Reactivity differences of Sc$_3$N@C$_{2n}$(2n = 68 and 80). Synthesis of the first methanofullerene derivatives of Sc$_3$N@D$_{5h}$-C$_{80}$.

This article can be cited before page numbers have been issued, to do this please use: X. Tian, Y. Zhu, M. Zhang, L. Luo, J. Wu, H. Zhou, L. Guan, G. Battaglia and Y. Tian, Chem. Commun., 2017. DOI: 10.1039/C6CC09470H.
Localization Matters: A Nuclear Targeting Two-Photon Absorption Iridium Complex Induced Intracellular Immigration and Dual-damage in Photon Dynamic Therapy

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Dedicated to Professor Yupeng Tian for celebration of his 60th birthday.

We present a two-photon (2P, 800nm) PDT cyclometalated iridium (III) complex (Ir-Es) that targets intracellular nucleus, it is capable of immigrating sequentially from nucleus to mitochondria and inducing dual-damage under light exposure. It is suggest that with minor modification of complexes terminal moieties, their final intracellular destinations and PDT efficiency can significantly be impacted.

Photodynamic therapy (PDT) has drawn increasing attention over the past decades and has been successfully applied in treatment of certain types of cancer. PDT is considered as a non-invasive treatment and relies on the use of combination of a photosensitizer (PS), light, and oxygen. Generally, an ideal PS should present non-toxicity in absence of light. When excited under the selected laser, it reacts with the molecular oxygen (O2) at ground state, consequently, generating 1O2 and other reactive oxygen species (ROS), which are considered as the primary toxic species at subcellular organelles or vasculature damage. Although several PDT agents are clinically available, weak photostability, poor water solubility, high energy/shallow tissue penetration laser (excited from 400 to 700 nm) and excessive oxygen consuming still prevent their development.

Moreover, previous PDT agents including functionalized nanoparticles are restricted to damage in one subcellular organelle (e.g. mitochondria and endoplasmic reticulum), whereas nuclear or multi-organelle specific PSs are rarely existed. To tackle these barriers, two-photon (2P) PDT agents have been proposed to alter current PSs. Compared with traditional PDT agents, 2P-PDT agents apply low energy near-infrared laser as light source, which displays significant benefits including less photobleaching of PSs and deeper tissue penetration. Luminous cyclometalated Iridium (III) complexes with two-photon absorption (2PA) behaviors have been widely investigated, particularly for their bio-application, their utilization on PDT induced cell death was also well discussed. Their main merits can be concluded as follows: (i) High photostability allows continuous irradiation and real-time monitoring of intracellular trafficking. (ii) Long-lived triplet states result in long lifetime and provide possible reaction with oxygen to generate ROS. (iii) Large Stokes Shift can minimize the possible self-quenching effect even in high concentration. (iv) Compatibility with two-photon excitation achieves deeper tissue-penetration and excellent resolution.

Scheme 1. The molecular structure of Ir(III) complexes.

In this work, we report terpyridine-based cyclometalated Iridium (III) complex that can be used as 2P-PDT agents and Iridium (III) is chosen as considering below. C-Ir metal bond, constructed by 2-phenyl pyridine, was used to stabilize the energy levels of the Ir complexes. Subsequently, the photophysical properties were tuned by using terpyridine derivatives, which used as bidentate ligand. Finally, terminal substitutes were modified to obtain different Ir(III) complexes to adjust their pull/push electronic capability and bio-affinity. Intriguingly, we found that complex Ir-Es could specifically target cell nucleus via intracellular microtubules dependent endocytosis and induced DNA binding. Subsequent two-photon irradiation triggered the immigration of Ir-Es from nucleus to mitochondria in living cells with efficient dual PDT damage, thus shedding light on the further practical utilization of Ir-Es as an anti-tumour agent. Compared with commercially available PS agent Chlorin e6 (Ce6, 660 nm), Ir-Es displayed considerable inhibition (2P: 808 nm) towards solid tumour growth in vivo in a mouse model.

