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On the antitumor properties of novel cyclometalated benzimidazole Ru(II), Ir(III) and Rh(III) complexes†

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Smart design and efficient synthesis of benzimidazole Ru, Ir and Rh cyclometalated complexes are reported with promising cytotoxic activity against HT29, T47D, A2780 and A2780cisR cancer cell lines. Their apoptosis, accumulation, cell cycle arrest, protein binding and DNA binding effects are also discussed.

Since the end of the 1970s, platinum based metallodrugs such as cisplatin, carboplatin and oxaliplatin have become established chemotherapeutics for applying to various types of cancers.[1] Despite their remarkable versatility, platinum drug resistance to tumor represents a significant limiting factor and a continuing challenge.[2] Consequently, the discovery of novel metallodrugs with distinct structural and mechanistic profiles for drug development plays an important role in cancer drug research. To enhance the traditional paradigm of metallodrug discovery, organometallic compounds with properties somewhat intermediate between classical inorganic and organic drugs have recently been considered as promising alternatives. Moreover, organometallic compounds are suitable for rational drug design and thus they could solve many of the challenges in turning a structural lead into a drug candidate with improved efficacy and tolerability.[3] Concomitantly with platinum based metallodrugs, substantial efforts have been dedicated to develop Ru, Ir, Os and Rh organometallodrugs.[4]

Synthesis of small drug-like heterocyclic compounds and the use of these molecules as chelating ligands for synthesis of organometallic complexes have been very well realized for generating promising anticancer metallodrugs. The design concept of the presently synthesized target has originated from the recognition of the biological role of the benzimidazoles which exhibits a wide range of pharmacological properties including anticancer and HIV-1 integrase inhibition.[5] Moreover, the benzimidazole core can be easily tuned for the generation of a fused heterocyclic skeleton that has a substantial intellectual appeal.[6] Hence the benzimidazole moiety linked with a phenyl ring is selected as the main core of the design. As shown in Fig. 1, the C–N site is readily available for cyclometalation to construct organometallic complexes. Gratifyingly, the presence of an NH-functionality on benzimidazole can allow a simple installation of different moieties to modulate hydrophilicity. Easy derivatization of the phenyl ring can be achieved for probable SAR studies. The ester functionality was installed as a handle for intended functionalization of metallodrugs. Accordingly, in continued efforts to develop novel better metallodrugs,[7] here we disclose a novel series of *C*,*N*-cyclometalated ruthenium, iridium and rhodium antitumor complexes containing benzimidazole ligands. To the knowledge of the authors, the present strategy with these polyvalent ligands is completely innovative in the bibliography, and these studies open a new panoramic view for modeling metal drugs with diverse and simultaneous functions.

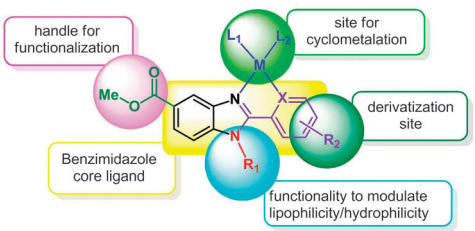

Fig. 1 Design of a novel ligand for metallodrugs.

The key intermediate **1** was efficiently synthesized from 4-fluoro-3-nitro-benzoic acid using reported procedures with few modifications (Scheme 1).[8] Acid catalyzed methyl esterification and nucleophilic aromatic substitution of the fluoro group by butyl amine affords methyl 4-(butylamino)-3-nitrobenzoate. The butyl group was chosen initially aiming to determine lipophilic properties of the final complex. Subsequently, reduction of the nitro group using zinc and ammonium formate in methanol affords **1** in 60% overall yield. The final ligand **2** was synthesized with construction of a benzimidazole ring by condensation of **1** and benzaldehyde. Reaction was carried out in acidic ethanol at room temperature for 24 h to obtain benzimidazole ligand **2** in good (67%) yield. The formation of a benzimidazole moiety was confirmed by spectroscopic methods. The downfield shifting of three

