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Metal Ion Complexes of Kaempferitrin as DNA Topoisomerase I Inhibitors and Its' Cytotoxicities

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In this study, four binuclear M(II) (M = Cu, Zn, Co and Cd) complexes of kaempferitrin were synthesized. Their structures were characterized by elemental analysis, infrared spectroscopy, TG-DTG analysis and MS spectra. The activities of kaempferitrin and its metal complexes on DNA topoisomerase I (TOPO I) were measured by agarose gel electrophoresis using plasmids pBR322 and pUC19 as model substrate DNA. The cytotoxicities of these compounds on MCF-7, SGC-7901, SK-OV-3 and HePG-2 cell lines were determined by MTT assay. The results showed that four complexes could significantly affect the performance of DNA TOPO I to promote its mediated DNA helicase or breakage. Copper complex was identified as an excellent TOPO I Inhibitor. Furthermore, copper complex can inhibit the proliferation of SGC-7901 and SK-OV-3 with the inhibition rate of 44.19 and 44.58 %, stronger than that of the kaempferitrin and other complexes. This study has proved that the kaempferitrin complexes have stronger inhibitory effects than kaempferitrin for potential synergistic effects.

Keywords: Kaempferitrin, Complex, Topoisomerase I Inhibitor, Cytotoxicity.

INTRODUCTION

Some naturally occurring flavonoids, such as rutin and apigenin, have been well-known for their medicinal properties for many years. In recent years, the discovery that their complexes with metal ions are more effective than flavonoids alone changed the course of drug research. Numerous studies showed that these complexes can be successfully used in a range of diseases such as diabetes mellitus[1], some bacterial infections[2] or even cancers[3]. The complexes can also influence the equilibrium of iron within a living organism, which is an important factor in the treatment of diseases[4].

Kaempferitrin (KR), a glycosylated flavonoid, is widely distributed in plants and is well known to be a powerful antioxidant[5]. Recent studies have shown that kaempferitrin exhibits anti-inflammatory[6,7] and anti-proliferation effects on several forms of cancer cells such as HeLa[8]. Furthermore, kaempferitrin can activate the insulin signaling pathway and stimulate secretion of adiponectin[9,10] and inhibit GLUT4 translocation and uptake glucose in 3T3-L1 adipocytes[11]. Moreover, kaempferitrin can exert immunostimulatory effects on immune responses mediated by splenocytes, macrophages, PBMC and NK cells[12]. Therefore, kaempferitrin is a good option for cancer treatment for its low toxicity in non-cancerigenic cells and its anticancer effects.

It is well known that TOPO I is a class of basic ribozyme which can maintain or alter DNA topology of the genetic process of cells replication, transcription, translation, restructuring and chromatid separation smoothly[13]. This enzyme was also used as a target enzyme for anticancer and antibacterial drug discovery[14]. Screening topoisomerase inhibitors used TOPO I as a target is an important method for efficient anticancer drugs[15].

Kaempferitrin is a good multiple electron donors and can effectively complex with various metal ions. However, to the best of our knowledge, metal complexes using kaempferitrin as ligand have not been reported so far. In this article, we report the synthesis of four complexes of kaempferitrin as TOPO I inhibitors and evaluated the cytotoxicities of kaempferitrin complexes on MCF-7, SGC-7901, SK-OV-3 and HePG-2 cell lines.

EXPERIMENTAL

Electrophoresis was measured using a Bio-rad Mini-Protean Tetra Electrophoresis System. The enzyme-linked immunosorbent assay was analyzed in a microplate ELISA reader (Bio-Rad, Model 450). Micrographs were taken at 25 °C using a fluorescent motorized microscope Zeiss Axiovert 200 (Zeiss, Goettingen, Germany).

Kaempferitrin was isolated from leaves of *Siraitia grosvenori* in previous research[16]. 0.2 mmol (0.1156 g) kaempferitrin was dissolved in 10 mL of anhydrous ethanol with stirring. A mixed solution of $NH_3 \cdot H_2O$ and CH_3CH_2OH (1:1) was added slowly to the kaempferitrin solution until a pH value of 8-9 was reached. 0.12 mmol metal ion such as $CuCl_2 \cdot 2H_2O$ or $Co(NO_3)_2 \cdot 6H_2O$, $Zn(NO_3)_2 \cdot 2H_2O$ and $Cd(CH_3COO)_2 \cdot 2H_2O$ in 5 mL ethanol were added to the solution. The reaction was taken 8 h at room temperature. The precipitates were repeatedly washed with distilled water and dried under vacuum to give a dark green solid.

