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A Novel Dinuclear Pd(II) Complex Dibridged by 2,2-Iminodibenzoic Acid: Synthesis, Characterization, and Biological Activity[1]

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Abstract–The novel dinuclear complex $[Pd_2(L)_2(Phen)_2]$ (Phen = 1,10-phenanthroline, H_2L = 2,2'-iminodibenzoic acid) was synthesized and characterized by IR spectra, element analysis, and X-ray single-crystal diffraction (CIF file CCDC no. 1469344). The competitive binding study with ethidium bromide (EtBr) was tested by fluorescence measurement. The result indicated that the complex has an ability to displace the EtBr bound to DNA. Gel electrophoresis assay demonstrated the ability of the complex to cleave the HL-60 DNA. Apoptotic research indicated that $[Pd_2(L)_2(Phen)_2]$ exhibited significant cancer cell inhibitory rate.

Keywods: Pd(II) complex, DNA binding, cleavage, apoptotic

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INTRODUCTION

Cisplatin was the first clinically successful platinum anticancer drug available for therapeutic of human tumors, itself and various analogues were found to be able to bind to cellular DNA, halting replication and inducing apoptosis [1, 2]. However, it has significant disadvantages including neurotoxicity, ototoxicity, nausea, etc. Thus, the design and synthesis of new complexes with low toxicity is practically important. Recently, there have been a number of reports highlighting the use of metal complexes as anticancer agents [3–6], which can interact noncovalently with DNA by intercalation, groove binding, or external electrostatic binding. The application of metal complexes in medicinal has become a field of increasing prominence [7–9] due to their fascinating topological structures and highly effective in treating cancers. As it is well-known, palladium(II) is an important transition-metal element with biologically accessible oxidation states, and its mixed-ligand complexes have been reported to intercalate between DNA base pairs, building blocks to behave as artificial DNA nucleases generating nicks at different DNA sites [10–13]. Furthermore, some studies show the Pd(II) complexes to produce significant cytotoxic and antiproliferative effects compared with control [14, 15].

Based on the structural analogy between Pt(II) and Pd(II) complexes, herein, we describe the synthesis, characterization, and crystal structure of mixed-ligand dinuclear palladium(II) complex (I) dibridged by anion of 2,2'-iminodibenzoic acid (L^{2-}) and 1,10-phenanthroline (Phen). The fluorescence quenching experiment study elucidated DNA binding ability. The DNA cleavage activities of the complex with HL-60 DNA have been carried out by gel electrophoresis. Apoptotic study showed the complex exhibite cytotoxic specificity and significant cancer cell inhibitory rate. The newly synthesized Pd(II) complex may be a potential antitumor agent due to its unique interaction mode with DNA.

EXPERIMENTAL

Materials and physical measurements. All chemicals and solvents used for synthesis and characterization of the complex were reagent-grade commercial and were used without further purification. The HL-60 cells, HeLa (human cervix epitheloid carcinoma) cells, and KB (human oral epithelial carcinoma) cells were obtained from American Type Culture Collection.

Elemental analysis (C, H, and N) was performed on a model Finnigan EA 1112 instrument. The IR spectra in the range of 4000–400 cm^{-1} were obtained as KBr pellets on a Nicolet IR 470 spectrometer. Fluorescence measurement was carried out on a Perkin-Elmer LS55 fluorescence spectrofluorometer. The entrance and exit slits were maintained at 10 and

[1] The article is published in the original.

Table 1. Selected crystallographic data, details of experiment, and refinement factors for complex **I**

Parameter	Value
Crystal system	Monoclinic
Space group	$P2_1/n$
a, Å	26.5485(13)
b, Å	13.5592(7)
c, Å	28.5707(14)
β deg	92.4960(10)
V, Å^3	10275.0(9)
Z	8
ρ_{calcd}, g/cm^3	1.574
μ, mm^{-1}	0.812
θ Range, deg	1.427 to 25.230
F(000)	4948
Index ranges	$-31 \le h \le 30$, $-14 \le k \le 16$, $-32 \le l \le 34$
Number of reflections measured	53137
unique (N) (R_{int})	18528 (0.0625)
Completeness, %	99.6
Refinement parameters	1347
GOOF	1.043
Final R indices ($I > 2\sigma(I)$)	$R_1 = 0.0858$, $wR_2 = 0.2421$
R indices (all data)	$R_1 = 0.1358$, $wR_2 = 0.2836$
$\Delta\rho_{max}/\Delta\rho_{min}$, e Å^{-3}	5.995 and −0.784

