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Effects of $[Cu(2\text{-}MeSNic)_2(MeNia)_2(H_2O)_2]\cdot H_2O$ and $[Cu(2\text{-}MeSNic)_2(H_2O)]_2$ on *Candida albicans*

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Abstract: Probable mode of action of new copper complexes of 2-methylthionicotinate (2-MeSNic) of composition $[Cu(2\text{-}MeSNic)_2(H_2O)_2]$ and $[Cu(2\text{-}MeSNic)_2(MeNia)_2(H_2O)_2]\cdot H_2O$ (where MeNia is N-methylnicotinamide) is described. Both partial growth inhibition of *Candida albicans* ($IC_{50} \geq 1.78$ mmol·L^{-1}, MIC ≥ 2.5 mmol·L^{-1}) and leak of proteins into the extracellular space (more than 80 %) were observed in the presence of these copper complexes. The membrane damage was detected by staining with Hoechst 33342, propidium iodide and methylene blue. Ascorbic acid potentiated antifungal activity of copper complexes approximately seven-fold and induced the oxidative stress, respectively. The production of intracellular reactive oxygen species was visualized by dichlorofluorescein. Thiobarbituric acid-reactive substances were formed as a by-product of lipid peroxidation.

Keywords: *Candida albicans*, copper 2-methylthionicotinate, membrane attack, mode of action, oxidative damage

Abbreviations

2-MeSNic — 2-methylthionicotinate
AA — ascorbic acid
DCF — 2´,7´-dichlorofluorescein
DCFH-DA — 2´,7´-dichlorofluorescein diacetate
DMSO — dimethyl sulfoxide
IC_{50} — concentration causing 50 % inhibition of growth compared to the control without inhibitor
MDA — malondialdehyde
MeNia — N-methylnicotinamide
MIC — minimum inhibitory concentration
ROS — reactive oxidative species
TBARS — thiobarbituric acid reactive substances

Introduction

The synthesis of drug complexes with copper atom in the molecule is preferred to a preparing of new bioactive agents. The complex formation often potentiate effects of the original substances, reduce the adverse side effects, and extend the spectrum of biological effects, respectively.
There are notable antimicrobial (Gar et al., 2008; Luo et al., 2008), antineoplastic (Williams et al., 2008), antimutagenic (Szabová and Mikulášová, 1999), anti-inflammatory (Kalia et al., 2007), antiulcer (Dillon et al., 2003), anticonvulsant (Morgant et al., 2000), antidiabetic (Vančo et al., 2008), antioxidant (Vančo et al., 2008) and radioprotective (Abou-Seif et al., 2003) activities of copper complexes, which may be of pharmacological relevance.
Newly synthesized copper complexes of 2-methylthionicotinate with bioactive ligands had inhibitory effects against *Staphylococcus aureus* (Dudová et al., 2001). The synthesis of nucleic acids was more affected than that of proteins during *Serratia marcescens* cells cultivation in the presence of the tested compounds. These compounds also did not increase the number of *Salmonella typhimurium*TA 98 and TA 100 revertants (Dudová et al., 2001). The inhibition by these compounds of *Alternaria alternata* sporulation, and morphological changes of *Botrytis cinerea* were observed in addition to their antifungal activity (Dudová et al., 2002; Segľa et al., 2004). In this work, we tested the effect of these compounds on the pathogenic fungus Candida albicans.

Materials and Methods

Antifungal activity of the tested compounds on *Candida albicans* CCM 8186 was determined by the macro-dilution method using Sabouraud broth (Hudecová et al., 1996); final concentrations of the compounds were in the range of 0.1 to 5 mmol·L^{-1}, final concentration of dimethyl sulfoxide (used as a solvent) was 1 %. The intensity of growth was determined by turbidity measurements; the inhibition effects were characterised by IC_{50} and MIC values, specific growth rates, these being calculated from the toxicity curves.

Changes in the membrane permeability during the incubation *Candida albicans* cells in the presence of the tested compounds were monitored by several methods. The amount of protein released into the extracellular space was determined by Lowry method (Lowry et al., 1951), vital staining was performed using methylene blue (Betina et al., 1987), fluorescent dyes Hoechst 33342 and propidium iodide, respectively.

Modulation of the cytotoxic effect of the tested compounds by the addition of ascorbic acid (final concentrations were 1, 7 and 15 mmol · L^{-1}) was performed using the modified procedure of Jantová et al. (2000). Intensity of membrane lipid peroxidation was determined by measuring concentrations of the thiobarbituric acid-reactive substances (TBARS) via malondialdehyde (MDA) $\varepsilon_{532} = 156\,000\ mol^{-1} \cdot L \cdot cm^{-1}$ (Kiššová et al., 2006). Reactive oxygen species, generated in *Candida albicans* cells during cultivation in the presence of the tested compounds and ascorbic acid, were detected using the fluorescent dye dichlorodihydrofluorescein diacetate (DCFH-DA) (LeBel et al., 1992).

