Synthesis of New Cu Complex Based on Natural 5Z,9Z-Eicosadienoic Acid: Effective Topoisomerase I Inhibitor and Cytotoxin

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ABSTRACT

The complex $(bipy)_2Cu(5,9\text{-eicd})$ was prepared by the reaction of $Cu(OAc)_2$ with 5Z,9Z-eicosadienoic acid and 2,2′-bipyridine in methanol. The new copper complex showed high antitumor activity in vitro toward A2780cis, A2780, Hek293, K562, HL60, Jurkat, and U937 cell lines and efficiently inhibited human topoisomerase I. Using flow cytofluorometry, $(bipy)_2Cu(5,9\text{-eicd})$ was studied for the effect on the cell cycle and apoptosis-inducing activity in tumor cells.

Keywords :

INTRODUCTION

In 1969, Rosenberg and co-workers reported the first results concerning the cytotoxicity of platinum compounds against murine tumors,[1] which initiated a new area of medicinal chemistry dealing with metal-containing antitumor agents. Currently, platinum-based compounds such as cisplatin, carboplatin, and oxaliplatin are widely used in medical practice.[2] Although cisplatin and its derivatives have been successfully included into the armory of anticancer chemotherapeutical agents, there are problems related to their high toxicity and low selectivity to malignant tumors and, in some cases, drug resistance of cancer cells. This stimulates extensive studies aimed at the development of new metal derivatives that would be free from the indicated drawbacks.[3] Among nonplatinum compounds, researchers are focused on less toxic copper-based compounds.[4] The properties exhibited by complexes are substantially dependent on the nature of ligands and the donor atoms that are coordinated to the metal.[5]

The relationship between the structure and biological activity of copper complexes has been investigated for as long as more than 5 decades. The biological activities of many copper compounds is governed by the chelating properties of the ligands towards transition-metal ions.[6] It is also known that metal-ion chelates show a much better uptake by living organisms than free metal ions. However, many issues concerning the behavior of copper ions in the intracellular space remain unsolved: it is absolutely unknown whether or not the structure of sophisticated complexes is retained in the cell as the copper ion is released and what happens when the copper valence changes from Cu(II) to Cu(I).[7] An increase in the copper-ion concentration inside the cells is known to induce general intoxication to inhibit the DNA synthesis and oxidative phosphorylation, and also to

result in total thioloxidation to disulfides.[8] The biological effects of copper complexes are highly diversified and selective, and all this complicates the search for and systematization of literature sources. All of the listed issues can apparently attest to the high diversity of biological effects of these compounds.

A fairly important factor for the design of modern antitumor compounds is the selection of the molecular target, most often, an enzyme that performs a key function in tumor cells. It is known that many copper complexes with bipyridine ligands that exhibit antitumor activity are effective inhibitors of topoisomerases, that is, key cell cycle enzymes.[4,5] Recently, it was also shown in a number of works that 5Z,9Z-dienoic acids are efficient nonspecific inhibitors of human topoisomerase.[9] In view of the foregoing, we hypothesized that copper carboxylates with pyridine ligands based on 5Z,9Z-dienoic acids could also exhibit high cytotoxicity via targeted action on topoisomerases.

This communication presents preliminary results on the synthesis of copper bipyridine complexes based on 5Z,9Z eicosadienoic acid, which exhibited the highest inhibitory activity toward topoisomerase I, and study of its antitumor properties in vitro on several tumor cell lines of various etiology by means of flow cytofluorometry.

RESULTS AND DISCUSSION

The complex $Cu(bpy)_2$(5,9-eicd) (bpy is 2,2′-bipyridyl, 5,9- eicd is 5Z,9Z-eicosadienoic acid) 1 was synthesized by the reaction of Cu(OAc)2·H2O with 2,2′-bipyridine (2 equiv) and 5Z,9Z-eicosadienoic acid (1 equiv) in methanol by a modified procedure (Figure 1).[10] A similar copper complex based on arachidic acid, $Cu(bpy)_2$(arach) (bpy is 2,2′-bipyridyl, arach is arachidic acid) 2, was prepared as a reference compound (Figure 1).