10 nm, respectively. The sample was incubated for 4 h at room temperature (20°C) before spectral measurement and excited at 526 nm, the emission range was set between 540 and 750 nm. The buffer used in the binding study was 50.0 mM Tris-HCl, pH 7.4, containing 10.0 mM NaCl. The measurement of ethidium bromide (EtBr, brominated 3,8-diamino-5-ethyl-6-benzene, phe-nanthrene tablets) binding to DNA-Pd(II) complex was studied by increasing the concentrations of complex **I** and measuring the change in fluorescence intensity. For the gel electrophoresis experiment, HL-60 DNA was treated with complex **I** in Tris-buffer (50.0 mL Tris-HCl, 10.0 mL NaCl buffer, pH 7.4). After incubated for 1.5 h at room temperature the sample was electrophoresed for 3 h at 90 V on 0.8% agarose gel. Then, the gel was stained with 1.0 mg/mL EtBr and photographed under UV light. The complex to induce apoptosis ability is evaluated in KB cell line using Annexin V conjugated with FITC and propidium iodide (PI) counterstaining by flow cytometry. The KB cells in a usable condition were seeded in a 6-well culture plate at 1×10^6 cells per well in a 3 mL culture medium. 6 and 12 h later the medium including the Pd(II) complex was given. After 6 h (or 12 h) incubation, cells were gathered, wash cells twice with cold phosphate-buffered saline (PBS) and then resuspend cells in 1× Binging Buffer at a concentration of 1×10^6 cells/mL. We added 5 μL of FITC Annexin V and 5 μL PI. Gently vortex the cells and incubate for 15 min at RT (25°C) in the dark. We added 400 μL of 1× Binding Buffer to each tube. Analyzed by flow cytometry (Accuri C6, USA) within 1 h.

Synthesis of complex I. An aqueous solution containing 1.5 mmol (10 mL) of H_2L was added dropwise into 10 mL water solution containing 1.5 mmol of $K_2[PdCl_4]$ and stirred at room temperature. After the solution was stirred overnight (12 h), a 10 mL ethanol solution of Phen (1.5 mmol), was added and stirred another 12 h. Then the solution was filtered off and allowed to stand in air. Several weeks later, the resulting red crystals were filtered, washed with ethanol and dried in vacuo.

For $C_{104}H_{68}N_{12}O_{16}Pd_4 \cdot 2H_2O$

anal. calcd., %:	C, 66.13;	H, 3.29;	N, 7.63.
Found, %:	C, 66.34;	H, 3.49;	N, 7.37.

IR (KBr; ν, cm^{-1}): ν(O–H) 3401 m; ν(=C–H) 3058 m; ν(C=O) 1603 s; ν(C=C) 1514 s, 1447 m; ν(C–N) 1317 s; ν(C=O) 1274 m; ν(C–H) 750.

X-ray diffraction. Single-crystal X-ray data of the complex were collected on a Brucker Smart 1000 CCD diffractometer with MoK_α radiation (λ = 0.71073 Å) at 293 K in the range of $1.427° < \theta < 25.230°$, ω-scan technique. The structure was solved by a direct method using SHELXL-97 and refined by full-matrix least-squares methods on F^2 [16, 17]. All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were located from different Fourier maps. Crystal data and structure refinement details are presented in Table 1. Selected bond lengths and angles are listed in Table 2.

Supplementary material for structure **I** has been deposited with the Cambridge Crystallographic Data Centre (no. 1469344; deposit@ccdc.cam.ac.uk or http://www.ccdc.cam.ac.uk).

Cell line and culture. The cell lines used in this experiment were routinely maintained in a RPMI-1640 medium supplemented with 10% (v/v) heat inactivated fetal bovine serum, 2 mmol L^{-1} of glutamine, 100 μg mL^{-1} of penicillin, and 100 μg mL^{-1} of streptomycin in a highly humidified atmosphere of 95% air with 5% CO_2 at 37°C.