TOPO I inhibitory activity test: 10 μL reaction mixture contained TOPO I (20 u μL^{-1}, obtained from TaKaRa), pBR322/pUC19 (0.5 μg μL^{-1}, purchased from Sigma-Aldrich). 1 μL 0.1 % BSA, 10 × DNA TOPO I buffer and 6 μL kaempferitrin (or its metal complexes) with different concentrations. The incubations were run for 1 h at 37 °C and the reaction was terminated by adding 2 μL loading buffer as a stop solution. The DNA products and pBR322 DNA/pUC19 were separated by electrophoresis using a 1 % agarose gel for 90 min at 5 V cm^{-1}. The gels were subsequently stained with 0.5 μg L^{-1} ethidium bromide (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd, China) for 0.5 h. The DNA-containing bands were visualized using an ultraviolet light box. All experiments were repeated at least three times.

Cells and medium: Four human cancer cell lines, namely MCF-7, SGC-7901, SK-OV-3 and HePG-2 purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), were used in this study. The cells were grown in RPMI1640 medium or DMEM medium (Gibco, NY, USA) at 37 °C in 5 % CO_2 for 24 h. Two culture mediums contained 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 10 % new born calf serum. Disaggregation was carried out using 15 min incubation at 37 °C with a 0.25 % solution of trypsin.

MTT assay: MTT (Amersco, USA) assay was carried out to determine the cytotoxicities of kaempferitrin complexes against studied cancer cell lines. That is, cells were plated in 96-well plates at a density of 1×10^4 cells per well and grown for 12 h, 10 μL of different concentrations of kaempferitrin and its' complexes were added to 200 μL mediums of wells for 48 h. 10 μL of MTT stock solution (5 mg L^{-1} in PBS) was added to each well and the microplate was wrapped with aluminum foil and incubated at 37 °C for 4 h and then the purple formazan product formed was dissolved by the addition of 150 μL DMSO to each well. The optical density of each well was read by microplate reader.

RESULTS AND DISCUSSION

Elemental analysis: Elemental analyses were carried out using a PE-2400 II elemental analyzer. Analyses indicated by the symbols of the elements were within ± 1.5 % of the theoretical values. As shown in Table-1, kaempferitrin was coordinated with M(II) at the ratio of 1:1.

Infrared spectroscopy analysis: Infrared spectroscopy was used to analyze the structural changes in samples and was recorded on a Nicolet EFP 360 FI-IR spectrometer as KBr pellets.

As shown in Table-2, some chemical bonds in four complexes, such as C-O-C, C-OH, did not show significant changes of the wave-numbers. However, the C=O bending vibrations were shifted to lower wave-numbers at 1625-1619 cm^{-1} region compared with kaempferitrin in 1695 cm^{-1}. The results proved that these bonds did not been destroyed in the reaction. Its' reaction site may be C=O at 4' and O-H at 5'.

TG-DTG analysis: TG-DTG analysis were measured under the nitrogen air atmosphere with PRIS Diamond TG/DTA thermal analyser at a heating rate of 10 °C min^{-1} in the temperature range 35-1000 °C for four complexes.

As shown in Fig. 1, TG-DTG analysis of four complexes loss weight through a different temperature gradient. The amount of weight loss at different temperatures may be inferred the peripheral portion of the molecule.

In conclusion, binuclear M(II) (M = Cu, Zn, Co and Cd) complexes, obtained by the reaction of metal (II) nitrate and kaempferitrin in a ratio of 1:1 were characterized by elemental analysis, infrared spectroscopy, TG-DTG analysis and MS spectra (Data not shown). Their possible chemical compositions were inferred as $[Cu(KR)(H_2O)_3Cl] \cdot 4H_2O \cdot NH_3$, $[Co(KR)(H_2O)_3(NH_3)] \cdot NO_3$, $[Zn(KR)(H_2O)_3 \cdot (NO_3)] \cdot 4H_2O$ and $[Cd(KR)(H_2O)_3 \cdot NH_3 \cdot (CH_3COO)] \cdot 2H_2O \cdot CH_3CH_2OH$.