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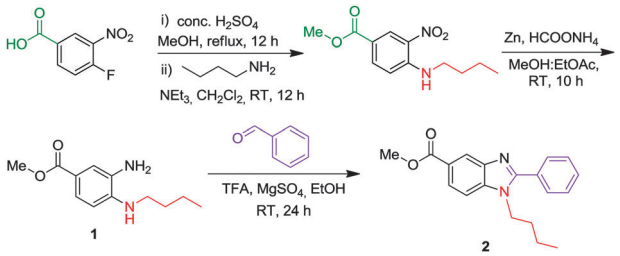
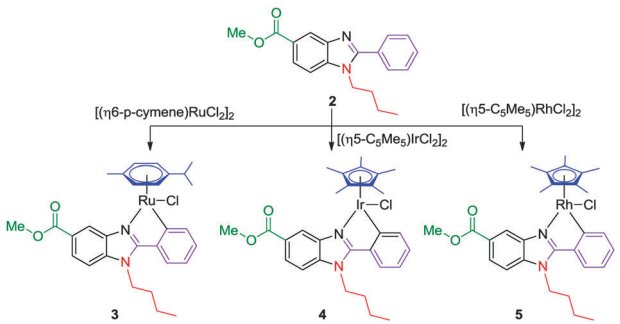
† Electronic supplementary information (ESI) available: Synthesis, characterization data and biological study details. CCDC 950001. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c3cc46239k



Scheme 1 Synthesis of a phenyl benzimidazole ligand.



Scheme 2 Synthesis of cyclometalated Ru–Ir–Rh complexes.

phenyl protons and the appearance of new phenyl ring peaks indicate formation of a benzimidazole ring.

With the key benzimidazole ligand in hand, we next focused on synthesis of organometallic complexes with ruthenium, iridium and rhodium metals. Cyclometalation was achieved by slight modification of methods used for other ligands.[9] Ligand **2** was treated with *para*-cymene ruthenium(II) $[RuCl_2(p\text{-cymene})]_2$ and sodium acetate in dichloromethane at room temperature for 24 h to obtain ruthenium complex **3** in 72% yield (Scheme 2). Formation of a ruthenium complex was confirmed by spectroscopic methods. In the ^{1}H NMR spectrum of **3**, disappearance of one aromatic proton and introduction of four doublets at 6.5–6.7 ppm, a singlet at 2.2 ppm, and two doublets at 0.9 ppm for six protons corresponding to *p*-cymene depicts the formation of ruthenium complex **3**. Similarly, half-sandwich iridium(III) complex **4** and rhodium(III) complex **5** were prepared using a similar method starting from the corresponding pentamethylcyclopentadienyl chlorido iridium(III) and rhodium(III) dimers, respectively, in good yield. The structures of **4** and **5** were also established by spectroscopic and analytical methods.

In addition, the structure of representative iridium complex **4** was unambiguously confirmed by the X-ray crystallographic study. Fig. 2 depicts the ORTEP diagram of complex **4** (X-ray data are supplied in ESI†). The single crystal X-ray analysis of compound **4** confirmed its "piano-stool" structure, showing that the two rings of the benzimidazole and phenyl moieties are not coplanar.

With the successful convergent synthesis of three organometallic complexes, our next intention was to synthesize analogs of these complexes to generalize the method. The first emphasis was placed on effects of different substitution on the benzimidazole nitrogen which probably modulates the lipophilicity and hydrophilicity of the metal complex and ultimately the cytotoxicity. As a model study we first chose the methyl and benzyl groups for *N*-substitution. Accordingly, a small series of complexes **6** to **11** was synthesized as depicted in Scheme 3 following a similar protocol. It is noteworthy to mention that yields for synthesis of iridium complexes are higher (85–90%)

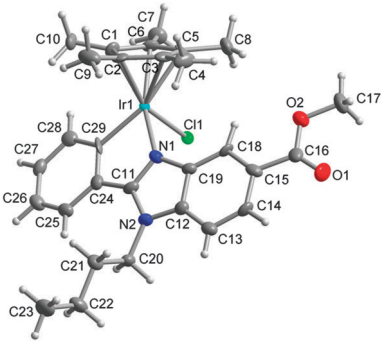
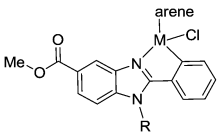


Fig. 2 X-ray crystallographic ORTEP diagram of complex **4**.



