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Highly Potent Extranuclear-targeted Luminiscent Iridium(III) Antitumor Agents Containing Benzimidazole-based Ligands with a Handle for Functionalization

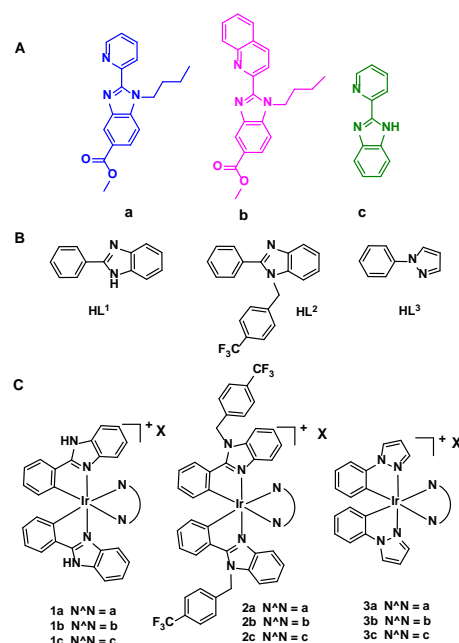
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A series of 6 substitutionally inert and luminiscent iridium(III) antitumor agents of the type $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\text{N}^{\wedge}\text{N})][\text{PF}_6]$ containing a benzimidazole $\text{N}^{\wedge}\text{N}$ ligand with an ester group as a handle for further functionalization has been prepared. They exhibit IC_{50} values in the high nanomolar range in some ovarian and breast cancer cell lines (approximately 100x more cytotoxic than cisplatin (CDDP) in MDA-MB-231) and are located in the actin cortex predominantly as shown by confocal luminescence microscopy. This discovery could open the door to a new large family of drug bioconjugates with diverse and simultaneous functions.

Platinum-based drugs such as CDDP, carboplatin, and oxaliplatin are widely used against various solid tumors including genitourinary, colorectal, and non-small cell lung cancers.¹ These complexes are known to exert their anti-cancer activity mainly via extensive DNA-adduct formation which triggers apoptotic cell death.² However, their effectiveness is still hindered by clinical problems, including acquired or intrinsic resistance,³ a limited spectrum of activity, and high toxicity leading to side effects.⁴ Other precious metals such as ruthenium and iridium have attracted increasing attention as therapeutic agents,^{5,6} as well as biomolecular and cellular probes.⁷ On the other hand, benzimidazole has been shown to be a widely used pharmacophore,⁸ and some benzimidazole half-sandwich iridium(III) compounds have been recently shown by us to act either as anti-angiogenic agents^{6d} or inhibitors of amyloid- β aggregation.^{6h} Accordingly, in continued efforts of developing novel better metallodrugs,

here we disclose a series of substitutionally inert and luminiscent iridium(III) antitumor agents of the type $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\text{N}^{\wedge}\text{N})][\text{PF}_6]$ (Chart 1) containing a benzimidazole $\text{N}^{\wedge}\text{N}$ ligand (**a** and **b** in Chart 1A) with an ester group as a handy tool for further functionalization and a butyl group for *N*-substitution chosen initially to modulate lipophilic properties of final complex, together with various $\text{C}^{\wedge}\text{N}$ ligands based on 2-phenylbenzimidazole (**HL**¹ and **HL**² in Chart 1B) and 1-phenylpyrazole (**HL**³). The iridium complexes **1c**, **2c** and **3c** containing the non-substituted $\text{N}^{\wedge}\text{N}$ ligand 2-(2-pyridyl)-benzimidazole have also been prepared for comparison purposes.

Chart 1 Structures of compounds included in this study.



The benzimidazole $\text{N}^{\wedge}\text{N}$ ligands **a** and **b**, containing an ester functionality, were synthesized by condensation of the intermediate methyl 3-amino-4-(butylamino)benzoate,^{6c,d} with

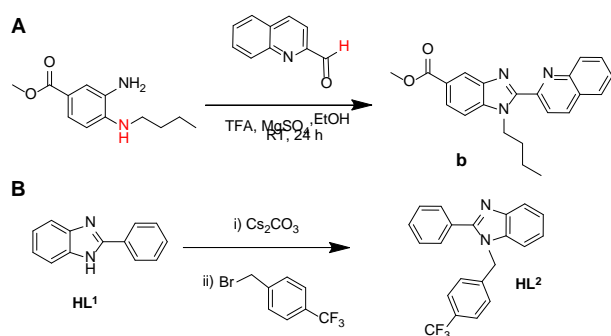
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the corresponding aldehyde in ethanol with catalytic amount of trifluoroacetic acid at room temperature for 24 h (Scheme 1A). The preparation of the new C[^]N proligand **HL²** containing a trifluoromethyl group is outlined in Scheme 1B.



