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Membrane-disrupting Iridium(III) Oligocationic Organometallopeptides

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A series of oligoarginine peptide derivatives containing cyclometallated iridium(III) units display remarkable cytotoxicity, comparable to that of cisplatin. *In vitro* studies with unilamellar vesicles support a membrane-disrupting mechanism of action

Cancer is one of the leading causes of death and morbidity worldwide.[1] Recent high-throughput sequencing studies have shown that thousands of different mutations can lead to cancer development, and that the mutational signature of cancer is highly dynamic and heterogeneous, even between parts of individual primary tumors.[2] These results suggest that anticancer strategies that target a single oncogene will likely fail to deliver effective treatments, and support the use of combination therapies.[3] Unfortunately, the convergence of most of the standard chemotherapeutic agents on a small number of pathways—particularly DNA synthesis and transcription—have hampered the development of successful, and much needed, combined treatments.[4] In this context, membrane-disrupting lytic peptides have surfaced as potential chemotherapeutic agents.[5] In contrast with current anticancer drugs, the mode of action of these anticancer peptides (ACPs) is not linked to any particular molecular target, but to the selective and fast damage of malignant cell membranes, which typically present altered composition,[6] thus avoiding the development of resistance and opening the possibility of synergistic combination therapies.[7]

ACPs are structurally diverse, but often contain cationic sequences with arginine residues and hydrophobic clusters.[8] While the role of the guanidine groups of the Arg side chains in the interaction and disruption of the lipid bilayer has been extensively studied,[9] the mechanistic influence of the hydrophobic residues is still less clear.[10] However, although not fully understood, it appears that tryptophan side chains, which combine an extended π-electron system with a high quadrupole moment, are crucial for the lipid bilayer disruption effect, [11] and that replacement of Trp by Gly, or even by other aromatic residue such as Phe, results in many cases in complete loss of activity.[12] Interestingly, cyclometalated iridium(III) complexes display extended aromatic surfaces and relatively low cationic charge, and therefore could potentially act as functional substitutes of Trp residues in ACPs. Moreover, cyclometalated Ir(III) complexes display intrinsic cytotoxicity and rich photophysical properties, e.g., high quantum yields, good photostability, and long-lived excited states, that can be easily modulated by appropriate selection of the ancillary ligands, thus providing a privileged platform for the exploration of novel ACPs with theranostic potential.[13] Considering the beneficial effects of conformational restriction and backbone rigidity in the properties of lytic pepides,[14] we also decided to explore the effect of cyclization in the activity of the iridium organometallopeptides. Therefore, we report herein the application of standard solid-phase peptide synthesis (SPPS) methods for the straightforward preparation of a set of cationic linear and cyclic oligopeptides featuring two, or three cyclometalated Ir(III) 2-phenylpyridine (ppy) complexes: **Ir$_2$-R$_8$**,[15] **(Ir-R$_3$)$_2$**, **(Ir-R$_3$)$_3$**, ***cyclo*-(Ir-R$_3$)$_2$**, and ***cyclo*-(Ir-R$_3$)$_3$** (Fig. 1), as well as the evaluation of their cytotoxicity and membrane transport properties.

The SPPS approach relies on the preparation of an appropriately derivatized 2,2'-bipyridine (bpy) ligand in the form of an Fmoc-protected amino acid.[16] Thus, the synthesis of

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the bpy amino acid building block was achieved according to previously reported procedures developed in house.[17] For solubility and reactivity reasons, the 2'-amino group in the bpy was modified with Fmoc-β-Alanine (Fmoc–βAla-bpy–OH **1**).

