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ORIGINAL ARTICLE

Evaluation of new Cu(II) complexes as a novel class of inhibitors against plant carbonic anhydrase, glutathione reductase, and photosynthetic activity in photosystem II

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Abstract Increasing inefficiency of production of important agricultural plants raises one of the biggest problems in the modern world. Herbicide application is still the best method of weed management. Traditional herbicides blocking only one of the plant metabolic pathways is ineffective due to the rapid growth of herbicide-resistant weeds. The synthesis of novel compounds effectively suppressing several metabolic processes, and therefore achieving the synergism effect would serve as the alternative approach to weed problem. For this reason, recently, we synthesized a series of nine novel Cu(II) complexes and four ligands, characterized them with different analyses techniques, and carried out their primary evaluation as inhibitors of photosynthetic electron transfer in spinach thylakoids (design, synthesis, and evaluation of a series of Cu(II) based metal–organic complexes as possible inhibitors of photosynthesis, J Photochem Photobiol B, submitted). Here, we evaluated in vitro inhibitory potency of these agents against: photochemistry and carbonic anhydrase activity of photosystem II (PSII); α-carbonic anhydrase from bovine erythrocytes; as well as glutathione reductase from chloroplast and baker's yeast. Our results show that all Cu(II) complexes excellently inhibit glutathione reductase and PSII carbonic anhydrase activity. Some of them also decently inhibit PSII photosynthetic activity.

Keywords Carbonic anhydrase inhibitors · Photosynthetic inhibitors · Glutathione reductase inhibitors

Abbreviations

PSII, PSI	Photosystem II and photosystem I, respectively
GSH	Reduced glutathione
GSSG	Oxidized glutathione disulfide
F_o, F_m	Minimum and maximum chlorophyll fluorescence intensity emitted by dark-acclimated samples, respectively
F_v/F_m	Maximum quantum yield of primary photosystem II photochemistry
EDTA	Ethylenediaminetetraacetic acid
TyrZ	Tyrosine Z, redox active tyrosines in the D1 protein of PSII
P_{680}	PSII reaction center chlorophyll dimer
GR	Glutathione reductase
DMSO	Dimethyl sulfoxide
CA	Carbonic anhydrase
Q_A, Q_B	Primary and secondary quinone electron acceptors of PSII

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NADFH	Nicotinamide adenine dinucleotide phosphate, reduced form
QSAR	Quantitative structure–activity relationship
IC_{50}	Half maximal inhibitory concentration
pIC_{50}	$-\log_{10}(IC_{50})$
HOMO	Highest occupied molecular orbital

Introduction

Weeds are still a limiting factor in crop production all over the world. Meanwhile, the use of herbicides is the best method to control weeds (Cobb and Reade 2010; Kingston 2011; Krämer and Wiley InterScience (Online service) 2012; Oerke 2006). Novel herbicides are still highly desired and even essential to protect crop quality in the future due to rapid-growing herbicide-resistant weeds and higher request of the eco-friendly behavior of compounds (Beckie and Tardif 2012; Vencill et al. 2012). So, researchers have to find novel herbicides and their Modes of Action, since commercially important herbicides affect only a very limited number of Modes of Action in plants, or develop novel solutions for weed control.

Copper (Cu) plays an important role in numerous metabolic processes in all photosynthetic organisms (i.e., cyanobacteria, algae and plants) (Yruela 2005). Although Cu acts as a significant cofactor of many important enzymes, free Cu ions induce oxidative damage of cells catalyzing the formation of reactive oxygen species (Murakami et al. 2014). The primary toxic effect of Cu may result from the intracellular reaction between Cu and reduced glutathione (GSH), leading to a lower GSH/oxidized glutathione disulfide (GSSG) ratio and suppression of mitosis. Accumulation of GSSG may shut off protein synthesis, which, in turn, inhibits cell division (Zhang et al. 1997). Previous research has shown that Cu reacts with enzyme SH-group and free thiols (e.g., GSH), which are essential for enzymatic activity and protein structure (Stauber and Florence 1987; Chen et al. 2012). Copper cations are known to be the important micronutrients for plant growth. At the same time, high concentrations of Cu(II) exhibit the highest toxicity among the cations of heavy metals, including the effect on photosynthetic organisms (Clijsters and Van Assche 1985). Copper inhibitory effects on photosynthetic electron transport are well-studied for higher plants, green algae, and cyanobacteria. The investigations in vitro revealed that the components of photosystem II (PSII) are more sensitive to inhibitory effect of Cu (Baron et al. 1995; Droppa and Horváth 1990) than these of photosystem I (PSI) (Ouzounidou et al. 1997). However, the exact location of Cu(II)-binding sites in PSII and the mechanisms of inhibitory effect are still vigorously investigated. It is supposed that both donor (Burda et al. 2003; Cedeno-Maldonado et al. 1972; Vierke and Struckmeier 1977) and acceptor sides of PSII are subjected to inhibitory effect of Cu (Yruela et al. 1996a, b). At the PSII acceptor side, Cu(II) affects Q_B-site (Mohanty et al. 1989), and Pheo-Fe-QA domain (Burda et al. 2003; Yruela et al. 1996a, b) in lower concentrations. In higher concentrations, Cu(II) also affects PSII donor side (Jegerschold et al. 1995; Schröder et al. 1994). In addition, Cu(II) is known to influence PSII reaction centers and decompose chlorophyll *a* (Küpper et al. 1996); it can inhibit oxidizing side (Cid et al. 1995) and PSII water-splitting complex (Mijovilovich et al. 2009). Also, Cu(II) is known to affect PSII photochemical activity and damage the structure of the thylakoid membranes (Deng et al. 2014), which may influence the conformation and function of the photosystems. It was suggested that such processes could include the destruction of polypeptide composition of water-splitting complex or the interactions with Mn^{2+}, Ca^{2+}, and Cl^- necessary for its normal functioning (Baszynski and Krupa 1995). Moreover, it has been assumed that Cu(II) can modify TyrZ, with electron transport between TyrZ and P_{680} being blocked (Schröder et al. 1994). Considering the inhibition on the PSII acceptor side, Yruela et al. suggested that in the presence of Cu(II), the inactivation of PSII occurs due to the destructive effect of hydroxyl radicals generated at the PSII acceptor side (Yruela et al. 1996a, b).

Generally, copper is present as organic chelate complexes in both environment (water, soil) and biological systems (Flemming and Trevors 1989). Besides non-organic copper complexes, EDTA-Cu(II) chelate was used to affect photosynthetic organisms. EDTA (Ethylenediaminetetraacetic acid) is a widespread chelating agent capable of forming chelates with both transition-metal ions and main-group ions. The constants of the complex formation were determined for EDTA with ions of different metals (Anderegg and International Union of Pure and Applied Chemistry. Commission on Equilibrium Data 1977; Martell and Smith 1982; Smith and Martell 1989). According to these data, the complex formation constant and stability constant of EDTA-Cu(II) complex are equal to 6.3×10^{18} and 18.8, respectively. EDTA-Cu(II) chelate is relatively resistant to chemical and bio-degradation due to the chemical composition of the ligand, chemical structure of the chelate, and high stability constant.

Organic ligands of chelate complexes with metal cations significantly increase the availability of the biosystem regions sensitive to the metal effect; thus, the inhibitory activity of such complexes is increased as well. Copper complexes with organic ligands better inhibit photosynthesis and other cellular reactions. It has been demonstrated that complexes based on the copper salts of phenoxyacetic

acid and its derivatives can inhibit several biological activities (Goebel and Doedens 1971; Smith et al. 1981; Prout et al. 1968).

