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ARTICLE TYPE

Dinuclear Zinc(II) Complexes Containing (Benzimidazo1-2-yl)benzene That Overcome Drug Resistance in Hepatocellular Carcinoma Cells through Induction of Mitochondria Fragmentation

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Herein we demonstrated that dinuclear zinc complexes could overcome the drug resistance in R-HepG2 drug resistance hepatocellular carcinoma cells by induction of mitochondria-mediated apoptosis through triggering mitochondria fragmentation, depletion of the membrane potential and intracellular ATP levels.

Transition-metal complexes have been widely used in the diagnosis, medicine, and chemotherapy.[1] However, the limitations of cisplatin-based chemotherapy, such as undesirable side effects and treatment failure due to the development of drug resistance, have stimulated the search for alternative transition metal complexes with higher activities and lower toxicity.[2] Non-platinum complexes exhibit superior properties for drug design, such as diversified geometries and coordination characteristic, various oxidation states, better solubility, feasible substitution kinetic pathways, and so on.[3, 4] Therefore, many efforts have been made to develop non-platinum anticancer complexes.[3, 5] Zinc (Zn) is one of the most essential trace elements in human body by acting as structural and functional cofactors of many intracellular proteins.[6] Many Zn-containing compounds exhibited application potentials as radioprotective agents, cancer photosensitizers, antidiabetic and antibacterial agents.[7] Interestingly, studies also found that synthetic Zn(II) complexes displayed novel antiproliferative activities against various human cancer cells.[8-14] For instance, Terenzi et al found that Zn(II) complexes of a 2,5-diphenyl[1,3,4]oxadiazole derivative could bind to DNA and inhibit the proliferation of human carcinoma cells.[13] Tabassum and coworkers synthesized a series of Zn(II)-based potential complexes that displayed DNA-binding and cleavage properties and antimicrobial activity.[12] However, the underlying molecular mechanisms and the signaling pathways induced by Zn(II) complexes remain elusive.

An interesting class of metallodrugs that exhibit novel biological properties is polynuclear metal complexes with flexible or sterically rigid linking groups .[15, 16] In the past years, substantial interest has been paid to the use of inert multi-nuclear complexes for biological applications, and a variety of studies have showed that these kind of complexes exhibited significant cytotoxicities toward cancer cells through interactions with nucleic acids, like DNA.[5, 8, 17-22] For example, Pisani and coworkers found that inert dinuclear polypyridylruthenium(II) complexes acted as highly cytotoxic lipophilic cations that could cause cell death by apoptosis.[19] Study also found that dinuclear copper(I) complexes could effectively inhibited the proliferation of human cervical and breast cancer cells.[18] Moreover, Anbu and coworkers showed that oxyimine-based macrocyclic dinuclear Zn(II) complexes could enhance phosphate ester hydrolysis, DNA binding, DNA hydrolysis, and lactate dehydrogenase inhibition and induce apoptosis in human cancer cells.[8] Interestingly, in our previous studies, we found that, Zn(II) complexes containing bis-benzimidazole derivatives were able to induce p53-dependent apoptosis in cancer cells by triggering DNA damage in an intercalating mode.[23] However, these studies are mainly limited to mononuclear complexes, and it is anticipated that dinuclear Zn(II) complexes of similar ligands may exhibit better anticancer performance. It was of interest, therefore, in the present work, to synthesize a dinuclear Zn complex (Fig. 1A), $Zn_2(tbb)Cl_4$ [bbb= bis(2-benzimidazolyl)benzene, tbb= (1, 2, 4, 5 –tetrakis (benzimidazo1-2-yl)benzene)], evaluate their *in vitro* anticancer activities, and elucidate the underlying molecular mechanisms.

The complexes were synthesized following the route shown in Scheme S1 and characterized by various methods to confirm the structure and purity. The appearance of N-H stretching band in IR spectrum and NH hydrogen chemical shift at δ=13.98 ppm in the 'HNMR spectrum of the complexes suggested that the two N atoms in C=N groups were coordinated to Zn(II). The crystal structure was analyzed on a Siemens Smart-CCD diffractometer and summarized in Table S1-3. For the dinuclear complex 2, each Zn ion adopted distorted tetrahedron geometry, and the bond angle for N1-Zn1-N4 was 90.87(7)° constrained by the bite of the bischelating ligand. Selected bond lengths are listed in Table S3. The four benzimidazole rings and the central phenyl ring in the ligand were not in the same plane. The clear characterization of the chemical structure could provide useful information for the future design of anticancer drugs based on Zn complexes.

