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Single-crystal structure and intracellular localization of Zn(II)-thiosemicarbazone complex targeting mitochondrial apoptosis pathways

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ABSTRACT

Tracking of drugs in cancer cells is important for basic biology research and therapeutic applications. Therefore, we designed and synthesised a Zn(II)-thiosemicarbazone complex with photoluminescent property for organelle-specific imaging and anti-cancer proliferation. The Zn(AP44eT)$(NO_3)_2$ coordination ratio of metal to ligand was 1:1, which was remarkably superior to 2-((3-aminopyridin-2-yl) methylene)-N, *N*-diethylhydrazinecarbothioamide (AP44eT·HCl) in many aspects, such as fluorescence and anti-tumour activity. Confocal fluorescence imaging showed that the Zn(AP44eT)$(NO_3)_2$ was aggregated in mitochondria. Moreover, Zn(AP44eT)$(NO_3)_2$ was more effective than the metal-free AP44eT·HCl in shortening the G2 phase in the MCF-7 cell cycle and promoting apoptosis of cancer cells. Supposedly, the effects of these complexes might be located mainly in the mitochondria and activated caspase-3 and 9 proteins.

Mitochondria play a key role in most eukaryotic cells, from the production of energy to the regulation of apoptosis[1–3]. Under normal conditions, cell proliferation is controlled by a variety of signalling mechanisms[3–5]. For example, cellular structure and molecular damage can lead to programmed cell death or apoptosis[2]. However, the mechanism of uncontrolled cell division and the formation of tumours are abnormal in cancer cells[6]. Mutations in mitochondrial genes and high levels of intracellular reactive oxygen species (ROS) could induce tumour[2,7]. The ROS that were generated and accumulated during mitochondrial respiration induced the impairment of electron transport chain[4,8]. As the research progressed, researchers designed and synthesised a series of metal-based drugs targeting mitochondria, but these drugs are only in the basic research stage[9–12]..

Zinc is an essential trace element in organisms which is involved in many biological processes. The thiosemicarbazide chelator is mainly responsible for disrupting the iron metabolism of tumour cells against cell proliferation[13–15]. 3-aminopyridinecarbaldehyde thiosemicarbazone (3-AP), one of the most representatives, is applied to clinical trials and achieved a remarkable therapeutic effect[14,16]. Recent studies showed that its biological and anti-tumour activities are considerably enhanced by modifying lipophilic groups on the N4 groups of thiosemicarbazones[17,18]. The inherent NNS tridentate coordination scaffolds leading to organic thiosemicarbazones are excellent chelators to metal ion (Fe, Cu, Mn, Zn, Ga, etc.), which prevents the formation of hydroxide and improve their anti-cancer activity and bioavailability[19–23]. The coordination with metals also increased the membrane affinity and anti-tumour activity of thiosemicarbazones[24–26].

The interesting photophysical properties of Zn complexes can be applied to biotechnology, biology and potential pharmaceuticals[27]. Therefore, AP44eT·HC (la thiosemicarbazone ligand) and Zn(AP44eT)$(NO_3)_2$ were designed and synthesised as imaging and therapeutic agents (Scheme 1). Their targets were researched by laser confocal microscopy. The effects of AP44eT·HCl and Zn(AP44eT)$(NO_3)_2$ on cytotoxicity and anti-tumour mechanisms were specifically studied.

Design and structure of Zn(AP44eT)$(NO_3)_2$

The thiosemicarbazide ligand (AP44eT·HCl) which was synthesised by the classical Schiff base reaction has a high purity and does not require further purification[23,28]. AP44eT·HCl is slightly soluble in water but soluble in organic solvents, including ethanol, methanol and DMSO. AP44eT·HCl and zinc (II) complex crystallise for suitable x-ray single-crystal diffraction are crystallised from a slowly volatilised ethanol solution. The crystal structure of the X-ray diffraction analysis is presented in Fig. 1, the structure refinement and crystal data for AP44eT·HCl and Zn(AP44eT)$(NO_3)_2$ are shown in Table S1, and the bond length and angle of the crystal are shown in Table S2-3. AP44eT·HCl and Zn(AP44eT)$(NO_3)_2$ were crystallised in the monoclinic

