minutes at 50 µM (2% ethanol), co-stained with Nile Red at 100 ng/mL a) DIC micrograph, b) green channel with λ_{ex} = 405 nm, red channel with λ_{ex} = 543 nm and d) is an overlay of the previous three images. Where green represents fluorescence emission from compound **4**, red is emission from Nile Red and yellow represents colocalisation of both dyes.

Conclusions

The pyrene-substituted carbene chromium complex **4** is an effective CO-releasing molecule which is emissive in both organic and aqueous media. Microscopy studies have demonstrated that the addition of **4** to HeLa cells also results in emissive behaviour, which is not observed in the case of **1**. Although these data do not unambiguously demonstrate that **4** is intact in cells, the observation of emission from the pure complex in water which is similar to that observed in CH_2Cl_2 (in which the complex is stable) and the lack of emission in the microscopy measurement from 1-Bromopyrene appears to indicate that this behaviour is related to the complex. Localisation occurs within both lipid droplets and mitochondria, the latter being especially important, as cytochrome *c*-oxidase (the terminal oxidant in the respiratory chain) is a known target of CO.[18] Further, the application of FLIM and TSCPC has allowed us to better understand the fate of the complex in live cells.

Experimental

All manipulations were accomplished using standard Schlenk line and Glove Box apparatus. The THF was distilled from sodium benzophenone/ketyl prior to use. All other solvents were AR grade and used without further purification. Complexes $[Cr(=C\{OCH_3\}R)(CO)_5]$ (R= CH_3, C_6H_5) were prepared according to literature procedures.[19] $[Me_3O]BF_4$, 1-bromopyrene, **1**, and $Cr(CO)_6$ were purchased from Aldrich the latter was sublimed prior to use. Myoglobin, light mineral oil and PBS were purchased from Sigma and sodium dithionite from Alfa Aeasar. Ultra violet-visible spectra were recorded using a JASCO V-560 instrument. IR spectra were acquired on a Mattson Research Series FTIR spectrometer using CsCl solution cells. NMR spectra were recorded on a Bruker AMX 300 Spectrometer (Operating frequencies ^{1}H 300.13 MHz; ^{13}C 76.98 MHz). For ^{13}C NMR data, "*cis*" and "*trans*" carbonyl resonances refer to orientation related to the carbene substituent. Mass spectra were obtained on a Bruker microTOF instrument. Emission spectra were obtained on Toshiba F-4500 FL Spectrophotometer. Assessment of CO-release by the myoglobin assay was performed as detailed previously.[15] Confocal microscopy and FLIM measurements were performed on a modified Nikon TE2000-U at the Rutherford Appleton Laboratory.

Synthesis of $[Cr(CO)_5(=C\{OMe\}C_{16}H_9)]$ 4. 1-Bromopyrene (0.33g, 1.17 mmol) was dissolved in THF (10 ml) and the solution cooled to -78 °C. A solution of Li^{n}Bu (1 ml, 1.2 M diluted in 10 ml THF) was added dropwise. After 30 mins, $Cr(CO)_6$ (0.303 g, 1.38 mmol) was added and the solution stirred for 20 mins and warmed to 0 °C and stirred for a further 30 min, during which time the solution changed colour from yellow to orange/brown. Following addition of $[Me_3O]BF_4$ (0.191 g, 1.29 mmol) the solution was stirred for at 0 C for 20 min before warming to room temperature and stirring for a further 30 mins, during which time a colour change to dark red occurred. The reaction was

then quenched with 0.2 M $NaHCO_3$ before extracting with hexane. After removal of the solve the resulting residue was purified by chromatography on silica gel using a 4:1 (v/v) mixture of hexane:CH_2Cl_2 as eluent. Complex **4** was isolated as a dark red solid. Yield 112 mg, 25 %. ^{1}H NMR (300 MHz, $CDCl_3$) δ(ppm): 4.18 (br, 3H, O***Me***), 7.60-8.25 (m, 9H, pyrene), $^{13}C\{^1H\}$ NMR (100 MHz, $CDCl_3$) δ 66.8 (O***Me***), 119.8~147.9 (pyrene), 216.4 (*cis*-CO) , 225.3 (*trans*-CO), 358.9 (Cr=***C***); IR (CH_2Cl_2, cm^{-1}): 2063s, 1948vs; HRMS (ESI): m/z 437.0125 (437.0123 calculated for $C_{23}H_{13}CrO_6$ $[M+H]^-$).

Cell culture of HeLa cells was carried out in T75 flasks with Eagle's Minimum Essential Media (EMEM) containing 10% foetal calf serum (FCS), this was supplemented with final concentrations of 1% L-glutamine (200 mM), 0.5% penicillin/streptomycin (10 000 IU mL^{-1}/10 000 mg mL^{-1}). After the EMEM containing FCS was aspirated phosphate buffered saline (PBS) was used (3 x 5 mL) to wash the cells, which was subsequently aspirated. To this a 2.5% trypsin solution was added to detach the cells from the flask, which was incubated at 37 °C for 5-10 minutes, after which 5 mL EMEM containing FCS was added. Next, the cells were seeded in glass bottomed dishes for 12 hours. Cells were washed 5 times with 1 mL Hank's Balanced Salt Solution (HBSS) and subsequently 980 µL serum free EMEM was added to the cells followed by 20 µL from a 2.5 mM ethanol solution of the compound (giving a final concentration of 50 µM, 2% ethanol). This was incubated for 15 minutes and washed 3 times with 1 mL of HBSS before being returned to serum free EMEM prior to imaging.

Details on RAW 264.7 murine macrophage culture is in the supplementary information.

Colocalisation staining was performed as follows. Cells were washed 5 times with 1 mL Hank's Balanced Salt Solution (HBSS) and subsequently 990 µL serum free EMEM was added to the cells followed by 10 µL of the colocalisation dye in DMSO. Nile Red (*Invitrogen*) Mitotracker Red® were prepared as final concentrations of 100 ng/mL and 200 nM respectively and incubation times of 10 minutes and 30 minutes correspondingly. After washing a further 3 times, cells were returned to serum free EMEM and complex **4** or its ligand precursor were added as described above.

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Notes and references

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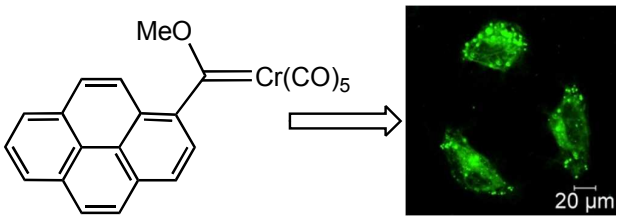

Graphical Abstract