

Metal-Based Drugs

Iridium(I) Compounds as Prospective Anticancer Agents: Solution Chemistry, Antiproliferative Profiles and Protein Interactions for a Series of Iridium(I) N-Heterocyclic Carbene Complexes

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Abstract: A series of structurally related mono- and bis-NHC–iridium(I) (NHC: N-heterocyclic carbene) complexes have been investigated for their suitability as potential anticancer drugs. Their spectral behaviour in aqueous buffers under physiological-like conditions and their cytotoxicity against the cancer cell lines MCF-7 and HT-29 are reported. Notably, almost all complexes exhibit significant cytotoxic effects towards both cancer cell lines. In general, the cationic bis-carbene complexes show higher stability and greater anticancer activity than their neutral mono-carbene analogues with IC₅₀ values in the high nanomolar range. Furthermore,

to gain initial mechanistic insight, the interactions of these iridium(I)–NHC complexes with two model proteins, namely lysozyme and cytochrome c, were explored by HR-ESI-MS analyses. The different protein metalation patterns of the complexes can be roughly classified into two distinct groups. Those interactions give us a first idea about the possible mechanism of action of this class of compounds. Overall, our findings show that iridium(I)–NHC complexes represent very interesting candidates for further development as new metal-based anticancer drugs.

Introduction

In the field of chemotherapy, the metal-containing compound cisplatin and its derivatives carboplatin and oxaliplatin are currently used in more than 50% of all cancer treatments.^[1] Despite their great success, Pt-based chemotherapeutics still bear several drawbacks, such as a remarkable toxicity against healthy cells and the development of platinum-resistant tumors.^[2,3] These disadvantages underline the urgent need for further development of new anticancer drugs.

Organometallic compounds are among the most promising candidates in this area of research.^[4–8] Due to their wide range of coordination numbers and geometries they offer a great structural variety and, accordingly, a large variety of biological mechanisms. Indeed, a suitable ligand combined with the appropriate metal centre often allows a fine-tuning of the anticancer activity with higher cancer selectivity. Concerning the ligand system, N-heterocyclic carbenes (NHCs) have attracted

great attention owing to their high stability and facility of derivatisation. Thus, in recent years, a large number of metal–NHC complexes has been synthesised, and investigated as prospective anticancer drugs.^[9–12] In particular, numerous gold(I)–NHC complexes have shown remarkable antiproliferative effects against several cancer cell lines.^[13–16]

An interesting element that has recently received significant attention is iridium.^[5,17–19] The majority of Ir compounds for biological investigations feature either cyclopentadienyl^[20–22] (especially the Cp* ligand) or diimine ligands.^[23–25] The element, in all these compounds, is in its +III oxidation state. In this oxidation state, Ir is one of the most kinetically inert metal ions that exist under physiological conditions. It is, therefore, highly unlikely that Ir^{III} compounds will covalently interact with biomolecules. In contrast, iridium in the lower oxidation state +I is kinetically more reactive but, as yet, rather poorly investigated for applications in medicinal organometallic chemistry. Although a wide number of iridium(I)–NHC complexes is known, biological investigations remain extremely rare.^[26] In a recent publication, we reported on the cytotoxic activity of a new iridium(I) complex containing a simple ligand system.^[27] We discovered that our iridium(I) complex most likely causes its cytotoxic effects through binding to biologically relevant proteins. However, it is indispensable to investigate a greater number of iridium(I) complexes to establish a first detailed structure–activity relationship (SAR).

This prompted us to synthesise, characterise and evaluate a whole series of new iridium(I) compounds as prospective cytotoxic and anticancer agents. These compounds were prepared and screened for their cytotoxic activity against two representative cancer cell lines. Particular attention was paid to

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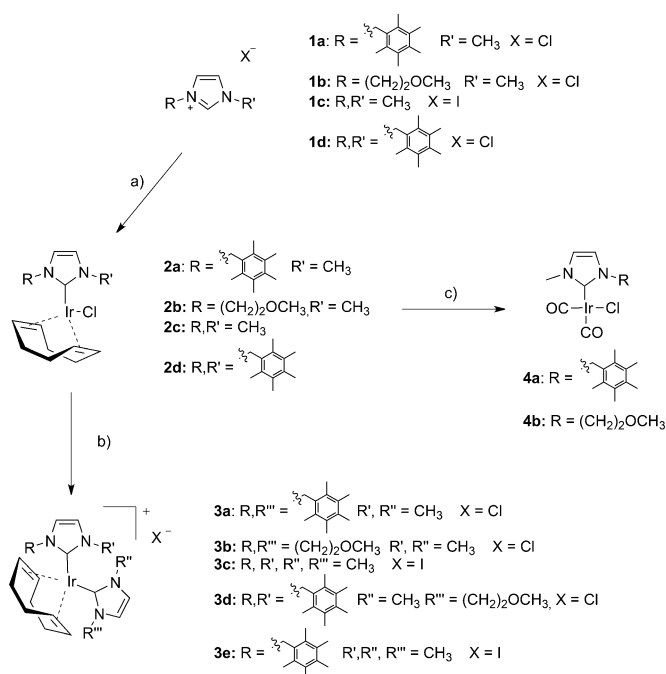
Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under <http://dx.doi.org/10.1002/chem.201601542>.

the analysis of their behaviour and stability in solution, and to their interactions with proteins, which seems to be mechanistically relevant. In fact, we wanted to ascertain whether these metal complexes exert their biological actions as intact species or undergo chemical activation and transformation prior to performing their biological effect, thus behaving as classical prodrugs.

Results and Discussion

Synthesis and characterisation

The iridium(I)–NHC complexes in the present study were prepared according to well-established procedures, depicted in Scheme 1. The ligands differ in the lipophilicity of their imidazole side-chains, in order to understand whether lipophilicity has a significant influence on the anticancer activity. Further, Ir^I–NHC compounds with chlorido ligands were compared to bis-NHC complexes devoid of such a labile ligand.



Scheme 1. Synthesis of **1a–4b**. a) 0.5 equiv Ag₂O, 0.5 equiv [Ir(COD)Cl]₂ in CH₂Cl₂ over night; b) imidazolium salt, 1 equiv K₂CO₃ in CH₂Cl₂; c) CO atmosphere in CH₂Cl₂, 10 min.

For the preparation of the mono-carbene complexes, the respective silver carbene complexes were generated in situ by treatment of the imidazolium salts with 0.5 equivalents of Ag₂O. Addition of [Ir(COD)Cl]₂ led to the corresponding iridium carbenes. The products were purified by column chromatography and full characterisation was carried out using ¹H NMR and ¹³C NMR spectroscopy, mass spectrometry and elemental analysis. The formation of the complexes was confirmed by the disappearance of the C2 proton signal in the ¹H NMR spectra and by a strong downfield shift of the C2

carbon signal in the ¹³C NMR spectra. To obtain the iridium(I)–bis-carbene complexes, the mono-carbene complexes were reacted with the corresponding imidazolium salt in the presence of 1 equivalent of a mild base and purified by column chromatography. ¹H NMR and ¹³C NMR spectroscopy and mass spectrometry confirmed the formation of the complexes with characteristic upfield shifts of the signals originating from the unsaturated cyclooctadiene carbon atoms between 5 and 10 ppm.