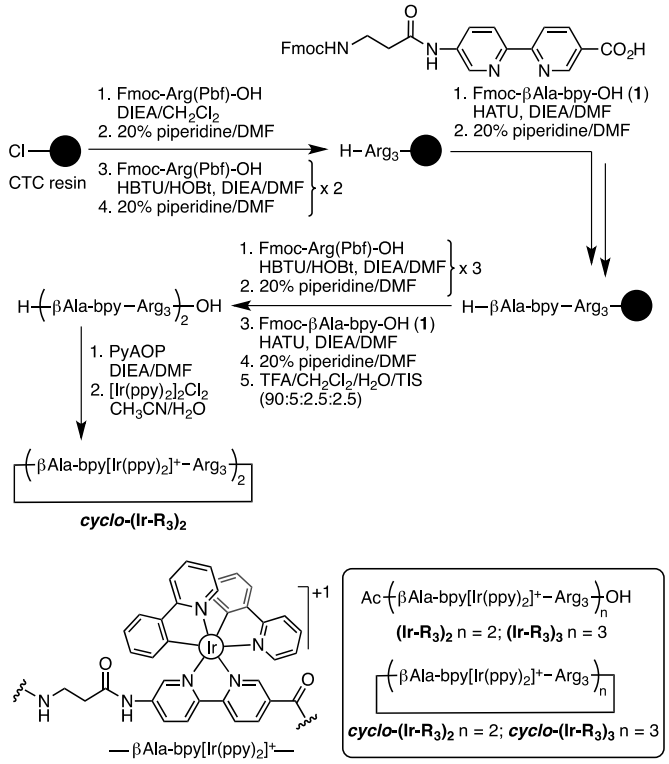

Fig 1. Top: Synthesis of the iridium(III) ***cyclo*-(Ir-R$_3$)$_2$** peptide. Bottom: sequences of the peptides used in this study. Structure of the βAla-bpy[Ir(ppy)$_2$]$^+$ unit. CTC resin = Chlorotrityl chloride resin.

Having at hand the desired bpy building block, we carried out the synthesis of the cyclometalated iridium(III) peptides following standard Fmoc/*t*Bu solid-phase protocols,[18] as exemplified with the synthesis of the ***cyclo*-(Ir-R$_3$)$_2$** peptide (Fig. 1). Brifely, after the assembly of the peptidic chain and its cleavage/deprotection using the standard acidic cocktail (ESI†), the peptide chain was purified by HPLC and subjected to the macrocyclization conditions by treatment with PyAOP/DIEA in DMF. The resulting cyclopeptide was reacted in CH_3CH/H_2O with the *ortho*-metalated dimer precursor $[Ir(ppy)_2Cl]_2$, obtained by a modification of the procedure reported by Sprouse.[19] The reaction proceeds in mild conditions, and the final peptide was obtained in good yields after HPLC purification. Similar procedures were followed for the synthesis of the rest of the iridium(III) metallopeptides (ESI†). Additionally, the tryptophan analog ***cyclo*-(W-R$_3$)$_2$** was also synthesized as control.

Once we synthesized the set of iridium(III) metallopeptides, we studied their cytotoxicity in two tumoral cell lines, NCI-H460, a lung cancer cell line,[20] and NCI/ADR-RES, which has been widely used as a multidrug-resistant breast cancer cell model in cancer research,[21] as well as in non-transformed lung fibroblasts (MRC-5).[22] The dose-response curves were analyzed and the results, summarized in Table 1, show that the IC_{50} values of all the cyclometalated iridium peptides are in the same range to that of cisplatin.[23] Thus, for example, the linear peptides **Ir$_2$-R$_8$** and **(Ir-R$_3$)$_2$** have an IC_{50} values of 15 and 13 μM for NCI/ADR-RES, respectively, matching the IC_{50} obtained for cisplatin within the error of the experiment. Likewise, the cyclic peptides ***cyclo*-(Ir-R$_3$)$_2$** and ***cyclo*-(Ir-R$_3$)$_3$** display IC_{50} values in a similar same range (19 and 21 μM in NCI/ADR-RES cell lines). The peptides display roughly the same toxicity against the NCI-H460 cells, and a slight reduction in IC_{50} values against normal fibroblasts. Interestingly, while the potency of these iridium metallopeptides evidenced by the IC_{50} values is fairly similar to that of cisplatin, their Emax was in all cases significantly higher than those of cisplatin. For instance, the measured Emax values for cisplatin were ≈ 83 μM and ≈ 68 μM for the two cancer lines, whereas all the iridium(III) peptides showed Emax values over 90, showing higher potency than cisplatin for the tumor lines under the same experimental conditions.[24] Curiously, in contrast with similar Arg/Trp rich peptides,[25] these metallopeptides did not display significant antibacterial properties (data not shown).

Table 1. MTT cytotoxicity assays of the cyclometalated peptides. IC_{50} values are reported in μM, and Emax in %.

