A lipophilic copper(II) complex as an optical probe for intracellular detection of NO

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Abstract

A new chemical sensor for cellular imaging of NO is presented. This cell-permeable probe is based on a complex where copper(II) is coordinated to a tridentate ligand substituted with a fluorophore (NBD) and an octyl group. The fluorescent response of this complex towards a range of reactive species (namely NO, NO_2^-, NO_3^-, H_2O_2, ClO^-, O_2^- and $ONOO^-$) has been studied *in vitro* showing that the probe is highly selective for NO. The probe is readily taken up by cells and is able to image the cellular concentrations of NO.

Introduction

Nitric oxide (NO) is known to bind readily to transition metals and its coordination chemistry has been extensively studied.[1,2] Interest in the chemistry of this small molecule increased dramatically with the discovery, over 25 years ago, that NO is the endothelium-derived relaxing factor which promotes smooth muscle relaxation.[3] Since this seminal discovery, NO has been found to play important signalling roles in the cardiovascular, immune and nervous system. Furthermore, misregulation in the production of NO has been associated with a range of disease states such as cardiovascular diseases,[4] cancer[5,6] and inflammation.[7] Considering its biological relevance, it is not surprising that over the past few years, great efforts have been directed at developing molecular probes that can selectively detect and image NO under physiological conditions.[8,9] This is a challenging task since NO is produced in low concentrations *in vivo* and diffuses rapidly away from the site of production. In addition, NO is highly reactive, being scavenged by reaction with haemoglobin or by reaction with oxygen to yield nitrite and nitrate in the cell. Such reactivity gives NO an average life span in the range of milliseconds to seconds. These issues of short lifetime and low concentration have therefore placed high demands on the technology required for *in vivo* cellular NO imaging.

Arguably, the most successful NO probes developed to date are based on fluorescent molecules. Examples of these are probes based on coupling fluorescent groups (such as fluorescein, BODIPY or cyanine dyes) to a phenylendiamine moiety which reacts with NO to yield highly emissive triazole derivatives.[10,11] On the other hand, Lippard has developed several elegant approaches to detect NO using transition metal complexes.[8,12,13] One of these approaches is based on copper(II) coordinated to fluorescent ligands, which due to the unpaired electrons on this metal cation, quenches the emission of the fluorophore. However in the presence of NO, copper(II) (paramagnetic) is reduced to copper(I) (diamagnetic) under

physiological conditions, which removes the quenching effect and thus effects a 'switch-on' of the fluorescence. This approach has led to the development of several copper(II) complexes that can be used for *in vitro* detection of NO.[14-20] While some of these complexes have also been successfully employed for the detection of NO *in vivo*,[21] not every NO sensor operative under *in vitro* conditions can be employed in cells often due to low uptake. In addition, many optical probes for cellular imaging lack the localisation potential of the equivalent biological sensors often used in cell biology experiments.

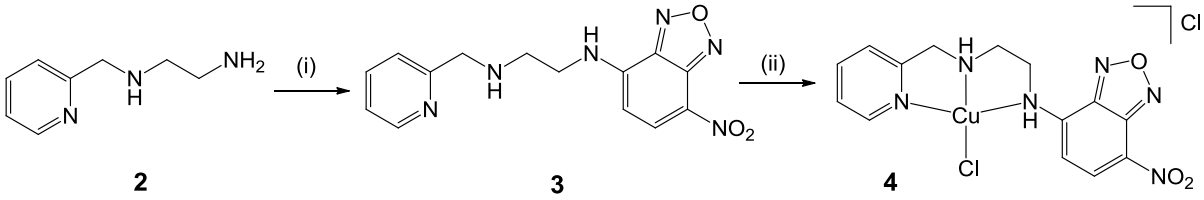
Employing the NO sensing principle pioneered by Lippard, we have developed a new copper-based NO sensor, which was subsequently modified to aid cell permeability. This was achieved by the introduction of a hydrophobic lipid tail (C_8 alkyl group), creating a highly NO-selective and membrane permeable NO sensor. In contrast to the parent molecule, the octylated probe showed high cell permeability, demonstrating that the introduction of short-chain alkyl substituents can facilitate the cellular uptake of otherwise non-permeable compounds. Herein we report the syntheses of these NO-sensing copper(II) complexes, their *in vitro* selectivity profile towards NO and several other analytes, and their applicability to cell imaging experiments.