The carbonyl-substituted iridium(I) complexes [Ir(NHC)(CO)₂Cl] were obtained by passing carbon monoxide gas through a solution of the [Ir(NHC)(COD)Cl] complexes in dichloromethane at room temperature resulting in quantitative replacement of the COD ligand by two equivalents of CO.

Suitable crystals of complexes **2b** and **4a** for single-crystal X-ray diffraction analysis were obtained by slow diffusion of hexane into a solution of the complexes in dichloromethane at 4 °C (Figure 1).

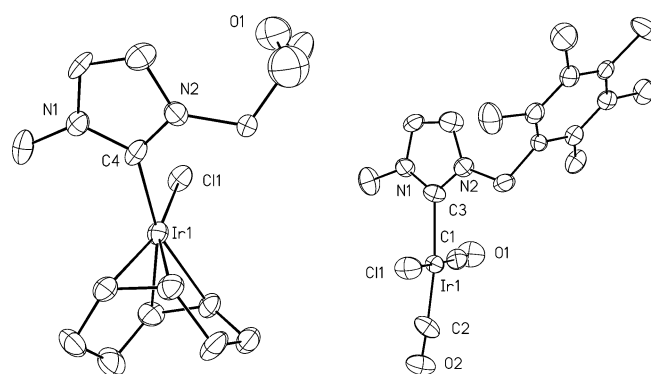


Figure 1. Molecular structure of **2b** (left) and **4a** (right). Heavy atoms are displayed at 30% probability, hydrogen atoms were omitted for clarity. Selected bond lengths (Å) for **2b**: Ir1–Cl1 2.372(4), Ir1–C4 2.05(1), C4–N1 1.36(2), C4–N2, 1.36(2); and for **4a**: Ir1–Cl1 2.334(4), Ir1–C1 1.85(1), Ir1–C2 1.89(2), Ir1–C3 2.11(1), C1–O1 1.13(2), C2–O2 1.14(2), C3–N1 1.33(2), C3–N2, 1.32(2).

Solution studies

When considering a possible use of iridium complexes as anti-cancer drugs, it is important to understand their behaviour in aqueous solutions. The solution behaviour of the compounds was therefore analysed by time-dependent UV/Vis absorption spectroscopy in DMSO and in aqueous buffers under physiological-like conditions. Final concentrations of 200 μM or 500 μM of the individual complexes were diluted in the reference buffer (20 mM ammonium acetate, pH 6.8 or 20 mM phosphate buffer, pH 7.3) with an addition of DMSO (50% for **2a**, **2b** and **4a**, and 10% for the bis-carbene complexes **3a–3e**) due to poor water solubility. The changes in the spectral profiles were monitored over 48 h at room temperature for all compounds (Figure 2).

The neutral complexes bearing a chlorido ligand show only small changes in their spectral profile in neat DMSO, expressed by a progressive decrease in absorption intensity. As shown in previous work,^[27] the observed spectral changes for the complexes can be traced back to the slow dioxygen-caused oxida-

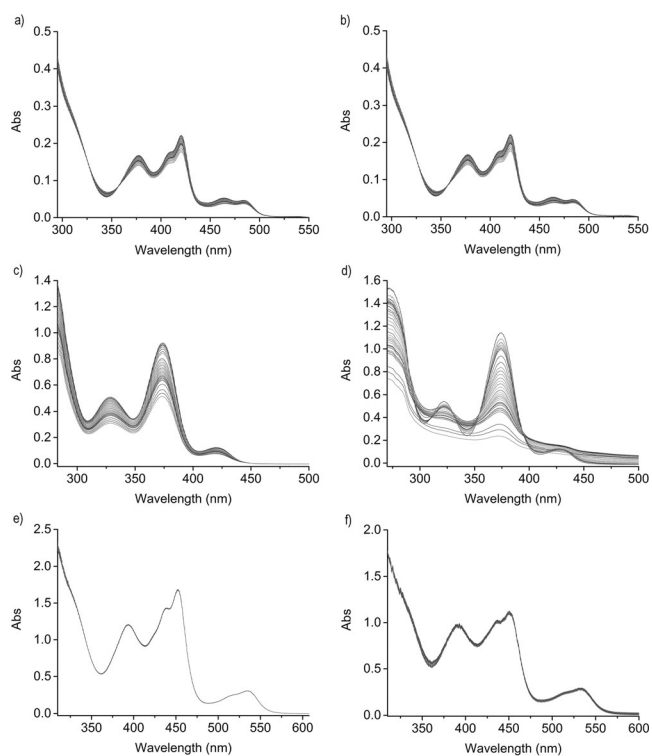


Figure 2. Spectral profiles of **2b**: a) 200 μM DMSO, b) 200 μM in ammonium acetate, pH 6.8, 50% DMSO. Spectral profiles of **4a**, c) 200 μM DMSO, d) 200 μM in ammonium acetate, pH 6.8, 50% DMSO. Spectral profiles of **3d**, e) 500 μM DMSO, f) 500 μM in ammonium acetate, pH 6.8, 10% DMSO.

tion presumably of Ir^{I} to Ir^{III} . Among the mono-carbene complexes, the order $2\text{b} > 4\text{a} > 2\text{a}$ is established for the stability of the complexes towards oxidation in DMSO, while in ammonium acetate buffer, **4a** is less affected followed by **2b** and **2a**.

Substitution of the chlorido ligand by another NHC ligand greatly increases the stability towards oxidation in solution. The cationic compounds **3a–3e** show no oxidation in DMSO even upon diluting DMSO solutions with aqueous buffers. Spectral profiles of **3b** and **3e** are reported in the supporting information (Figure S2–S8).

Since the bis-carbene complexes show no oxidation by air in aqueous solutions, we conclude that the oxidation state +I is much more stable in this class of compounds. This was further probed by exposing them to an excess of hydrogen peroxide. To this end, the spectral change of the complexes in the presence of 10 equivalents of hydrogen peroxide over a period of 18 h was recorded. The intensity at the highest maximum in the visible range at 451 nm as a function of time, with and without the presence of H_2O_2 for **3e**, is depicted in Figure 3.

The intensity is described as a linear dependence on the time. In the absence of H_2O_2 the intensity shows a steady behaviour. A different behaviour is observed in the presence of 10 equivalents of H_2O_2 . Here, the intensity decreases to 65% after 18 h, which can be traced back to a change of the oxidation state of iridium from Ir^{I} to Ir^{III} .