RESULTS AND DISCUSSION

The crystal structure of complex **I** was determined by X-ray crystallography, and is shown in Fig. 1. Both Pd(II) atoms are four coordinated, while each Pd(II) atom is coordinated by two nitrogen atoms from the phen, and two oxygen atoms from two different

ligands L^{2-}. Stacking takes place between two Phen, with a dihedral angle between them of 7.39° and 3.78° by an offset face-to-face mode arrangement, indicated that an approximate π–π interaction exists in the complex [18]. The Pd···Pd distance in the dinuclear complex **I** is 3.192 Å (Pd(1)···Pd(2)) and 3.271 Å (Pd(3)···Pd(4)). It was revealed that there are weak metal–metal interactions, but larger than Pd···Pd distance observed in some dinuclear palladium complexes [19–21]. Herein, the final structure of the complex may be the result of the sum of the different interactions. The packing structure of the complex is provided in Fig. 2.

Fluorescence quenching measurement used as a monitor metal binding [22]. Ethidium bromide (EtBr) is a highly sensitive fluorescent dye. It has conjugate aromatic nucleus perfect planar molecule and its fluorescence intensity is very weak. But when EtBr is specifically intercalated into the base pairs of double stranded the emit fluorescence light is greatly increased [23, 24]. Competitive binding studies with EtBr were implemented to obtain support for the mode of the complex binding with DNA. The research includes the addition of the complex with EtBr ([DNA]/[EtBr] = 2.5) to DNA and the measurement of the intensity of emission. The result in Fig. 3 showed that the fluorescence intensity of HeLa-DNA–EtBr decreased remarkably with the addition of palladium complex, which indicated that the Pd(II) complex can bind to DNA and replace EtBr from the HeLa-DNA–EtBr system.

Gel electrophoresis is a general method to evaluate the binding mode of small molecules and DNA base pairs [25–27]. In this paper, we used the HL-60 cells DNA, which was extracted by ourselves, so the mechanism can coincide with the activity. When HL-60 cells DNA is subject to electrophoresis, relatively fast migration will be observed for the intact supercoil

Table 2. Selected bond lengths (Å) and angles (deg) for complex **I**

Bond	*d*, Å	Bond	*d*, Å
Pd(1)–N(8)	1.998(8)	Pd(1)–N(7)	2.008(8)
Pd(1)–O(9)	2.009(6)	Pd(1)–O(13)	2.011(6)
Pd(1)–Pd(2)	3.192(11)	Pd(2)–N(6)	2.004(8)
Pd(2)–N(5)	2.018(9)	Pd(2)–O(12)	2.002(7)
Pd(2)–O(16)	2.021(7)	Pd(3)–N(9)	1.999(8)
Pd(3)–N(10)	2.007(8)	Pd(3)–O(6)	2.019(7)
Pd(3)–O(3)	1.984(7)	Pd(3)–Pd(4)	3.271(11)
Pd(4)–N(11)	1.988(8)	Pd(4)–N(12)	2.003(9)
Pd(4)–O(7)	2.001(8)	Pd(4)–O(2)	2.005(7)
Angle	**ω, deg**	**Angle**	**ω, deg**
N(8)Pd(1)N(7)	82.5(3)	N(8)Pd(1)O(9)	172.8(3)
N(7)Pd(1)O(9)	91.9(3)	N(8) Pd(1)O(13)	95.1(3)
N(7)Pd(1)O(13)	176.9(3)	O(9)Pd(1)O(13)	90.3(3)
N(6)Pd(2)N(5)	81.1(4)	O(12)Pd(2)N(6)	173.5(3)
O(12)Pd(2)N(5)	93.5(3)	N(6)Pd(2)O(16)	95.1(3)
N(5)Pd(2)O(16)	174.3(3)	O(12)Pd(2)O(16)	90.0(3)
N(9)Pd(3)N(10)	82.7(4)	O(3)Pd(3)N(9)	91.3(3)
O(3)Pd(3)N(10)	173.2(3)	N(9)Pd(3)O(6)	177.2(3)
N(10)Pd(3)O(6)	95.2(3)	O(3)Pd(3)O(6)	91.0(3)
N(11)Pd(4)N(12)	81.9(4)	N(11)Pd(4)O(7)	168.4(3)
N(11)Pd(4)O(2)	96.7(3)	O(7)Pd(4)N(12)	89.4(4)
N(12)Pd(4)O(2)	178.0(4)	O(7)Pd(4)O(2)	91.8(3)