Scheme 1 A) Synthesis of the new neutral N[^]N ligand **b**. B) Synthesis of the new C[^]N proligand **HL²**.

The iridium(III) complexes (Chart 1C) can be obtained with high isolated yields, via a two steps synthesis following known procedures. The starting dinuclear bis-C[^]N complexes [Ir₂(C[^]N)₄Cl₂] were synthesized using a modified literature method⁹ by reaction of iridium chloride with the corresponding HC[^]N proligand. The synthesis of the mononuclear complexes **1a-c**, **2a-c**, and **3a-c** (as PF₆⁻ salts) was achieved by reaction of the corresponding dinuclear bis-C[^]N complex [Ir₂(C[^]N)₄Cl₂] with the appropriate N[^]N ligand, followed by the addition of sodium hexafluorophosphate. All complexes were characterized using ¹H NMR (Figures S4-S15) and ¹³C NMR spectroscopy, and elemental analysis. The positive ion ESI-MS spectra displayed the [M – PF₆]⁺ peaks with the expected isotopic distribution pattern. All complexes were synthesized and tested as racemic mixtures of enantiomers. The stability in DMSO-d₆ of these complexes was confirmed by the ¹H-NMR spectra of **3a** in DMSO-d₆ over 48 h at RT (Figure S7). The UV/Vis absorption spectra of **3a** in DMSO/H₂O at RT remained also unaltered over 5 days (Figure S19).

In addition, the structures of representative iridium complexes **1a** and **3a** were unambiguously confirmed by the X-ray crystallographic study, confirming the anticipated molecular structure (Figure 1), the N atoms of the C[^]N ligands being trans to each other. The iridium cation of **1a** crystallized with one PF₆⁻ anion and three methylene chloride solvent molecules. In comparison to the disordered structure of **3a**, it is obvious that the stronger N-H...F bonds in **1a** prevent orientational ligand disorder of the 2-phenyl-1H-benzo[d]imidazole ligands. The iridium cation of **3a** crystallized with two half-occupied PF₆⁻ anions and methylene chloride solvent molecules. Only one half-occupied CH₂Cl₂ molecule could be reasonably refined.

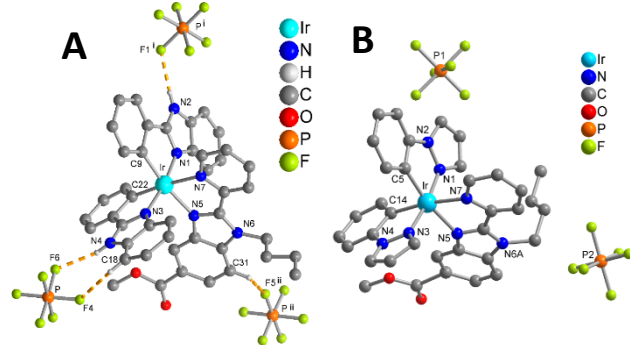


Fig. 1 Molecular structures with atom numbering schemes for (A) **1a**, showing the iridium cation and three surrounding PF₆⁻ anions with N-H...F and C-H...F hydrogen bonds (dashed orange lines), and (B) **3a**, showing the iridium cation and the two half-occupied PF₆⁻ anions).

The cytotoxicity of the new complexes **1a-c**, **2a-c**, and **3a-c** was evaluated towards a panel of human cancer cell lines, including A2780 (human ovarian cancer cells), A2780cisR (acquired resistance to CDDP), and the breast tumor cell lines MCF-7 (ER+) and MDA-MB-231 (triple negative). Because of the low aqueous solubility of the complexes, the tested compounds were dissolved in DMSO first and then serially diluted in complete culture medium such that the effective DMSO content did not exceed 0.4%. CDDP, diluted in water, was used as positive control. As shown in Table 1, the 6 iridium compounds containing a butyl group attached to the benzimidazole N[^]N ligand and a handle for functionalization are highly cytotoxic in A2780 cancer cells (**2a** and **3b** being approximately 7x more cytotoxic than CDDP) and also in breast cancer cells (approximately 100x more cytotoxic than CDDP in MDA-MB-231 cells in most cases), the ligands **a**, **b**, **HL¹** and **HL²** being not cytotoxic (IC₅₀ values higher than 10 μM for all cancer cell lines). The less active iridium derivatives were **1c**, **2c** and **3c**, i.e., those complexes containing the less lipophilic 2-(2-pyridyl)-benzimidazole **c** as a neutral N[^]N ligand. In addition, most complexes overcome the acquired resistance to CDDP in A2780cis cell line (Table 1) and their resistance factors (RFs) are much lower than that of CDDP. On the other hand, differential selectivity of an anticancer drug towards cancer cells versus normal cells increases the likelihood of tumor-specific cytotoxicity, reducing side effects in patients. Hence, antiproliferative activity of iridium compounds and CDDP was also evaluated in a kidney healthy cell line, BGM (african green monkey kidney, Table 1). All complexes (except **1b**) were found to be less toxic than CDDP in the kidney cell line. That may contribute to overcome nephrotoxicity, which is one of the most aggressive side effects of chemotherapy.