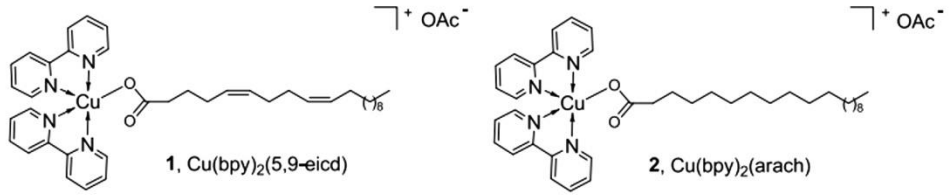

Figure 1. Structures of complexes $Cu(bpy)_2$(5,9-eicd) (**1**) and $Cu(bpy)_2$(arach) (**2**).

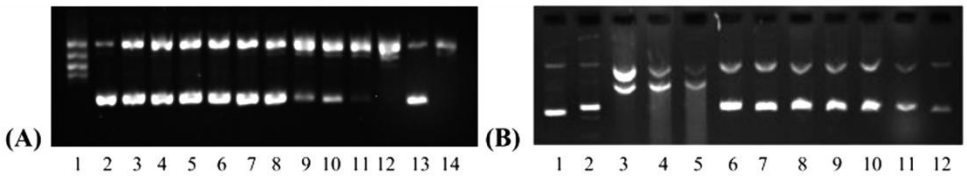

Figure 2. Electropherogram of the products of relaxation of supercoiled plasmid DNA in vitro under the action of topoisomerase I (Topogen) and $Cu(bpy)_2$(5,9-eicd) (**A**) without ethidium bromide and (B) with ethidium bromide. Lane: (**1**). Relaxed plasmid DNA (pHOT1) (**2**). Supercoiled plasmid DNA (pHOT1). (**3–12**). Supercoiled plasmid DNA + topoisomerase I (1 unit) + compound **1** at various concentrations (**3**: 100 nM, **4**: 80 nM, **5**: 20 nM, **6**: 5 nM, **7**: 4 nM, **8**: 3 nM, **9**: 2 nM, **10**: 1 nM). (**11**). Supercoiled plasmid DNA + topoisomerase I (1 unit) + camptothecin (10 μM). (**12**). Supercoiled plasmid DNA + topoisomerase I + dimethyl sulfoxide (DMSO) (3%).

Table 1. Inhibition of Jurkat, HL-60, K562, U937, A2780cis, A2780, and HEK293 Cell Viability by the Complexes $Cu(bpy)_2(5,9\text{-eicd})$ (1) and $Cu(bpy)_2(arach)$ (2), CC_{50} (μM) ± SE (μM)

	Jurkat	HL-60	K562	U937
CC_{50} (1)	0.05 ± 0.001	0.04 ± 0.001	0.10 ± 0.007	0.08 ± 0.006
CC_{50} (2)	11.26 ± 1.09	9.38 ± 0.91	15.28 ± 1.43	13.17 ± 1.29
CC_{50} (cisplatin)	0.12 ± 0.004	0.10 ± 0.003	0.21 ± 0.006	0.18 ± 0.006
	HEK293	A2780cis	A2780	Fibrobl.
CC_{50} (1)	0.22 ± 0.008	0.47 ± 0.006	0.23 ± 0.004	1.07 ± 0.018
CC_{50} (2)	18.47 ± 1.82	26.89 ± 2.14	22.11 ± 2.24	76.32 ± 2.44
CC_{50} (cisplatin)	0.32 ± 0.015	0.94 ± 0.028	0.35 ± 0.006	2.26 ± 0.029

We studied the ability of the compounds $Cu(bpy)_2(5,9\text{-}$ eicd) (bpy: 2,2′-bipyridyl, 5,9-eicd: 5Z,9Z-eicosadienoic acid) 1 and $Cu(bpy)_2(arach)$ (bpy: 2,2′-bipyridyl, arach: arachidic acid) 2 synthesized in this work to inhibit topoisomerase I.

