Cytotoxicity of copper(II) complexes of N-salicylidene-L-glutamate: modulation by ascorbic acid

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Cytotoxic/cytostatic activity of N-salicylidene-L-glutamato diaqua copper(II) complex (CuC) against mice leukemia cells L1210 has been estimated and their bioactivity was enhanced by addition of ascorbic acid. The Cu-complex with isoquinoline ligand (IQ-CuC) had stronger cytostatic effect (IC_{50} =15.6 μM) than parental complex (CuC) and its cytotoxicity several times increased in the presence of 0.1 mM ascorbic acid (IC_{50} =1.0 μM). The cytotoxicity has been caused by oxidative stress, enhanced creation of TBARS has been confirmed, and formation of 2',7'-dichlorofluorescein from 2',7'-dichlorodihydrofluorescein has been observed, also. Some hallmarks of apoptotic/necrotic death of L1210 cells have been observed by fluorescent microscopy after dyeing of cell with propidium iodide and Hoechst 33342. In addition, it was confirmed that both complexes in the presence of ascorbic acid cleavaged of pDNA. Although these copper complexes were initially prepared as substances with antioxidant properties we have showed that combined treatment of L1210 cells with IQ-CuC and ascorbic acid induced strong oxidative stress and death of cells. Our results confirmed that physiological concentration of ascorbic acid increases the cytostatic/cytotoxic efficiency of N-salicylidene-L-glutamato diaqua copper(II) complexes.

Key words: copper complex, ascorbate, leukemia, anticancer, oxidative stress

Some copper-based drugs have exhibited antineoplastic capacity and certain copper-complexes catalyze radical formation while others seem to have antioxidant efficacy, therefore there has been substantial interest in the synthesis of novel copper-based drugs [1–6]. Different behavior of Cu-complexes depends upon the chemical environment and the nature of the chelating agent [7–9]. Schiff-base copper(II) complexes derived from salicylaldehyde have been synthesized for their antimicrobial properties and some of them have antioxidative properties [10]. Valent et al. [11] prepared and characterized the product of reaction of N-salicylidene-L-glutamato diaqua copper(II) monohydrate (CuC, Fig. 1) with isoquinoline, and they showed that both, parental complex and a complex with isoquinoline ligand (IQ-CuC), have antimicrobial effects.

The antioxidant property of ascorbic acid is often considered responsible for its protective effect against certain types of cancer. However, this idea remained controversial. Earlier studies have established that several antioxidants are themselves capable of inducing oxidative DNA cleavage either alone or in the presence of transition metal ions specially copper [12]. It was also established that oxidant/antioxidant balance is important factor for initiation and progression of cancer [13]. The other way around, the antioxidant properties might be associated with therapeutic efficacy of any antineoplastic drugs [14, 15], and any cancer cells showed to be more sensitive than normal cells [16].

In the present paper we have focused our attention on cytotoxic/cytostatic activities of CuC and IQ-CuC against mice leukemia cells L1210. Copper complexes are redox active substances and they can have prooxidat properties in the presence of reductant therefore cytotoxicity of both complexes has been evaluated in the presence of ascorbic acid, also.

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Abbreviation: CuC – [Cu(N-salicylidene-L-glutamato)·2(H_2O)].H_2O, IQ-CuC- [Cu(N-salicylidene-L-glutamato) (isoquinoline)]·H_2O, H_2A- ascorbic acid.

Materials and methods

Reagents: The derivatives of copper complexes were prepared as desribed earlier [11]. Propidium iodide, Hoechst 33342, 2',7'-dichlorodihydrofluorescein, ethidium bromide, thiobarbituric acid, ascorbic acid, and Triton X-100 were purchased from Sigma-Aldrich Chemie (Germany). Other chemicals were purchased from Lachema (Czech Republic).

Methods

Cell culture: Murine leukemia cell line (L1210) was obtained from the American Type Culture Collection (Rockville, MD, USA). The cell line was kept in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (Grand Island Biological Co., Grand Island, NY, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2:95% air.