Hepatocellular carcinoma (HCC) is the fifth common malignant cancer that affects approximately one million people worldwide every year. However, drug resistance has greatly limited the

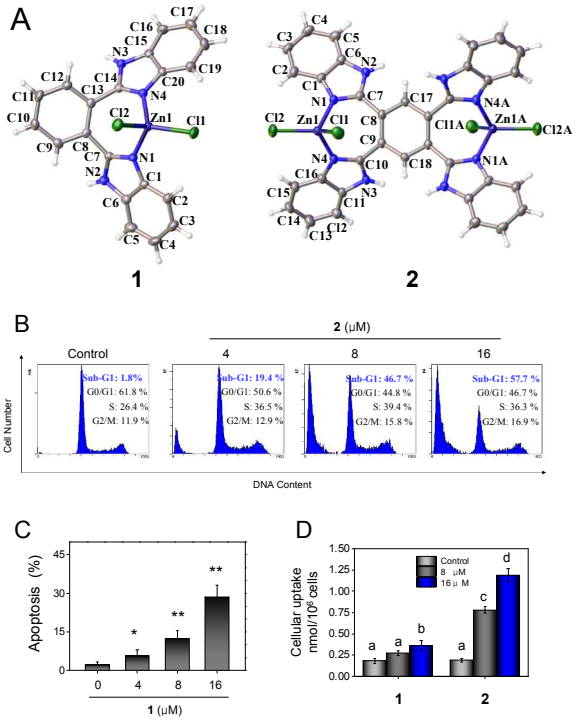

Fig. 1. Crystal structures of the complexes (A) and the effects on cell cycle distribution of R-HepG2 (B) and HepG2 (C) cells for 72 h. (D) Cellular uptake of the complexes in R-HepG2 cells (24 h). Bars with different characters are statistically different at the $P < 0.05$ level.

efficiency of anticancer drugs against HCC. Therefore, the discovery of effective agents that could overcome drug resistance of HCC and thus induce cancer cell death has kindled great interest of scientists. Many studies have demonstrated that metal complexes could act by enhancing the delivery of the active ligands to targeting sites inside the cells, and thus exhibit synergistic effects between the metal ions and the ligands.24 In this study, the in vitro anticancer activities of the Zn(II) complexes against HepG2 and R-HepG2 drug-resistant HCC cells were evaluated by comparing with the ligand bbb, cisplatin and doxorubicine (Dox). As summarized in Table 1, Dox showed high cytotoxic effect on HepG2 cells and low effect on R-HepG2 cells with IC_{50} values at 0.71 μM and 226.5 μM respectively, indicating the drug-resistant ability of R-HepG2 cells to Dox with resistance index (RI) of 319.0. The cells were also resistant to cisplatin, with RI value of 3.8. In contrast, the synthetic Zn(II) complexes exhibited effective growth inhibition on both HCC cells. Especially the dinuclear complex **2**, it was more active toward the drug-resistant R-HepG2 cells than complex **1**, ligand, Dox and cisplatin. The IC_{50} and RI values were decreased to 10.5 μM and 0.8. The efficacy of this complex was further confirmed by the dose-dependent growth inhibition and morphological change (Fig. S7).

Generally, anticancer drugs inhibit cancer cell proliferation through induction of apoptosis or triggering cell cycle arrest.[23] Apoptosis has been identified as an important mechanism accounting for the anticancer action of metal complexes.[1, 4, 8, 23, 25] Therefore, in the present study, PI-flow cytometric analysis was employed to elucidate the action mechanisms of dinuclear Zn(II) complex in R-HepG2 cells. As shown in Fig. 1B**,** dose-dependent increase in apoptotic sub-G1 cell population from 1.8% (control)

Table 1. Cytotoxic effects of Zn(II) complexes.

Compounds	IC_{50} (μM)		
	R-HepG2	HepG2	RI
Complex 1	27.2	11.4	2.4
Complex 2	10.5	13.7	0.8
Ligand bbb	>160	>160	-
DOX	226.5	0.71	319.0
Cisplatin	49.2	13.1	3.8

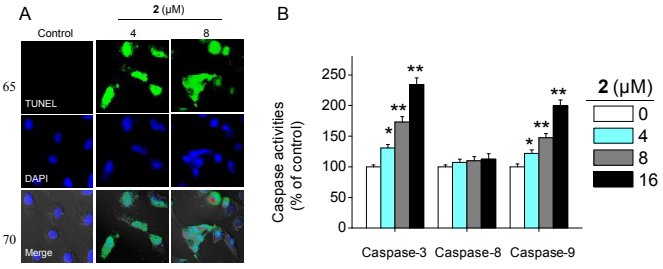

Fig. 2. Examination of cell apoptosis by TUNEL-DAPI assay (A) and caspases activation (B) induced by complex 2. In R-HepG2 cells for 72 h. * $P<0.05$; ** $P<0.01$ vs control.

to 19.4%, 46.7% and 57.7% was observed in cells exposed to complex **2**, while no significant changes in G0/G1, S and G2/M phases were observed. Similar changes were also observed in cells exposed to complex **1** (Fig. 1C). The higher apoptosis-inducing efficacy of complex **2** should be due the higher cellular uptake in R-HepG2 cells (Fig. 1D). This finding was further confirmed by TUNEL-DAPI co-staining assay [26]. As shown in Fig. 2A, treatment of the cells with complex **2** resulted in a dose-dependent increase in DNA fragmentation and nuclear condensation. Therefore, apoptosis is the major mode of cell death induced by dinuclear Zn(II) complex against drug resistant HCC cells.