Results and Discussion

$[Cu(2\text{-}MeSNic)_2(H_2O)_2]$ and $[Cu(2\text{-}MeSNic)_2(MeNia)_2(H_2O)_2] \cdot H_2O$ had higher anti-yeast effects against *Candida albicans* in the comparison to 2-MeSnicH or MeNia, respectively (Table. 3). Prolonged lag phase and reduced maximal number of cells in the stationary phase were observed in the presence of both complexes. Also, generation time had increased and specific growth rate had decreased proportional to the concentrations of tested compounds (Fig. 1A). If $[Cu(2\text{-}MeSNic)_2(MeNia)_2(H_2O)_2] \cdot H_2O$ (final concentrations were 2.5 and 5 mmol · L^{-1}) was added to *Candida albicans* cells in the exponential phase, this had static effect on the replication of cells, or even, a decrease in absorbance indicated cell lysis, was observed (Fig. 1B).

Based on these observations, the suspension of *Candida albicans* cells has been incubated in the presence of copper complexes. Then, proteins in the extracellular space as marker of change permeability membranes were determined. $[Cu(2\text{-}MeSNic)_2(MeNia)_2(H_2O)_2] \cdot H_2O$ (final concentration of 1 mmol · L^{-1}) caused the leak of almost a quarter of proteins after 1.5 hours incubation and four fifths of released proteins were in the extracellular space of *Candida albicans* cells after 24 hours of incubation (Tab. 1.).

The cells obtained under the conditions mentioned above had been subjected to two staining techniques. The combination of fluorescent dyes Hoechst 33342 and propidium iodide is used in detection of cells with damaged membranes. The results (Tab. 2) show that there had been 70 % of *Candida albicans* cells with damaged membranes after 24 hours incubation in the presence of $[Cu(2\text{-}MeSNic)_2(MeNia)_2(H_2O)_2] \cdot H_2O$ (1 mmol · L^{-1}). However, methylene blue had stained 50 % of the cells in parallel staining. So, there were cells with damaged membranes but maintaining vital functions in the culture.

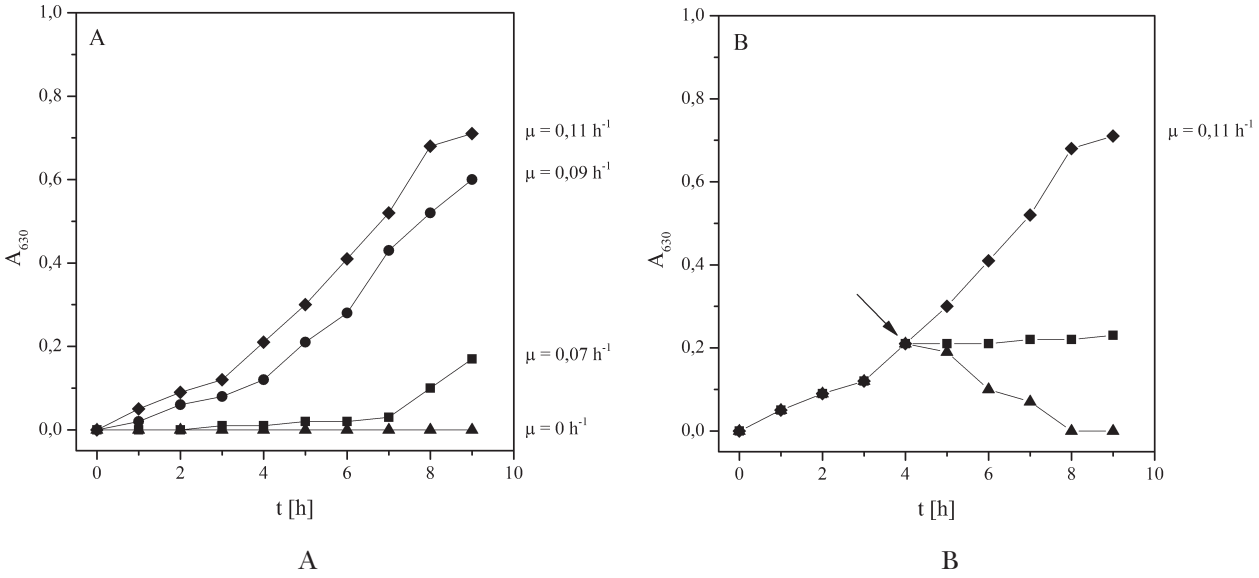

Fig. 1. Growth of *Candida albicans* in the presence of $[Cu(2\text{-}MeSNic)_2(MeNia)_2(H_2O)_2] \cdot H_2O$ (final concentrations: ▲ 5, ■ 2.5, ● 1.25 and ♦ 0) added during lag (A) or exponential (B) phase of growth; right of the chart, the values of the specific growth rate.