In addition, it is known that 4-chlorine- and 4-chlorine-2-methylphenoxyacetic copper complexes with several *O*- and *N*-donor ligands have herbicidal activity and fungicidal activity against phytopathogenic fungi (Kráľová et al. 1994). Moreover, it was shown that several carboxylate Cu(II)-containing complexes and 3,5-dimethylpyrazole-aryloxy acetate copper complexes soluble in water express their antimicrobial activity effectively suppressing the growth of such pathogens as *Escherichia coli, Candida albicans, Staphylococcus aureus* (Kráľová et al. 1994). The biological activity of salicylate tetrahydrate copper complexes was revealed, and the effect of several Cu(II)-containing complexes with hydroxyquinoline derivatives on photosynthesis, structure, and permeability of biological membranes was investigated. Also, Kráľová et al. studied the effect of some Cu(II) complexes on the chlorophyll content in *Chlorella vulgaris* and the inhibition of photosynthetic electron transport in spinach chloroplasts (Kráľová et al. 2004). In their work, they used ligands derived from α-alanine or β-alanine, valine, phenylalanine, *N*-salicylidene-β-alanine, imidazole, pyrazole, pyridine, quinoline, urea, and thiourea (Kráľová et al. 2004). In green alga *Scenedesmus quadricauda*, the Cu(II)-containing complexes effectively suppress reactions of photosynthetic oxygen release; the extent of growth suppression depends on the concentration of the complexes. It was found that in the form of organic complexes Cu(II) cations are extremely toxic even at low concentrations (Fangstrom 1972).

As the aim of the study is to find new versatile inhibitors of several key reactions, we also studied the effect of Cu(II) complexes on glutathione reductase activity and the carbonic anhydrase activity of PSII. In plants, glutathione reductase (GR) is a major cellular antioxidant enzyme catalyzing the reduction of GSSG to GSH using NADPH as an electron donor. It has been reported that GR regulates under stresses such as salinity, drought, high light intensity, mechanical wounding, chilling, air pollution, and herbicides (Alscher 1989; Noctor et al. 2012). Moreover, it was essential to study the effect of new compounds on PSII activity. Being the one of the main sites of reactive oxygen species generation (Pospíšil 2009), PSII also exhibits carbonic anhydrase activity related to the presence of manganese cluster. As it has been demonstrated, intact PSII and/or 33 kDa extrinsic protein displays carbonic anhydrase activity in the presence of manganese (Shitov et al. 2009, 2011).

There is a great number of investigations of Cu(II) complexes effect on the different reactions of plant and animal cell. In all such studies, Cu(II) cations as organic complex compounds are generally considered to be effective inhibitors of cellular reactions. In our previous study, we showed that antimony(III) complexes act as excellent PSII inhibitors (Karacan et al. 2014, 2016). To continue and expand this study, nine novel Cu(II) complexes and four ligands were synthesized, characterized by means of different analyses techniques and evaluated as inhibitors of photosynthetic electron transfer in spinach thylakoids [design, synthesis and evaluation of a series of Cu(II)-based metal–organic complexes as possible inhibitors of photosynthesis. JPPB-B, submitted]. In this work, we in vitro evaluated inhibitory potency of these agents against photochemistry and carbonic anhydrase activity of PSII; α-carbonic anhydrase (α-CA) from bovine erythrocytes; and glutathione reductase from chloroplasts and baker's yeast.

Materials and methods

Highly purified glutathione reductase from baker's yeast (*Saccharomyces cerevisiae*; type IV, E.C. 1.6.4.2; 219 units per mg protein), oxidized glutathione disulfide, NADPH, and α-Carbonic anhydrase isozyme II (Sigma-C2522) from bovine erythrocytes (lyophilized powder, 2500 W-A units per mg protein) were purchased from Sigma-Aldrich (USA) and used without further purification. Stock solutions of Cu(II) complexes were prepared by dissolving in DMSO.

Isolation of PSII-preparations

PSII-enriched photochemically active thylakoid membrane fragments (PSII-preparations) were isolated from leaves of the greenhouse spinach (*Spinacia oleracea* L.) as described earlier (Schiller and Dau 2000). The chlorophyll concentration was 220–250 molecules per reaction center and the preparations were capable to evolve photosynthetic oxygen with rates of 400–500 μmol O_2 mg Chl^{-1} h^{-1} under saturating light in the presence of 0.1 mM 2,5-dichloro-*p*-benzoquinone and 1 mM $K_3Fe(CN)_6$ as electron acceptors (Klimov et al. 1982). The PSII-preparations were fast frozen in liquid nitrogen for storage at −80 °C. The total chlorophyll concentration of the PSII-preparations was determined using 96% (v/v) ethanol as described earlier (Arnon 1949).

PSII photochemical activity

To analyze PSII photochemical activity, the kinetics of photoinduced changes of the PSII chlorophyll fluorescence yield (F_v) related to photoreduction of the PSII primary electron acceptor, plastoquinone Q_A, were measured using 1-cm cuvette at 20 °C by a pulse-amplitude modulation fluorimeter (XE-PAM, Heinz Walz, Germany) accompanied by the Power Graph Professional 3.3 software.

Measuring medium contained 25 mM MES-NaOH (pH 6.5), 10 mM NaCl, and 300 mM sucrose. Total value of measuring mixture was 1 mL. The Chl concentration was 10 μM mL^{-1}. During the measurements, the characteristic values F_o, F_m—minimum, and maximum fluorescence yield of chlorophyll, $F_v = F_m - F_o$ and maximum quantum photochemical yield of PSII (F_v/F_m) were identified. F_o was measured under weak probe pulses of measuring light ($\lambda = 490$ nm; 4 μmol photons m^{-2} s^{-1}) in dark-adapted samples. To record F_m, the dark-adapted samples were subjected to the light of saturating intensity (1000 μmol photons m^{-2} s^{-1}).

Inhibition of PSII photochemical activity was recorded after 3 min of dark incubation of PSII-preparations in the presence of the Cu(II) complex or 1% DMSO.

Carbonic anhydrase activity

According to the electrometric procedure of Wilbur and Anderson (Anderson and Wilbur 1948), the carbonic anhydrase activity was measured as the rate of pH change in CO_2 hydration by means of Mettler Toledo InLab 413 pH electrode and cpX-2 pH/ion meter (Institute of Biological Instruments (IBI) of the Russian Academy of Sciences, Pushchino). All measurements were conducted in the medium containing 25 mM veronal (pH 8.6), 50 mM KCl, and 15 mM $MgCl_2$ at 1.5–2 °C, according to (Karacan et al. 2016). To analyze the inhibition of PSII carbonic anhydrase activity or α-Carbonic anhydrase isozyme II from bovine erythrocytes, an agent (at final concentration of 100 μM) was added to the preparations. The measurements were carried out after 1 min incubation of the mixture in the cell.

Glutathione reductase activity

Glutathione reductase activity measurements of enzyme extract from spinach chloroplasts or GR from baker's yeast were performed as described earlier (Carlberg and Mannervik 1985). Stock solutions of baker's yeast GR and GSSG (20 mM) were prepared in 100 mM potassium phosphate buffer (pH 7.2), and NADPH stock solution (4 mM) was prepared in 10 mM Tris–HCl buffer (pH 7.0). The activity of the enzyme was expressed as μmole of NADPH oxidized per minute per mg protein, at pH 7.2 and 25 °C, with NADPH molar extinction coefficient of 6.2 mM^{-1} cm^{-1} at 340 nm. The decrease of NADPH absorption at 340 nm due to its oxidation was monitored by means of a photodiode array Agilent 8453 spectrophotometer (USA) accompanied with UV–Visible ChemStation software. The standard GR reaction medium contained 100 mM potassium phosphate buffer (pH 7.2), 2 mM EDTA (pH 7.2), enzyme extract from spinach chloroplasts or GR from backer's yeast, 0.2 mM NADPH, and 1 mM GSSG, with the final mixture volume of 2 mL. The reaction mixture was constantly stirred. Enzyme was incubated at the presence of the inhibitory agent for 3 min at 25 °C. Time-dependent changes in NADPH absorption were monitored for 100 s in the quartz cuvette with a 1-cm light path. The initial rate of linear GR reaction was measured by the slope of recorded tracing. Control measurements were done in the presence of DMSO only. The enzyme activity in the absence of investigated chemicals was taken as 100%.