Determination of cell proliferation and cytotoxicity by dye exclusion test: Cell proliferation, growth curves and cytotoxic/cytostatic potential of the copper complex were determined by the trypan blue dye exclusion test. For the 3-day experiments, cells were seeded at 1 × 10^5 cells/ml in 60-mm Petri dishes and a treatment of cells with Cu-complexes and/or ascorbic acid has been done in 24 hours (during the exponential phase of cell growth), and viable and non-viable cells were counted daily using trypan blue exclusion. Growth curves (each point in the growth curves was obtained from tree repeated experiments) have been obtained and inhibition concentrations of Cu-complexes (IC_{50}) were determined.

Measurement of oxidative stress: The determination of intracellular oxidant production was based on the oxidation of 2',7'-dichlorodihydrofluorescein (H_2DCF) to a fluorescent 2',7'-dichlorofluorescein (DCF) [17]. Following treatment of L1210 with Cu-complexes and ascorbic acid, the medium was aspirated and cells were washed twice with PBS (PBS – phosphate-buffered saline, pH=7.2). H_2DCF was added at a final concentration of 10 μM and incubated for 5 min. The cells were then washed once with PBS and maintained in a 1 ml PBS. The fluorescence was monitored using a fluorescence microscope (JENA LUMAR, Carl Zeiss, Germany).

Microscopic analysis for dead cells: Cell death (apoptosis/necrosis) induced by copper complex without or in the presence of ascorbic acid was determined by fluorescent microscopy after staining with Hoechst 33342 and propidium iodide (PI). After treatment (24 h) the medium was removed and cells were washed twice with PBS and incubated with 10 μg/ml Hoechst 33342 for 15 min at 37 °C and with 10 μg/ml PI for 15 min at 37 °C. Dual-stained L1210 cells were examined using a fluorescent microscope (JENA LUMAR, Carl Zeiss, Germany). Photographic images (Olympus Camedia C-4000) were taken from four random fields. Viable cells take up green dye (Hoechst 33342) and red-stained cells (PI) were considered late apoptotic (condensed chromatin) or necrotic cells. Technique has been described in detail previously [18].

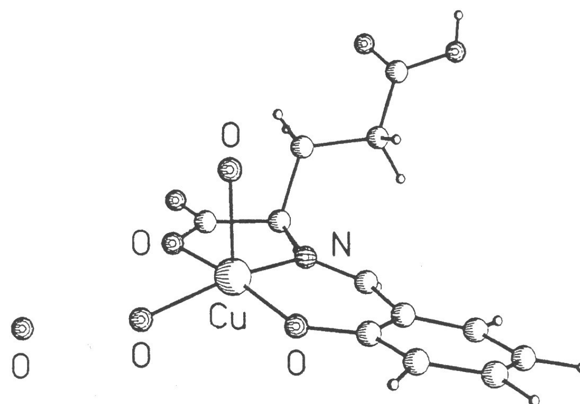


Figure 1. Structure of diaqua-(N-salicylidene-L-glutamato)copper(II) monohydrate, for clarity the hydrogen atoms of H_2O molecules are omitted [12].

Lipid peroxidation assays: The occurrence of free radical lipid peroxidation during incubation of cells was monitored by thiobarbituric acid assay [19]. Cells used for this measurement were grown for 1 h in the presence of copper complex and ascorbic acid. Cell suspensions were mixed with 140 μl of 30% (w/v) trichloroacetic acid and after centrifugation to remove precipitated proteins, a 500 μl aliquot of each supernatant fraction was mixed with 500 μl 2-thiobarbituric acid (TBA). The mixture was heated at 80°C for 20 min, cooled rapidly to room temperature, and absorbance at 532 nm was recorded, using a spectrophotometer (PU 8750 UV/Vis-spectrophotometer, Philips). Absorbance readings were converted to TBARS (thiobarbituric acid-reactive substances) values by using an extinction coefficient of 157 $mM^{-1}cm^{-1}$.