The detailed synthesis and characterization of Ir(III) complexes including Ir-Es, Ir-Me, Ir-Pn, Ir-Pc and Ir-Cz used in this study were stated in the Supporting information (Scheme S1, Fig. S1-S5). As expected, these complexes displayed singlet and triplet metal-to-ligand charge transfer (MLCT) ranging from 350 nm to 520 nm (Fig. S6 and Table S2), and the emission bands were located in range of 570 - 600 nm (shift > 25 nm, Fig. S6). These
five complexes displayed varied luminescent lifetimes and quantum yields, indicating tunable emission properties of Ir(III) complexes. The 2PA cross sections (χ) of the five iridium complexes from 700-900 nm were shown in Fig. S7. The largest 2PA cross-sections of Ir complexes were located around 800 ± 30 nm with δ values between 60 - 110 GM.

The precise location in subcellular organelles was further confirmed by incubating AdP (100 μM dissolved in PBS mixed with 5 μM Ir-ES and Ir-Me, respectively) with laser exposure for 0, 0.5, 1, 5, 10, 15 min. The impact of iridium complexes treated cells under dark and irradiation condition was firstly evaluated by MTT assay. The cell viability of HepG2 (human liver cancer cell) (Fig. 1a and S8) as well as on non-cancerous HELF (human embryo liver fibroblast, Fig. S9) suggested that under dark incubation, Ir-ES displayed less invasive property (~85% viability, 15 μM, 24 hours), whereas under UV irradiation a significant reduction in cell viability was detected. Compared with Ir-Me treated cells as showed in Fig. 1a, it is apparent that Ir-ES (~4% viability) possess more potency of phototoxic effects under UV condition than Ir-Me complex (~35% viability). This difference was interesting since the capability of singlet-oxygen generation form Ir-ES and Ir-Me showed no difference (Fig. 1b and Fig. S10), which indicated a chemical based method by monitoring the deduction of absorption of ADPA (9,10-Anthracenedipropionic acid),5c with \( \Delta_{abs} = 0.2755 \) (Ir-ES, 15min) and 0.2875 (Ir-Me, 15min), respectively. Considering the significant oxygen consumption required by Ce6, moderate ROS generation nature of Ir(III) complexes might have more advantages particularly against hypoxic solid tumour, which bears by an inadequate oxygen supply.55

We therefore decided to evaluate the intracellular distribution of Ir-ES. The HepG2 cells were incubated with 5 μM complexes for 1 hour and MLCT (metal-to-ligand charge transfer) emission and 2PA confocal micrographs in living cells were successfully achieved (Fig. S11 and S12). It is notable that either from confocal images (Fig. 2a and 2b) or the single cell intensity profiles (Fig. S13), Ir-ES located in different subcellular compartment by contrast to the other Ir-complexes (Ir-Me, Ir-Pn, Ir-Pc and Ir-Cz). Ir-ES dominantly located in nuclear region, while other Ir(III) complexes distributed only in cytosolic space. The precise location in subcellular organelles was further confirmed by colocalization experiments using nuclear stains Hoechst 33342 (Hoechst) and mitochondrial marker Mitotracker Far-Red (MT-FR) (Fig. 2a and 2b). The confocal micrographs (and 3D micrographs, Fig. S14) strongly suggested that Ir-ES targeted intracellular nucleus (Pearson Correlation Coefficient \( r = 0.8384 \)); in contrast, Ir-Me as a representative for the other four complexes, showed much less overlapping with nucleus but strong overlap with mitochondria (Fig. S15). We speculated that the variation in cell uptake were due to different terminal moiety on Ir-ES and other Ir(III) complexes, consequently, leading to the disparate entry mechanism.

Therefore, cell entry inhibition studies were performed (Fig. S16-S18) and the intracellular intensity analysis suggested that Ir-ES cell entry could be considerably decreased by colchicine (microtubule-dependent endocytosis), a chemical could disrupt the polymerization of microtubules and further hinder endosomal trafficking; whereas the Ir-Me cell uptake was significantly reduced by several other inhibitors including choeroquine (lysosomalotropic agent), amonia chloride (lysosomalotropic agent) and chlorpromazine (clathrin-mediated endocytosis). As Ir-ES and Ir-Me shared the same backbone besides terminal substituent, we presumed that complexes’ ester (Ir-ES) or methyl (Ir-Me) terminal might trigger specific microtubules dependent endocytosis or clathrin-mediated endocytosis, subsequently the former rerouted to cell nucleus and the latter accumulated within mitochondria, respectively. Their energy dependent cell entry pathways were confirmed by incubating Ir-ES and Ir-Me with pre-fixed cells (Fig. S19). Compare to in cellulo results, both Ir-ES and Ir-Me showed a generalized cytosolic staining with no mitochondrial and nuclear specificity.