The results presented in Figure 2A,B indicate that the relaxation of supercoiled plasmid DNA with inhibition of topoisomerase I (Topogen) by the complex Cu(bpy)2(5,9- eicd) (in this case, 3 enzyme units of topoisomerase I are inhibited by 2 nM the test compound) results in decrease in the residual amount of the supercoiled plasmid DNA and increase in the number of formed topoisomers (lanes 2−10) as the concentration of the test compound is increased from 1 to 100 nM. When the compound concentration is in the range from 1 to 20 nM, the supercoiled plasmid is predominantly accumulated upon addition of ethidium bromide to the gel (Figure 1B, samples 8−12), while at concentrations above 20 nM (Figure 2B, samples 3−5), the level of the open circular form increases. These results lead to the conclusion that Cu(bpy)2(5,9-eicd) suppresses the topo I catalytic activity; however, its action is dose-dependent, and in concentrations of 20 nM and higher, it can also influence the formation of covalent complexes of DNA with topo I. Compound 2 did not show an inhibitory effect on the enzyme topoisomerase I. Thus, the synthesized copper complex has more than 10 times higher human topoisomerase inhibitory activity than the initial unsaturated acid.

The quantitative and qualitative analyses of cell viability, cell cycle, and apoptosis-inducing activity of $Cu(bpy)_2(5,9\text{-eicd})$ (bpy is 2,2′-bipyridyl, 5,9-eicd is 5Z,9Z-eicosadienoic acid) were performed by means of Guava Nexin Reagent, Guava Cell Cycle, and Guava ViaCount kits (Millipore).

The cytotoxic activity of the complex $Cu(bpy)_2(5,9\text{-eicd})$ (bpy is 2,2′-bipyridyl, 5,9-eicd is 5Z,9Z-eicosadienoic acid) in vitro against the Jurkat, HL-60, K562, and U937 human leukemia cells and HEK293 kidney cancer cells was tested using the Guava ViaCount kit (Millipore) (Table 1).

The complex Cu(bpy)2(5,9-eicd) in concentrations from 0.05 to 0.22 μM exhibited a clearly pronounced cytotoxic effect against all types of cancer cells used in the tests. However, the highest CC50 value was found for the A2780cis cell line (0.47 μM), whereas the CC50 values for Jurkat, K562, HL-60, and U937 cells were 0.05, 0.1, and 0.08 μM, respectively. It is noteworthy that the arachidic acid-based copper complex 2 and cisplatin taken as reference compounds showed substantially lower antitumor activities in vitro than complex 1 (Table 1).

It should be noted that the synthesized compounds exhibit good selectivity index (SI) (SI = CC50 fibroblasts/CC50 cancer cells); in particular for compound 1, the selectivity index varies from 2 to 18 with respect to all tumor cells.

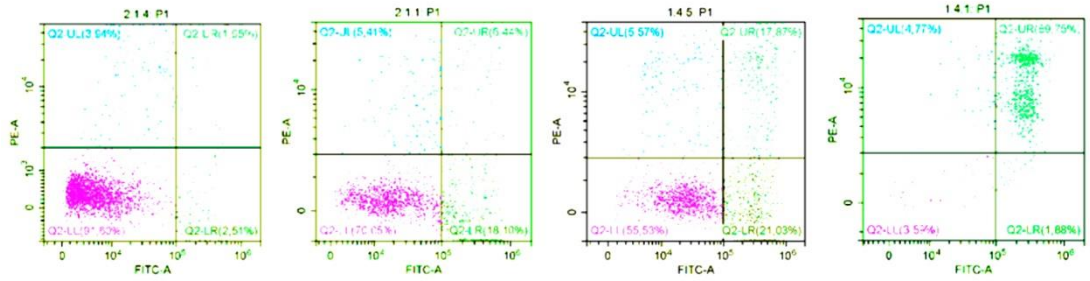

Figure 3. HEK293 cells treated with different concentrations of complex $Cu(bpy)_2(5,9\text{-eicd})$ were double-stained with annexine V/PI and analyzed by flow cytometry. (A) Control; (B) **1** (0.025 μM); (C) **1** (0.05 μM); and (D) **1** (0.1 μM).