Complex	M	R	arene
6	Ru	Methyl	*p*-Cymene
7	Ru	Benzyl	*p*-Cymene
8	Ir	Methyl	C_5Me_5
9	Ir	Benzyl	C_5Me_5
10	Rh	Methyl	C_5Me_5
11	Rh	Benzyl	C_5Me_5

Scheme 3 Synthesis of analogs of metal complexes.

than those corresponding to ruthenium complexes (65–75%), while rhodium complexes are obtained in medium yields (50–60%). The structures of all complexes were confirmed by ^{1}H NMR (1D and 2D) and ESI-MS while their high degree of purity was determined by elemental analysis (see the ESI†). Moreover stability of metal complexes was determined in DMSO, DMSO–water and 100 mM chloride ion concentration in DMSO–water for 24 h at 37 °C.

The cytotoxicity of all the compounds was evaluated toward a panel of human cancer cell lines representative of epithelial ovarian carcinoma A2780 and A2780cisR (acquired resistance to cisplatin), breast cancers (T47D) and colon cancers (HT29). For comparison purposes the cytotoxicity of cisplatin and the free ligand **2** was also evaluated. As depicted in Table 1, the majority of complexes are more active than cisplatin towards HT29 and T47D. Noteworthy, complexes **4** and **9** are about as active as cisplatin towards A2780. On the other hand, A2780cisR encompasses all of the known major mechanisms of resistance to cisplatin: reduced drug transport, enhanced DNA repair/tolerance, and elevated GSH levels.[10] The ability of most of the new complexes to circumvent cisplatin acquired

Table 1 Cytotoxic activity of complexes expressed as IC_{50} values [μM]

Comp	HT29	T47D	A2780	A2780cisR[a]
2	>50	>100	>100	>100
3	2.18 ± 0.39	5.48 ± 0.17	6.61 ± 0.12	6.42 ± 0.13 (1.0)
4	0.98 ± 0.02	2.27 ± 0.04	1.87 ± 0.04	1.77 ± 0.04 (0.9)
5	7.76 ± 0.04	6.41 ± 0.23	7.12 ± 0.14	4.67 ± 0.07 (0.7)
6	>50	15 ± 1	69 ± 7	36 ± 2 (0.5)
7	2.40 ± 0.13	4.37 ± 0.11	7.40 ± 0.04	7.46 ± 0.12 (1.0)
8	9.16 ± 0.98	6.67 ± 0.29	6.09 ± 0.18	4.43 ± 0.12 (0.7)
9	2.40 ± 0.07	2.34 ± 0.22	1.82 ± 0.02	2.07 ± 0.06 (1.1)
10	>50	8.97 ± 0.24	8.05 ± 0.09	5.27 ± 0.16 (0.6)
11	5.37 ± 0.02	22 ± 1	6.64 ± 0.08	4.36 ± 0.08 (0.6)
CisPt	9.5 ± 0.2	38 ± 2	1.54 ± 0.07	15 ± 1 (9.7)

[a] The numbers in parentheses are the resistance factors.