Inhibitory treatment

Stock solutions of 10 mM inhibitors were prepared in 1 mL of DMSO at room temperature. Successive inhibitor dilutions were prepared from the above stock solutions. In all measurements, the final concentration of DMSO did not exceed 1.0% (v/v) and induced no damage of the samples.

Results and discussion

Previously (submitted), we synthesized a series of nine Cu(II) complexes and four ligands and characterized them with different analyses techniques. We evaluated these inhibitory agents by its action on the uncoupled basal noncyclic electron transport from water to methyl viologen in spinach thylakoids. Structures of the Cu(II) complexes and the ligands, as well as chemical formulas and their designation, are presented in Fig. 1 and Table 1.

Inhibition of PSII photochemical activity

The effects of new inhibitory agents on the PSII photosynthetic activity were studied by photoinduced changes of the PSII chlorophyll fluorescence yield related to photoreduction of the primary electron acceptor, plastoquinone Q_A, as it was shown earlier (Klimov et al. 1982). The changes in PSII maximum quantum photochemical yield of the dark-adapted samples (F_v/F_m) in the presence of the studied inhibitors were used as a measure of PSII photosynthetic activity. F_v was calculated as $F_m - F_o$, maximum, and minimum fluorescence yield of chlorophyll *a* in dark-adapted samples, correspondingly. Before each measurement, the PSII-preparations were preliminary incubated in the presence of inhibitory agents (or solvent (DMSO) at control) for 3 min in the dark under gentle stirring to ensure the formation of complex between inhibitor and PSII-preparations. Furthermore, the possible effects of inhibitory agents on F_o level, F_m value, the rate of photoinduced F_v growth, and F_m dark relaxation rate were also analyzed. The characteristic kinetics of the photoinduced changes of the PSII chlorophyll fluorescence yield in the absence of inhibitory agents (kinetic 1), the PSII photochemical activity in

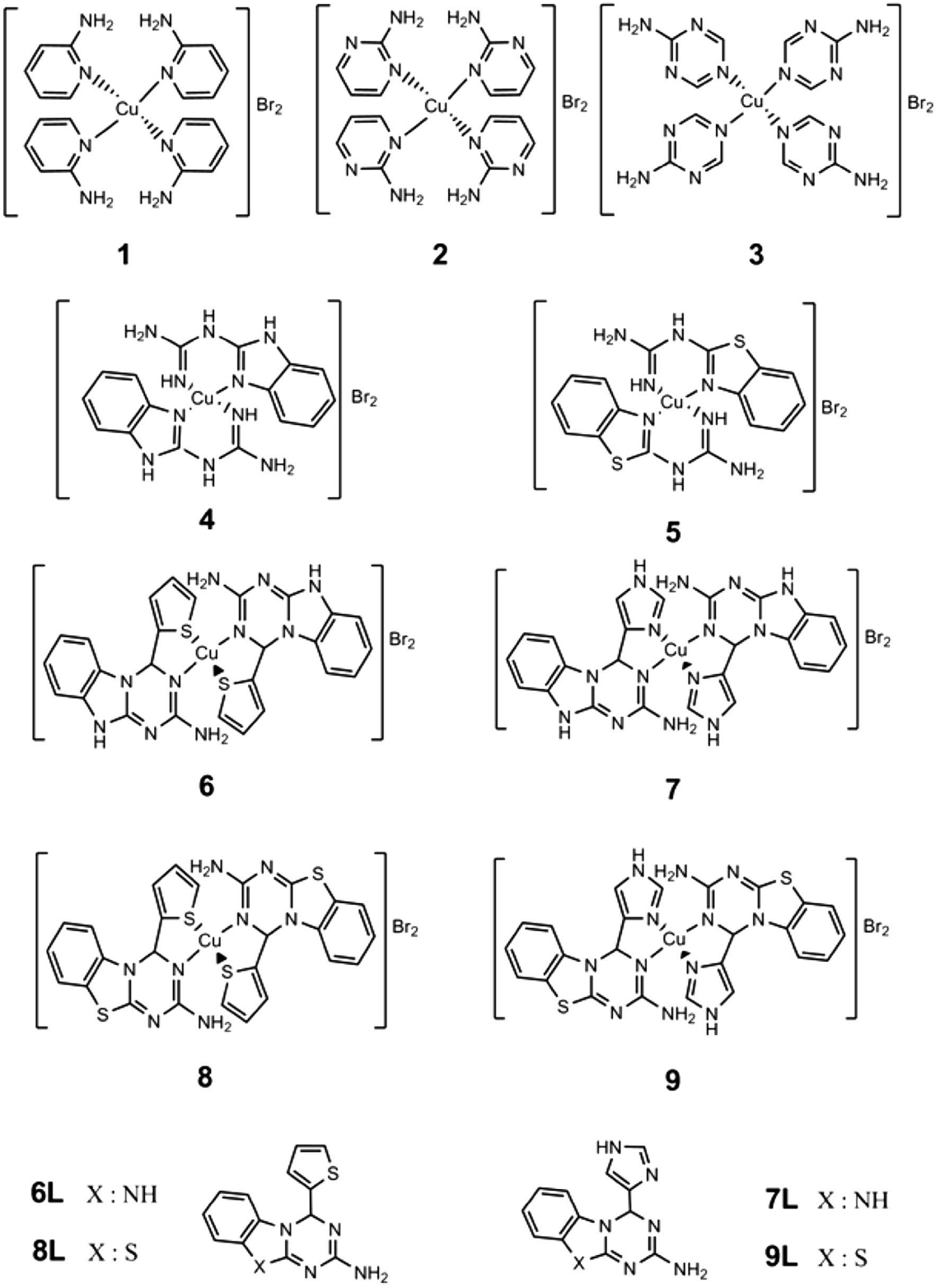

Fig. 1 Structures of the ligands and the copper(II) complexes

Table 1 The chemicals and its designations

Designation	Chemical
Ligands	
6L	1,3,5-triazino[1,2-a]benzimidazole-2-amine,3,4-dihydro-4-(2-thiophene)
7L	1,3,5-triazino[1,2-a]benzimidazole-2-amine,3,4-dihydro-4-(4-imidazole)
8L	4H-1,3,5-triazino[2,1-b]benzothiazole-2-amine,4-(2-thiophene)
9L	4H-1,3,5-triazino[2,1-b]benzothiazole-2-amine,4-(2-imidazole)
Cu(II) complexes	
1	Tetrakis(2-aminopyridine)copper(II) bromide
2	Tetrakis(2-aminopyrimidine)copper(II) bromide
3	Tetrakis(2-amino-1,3,5-triazine)copper(II) bromide
4	Bis(2-guanidobenzimidazole)copper(II) bromide
5	Bis(2-guanidobenzothiazole)copper(II) bromide
6	Bis{1,3,5-triazino[1,2-a]benzimidazole-2-amine,3,4-dihydro-4-(2-thiophene)}copper(II) bromide
7	Bis{1,3,5-triazino[1,2-a]benzimidazole-2-amine,3,4-dihydro-4-(2-imidazole)}copper(II) bromide
8	Bis{4H-1,3,5-triazino[2,1-b]benzothiazole-2-amine,4-(2-thiophene)}copper(II) bromide
9	Bis{4H-1,3,5-triazino[2,1-b]benzothiazole-2-amine,4-(2-imidazole)}copper(II) bromide