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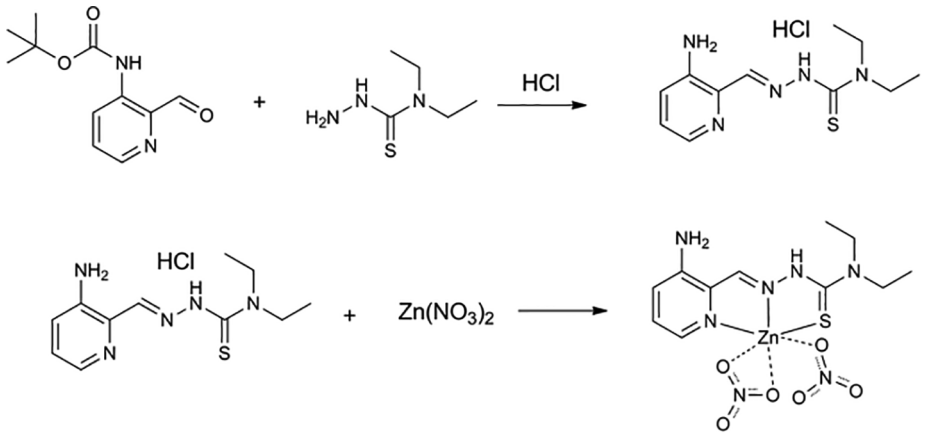

Scheme 1. Synthesis routes for AP44eT·HCl and Zn(AP44eT)$(NO_3)_2$.

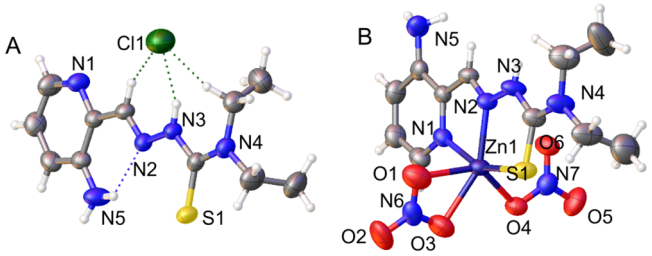

Fig. 1. (A) Molecular structure of AP44eT·HCl; (B) Molecular structure of Zn(AP44eT)$(NO_3)_2$.

space group P_{21}/n. AP44eT•HCl adopted an E-isomeric to form a hydrogen bond between N2 and N5, and a free chloride ion formed hydrogen bond with C6, N3 and C8 simultaneously. In the Zn(AP44eT)$(NO_3)_2$, the coordination polyhedron was a deformed octahedron in which AP44eT·HCl was coordinated to Zn by two nitrogen atoms of pyridine, a thiosemicarbazide, sulfur donor and two nitrates were coordinated from the sides of the metal. The C—S bond and the C—N bond of AP44eT·HCl did not change notably after coordination with the zinc atom, but the configuration changed from the E-configuration to the Z-isomeric form.

Spectroscopic properties

AP44eT·HCl and Zn(AP44eT)$(NO_3)_2$ were dissolved in DMSO as 10 mM stock solutions and diluted in cell culture medium to different concentrations for the involved biological experiment. Therefore, the UV–Vis spectra of AP44eT·HCl and Zn(AP44eT)$(NO_3)_2$ were tested in 0.1% DMSO PBS solution (pH 7.4), and the results are shown in Fig. S1 and S2. The results showed that the Zn(AP44eT)$(NO_3)_2$ was stable in 0.1% DMSO PBS solution (pH 7.4) for 48 h (Fig. S2). The AP44eT·HCl had a strong absorption in the 300–400 nm region and the maximum absorption band (λ_{max}) was 352 nm while Zn(AP44eT)$(NO_3)_2$ showed a stronger absorption bands around 340–500 nm and the maximum absorption band (λ_{max}) was 426 nm (Fig. S1). In the PBS solution, AP44eT·HCl emitted a blue emission centred at 435 nm upon excitation at 320 nm (Fig. 2A) while the Zn(AP44eT)$(NO_3)_2$ emitted a blue emission centred at 461 nm upon excitation at 355 nm (Fig. 2B).