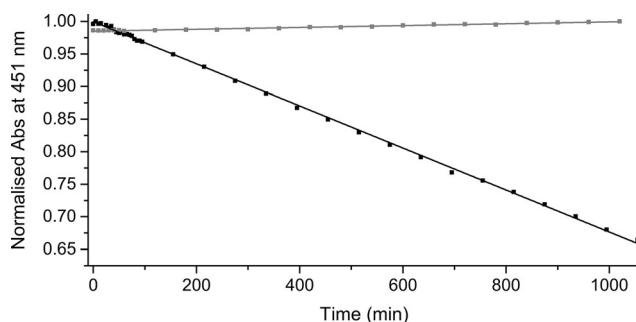


Figure 3. Normalised absorption at 451 nm of **3e** in ammonium acetate, pH 6.8, 10% DMSO with a concentration of 500 μM (top line) and of 100 μM in the presence of 10 equiv H_2O_2 (bottom line). Spectra were recorded at room temperature.

Antiproliferative activity in vitro

The in vitro anticancer activity of the iridium complexes was determined in MCF-7 (human breast adenocarcinoma) and HT-29 (colon adenocarcinoma) cells using the MTT assay with an incubation time of 48 h (see Table 1).

Table 1. Antiproliferative effects of the complexes towards MCF-7 and HT-29 cells. IC_{50} values are expressed as the mean [μM] (\pm standard deviation) of three independent experiments.^[a] Numbers are reported to two relevant digits in all cases for consistency, thus resulting in apparently different precision.

Complex	MCF-7	HT-29
2a	14.6 \pm 2.5[18]	19.3 \pm 3.8[18]
3a	0.61 \pm 0.11	0.35 \pm 0.19
4a	64.2 \pm 9.8	95.6 \pm 2.5
2b	38.7 \pm 5.9	49.9 \pm 7.6
3b	4.85 \pm 0.91	6.3 \pm 1.5
2c	46.920 \pm 0.085	43.8 \pm 4.8
3c	8.6 \pm 1.3	6.4 \pm 1.2
3d	0.492 \pm 0.031	0.261 \pm 0.046
3e	0.54 \pm 0.27	0.470 \pm 0.063

[a] Examples of dose-response curves are provided in the Supporting Information (Figures S9–S12).

The biological activity of complexes **2d** and **4b** could not be studied since **2d** showed poor solubility in DMSO and **4b** decomposed too rapidly under the assay conditions.

The neutral mono-carbene complexes **2a**, **2b** and **2c** of the type $[\text{Ir}(\text{NHC})(\text{COD})\text{Cl}]$ show moderate cytotoxicity in the low micromolar range against both cell lines. Replacement of the COD ligand in **2a** by CO leads to a noteworthy decrease of the activity, resulting in around 4-fold higher IC_{50} values.

Remarkably, the introduction of a second carbene ligand produces a strong increase of the cytotoxic effects towards both cell lines with IC_{50} values in the high nanomolar range. Accordingly, the bis-carbene complexes **3a**, **3d** and **3e** show significantly higher cytotoxicity than the well-established drug cisplatin.^[28,29] It is noted, that complexes containing the more lipophilic pentamethylbenzene in their side-chains (**2a**, **3a**, **3d** and **3e**) in general feature a significantly higher antiproliferative activity against both cell lines in both the mono- and bis-

carbene series. In this respect, the less lipophilic complexes **3b** and **3c** show significantly lower IC_{50} values, but still comparable to the value of cisplatin.

In terms of structure–activity relationships, we can draw the following conclusion from the cytotoxicity data. The bis-carbene complexes remain the most toxic derivatives in this class of compounds with IC_{50} values below $1 \mu\text{M}$. The corresponding neutral mono-carbene complexes display antiproliferative activity in the low micromolar range, whereas exchange of the COD ligand by CO further reduces the cytotoxic activity.

Reactions with model proteins

The identification of possible biological targets of anticancer metallodrugs may give a first insight into their mode of action. In a previous study, we could demonstrate that **2a** shows adduct formation with different model proteins. This binding occurs with simultaneous oxidation of Ir^{I} to Ir^{III} , by loss of the chlorido and cyclooctadiene ligand.

The same protein metalation studies were performed for all compounds presented in this work for two major reasons. Firstly, we wanted to address the question is adduct formation general between mono-carbene complexes and these proteins? The second reason was our inquisitiveness whether also the more oxidation-resistant bis-carbene complexes show the same behaviour in spite of their greater stability and intriguingly higher antiproliferative effects.

Accordingly, the reactivity of all new complexes with the proteins horse heart cytochrome c and hen egg white lysozyme (HEWL) has been systemically investigated. In this study, they serve primarily as small model proteins. However, both proteins are present in the human body as well and are involved in different cellular processes. Whereas cytochrome c is mainly found in the membranes of mitochondria, lysozyme is an important part of the inherent immune system. The interaction between these proteins and the metal complexes has been studied by using high-resolution electrospray ionisation mass spectrometry (HR-ESI-MS), which allows rapid and straightforward identification and characterisation of the resulting metallodrug–protein adducts. Solutions of the complexes in ammonium acetate (10% DMSO for compounds **3a–3e**; 50% DMSO for **2a + 2b + 4a**) were incubated with the protein (3:1 and 10:1 ratio) at 37°C . At selected time points (24 h, 48 h, 72 h), aliquots were taken and analysed by HR-ESI-MS. All mono-carbene complexes show extensive adduct formation with both proteins. Among these compound **4a** appears to be the most reactive, producing the largest amounts of protein adducts as judged by the absolute intensity of the MS signals. As an example, the mass spectrum of cytochrome c after incubation with **4a** for 72 h is depicted in Figure 4.

In total, two specific adducts could be identified. The assignments were proven by comparing the observed isotope patterns with the calculated ones. The adduct with the highest intensity belongs to the iridium(III)–NHC fragment binding to cytochrome c with concurrent loss of the chlorido ligand and both carbonyl groups. This observation differs from the adduct formation with HEWL, in which one CO is still bound to the iri-

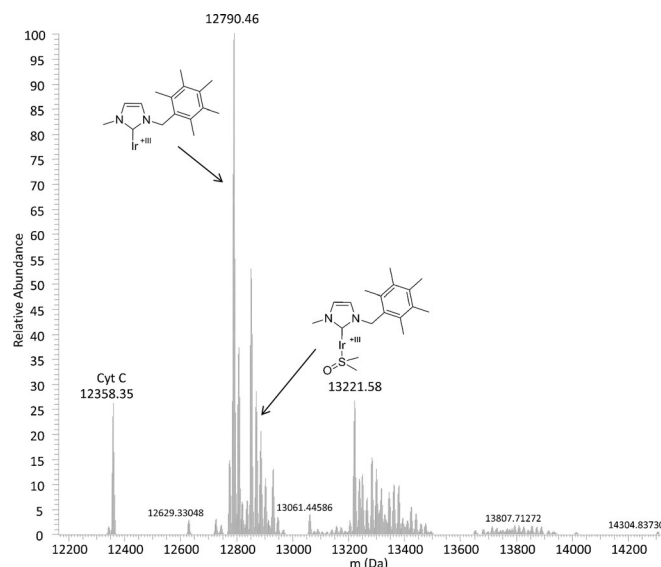


Figure 4. HR-ESI-MS of cytochrome c treated with $300 \mu\text{M}$ of **4a** (complex/protein = 3:1) in 20 mM ammonium acetate buffer (50% DMSO), pH 6.8 recorded after 24 h of incubation at 37°C .

dium centre (see Supporting Information, Figure S15). Another adduct formed between **4a** and cytochrome c can be assigned to the iridium(III)–NHC fragment containing only one DMSO molecule, most probably bound to the iridium centre. However, in all cases the oxidation state of iridium changes from +I to +III, which is in good agreement with our earlier work.