	NCI/ADR-RES		NCI-H460		MRC-5	
	IC_{50}	E_{max}	IC_{50}	E_{max}	IC_{50}	E_{max}
cisplatin	14(1)	83(1)	~6(0.1)	68(2)	~7(0.3)	91(1)
Ir$_2$-R$_8$	15(1)	92(1)	15(0.1)	91(1)	22(2)	89(1)
(Ir-R$_3$)$_2$	13(1)	91(1)	21(1)	91(1)	26(2)	91(1)
(Ir-R$_3$)$_3$	45(1)	91(1)	34(1)	93(2)	~4(0.1)	84(1)
***cyclo*-(Ir-R$_3$)$_2$**	19(1)	92(1)	36(1)	91(1)	16(1)	90(1)
***cyclo*-(Ir-R$_3$)$_3$**	21(1)	91(1)	17(1)	92(1)	17(2)	91(1)
***cyclo*-(W-R$_3$)$_2$**	41(1)	87(1)	47(1)	94(1)	50(3)	95(2)

Intrigued by the high cytotoxicity of these metallopeptides we decided to study them by fluorescence microscopy. Thus, Vero cells in DMEM medium were incubated for 30 min with peptides **(Ir-R$_3$)$_2$**, ***cyclo*-(Ir-R$_3$)$_2$**, and ***cyclo*-(Ir-R$_3$)$_3$** at 37 °C, and observed in the fluorescence microscope. The resulting micrographs show that the compounds have a strong lytic effect, and that the cell membranes are rapidly disrupted in the presence of the metallopeptides, which aggregate exclusively in the surface of the cells forming highly luminescent membrane protrusions (Fig. 2).

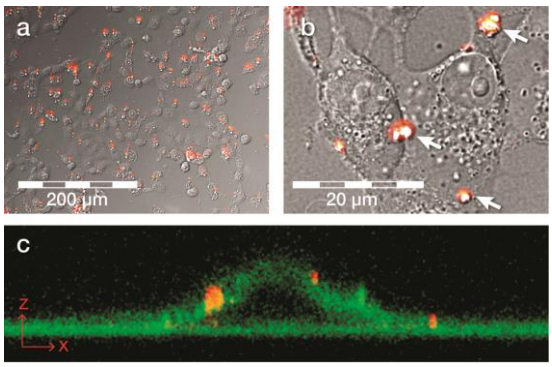

Fig 2. a) Representative fluorescence images of the iridium metallopeptides, in this case with Vero cells incubated with 20 μM ***cyclo*-(Ir-R$_3$)$_2$** for 30 min. Similar

experiments have been performed with all the cyclopeptides with equivalent results. a) Aggregation of the cyclometallopeptide in the cells and not in the culture medium or the plate surface; b) detail of the cells showing that the peptide is concentrated at particular spots in the surface of the cells (white arrows); c) confocal XZ-section of Fluorescein-conjugated concanavalin A-stained cell membrane (green),[26] costained with ***cyclo*-(Ir-R$_3$)$_2$** (red) showing the peptide colocalizing with the membrane marker.

Membrane integrity was further studied with the trypan blue exclusion assay, in which cells with damaged membranes are stained in a characteristic blue color readily observed under the microscope.[27] Vero cells were incubated for 1 h with 20 μM ***cyclo*-(Ir-R$_3$)$_2$**, washed, stained with trypan blue, and monitored for 1 h. Gratifyingly, the cells in the preparation quickly internalized the trypan blue, and were progressively stained so that all of them were blue after 1 h, thus demonstrating that the cyclometallopeptides lead to plasma membrane permeabilization (ESI†). The morphology of these peptide aggregates on the membranes suggests a detergent-type membrane lytic mechanism in which the peptides localize and aggregate on the cell membrane, until they reach a threshold local concentration that allows them to behave like detergents and thus degrade the phospholipid bilayer causing cell death.[28] These observations support a plausible mechanism of action based on the fast physical degradation of the lipid bilayer, which occurs at a much faster timescale than the toxicity effects of cisplatin.

