Copper complexes: cytotoxicity and transport possibilities

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A copper complex with *N*-(5,6-dihydro-4*H*-1,3-thiazin-2-yl)benzamide was synthesized for the first time and characterized by ^{1}H NMR spectroscopy, LETDY mass spectrometry, and elemental analysis. A comparative study of the obtained complex and copper 2-aminopyrimidine complex was performed by flow cytometry on normal lymphocytes and *Jurkat* cells. Complexes with radionuclides ^{64,67}Cu (with and wihout a carrier) were prepared, and distribution of the labeled complexes in the organs of mice was considered. Packing in carboxymethylcellulose-based microgels was proposed as a transport form for the complexes. The results show a promise of the obtained materials as potential radiopharmaceutical agents.

Key words: copper *N*-(5,6-dihydro-4*H*-1,3-thiazin-2-yl)benzamide complex, copper 2-aminopyrimidine complex, flow cytometry, mouse model, *Jurkat* cell line, carboxymethylcellulose.

A large number of organic copper complexes have been reported in the literature, some of them being considered as potential pharmaceutical agents. The mechanism of action of such complexes on the cells is not always clear. Some copper complexes influence the cell division mechanism of cancer cells. Other copper compounds bind to DNA and trigger the mechanism of redox processes, resulting in single- and even double-strand breaks. These cells cannot pass the G2/M phase border and proceed to early apoptosis.[1,2] Copper thiosemicarbazonate complexes inhibit DNA replication and also promote genotoxic effects.[3] Many copper complexes exhibit antibacterial activity, in particular, complexes with ciprofloxacin[4] and with various Schiff bases.[5] Thiopurines inhibit the DNA and RNA synthesis and are used for the treatment of acute leukemias.[6] Numerous complexes with aminopyrimidine,[7] thiazine,[8] thiazole,[9] and thiourea[10] derivatives exhibiting various biological, including anticancer, activities have been synthesized. A special place in the studies belongs to ligands for copper radionuclides. The ^{64}Cu β^+-emitter is used for positron emission tomography (PET). Complexes of ^{64}Cu with 2-nitroimidazole, metronidazole, and diacetyl-bis (*N*4-methylsemicarbazone) ([^{64}Cu-ATSM]) are known;[11] lately, complexes with cyclam- and cyclen-based macrocyclic chelators have become popular,[12—14] because of their kinetic stability. These macrocycles are conjugated with monoclonal antibodies, for example, ^{64}Cu-SurAr-mAb {1-*N*-(4-aminobenzyl)-3,6,10,13,16,19-hexaazabicyclo[6.6.6]eicosane-1,8-diamine) anti-GD2 monoclonal antibody}. The ^{67}Cu isotope is used in the therapy of neuroblastoma; the ChCE7 antibodies with the bifunctional 4-(1,4,8,11-tetraazacyclotetradec-1-yl)methylbenzoic acid tetrahydrochloride ligand serve as the chelators.[15] Both Cu^{II} and Cu^{I} complexes are used. Their structures are highly diverse, including frameworks (*e.g.*, with aminopyrimidines[16]) and stair-step structures, *e.g.*, Cu^{I} complexes with thioureas[10] (Fig. 1).

Fig. 1

In this study, copper radionuclides were obtained and isolated by a simple method; their complexes with 2-aminopyrimidine (AP) and thiazine derivative (L) were prepared and studied for biochemical performance *in vitro* and *in vivo*, in particular, using microgels based on carboxymethylcellulose as transport forms of the complexes. The agents are designed both with a non-radioactive carrier and without a carrier.

Experimental

Materials. Commercial 2-aminopyrimidine (AP), $CuCl_2$, HCl, sodium carboxymethylcellulose (CMC) with a molecular mass of 90000 Da (Sigma, US), and Cu-resin sorbent (Triskem, France) were used.

^{1}H NMR spectra were recorded on a Bruker CXP-200 instrument (Germany); the chemical shifts are given in the δ-scale and referred to Me_4Si.

Isolation of copper radionuclides. A zinc target irradiated with bremsstrahlung photons with E = 55 MeV on an electron cyclotron was dissolved in 36% HCl and subjected to separation by ion exchange chromatography on a column with a Cu-resin sorbent (3 mL), in which copper was sorbed at pH 2.4 and zinc was washed out of the column. After zinc elution, copper desorption was carried out with 5 *M* HCl. The result is presented in Fig. 2. Solutions containing radionuclides ^{64}Cu + ^{67}Cu ($T_{1/2}$ = 12.7 and 61.8 h, respectively) were used; this was monitored on a gamma-ray spectrometer with a Canberra GC

3020 semiconductor detector (US). The characteristics of copper radionuclides are summarized in Table 1.