Zn(AP44eT)$(NO_3)_2$ localised in mitochondria

The good cell permeability and excellent photo-stability of Zn(AP44eT)$(NO_3)_2$ made it easier to know their location within the cell, providing vital information about their potential mechanism of action. The blue AP44eT·HCl fluorescence at 435 nm and Zn(AP44eT)$(NO_3)_2$ at 461 nm were again seen in MCF-7 cells (Fig. 3). As the mitochondria are surrounding the nucleus, MitoDeedRed (mitochondria) was used for staining the MCF-7 cell line to locate mitochondria (Fig. 3). After the cells were incubated with AP44eT·HCl and Zn(AP44eT)$(NO_3)_2$, perinuclear punctuated green fluorescence was seen under 465 nm excitation (Fig. 3), which was consistent with the location of the MitoDeedRed. The correlation coefficients of Pearson were 0.79 and 0.85 for AP44eT·HCl and Zn(AP44eT)$(NO_3)_2$, indicating that AP44eT·HCl and Zn(AP44eT)$(NO_3)_2$ were localised in mitochondria.

Anti-cancer activity

Studies showed that 3-aminopyridinecarbaldehyde thiosemicarbazone (3-AP) has a strong anti-tumour activity against a variety of tumour cells, and the anti-tumour activity of the N4 group lipoprotein (alkyl) modification is remarkably improved[14,29,30]. More so, the anti-tumour activity of thiosemicarbazones could be increased by coordination with metal ions (Cu^{2+}, Ga^{3+}, Zn^{2+}, etc.)[16]. The colorimetric method of 3- (4, 5-dimethylthiazole-2-yl) −2, 5-diphenyltetrazolium ammonium bromide (MTT) was used to detect the cytotoxicity of ligands and complexes. The antiproliferative of AP44eT·HCl and Zn(AP44eT)$(NO_3)_2$ were examined by using MCF-7 Cells (human breast adenocarcinoma cell line), and half-maximal inhibitory concentration (IC_{50}) was evaluated. Therefore, we designed and synthesised a thiosemicarbazone ligand (AP44eT·HCl) which was modified at the N4 position of 3-AP to form a new ligand. The Zn(II) nitrate (60 μM) did not show notable antiproliferative activity against the MCF-7 cells. As a vehicle control, cisplatin showed a relatively low anti-tumour activity (IC_{50} = 15.16 ± 1.75 μM). The anti-tumour activity of AP44eT·HCl (IC_{50} = 0.68 ± 0.04 μM) was significantly ($p < 0.001$) higher than that of 3-AP (IC_{50} = 4.88 ± 0.31 μM). The IC_{50} values of Zn(AP44eT)$(NO_3)_2$ was 0.23 ± 0.01 μM, meaning that the Zn(AP44eT)$(NO_3)_2$ showed considerably ($p < 0.05$) higher anti-cancer activity than that of the free AP44eT·HCl. The Zn (II) thiosemicarbazone complexes tend to be more cytotoxic than thiosemicarbazone ligands alone, and this experimental result is consistent with studies from other laboratories[22,27,31,32]. It is known that the toxicity of Zn (II) complex of different cell lines depends on the distribution of sub-cellular layers in tumour cells[27].

Promoting apoptosis mechanism

Mitochondria are structures that provide energy in cells and the main places for cells to breathe aerobically[33]. Therefore, it is important to study the mechanism of mitochondria-targeted drugs in promoting apoptosis[34–36]. Mitochondria-mediated apoptosis is usually associated with mitochondrial membrane changes that cause the leakage of apoptotic factors[37]. The lipophilic fluorescent probe JC-1 (5, 5, 6, 6′-