In addition to photoluminescence confocal microscopy, transmission electron microscopy (TEM) image of Ir-ES and Ir-Me complexes was used to confirm cellular distribution owing to the scattering electron capability of transition metal complex specifically accumulated at subcellular compartments.17 HepG2 cells stained with osmium tetroxide (OsO₄) were used as control groups to show detailed information of intracellular membrane composition, such as nuclear membrane, vesicles and bilayer mitochondria structure (Fig. 2e, left). Compared to osmium tetroxide treated cells, Ir(III) complexes solely treated cells without OsO₄ showed much weaker membrane contrast. However, Ir-ES treated cells presented reduced contrast in cytosolic regions, while significant greater contrast was detected in the nuclear space, suggesting that Ir-ES complex accumulated within cell nucleus (Fig. 2e, right). Ir-Me intracellular distribution (Fig. S20) displayed abundant cylinder-like structures in cytosol, which were highly correspondent to mitochondria. Considering the singlet-oxygen generation and living cell staining results obtained from Ir-ES complex, the PDT on HepG2 cells was carried out. Ir-Me did not present significant cell damage after initial (30-times, 800 nm) laser irradiation (Fig. S21). Its analogue Ir(bpy) (Figure S2 and S4 for characterization) also indicated relatively lower PDT efficiency with mitochondrial staining (Fig. S22 and S23). On the contrary, cells incubated with Ir-ES complex showed nuclear uptake and presented healthy morphology before light treatment (Fig. 3a). Once a smaller region was specifically selected (red line box) for laser irradiation (Fig. 3b), drastic morphology changes of cells could be clearly observed (Fig. 3c and 3d, black-dot box).
osmium tetroxide; Abbreviations: \( n \) = nucleus, \( mt \) = mitochondria. TEM scale bar = 5 \( \mu m \).

(3h) was clearly observed, this also proved by ICP-MS evaluation. Additionally, the PDT impact on cell viability after irradiation under \( Ir\)-Es and \( Ir\)-Me treatments were also quantitatively confirmed by mitochondria activity and nuclear permeability using classic JC-1\(^{18}\) labelling kit and ANNEXIN-V FITC/PI (propidium iodide) double staining\(^{19}\) kit, respectively. Both \( Ir\)-Es and \( Ir\)-Me treated cells showed significant mitochondrial damage after irradiation (Fig. 2S7) and emitted in green channel (\( \lambda_{ex}=488 \) nm, \( \lambda_{em}=500-520 \) nm). Whereas extensive FITC/PI double positive signals after continued two-photon treatment (800 nm, 180 s) demonstrated an effective PDT outcome (Fig. 2S8) that induced significant cell death and nucleus damage with permeable nuclear membrane; and relatively lower FITC and negligible PI signal treated with \( Ir\)-Me under the same condition suggested why it was the localization difference matters. These results were further strengthened by TEM micrographs. The cells here were incubated with \( Ir\)-Es and received 4 illuminations within two hours incubation (illumination=30 s, interval=30 min), as comparison the \( Ir\)-Me group was also added (Fig. 2S9). In order to highlight the intracellular structure and membrane damage, we performed the second fixation using Os\(_2\)O\(_4\) for 1 hour. Compared to previous untreated cells under TEM (Fig. 2e, left), it clearly showed that after UV irradiation, \( Ir\)-Es treated cells contained abundant vacuoles, an organelle that plays a major role in early autophagy, leading to apoptotic cell death\(^{20}\) (Fig. 3i, left). It had also clearly showed swollen mitochondria and misshapen nuclear structures in \( Ir\)-Es treated cells (Fig. 3i, right). In a good agreement with above confocal studies, the \( Ir\)-Es treated cells not only demonstrated considerable pre-autophagosomal structures (PAS) in cytosolic region, but also displayed much less contrast in nuclear region with a certain degree of nucleus misshapen or damage (red star). The above results highly suggested that an effective double damage had been occurred in \( Ir\)-Es treated cell sequentially from nucleus to mitochondria under irradiation, which was in a good agreement with the initial MTT assay that \( Ir\)-Es displayed a much stronger phototoxic response.