Fig. 2

Table 1

The $(AP)_2CuCl_4$ complex (yellow-colored) was synthesized by a previously reported method.[17] The results of elemental analysis and ^1H NMR spectroscopy are consistent with literature data.[17]

Copper complexes with *N*-(5,6-dihydro-4*H*-1,3-thiazin-2-yl)benzamide (L). The $LCuCl_2$ complex was prepared in the following way: ligand L (0.14 g, 0.6 mmol) was dissolved in a 2 : 1 ethyl alcohol (95%)—isopropyl alcohol mixture (12 mL) and then $CuCl_2 \cdot 2H_2O$ (0.9 g, 0.5 mmol) was added with stirring. The precipitate that formed was collected on a filter to give 0.12 g (67%) of a bright-green compound. Found (%): C, 37.50; H, 3.58; N, 7.89. $C_{11}H_{12}Cl_2CuN_2OS$. Calculated (%): C, 37.24; H, 3.41; N, 7.90. ^1H NMR (200 MHz, DMSO-d_6), δ: 1.7—2.6 (m, 2 H, $SCH_2CH_2CH_2N$); 3.2—3.6 (m, 2 H, $SCH_2CH_2CH_2N$); 3.7—4.1 (m, 2 H, $SCH_2CH_2CH_2N$); 7.3—7.6 (m, 3 H, H_{arom}); 7.8—8.3 (m, 2 H, $H_{аром}$); 10.8—11.2 (br.s, 1 H, NH).

The L_2CuCl_2 complex was prepared in a similar way by adding a solution of $CuCl_2$ $2H_2O$ (0.9 g, 0.5 mmol) in 95% ethyl alcohol (1 mL) to a solution of ligand L (0.24 g, 1.1 mmol) in 95% ethyl alcohol (20 mL). This gave 0.06 g (21%) of a bright-green crystalline product. Found (%): C, 45.85; H, 4.91; N, 9.70. $C_{22}H_{24}Cl_2CuN_4O_2S_2$. Calculated (%): C, 45.95; H, 4.21; N, 9.74. ^1H NMR (200 MHz, DMSO-d_6), δ: 1.9—2.3 (m, 2 H, $SCH_2CH_2CH_2N$); 3.2—3.5 (m, 2 H, $SCH_2CH_2CH_2N$); 3.7—4.0 (m, 2 H, $SCH_2CH_2CH_2N$); 7.4—7.7 (m, 3 H, H_{arom}); 7.9—8.2 (m, 2 H, H_{arom}); 10.3—11.3 (br.s, 1 H, NH). Acording to ^1H NMR spectra, the ligand did not decompose during the synthesis and isolation of the complexes.

The polymeric microgels (PMGs) based on carboxymethylcellulose (CMC) containing AP and Cu^{II} were prepared using a method reported previously.[18] The PMGs with the Cu : AP : CMC ratio of 1 : 2 : 7 (**1**) and 1 : 2 : 15 (**2**) were used.

Laser-induced electron transfer desorption/ionization (LETDI) mass spectrometry. The scheme of the mass spectrometric setup was described in detail in a previous publication.[19] The time-of-flight mass spectrometer was assembled as a linear circuit with a free path of 0.65 m and accelerating gap of 14 mm. A laser system based on a diode-pumped Nd:YAG-laser (RL-02.355, manufactured by ELS-94, Russia) operating at a repetition frequency of 300 Hz, with pulse duration of 0.37 ns and the maximum energy per pulse of 30 mkJ, was used as the radiation source. Silicon substrates for ion emitters were used; the substrates were pretreated with a 5% solution of HF, washed with distilled water, and placed into the ion source.

Experiments *in vivo*. Normal male laboratory white mice weighing 28—33 g were used. Prior to experiments, the mice were kept under standard conditions on a 12-hour cycle. The $^{64,67}Cu$-labeled $(AP)_2CuCl_4$ (**3**) and L_2CuCl_2 (**4**) agents were prepared by isotope exchange (30 min) in 36% HCl or (without a carrier) by direct synthesis with the radionuclide in 36% HCl (by reported procedures) followed by evaporation in a crucible on a water bath. The agents (100 μL) were administered intraperitoneally; the mice were sacrificed by cervical dislocation. The activities of radionuclides are summarized in Table 2.

Table 2

Flow cytometry. Prior to the experiment, the cells were washed twice with a cold phosphate-buffered saline (PBS) and the cell concentration was brought to $1 \cdot 10^6$ cells mL^{-1} in the binding buffer. The measurements were performed using standard FITC Annexin V Apoptosis Detection Kit I (Becton Dickinson, US, see Ref. 20) and a FACScan flow cytometer (Becton Dickinson, US) with fluorescence excitation by an argon laser (λ = 488 nm).

