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β-Ketoiminato iridium(III) organometallic complexes: Selective cytotoxicity towards colorectal cancer cells HCT116 p53-/-

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Abstract: This report presents a new library of organometallic iridium(III) compounds of the type [Cp*IrCl(N,O)] (Cp* = pentamethylcyclopentadienyl and N,O = a functionalized β-ketoiminato ligand) showing moderate to high cytotoxicity against a range of cancer cell lines. All compounds show increased activity towards colorectal cancer, with preferential activity observed against the immortalized p53-null colorectal cell line, HCT116 p53/-, with sensitivity factors (SF) up to 26.7. Additionally, the compounds have excellent selectivity for cancerous cells when tested against normal sensitivity factors (SF) up to 26.7. Contrarily, the work by Gasser et al. has highlighted new iridium(III) COD, showing different cytotoxic potentials depending on the solvent used during the assays (DMSO vs. DMF).[16] Both works show that the cellular modes of action are hard to identify, and small changes in either the experimental setup or the cellular

To date, the design of organometallic iridium compounds as potential therapeutics is still in its infancy, and this is in part due to the reported inert nature of the low-spin d6 metal ion. This is particularly well characterized for the slow exchange of aqua ligands on [Ir(H2O)3]3+, which is known to take hundreds of years.[19] On the other hand, it is also known that water exchange in [Cp*Ir(H2O)]3+ is approximately 1014 times faster.[11] Saldar et al. first reported the half-sandwich iridium(III) complexes of the type [Cp*Ir(X,Y)Cl]3+ (Cp* = functionalized cyclopentadienyl and X,Y = N,N-dimethylphenylazopridine), in which the complexes are labile towards hydrolysis and inactive anti-cancer agents.[12] However, when the Cp* ligand was extended to contain more phenyl rings, the complexes showed increased intercalation with DNA bases and had good potency towards cancerous cells. Additionally, when changing the chelating N,N-ligand for a chelating N,C-ligand, the extension to the Cp* ligand was no longer required for the complex to be cytotoxic, highlighting the importance of the bidentate ligand. Such compounds were shown to have polypharmacologic properties, which increase the amount of reactive oxygen species (ROS), damage DNA and disrupt protein synthesis.[12,13] Dyson et al. then reported that the cyclopentadienyl ligands can stabilize Ir(III), and confer kinetic lability on trans monodentate halide ligands, again showing that the addition of phenyl substituents on the Cp* ring has a major effect on the behavior of complexes. Iridium organometallic compounds incorporating the naturally occurring curcumin, both with and without their well-known PTA (1,3,5-triaza-7-phosphatricyclo[3.3.1.1]decane) ligands, were reported, however, these complexes were less active than curcumin alone.[14]

More recently, researchers have reported on iridium COD (COD = 1,5-cyclooctadiene) compounds showing good in vitro potential. Metzler-Nolte et al. reported iridium(I) COD compounds with the incorporation of NHCs (N-heterocycle carbenes), in which they suggest that the neutral complexes cause cell death through interactions with proteins, whereas the cationic complexes are likely to exert their biological effect without reacting with other cellular components. The cationic compounds have high nanomolar cytotoxicity, with high stability towards oxidation and no reaction with model proteins. Though in the presence of excess H2O2, the I(II) slowly oxidizes to Ir(III), increasing its cytotoxic potential, by inducing redox chemical effects and allowing for a better coordination and ultimately changing the potential cellular mode of action by changing the oxidation state.[15] Contrarily, the work by Gasser et al. has highlighted new iridium(III) COD, showing different cytotoxic potentials depending on the solvent used during the assays (DMSO vs. DMF).[16] Both works show that the cellular modes of action are hard to identify, and small changes in either the experimental setup or the cellular

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Supporting information for this article is available via a link at the end of the document.
environment may induce a drastic change in the compounds potential as a drug.