The mass spectra of the proteins incubated with the COD containing mono-carbene complexes **2a–c** show the same main adducts corresponding to the iridium(III)–NHC fragment.

The substitution of chloride by another NHC ligand greatly increases the stability in solution and reduces the reactivity with biomolecules. Accordingly, no adduct formation with either of the two model proteins was observed for the cationic bis-carbene complexes **3a–e** by mass spectrometry even upon long incubation times and a protein–complex ratio of 1:10. (Examples are provided in the Supporting Information, Figures S13 and S14.) It seems that substitution of the most labile chlorido ligand is the initial step for protein interactions, and is in fact required before oxidation of the iridium(I) centre to iridium(III).

Therefore, our findings strongly suggest that the loss of labile ligands such as chloride, followed by the oxidation of the metal centre, paves the way for protein binding. Clearly, this reaction cannot happen in the bis-carbene complexes **3**, which consequently do not interact with proteins easily. Apparently, protein binding results in a drastic attenuation (but not loss) of the cytotoxic activity. Overall, these observations indicate different modes of actions for the neutral mono-carbene complexes on the one hand, and for the cationic bis-carbene complexes on the other hand.

Conclusion

The synthesis, structural aspects, solution behaviour, antiproliferative activity and the interaction with model proteins for a series of novel iridium(I)-NHC complexes were comprehensively investigated in this study. The synthesised compounds can be roughly categorised into two groups, that is, cationic and neutral Ir^I-NHC complexes. These two classes of compounds show markedly different behaviour in solution, as well as different metalation behaviour with proteins.

Almost all investigated compounds show antiproliferative effects at least against two representative cell lines, for which the cytotoxicity of the cationic compounds in the high nanomolar range stands out in particular. Solution studies of the latter revealed a high stability toward oxidation and no reaction with model proteins. Only the presence of an excess of H₂O₂ leads to slow oxidation of Ir^I to most likely Ir^{III}. The neutral complexes however, undergo progressive oxidation in aerated solutions, being far less inert than their cationic counterparts both in DMSO and aqueous buffers. They bind to cytochrome c and lysozyme by oxidation to Ir^{III} with simultaneous loss of chlorido and cyclooctadiene/carbonyl ligands. Both cytochrome c as well as lysozyme, although having different properties, are good Ir^I models for other protein targets. It is therefore reasonable to assume that these Ir^I complexes with labile ligands have the potential of metalating and altering cellular proteins, thus leading to irreversible cell damage and death.

The more oxidation-resistant cationic bis-carbene complexes, while showing no interactions with proteins, produce far greater cytotoxic effects. This is an indication for different modes of action for this respective class of compounds. While the neutral complexes seem to cause cell death through interactions with several proteins (likely with little target specificity), the cationic complexes most probably exert their biological effect as intact molecules. Their higher activity may therefore arise from a more effective targeting, since they might not react with other cellular components before reaching their actual target(s). Moreover, it is hypothesised that they work by causing selective mitochondrial damage, given their structures as large, delocalised lipophilic cations. In line with this thought, the higher activity of the more lipophilic derivatives in both series of compounds likely results from a better cellular uptake.

Experimental Section

General: Reactions were carried out under N₂ by using standard Schlenk techniques. All solvents were of analytical grade. Chemicals were obtained from commercial sources and used without further purification.

¹H and ¹³C NMR spectra were recorded at room temperature on Bruker DPX 200, DPX 250 and DRX 400 spectrometers, respectively, in deuterated solvents which were used as internal reference. The chemical shifts are reported in ppm (parts per million) relative to TMS. Coupling constants *J*, are reported in Hz, multiplicities being marked as: singlet (s), doublet (d), triplet (t) or multiplet (m). IR spectra were measured on a Bruker Tensor 27 instrument equipped

with an attenuated total reflection (ATR) unit at 4 cm⁻¹ resolution. ESI mass spectra were measured on a Bruker Esquire 6000 mass spectrometer. FAB and EI high resolution mass spectrometry were performed with a Fisons VG Instruments Autospec spectrometer. High-resolution mass spectra were recorded with an LTQ Orbitrap high-resolution mass spectrometer (Thermo Scientific, San Jose, CA, USA) equipped with a conventional ESI source. The mass to charge relation (*m/z*) is given as a dimensionless number. UV-Vis absorption spectra were recorded on a Varian Cary 50 spectrophotometer. Elemental analyses were carried out on a Vario EL (Elementar Analysensysteme GmbH, Hanau, D) in C, H, N mode.

Cell culture and cytotoxicity: Dulbecco's Modified Eagle's Medium (DMEM), containing 10% fetal calf serum, 1% penicillin and streptomycin, was used as growth medium. MCF-7 and HT-29 cells were detached from the wells with trypsin and EDTA, harvested by centrifugation and resuspended again in cell culture medium. The assays have been carried out on 96 well plates with 6000 cells per well for both cell lines, MCF-7 and HT-29. After 24 h of incubation at 37 °C and 10% CO₂, the cells were treated with the compounds (with DMSO concentrations of 0.5%) with a final volume of 200 μL per well. For a negative control, one series of cells was left untreated. The cells were incubated for 48 h followed by adding 50 μL MTT (2.5 mg mL⁻¹). After an incubation time of 2 h, the medium was removed and 200 μL DMSO were added. The formazan crystals were dissolved and the absorption was measured at 550 nm, using a reference wavelength of 620 nm. Each test was repeated in quadruplicates in three independent experiments for each cell line.

Interaction with cytochrome c (lysozyme): Solutions of the compounds (100 μM) with cytochrome c (lysozyme) (10:1 complex/protein molar ratio) in 10% (50%) ammonium acetate buffer (20 μM, pH 6.8) and 90% (50%) DMSO were incubated at 37 °C. After 24 h (48 h, 72 h) and 20-fold dilution with water ESI MS spectra were recorded by direct introduction at 5 μL min⁻¹ flow rate in an Orbitrap high-resolution mass spectrometer (Thermo, San Jose, CA, USA), equipped with a conventional ESI source. The working conditions were as follows: spray voltage 3.1 kV, capillary voltage 45 V, capillary temperature 220 °C, tube lens voltage 230 V. The sheath and the auxiliary gases were set, respectively, at 17 (arbitrary units) and 1 (arbitrary units). For acquisition, Xcalibur 2.0. software (Thermo) was used and monoisotopic and average deconvoluted masses were obtained by using the integrated Xtract tool. For spectrum acquisition a nominal resolution (at *m/z* 400) of 100 000 was used.