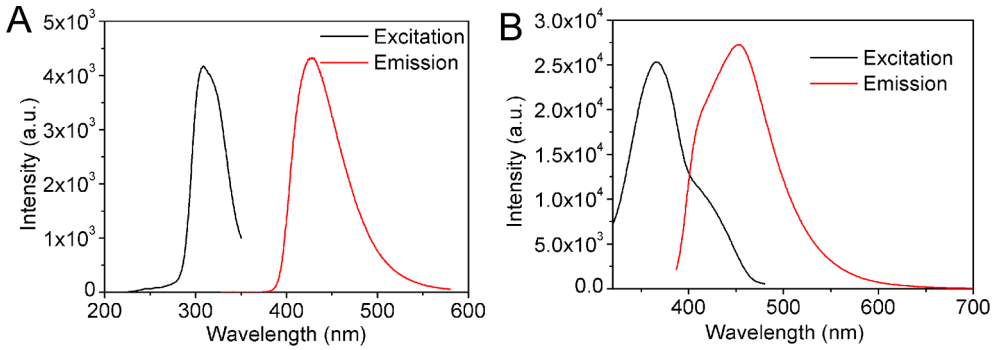

Fig. 2. (A) Photoluminescence (PL) emission spectra of AP44eT·HCl; (B) Photoluminescence (PL) emission spectra of the $Zn(AP44eT)(NO_3)_2$.

tetrachloro-1, 1′, 3, 3′-tetraethyl-imidacarbocyanine iodide) was used to analyse the mitochondrial membrane potential changes (Δψm). The reduction of JC-1 aggregates (red fluorescence) and increment of JC-1 monomers (green fluorescence) showed the decrease of Δψm. The JC-1 probe in the control cell was mainly in the form of an aggregated state, indicating that the mitochondrial membrane potential of the cells was at a normal level (Fig. 4A). After MCF-7 cells incubated with 10 μM of AP44eT·HCl and $Zn(AP44eT)(NO_3)_2$ for 30 mins, more red fluorescence was replaced by the green fluorescence compared to the control (Fig. 4A). Mitochondria appeared orange after AP44eT·HCl treatment (suggesting a partial loss of mitochondrial membrane potential) while the JC-1 aggregates (red fluorescence) almost completely disappeared after treatment with the $Zn(AP44eT)(NO_3)_2$ (which means the complete loss of the mitochondrial membrane potential). Importantly, the mitochondrial membrane potential completely collapsed and the morphology of the cells changed from shuttle to round after the incubation with $Zn(AP44eT)(NO_3)_2$ (Fig. 4A). The collapse of mitochondrial membrane potential caused irreversible release of pro-apoptotic factors. After the MCF-7 cells were exposed to 2 μM of AP44eT·HCl and $Zn(AP44eT)(NO_3)_2$ for 24 h, the expression levels of p53, Bax and Cyt C proteins increased observably while the expression of anti-apoptotic protein (Bcl-2) decreased (Fig. 5).

In the process of apoptosis, the mitochondrial transmembrane potential dissipation is the major manifestation of increased mitochondrial membrane permeability[38]. Studies showed that mitochondrial permeability transition pore (PT pore) is necessary and sufficient for apoptosis, such as activated caspase-3 and 9[19]. The pro-apoptotic factor released by the opening of the PT pore leads to apoptosis while PT pore closure prevents apoptosis. The effect of AP44eT·HCl and $Zn(AP44eT)(NO_3)_2$ on promoting caspase-3 and 9 activities were assayed by flow cytometry. After incubation with the control, AP44eT·HCl and $Zn(AP44eT)(NO_3)_2$, the activated caspase-3 protein levels increased to 11.29%, and 30.59% as per the control, and the activated caspase-9 protein levels increased to 15.29%, and 37.09% as per the control, respectively (Fig. 4B and C).

$Zn(AP44eT)(NO_3)_2$ significantly promotes apoptosis

The activation of the caspase family proteins may lead to cell apoptosis. We used Annexin V FITC/PI staining to analyse the effect of AP44eT·HCl and $Zn(AP44eT)(NO_3)_2$ on promoting the MCF-7 cell apoptosis. After MCF-7 cells were incubated with 2 μM of AP44eT·HCl and $Zn(AP44eT)(NO_3)_2$ for 24 h, the treated cells were collected and analysed by flow cytometry. Quantification of the result showed that the MCF-7 cell induced cell early apoptosis of 7.68% for AP44eT·HCl and 29.46% for $Zn(AP44eT)(NO_3)_2$ which were more effective than the vehicle-treated control (Fig. 6A). The results of the apoptosis assay showed that the coordination with the Zn atom considerably increased the activity of the AP44eT·HCl.