In 2012, alongside its ruthenium analogue, the authors reported the organometallic iridium(III) complex A (Figure 1), which incorporates a β-ketoiminato ligand. The cytotoxicity against a range of cell lines showed that the binding mode of the chelating ligand affects the activity, whereby \(\text{N}(\text{N}) > (\text{O}) > (\text{N}, \text{N})\). Simple β-diketonate (O,O) iridium(III) complexes similar to B (Figure 1) have been shown to be potent antibacterial agents, however, we observed a 16-fold decrease in cytotoxicity when changing the ligand from a β-ketoiminato (N,O) (A) to a β-diketonato (O,O) (B) or eliminating the aniline ring from the β-ketoiminato ligand (C) (Figure 1). To date, the β-ketoiminato complexes are the most promising class of ligands, exhibiting high cytotoxicity against a range of cell lines and inducing dose-dependent single strand DNA breakage. Additionally, organometallic iridium(III) complexes with different length alcohol tethers introduced onto the Cp\(^\beta\) substituent have been reported, in which the cytotoxicity increased with tether length, but was independent of the chelation.

Herein, we have synthesized a library of compounds related to compound A. This range of compounds were designed using our understanding of similar ruthenium complexes, in which the complexes showed promising cytotoxicity against the colorectal cancer cell line HT-29. The compounds reported here are selective for colorectal cancer, especially immortalized colorectal cancers with null p53 function. As the p53 plays a role in apoptosis, and the increased activity of these compounds against null-p53 cells gives rise to a potential p53-independent pathway of cell death. Additionally, we report chemosensitivity studies against normal cell types, which show these compounds to be non-toxic towards normal cells, contrary to that of cisplatin which remains cytotoxic. There are reports on small molecules which target the p53 pathway, but these ruthenium compounds which have a greater radio-sensitizing activity in p53-wildtype cells compared with p53-null or p53-mutated, and novel ruthenium compounds which induce S-phase arrest and apoptosis in HepG2 cells through a p53-independent pathway. Yet to date, very little work has been reported on iridium complexes which are both selective towards cancer cells types and are active through p53-independent pathways. However, work by Sadler et al. has begun to underpin the modes of action of such iridium organometallic compounds, including increases in ROS, DNA damage, and disruption of protein synthesis.

Results

Synthesis and Characterization of Iridium(III) Metal Complexes

The β-ketoiminato ligands and their respective iridium complexes were synthesized according to our previous method (Scheme 1). The iridium dimer, [Cp\(^\beta\)IrCl\(_2\)] (0.5 eq.) was added to a stirring mixture of functionalized ligand (N,O) (1 eq.) and triethylamine (1 eq.) in dichloromethane at room temperature. Complexes of the type [Cp\(^\beta\)IrCl(N,O)] were isolated by removing the solvent by vacuum and recrystallization from methanol to yield analytically pure products in moderate yields. All complexes have been characterized by \(^1\)H and \(^13\)C\(^{1}\)H NMR spectroscopy, mass spectrometry, single crystal X-ray diffraction and microanalysis.

Orange/red single crystals of complexes 1-4 and 6-9, suitable for X-ray crystallographic analysis, were obtained from slow evaporation from the methanolic solutions. X-ray solutions were performed in a triclinic (1-4, 6 and 7), monoclinic (8) or orthorhombic (9) space group, and the crystallographic data is present in Table S1 (1-4) and Table S2 (6-9). All of the bond lengths and bond angles around the metal center show the geometry expected for pseudo octahedral complexes, which are common for these half-sandwich structures (Tables S3-S6). The angles between the metal and the pseudo equatorial ligands are in the range of 83.2(2) - 89.70(19)°, with the remaining three coordination sites occupied by the Cp\(^\beta\) ligand and the angles observed axial to the pseudo equatorial ligand range between 97.26 - 136.51°. Molecular structures for complexes 1-4 and 6-9 are presented in Figure 2, with displacement ellipsoids placed at the 50% probability level. The lower yields have been attributed to the presence of the by-product [Cp\(^\beta\)IrCl(\text{an})] (an = aniline, \(\text{NH}_2\text{C}_6\text{H}_4\)), which recrystallized from several reactions. This structure was confirmed by NMR spectroscopy and a cell check against the CCDC database.