Results and Discussion

Synthesis of NO probes. Two copper(II) complexes coordinated to fluorescent ligands **3** and **6** (Schemes 1 and 2) were developed as potential NO optical probes. The rationale behind the choice of metal, as described in the Introduction, was so that the emission of the ligands would be quenched when coordinated to copper(II) but upon reduction to copper(I) in the presence of NO, the emission would be restored. The two tridentate ligands were designed to study the

difference in cellular uptake and localisation of the resulting copper(II) complexes with the octyl-containing ligand (more lipophilic) vs. that with the unsubstituted ligand.

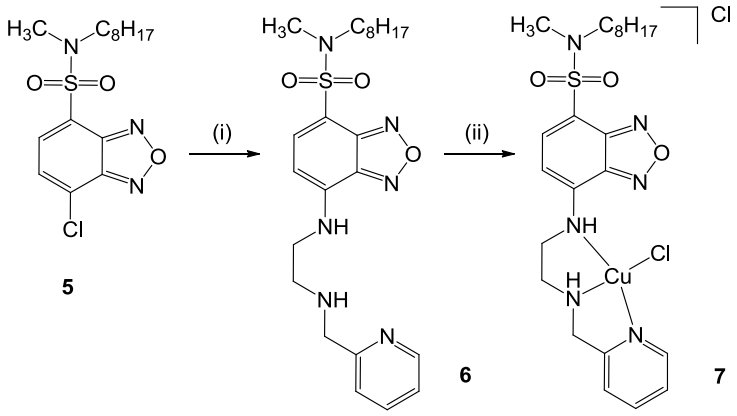
The synthesis of complex **4** was carried out as detailed in Scheme 1. NBD chloride was reacted with **2** (prepared as previously reported[22,23]) in the presence of K_2CO_3 and under a nitrogen atmosphere. The resulting crude product was purified by silica gel column chromatography to yield compound **3**. The ^{1}H NMR spectrum of this compound (see Figure S1) showed two doublets at δ 8.50 and 6.20 ppm corresponding to the NBD whose integration with respect to the aliphatic signals and the pyridyl group was consistent with the proposed formulation. Mass spectrometry and elemental analysis confirmed the successful synthesis of the new ligand **3**. This compound was shown to be emissive (λ_{ex} = 450 nm and λ_{em} = 520 nm) in dichloromethane. The copper(II) complex **4** was obtained by addition of $CuCl_2$ in methanol to a solution of ligand **3** in the same solvent. The formation of the complex was confirmed by elemental analyses.



Scheme 1. Synthetic scheme for the preparation of ligand **3** and the corresponding copper(II) complex **4**. (i) Dry methanol, K_2CO_3, NBD-Cl; (ii) Methanol, $CuCl_2$.

A second copper complex (**7**) coordinated to ligand **6** which contains an octyl substituent, was developed with the aim of rendering the probe more lipophilic and hence cell permeable. The synthesis was carried out as outlined in Scheme 2. Compound **5** was synthesised according to a previously reported procedure[24] while ligand **6** was synthesised by addition of **2** to **5** in the presence of triethylamine. In the ^{1}H NMR spectrum of compound **6** (see Figure S2), two

doublets at 8.70 and 7.90 ppm corresponding to the NBD ring were observed. These integrated correctly with the pyridine and the alkyl resonances, consistent with the formulation of **6**. Mass spectrometry and elemental analyses were used to confirm the successful synthesis of the ligand. The synthesis of copper(II) complex **7** was carried out using an analogous procedure to that used for the synthesis of complex **4** and the formation of the copper(II) complex was confirmed by elemental analyses.



Scheme 2. Synthetic scheme for the preparation of ligand **6** and the corresponding Cu complex **7**. (i) Under N_2, dry acetonitrile, NEt_3, $H_2NCH_2CH_2NHCH_2Py$; (ii) Methanol/Acetonitrile, $CuCl_2$.

Optical properties. To investigate the effect that the paramagnetic copper(II) ion had on the fluorescence of ligands **3** and **6,** increasing amounts of $CuCl_2$ in PIPES buffer were added to the corresponding ligand and the emission recorded. As expected, due to the unpaired electron in copper(II), the emission of NBD was greatly reduced upon addition of $CuCl_2$ (see Figure 1a for titration of **6**) and reached a minimum when 1 eq. of copper(II) had been added – indicating the formation of a 1:1 complex.

