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 have been reported with promising cytotoxic activity against HT29, T47D, A2780 and A2780*cis* cancer cell lines. Their apoptosis, accumulation, cell cycle arrest, protein binding and DNA binding effects are also discussed.

Since the end of the 70s, platinum based metallodrugs such as cisplatin, carboplatin and oxaliplatin have become an established chemotherapeutics for applying to various types of cancers.[1] Despite their remarkable versatility, platinum drug resistance to tumour represents the 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 designed concept of the presently synthesized target has been 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 to the generation of a fused heterocyclic skeleton that has a substantial intellectual appeal.[6] Hence the benzimidazole moiety linked with a phenyl ring selected as the main core of the design. As shown in Figure 1, 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 on the phenyl ring can achieve for probable SAR studies. The ester functionality was installed as a handle for intended functionalization of metallodrugs. Accordingly, in continued efforts of developing novel better metallodrugs,[7] here we disclose a novel series of C,N-cyclometalated ruthenium, iridium and rhodium antitumor complexes containing benzimidazole ligand. 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.

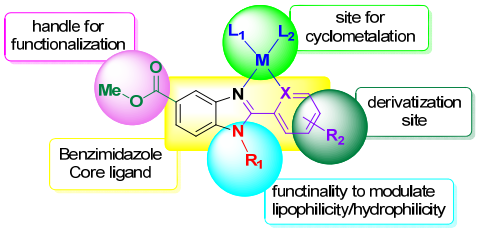
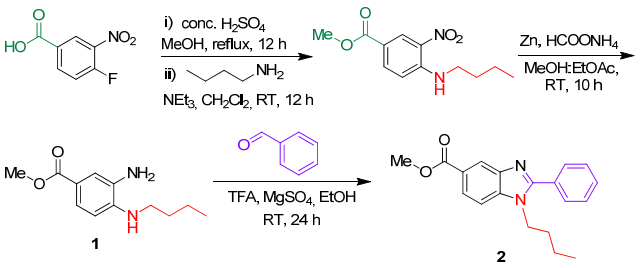


Fig. 1 Design of novel ligand for metallodrugs

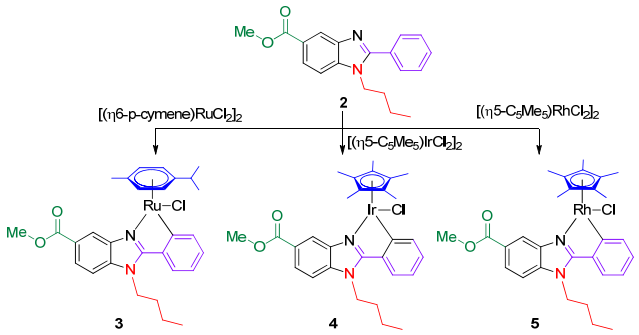
The key intermediate **1** was efficiently synthesized from 4-fluoro-3-nitro-benzic acid using reported procedures with few modifications (Scheme 1).[8] Acid catalyzed methyl esterification and nucleophilic aromatic substitution of fluoro group by butyl amine affords methyl 4-(butylamino)-3-nitrobenzoate. Butyl group was chosen initially aiming to lipophilic properties of final complex. Subsequently, reduction of nitro-group using zinc and ammonium formate in methanol affords **1** in 60% overall yield. The final ligand **2** was synthesized with construction of 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 benzimidazole moiety was confirmed by spectroscopic methods. The downfield shifting of three phenyl protons and the appearance of new phenyl ring peaks indicate formation of benzimidazole ring.



Scheme 1 Synthesis of phenyl benzimidazole ligand

With the key benzimidazole ligand in hand, we next focused to synthesize 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) $[RuCl_2(p\text{-cymene})_2]_2$ and sodium acetate in dichloromethane at room temperature for 24 h to obtain ruthenium complex **3** in 72% yield (Scheme 2). Formation of 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.



Scheme 2 Synthesis of cyclometalated Ru/Ir/Rh complexes

In addition, the structure of representative iridium complex **4** was unambiguously confirmed by the X-ray crystallographic study. Figure 2 depicts the ORTEP diagram of complex **4** (X-ray data are supplied in Supporting Information). 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 no coplanar.