Chemosensitivity Studies

Chemosensitivity studies were conducted against human colon colorectal carcinomas p53-wildtype (HCT116 p53\(^+\)) and p53-null (HCT116 p53\(^-\)), human pancreas carcinoma (MIA PaCa-2), and novel ruthenium compounds which induce S-phase arrest and apoptosis in HepG2 cells through a p53-independent pathway. Yet to date, very little work has been reported on iridium complexes which are both selective towards cancer cells types and are active through p53-independent pathways. However, work by Sadler et al. has begun to underpin the modes of action of such iridium organometallic compounds, including increases in ROS, DNA damage, and disruption of protein synthesis.
normal human prostate cells (PNT2) and human lung carcinoma (A549). In order to assess selectivity towards cancerous cells, the compounds were screened against normal prostate (PNT2) and normal retinal epithelial cells (ARPE-19). The HCT116 cells are human colorectal cancer cell lines that are genetically identical (isogenic) except for the presence or absence of functional p53.\(^\text{[25]}\)

All cells were firstly incubated for 120 h with compounds 1-9 or cisplatin for reference, and all \(\beta\)-ketoiminato ligands were tested and are inactive at the tested threshold concentration (IC\(_{50}\) > 100 \(\mu\)M). Additionally, the by-product \([\text{Cp}^*\text{IrCl}_2(\text{an})]\) was isolated and tested, and is also inactive at the tested threshold concentration (IC\(_{50}\) > 100 \(\mu\)M). The results are shown Table 1 and Figure S1, and highlight a general trend whereby compounds 1-9 have low to no activity against MIA PaCa-2 or A549 cells, and are >8 times less active than cisplatin. The most significant results are observed against colorectal cancer cells, whereby compounds 1-9 all show an increased cytotoxicity towards the isogenic HCT116 cell lines, in particular towards the p53-null line, HCT116 p53-/-.

This is the opposite trend observed for cisplatin, which is more cytotoxic towards the p53-wildtype cells, HCT116 p53+/. The HCT116 p53-/- are immortalized, therefore any increase in activity compared to HCT116 p53+/+ suggests an activity that is independent of p53. The Sensitivity Factors (SFs) have been calculated for the HCT116 cell lines, and shows all iridium compounds 1-9 are sensitive to the HCT116 p53-/- cell line, with SF values ranging from 1.07-26.71 (Table S8). The most striking result is observed for compound 4 against HCT116 p53-/-, which has a SF > 26, compared to cisplatin which is more sensitive towards the HCT116 p53+/+ cell line (SF = 0.43). Additionally, compound 4 is > 2.8 times more cytotoxic than cisplatin against HCT116 p53-/- (2.8 ± 0.2 \(\mu\)M (4) versus 8.1 ± 1.8 \(\mu\)M (cis)).

Similar trends have been observed with iron helicate complexes, whereby the compounds are more sensitive to HCT116 p53-/-, and have IC\(_{50}\) values in the nanomolar range, however, a mechanistic understanding has not been reported yet.\(^\text{[26]}\) The iridium compounds presented herein have the potential to be cytotoxic towards cisplatin resistant lines; however, further mechanistic studies are required to underpin the observed sensitivity and selectivity.

Cytotoxicity after 24 and 48 hours

Cell viability assays were conducted for selected compounds to assess their potential after short incubation periods. Compounds 1, 4 and 9 were incubated for 24 h and 48 h with HCT116 p53-/- cells, and then a 96 h or 72 h recovery period was employed respectively. Even though the compounds are cytotoxic after 120 h, they exhibit low to no activity after exposure for 24 or 48 h (IC\(_{50}\) = 63-100 \(\mu\)M) contrary to cisplatin which is cytotoxic after these exposure times (IC\(_{50}\) = 10-15 \(\mu\)M) (Figure S7).