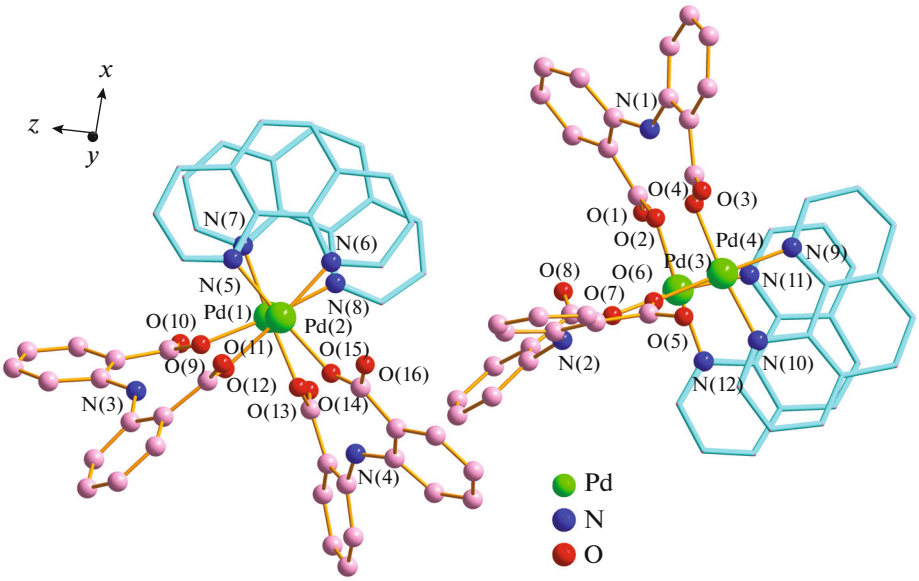

Fig. 1. The two independent molecules of complex **I** with numbering of atoms (H atoms were omitted for clarity).

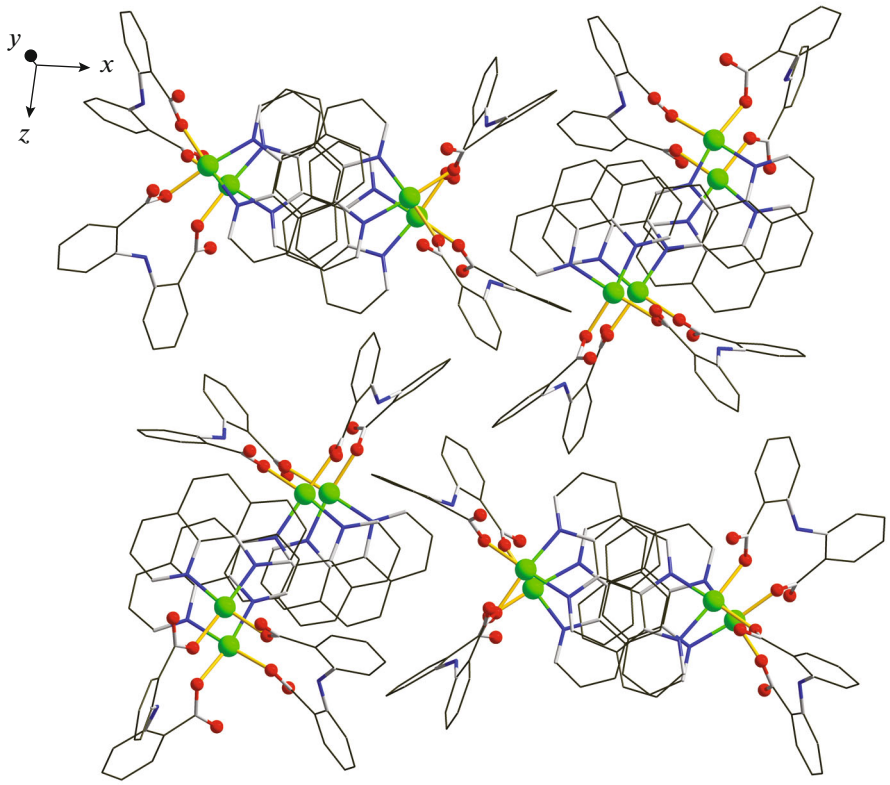

Fig. 2. Packing of the structure of **I** (H atoms were omitted for clarity).

form (Form I). If scission occurs on one strand (nicking), the supercoil will relax to generate a slower-moving open circular form (Form II). If both strands are cleaved, a linear form (Form III) that migrates between Form I and Form II will be generated [28].