Plasmid DNA degradation: Typically incubations in PBS contained 500 ng of plasmid DNA (pGEM t-EASY DNA, 3.015 kb, Promega, U.S.A.), Cu-complexes at a final concentration of 10 μM or 100 μM and ascorbic acid at a final concentration 1 mM or 10 mM. The mixture was incubated at 37 °C for 1 h. Damage of plasmid DNA was determined by horizontal electrophoresis (0.8% agarose). DNA was post-stained with ethidium bromide for 15 min and visualized under UV light.

Statistical analysis: Data obtained were analyzed using descriptive statistics, single factor analysis of variance (ANOVA) and presented as mean value ± SD from the minimum of three independent measurements.

Results

Cytotoxicity of both substances has been evaluated by direct counting of viable cells (trypan blue exclusion assay) for 48-h and 72-h tretment of L1210 cells with CuC or IQ-CuC. The values of IC_{50} are given in the Table1. The Cu-complex with isoquinoline ligand was more effective cytostatic substance than parental complex. The cytotoxicity of both

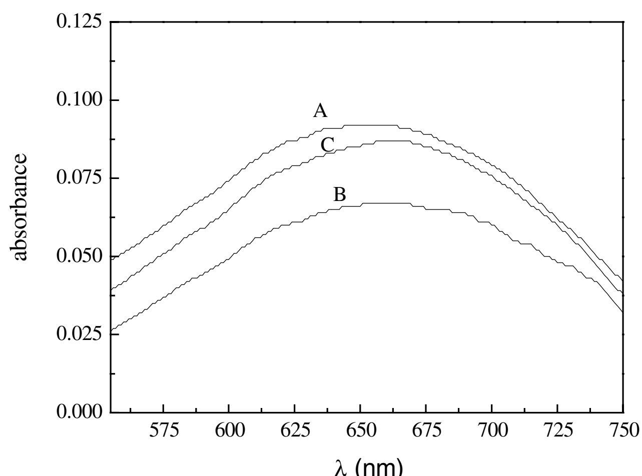


Figure 2. Visible spectral traces showing the conversion of 1 mM IQ-Cu(II)C (spectrum A) to IQ-Cu(I)C (spectrum B) on reduction with 10 mM ascorbic acid in DMSO/aqua=1/1, and the regeneration of the IQ-Cu(II)C after 5 min (spectrum C).

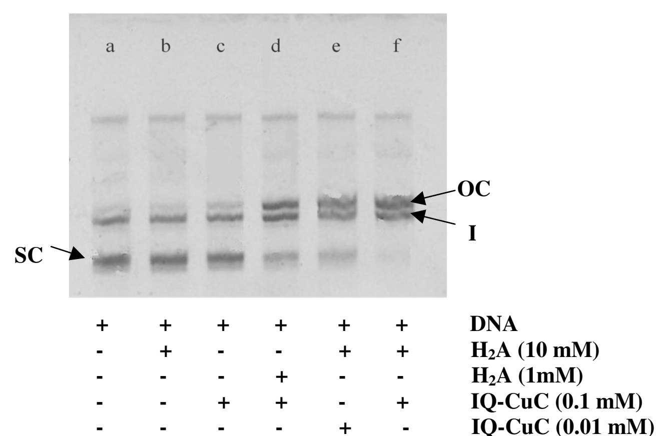
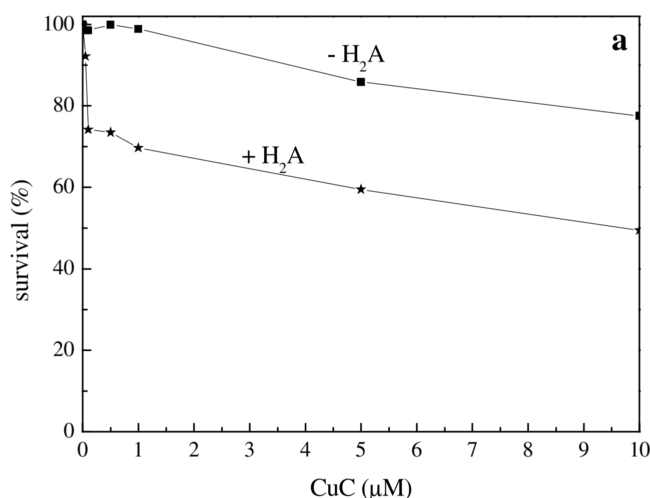