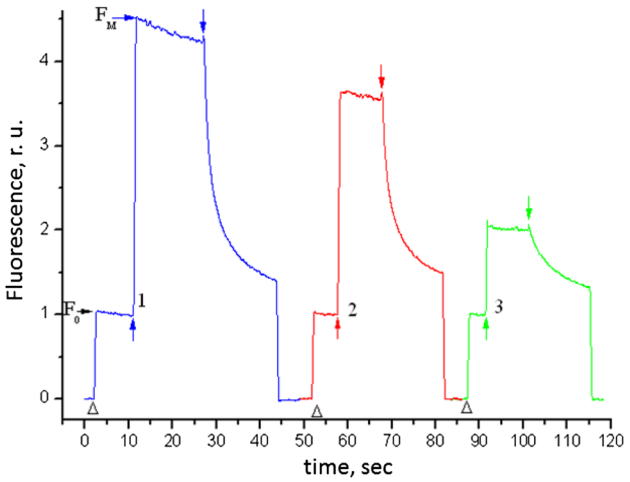

Fig. 2 Effects of 100 μM Cu(II) complex **3** (kinetic 2) and 100 μM Cu(II) complex **9** (kinetic 3) on kinetics of the photoinduced changes of the PSII chlorophyll fluorescence yield. Kinetic 1 was measured only in the presence of 1% DMSO. *Triangle symbol* indicates the moment of switching on the measuring light ($\lambda = 490$ nm, 4 μmol photons m^{-2} s^{-1}) exciting PSII chlorophyll fluorescence, F_o ($\lambda \geq 650$ nm). The *upward* and *downward arrows* indicate the moment of respective switching on and off the actinic light ($\lambda > 600$ nm, 1000 μmol photons m^{-2} s^{-1}). Chlorophyll concentration was 10 μg mL^{-1}. Each trace is at least an average of seven separate measurements

control measurement, and effects of 100 μM Cu(II) complexes **3** (kinetic 2) and **9** (kinetic 3) are presented at Fig. 2.

In the presence of inhibitory agents, a sharp decrease of the F_m value versus control by 23% for 100 μM complex 3 and by 69% for 100 μM complex 9 was observed. It is important that no increase of F_o level, the rate of photoinduced F_m growth, and slowing down of the F_m dark relaxation rate related to dark reoxidation of Q_A^- occur, as it usually takes place in case of electron transfer inhibition on the PSII acceptor side between Q_A and Q_B caused by diuron (not shown) or in the presence of exogenous PSII electron donors. Furthermore, no increase of the F_m dark relaxation rate also occurred in the presence of PSII electron acceptors (potassium ferricyanide), accelerating dark reoxidation of reduced Q_A.

Each from the new inhibitory agents was studied by its action on the PSII photosynthetic activity in similar experiments. In general, partially the same effects on kinetics of PSII chlorophyll fluorescence were observed in the presence of other Cu(II) complexes and their four ligands. Values of inhibition (%) of PSII photosynthetic activity by Cu(II) complexes (**1**–**9**) and four ligands (**6L**–**9L**) studied by above-mentioned methods are given in Table 2.

As shown in Table 2, Cu(II) complexes **5, 8**, and **9** showed the highest inhibitory activity (63.4, 67.6, and 69.2%, respectively). The Cu(II) complex **3** and ligand **9L** have a relatively middle inhibitory efficiency. The former inhibits the PSII photochemistry by 23.9%, whereas potency of the later is only 13.4%. The remaining Cu(II) complexes **1, 4, 6, 7**, as well as the ligands **6L, 7L,** and **8L** decreased the F_v/F_o ratio with low potency: by 3.2, 6.3, 4.3, 1.1, 3.2, 2.7, and 5.2%, respectively. Agent **2** practically did not affect the PSII maximum quantum photochemical yield. It is important to note that addition of 4 mM EDTA, known as chelator of divalent metals, did not eliminate inhibition of the PSII photochemistry.

The comparison of inhibitory efficiency of Cu(II) complexes **1**–**3** with monodentate ligands shows that on the one hand, an increase in the number of nitrogen atoms replacing the carbon atoms in the ligand aromatic benzene-like

Table 2 Inhibition (%) of PSII photosynthetic activity, PSII carbonic anhydrase activity, and α-carbonic anhydrase from bovine erythrocytes by the studied Cu(II) complexes (**1**–**9**) and four ligands (**6L**–**9L**)

Cu(II) complexes (**1**–**9**) and ligands (**6L**–**9L**)	Inhibition of PSII F_v/F_o (% of inhibition)	Inhibition of CA of PSII (pea) (% of inhibition)	Inhibition of CA of α-CA of bovine erythrocytes (% of inhibition)
Control	0.0	0.0	0.0
1	3.2	14.7	0.0
2	0.0	100 ± 2	90.0
3	23.9	81.1	66.7
4	6.3	42.7	23.2
5	63.4	83.1	73.3
6	4.3	64.5	4.3
7	1.1	52.8	69.5
8	67.6	100 ± 3	88.2
9	69.2	100 ± 2	84.2
6L	3.2	78.9	50.9
7L	2.7	39.4	33.4
8L	5.2	48.0	5.6
9L	13.4	51.0	17.8

heterocyclic ring does not significantly affect the photochemistry of PSII. For example, there is slight difference in activity between Cu(II) complex **1** with one N-atom in 2-aminopyridine ligand and complex **2** with two *N* atoms in 2-aminopyrimidine ligand. However, this assumption is disproved in case of complex **3**, which already has three *N* atoms in 2-amino-1,3,5-triazine ligand and its efficiency increases sharply to 23.9%. However on the other hand, the position of the side amino group (R-NH_2) in heterocyclic ring relative to N-atom liganding Cu(II) ion may also be important. In agents **1** and **2**, side amino group is located directly close to N-atom liganding Cu(II) ion, whereas in agent **3** it is strictly opposite.

The comparison of 2-guanidobenzimidazole ligand of agent **4** and **2**-guanidobenzothiazole ligand of agent **5** shows that their single difference is that in 5-membered thiazole ring (agent **5**) there is more electronegative S-atom instead of N-atom of imidazole ring (agent **4**). According to Pauling scale, electronegativity of S-atom (2.58) is lower than that of N-atom (3.04). However, it strongly effects on their PSII inhibitory potency. The inhibitory efficiency of agent **5** is about ten times higher than that of agent **4** (63.4 and 6.3% in the case of agent **5** and **4**, respectively, Table 2).

A similar trend is observed comparing the potency of two pairs of Cu(II) complexes (**6** and **8, 7,** and **9**) with bidentate ligands differing from each other only by the presence of S-atom (benzothiazole moiety of complexes **6** and **7**) instead of the N-atom (in benzimidazole moiety of complexes **8** and **9**). However, in both cases, such replacement is accompanied by drastic increase of inhibitory potency from 4.3 to 67.6% (complexes **6** and **8**) and from 1.1 to 69.2% (complexes **7** and **9**).

On the other hand, the nature of the Cu(II) ion ligand apparently does not have a significant effect on the inhibitory potency. In complex **6**, Cu(II) is liganded by two S atoms of thiophene moiety and two N atoms of imidazole moiety (one S and one N from two bidentate ligand **6L**), whereas in complex **7**, Cu(II) is liganded by four N atoms of imidazole moiety (two N from two bidentate ligands **7L**). However, the comparison of the inhibition efficiency of complexes **6** and **7** shows that they have comparable low efficiency (4.3 and 1.1%). A similar trend is revealed if one compares the inhibitory potency of complexes **8** and **9**.