Synthesis of 1a: To a flame-dried schlenk flask 1-(chloromethyl)-2,3,4,5,6-penta-methylbenzene (2 g, 10.2 mmol) and 1-methylimidazole (803 μL, 10.2 mmol) were dissolved in dry THF (20 mL) and stirred under reflux for 48 h. The precipitated solid was washed three times with THF to obtain **1a** as a white solid (2.23 g, 79.0%). ¹H NMR (250 MHz, CD₃OD): δ = 8.56 (s, 1H, N=CH-N), 7.57 (d, *J* = 2.0 Hz, 1H, N-CH=CH-N), 7.46 (d, *J* = 2.0 Hz, 1H, N-CH=CH-N), 5.52 (s, 2H, N-CH₂), 3.88 (s, 3H, H_{NMe}), 2.31–2.23 (m, 15H, Me); MS (FAB⁺): *m/z*: 243.1 [M–Cl]⁺.

Synthesis of 1b: To a flame-dried schlenk flask 2-methoxyethyl chloride (400 mg, 4.23 mmol) and 1-methylimidazole (337 μL, 4.23 mmol) were dissolved in acetonitrile (15 mL) and stirred under reflux for 24 h. The solvent was removed and the remaining yellow oil precipitated adding 10 mL of diethyl ether. **1b** was obtained as a beige solid (608 mg, 81.3%). ¹H NMR (200 MHz, D₂O): δ = 8.75 (s, 1H, N-CH=N), 7.52 (d, *J* = 1.7 Hz, 1H, N-CH=CH-N), 7.46 (d, *J* = 1.7 Hz, 1H, N-CH=CH-N), 4.40 (t, *J* = 5.0 Hz, 2H, N-CH₂), 3.91 (s, 3H, H_{NMe}), 3.84 (t, *J* = 5.0 Hz, 2H, O-CH₂), 3.39 ppm (s, 3H, H_{OMe}); ¹³C NMR (50 MHz, D₂O): δ = 136.3 (N-CH=N), 123.5 (NCH=CHN), 122.4 (NCH=CHN), 69.8 (O-CH₂), 58.2 (C_{OMe}), 48.9 (N-CH₂), 35.7 ppm (C_{NMe}).

Synthesis of 1c: To a flame-dried schlenk flask 1-methylimidazole (971 μL , 12.2 mmol) and iodomethane (1.35 mL, 14.6 mmol) were dissolved in dry THF (15 mL) and stirred under reflux for 48 h. The precipitated solid was washed three times with THF to obtain **1c** as a white solid (2.55 mg, 93.4%). ^1H NMR (200 MHz, CD_2Cl_2): δ = 9.08 (s, 1H, N-CH=N), 7.70 (d, J = 1.6 Hz, 2H, N-CH=CH-N), 3.85 ppm (s, 6H, H_{NMe}); ^{13}C NMR (50 MHz, DMSO) δ = 136.9 (N-CH=N), 123.3 (NCH=CHN), 35.8 ppm (C_{NMe}).

Synthesis of 1d: To a flame-dried schlenk flask, NaH (24.0 mg, 1.0 mmol) was dissolved in dry THF (20 mL). After 10 min imidazole (68.2 mg, 1.0 mmol) was added and the mixture was stirred for 15 min. Then 1-(chloromethyl)-2,3,4,5,6-pentamethylbenzene (393.4 mg, 2.0 mmol) was added and the reaction mixture was stirred for another 30 min. Water (50 mL) and diethyl ether (50 mL) were added, the phases were separated and the aqueous phase was extracted with diethyl ether (2 \times 50 mL). The combined organic phases were washed with water and dried over magnesium sulfate. The residue was purified by column chromatography on silica gel using mixtures of dichloromethane/methanol to give **1d** (289 mg, 68.0%) of a white solid. ^1H NMR (200 MHz, CD_3OD): δ = 8.40 (s, 1H, N-CH=N), 7.28 (s, 1H, NCH=CHN), 7.27 (s, 1H, NCH=CHN), 5.48 (s, 4H, N-CH₂), 2.26 (s, 6H, Me), 2.23 (s, 12H, Me), 2.19 ppm (s, 12H, Me); ^{13}C NMR (50 MHz, CD_3OD): δ = 138.2 (N-CH=N), 134.9 ($\text{C}_{\text{Ar-1}}$), 134.8 ($\text{C}_{\text{Ar-2}}$), 127.2 ($\text{C}_{\text{Ar-3,Ar-4}}$), 123.4 (NCH=CHN), 52.8 (N-CH₂), 17.3 (Me), 17.0 (Me), 16.6 ppm (Me); MS (ESI⁺): m/z : 389.0 [$M-\text{Cl}$]⁺.

General method for [Ir(COD)(NHC)(Cl)]—method A: A Schlenk flask was charged with the imidazolium salt and 0.5 equivalents of silver oxide in dry dichloromethane and stirred at rt for 1 h. 0.5 equivalents of [[Ir(COD)Cl]₂] was added and the resulting mixture was stirred for 24 h. The resulting suspension was filtered through celite, and the filtrate was concentrated to dryness. The crude product was purified by column chromatography on silica using mixtures of dichloromethane and methanol.

Data for 2a synthesised by method A: Compound **1a** (130.0 mg, 466 μmol), Ag₂O (54.0 mg, 233 μmol) and [Ir(COD)Cl]₂ (156.6 mg, 233 μmol) gave **2a** (226.0 mg, 83.9%) as a yellow solid. ^1H NMR (400 MHz, CD_2Cl_2): δ = 6.72 (d, J = 2.0 Hz, 1H, N-CH=CH-N), 6.24 (d, J = 2.0 Hz, 1H, N-CH=CH-N), 5.79 (d, J = 14.5 Hz, 1H, N-CH₂), 5.32 (d, J = 14.5 Hz, 1H, N-CH₂), 4.55–4.49 (m, 2H, COD), 3.96 (s, 3H, H_{NMe}), 3.17 (td, J = 7.1, 2.8 Hz, 1H, COD), 3.09 (td, J = 6.8, 2.8 Hz, 1H, COD), 2.34–2.21 (m, 4H, COD), 2.27 (s, 3H, Me), 2.24 (s, 6H, Me), 2.21 (s, 6H, Me), 1.79–1.71 (m, 2H, COD), 1.70–1.60 ppm (m, 2H, COD); ^{13}C NMR (101 MHz, CD_2Cl_2): δ = 180.9 (NCN), 136.4 ($\text{C}_{\text{Ar-1}}$), 134.5 ($\text{C}_{\text{Ar-2}}$), 133.7 ($\text{C}_{\text{Ar-3}}$), 128.8 ($\text{C}_{\text{Ar-4}}$), 121.2 (N-CH=CH-N), 119.5 (N-CH=CH-N), 84.5 (COD), 84.5 (COD), 51.9 (COD), 51.4 (COD), 50.1 (N-CH₂), 38.1 (C_{NMe}), 34.2 (COD), 34.2 (COD), 30.2 (COD), 17.4 (Me), 17.1 ppm (Me); MS (ESI⁺): m/z : 543.0 [$M-\text{Cl}$]⁺; MS (FAB⁺): m/z : 578.0 [M]⁺, 543.1 [$M-\text{Cl}$]⁺, 470.0 [$M-\text{COD}$]⁺; elemental analysis calcd for C₂₄H₃₄ClIrN₂: C 49.85, H 5.93, N 4.84; found: C 49.47, H 6.19, N 4.68.