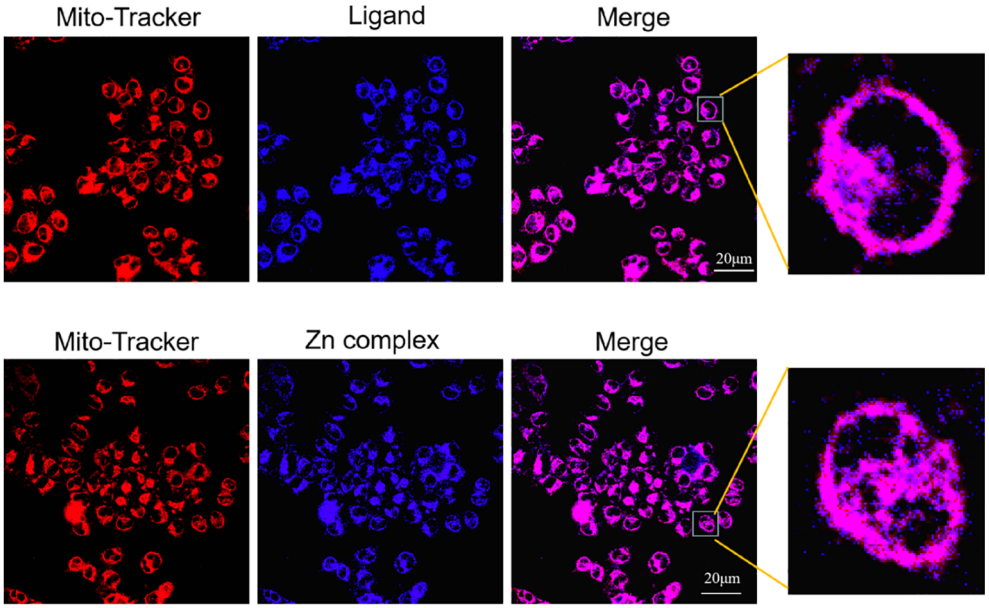

Fig. 3. The intracellular localisation of AP44eT·HCl (5 μM) and $Zn(AP44eT)(NO_3)_2$ (5 μM) in the MCF-7 cell line.

