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Highly potent extranuclear-targeted luminescent iridium(III) antitumor agents containing benzimidazole-based ligands with a handle for functionalization†

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A series of 6 substitutionally inert and luminescent iridium(III) antitumor agents of the type [Ir(C4N2N4N)][PF6] containing a benzimidazole N4N ligand with an ester group as a handle for further functionalization has been prepared. They exhibit IC50 values in the high nanomolar range in some ovarian and breast cancer cell lines (approximately 100 times 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. These complexes are known to exert their anti-cancer activity mainly via extensive DNA-adduct formation, which triggers apoptotic cell death. However, their effectiveness is still hindered by clinical problems, including acquired or intrinsic resistance, a limited spectrum of activity, and high toxicity leading to side effects. Other precious metals such as ruthenium and iridium have attracted increasing attention as therapeutic agents, as well as biomolecular and cellular probes. On the other hand, benzimidazole has been shown to be a widely used pharmacophore, and some benzimidazole half-sandwich iridium(III) compounds have been recently shown by us to act as either anti-angiogenic agents or inhibitors of amyloid-β aggregation. Accordingly, in continued efforts of developing novel better metallotherapeutics, here we disclose a series of substitutionally inert and luminescent iridium(III) antitumor agents of the type [Ir(C4N2N4N)][PF6] (Chart 1) containing a benzimidazole N4N 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 the lipophilic properties of the final complex, together with various C4N ligands based on 2-phenylbenzimidazole (HL1 and HL2 in Chart 1B) and 1-phenylpyrazole (HL3). The iridium complexes 1c, 2c and 3c containing the non-substituted N4N ligand 2-(2-pyridyl)benzimidazole have also been prepared for comparative purposes.

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† Electronic supplementary information (ESI) available: Synthesis, characterization data and biological study details. CCDC 1483570 and 1483571. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c6cc07909a

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The benzimidazole N^N ligands a and b, containing an ester functionality, were synthesized by condensation of the intermediate methyl 3-amino-4-(butylamino)benzoate, with the corresponding aldehyde in ethanol with a catalytic amount of trifluoroacetic acid at room temperature for 24 h (Scheme 1A). The preparation of the new C^N ligand HL^2 was synthesized using a modified literature method by reaction of iridium chloride with the corresponding HC\textsubscript{F}\textsubscript{3}O\textsubscript{2} peaks with the expected isotopic distribution pattern. All complexes were synthesized and characterized using 1H-NMR spectra of iridium compound 2a–c, and 3a–c (as PF\textsubscript{6}– salts) was achieved by reaction of the corresponding dinuclear bis-C^N complex [Ir\textsubscript{2}(C^N)]\textsubscript{4}Cl\textsubscript{2} with the appropriate N^N ligand, followed by the addition of sodium hexafluorophosphate. All complexes were characterized using 1H-NMR (Fig. S4–S15, ESI^†) and 13CN M spectroscopy and elemental analysis. The positive ion ESI-MS spectra displayed the [M – PF\textsubscript{6}]+ peaks with the expected isotopic distribution pattern. All complexes were synthesized and tested as racemic mixtures of enantiomers. The stability in DMSO-d\textsubscript{6} of these complexes was confirmed by the 1H-NMR spectra of 3a in DMSO-d\textsubscript{6} over 48 h at RT (Fig. S7, ESI^†). The UV/Vis absorption spectra of 3a in DMSO/H\textsubscript{2}O at RT also remained unaltered over 5 days (Fig. S19, ESI^†).

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 (Fig. 1), the N atoms of the C^N ligands being trans to each other. The iridium cation of 1a crystallized with one PF\textsubscript{6} 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 PF\textsubscript{6} anions and methylene chloride solvent molecules. Only one half-occupied CH\textsubscript{2}Cl\textsubscript{2} molecule could be reasonably refined.