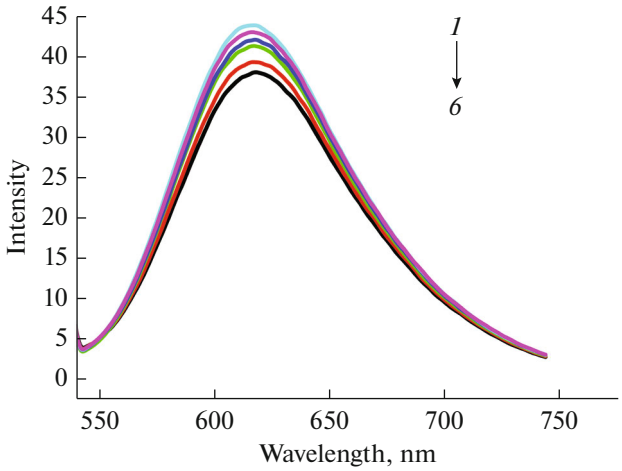

Fig. 3. Fluorescence spectra of the binding of EtBr to DNA in the absence (line *1*) and presence (lines *2*–*6*) of increasing amounts of complex **I**.

The electrophoretogram of the DNA sample treated with different concentration ratios of the complex was illustrated in Fig. 4. This electrophoretogram showed that, with the decreasing of the complex concentration, the intensity of the circular supercoiled DNA (Form I) band diminished gradually, while that of nicked (Form II) band apparently increased (Lanes 2–4). The complex induced obvious cleavage of the HL-60 DNA at 15, 7.5 and 3.75 μM. HL-60 DNA was cutted into small molecular DNA strands, and each lane has obviously towed the marks. From Fig. 4, we can know that complex **I** has an effect on the DNA cutting and the capability for cleavaging is stronger at higher concentrations than it is at low concentrations [29–32].

Annexin V-FITC was used to determine the mode of cell death (apoptosis/necrosis) resulted from the activity of the Pd(II) complex. Respectively, in 6 and 12 h the medium including the Pd(II) complex was given for KB cells (Fig. 5). Combinated with the analysis results can distinguish living and dead cells [33]. The percentage of apoptotic cells (early apoptotic cells Ann+/PI– and late apoptotic cells Ann+/PI+), after 6 h of incubation with the complex was 55.3%. After 12 h, the percentage of apoptotic cells (early apoptotic cells Ann+/PI– and late apoptotic cells Ann+/PI+) of incubation with the complex was 76.9%. By comparing, we know that the number of the dead cancer

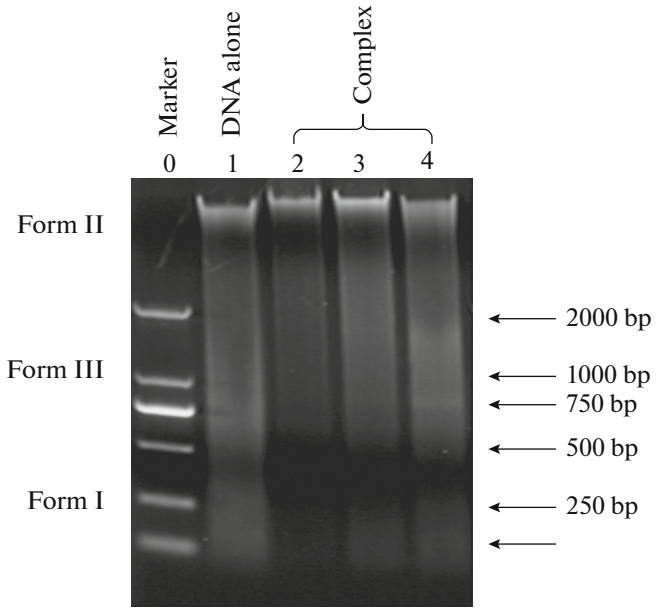

Fig. 4. Cleavage of HL-60 DNA in the present of complex **I**: Marker (0); DNA (1); DNA with different concentration of **I**: 15 (2), 7.5 (3), 3.75 μM (lane 4).

cells increases gradually with the time increase in early apoptotic (lower right quadrant) or late apoptotic/necrotic (upper right quadrant).

Apoptosis is the most common and well defined form of programmed cell death, which plays a critical role in tissue homeostasis and elimination of unwanted cells without affecting normal/unaffected cells [34, 35]. Necrosis is a messy, unregulated process of traumatic cell destruction, which is followed by the release of intracellular components when compare with the self-contained nature of apoptotic cell death [36]. Under the light microscope, in which way the new complex produced cellular death (necrosis or apoptosis) has been studies on KB cells (Fig. 6).