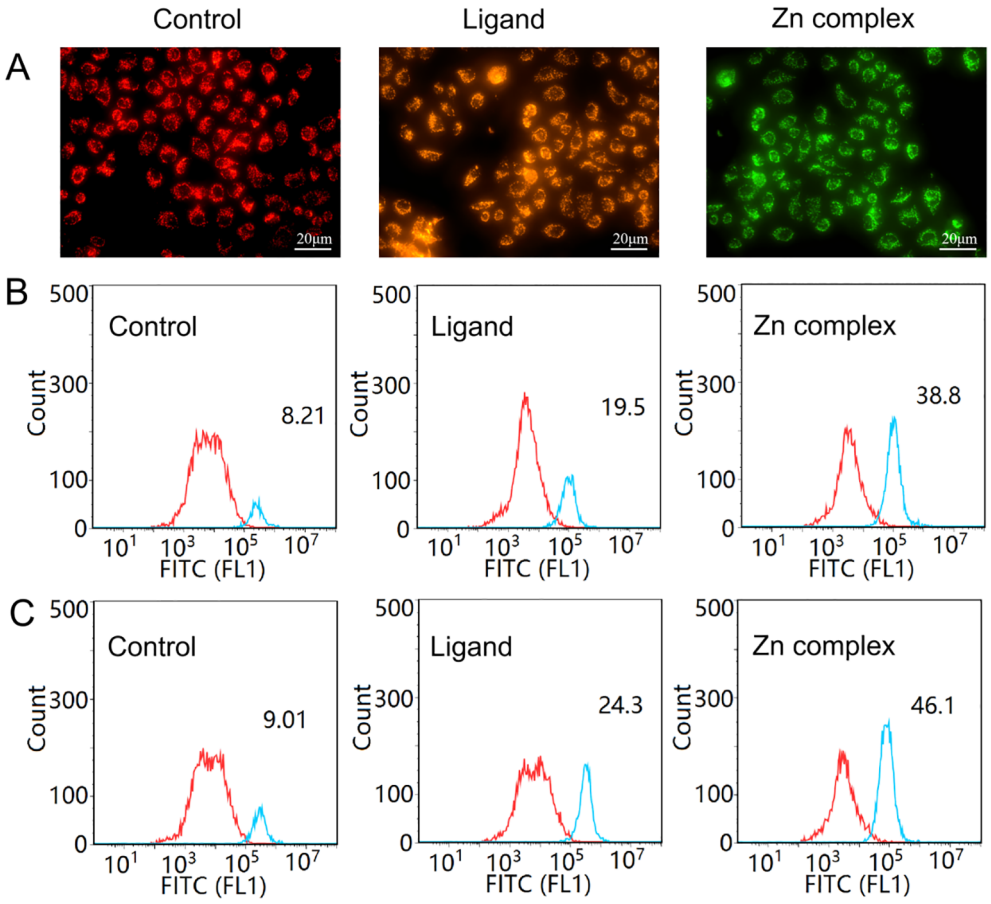

Fig. 4. (A) Assay of the MCF-7 cells mitochondrial membrane potential with JC-1 as fluorescence probe staining method; (B) The effect of the caspase-3 activation of the MCF-7 cells; (C) The effect of the caspase-9 activation of the MCF-7 cells. The cell line was treated with the control, AP44eT·HCl and $Zn(AP44eT)(NO_3)_2$.

$Zn(AP44eT)(NO_3)_2$ significantly inhibits the cell cycle

The relationship between the cell cycle and tumour has always been one of the hot topics in life science research. Tumour is a clonal population formed by the infinite proliferation of cells that have lost cell cycle control[39,40]. Thus, we used the PI staining of MCF-7 cell DNA to examine the effect of AP44eT·HCl and $Zn(AP44eT)(NO_3)_2$ on the cell cycle (Fig. 6B). After treated with 2 μM of AP44eT·HCl and $Zn(AP44eT)(NO_3)_2$ for 24 h, the percentage of the MCF-7 cells in the G2 phase decreased by 4.39% and 13.85% compared with the vehicle-treated control, respectively; and G1 increased by 9.8% and 19.62% compared with the vehicle-treated control (Fig. 6B). These results showed that the $Zn(AP44eT)(NO_3)_2$ anti-cell proliferation property was via inhibiting or delaying cell cycle progression in the G1 and S phases. The results of apoptosis and cell cycle analysis were line with the cytotoxicity experiments.

In summary, we have synthesised a novel thiosemicarbazone AP44eT·HCl with fluorescent properties and high anti-tumour activity. The fluorescence emission wavelength (435 nm) of AP44eT·HCl presented a redshift (461 nm) after the coordination with Zn(II).