The comparison of the inhibitory potency of ligands (**6L**–**9L**) with their Cu(II) complexes shows that ligands (**6L**–**9L**) exhibit low activity in the range of 2.7–13.4% versus the effect of Cu(II) complexes. This fact testifies that Cu(II) is evidently essential for inhibition of PSII photochemistry as a part of Cu(II) complexes.

One may propose that the found inhibitory effect of the agents on the PSII maximum quantum photochemical yield revealed as quenching of F_m value is a result of inhibition by free Cu(II), not by Cu(II) as a part of complex. It can be assumed that during the measurements the destruction of Cu(II) complexes and release of free Cu(II) ions may induce the observed inhibitory effects. To check this assumption, we studied absorption spectra (and their possible changes) of these Cu(II) complexes and their ligands in different buffer solutions used in measurements of PSII photochemistry, CA and glutathione reductase activity during 24 h. No changes in absorption spectra of these inhibitory agents occur (data not shown). These data prove the stability of new chemicals at least for the time enough for carrying out the investigations. Absorption spectra of inhibitor stock solutions in DMSO are stable during the whole

time of working with these agents (more than a year). Furthermore, as the addition of 4 mM EDTA to Cu(II) complexes did not eliminate inhibition of the PSII photochemistry, this assumption was disproved. One may propose that observed decrease of the F_m value induced by the studied inhibitory agents is a probable result of chlorophyll fluorescence quenching due to its reabsorption by the agents, or screening of measuring, or actinic light exciting fluorescence, or PSII photochemistry, respectively. However, studied absorption spectra of the inhibitory agents indicate that it is not for this case. On the other hand, if inhibition was associated only with the action of free Cu(II) ions, then there would have been no inhibitory action of the ligands. However, we see that ligand **9L** effectively suppresses PSII photochemistry. At the same time, among the nine studied Cu(II) complexes, only four have sufficiently high activity. If the inhibitory activity of the complexes was only due to the action of free Cu(II), then in this case, all complexes would have had a sufficiently high efficiency. However, it is the case. Nevertheless, it is known that copper induces inhibitory effects on photosynthetic electron transport in higher plants, green algae, and cyanobacteria (see "Introduction" section).

Inhibition of PSII carbonic anhydrase activity

A range of studies testifies that carbonic anhydrase activity of PSII is probably required for its maximum electron transport rate (Shutova et al. 2008; Shitov et al. 2009, 2011). Suppression of PSII carbonic anhydrase activity by a known inhibitor of α-carbonic anhydrases, acetazolamide (Shitov et al. 2011), or antimony(III) complexes (Karacan et al. 2016) is accompanied by a decrease of rates of reactions related to the electron transfer on the PSII donor side. We proposed that Cu(II) complexes could affect not only PSII photochemistry but also its CA activity. Therefore, all novel chemicals were screened by their action on carbonic anhydrase activity of PSII-preparations. Typical kinetic traces of the pH changes reflecting uncatalyzed spontaneous hydration of CO_2 (trace 6) as well as PSII carbonic anhydrase activity in the absence of inhibitory agents (trace 1, control) and in the presence of 100 μM of some Cu(II) complexes: **2, 8, 9,** or **5** (trace 2, 3, 4, and 5, respectively) are shown at Fig. 3. Control trace was measured in the presence of 1% DMSO used as solvent for Cu(II) complexes.

Efficiency of PSII carbonic anhydrase activity suppression by 100 μM Cu(II) complexes was evaluated versus carbonic anhydrase activity of PSII-preparations measured in the absence of inhibitory agents (0% of inhibition). The obtained data are presented in Table 2.

Generally, all Cu(II) complexes were shown to be the potent inhibitors of PSII carbonic anhydrase activity. Among them, there were the compounds with the highest

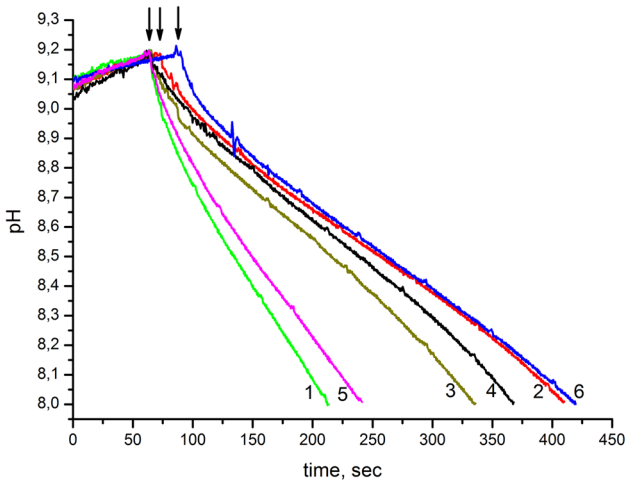

Fig. 3 Typical kinetic traces of the pH changes due to hydration of CO_2 catalyzed by carbonic anhydrase activity of PSII-preparations (100 μg Chl, at final concentration of Chl 44.4 μg mL^{-1}) in the absence of inhibitory agents (trace 1, control); or in the presence of 100 μM of Cu(II) complexes **2, 8, 9,** or **5** (traces 2, 3, 4, and 5, respectively). Control kinetics was measured in the presence of 1% DMSO used as solvent for Cu(II) complexes. Trace 6 shows an uncatalyzed spontaneous reaction of CO_2 hydration occurring in the measuring medium in the absence of PSII without any additions. *Downward arrow* indicates the moment of CO_2-saturated water addition. Each trace is an average of at least seven separate measurements. 100% PSII carbonic anhydrase activity (without any additions) was equal to twelve Wilbur-Anderson units per 1 mg Chl

activity: complexes **2, 8**, and **9**. They totally suppress PSII carbonic anhydrase activity. Compounds **3, 5**, and ligand **6L** have the second highest effect (inhibition on 81.1, 83.1, and 78.9%, respectively). However, the majority of ligands (**7L–9L**) exhibits the inhibitory activity lower (39.4, 48.0, and 51.0%) than that of corresponding complexes (**7–9**) in the range of 39–51% (Table 2). Cu(II) complex **1** is the only agent with lowest but nevertheless relatively perceptible potency (14.7%). As in the case of PSII photochemistry measurements, subsequent addition of 5 mM EDTA did not eliminate inhibition of PSII carbonic anhydrase activity. In preliminary experiments, we clarified that 1% DMSO had no effect on PSII carbonic anhydrase activity.

The comparison of the data presented in column 2 and 1 of Table 2 revealed that generally almost all Cu(II) complexes and ligands including those which nearly have no effect on the PSII photochemical activity do significantly suppress PSII carbonic anhydrase activity, and even stronger than they inhibit PSII photochemistry. The potencies of inhibition of PSII photochemistry and CA activity by various complexes and ligands are strongly different. The complexes and ligands may be arranged in the next row by the decreasing order of their potency difference: Cu(II) complexes **2, 6, 3, 7, 4, 8, 9, 1** (100, 60.2, 57.2, 51.7, 36.4,

32.4, 30.8 11.5%) and ligands **6L, 8L, 9L** and **7L** (75.7, 42.8, 37.6, 36.7%). Even agents **5** (63.4%), **8** (67.6%) and **9** (69.2%) inhibiting PSII photochemistry with rather high efficiency nevertheless suppress PSII CA activity more efficiently by 83.1, 100, and 100%, respectively. This correlation confirms the assertion that the CA activity of PSII components is required for its maximum photochemical activity, and a decrease in the PSII CA activity is accompanied by only a partial (not complete) suppression of electron transfer (Shitov et al. 2011; Karacan et al. 2016).