To get further insights into the potential membrane disruption mechanism of the Ir(III) metallopeptides, we decided to perform model experiments in fluorogenic artificial vesicles. Therefore, we prepared zwiterionic large unilamellar vesicles from egg yolk phosphatidylcholine (EYPC LUVs) with entrapped carboxyfluorescein (CF) at self-quenched concentration (see the ESI†). Addition of membrane lytic compounds to isosmotic buffered suspensions of these liposomes triggers the escape, dilution and subsequent enhance of the fluorescence emission of the released CF.[29] CF leakage experiments confirmed the very strong lytic activities for all metallopeptides reported in this study (Fig. 3). The values of the maximal activity (Y_{Max}) and the concentration required to achieve half of the maximal activity (EC_{50}) matched fairly well with the observed cellular activity so that, as observed in cytotoxicity assays, the linear peptides showed a slightly higher membrane leakage than their cyclic counterparts (Fig. 3, Table 1).

Remarkably, the nanomolar activity observed for the assayed metallopeptides is three orders of magnitude higher than membrane disruption strength of the potent detergent Triton-X (EC_{50} = 100 μM, ESI†). This outstanding membrane disrupting activity might be related with an *in-plane* disruption mechanism in which very small amounts of metallopeptides induce wave-like membrane perturbations that feedback and reinforce each other leading to strong membrane curvature and membrane collapse.[30] Interestingly, all the Ir(III) metallopeptides display higher maximal activity (Y_{Max}) and lower EC_{50} than the tryptophan control ***cyclo*-(W-R$_3$)$_2$**, which lacks the metal centres. This result highlights the beneficial role of the cyclometalated Ir(III) complex in the membrane disruption mechanism and it is consistent with the higher cytotoxicity of the iridium metallopeptides.

In summary, we have synthesized a new family of cyclometalated Ir(III) oligocationic peptides that display potent lytic properties, and antitumoral activity comparable to that of cisplatin. The luminescent properties of these peptides allowed us to observe their aggregation on the cell membranes, highlighting the analytical potential of these metallopeptides. Furthermore, the peptidic nature of these cytotoxic probes opens the door the straightforward modulation of their cytotoxicity through judicious modification of the peptide sequence, or repositioning of the metal centers, so that their activity against non-tumoral cells is reduced.

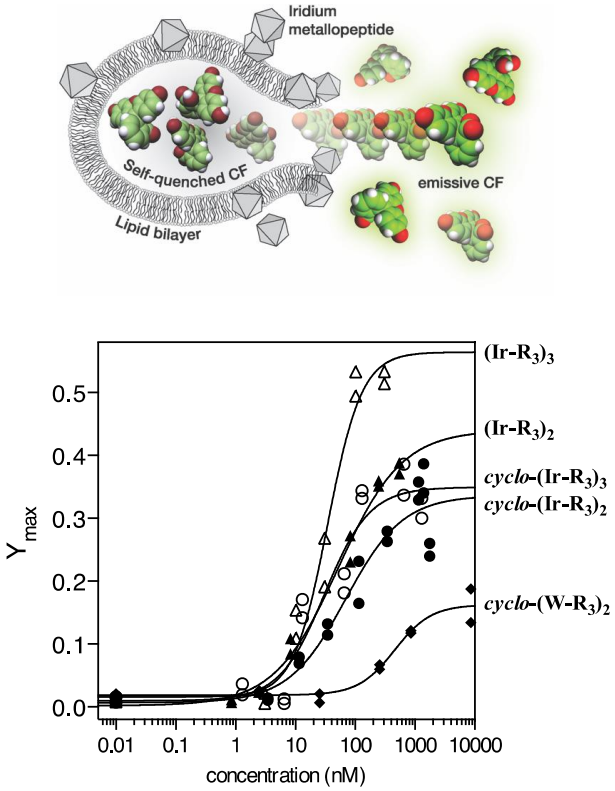

Fig 3. Top: cartoon showing the basics of the membrane disruption experiment in which CF increases its emission upon LUV disruption. Bottom: Dose-response curves of carboxyfluorescein efflux from EYPC LUVs. Each curve was obtained by fitting the data to the *Hill* equation (see ESI†). **(Ir-R$_3$)$_3$** (△), **(Ir-R$_3$)$_2$** (▲), ***cyclo*-(Ir-R$_3$)$_3$** (○), ***cyclo*-(Ir-R$_3$)$_2$** (●), and ***cyclo*-(W-R$_3$)$_2$** (◆).

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