The next step was to determine whether the presence of NO would restore the fluorescence of the compounds (by reducing the copper(II) to copper(I) as described for other copper-based

NO sensors). Thus, **4** and **7** (0.01 mM) were exposed to NO generated from the well-known NO donor 2-(*N*,*N*-diethylamino)-diazenolate (DEA-NO).[25] This method was preferred rather than directly bubbling NO through since it was much easier to control the amount of gas delivered to the system. After 5 minutes of exposing **4** and **7** to NO, restoration of the emission was observed (see Figure 1b for **7** and S4 for **4**).

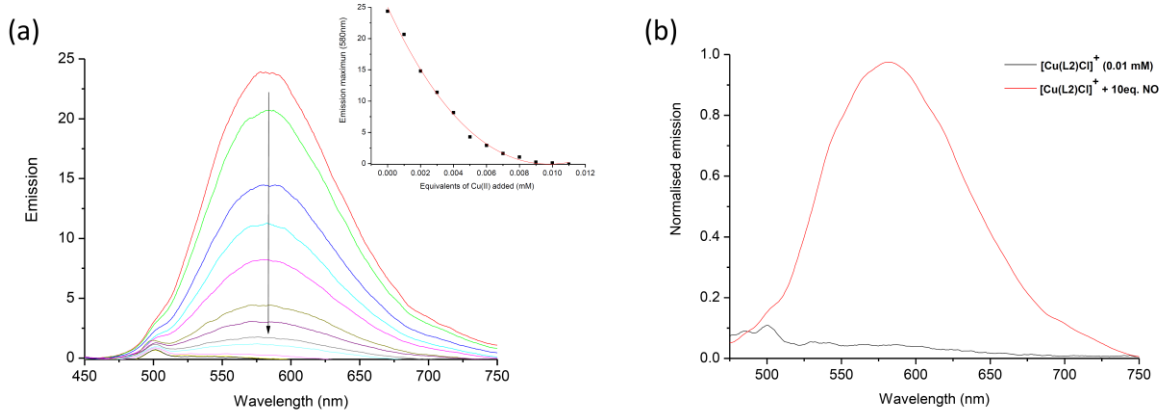


Figure 1. (a) Emission spectra (λ_{ex} = 440 nm, λ_{em} = 580 nm) of ligand **6** (0.01 mM) upon addition of copper(II) (0.1 – 1.1 eq.) (in 50 mM PIPES, 100 mM KCl, pH 7.0). Inset shows a plot of the emission maximum vs. concentration of added copper(II). (b) Spectra showing the restoration of fluorescence of complex **7** upon addition of 10 equivalents of NO generated from DEA-NO (50 mM PIPES, pH 7.0, 100 mM KCl).

Having determined the ability of complexes **4** and **7** to detect the presence of NO, their selectivity for NO over several other reactive species (namely NO_2^-, NO_3^-, H_2O_2, ClO^-, O_2^- and $ONOO^-$) of biological relevance was investigated. For this, 100 equivalents of each competing species were added to a 10 μM solution of the corresponding complex in PIPES buffer and the fluorescence recorded after 1 hour. Both complexes were highly selective for NO over the other analytes investigated (see Figure 2 for complex **7** and S5 for complex **4**).