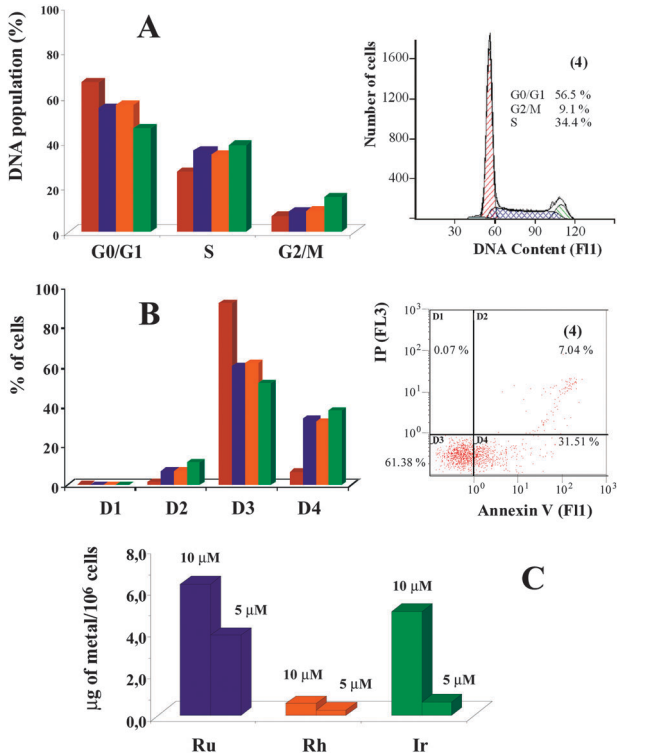


Fig. 3 Results corresponding to the arrest cell cycle (A), apoptosis (B) with HT29 cells and metal accumulation (C) assays in T47D cell line (C). Right panels in A and B correspond to the experimental data for compound **4**. In the three panels brown, blue, orange and green colors correspond to control and complexes **3**, **4**, and **5**, respectively. For cell cycle experiments (A) the results are given as the percentage of DNA found in each of the phases. Metal accumulation (C) shows the µg of metal inside the cell per one million cells when either 10 or 5 µM of the compounds **3**, **4**, and **5** were added to the RPMI 1640 medium.

resistance was determined from the resistance factor (RF) defined as the ratio of the IC_{50} resistant line to the IC_{50} parent line, a very low RF value being observed at 48 h (RF = 0.5, Table 1). An RF value of <2 is considered to denote noncross-resistance.[11] Notably, butyl substituted complexes are more active than their benzyl and methyl derivatives in all studied cell lines. The IC_{50} value of free ligand **2** was higher than 50 µM in all studied cancer cell lines.

To understand the impact of the new complexes on cell growth we examined the effect of the most active compounds (*i.e. N*-butyl substituted complexes) on the cell cycle. Treatment of HT29 cell lines with compounds **3**, **4** and **5** at their IC_{50} concentrations led to a 11%, 10% and 20% decrease, respectively, in the number of cells in the G0/G1 phase (Fig. 3A). The number of cells accumulated in the S-phase improved from 27% (control cells) to 36% (cell treatment with **3**), 34% (**4**) and 39% (**5**) at 48 h. These results show that these compounds are able to arrest the S cell cycle.

Apoptotic studies were also carried out with HT29 cells by flow cytometric assay following exposure of phosphatidylserine with the propidium iodide/Annexin V-FLUOS staining kit (Roche). The results are shown in Fig. 3B. Compounds **3**, **4**, and **5** show nearly one third of the total population of cells (33.20, 31.51, and 37.44%, respectively) in the lower right (D4) quadrant; this clearly indicates that all of them induce early apoptosis.

Metal accumulation inside the cells has also been determined (Fig. 3C). After exposing a T47D cell with complexes **3**, **4**, and **5** (5–10 µM) for 48 h, accumulation of Ru, Ir and Rh inside the cell was determined by atomic absorption spectroscopy. Compounds **3** and **4** display higher levels of metal accumulation (*ca.* 2–3 times) than that we have recently found for cisplatin in this cell line.[7a] It is remarkable that Rh accumulation is lower than either Ru or Ir compounds. Thus a relationship between cytotoxicity and the mode of action of the drug is not evident; very similar for the three compounds, and the accumulation in the cell.

Reactions of anticancer metallodrugs with proteins and DNA are of considerable interest as they play a crucial role in the biodistribution, toxicity, and their mechanism of action.[12] We studied the interaction of these complexes with HSA and the ct-DNA by means of competition experiments using fluorescence spectroscopy. Complexes **3** and **4** interact with HSA at site I (warfarin binding) as well as at site II (dansyl glycine site). They are also able to bind DNA at the minor groove, since both displace Hoechst 33258 (ESI,† Fig. S8–S13 and Table S3).

In conclusion, we have successfully synthesized a series of benzimidazole cyclometalated complexes exhibiting good anticancer activity against HT29, T47D, A2780 and A2780cisR cancer cell lines. Representative complexes show high apoptosis, good accumulation and S-phase cell arrest and strongly bind to HSA at sites I and II and also weakly bind to DNA at the minor groove.

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