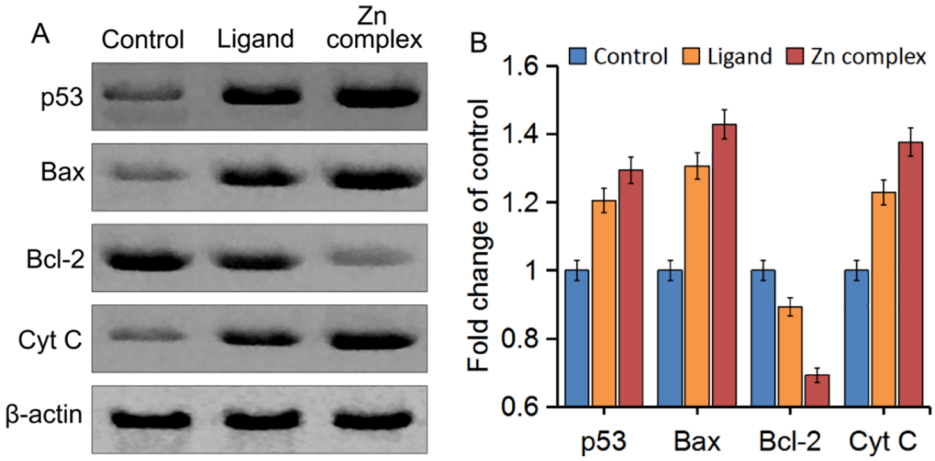

Fig. 5. Western blot analysis of p53 and apoptosis-related proteins (Bax, Bcl-2 and Cyt C) in the MCF-7 cells treated by the control, AP44eT·HCl and $Zn(AP44eT)(NO_3)_2$ for 12 h.

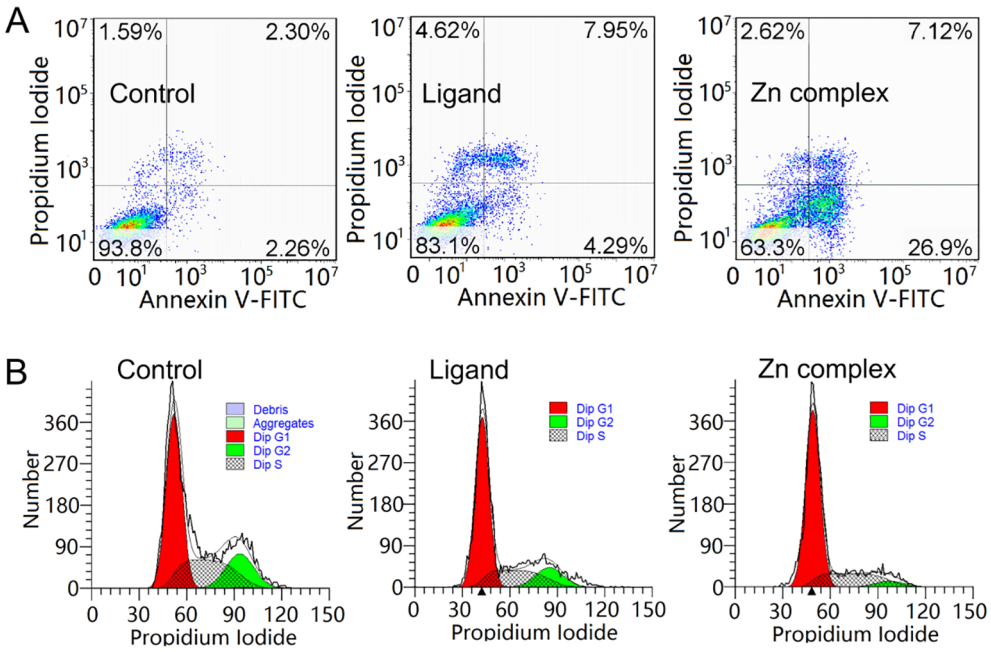

Fig. 6. (A) The representative dot plots of PI and Annexin V double staining on the MCF-7; (B) The effect of the cell cycle of MCF-7. The cell line was treated with the control, AP44eT·HCl and $Zn(AP44eT)(NO_3)_2$.

AP44eT·HCl and $Zn(AP44eT)(NO_3)_2$ were localised in mitochondria organelles. Compare to AP44eT·HCl, $Zn(AP44eT)(NO_3)_2$ was more effective in causing mitochondrial membrane potential collapse, promoting caspase-3 and 9 activation, resulting in cell cycle arrest and apoptosis. Briefly, $Zn(AP44eT)(NO_3)_2$ has a higher advantage in the fluorescence emission wavelength, mitochondrial-targeted anti-tumour activity, the collapse of mitochondrial membrane potential, promotion of apoptosis and inhibition of cell cycle, etc. These experimental results may be useful in the development of novel $Zn(AP44eT)(NO_3)_2$ as tumour therapy and imaging agents.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2020.127340.

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