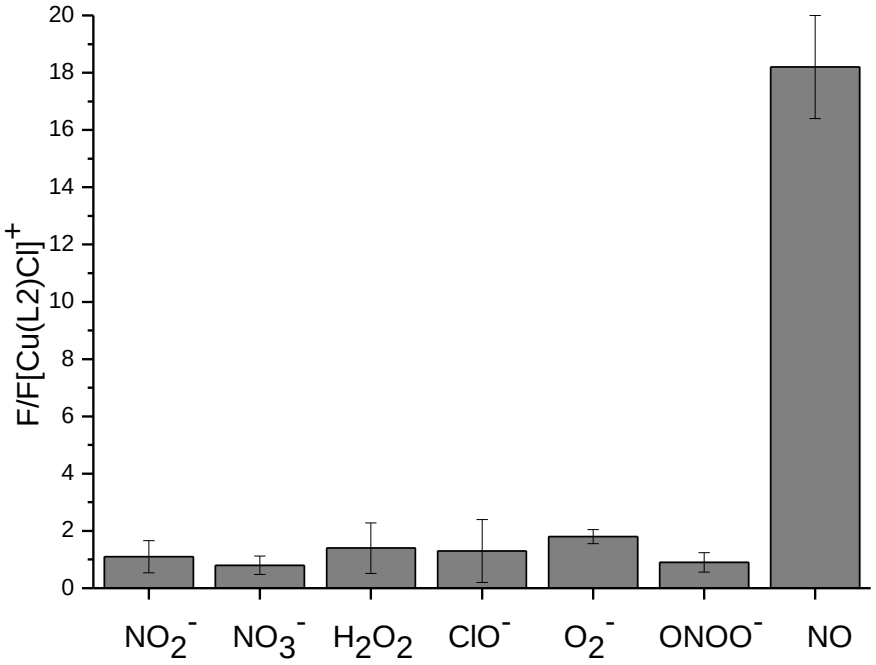


Figure 2. Emission intensity (recorded at 580 nm) of 10 μM solutions of complex **7** after addition of 100 eq. of NO_2^-, NO_3^-, H_2O_2, ClO^-, O_2^- and NO and incubating for 1 hour in buffered PBS (pH 7). Average emissions (triplicate +/- SD) were normalised with respect to the emission of **7** (10 μM).

Cellular uptake and confocal microscopy studies of copper(II) complexes 4 and 7. Prior to the evaluation of the two new complexes as cellular NO probes, the cytotoxicity of **4** and **7** in live NIH 3T3 mouse fibroblasts cells was determined. Both compounds were non-toxic up to concentrations of 10 μM, which was subsequently employed for the cellular uptake assays (see Figures S6 and S7). Complex **4** became cytotoxic at the much higher concentration of 100 μM, which probably reflects its reduced cellular uptake as compared to complex **7** (*vide infra*).

Cellular uptake experiments were conducted by incubating NIH 3T3 mouse fibroblast cells with both compounds, followed by UV/Vis spectroscopic determination of the cell lysate (Figure 3) and the culture media (Figure S8). It was demonstrated in both cases (Figure 3 and S7) that the uptake of **4** was very limited (< 10%), whereas >40 % of the alkyl-substituted

complex **7** was taken up by the cells. The latter observation demonstrates that the presence of the octyl substituent improves significantly the cellular uptake of this copper(II) complex.

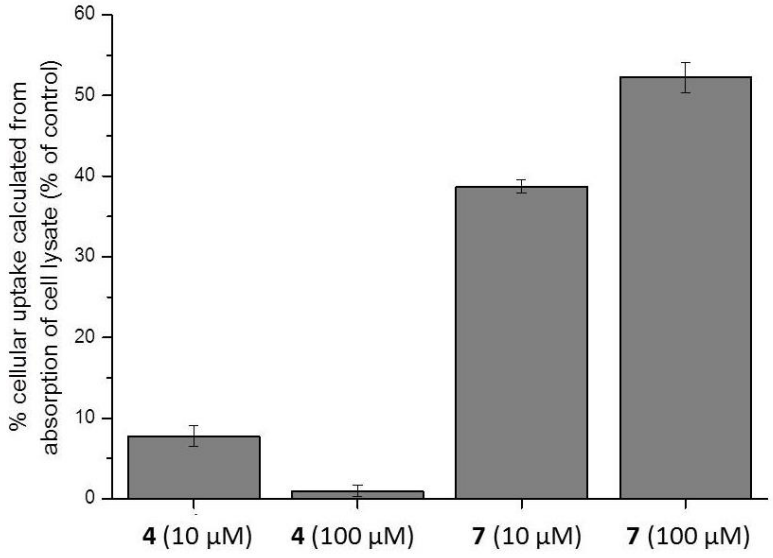
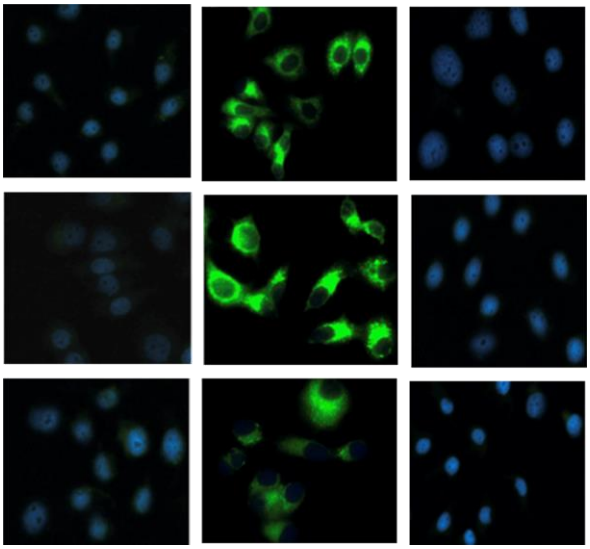