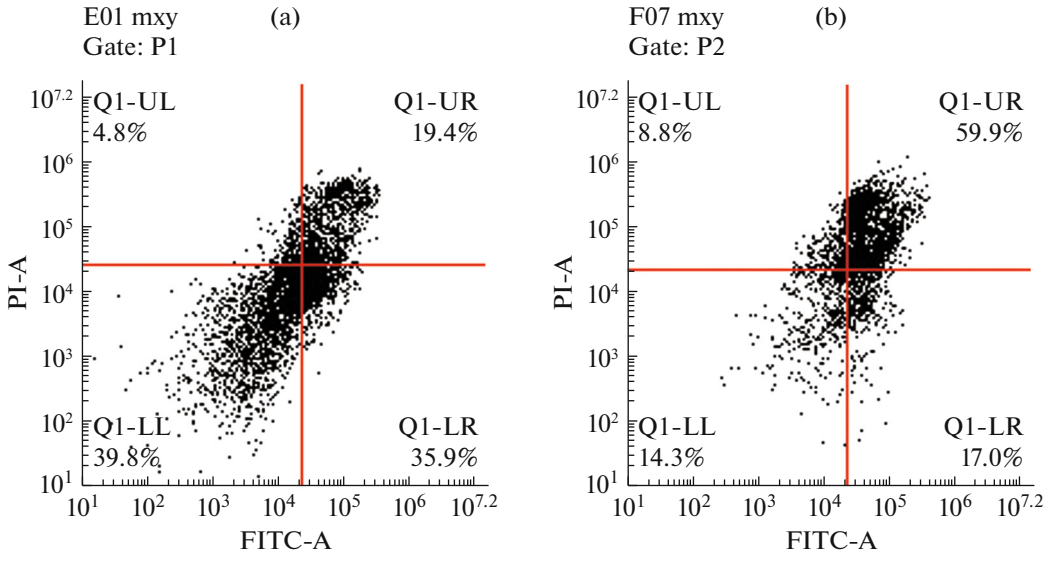

Fig. 5. The KB cells (human oral epithelial carcinoma) cultured with 1000 μg/mL of **I** for 6 (a) and 12 h (b) were measured by surface expression of phosphatidyl serine using FITC-conjugated Annexin V antibody. Membrane permeability was assessed by propidium iodide exclusion (PI), analyzed by flow cytometry. Dot plots show percentages representing the population of cells that are non-apoptotic (lower left quadrant), early apoptotic (lower right quadrant) or late apoptotic/necrotic (upper right quadrant). Quadrants were established using controls.

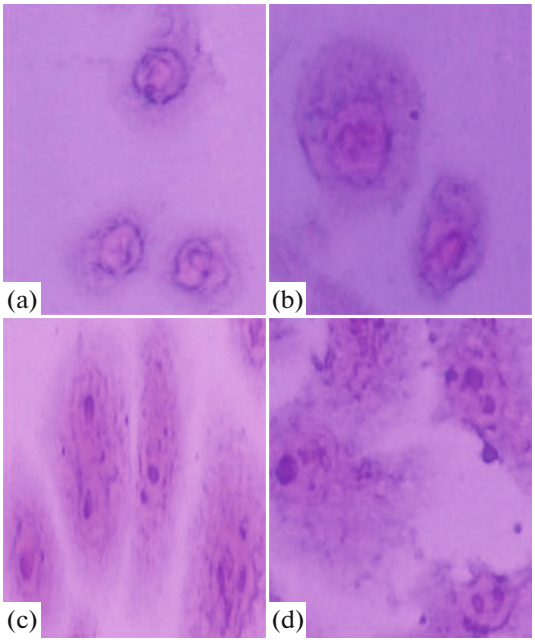

Fig. 6. Morphological changes of KB (human oral epithelial carcinoma) cells: normal KB cells for 6 (a) and 12 h (b); KB cells treated with complex **I** for 6 (c) and 12 h (d).

Figures 6a, 6b were the normal cells for 6 and 12 h. It exhibited a dense state and an intercellular tight junction, the single cell is irregular cell morphology and cytoplasm has a clear appearance. After addition of complex **I** for 6 h (Fig. 6c) and 12 h (Fig. 6d) the cells appeared small size, which different from the apoptotic cells, the cell nucleus became pyknotic (shrunken and dark). The nuclear fragmentation indicated that the complex has the ability to apoptotic canner cell.

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