Selectivity towards cancer cells

As with cisplatin, a major limitation of new drug candidates is the cytotoxicity towards normal cells; this can limit the administrable dosages and can result in an increase of harmful side-effects. Compounds 1-9 were tested against normal prostate (PNT2) and normal retinal epithelial (ARPE-19) cell lines, to gain a preliminary indication of the compounds’ cancer selectivity (Table 1). All complexes are only moderately cytotoxic towards PNT2, and a significant decrease in activity is observed against ARPE-19. As such, the compounds are confirmed to be non-toxic against ARPE-19 when tested at the maximum concentration (IC\(_{50}\) values = 71 ± 2 \(\mu\)M (6) and > 100 \(\mu\)M (1-5, 7-9)). Unlike cisplatin, which is toxic towards ARPE-19 (IC\(_{50}\) = 6 ± 1 \(\mu\)M), the iridium(III) compounds presented herein are > 11.8 times less cytotoxic towards this cell line, highlighting a potentially different mode of action. Additionally, the results have been expressed as Selectivity Ratios (SRs), which is defined as the ratio of the mean IC\(_{50}\) values in the nanomolar range , however, a minimal possible SR value. The most significant results are observed for compounds 4 and 9, which can be described as cancer selective, having a SR > 1, indicating a preferential selectivity towards tumor cells compared to normal cells. The SRs values against HCT119 p53-/- for 4 and 9 are 14.07 and 3.97 (PNT2) and 35.59 and 20.28 (ARPE-19), respectively. The SR for cisplatin shows no selectivity for either of the normal cells and very low selectivity over cancerous cells, with SR ranging from 0.82-2.30 (PNT2) and 0.74-1.99 (ARPE-19). This further highlights these iridium compounds are active on different cell types than cisplatin, and could potentially combat current issues of platinum resistance in cancer cells, without affecting normal cells..
Table 1. IC₅₀ values (µM) ± SD for cisplatin and compounds 1-9 after a 120 h exposure with HCT116 p53+/+, HCT116 p53-/-, MIA PaCa-2, PNT2 and ARPE-19. Results are the average of triplicate repeats.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC₅₀ values (µM) ± SD</th>
<th>MIA PaCa-2</th>
<th>A549</th>
<th>PNT2</th>
<th>ARPE-19</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HCT116 p53+/+</td>
<td>HCT116 p53-/-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>42 ± 2</td>
<td>37 ± 2</td>
<td>67 ± 2</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>2</td>
<td>80 ± 1</td>
<td>47 ± 2</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>55 ± 2</td>
</tr>
<tr>
<td>3</td>
<td>19 ± 1</td>
<td>17.6 ± 0.3</td>
<td>37 ± 1</td>
<td>&gt; 100</td>
<td>10.8 ± 0.3</td>
</tr>
<tr>
<td>4</td>
<td>75 ± 2</td>
<td>2.8 ± 0.2</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>40 ± 4</td>
</tr>
<tr>
<td>5</td>
<td>75 ± 4</td>
<td>23 ± 2</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>44 ± 2</td>
</tr>
<tr>
<td>6</td>
<td>33 ± 1</td>
<td>6.6 ± 0.9</td>
<td>&gt; 100</td>
<td>43 ± 1</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>7</td>
<td>&gt; 100</td>
<td>69 ± 2</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>8</td>
<td>20.7 ± 0.2</td>
<td>17 ± 1</td>
<td>31.5 ± 0.7</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>9</td>
<td>24 ± 2</td>
<td>4.9 ± 0.6</td>
<td>35 ± 1</td>
<td>52 ± 2</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>cisplatin</td>
<td>3.5 ± 0.5</td>
<td>8.1 ± 1.8</td>
<td>3.6 ± 0.7</td>
<td>3.0 ± 0.1</td>
<td>6.9 ± 0.3</td>
</tr>
<tr>
<td>[Cp*IrCl₂(an)]</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
</tbody>
</table>

Figure 3. The selectivity ratio is defined as the IC₅₀ in normal cells divided by IC₅₀ relevant cancerous cells. An SR = 1 indicates equitoxic potency against tumor and normal cells. An SR > 1 indicates preferential selectivity for tumor cells compared to normal cells, whereas an SR < 1 indicates poor selectivity and a greater cytotoxicity towards normal cells. Bar-charts show SR for a) PNT2 and b) ARPE-19.