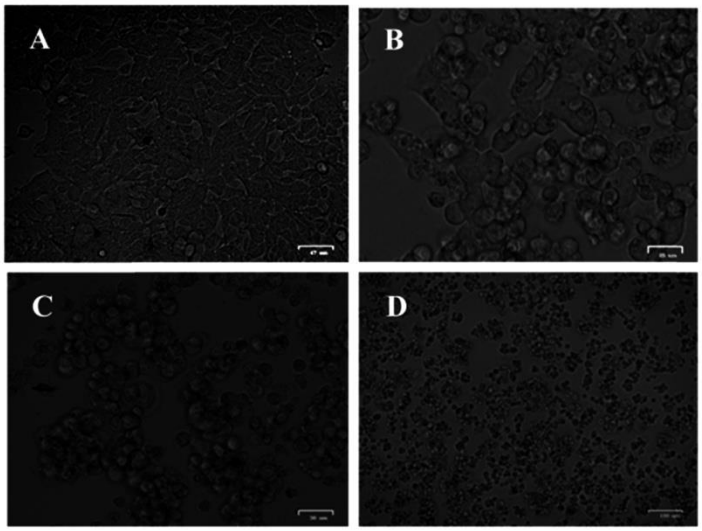

Figure 4. Conventional light microscopy of HEK293 cells treated with different concentrations of the test compound after 24 h of exposure. (A) Control; (B) **1** (0.025 μM); (C) **1** (0.05 μM); and (D) **1** (0.1 μM).

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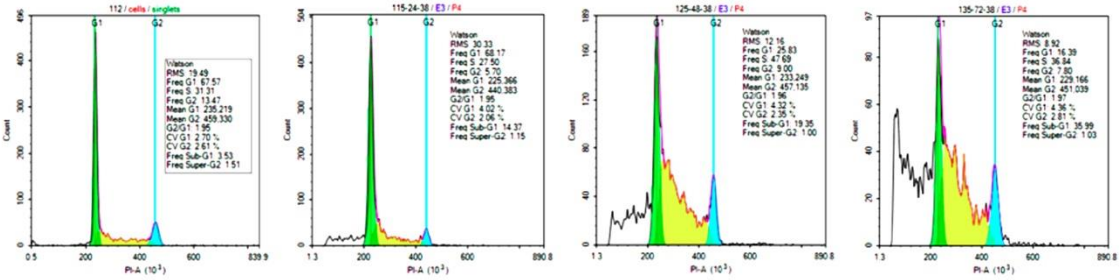

Figure 5. Results of cell-cycle analysis by flow cytometry and representative of the profiles of cell cycle distribution in three independent experiments is shown. Jurkat cells treated with complex $Cu(bpy)_2(5,9\text{-eicd})$ for (A) absence of complex, (B) 24 h, (C) 48 h, and (D) 72 h. The percentage of Jurkat cells after staining with propidium iodide. Data are presented as mean ± standard deviation of three independent experiments.

The Cu(bpy)2(5,9-eicd)-induced apoptosis in the Jurkat, HL-60, K562, U937, and HEK293 cell cultures was estimated by the detection of phosphatidylserine externalization on the plasmatic membrane after treatment of cell cultures with the test compound. It is worth noting that the effect of the complex Cu(bpy)2(5,9-eicd) on the induction of apoptosis in Jurkat cells is more pronounced than in other types of cells, which is in line with the higher cytotoxicity of this compound against this cell line. As can be seen from Figure 3, the action of Cu(bpy)2(5,9-eicd) on the HEK293 tumor cell culture induces a substantial dose-dependent increase in the

number of apoptotic cells occurring at early and late apoptosis stages The highest percentage of early and late apoptosis (~91%) was observed at a compound concentration of 0.1 μM. In particular, as shown in Figure 3, both early and late apoptosis stages for the Jurkat cells increase as compared with HL60 ($p \leq 0.0003$), U937 ($p \leq 0.0005$), HEK293 ($p \leq 0.0007$), and K562 ($p \leq 0.00019$).

Under conventional light microscopic examination, HEK293 cells treated with the complex $Cu(bpy)_2$(5,9-eicd) differed from the control untreated sample by the presence of clear-cut morphological changes. The complex $Cu(bpy)_2$(5,9-eicd) induced nuclear condensation and apoptotic body formation. These morphological changes, characteristic of early apoptosis, were visible as soon as 3 h after treatment. Figure 4 shows cells with different concentrations of the test compound after 24 h of exposure.