Data for 2b synthesised by method A: Compound **1b** (150.0 mg, 849 μmol), Ag₂O (98.4 mg, 425 μmol) and [Ir(COD)Cl]₂ (285.2 mg, 425 μmol) gave **2b** (303.2 mg, 75.0%) as a yellow solid; ^1H NMR (250 MHz, CD_2Cl_2): δ = 7.02 (d, J = 1.9 Hz, 1H, N-CH=CH-N), 6.83 (d, J = 1.9 Hz, 1H, N-CH=CH-N), 4.79–4.59 (m, 1H, N-CH₂), 4.59–4.45 (m, 2H, COD), 4.45–4.30 (m, 1H, N-CH₂), 3.92 (s, 3H, H_{NMe}), 3.86–3.71 (m, 2H, O-CH₂), 3.34 (s, 3H, H_{OMe}), 3.08–2.83 (m, 2H, COD), 2.36–2.12 (m, 4H, COD), 1.86–1.51 ppm (m, 4H, COD); ^{13}C NMR (63 MHz, CD_2Cl_2): δ = 180.8 (NCN), 122.2 (N-CH=CH-N), 121.9 (N-CH=CH-N), 84.5 (COD), 72.6 (O-CH₂), 59.3 (C_{OMe}), 51.1 (COD), 51.9 (COD), 50.7 (N-CH₂), 37.8 (C_{NMe}), 34.2 (COD), 34.0 (COD), 30.2 (COD), 30.0 ppm (COD); MS (ESI⁺): m/z : 441.0 [$M-\text{Cl}$]⁺, 476.9 [M]⁺; ele-

mental analysis calcd for C₁₅H₂₄ClIrN₂O: C 37.85, H 5.08, N 5.88; found: C 37.44, H 5.41, N 5.65.

Data for 2c synthesised by method A: Compound **1c** (150 mg, 670 μmol), Ag₂O (77.5 mg, 335 μmol) and [Ir(COD)Cl]₂ (225 mg, 670 μmol) gave **2c** (226.3 mg, 78.2%) as a yellow solid; ^1H NMR (200 MHz, CD_2Cl_2): δ = 6.85 (s, 2H N-CH=CH-N), 4.51–4.42 (m, 2H, COD), 3.91 (s, 6H, H_{NMe}), 3.01–2.91 (m, 2H, COD), 2.28–2.12 (m, 4H, COD), 1.75–1.57 ppm (m, 4H, COD); ^{13}C NMR (50 MHz, CD_2Cl_2): δ = 181.1 (NCN), 122.1 (N-CH=CH-N), 84.4 (COD), 51.7 (COD), 37.7 (C_{NMe}), 34.1 (COD), 30.1 ppm (COD).

Data for 2d synthesised by Method A: Compound **1d** (150 mg, 353 μmol), Ag₂O (40.9 mg, 177 μmol) and [Ir(COD)Cl]₂ (119 mg, 177 μmol) gave **2d** (237 mg, 92.7%) as a yellow solid; ^1H NMR (400 MHz, CD_2Cl_2): δ = 6.12 (d, J = 2.9 Hz, 2H, N-CH=CH-N), 5.88 (d, J = 14.5 Hz, 2H, N-CH₂), 5.37 (d, J = 14.5 Hz, 2H, N-CH₂), 4.63–4.51 (m, 2H, COD), 3.39–3.25 (m, 2H, COD), 2.43–2.28 (m, 4H, COD), 2.39–2.23 (m, 6H, Me), 2.23 (s, 12H, Me), 2.22 (s, 12H, Me), 1.87–1.63 ppm (m, 4H, COD); ^{13}C NMR (101 MHz, CD_2Cl_2): δ = 180.6 (NCN), 136.2 ($\text{C}_{\text{Ar-1}}$), 134.6 ($\text{C}_{\text{Ar-2}}$), 133.6 ($\text{C}_{\text{Ar-3}}$), 129.0 ($\text{C}_{\text{Ar-4}}$), 118.5 (N-CH=CH-N), 84.4 (COD), 54.4 (N-CH₂), 51.6 (COD), 50.5 (COD), 34.3 (COD), 30.2 (COD), 17.4 (Me), 17.2 (Me), 17.1 ppm (Me); MS (ESI⁺): m/z : 689.1 [$M-\text{Cl}$]⁺.

General method for [Ir(COD)(NHC)₂]₂—method B: A Schlenk flask was charged with [Ir(COD)(NHC)(Cl)], the corresponding imidazolium salt and 1 equivalent of K₂CO₃ in dry dichloromethane and stirred at RT for 48 h. The resulting suspension was filtered through celite and the filtrate was concentrated to dryness. The crude product was purified by column chromatography on silica using mixtures of dichloromethane and methanol.

Data for 3a synthesised by method B: Compound **2a** (100 mg, 172.9 μmol), K₂CO₃ (15.9 mg, 172.9 μmol) and **1a** (48.2 mg, 172.9 μmol) gave **3a** (50.1 mg, 35.3%) as an orange solid. ^1H NMR (400 MHz, CD_2Cl_2): δ = 7.06 (d, J = 2.0 Hz, 2H, N-CH=CH-N), 6.34 (d, J = 1.9 Hz, 2H, N-CH=CH-N), 5.20 (d, J = 13.9 Hz, 2H, N-CH₂), 4.99 (d, J = 14.0 Hz, 2H, N-CH₂), 4.52 (t, J = 6.6 Hz, 2H, COD), 4.19 (s, 6H, H_{NMe}), 3.58 (dd, J = 13.3, 7.7 Hz, 2H, COD), 2.52 (dtd, J = 16.1, 9.7, 6.6 Hz, 2H, COD), 2.37–2.17 (m, 4H, COD), 2.26 (s, 6H, Me), 2.21 (s, 12H, Me), 1.94 (s, J = 3.8 Hz, 12H, Me), 1.78 ppm (dt, J = 14.2, 8.7 Hz, 2H, COD); ^{13}C NMR (101 MHz, CD_2Cl_2): δ = 177.8 (NCN), 137.3 ($\text{C}_{\text{Ar-1}}$), 134.2 ($\text{C}_{\text{Ar-2}}$), 133.8 ($\text{C}_{\text{Ar-3}}$), 127.2 ($\text{C}_{\text{Ar-4}}$), 122.7 (N-CH=CH-N), 120.7 (N-CH=CH-N), 80.3 (COD), 74.2 (COD), 50.0 (N-CH₂), 39.5 (C_{NMe}), 35.6 (COD), 28.6 (COD), 17.5 (Me), 17.1 (Me), 16.4 (Me); MS (ESI⁺): m/z : 785.1 [$M-\text{Cl}$]⁺; MS (FAB⁺): m/z : 785.1 [$M-\text{Cl}$]⁺.