The cytotoxicity of the new complexes 1a–c, 2a–c, and 3a–c was evaluated towards a group 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 a 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 7× more cytotoxic than CDDP) and also in breast cancer cells (approximately 100× more cytotoxic than CDDP in MDA-MB-231 cells in most cases), the ligands a, b, HL\textsuperscript{1} and HL\textsuperscript{2} being not cytotoxic (IC\textsubscript{50} 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 the A2780cis cell line (Table 1) and their resistance factors (RFs) are much lower than that of CDDP. On the other hand, the 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, the 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 overcoming nephrotoxicity, which is one of the most aggressive side effects of chemotherapy.

Confocal microscopy studies were performed with human ovarian carcinoma cells A2780 treated with the phosphorescent iridium compound 2a for 3 h (after 3 h-treatment IC\textsubscript{50} is expected to be considerably higher than the value shown for 2a at 48 h (Table 1)).

The stable and bright luminescence of 2a allowed us to evaluate cellular localization after the treatment of the cells. We illuminated the cells in a confocal microscope under 405 nm. This wavelength does not provide the best luminescence quantum yield according to the spectral parameters of iridium compound 2a, but it is still the nearest wavelength to the absorption maxima of this compound (Fig. S20 in the ESI^†). As shown in Fig. 2, the predominant accumulation of compound 2a was observed in the cytoplasm of the cells and it is interesting to note that the iridium...
complexes localized mainly in the actin cortex (Fig. 2A), which 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 (Fig. 2B and C).\(^{11}\)

A factor relevant for cell uptake and anticancer activity is the partition coefficient (log \(P\)) for octanol–water partition, which provides a measure of hydrophobicity. For several anticancer metalodrugs, 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. Complexes 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 and 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(m) complexes.

The hydrophobicity data for these Ir(m) complexes prompted us to examine accumulation of complexes 2a and 3a in cells. The total cellular accumulation of the Ir(m) 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 \(\mu\)M. As shown in Table 2, the total cellular accumulation of iridium from 2a or 3a in MCF-7 cells treated with these highly cytotoxic Ir(m) complexes (after 24 h) was approximately 8-fold greater than that of platinum from CDDP. Thus, the cancer cell activity in MCF-7 (Table 1) and cell accumulation of the iridium 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 CDDP at 0.5 \(\mu\)M. 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 and 3a and platinum from CDDP associated with total cellular RNA was not very different. Hence, the 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).

In conclusion, a series of substitutionally inert and luminescent bis-CN-cyclometalated iridium(m) antitumor agents of the type [Ir(C=N)\(_2\)(N=C=N)]\(_2\)PF\(_6\) containing a handle for functionalization in the benzimidazole N=N ligand has been prepared. Compounds show very high cytotoxicity in A2780 cells (2a and 3b being approximately 7\(\times\) more cytotoxic than CDDP), and, interestingly, they are also very active towards the breast tumor cell line, MDA-MB-231.

### Table 1 IC\(_{50}\) (\(\mu\)M) for CDDP and compounds 1a–c, 2a–c, and 3a–c at 48 h

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

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

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>pmol/10(^6) cells(^a)</th>
<th>pg/(\mu)g DNA(^b)</th>
<th>pg/(\mu)g RNA(^c)</th>
<th>log (P)(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a (0.5 (\mu)M)</td>
<td>165 ± 6</td>
<td>1.9 ± 0.5</td>
<td>1.8 ± 0.1</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>3a (0.5 (\mu)M)</td>
<td>190 ± 8</td>
<td>1.0 ± 0.4</td>
<td>1.2 ± 0.1</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td>CDDP (0.5 (\mu)M)</td>
<td>22 ± 4</td>
<td>10 ± 2</td>
<td>2.3 ± 1.0</td>
<td>-2.36 ± 0.05</td>
</tr>
</tbody>
</table>

\(^a\) 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 using the “shake flask” method at room temperature. Results are expressed as the mean ± SD from three independent experiments.
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 an 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 suggests that they exert their toxic effects in tumor cell lines by a mechanism not involving coordinate 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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Notes and references