Inhibition of α-carbonic anhydrase from bovine erythrocytes

Since α-carbonic anhydrase from bovine erythrocytes has a structure and activity properties which are relatively close to those of plant α-CA-s, we also screened our Cu(II) complexes by their effects on the activity of this enzyme. If the effects of novel inhibitory agents on PSII CA activity were similar to those on α-CA from bovine erythrocytes, it would be relative indicator of the comparability of the CA enzyme carrier properties. It would allow to get more information about CA properties of PSII particles that are not enough studied yet and have some features not revealed in other CA enzymes. CA activity of some components of PSII-preparations are activated by Mn^{2+} ions (Dasgupta et al. 2008). Figure 4 shows the effects of novel Cu(II) complexes on activity of α-CA from bovine erythrocytes. As shown in Fig. 4, all presented Cu(II) complexes suppress α-CA activity with good potency.

Efficiency of α-CA activity suppression by 100 μM Cu(II) complexes was evaluated versus activity of α-CA measured in the absence of inhibitory agents (0% of inhibition). The obtained data are presented in Table 2.

Data from Table 2 testify that all nine Cu(II) complexes (excluding **1** and **6**—0 and 4.3% of inhibition, respectively) and all ligands (excluding **8L**, only 5.6% of inhibition) are relatively potent inhibitors of α-CA activity (inhibition in the range of 23.3−90.0%). Among them, six complexes (**3, 7, 5, 9, 8,** and **2** with inhibition potency equal to 66.7, 69.5, 73.7, 84.2, 88.2, and 90.0%, respectively) and one ligand (**6L** with inhibition potency equal to 50.9%) revealed inhibition higher than 50%. As well as inhibition of CA activity of PSII-preparations, complex **2** also exhibits the best inhibitory activity against α-CA of bovine erythrocytes.

Among complexes with six-membered benzene-like heterocyclic ring containing different quantity of N atoms as ligands (2-aminopyridine, 2-aminopyrimidine and 2-amino-1,3,5-triazine in complexes **1, 2, 3**, respectively), complex **2** has the best potency (90.0% of inhibition); complex **3** has lower depth of inhibition (66.7%), whereas complex **1** does not affect the α-CA activity at all (0.0% of inhibition).

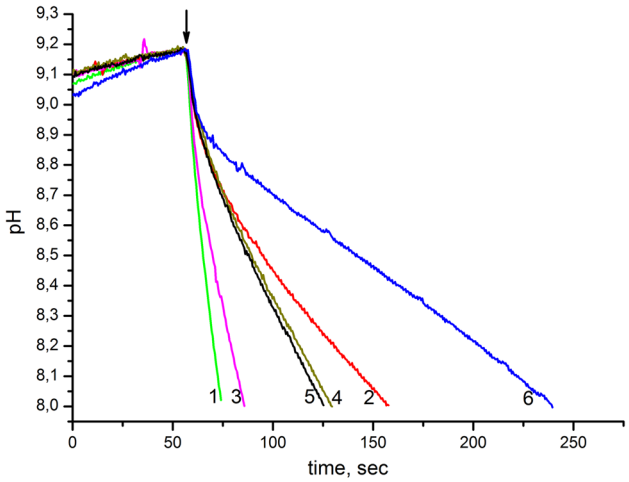

Fig. 4 Kinetic traces of pH changes characterizing α-CA activity (1.125 μg, final concentration of protein 0.5 μg mL^{-1}) in the absence of inhibitory agents (trace 1, used as control) and in the presence of 100 μM the Cu(II) complexes **2, 5, 8**, or **9** (traces 2, 3, 4, and 5, respectively). Control trace was measured in the presence of 1% DMSO. Trace 6 shows an uncatalyzed spontaneous reaction of CO_2 hydration occurring in the measuring medium without α-CA. *Downward arrow* indicates the moment of CO_2-saturated water addition. Each trace is an average of at least seven separate measurements. 100% carbonic anhydrase activity of protein (without additions) was equal to 2500 Wilbur-Anderson units per 1 mg of protein. Measurements were carried out in veronal buffer (pH 8.6)

This fact evidently indicates that quantity of N atoms in structure of ligands influences on the potency. On the other hand, weaker inhibitory efficiency of complex **3** (three N atoms) compare to complex **2** (two N atoms) may be due the influence of side amino group ($R\text{-}NH_2$) in heterocyclic ring relative to N-atom liganding Cu(II) ion. In complex **3** with maximal quantity of N atoms among complexes, **1**–**3** side amino group is located strictly opposite to N-atom liganding Cu(II) ion unlike to complexes **1** and **2**, whereas this group is directly nearby to it. The similar influence of quantity of N and side amino group in ligands on potency of inhibition of CA activity is also revealed on PSII particles.

Complexes **4** and **5** with nine-membered atoms of the bicycle as ligands (2-guanidobenzimidazole and 2-guanidobenzothiazole in complex **4** and **5**) are highly different: agent **5** (73.3% of inhibition) is at least three times more potent that agent **4** (23.2%).

Ligands of complexes **6** and **7** only differ in N-atom replaced by S-atom in separate five-membered aromatic heterocyclic ring: thiophen ring (complex **6**) instead of imidazole ring (complex **7**), and therefore Cu(II) is ligated by four N atoms in complex **7**, but—two N atoms and two S atoms in complex **6**. As it was mentioned above, electronegativity of S-atom (2.58) is lower than that of N-atom (3.04). Probably, in the result of this

replacement, the potency of complex **6** (4.3%) is 16 times lower than that of complex **7** (69.5%).

At the same time, complexes with similar replacement in five-membered aromatic heterocyclic ring of ligands [thiophen ring (complex **8**) instead of imidazole ring (complex **9**)] and consequent differences in nature of atoms ligated to Cu(II) have very close potency: 88.2 and 84.2%, respectively.

It is interesting to compare two pairs of complexes (**6** and **8**) and (**7** and **9**). In these pairs, ligands only differ in the replacement of benzimidazole in complexes **6** and **7** by benzothiazole in complexes **8** and **9**. In both pairs, both complexes have the same atoms ligated to Cu(II): four S atoms or four N atoms in pair complexes (**6** and **8**) and (**7** and **9**), respectively. But besides the significant potency difference in pair complexes **6** (4.3%) and **8** (88.2%), in pair complexes **7** and **9**, the potency is more comparable (69.5 and 84.2%).

If one compares the effect of complexes and their ligands separately, it is obvious that complexes (**7–9**) show higher inhibitory activity than their corresponding ligands (**7L–9L**) (Table 2). This difference is especially pronounced, if we compare potency of Cu(II) complex **8** and its ligand **8L** (88.2 and 5.6%). Cu(II) complex **6** and its ligand **6L** that is much stronger that metal complex (4.3 and 50.9%) are the exception from such regularity.

As in case of PSII CA activity suppression, all these new Cu(II) complexes and four ligands suppress α-CA activity much stronger that PSII photochemical activity. Nevertheless, all compounds show decreased effect in comparison with the inhibition values of PSII carbonic anhydrase activity.