Figure 3. Cleavage of pDNA (0.5 μg) by IQ-CuC (0.1 or 0.01 mM) in the presence of ascorbic acid (1 or 10 mM H_2A) after 1-h incubation at 37 °C. Lane a, DNA control; Lane b, DNA+10 mM H_2A; Lane c, DNA+0.1 mM IQ-CuC; Lane d, DNA+1 mM H_2A+0.1 mM IQ-CuC; Lane e, DNA+10 mM H_2A+0.01 mM IQ-CuC; Lane f, DNA+10 mM H_2A+0.1 mM IQ-CuC. SC – supercoiled form, OC – open circular form, and L- linear DNA.

Cu-complexes could be enhanced by a suitable reductant, we have supposed that if the $Cu^{(2+)}$ ions in CuC or IQ-CuC are reduced by ascorbic acid than free radicals could be generated in reactions of $Cu^{(1+)}$ with oxygen, and these radicals could

Table 1. IC_{50} values estimated by direct counting of cells.

	IC_{50} (μM)	
	48 h	72 h
CuC	50.5±1.6	35.2±1.0
IQ-CuC	20.2±0.9	15.6±0.8



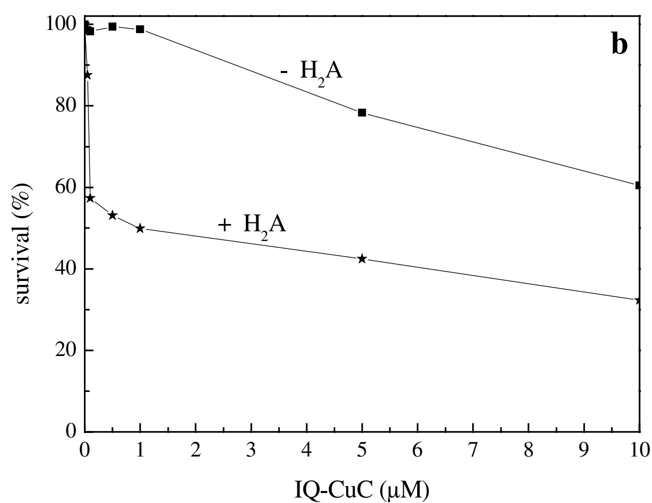


Figure 4. The effect of ascorbic acid (H_2A) on cytotoxicity of copper complexes CuC and IQ-CuC. L1210 cells were treated with CuC (0 – 10μM, Fig. 4a) or IQ-CuC (0 – 10μM, Fig 4b) together with/without H_2A (100 μM) for 72 h.

induce oxidative stress and consequently inhibition of cell proliferation or apoptosis/necrosis of cells.

At first, the reactions of the Cu-complexes with ascorbic acid were monitored by visible spectroscopy in aqua solution of DMSO at 25°C. As seen in Fig. 2, the IQ-CuC (spectrum A) reacted readily with ascorbic acid (H_2A) to form an unstable copper(I) species (spectrum B) which on exposure to air converted to the parent copper(II) complex (spectrum C).

To demostrate that ascorbic acid can increase oxidative stress induced by the copper complex the ability of the IQ-CuC, in the presence of ascorbic acid (H_2A), in effecting pDNA cleavage has been verified by gel electrophoresis using supercoiled pDNA in PBS (pH 7.2). Control experiments using only IQ-CuC or H_2A do not show any apparent cleavage of DNA (Fig. 3 lanes a-c). Both Cu-complexes in presence