Figure 3. Uptake determination in cell lysate of **4** vs. **7** by NIH 3T3 mouse fibroblast cells measured via UV/Vis. spectroscopy. The values were compared with the controls (known concentration of compound in cell free, phenol red free media) in order to determine the average percentage of cellular uptake values (triplicate +/- SD) of each compound.

Having established that complex **7** can detect NO *in vitro* and that it is cell permeable, we investigated its ability to detect endogenously generated NO in cells. To this aim, **7** and the nitric oxide stimulating hormone 17β estradiol were incubated with MCF-7 human breast adenocarcinoma cells (Figure 4). This led to a 12-fold enhancement of fluorescence intensity as compared to the untreated cells (Figure 4b). To demonstrate that the increase in fluorescence was indeed due to NO, the following control was carried out. Since the enzyme nitric oxide synthase (NOS) is responsible for the synthesis of endogenous nitric oxide, cells incubated with probe **7**, were treated with 17β estradiol and the NOS inhibitor (L-NG-Nitroarginine methyl ester, L-NAME).[26] Figure 4 clearly shows that upon addition of this inhibitor the emission is reduced to the same level of the untreated control cells. The differences in

fluorescence responses of **7,** stimulated with and without estradiol and in the presence of an NOS inhibitor (Figure 4), clearly demonstrates that **7** is an effective probe for imaging endogenously generated nitric oxide. Interestingly, complex **7** seems to be localised in the perinuclear region of the cell, which is not dissimilar to the region where eNOS is localised. The latter enzyme is known to be myristylated and palmitoylated, a modification that has strong similarities with the alkyl chain introduced to the probe tested here.[27,28]

(a)



7 (10 µM)	+	+	+
Estradiol (10 nM)	-	+	+
L-NAME (10 mM)	-	-	+

(b)

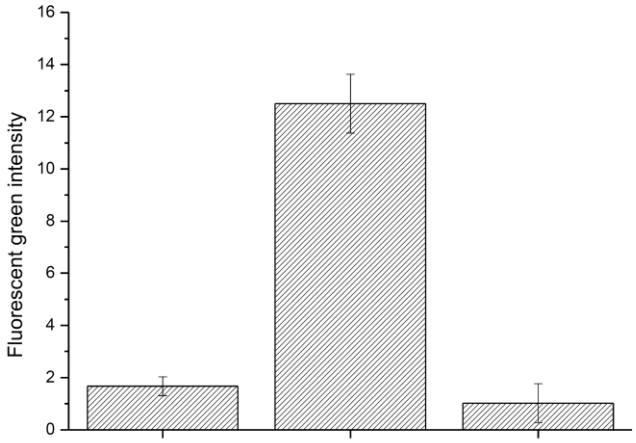


Figure 4. (a) Cellular confocal microscopy images of **7** in response to NO stimulated endogenously via estradiol (10 nM) in MCF-7 cells in the presence and absence of the NOS inhibitor L-NAME. MCF-7 cells were incubated with: 500 µl of 10 µM **7** (control, first image); 500 µl of 10 µM **7** plus 10 nM estradiol (second image); and 500 µl of 10 µM **7** plus 10 nM estradiol and 10 mM NOS inhibitor L-NAME (third image). All samples were incubated in DMEM for 1 hour at 37 ^{o}C. (b) Quantification of the relative fluorescent intensities (mean +/- SD) of the images presented in (a).