Inhibition of glutathione reductase from spinach chloroplasts and 2D-QSAR analysis

Glutathione reductase and glutathione are present in spinach (*Spinacia oleracea* L.) (Foyer and Halliwell 1976; Halliwell and Foyer 1978) and pea (*Pisum sativum*) chloroplasts (Kalt-Torres et al. 1984; Connell and Mullet 1986). Glutathione reductase and glutathione are the parts of anti-oxidative defense systems that play a central role in the environmental acclimation of plants. Glutathione also participates in photosynthesis regulation in chloroplasts (Wolosiuk and Buchanan 1977) and also at the level of photosystems. With gold particles bound to glutathione, it was detected in the stroma and inside the lumen of thylakoids (Heyneke et al. 2013). Therefore, all nine Cu(II) complexes (**1–9**) and four ligands (**6L–9L**) were screened by their effect on the activity of GR from chloroplasts. Effect of investigated inhibitory agents expressed as IC_{50} and pIC_{50} values is presented in Table 3.

In general, all complexes showed good inhibitory effect at the nanomolar level. Among the first three complexes, triazine Cu(II) complex **3** proved to be the most potent inhibitor with $IC_{50} = 0.025$ μM. It can be seen that complexes **6–9** with the similar molecule structure had the greatest inhibitory effect. Among them, compound **7** was found to be the most potent inhibitor, with an IC_{50} value of 0.025 μM. Compound **6** has the second-best activity ($IC_{50} = 0.037$ μM). These two complexes are synthesized from the derivatives (**6L** and **7L**) of the guanidobenzimidazole ligands; however, guanidobenzimidazole-copper complex **4** showed less activity than **6** and **7**. Nevertheless, Cu(II) complexes **6–9** exhibit stronger inhibitory activity than corresponding ligands (**6L–9L**).

Table 3 Glutathione reductase inhibitory activity values of the compounds, and their molecular descriptors used in QSAR equation

Compounds	GR inhibitory activity				Descriptors		
	IC_{50} (μM)	pIC_{50} (obs)	$pIC_{50\ (pred)}$	$\Delta_{obs-pred}$	NoN	MNRICu	HOMO
1	1.050	5.9815	5.9790	0.0025	8	1.9706×10^{-4}	−12.0229
2	0.085	7.1156	7.0710	0.0446	12	9.1746×10^{-4}	−12.9242
3	0.025	7.6369	7.6020	0.0369	16	1.5053×10^{-4}	−13.1895
4	0.925	5.0205	6.0340	−0.1135	10	1.7328×10^{-4}	−11.8196
5	1.350	5.9621	5.8700	0.0921	8	1.2651×10^{-4}	−12.2382
6	0.037	7.3764	7.4320	−0.0556	10	2.4436×10^{-3}	−11.4357
7	0.025	7.5593	7.6020	−0.0427	14	2.5115×10^{-4}	−10.7606
8	0.282	6.5482	6.5500	−0.0018	8	1.7207×10^{-4}	−11.6996
9	0.191	6.7564	6.7190	0.0374	12	2.5887×10^{-4}	−11.1121
6L	0.324						
7L	0.423						
8L	0.528						
9L	0.673						

2D-QSAR analysis

In addition, we also performed 2D-QSAR analysis in order to find quantitative relationships between inhibition of GR activity and physical parameter of the Cu(II) complexes. For this reason, geometry optimization of the cationic complexes was performed using DFT/B3LYP/LanL2DZ method to generate lowest energy structure. Then average 370 descriptors were calculated with CODESSA software. For regression analysis, BMLR (Best Multi Linear Regression) method was used with the same software. Obtained two-parameter equation is as follows:

$$\mathrm{pIC}_{50} = 2.35\left(\pm 2.4 \times 10^{-1}\right)\mathrm{NoN} + 4.84 \times 10^{-1} \left(\pm 8.40 \times 10^{-2}\right)\mathrm{MNRICu} + 1.87\left(\pm 2.9 \times 10^{-2}\right)$$

$$n = 9, r^2 = 0.9921, F = 210.04, s^2 = 0.0062, R^2cv = 0.9524$$

In the above-mentioned equation, according to t test value, the most significant descriptor is number of N atoms (NoN). The positive sign of coefficient implies that the increase in N atoms would be favorable for the activity of the compounds. The second descriptor is minimum nucleophilic reactivity index for Cu atom (MNRICu). Fukui atomic nucleophilic reactivity index is defined as

$$N'_A = \sum_{i \in A} C^2_{iHOMO}$$

It relates to electron population of the highest occupied molecular orbital of a particular atom in a molecule. It estimates the relative reactivity of the Cu atoms in the molecule. Its positive coefficient in the equation implies that increasing value of this descriptor can lead to the higher activity of Cu(II) complexes. The relationship between experimental and predicted pIC_{50} values can be displayed graphically (Fig. 5). Molecular parameters, observed and predicted pIC_{50} values are listed in Table 2. Correlation matrix also was used to determine if there is a correlation between descriptors, and no correlation was found.

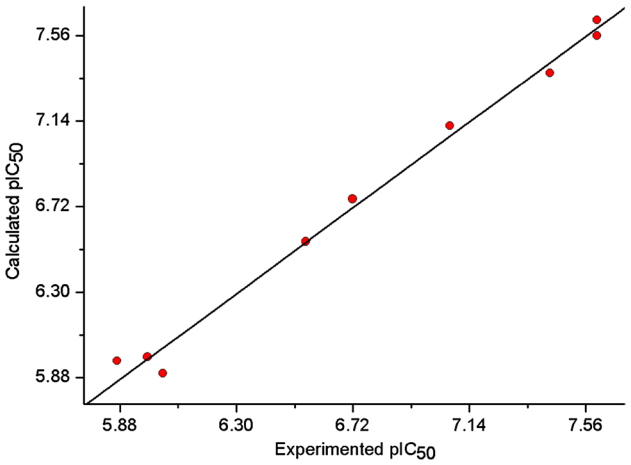

Fig. 5 Correlation of observed and predicted pIC_{50} values

Table 4 Time-dependence of inhibition of oxidized and reduced glutathione reductase by Cu(II) complexes (GR from *S. cerevisiae*)

Compounds	Inhibition of reduced GR (%)		Inhibition of oxidized GR (%)
	Incubation 3 min	Incubation 20 min	Incubation 3 min
Control	0	0	0
1	0	42.32	0.24
2	83.29	85.86	96.51
3	73.52	76.38	41.51
4	65.52	78.71	25.05
5	91.57	90.34	94.09
6	44.01	64.95	34.72
7	84.29	84.50	73.65
8	95.20	99.12	95.41
9	90.11	92.09	94.56
6L	0	6.89	18.64
7L	0	43.39	39.23
8L	0	19.91	19.19
9L	0.24	32.61	16.38

Inhibition of glutathione reductase from baker's yeast

In many respects, GR from baker's yeast is similar to that from pea and spinach chloroplasts, including a molecular weight of about 120 kDa and containing two moles of FAD per mole of protein (Mapson and Isherwood 1963). To compare the inhibitory activity on nine novel Cu(II) complexes and four ligands with known GR inhibitors, and to test the specificity of their action on the GR activity from various sources, these agents were screened by their effects on GR from baker's yeast as known model object. As control, we used the GR activity measured in the absence of inhibitor (100% of activity) but in the presence of 1% DMSO. The data characterizing effect of investigated agents in concentration of 100 μM on the activity of GR from baker's yeast are presented in Table 4 as (%) of inhibition.

In addition, we compared the inhibitory effect of the new agents on oxidized and reduced forms of GR (Table 4). According to the data, nearly all Cu(II) complexes (exclude **1**) are relatively strong inhibitors of reduced GR after 3 min incubation with reduced enzyme and can be arranged in decreasing order **8, 5, 9, 7, 2, 3**, and **4** with potency 95.20, 91.57, 90.11, 84.29, 83.29, 73.52, and 65.52%, receptively. Only complex **6** has lower, but appreciable inhibition equal

to 44.01%. It should be noted that all ligands (**6L–9L**) have practically no effect on GR.