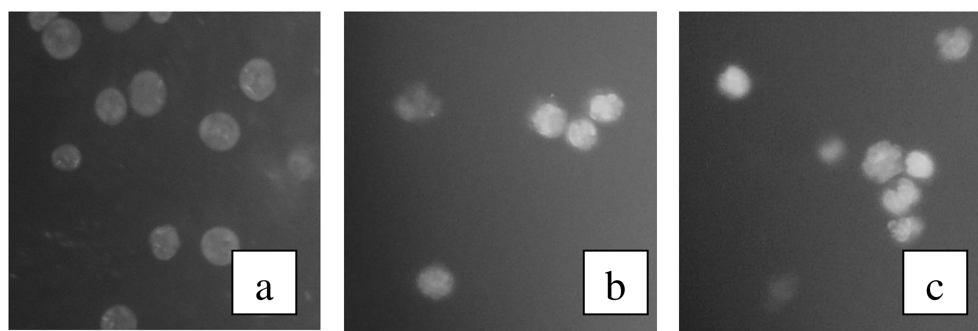


Figure 5. IQ-CuC induced apoptosis/necrosis in L1210 in the presence ascorbic acid. L1210 cells were stained with Hoechst 33342 (green) and PI (red) after 24- treatment: a – control, b -25 μM IQ-CuC, c – 2.5 μM IQ-CuC and 100 μM H_2A. Green stained cells have intact function of cell membrane. Cells with condensed nucleus are early apoptotic cells – bright green cells, and necrotic or late stage apoptotic cells – red or orange cells. Images are representative of three independent experiments.

of ascorbic acid showed pseudo-nuclease activity. The cleavage efficiency of IQ-CuC in the presence of ascorbic acid depends on ascorbic acid/Cu-complex molar ratio as shown in Fig. 3. The Cu-complex was able to convert supercoil (SC) to nicked open circular DNA (OC) and linear DNA (L) in the presence of ascorbic acid. If the molar ratio H_2A /IQ-CuC was 10:0.1 than almost 100% of the initial SC form was converted to OC and L form (lane f), whereas if the ratio H_2A / IQ-CuC was 10:0.01 (lane e) or 1:0.1 (lane d), only part of the SC was converted.

Combined action of Cu-complex (IQ-CuC) and ascorbic acid induced damage of pDNA and the aim of our further study was to elicit whether the addition of ascorbic acid into medium increase antiproliferative effect of these CuC and IQ-CuC complexes (Fig. 4). The cytotoxic effect of CuC (0 -10 μM) in the presence of 0.1 mM H_2A was tested, and as seen in Fig. 4a proliferation of cells decreased about 25% even in the presence 0.1 μM CuC. The effect of ascorbic acid on toxicity of IQ-CuC was even biger, co-treatment of cells with 0.1 μM IQ-CuC and 0.1 mM H_2A inflicted inhibition of cell proliferation about 42% (Fig 4b).

To estimate mode of death the L1210 cells were stained with Hoechst 33342 and propidium iodide after 24-h treatment of cells with copper complex in the presence of ascorbic acid. Dual-stained cells showed that condensed chromatin was formed after incubation of cells with 25 μM IQ-CuC, and if cells were co-treated with 2.5 μM Cu-complex and ascorbic acid (100 μM H_2A), late apoptotic (red) cells were observed also (Fig. 5). L1210 cells showed chromatin aggregation and cell membrane blebbing that could be ascribed to apoptosis.

We supposed that co-treatment of cells with IQ-CuC and ascorbic acid can induce oxidative stress therefore the dichlorofluorescein staining technique has been used for monitoring the intracellular oxidant production. This assay is based on the oxidation of H_2DCF by intracellular peroxide and other oxidants to a fluorescent compound, DCF. Microphotos (Fig. 6) indicate that there is an increase in the intensity of DCF staining in L1210 co-treated with 250 μM ascorbic acid and 100 μM IQ-CuC for 1 hour. This result was confirmed by estimation of TBARS in L1210 cells after short-treatment with IQ-CuC and H_2A (Fig. 7). The level of TBARS was increased only if co-teatment of cells had been applied. Pre-treatment of the cells with antioxidant vitamin E, significantly decreased the cytotoxic effect as compared with cells co-treated with IQ-CuC and H_2A (Fig. 8).