The assays were conducted on normal lymphocytes and on *Jurkat* leukemia cells. The statistical treatment of the results was done using the Statistica 5.11 software.

MTT assay. The method is based on the ability of dehydrogenases present in living cells to convert 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) to insoluble formazan crystals. After extraction into isopropyl alcohol, the produced formazan was determined by spectrometry using a Microplate Reader 550 (Bio´Rad) at λ = 550 nm. The method was described in detail previously.[21,22] Normal lymphocytes (mononuclear cells) and *Jurkat* cells were used. At least five experiments were carried out for each cell line. The results were treated according to the Mann—Whitney U-test ($p < 0.05$).

Results and Discussion

The radionuclide ^{64}Cu is a β^+-emitter, *i.e.*, it can be used in PET diagnosis. The radionuclide ^{67}Cu is a β^--emitter and is of interest as a therapeutic agent (see Table 1). Thus, simultaneous isolation and introduction into pharmaceuticals of both isotopes may be useful for theranostics if an appropriate chelator or transporter for delivery into tumor tissue is present. The procedure that we propose for isolation of copper radionuclides is simple and fast. The ligand L was described previously as an antitumor, antishock, and radioprotective agent.[23—26] In addition, it is a potent inhibitor of inducible NO synthase (iNOS), which is overexpressed in some tumors.[27] As discussed earlier,[23] compounds of thiazine-thiazoline and thiourea classes, most of which are also radioprotectors with different dose modifying factor (DMF) and NOS-inhibitory activity characteristics, may be useful, in some cases, in combined therapy comprising radiotherapy and chemotherapy.

The positive ion mass spectrum of the newly synthesized complex **4** recorded with droplet deposition from a methanol solution onto a silicon substrate (Fig. 3) exhibits two major groups of peaks. One group corresponds to ions of the protonated ligand (L) molecules and the other belongs to the complex molecular ions with the ratio Cu : L = 1 : 2. The most intense peaks in these groups have *m*/*z* 221 and 501, respectively.

Fig. 3

We obtained for the first time copper radionuclide-labeled complexes **3** and **4** and characterized their distribution in the organs of mice under different conditions of exposure (Tables 3 and 4). It can be seen that the distribution times of complexes **3** and **4** in organs are different and also differ from that of $CuCl_2$, which indicates the presence of physiological transporters in the body. The absence of a carrier (in the case of **3**) leads to more rapid clearance of the agent. In the case of **4** with a carrier, no difference in the distribution was observed

between 30 and 60 min exposure times in mice, like in the case of the control solution of $CuCl_2$.

Table 3

Table 4

The specific activity of complex **4** in brain, lungs, and heart is too low to consider the differences. However, while examining the liver to kidneys activity ratio, one can see that this value increases with increasing exposure time in mice. Comparison of the values shows a longer clearance for complex **4**. Analysis of these data suggests the presence of a biological target in liver.

Flow cytometry (table. 5) gives an idea of the mechanism of action of the agents. Without the agents, small numbers of both normal and leukemic cells die showing late apoptosis (Fig. 4). The PMG-based agents increase the percentage of normal cells showing early apoptosis, especially in the case of compound **1** (Fig. 5), which has a higher percentage of copper atoms. Enhanced early apoptosis is also observed for leukemia cells; however, in this case, on the contrary, a higher percentage of apoptosis occurs for complex **2** (Fig. 6). Evidently, this is attributable to pronounced uptake of particles with high CMC content by leukemia cells. Generally, inclusion of copper into the agent can considerably promote apoptotic events; for example, complex **3** has a 5 times higher percentage of apoptosis (Fig. 7) than the ligand it contains (see Fig. 4).

Table 5

Figs 4—7

The results demonstrate a high promise for copper complexes, especially **4**, for combined (radio and chemo) treatment of tumor cells, the transport to which may be manly due to the high uptake of the CMC shell by particularly cancer cells.

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Graphical Abstract

Fig. 7 с подписью

Flow cytometry method: early and late apoptosis of *Jurkat* cell line under the action of complex $(AP)_2CuCl_4$ (AP is 2-aminopyrimidine); (*a*) cell gate for forward (FSC-H) and side (SSC-H) light scatter plots under the action of $(AP)_2CuCl_4$, P1 is selected cell gate; (*b*) P2 are early apoptotic cells; (*c*) P3 are late apoptotic cells.