To find out whether the retarding effect exerted by complex $Cu(bpy)_2$(5,9-eicd) is caused by the cell cycle arrest, we studied the distribution of cell cycle phases for five cell lines, Jurkat, HL60, K562, and U937, and the adhesion cell culture HEK293 after the appropriate treatment with the test compound by flow cytometry. According to cell cycle studies by the Guava Cell Cycle Reagent, the complex $Cu(bpy)_2$(5,9- eicd) proved to be a potent inducer of hypodiploid cell population (sub-G1 phase) in all five cell lines. As shown in Figure 5, incubation of Jurkat cells for 24, 48, or 72 h after treatment with the complex resulted in 14.37 ± 1.02, 19.35 ± 1.33, or 35.99 ± 2.38% of hypodiploid cells, respectively. The percentage of cells in the G1 phase decreased compared with the control from 67.57 to 16.39%, whereas the percentage of cells in the S-phase substantially increased (from 27.50 to 47.69%) in the samples incubated for 72 and 48 h. These results indicate that the complex $Cu(bpy)_2$(5,9-eicd) arrests the cell cycle in the S phase depending on the time of incubation.

It was shown that the complex $Cu(bpy)_2$(5,9-eicd) is absorbed rather rapidly by cancer cells and induces apoptosis phenomena as soon as after a 3 h incubation. The presence of the 5Z,9Z-eicosadienoic acid moiety in this complex markedly increases the stability and lipophilicity of the molecule, which may be significant for penetration through the tumor cell membrane. The complex $Cu(bpy)_2$(5,9-eicd) showed an exceptionally high cytotoxicity on a panel of five tumor cell lines, the IC 50 values being in the range from 0.05 to 0.22 μM; this is an order of magnitude lower than this value for cisplatin.

It was found that compound 1 significantly reduces the proliferative activity and viability of cisplatin-resistant A2780cis cells, as shown in a study of the cytotoxicity of the compounds under study (Table 1). According to real-time cell analysis (RTCA), $Cu(bpy)_2$(5,9-eicd) is a substance that exhibits a strong inhibitory effect even at the lowest concentration on A2870cis-resistant ovarian cancer cells on cisplatin cells (Figure 6). At the same time, cisplatin itself, added at a concentration of 1 μM (CC_{50} = 0.94 ± 0.028 for A2780cis cells), also slightly reduces the proliferative ability of the A2870cis line in the first two days; however, by the third day, the cells regain their ability to grow, which is confirmed by an increase in cell index (CI).

Thus, it can be argued that the introduction of 1 into the culture medium reduced the proliferation and viability of cisplatin-resistant ovarian cancer for four days, indicating a potential antiproliferative effect of 1 for cisplatin-resistant ovarian cancer.

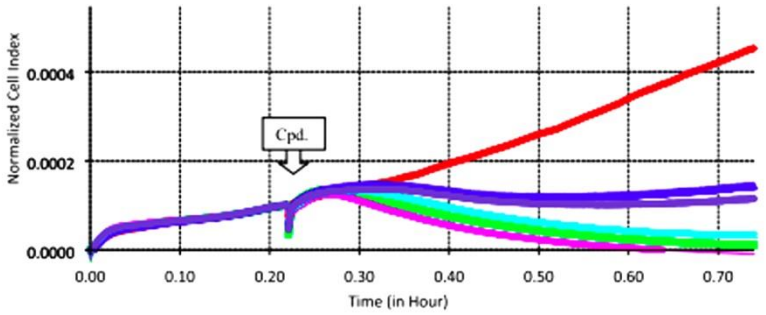

Figure 6. Effect of different sample concentrations of cisplatin and $Cu(bpy)_2(5,9\text{-eicd})$ on real-time cell analyzer curves xCELLigence RTCA system generated with A2870cis cells. Effect of cisplatin 0.5 μM/mL (blue line), cisplatin 1.0 μM (gray line), $Cu(bpy)_2(5,9\text{-eicd})$ 0.3 μM (turquoise line), $Cu(bpy)_2(5,9\text{-eicd})$ 0.5 μM (green line), $Cu(bpy)_2(5,9\text{-eicd})$ 1.0 μM (purple line), and control (red line, control group: untreated cells).