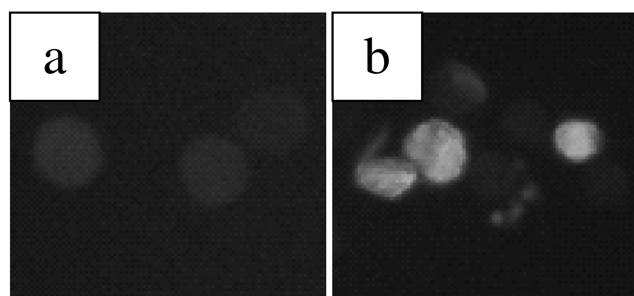


Figure 6. The combined effect of ascorbic acid and IQ-CuC on oxidative stress as measured by dichlorofluorescein staining. L1210 cells ($5x10^5$/ ml) were treated with 100 μM IQ-CuC (a) and with 250 μM ascorbic acid and 100 μM IQ-CuC (b) for 1 hour as indicated under "Experimental Procedures." Following 1 h incubation, the medium was aspirated and cells were washed twice with PBS and incubated with 10 μM H_2DCF for 5 min. The cells were then washed once with PBS and maintained in 1 ml of the culture medium. Microphoto (b) indicate that there is an increase in the intensity of DCF staining (see Results).

Discussion

The present study indicate that both N-salicylidene-L-glutamato diaqua copper(II) complexes have antiproliferative activity (Table 1). The Cu-complex with isoquinoline ligand has stronger cytostatic effect (IC_{50} =15.6±0.8 μM) than parental complex (IC_{50} =35.2±1.0 μM) however if CuC was applied together with isoquinoline (20 μM isoquinoline –

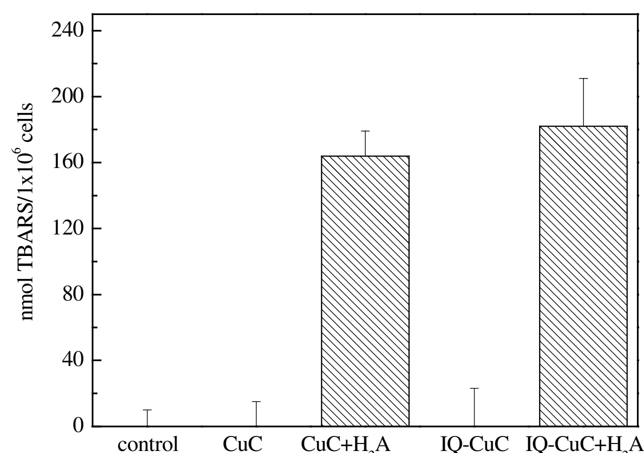


Figure 7. The effect of ascorbic acid and Cu-complexes on lipoperoxidation in L1210 cells expressed as TBARS production (nmol/10^6 cells). Cells were incubated with 50 μM Cu-complexes without or with 500 μM H_2A for 60 min at 37°C.

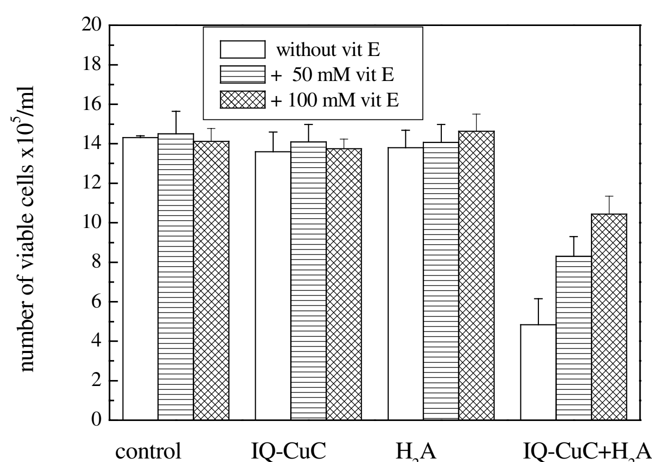


Figure 8. Effect of vitamin E on the cytotoxicity IQ-CuC in the presence of ascorbic acid (H_2A). L1210 cells were treateted (48 h) with IQ-CuC (2.5 μM) and ascorbic acid (0.25 mM) without or in the presence of vit E (vit E was added 1 h before treatment with IQ-CuC and ascorbic acid).