Confocal microscopic studies were performed with human ovarian carcinoma cells A2780 treated with the phosphorescent iridium compound **2a** for 3 h (after 3 h-treatment IC₅₀ is expected to be considerably higher than the value shown for **2a** at 48 h (Table 1)).

Table 1 IC₅₀ (μM) for CDDP and Compounds **1a-c**, **2a-c**, and **3a-c** at 48 h

| Compound | A2780 | A2780cisR | MCF-7 | MDA-MB-231 | BGM |
|-----------|---------------|---------------|---------------|---------------|--------------|
| 1a | 2.49 ± 0.194 | 1.94 ± 0.1 | 3.95 ± 0.12 | 1.33 ± 0.036 | > 20 |
| 1b | 0.352 ± 0.008 | 0.562 ± 0.01 | 0.182 ± 0.071 | 0.374 ± 0.022 | 0.43 ± 0.08 |
| 1c | >10 | >10 | >10 | >10 | >20 |
| 2a | 0.197 ± 0.037 | 0.261 ± 0.011 | 0.459 ± 0.047 | 0.305 ± 0.016 | >20 |
| 2b | 0.329 ± 0.007 | 0.319 ± 0.008 | 0.562 ± 0.052 | 0.283 ± 0.021 | 10.28 ± 0.37 |
| 2c | >10 | >10 | >10 | >10 | >20 |
| 3a | 0.322 ± 0.005 | 0.518 ± 0.009 | 0.331 ± 0.005 | 0.724 ± 0.027 | >20 |
| 3b | 0.184 ± 0.008 | 0.077 ± 0.015 | >10 | 0.248 ± 0.026 | 6.03 ± 0.21 |
| 3c | >10 | >10 | >10 | >10 | >20 |
| CDDP | 1.41 ± 0.05 | 34.12 ± 6.81 | 7.15 ± 0.1 | 24.31 ± 4.17 | 5.45 ± 0.15 |

Stable and bright luminiscence of **2a** allowed us to evaluate cellular localization after the treatment of the cells. We illuminated the cells in confocal microscope under 405 nm. This wavelength does not provide the best luminiscence quantum yield according to the spectral parameters of iridium compound **2a**, but it is still the nearest wavelength to absorption maxima of this compound (Figure S20 in Supp. Info.). As shown in Figure 2, the predominant accumulation of compound **2a** was observed in the cytoplasm of the cells and it is of interest that the iridium complexes localized mainly in the actin cortex (Figure 2A), that is a specialized layer of cytoplasmic protein (actin-rich network) on the inner face of the plasma membrane of the cell periphery,¹⁰ which functions as a modulator of plasma membrane behavior and cell surface properties. In addition, the suggestion that the iridium complexes localized mainly in the actin cortex is supported by the origin of cellular blebs observed after the treatment (Figures 2B,2C).¹¹

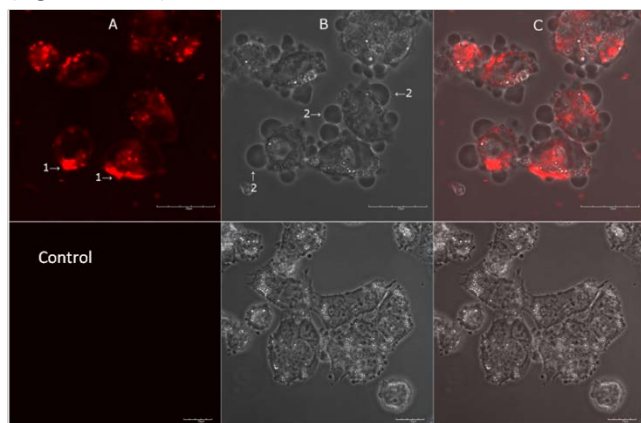


Fig. 2 Confocal microscopic studies of A2780 cells treated with **2a**. Cells were exposed to the Ir complex (5 μM) for 3 h (upper row); 1 in Fig. 2A designates accumulation in actin cortex, 2 in Fig. 2B designates membrane protrusions (blebs). Controls (bottom row) were treated with corresponding amount of DMSO (only one representative figure is shown). A. Luminiscence channel; B. bright field channel; C. merge of the luminiscence and bright field channels.