These agents also strongly inhibited oxidized GR after 3 min incubation with potency comparable to that of reduced GR inhibition. In this case, they should be organized in other row: **2, 8, 9, 5**, and **7** with potency 96.51, 95.41, 94.56, 94.09, 73.65%, receptively. However, complexes **3, 6**, and **4** have activity lower than 50% (41.51, 34.72, and 25.05%). Furthermore, the efficiency of (**6L, 8L**, and **9L**) is appreciable higher than in case of reduced GR (18.64, 19.19, and 16.38%) and especially in case of ligand **7L** (39.23%) even after 3 min incubation.

The comparison of inhibition potency of reduced and oxidized enzyme by new agents testifies that, besides the ligands, some complexes affect these forms with different efficiency. Complex **2, 5**, and **9** are more efficient inhibitors of oxidized GR by about 13, 3, and 5%, whereas complexes **3, 4, 6, 7** have more potency of reduced form inhibition by about 32, 40, 10, 10%. Complex **8** suppresses activity of both reduced and oxidized forms of enzyme with similar efficiency (95.20 and 95.41%). In any case, among new agents, there are some complexes (**8, 9, 5, 2**, and **7**) with good percentage of GR inhibition of both enzyme forms.

The facts that reduced form is inhibited only by Cu(II) complexes, but oxidized form is inhibited by both complexes and their ligands may indicate two different inhibition mechanisms. Oxidized form is suppressed by two ways: by whole Cu(II) complexes [not free Cu(II) ions] and probably there is additional effect of ligand on the other component(s) of the enzyme. Whereas, in case of reduced GR, additional effect of ligand is eliminated probably due to the structural change of enzyme as a result of its reduction. Influence of free Cu(II) ions is excluded as EDTA is always present in the measuring medium.

It is known that increase of inhibitory potency depending on prolongation of incubation in the presence of enzyme is evident indicator of inhibition irreversibility (Deponte et al. 2005). Therefore, the new agents were screened by reversibility of their inhibitory potency on oxidized GR in a time-dependent manner. In general, potency of inhibition of reduced GR increased after 20 min of incubation (complexes **1, 2, 3, 4, 6, 8**, and **9**). It is especially pronounced in case of complex **1** (from 0 to 42.32%) and all ligands (also from 0 to 6.89, 43.39, 19.91, 32.61%). Latter fact also testifies in favor of proposition that there are two different inhibition mechanisms. One may propose that as the first mechanism there is an oxidation of some enzyme components by Cu(II) ions namely as part of Cu(II) complexes, and then the ligands induce additional irreversible destruction of enzyme. The fact that the potency of inhibition of reduced GR is weakly depended on types of ligands testifies in favor of this assumption. Statement that Cu(II) ions act namely as part of Cu(II) complexes is proved by complex **1** requiring time (20 min) for visible inhibition due to oxidation of reduced GR. In case of free Cu(II) ions, oxidation evidently would be achieved faster. The data that all ligands suppress oxidizing GR and reduced GR but only after 20 min of incubation indicate that ligands themselves are capable of inhibiting even reduced GR, but need more time.

To check whether Cu(II) complexes are capable of oxidizing the reduce form of GR (like GSSG does), we studied the effect of complex **5** on absorption spectra of initial enzyme, oxidized GR; GR reduced by addition of NADPH; the reduced GR oxidized by further addition of GSSG or Cu(II) complex **5** (Fig. 6).

According to the obtained spectra (Fig. 6), complex **5** is capable of oxidizing the enzyme, thus behaving as a substrate of the reaction (GSSG). This could explain the inhibitory activity of the complexes—they could suppress the further reaction with GSSG by taking the protons from the enzyme.

As it was obtained from the experimental data, the structure of the compounds plays significant role in the determination of their activity. Thus, among the complexes with monodentate ligands (**1–3**), the activity increases with the increasing of the number of N atoms on the aromatic ring. Comparing complexes with bidentate ligands, we may see that Cu(II) complexes bearing benzimidazole group (**4, 6, 7**) have higher activity than the compounds bearing benzothiazole ring (**5, 8, 9**). Furthermore, complex **7** with benzimidazole ligands containing 2-imidazole moiety exhibits higher activity than the compound **6** with 2-thiophene moiety. In their structure, the most potent inhibitors of both reduced and

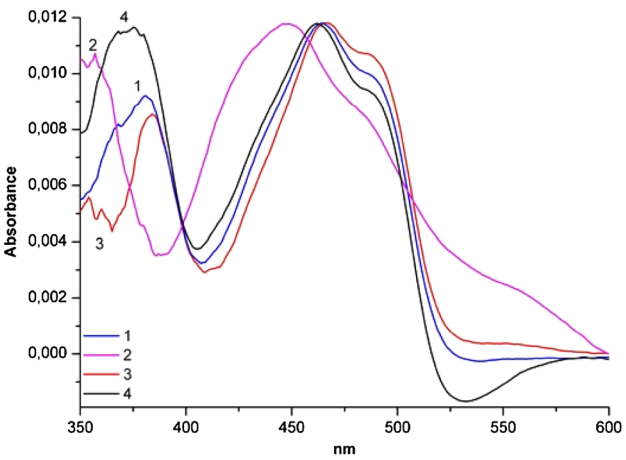

Fig. 6 Changes in absorption spectrum of GR from baker's yeast in accordance with the addition of reaction components: *1* oxidized GR; *2* GR reduced by addition of NADPH; *3, 4* the reduced GR oxidized by further addition of GSSG or Cu(II) complex **5**, respectively

oxidized GR from baker's yeast have bidentate ligands and *N*-donors for the central Cu atom. The complex **5** bearing benzothiazole ring shows the highest activity.

Also, it has been shown that the inhibitory effect of the Cu(II) complexes can increase and sometimes decrease in time. The decrease may occur due to the loss of stability to hold the electrons from NADPH by inhibitory complexes, and it requires more detailed study. However, generally, all complexes more or less inhibit GR from chloroplasts and yeast. We can assume that some Cu(II) complexes may disrupt GSSG-binding site of GR, with NADPH-binding site remaining active. So, the enzyme is still capable of performing its diaphorase activity and can transport the electrons from NADPH to the complexes, serving as electron acceptors due to their structures (Paulíková and Berczeliová 2005). However, the mechanism of such reactions still needs to be studied in details.

The ligands (**6L–9L**) have specific behavior: after 3 min of incubation with oxidized GR, they show almost no inhibitory activity, which nevertheless appears after the time of incubation was prolonged. Along with this, the ligands have the effect on oxidized GR, though rather low in comparison with Cu(II) complexes. So, one should use the complexes in order to obtain the acceptable GR inhibition.

Conclusion

Four new ligands (**6L–9L**) and nine new copper(II) complexes (**1–9**) were screened by their action on photochemistry and carbonic anhydrase activity of photosystem II (PSII) particles; α-carbonic anhydrase from bovine erythrocytes; as well as glutathione reductase from chloroplast and baker's yeast. Our results showed that all copper(II) complexes exhibited excellent inhibitory effect on both carbonic anhydrase and glutathione reductase. Some of them also performed good inhibition of PSII photosynthetic activity. The Cu(II) complexes **5, 8**, and **9** are the most versatile inhibitors of all mentioned activities. The compounds **2** and **7** exhibit good inhibitory effect on carbonic anhydrase and glutathione reductase, but do not affect much photosynthetic activity. The ligands were shown to have lower inhibitory effect than that of the Cu(II) complexes, but nevertheless the activity does not depend on copper content. As these compounds were synthesized for the first time, the mechanisms and features of their action require detailed investigation. However, this study allows to find such compounds that will help people to solve urgent agricultural and medical problems in the future.

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