Inhibitory activities of kaempferitrin and its' complexes on TOPO I: The activities of kaempferitrin and its metal complexes on TOPO I were measured by agarose gel electrophoresis using plasmids pBR322 and pUC19 as model substrate DNA.

TABLE-1
ELEMENTAL ANALYSIS FOR FOUR METAL COMPLEXES

Complexes	C (%)		H (%)		N (%)		M^1 (%)	
	Found	Calcd	Found	Calcd	Found	Calcd	Found	Calcd
KR-Cu(II)	42.12	42.43	5.28	5.61	1.47	1.70	7.74	7.75
KR-Co(II)	42.23	42.14	4.91	4.94	3.59	3.64	7.02	7.66
KR-Zn(II)	39.65	39.02	5.34	5.18	1.83	1.68	7.36	7.87
KR-Cd(II)	41.66	41.26	5.42	5.65	1.51	1.55	11.78	12.46

TABLE-2
KEY INFRARED BANDS (cm^{-1}) OF KR AND ITS METAL COMPLEXESES

Complexes	ν(C=O)	ν(C=C)	ν(C-OH)	ν(C-O-C)	ν(O-H)
KR	1659	1603	1355	1180	3435
KR-Cu(II)	1625	1560	1351	1182	3425
KR-Co(II)	1627	1575	1347	1177	3435
KR-Zn(II)	1619	1576	1350	1180	3394
KR-Cd(II)	1619	1591	1348	1175	3421