Inhibition of Thioredoxin Reductase 1 (Trx-R)

The results of the MTT assay described above give a good indication of inhibition, but they do not provide information of cell growth, cell death or cell targets. As the biological effects of Trx-R have been shown to contribute to tumor growth and progression,[27] and the enzyme is over-expressed in several tumor types, the targeting of this enzyme can have important therapeutic results.[28–30] To gain a preliminary understanding of the potential targets, the inhibition of Trx-R was determined using the substrate 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) as described elsewhere.[31] Compounds 4 and 9 were incubated with 0.232 units of recombinant rat enzyme, however, these compounds did not show any inhibition of Trx-R, with IC₅₀ values greater than the tested concentration (> 20 µM), contrary to our previously published work.[19]

Conclusions

This report presents a series of iridium complexes that have the potential to combat current issues of platinum toxicity towards normal cells. We have also highlighted that these compounds do not inhibit Trx-R, indicating a different mode of action as previously report iridium compounds. The most significant findings are the sensitivity of these compounds when tested against an isogenic pair of colorectal cancer, HCT116, in particular those which are p53-null, HCT116 p53-/-, showing a Sensitivity Factor (SF) up to 26 (compared to cisplatin SF = 0.43). Importantly, these compounds are non-toxic towards normal cell types, contrary to cisplatin which remains cytotoxic. We propose that these compounds are active via a p53 independent pathway, and as p53 is mutated in many cancer types, especially colorectal
cancers, we have reason to believe these compounds could help target issues with mutant cell types.

### Experimental Section

**Synthesis of complexes 1-9.** Complexes were synthesized by addition of \( [\text{Cp}^\ast\text{IrCl}_2] \) (0.5 eq.), to a stirring solution of a functionalized \( \text{Pt} \)-ketiminato ligand (1 eq.) and Et2N (1 eq.) in dichloromethane (30 mL). The mixtures were stirred overnight at room temperature and then the solvent removed under reduced pressure. The products were all recrystallized from methanol and obtained in 29-77% yields.

**X-ray Crystallography.** A suitable single crystal was selected and immersed in an inert oil. The crystal was then mounted on a glass capillary or nylon loop and attached to a goniometer head on a Bruker X8 Apex diffractometer using graphite mono-chromated Mo-K\( \alpha \) radiation \((\lambda = 0.71073 \text{ Å})\) or an Agilent SuperNova diffractometer using mirror monochromated Mo-K\( \alpha \) radiation \((\lambda = 0.71073 \text{ Å})\), using 1.0° \( \phi \)-rotation frames. The crystal was cooled to between 100-173 K by an Oxford Cryostream low temperature device.[30] The full data sets were recorded and the images processed using DENZO and SCALEPACK programs[32] or CrysAlis Pro software.[33] Structure solution by direct methods was achieved through the use of SHELXS programs,[33] and the structural models refined by full matrix least squares on F2 using SHELEX[34] unless otherwise stated. Hydrogen atoms were placed using idealized geometric positions (with free rotation for methyl groups), allowed to move in a “riding model” along with the atoms to which they were attached, and refined isotropically. Molecular graphics, editing of CIFs and construction of tables of bond lengths and angles were all achieved using OLEX2.[36]