Running title

Copper complexes labeled by radionuclide

Figure captions and Tables

Fig. 1. Conventional stair-step chains in the $Cu^{I}I$ complex with *N*-(pyrimidin-2-yl)benzo[*d*]thiazole-2-amine ($(CuI)_2L'$). The copper atom with the coordination number of four forms a network *via* lateral bridging bonds (taken from Ref. 10); L´ is ligand.
Простая лестничная цепь Conventional stair-step chain
Fig. 2. Chromatogram for the isolation of copper from irradiated target on the Cu-resin sorbent.
Бк Bq
мл mL
Fig. 3. LETDI mass spectrum of complex **4** recorded in the positive ion mode.
Ионный сигнал (отн.ед.) Ion signal (rel.u.)
Fig. 4. Flow cytometry study of early and late apoptosis of normal lymphocytes (*a*—*c*) and *Jurkat* cell line (*d*—*f*) induced by AP: (*a, d*) cell gate for forward (FSC-H) and side (SSC-H) light scatter plots under the action of AP, P1 is the selected cell gate; (*b, e*) P2 are early apoptotic cells, I_{an} is fluorescence intensity upon the addition of FITC annexin; (*c, f*) P3 are late apoptotic cells, $I_{PI\text{-}PE}$ is fluorescence intensity upon the addition of PI-PE. Here and in Figs 5—7, *N* is the number of cells.
(отн.ед.) (rel.u.)
Fig. 5. Flow cytometry study of early and late apoptosis of normal lymphocytes (*a*—*c*) and *Jurkat* cell line (*d*—*f*) induced by complex **1**: (*a, d*) cell gate for forward (FSC-H) and side (SSC-H) light scatter plots under the action of complex **1**, P1 is the selected cell gate; (*b, e*) P2 are early apoptotic cells; (*c, f*) P3 are late apoptotic cells.
(отн.ед.) (rel.u.)
Fig. 6. Flow cytometry study of early and late apoptosis of normal lymphocytes (*a*—*c*) and *Jurkat* cell line (*d*—*f*) induced by complex **2**: (*a, d*) cell gate for forward (FSC-H) and side (SSC-H) light scatter plots under the action of complex **2**, P1 is the selected cell gate; (*b, e*) P2 are early apoptotic cells; (*c, f*) P3 are late apoptotic cells.
(отн.ед.) (rel.u.)
Fig. 7. Flow cytometry study of early and late apoptosis of *Jurkat* cell line induced by complex **3**: (*a*) cell gate for forward (FSC-H) and side (SSC-H) light scattering induced by complex **3**, P1 is the selected cell gate; (*b*) P2 are early apoptotic cells; (*c*) P3 are late apoptotic cells.
(отн.ед.) (rel.u.)

Table 1. Characteristics of copper radionuclides
Радионуклид Radionuclide
$T_{1/2}$/ч $T_{1/2}$/p
Основные пути образования Principal formation routes
Порог реакции/МэВ Reaction threshold (MeV)
Выход на EOB* /Бк • мкА$^{-1}$ • ч$^{-1}$/(г • см$^{-2}$) Yield at EOB* /Bq μA^{-1} h^{-1}/(g cm^{-2})
* EOB is end of bombardment.
Table 2. Activities of ^{64,67}Cu introduced into mice in *in vivo* experiments
Препарат Agent
Введенная радиоактивность /Бк Introduced radioactivity/Bq
Экспозиция мышей после введения препарата/мин Mice exposure after administration of the compound/min
Изотопный обмен 30 мин Isotope exchange for 30 min
без носителя without a carrier
Table 3. Distribution of specific radioactivity of complex **3** in the liver and kidneys of mice
Время экспозиции изотопного препарата/мин Exposure time of the isotope compound/min
Печень Liver
Почки Kidneys
Печень : почки Liver : kidneys
Table 4. Specific radioactivity (A_{sp}) of complex **4** and $CuCl_2$ in the organs of mice
Препарат

Время экспозиции препарата без носителя в мыши/мин — Exposure time of the agent without a carrier in mice /min

Бк • ч$^{-1}$	Bq h^{-1}
Мозг	Brain
Сердце	Heart
Легкие	Lungs
Печень	Liver
Почки	Kidneys
Печень : почки	Liver : kidneys

Table 5. Cytotoxicity (LC_{50}) and quantitative data for separation of early and late apoptosis obtained by flow cytometry for normal lymphocytes (HD) and *Jurkat* leukemic cells treated with the agents

Клеточная линия	Cell line
Соединение	Compound
Число клеток	Number of cells
Апоптоз	Apoptosis
ранний	early
поздний	late
Без препарата	None