Data for 3b synthesised by method B: Compound **2b** (30 mg, 63.4 μmol), K₂CO₃ (8.79 mg, 63.4 μmol) and the **1b** (11.2 mg, 63.4 μmol) gave **3b** (32.0 mg, 81.8%) as an orange solid. ^1H NMR (400 MHz, CD_2Cl_2): δ = 7.23 (d, J = 1.9 Hz, 1H, N-CH=CH-N), 7.20 (d, J = 1.9 Hz, 1H, N-CH=CH-N), 7.09 (d, J = 2.0 Hz, 1H, N-CH=CH-N), 7.09 (d, J = 2.0 Hz, 1H, N-CH=CH-N), 4.71–4.60 (m, 1H, N-CH₂), 4.46 (ddd, J = 13.9, 6.7, 4.0 Hz, 1H, N-CH₂), 4.31 (ddd, J = 13.1, 5.9, 3.6 Hz, 2H, N-CH₂), 4.16–4.07 (m, 1H, COD), 3.99 (s, 3H, H_{NMe}), 3.93 (s, 3H, H_{NMe}), 3.92–3.87 (m, 1H, COD), 3.86–3.80 (m, 1H, COD), 3.73–3.69 (m, 1H, O-CH₂), 3.66–3.56 (m, 4H, COD, O-CH₂), 3.35 (s, 3H, H_{OMe}), 3.30 (s, 3H, H_{OMe}), 2.34–2.18 (m, 4H, COD), 2.10–1.79 ppm (m, 4H, COD); ^{13}C NMR (101 MHz, CD_2Cl_2): δ = 177.7 (NCN), 177.7 (NCN), 123.7 (N-CH=CH-N), 123.7 (N-CH=CH-N), 122.1 (N-CH=CH-N), 121.8 (N-CH=CH-N), 78.4 (COD), 77.0 (COD), 76.8 (COD), 75.5 (COD), 71.7 (O-CH₂), 71.5 (O-CH₂), 59.5 (C_{OMe}), 51.1 (N-CH₂), 51.0 (N-CH₂), 38.7 (C_{NMe}), 38.6 (C_{NMe}), 33.4 (COD), 31.9 (COD), 31.9 (COD), 30.4 ppm (COD); MS (ESI⁺): m/z : 581.0 [$M-\text{Cl}$]⁺; MS (FAB⁺): m/z : 581.3 [M]⁺; elemental analysis calcd for C₂₂H₃₆IrN₄O₂Cl: C 42.88, H 5.89, N 9.09; found: C 41.42, H 6.25, N 8.62.

Data for 3c synthesised by method B: Compound **2c** (20.3 mg, 47 μmol), K_2CO_3 (6.5 mg, 47 μmol) and **1c** (10.5 mg, 47 μmol) gave **3c** (26.3 mg, 90.4%) as an orange solid; $^1\text{H NMR}$ (400 MHz, CD_2Cl_2): $\delta = 7.07$ (s, 4H, N-CH=CH-N), 3.91 (s, 12H, H_{NMe}), 3.86–3.78 (m, 4H), 2.35–2.19 (m, 4H, COD), 1.98–1.88 ppm (m, 4H, COD); $^{13}\text{C NMR}$ (101 MHz, CD_2Cl_2): $\delta = 177.9$ (NCN), 123.5 (N-CH=CH-N), 76.7 (COD), 38.5 (C_{NMe}), 31.9 ppm (COD); MS (ESI⁺): m/z : 492.8 [M–I]⁺.

Data for 3d synthesised by method B: Compound **2d** (50 mg, 69 μmol), K_2CO_3 (9.5 mg, 69 μmol) and **1b** (12.2 mg, 69 μmol) gave **3d** (36.9 mg, 61.9%) as an orange solid; $^1\text{H NMR}$ (400 MHz, CD_2Cl_2): $\delta = 7.42$ (d, $J = 1.8$ Hz, 1H, N-CH=CH-N), 7.28 (d, $J = 1.7$ Hz, 1H, N-CH=CH-N), 6.22 (d, $J = 1.8$ Hz, 1H, N-CH=CH-N), 6.19 (d, $J = 1.8$ Hz, 1H, N-CH=CH-N), 5.49 (d, $J = 14.1$ Hz, 1H, N-CH₂), 5.41 (d, $J = 14.1$ Hz, 1H, N-CH₂), 5.25 (dd, $J = 14.1$, 4.7 Hz, 2H, N-CH₂), 4.87 (ddd, $J = 13.7$, 6.0, 3.6 Hz, 1H, N-CH₂-CH₂), 4.29 (dd, $J = 6.9$, 3.6 Hz, 1H, N-CH₂-CH₂), 4.25–4.19 (m, 1H, COD), 4.19–4.12 (m, 1H, COD), 4.11 (s, 3H, H_{NMe}), 4.09–4.02 (m, 1H, COD), 3.87–3.83 (m, 1H, COD), 3.83–3.74 (m, 2H, O-CH₂), 3.31 (s, 3H, H_{OMe}), 2.47–2.24 (m, 6H, COD), 2.26 (d, $J = 3.2$ Hz, 6H, Me), 2.22 (d, $J = 4.8$ Hz, 12H, Me), 2.04 (d, $J = 4.4$ Hz, 12H, Me), 2.01–1.91 ppm (m, 2H, COD); $^{13}\text{C NMR}$ (101 MHz, CD_2Cl_2): $\delta = 178.7$ (NCN), 177.9 (NCN), 137.2 ($\text{C}_{\text{Ar-1}}$), 134.1 ($\text{C}_{\text{Ar-2}}$), 134.0 ($\text{C}_{\text{Ar-3}}$), 127.7 ($\text{C}_{\text{Ar-4}}$), 127.6 ($\text{C}_{\text{Ar-4}}$), 124.2 (N-CH=CH-N), 122.2 (N-CH=CH-N), 119.6 (N-CH=CH-N), 119.3 (N-CH=CH-N), 78.0 (COD), 77.3 (COD), 76.8 (COD), 75.2 (COD), 71.6 (CH₂-O), 59.6 (C_{OMe}), 51.7 (N-CH₂-CH₂), 50.8 (N-CH₂), 50.8 (N-CH₂), 39.1 (C_{NMe}), 33.0 (COD), 32.7 (COD), 31.4 (COD), 31.2 (COD), 17.5 (Me), 17.5 (Me), 17.2 (Me), 17.2 (Me), 17.0 (Me), 16.8 ppm (Me); MS (ESI⁺): m/z : 829.1 [M–Cl]⁺; MS (FAB⁺): m/z : 829.2 [M–Cl]⁺.