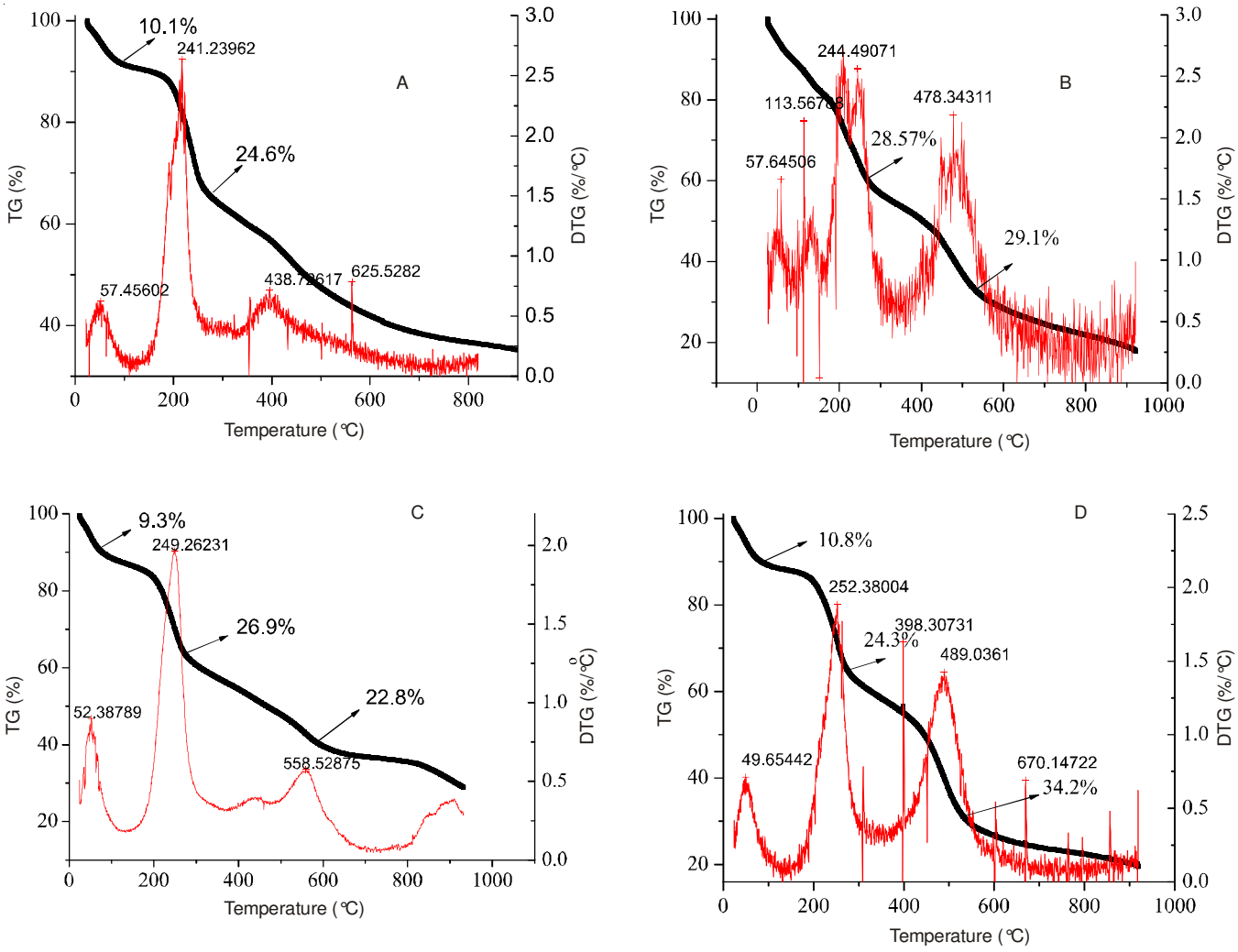

Fig. 1. TG-DTG analysis of four complexes. A: KR-Cu(II); B: KR-Co(II); C: KR-Zn(II); D: KR-Cd(II))

We can determine the activity of metal complexes on the TOPO I by the TOPO I mediated pBR322 and pUC19 helicase.

A typical agarose gel electrophoresis of pBR322 and pUC19 was shown in Figs. 2 and 3, which were incubated with kaempferitrin and its complexes and HCPT used as a positive control. As shown in Fig. 2, two forms (super-coiled, SC; relaxed coil, RE) of pBR322 were separated by electrophoresis on an agarose gel (lane 3). 1 u TOPO I can catalyze the reaction of pBR322 completely from SC form to RE form (lane 2). HCPT can effectively inhibit helicase activity of TOPO I (lane 1). Kaempferitrin cannot suppress the TOPO I relaxation effects on pBR322, while its metal complexes showed good inhibitory activities. Super-coiled pBR322 is increased gradually with the increase of the molar concentration and inhibition of the activity-enhanced TOPO I. Cadmium complexes at 10 μM showed better inhibitory activity, while zinc, copper, cobalt complexes could be suppressed TOPO I to generate DNA relaxation effects at high concentrations (> 100 μM).

Furthermore, it is observed that the four metal complexes have strong inhibition on TOPO I using the super-coiled pUC19 DNA as substrate in Fig. 3. Among them, copper and cadmium complexes at 100 μM (lanes 7 and 11) can effectively suppress TOPO I on pUC19 and the cadmium complex at

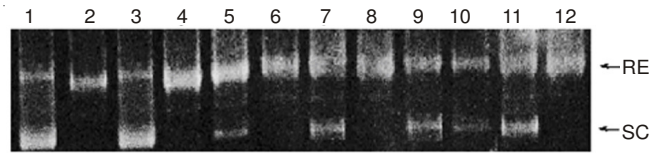

Fig. 2. A typical agarose-gel electrophoresis of plasmid pBR322 DNA incubated with kaempferitrin (KR) and its complexes at various concentrations. Lane 1. pBR322, TOPO I and HCPT; lane 2. pBR322 and TOPO I; lane3. pBR322 only; lane 4 to11: Zn (10 μM), Zn (100 μM), Cu (10 μM), Cu (100 μM), Co (10 μM), Co (100 μM), Cd (10 μM), Cd (100 μM) with pBR322 and TOPO I; 12. KR (100 μM) only

50 μM has the inhibitory activity (lane 10). Cobalt and zinc complexes were also identified as good inhibitors. With the increase of the concentration of metal complexes, the form of super-coiled DNA was decreased, the inhibitory activities of TOPO I were weakened. Therefore, the complexes have a significant effect on the helicase-mediated pBR322 and pUC19 in the cell-free system. This inhibitory activity exhibited a significant dose-response relationship.