Conclusions

The new copper(II) complex **7** has been synthesised and shown to be an effective nitric oxide probe. *In vitro* experiments have demonstrated that this copper complex (as well as **4**) enhances significantly its fluorescence upon exposure to NO but not in the presence of several other common analytes. This indicates that the system is highly selective for NO sensing as compared to several other small molecules and ions (i.e. namely NO, NO^{2-}, NO^{3-}, H_2O_2, ClO^-, O_2^- and $ONOO^-$). The observed enhanced emission is due to the reduction of copper(II) to copper(I) with the concomitant switch-on of the emission. A comparison between the behaviour of complexes **4** and **7** in a cellular environment, indicates that the presence of the octyl substituent in **7** is necessary for cellular uptake. Complex **7** does not exhibit significant cytotoxicity within its working fluorescent range (1-10 μM) and hence can be successfully used for cellular imaging of NO. This was demonstrated by using this probe to successfully detect endogenous nitric oxide in cells generated from the nitric oxide stimulating hormone 17β estradiol. Interestingly, the presence of the NOS-inhibitor L-NAME was shown to diminish the fluorescent response of **7** due to the inhibition of NO synthesis. The probe displayed higher fluorescence throughout the cytoplasm than in the nuclear region, which is consistent with the localisation of the nitric oxide generating NOS. This implies that the probe is switched-on close to the nitric oxide generation centres in the cell, confirming the nitric oxide dependent mode of action of this probe. While these preliminary studies are very encouraging, further studies will need to be carried out to determine whether the labile chloride coordinated to compound **7** exchanges with other ligands present in the cell, which in turn could lead to differences in cellular localisation of the probe.

Experimental Details

General. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 Ultrashield NMR spectrometer using the solvent peak as the internal reference. Chemical shifts are reported in parts per million using residual proton impurities in the solvent. Mass spectra were recorded on a micromass autospec Q spectrometer. Characteristic isotope patterns were used to confirm assignments where appropriate. UV-Vis. absorption spectra were monitored at room temperature using a Thermo Genesys 10 UV-Vis. Spectrophotometer**.** Emission spectra were measured using a Varian Cary eclipse fluorescence spectrometer. Cell preparations were observed under a Nikon TE 2000 fluorescence microscope using 100x fluor oil lens. FITC fluorescent filters were used with excitation wavelength of 465-495 and emission wavelengths of 515-555 nm. Images were digitally acquired with a CCD camera (Hamamatsu) and processed using IPLab software v 3.65a. N^1-(pyridyl-2-ylmethyl)ethane-1,2-diamine hydrochloride (**2**) and 4-*N*-octyl-*N*-methylaminosulfonyl-7-chloro-2,1,3-benzoxadiazole (**5**) were prepared following previously reported synthetic procedures.[22-24] The release of NO from DEA-NO was carried out as previously reported.[25]

N^1-(7-nitrobenzo[c][1,2,5]oxadiazol(-4-yl)-N^2-(pyridyl-2-ylmethyl)ethane)-1,2-diamine (3). To a stirred solution of **2**·3HCl (300 mg, 1.1 mmol) in dry methanol (10 mL) 4-chloro-7-nitrobenzofurazan (180 mg, 0.9 mmol) and K_2CO_3 (552 mg, 4.0 mmol) were added under nitrogen. The solution turned brown and was left to stir overnight at room temperature. The resulting mixture was purified by column silica gel chromatography (10% MeOH/CH_2Cl_2) to yield an orange solid (156 mg, 0.5 mmol, 55%), mp 146-148 °C. ^{1}H NMR ($CDCl_3$/CD_2Cl_2): δ

8.63 (d,$^3J_{HH}$ = 7.6 Hz, 1H, *Py(6)*) 8.50 (d, $^3J_{HH}$ = 8.5 Hz , 1H, *NBD(H)*), 7.60 (ddd, $^3J_{HH}$ = 7.7 Hz, $^3J_{HH}$ = 7.6 Hz ,$^4J_{HH}$ = 1.8 Hz, 1H, *Py(5)*), 7.20 (d, $^3J_{HH}$ = 7.9 Hz , 1H, *Py(3)*), 7.17 (ddd, $^3J_{HH}$ = 7.9 Hz, $^3J_{HH}$ = 7.7 Hz , $^4J_{HH}$ = 1.7 Hz 1H, *Py(4)*), 6.10 (d,$^3J_{HH}$ = 8.5 Hz, 1H, *NBD(H)*), 3.95 (s, 2H, Py*CH*$_2$), 3.50 (br, 2H, PyCH$_2$NH$_2$CH$_2$*CH*$_2$), 3.05 (t, $^3J_{HH}$ = 5.6 Hz, 2H, PyCH$_2$NH$_2$*CH*$_2$). UV-VIS (CH_2Cl_2) λ, nm (Є, $M^{-1}cm^{-1}$): 460 (18710), 332 (7010). Excitation λ_{max} (480 nm), emission λ_{max} (520 nm). Elemental analysis: calculated for $C_{14}H_{14}N_6O_3$; C, 53.50, H 4.49, N 26.74; found C 53.56, H 4.39, N 26.89. ES MS; m/z: 315 a.m.u. $[M+1]^+$.