Data for 3e synthesised by method B: Compound **2a** (77.5 mg, 134 μmol), K_2CO_3 (18.5 mg, 134 μmol) and the **1c** (30.0 mg, 134 μmol) gave **3e** (87.4 mg, 85.2%) as an orange solid; $^1\text{H NMR}$ (400 MHz, CD_2Cl_2): $\delta = 7.15$ (d, $J = 1.9$ Hz, 1H, N-CH=CH-N), 7.10 (d, $J = 1.9$ Hz, 1H, N-CH=CH-N), 6.96 (d, $J = 2.0$ Hz, 1H, N-CH=CH-N), 6.28 (d, $J = 2.0$ Hz, 1H, N-CH=CH-N), 5.26 (d, $J = 13.9$ Hz, 1H, N-CH₂), 5.01 (d, $J = 13.8$ Hz, 1H, N-CH₂), 4.30 (dd, $J = 11.0$, 5.5 Hz, 1H, COD), 4.18 (td, $J = 7.2$, 2.5 Hz, 1H, COD), 4.12 (s, 3H, H_{NMe}), 4.09 (s, 3H, H_{NMe}), 3.83 (s, 3H, H_{NMe}), 3.69 (dd, $J = 9.9$, 5.2 Hz, 1H, COD), 3.62 (dd, $J = 9.9$, 5.2 Hz, 1H, COD), 2.47–2.35 (m, 2H, COD), 2.26 (s, 3H, Me), 2.22 (s, $J = 4.4$ Hz, 6H, Me), 2.29–2.12 (m, 4H, COD), 1.99 (s, 6H, Me), 1.85 ppm (tt, $J = 13.4$, 6.8 Hz, 2H, COD); $^{13}\text{C NMR}$ (101 MHz, CD_2Cl_2): $\delta = 178.1$ (NCN), 177.9 (NCN), 137.2 ($\text{C}_{\text{Ar-1}}$), 134.1 ($\text{C}_{\text{Ar-2}}$), 133.9 ($\text{C}_{\text{Ar-3}}$), 127.4 ($\text{C}_{\text{Ar-4}}$), 124.2 (N-CH=CH-N), 123.2 (N-CH=CH-N), 122.5 (N-CH=CH-N), 120.4 (N-CH=CH-N), 79.1 (COD), 78.5 (COD), 75.6 (COD), 74.8 (COD), 50.1 (N-CH₂), 39.4 (C_{NMe}), 39.0 (C_{NMe}), 38.6 (C_{NMe}), 34.1 (COD), 34.0 (COD), 30.0 (COD), 29.9 (COD), 17.5 (Me), 17.2 (Me), 16.6 ppm (Me); MS (ESI⁺): m/z : 639.1 [M–Cl]⁺; MS (FAB⁺): m/z : 639.1 [M–Cl]⁺.

General method for [Ir(CO)₂(NHC)(Cl)]—method C: The corresponding [Ir(COD)(NHC)(Cl)] complex was dissolved in dichloromethane and CO gas was passed through the solution for 10 min. The solution was evaporated to dryness at RT and the remaining solid was dried in vacuo.

Data for 4a synthesised by method C: Compound **2a** (70 mg, 121 μmol), gave **4a** (62.4 mg, 98.0%) as a slight yellow solid. $^1\text{H NMR}$ (250 MHz, CD_2Cl_2): $\delta = 6.86$ (d, $J = 2.0$, 1H, N-CH=CH-N), 6.42 (d, $J = 2.0$, 1H, N-CH=CH-N), 5.48 (s, 2H, N-CH₂), 3.90 (s, 3H, H_{NMe}), 2.27 (s, 3H, Me), 2.23 (s, 6H, Me), 2.22 ppm (s, 6H, Me); IR (ATR): $\tilde{\nu} = 2052$ (s, $\nu(\text{C}=\text{O})$), 1976 cm^{-1} (s, $\nu(\text{C}=\text{O})$); MS (ESI⁺): m/z : 548.8 [M+Na]⁺, 490.9 [M–Cl]⁺, 462.9 [M–Cl–CO]⁺; MS (EI⁺): m/z : 470.1 [M–CO–CO]⁺; MS (FAB⁺): $m/z = 527.0$ [M+H]⁺, 491.0 [M–Cl]⁺, 470.0 [M–CO–CO]⁺; elemental analysis calcd for $\text{C}_{18}\text{H}_{22}\text{ClIrN}_2\text{O}_2$: C 41.10, H 4.22, N 5.33; found: C 41.25, H 4.39, N 5.16.

Data for 4b synthesised by method C: Compound **2b** (140 mg, 294 μmol), gave **4b** (124.6 mg, 100%) as a slight yellow solid; $^1\text{H NMR}$ (250 MHz, CD_2Cl_2): $\delta = 7.14$ (d, $J = 1.9$ Hz, 1H, N-CH=CH-N), 6.97 (d, $J = 1.9$ Hz, 1H, N-CH=CH-N), 4.50–4.36 (m, 2H, N-CH₂), 3.87 (s, 3H, H_{NMe}), 3.75 (t, $J = 5.1$ Hz, 2H, O-CH₂), 3.33 ppm (s, 3H, H_{OMe}); $^{13}\text{C NMR}$ (63 MHz, CD_2Cl_2): $\delta = 182.4$ (NCN), 174.1 (C=O), 168.8 (C=O), 123.3 (N-CH=CH-N), 122.9 (N-CH=CH-N), 72.1 (O-CH₂), 59.3 (C_{OMe}), 51.6 (N-CH₂), 38.8 ppm (C_{NMe}); IR (ATR): $\tilde{\nu} = 2056$ (s, $\nu(\text{C}=\text{O})$), 1969 cm^{-1} (s, $\nu(\text{C}=\text{O})$).

Acknowledgements

Y.G. thanks Elena Michelucci for measurements and simulations of the ESI-MS spectra and Klaus Merz for his help with the crystal structures. T.M. thanks AIRC-FIRC (Fondazione Italiana per la Ricerca sul Cancro, 3-years Fellowship for Italy Project Code: 18044). The authors also gratefully acknowledge financial support from the COST action CM1105 (STSM to Y.G.), Beneficentia Stiftung (Vaduz) and CIRCMSB (Bari, Italy). Further, financial support to Y.G. by the Ruhr University Research School PLUS, funded by Germany's Excellence Initiative (DFG GSC 98/3) is gratefully acknowledged.

Keywords: antitumor agents • carbenes • cytotoxicity • iridium • medicinal organometallic chemistry • metal-based drugs • protein interactions

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Received: April 3, 2016

Published online on July 22, 2016