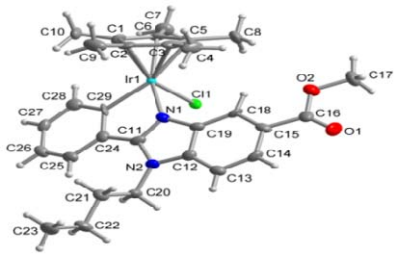
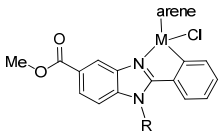


Fig. 2 X-ray crystallographic ORTEP diagram of complex **4**

With the successful convergent synthesis of three organometallic complexes, our next intention was to synthesize analogs of these complexes to generalize method. First emphasis was placed on effects of different substitution on the benzimidazole nitrogen which probably modulates the lipophilicity and hydrophilicity of metal complex and ultimately the cytotoxicity. As a model study we first choose the methyl and benzyl groups for *N*-substitution. Accordingly, small series of complexes **4** to **9** 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%) 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 spectrometry while their high degree of purity was determined by elemental analysis (see the Supporting Information). 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.



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

The cytotoxicity of all the compounds was evaluated toward a panel of human cancer cell lines representative of epithelial ovarian carcinoma A2780 and A2780*cis* (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, 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, A2780*cis* 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 resistance was determined from the resistance factor (RF) defined as the ratio of IC_{50} resistant line to IC_{50} parent line, a very low RF value being observed at 48 h (RF = 0.5, Table 1). An RF of < 2 is consider 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 for free ligand **2** was higher than 50 μM in all studied cancer cell lines.

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

Comp	HT29	T47D	A2780	A2780*cis*[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.

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 IC50 concentrations led to a 41%, 44% and 37% decrease, respectively, in the number of cells in the G0/G1 phase (Figure 3A). The number of cells accumulated in the S-phase was 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 S cell-cycle.

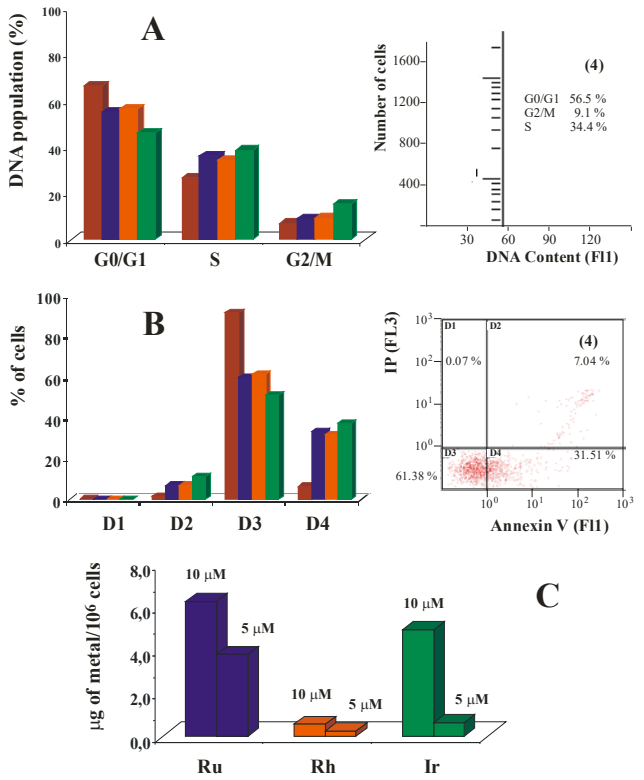


Fig. 3. Results corresponding to arrest cell cycle (A), apoptosis (B) with HT29 cells and metal accumulation (C) assays T47D cell line. Right panels in A and B correspond to the experimental data for compound **4**. In the three panels brown, blue, orange and green colors correspond to control, and complexes **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 of cells when either 10 or 5 µM of the compounds **3**, **4**, or **5** where added to the RPMI 1640 medium.

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 Figure 3B. Compounds **3**, **4**, and **5** shows nearly one third of total population of cells (33.20, 31.51, and 37.44 % respectively) in lower right (D4) quadrant; that clearly indicates all of them induce early apoptosis.

Metal accumulation inside the cells has been also determined (Figure 3C). After exposing T47D cell with complexes **3**, **4**, and **5** (5-10 µM) for 48h, 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 it is not evident a relationship between cytotoxicity and the way of action of the drug; 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 for 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 applying fluorescence spectroscopy. Complexes **3** and **4** interact with HSA at site I (warfarin binding) as well as the site II (dansyl glycin site). They are also able to bind DNA at the minor groove, since both displaces Hoechst 33258 (Supp. Info. Fig. S8-13, Table S3).

In conclusion, we have successfully synthesized a series of benzimidazole cyclometalated complexes exhibiting good anticancer activity against HT29, T47D, A2780 and A2780*cis* 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 to DNA at the minor grove.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [synthesis, characterization data and biological study details supplied].

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