A factor relevant for cell uptake and anticancer activity is partition coefficient (log *P*) for octanol–water partition, which

provides a measure of hydrophobicity. For several anticancer metallodrugs, a correlation between increased hydrophobicity and increased cytotoxic activity has been reported.¹² The log *P* values for complexes **2a** and **3a** and CDDP are shown in Table 2. Complex **2a** and **3a** are approximately equally hydrophobic while CDDP is hydrophilic (partitions preferentially into water). The hydrophobicity and cancer cell activity (Table 1) of complexes **2a**, **3a** and CDDP correlate in this study. The hydrophobicity difference appears to contribute to the higher cytotoxicity of complexes **2a** and **3a**. In addition, it seems reasonable to suggest that complexes **2a** and **3a** are hydrophobic enough to partition efficiently into cells so that it is very likely that this difference results in efficient cancer cell uptake of complexes **2a** and **3a** and consequently also toxicity in tumor cells of these Ir(III) complexes.

The hydrophobicity data for these Ir(III) complexes prompted us to examine accumulation of complexes **2a** and **3a** in cells. The total cellular accumulation of the Ir(III) complexes **2a** and **3a**, and CDDP in the MCF-7 cancer cell lines was investigated to reveal a possible relationship between cellular uptake and cytotoxicity. Cellular concentrations were determined by ICP-MS after 24 h of exposure to **2a**, **3a** or CDDP at 0.5 μM. As shown in Table 2, total cellular accumulation of iridium from **2a** or **3a** in MCF-7 cells treated with these highly cytotoxic Ir(III) complexes (after 24 h) was approximately 8-fold greater than that of platinum from CDDP. Thus, cancer cell activity in MCF-7 (Table 1), and cell accumulation of the metals from the metal complexes (Table 2) correlate significantly. In addition, metal levels on nuclear DNA and total cellular RNA were determined after the exposure of MCF-7 cells to **2a**, **3a**, or to CDDP at 0.5 μM for 24 h. The data in Table 2 show that the amount of iridium from **2a** and **3a** associated with nuclear DNA was considerably lower (5-10-fold) than that of platinum from CDDP. The amount of iridium from **2a**, **3a** and platinum from CDDP associated with total cellular RNA was not too different. Hence, toxicity of **2a**, **3a**, and CDDP in MCF-7 (Table 1) does not correlate with the amount of the metals associated with DNA or RNA (Table 2).

Table 2 Accumulation of **2a** and **3a**, and CDDP in MCF-7 cells treated for 24 h.

| Compound | pmol/10 ⁶ cells ^a | pg/μg DNA ^b | pg/μg RNA ^c | log P ^d |
|--------------------|---|------------------------|------------------------|--------------------|
| 2a (0.5 μM) | 165 ± 6 | 1.9 ± 0.5 | 1.8 ± 0.1 | 2.6 ± 0.1 |
| 3a (0.5 μM) | 190 ± 8 | 1.0 ± 0.4 | 1.2 ± 0.1 | 2.7 ± 0.1 |
| CDDP (0.5 μM) | 22 ± 4 | 10 ± 2 | 2.3 ± 1.0 | -2.36 ± 0.05 |

^a Total cellular uptake of tested compounds.^b Ir/Pt content of genomic DNA isolated from cells.^c Ir/Pt content of total RNA isolated from cells.^d Log P (octanol/water) values measured by "shake flask" method at room temperature.

Results are expressed as the mean ± SD from three independent experiments.

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In conclusion, a series of substitutionally inert and luminescent bis-C,N-cyclometalated iridium(III) antitumor agents of the type [Ir(C^N)₂(N^N)](PF₆) containing a handle for functionalization in the benzimidazole N^N ligand have been prepared. Compounds show very high cytotoxicity in A2780 cells (**2a** and **3b** being approximately 7× more cytotoxic than CDDP), and, interestingly, they are also very active towards the breast tumor cell lines MDA-MB-231 (approximately 100× more cytotoxic than CDDP in many cases), and MCF-7, while most of them show low toxicity in the nontumorigenic BGM cells. In addition, they are able to overcome the CDDP resistance in A2780cisR cells with a RF below 2. According to confocal luminescence imaging studies **2a** was located in the actin cortex predominantly. Quantification by ICP-MS of metal levels on nuclear DNA and total cellular RNA in MCF-7 cells suggest that they exert their toxic effects in tumor cell lines by a mechanism not involving coordinative binding to nucleic acids. These preliminary results have the potential to open up the door to a new large family of drug bioconjugates and theranostic agents. In addition, the identification of the molecular target of these complexes is underway.

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