Caspase family members play important roles in the initiation and execution of cell apoptosis.[27] They amplified the apoptotic signals by proteolytic cleavage of several specific substrates, such as PARP and Lamin A/C.[27] Generally, apoptosis could be regulated by two signalling pathways, including extrinsic (death receptor-mediated) and intrinsic (mitochondrial-mediated) pathways. In this study, fluorometric analysis was employed to examine the apoptotic pathways involved in the anticancer action of complex **2**. As shown in Fig. 2B, exposure of R-HepG2 cells to complex **2** resulted in dose-dependent increase in the activities of caspase-3 and -9, while no detectable activation of caspase-8 was observed, which indicate the important role of mitochondria in apoptosis induced by dinuclear Zn(II) complex.

Furthermore, living cell imaging was used to examine the effects of the synthetic Zn(II) complex on the status of mitochondria. As shown in Fig. 3, the mitochondrial network in the healthy R-HepG2 cells was extensively interconnected and appeared filamentous extended throughout the cytoplasm. However, in the treated cells, large-scale mitochondrial fragmentation and release of mitochondrial contents into cytosol were observed. Incubation of the cells with complex **2** also resulted in depletion of

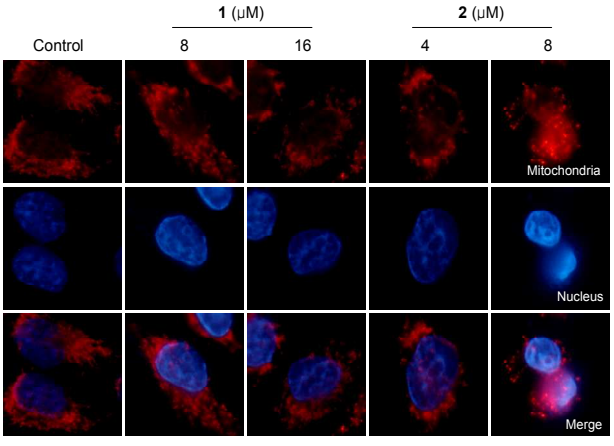

Fig. 3. Mitochondrial fragmentation induced by dinuclear Zn(II) complex.

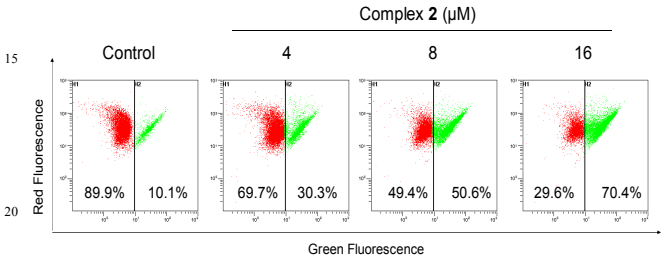

Fig. 4. Depletion of mitochondrial membrane potential in R-HepG2 cells by dinuclear Zn(II) complex (72 h).

mitochondrial membrane potential as demonstrated by the shift of fluorescence from red to green (Fig. 4) and intracellular ATP (Fig. S8). These findings provide direct evidence that dinuclear Zn(II) complex overcomes drug resistance in R-HepG2 cells through induction of mitochondrial fragmentation.

The stability of metal complexes is an important factor affecting their medicinal applications. We have detected the stability of the synthesized complexes **1** and **2** during incubation in aqueous solution at 25℃ for 72 h by UV-Vis. As shown in Fig. S9 A, B, the complexes kept stable at least for 72 h. No change in the UV-Vis spectra of the complexes was observed during incubation. These results suggest that hydrolysis did not occur in aqueous solutions. This stability supports their future applications in the treatment of cancers.

Conclusions

We presented the synthesis and characterization of a dinuclear Zn(II) complex and elucidate the in vitro anticancer activities and action mechanisms. The complex could overcome the drug resistance in R-HepG2 cells by induction of mitochondria-mediated apoptosis through triggering mitochondria fragmentation. Our results suggest that, dinuclear Zn(II) complexes could be further developed as candidates for treatments of drug-resistant HCC.

Notes and references

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Graphical Abstract

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