As \( Ir\)-Es located within nuclear in living cells, a model DNA was used to evaluate the capability of \( Ir\)-Es induced singlet-oxygen-mediated damage under light irradiation in cell-free experiments. The supercoiled pBR322 DNA treated with 30 \( \mu M \) \( Ir\)-Es and \( Ir\)-Me followed by 10 min illumination and 1 hour 37 \(^\circ\)C incubation. Significant DNA cleavage was observed for \( Ir\)-Es treated group as the intensity of \( Lane-I \) (~2000 bp) was decreased and the intensity of \( Lane-II \) (~150 bp) was sharply increased (Fig. 3j). In contrast, at the same illuminated condition, \( Ir\)-Me complex with non-nuclear binding specificity showed much less DNA cleavage effect. The DNA cleavage effect of \( Ir\)-Es was confirmed via DNA binding experiments either in \textit{in vitro} buffer solution (Fig. S30) and molecular modelling calculations using Discovery Studio Software (~9 fold stronger than DAPI, Fig. 3k and 3l, Fig. S31). Subsequently, on the basis of all of the results obtained above, we proposed a possible PDT mechanism (Fig. 3m): (1) \( Ir\)-Es with suitable positive charge, lipophilicity and ligand targeting moieties readily triggers microtubules dependent endocytosis across the plasma membrane and nuclear membrane, colocalized with DNA-riched chromatin or chromosomes. (2) Generated ROS induce DNA cleavage under two-photon irradiation and \( Ir\)-Es were released from nucleus, penetrated into cytosolic region. (3) \( Ir\)-Es binds to intracellular...
mitochondria with high membrane potential ($\Delta \Psi_m$), induced secondary damage under continued 2P irradiation.

To further assess the capability of Ir-Es as a potential PDT agent in vivo, mouse models were performed. The mice with solid tumour planted ($n=6$) were treated with Ir-Es and Ir-Me, respectively. For comparison, Ce6 a clinical PDT agent was applied, PBS and solely irradiation treated (660nm, 1400mJ, Fig. 4 and Fig. S32) animals were also added as negative control. Fig. 4a directly presented the morphology of the tumour from mice after 21 days’ treatment. As it measured daily in Fig. 4b, the volume of tumour treated with Ir-Me and under irradiation (808 nm, 600mA) is not significant difference from that treated with PBS. This suggested that although Ir-Me displayed certain damage towards cancerous cells in vitro in living cells, its in vivo PDT effect was very limited. On the contrary, when treated with Ir-Es, the growth of tumour was significantly inhibited (Fig. 4c, anti-tumour rate, ATR: 41.58 %) and similar to the outcome of the commercial PDT agents Ce6 (ATR: 40.76 %). Predominated necrosis and apoptotic regions were also presented in both H&E staining and Tunnel staining tumour tissue sections (Fig. S33) from groups treated Ir-Es.

In summary, we have designed and synthesized series of novel Iridium complexes and invested their photophysical properties in detail. With slightly modification on terminal substitute, these Iridium complexes were rerouted in nucleus (Ir-Es) and the mitochondria (Ir-Me, Ir-Pn, Ir-Pc, Ir-Cz), respectively. In cellulo studies revealed that Ir-Es complex presented low cytotoxicity in dark and displayed high cell damage under light irradiation. In particularly, we firstly demonstrated that a nuclear targeting complex Ir-Es generated significant ‘double’ damage sequentially form nucleus to mitochondria under two-photon irradiation, then successfully applied as a solid tumour growth inhibitor as a PDT agent using two-photon laser power. This study not only offers a competitive PDT candidate for therapeutic purpose, but also provides an idea on how to regulate the intracellular targeting within metallic molecular system via minor modification.

This work was supported by grants from the National Natural Science Foundation of China (21602003, 51432001, 51372003, and 51472002), Anhui Provincial Natural science foundation of China (1708085MC08), Anhui University Doctor Startup Fund (J01001962). We thank Dr. Martin R Gill (University of Oxford, Department of Oncology) for useful suggestions and help discussions.

Notes and references