The ability of the complex 1 to inhibit topoisomerase I results in disruption of DNA synthesis, inhibition of transcription, and initiation of apoptosis by the extrinsic pathway. These results shed light on the key molecular mechanisms that underlie the anticancer activity of $Cu(bpy)_2(5,9\text{-eicd})$ and are important for the development of DNA-specific copper complexes as antitumor drug candidates.

CONCLUSIONS :

Thus, the newly synthesized copper complex $(bipy)_2Cu(5,9\text{- eicd})$ based on 5Z,9Z-eicosadienoic acid and 2,2′-bipyridine shows a high antitumor activity in vitro toward Hek293, K562, HL60, Jurkat, and U937 cell lines, being more efficient than the known anticancer drug cisplatin. Furthermore, $(bipy)_2Cu\text{- }(5,9\text{-eicd})$ was found to arrest the cell cycle in the S phase, depending on the incubation time, and to act as an efficient human topoisomerase I inhibitor, which can help to make assumptions on the possible mechanism of its antitumor activity.

REFERENCES :

1. Rosenberg, B.; VanCamp, L.; Trosko, J. E.; Mansour, V. H. Platinum Compounds: a New Class of Potent Antitumour Agents. Nature 1969, 222, 385−386.
2. Fuertes, M. A.; Alonso, C.; Perez, J. M. Biochemical Modulation of Cisplatin Mechanisms of Action: Enhancement of Antitumor Activity and Circumvention of Drug Resistance. Chem. Rev. 2003, 103, 645−662.
3. Rabik, C. A.; Dolan, M. E. Molecular Mechanisms of Resistance and Toxicity Associated with Platinating Agents. Cancer Treat. Rev. 2007, 33, 9−23.
4. Hartinger, C. G.; Nazarov, A. A.; Ashraf, S. M.; Dyson, P. J.; Keppler, B. K. Carbohydrate-metal complexes and their potential as anticancer agents. Curr. Med. Chem. 2008, 15, 2574−2591.

5. Ramakrishnan, S.; Rajendiran, V.; Palaniandavar, M.; Periasamy, V. S.; Srinag, B. S.; Krishnamurthy, H.; Akbarsha, M. A. Induction of cell death by ternary copper(II) complexes of L-tyrosine and diimines: role of coligands on DNA binding and cleavage and anticancer activity. Inorg. Chem. 2009, 48, 1309−1322.

6. Paterson, B. M.; Donnelly, P. S. Copper complexes of bis(thiosemicarbazones): from chemotherapeutics to diagnostic and therapeutic radiopharmaceuticals. Chem. Soc. Rev. 2011, 40, 3005− 3018.

7. Bica, L.; Meyerowitz, J.; Parker, S. J.; Caragounis, A.; Du, T.; Paterson, B. M.; Barnham, K. J.; Crouch, P. J.; White, A. R.; Donnelly, P. S. Cell cycle arrest in cultured neuroblastoma cells exposed to a bis(thiosemicarbazonato) metal complex. Biometals 2011, 24, 117− 133.

8. Chan-Stier, C. H.; Minkel, D.; Petering, D. H. Reactions of bis(thiosemicarbazonato) copper(II) complexes with tumor cells and mitochondria. Bioinorg. Chem. 1976, 6, 203−217.

9. Nemoto, T.; Yoshino, G.; Ojika, M.; Sakagami, Y. Amphimic acids and related long-chain fatty acids as DNA topoisomerase I inhibitors from an Australian sponge, Amphimedon sp.: Isolation, structure, synthesis, and biological evaluation. Tetrahedron 1997, 53, 16699−16710.

10. Du, M.; Cai, H.; Zhao, X.-J. A Two-Dimensional Hydrogen-Bonding Supramolecular Architecture of bis-(2,2′-bipyridine)[(p-phenyl-enedi-oxy)-diacetato]-copper(II) Tetrahydrate. Acta Crystallogr., Sect. E: Struct. Rep. Online 2004, 60, m1139− m1141.

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