Inhibition effects of kaempferitrin and its' complexes: Four common cancer types, SGC-7901, HepG-2, MCF-7 and SK-OV-3 cell lines in human are selected to test the anticancer activity for kaempferitrin and its complexes because of their high incidence. The results were shown in Fig. 4.

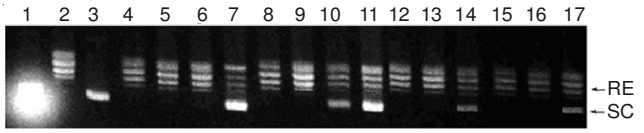

Fig. 3. A typical agarose-gel electrophoresis of plasmid pUC19 DNA incubated with kaempferitrin and its complexes at various concentrations. 1. pUC19, TOPO I and HCPT; 2. pUC19 and TOPO I ; 3. pUC19 only; 4. Cu complex (5 μM), TOPO I and pUC19; lane 5 to 7. Cu complex at 10, 50 and 100 μM; lane 8 to 11. Cd complex at 5, 10, 50 and 100 μM; lane 12 to 14. Co complex at 10, 50 and 100 μM; lane 15 to 17. Zn complex at 10, 50 and 100 μM

As shown in Fig. 4, kaempferitrin exhibits the weaker inhibition rate to all of cancer cells, but its complexes exhibit stronger inhibitory activities to SGC-7901 and SK-OV-3. Copper complex possesses significant inhibitory activity against SGC-7901 and SK-OV-3, stronger than that of the kaemp-feritrin and other complexes with the inhibition rate of 44.19 %, 44.58 %, respectively. Cadmium complex also exhibited moderate inhibition activity against other cells. Furthermore, inhibition rates of cobalt, zinc complexes against ovarian cancer SK-OV-3 were 22.37 and 28.92 %, higher than the kaempferitrin (13.25 %). The results indicated that the combination of flavonoids and mental ions for potential synergistic effects.

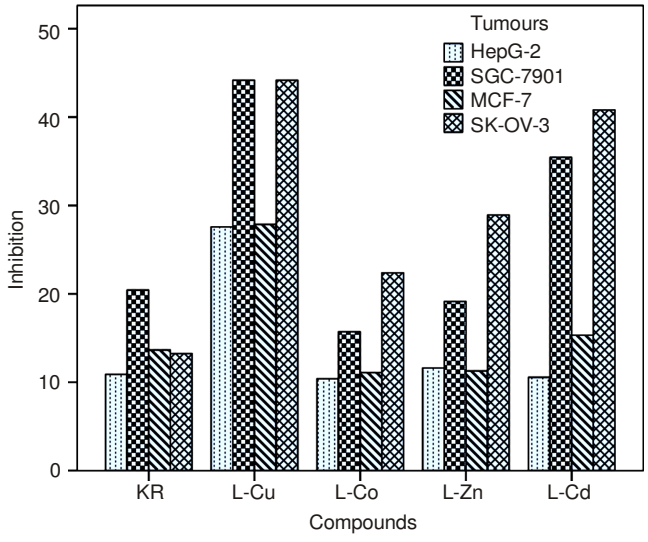

Fig. 4. Inhibition effects of kaempferitrin (KR) and its complexes on growth inhibition rate in HepG-2, SGC-7901, MCF-7 cell, and SK-OV-3 cells

Conclusion

In conclusion, this study shows that TOPO I could be inhibited by four metal complexes in pBR322 and pUC19. The inhibitory activity exhibited an obvious dose-response relationship. Copper complex was identified as a good inhibitor affecting on TOPO I. It could effectively inhibit TOPO I at 10 μM in pBR322 and at 50 μM in pUC19.

Kaempferitrin can induces apoptosis *via* the intrinsic pathway in HeLa cells and exerts anticancer effects[8]. However, comparative analysis of four metal complexes on four kinds of human cancer cell lines, we found that metal complexes shown the stronger inhibitory activities on various cancer strains than kaempferitrin. Metal ions should play an important role to improve the natural activity of flavonoid glycosides on cancer cells growth inhibition, unique efficacy and mechanism[17]. We can draw a conclusion that the combinations of kaempferitrin and metal ions have the potential synergy effects. Meanwhile, the activities of copper complexes against different cells were selective inhibition. Therefore, copper complex was promising cancer inhibitors that might provide a potential drug for the treatment of human cancers.

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