Complex [Cu(3)Cl]Cl (**4**). $CuCl_2$ (8.0 mg, 0.06 mmol) and ligand **3** (18.0 mg, 0.06 mmol) were each dissolved in methanol (10 mL); the ligand solution was added to the $CuCl_2$ solution and left stirring at room temperature for 3 h. A precipitate formed which was filtered under suction and washed with methanol to yield a brown solid (26.0 mg, 0.06 mmol, 96 %) UV-Vis (DMSO): λ_{max} = 487 nm; ε = 17534 $M^{-1}cm^{-1}$. Emission Spectroscopy (DMSO): λ_{ex} = 480 nm; λ_{max} = 520 nm. Anal. calcd for $C_{14}H_{14}N_6Cl_2CuO_3$; C 37.47, H 3.14, N 18.73 found: C 37.49, H 3.07, N 18.68%.

4-N-octyl-N-methylaminosulfonyl-2,1,3-benzoxadiazole-7-(ethylenediamine) methyl-pyridine (6). Compound **5** (172 mg, 0.48 mmol) and **2**·3HCl (124 mg, 0.48 mmol) were sealed in a Schlenk tube under nitrogen, to this was added acetonitrile (4 mL) and triethylamine (465 µL). The mixture was left to stir for 4 hours at 60 °C. The solvent from the reaction mixture was evaporated under reduced pressure and the residue purified by column chromatography on silica gel using a mixture of methanol and dichloromethane (MeOH: CH_2Cl_2 1:20) to yield a brown oil (153 mg, 0.31 mmol, 65%). ^{1}H NMR ($CDCl_3$) δ 8.67 (d, $^3J_{HH}$ = 8.0 Hz, 1H, *NBD(H)*), δ 7.90 (d, $^3J_{HH}$ = 7.9 Hz, 1H, Py(6)), 7.70 (ddd, $^3J_{HH}$ = 7.9 Hz, $^3J_{HH}$ = 7.9 Hz, $^4J_{HH}$ = 2.0 Hz, 1H, *Py(5)*), 7.31 (d, $^3J_{HH}$ = 7.60 Hz, 1H, *Py*(3), 7.23 (ddd, $^3J_{HH}$ 7.6 = Hz, $^3J_{HH}$ = 7.9 = Hz,

$^4J_{HH}$ = 2.0 Hz, 1H, *Py*(4)), 7.07 (br, 1H, *NH*), 6.10 (d, $^3J_{HH}$ =8.0 Hz , 1H *NBD(H)*), 4.05 (s, 2H, Py*CH*$_2$NH), 3.56 (m, 2H, -N*CH*$_2$(C_5H_{10})) 3.20-3.25 (m, 4H, PyCH$_2$NH*CH*$_2$*CH*$_2$), 2.88 (s, 3H, -N*CH*$_3$), 1.55 (br, 2H, -*CH*$_2$CH$_3$), 1.27 (br, 10H, -CH$_2$*C*$_5$*H*$_{10}$CH$_2$CH$_3$), 0.89 (t, $^3J_{HH}$ 6.4 Hz, 3H, -CH$_2$*CH*$_3$). ^{13}C NMR ($CDCl_3$): δ 149.5, 140.7, 139.1, 136.8, 122.5, 110.8, 98.7, 53.7, 50.3, 46.7, 42.8, 34.6, 31.8, 29.2, 28.0, 26.5, 22.6, 14.0. UV-VIS (DMSO): λ_{max} = 462 nm; ε = 18000 $M^{-1}cm^{-1}$. Emission spectrum: Excitation λ_{max} (450 nm), emission λ_{max} (490 nm). MS(CI) *m/z* = 474 [M^+]. Anal. calcd for $C_{23}H_{34}N_6O_3S$; C, 58.20, H 7.22, N 17.71; found C, 58.76, H 8.05, N 16.91%.