**Chemosensitivity Studies.** In vitro chemosensitivity tests were performed at the Institute of Cancer Therapeutics, Bradford, against human colon colorectal carcinoma, p53-wildtype (HCT116 p53+/+) and human lung carcinoma (A549) cell lines. Growth inhibitory effects were also tested against normal prostate (PNT2) and normal retinal epithelial cells (ARPE-19). ARPE-19 was obtained from the American Type Culture Collection and kindly donated by Professor Roger Phillips and Dr Simon Allison (University of Huddersfield). All cells were routinely maintained as monolayer cultures in appropriate medium: RPMI 1640 supplemented with 10% foetal calf serum for cell lines MIA PaCa-2 and ARPE-19; 10% foetal calf serum for cell lines HCT116 +/+ and HCT116 -/-; high glucose DMEM medium containing 10% foetal calf serum for cell lines p53-null (HCT116 p53-/-), human pancreas carcinoma (MIA PaCa-2) and human colon colorectal carcinoma, p53-wildtype (HCT116 p53+/+) and human colon colorectal carcinoma, p53-null (HCT116 p53-/-), human pancreas carcinoma (MIA PaCa-2) and human lung carcinoma (A549) cell lines. Growth inhibitory effects were also tested against normal prostate (PNT2) and normal retinal epithelial cells (ARPE-19). ARPE-19 was obtained from the American Type Culture Collection and kindly donated by Professor Roger Phillips and Dr Simon Allison (University of Huddersfield). All cells were routinely maintained as monolayer cultures in appropriate medium: RPMI 1640 supplemented with 10% foetal calf serum for cell lines HCT116 +/+ and HCT116 -/-; high glucose DMEM medium containing 10% foetal calf serum for cell lines MIA PaCa-2 and ARPE-19; low glucose DMEM medium containing 10% foetal calf serum for the A549 cells line. All media was additionally supplemented with sodium pyruvate (1 mM) and L-glutamine (2 mM). For chemosensitivity studies, cells were incubated in 96-well plates containing 100 \( \mu \)L cell suspensions at a concentration of 1 x 10^5 cells/well. The plates were incubated for 24 h at 37 °C in an atmosphere of 5% CO\( _2 \) prior to compound exposure (Lane 1 contains 100% media to serve as a blank). Complexes or cisplatin were each dissolved in DMSO to provide 100 mM stock solutions that were diluted to provide a range concentrations ranging from 200-1.56 \( \mu \)M. After 24 h, 100 \( \mu \)L of each compound/media mixture was added to the cells; so that the final DMSO concentrations were less than 0.1% (v/v) and final compound concentrations of 100-0.78 \( \mu \)M (Lane 2 contains 100% cell suspension to serve as a control). The cells were incubated with the compound/media mixture a period of time (24, 48 or 120 h, with a 96, 72 or 0 h recovery period respectively), at 37 °C in an atmosphere of 5% CO\( _2 \). After the incubation period, 20 \( \mu \)L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL) was added to each well and incubated for a further 3 hours at 37 °C in an atmosphere of 5% CO\( _2 \). All solutions were then removed via pipette and 150 \( \mu \)L of DMSO added to each well in order to dissolve the purple formazan crystals. Once mixed well via pipette, a Thermo Scientific Multiskan EX microplate photometer was used to measure the absorbance of each well at 540 nm. Using the blank and 100% cell control, the % cell viability was determined (abs of compound containing wells – abs media wells)/abs 100% cell suspension well control) and from this the % cell death (100% - % cell viability) can be calculated. On a logarithmic scale, the percentage cell death was plotted against the concentration of compound, and from this the half maximal inhibitory concentration (IC50) value was determined. Each of the experiments was performed in triplicate with mean values being stated as the IC50 ± Standard Deviation (SD).

**Inhibition of Thioredoxin Reductase Activity.** Thioredoxin reductase sourced from rat liver was obtained from Sigma Aldrich. It is a buffered aqueous glycerol solution, >100 units/ mg protein. The enzyme was dissolved in 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1 mM EDTA, and 10% glycerol. The rate of change of UV-vis absorbance was measured at 412 nm over 1 min to give the reaction velocity. The experiment was carried out using just the enzyme to get the control (no inhibitor) reaction velocity and then varying dilutions of the test compound were added up to a maximum of 20 \( \mu \)M. The reaction velocity in the presence of inhibitor was normalized relative to the control to generate % activity and plots of % activity versus concentration were constructed to obtain IC50 values (concentration that inhibited 50% of enzyme activity).

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**Keywords:** bioinorganic chemistry • cancer • colorectal cancer • iridium • organometallic


**Colorectal cancer selective iridium drugs.** This work presents new organometallic iridium(III) compounds which are sensitive to colorectal cancer cells, especially those with null p53 genes. Additionally, they are selective towards cancer cells over normal cells, contrary to cisplatin which is neither sensitive nor selective towards cancer.