nontoxic concetration) its cytotoxicity was the same as toxicity of IQ-CuC (results are not shown). The *in vitro* cytotoxicity of both N-salicyliden-L-glutamate copper complexes is lower than toxicity of known copper-based drugs which are used in chemotherapy of cancer [20]. The most of copper complexes initiate damage of biomembrane and/or DNA and proteins by mechanisms that involve formation of free radicals [21, 22] but some of them can have SOD-like activity. We supposed that low cytotoxicity of both studied complexes could be influenced partially by their SOD-mimic properties because Cu-complexes derived from tridentate Schiff bases of N-salicylideneaminoalcanoate type have SOD-activity [23].

Ascorbic acid plays broad biological roles, one of which is to prevent cancer as antioxidant in spite of that in the presence of metals this vitamin has prooxidative effects [24, 25]. We have tried to increase the production of free radicals in L1210 cells and to inhibit their proliferation by co-treatment cells with ascorbic acid and CuC or IQ-CuC. The generation of hydroxyl radicals (•OH) from the reaction of Cu(II) complexes with biological reductants such as ascorbic acid or glutathione was confirmed by Ueda et al. [26]. We showed that in the presence of ascorbic acid CuC or IQ-CuC formed an unstable copper(I) species which on exposure to air were converted to the parent copper(II) complex (Fig. 2). It was hypothesized that highly reactive radicals can be formed by a mechanism analogous to the metal-driven Haber-Weiss reaction, or the Fenton reaction: Cu(II) in the complex is reduced by ascorbic acid, Cu(I) reduces O_2 and Cu(II) is regenerated [25, 27]. The superoxide radical, hydrogen peroxide, the ascorbyl radical, and the hydroxyl radical are generated in these reactions. Although the creation of free radicals has not been prooving by direct methods, oxidative DNA-strand scission (Fig. 3) has been confirmed. DNA cleavage by Cu-complex (bis-phenanthroline copper complex) was first reported by Sigman in 1979 [28], and recently Rivero-Muller et al. [29] confirmed that copper based anticancer drugs, the casiopeinas, degraded DNA and RNA in the presence of ascorbic acid and that ROS were the major cause of the high cytotoxicity. We used low concentration (100 μM) of ascorbic acid because already Wagner et al. [30] had showed that physiological amounts of ascorbic acid in the presence of Fe^{+3} are able to generate free radical in L1210 cells and to induce cell death. Mode of cell death can be modulated by extent of oxidative damage and our results confirmed that physiological concentration of ascorbic acid changes the cytostatic/cytotoxic efficiency of N-salicylidene-L-glutamatodiaqua copper(II) complexes. If 100 μM ascorbic acid was added to L1210 cells than strong cytostatic effect of CuC or IQ-CuC was achieved even at their nontoxic concentration.

Combination of these complexes with ascorbic acid induced oxidative stress, and an increasing level of free radicals already after short-treatment of L1210 cells with ascorbic acid and Cu-complexes has been proven. Oxidative stress can induce apoptosis and we observed some hallmarks of apoptosis of L1210 cells: chromatin aggregation and cell membrane blebbing have been confirmed after co-treatment of cells with IQ-CuC and ascorbic acid.

Our study showed that *in vitro* copper(II) complexes of N-salicyliden-L-glutamate have antiproliferative activity, and these complexes together with ascorbic acid have the cytotoxic potential even if they are used at noncytoxic concentrations. Next, combinations of the Cu(II) complex with isoquinoline ligand in the presence of ascorbic acid induced apoptosis of L1210 cells. Ascorbic acid can improve biological activity of these complexes as potential anticancer agents, however further in-

formation are needed on the use of this novel anticancer compound for human clinical practice.

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