Complex [Cu(6)Cl]Cl (7). $CuCl_2$ (16 mg, 0.12 mmol) was dissolved in acetonitrile (2 ml) and a solution of **7** (48.0 mg, 0.10 mmol) in methanol (2 mL) was added to the former. The reaction mixture was stirred at room temperature for 3 h. A green precipitate formed which was filtered and washed with acetonitrile to yield the pure product (51 mg, 0.08 mmol, 80%). UV-VIS (DMSO): λ_{max} = 458 nm; ε = 19000 $M^{-1}cm^{-1}$. Anal. calcd for $C_{23}H_{34}N_6O_3SCl_2Cu\cdot 0.5H_2O$; C 44.05, H 5.76, N 13.48; found: C 44.81, H 5.71, N 13.59.

Cellular preparations and fluorescent microscopy. NIH 3T3 (mouse embryo fibroblasts) cells were cultured under sterile conditions in DMEM 4500 mg/mL and glucose 10% FCS, they were subsequently washed with PBS and split 1:10 when ~ 90% confluent, using EDTA free trypsin. Cells were subsequently treated as documented below and imaged using a Nikon TE 2000 fluorescence microscope. Cell preparations were observed under a Nikon TE 2000 fluorescence microscope using 100x fluor oil lens. FITC filter (ex/em 494/518 nm) was used to image optical probe **7** and a DAPI filter (ex/em 358/ 461 nm) for the DAPI (4',6-diamidino-

2-phenylindole). Images were digitally acquired with a CCD camera (Hamamatsu) and processed using IPLab software v 3.65a.

MTT proliferation assay. NIH 3T3 (mouse fibroblasts) cells were prepared as stated above and separated into wells of a 96 well-plate each containing 90 µL of phenol red free media. To each well was added 10 µL of compound (0.01 – 100 mM) which was subsequently incubated for 1 hour (37^{o}C). To each well was then added 20 µL of 5 mg/ml of thiazolyl blue tetrazolium bromide (yellow) and the cells were incubated for a further 3.5 hours (37 ^{o}C). The media was removed carefully via pipette and the precipitated formazon (purple) formed by mitochondrial reductase (healthy cells show higher levels of formazon) was dissolved in 150 µL MTT solvent (4 mM HCl, 0.1% Nondet P-40). The plate was read at 590 nM and compared against the control to determine cell viability in the presence of compound.

Evaluation of compound cellular uptake. NIH 3T3 (mouse fibroblasts) cells were prepared as stated above and separated into wells of 500 µL of phenol red free media. The media was replaced with 10 and 100 µM solutions of compound (500 µL) and were incubated for a further 1 hour, after which the cells were spun down and separated from the media. 100 µL aliquots of cell free media were transferred to a 96 well-plate and the UV absorbance was measured and compared with the control in order to determine the percentage of cellular uptake. The spun down cells were then treated with a triton lysate buffer to split them, after which they were again spun down and the UV absorbance of the media was recorded. The percentage of cellular uptake of each compound was subsequently calculated.

Protocol for cellular loading. NIH 3T3 (mouse fibroblasts) cells were prepared as stated above and separated into wells with 500 µL of DMEM media. The media was removed and the cells washed with PBS (x3) and incubated with 500 µL of compound at 37 °C, this process was repeated for each compound required for the experiment. After which the media was again removed and the cells washed with PBS (x3) and incubated with 250 µL of 2% PFA (paraformaldehyde) for 15 minutes at 37 °C, this allows the cells to fix to the microscope slide. The cells were again washed with PBS (x3) and incubated with 1µg/ml of the nucleus staining dye DAPI (4',6-diamidino-2-phenylindole) for 15 mins at room temperature. After a final wash with PBS (x3) and a dip wash with PBS and deionised water (x3) the cell slides were fixed to microscope cover slips.

Acknowledgements

The UK's Engineering and Physical Sciences Research Council (EPSRC) and GSK are thanked for funding

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