Tab. 1. Amount of released proteins after incubation of *Candida albicans* in the presence of selected compounds.

Compound	Concentration of proteins					
	1.5 h		6 h		24 h	
	[μg · mL^{-1}]	%*	[μg · mL^{-1}]	%*	[μg · mL^{-1}]	%*
Control (1 % DMSO)	31.46 ± 0.44	9	38.45 ± 0.50	11	41.95 ± 0.55	12
[Cu(2-MeSNic)$_2$(H$_2$O)]$_2$ [2.5 mmol · L^{-1}]	80.51 ± 1.12	15	96.86 ± 1.34	28	166.11 ± 2.30	81
[Cu(2-MeSNic)$_2$(MeNia)$_2$(H$_2$O)$_2$] · H$_2$O [1 mmol · L^{-1}]	91.05 ± 1.26	23	106.86 ± 1.48	35	167.20 ± 2.31	82
Amphotericin B [0,21 mmol · L^{-1}]	83.41 ± 1.17	20	88.68 ± 1.23	29	99.77 ± 1.38	35

*% — percentage referred to the amount of proteins released from the cells during autoclaving (120 kPa, 20 min).

Tab. 2. Ratios of stained *Candida albicans* cells after 24 hour incubation in the presence of selected compounds.

Compound	Hoechst 33342	Propidium iodide	Methylene blue	
			unstained	stained
Control (1 % DMSO)	79 %	21 %	84 %	16 %
[Cu(2-MeSNic)$_2$(H$_2$O)]$_2$ [2.5 mmol · L^{-1}]	61 %	39 %	66 %	34 %
[Cu(2-MeSNic)$_2$(MeNia)$_2$(H$_2$O)$_2$] · H$_2$O [1 mmol · L^{-1}]	30 %	70 %	51 %	49 %
Amphotericin B [0,21 mmol · L^{-1}]	48 %	52 %	63 %	38 %

Tab. 3. Antifungal activity of copper complexes characterised by IC$_{50}$ and MIC values [mmol · L^{-1}] in the presence of ascorbic acid.

Compound	Ascorbic acid [mmol · L^{-1}]							
	0		1		7		15	
	IC$_{50}$	MIC	IC$_{50}$	MIC	IC$_{50}$	MIC	IC$_{50}$	MIC
2-MeSNicH	> 5	> 5	4.35	> 5	3.42	> 5	2.69	> 5
[Cu(2-MeSNic)$_2$(H$_2$O)]$_2$	3.25	5 s	2.89	5 s	1.82	2.5 s	0.75	1 s
[Cu(2-MeSNic)$_2$(MeNia)$_2$(H$_2$O)$_2$] · H$_2$O	1.78	2.5 s	1.75	2.5 s	0.73	1 s	0.25	0.5 s
Cu^{2+} (CuSO$_4$)	3.44	> 5	1.43	2.5 s	0.60	1 s	0.35	1 s
MeNia	> 5	> 5	> 5	> 5	> 5	> 5	3.38	> 5
Amphotericin B	$4 \cdot 10^{-4}$	$1 \cdot 10^{-3}$	–	–	–	–	–	–

s — fungistatic effect, c — fungicidal effect, inactive compound IC$_{50}$> 5 mmol · L^{-1}, — unavailable.

It is clear that copper complex interacts with and effects function of the membranes. This hypothesis may be supported by R value 0.003 for [Cu(2-MeSNic)$_2$(Nia)$_2$(H$_2$O)$_2$] and *Serratia marcescens* (where Nia is nicotinamide; Dudová et. al., 2001). R value less than one is characteristic for biologically active substances that interfere with energy metabolism cells directly or indirectly by affecting the integrity of biological membranes (Majtán and Drobnica, 1982; Miko et al., 1991; Miko and Devínsky, 1993a; Miko and Devínsky, 1993b). The intense ramification of *Botrytis cinerea* hyphae in the presence of [Cu(2-MeSNic)$_2$(MeNia)$_2$(H$_2$O)$_2$] · H$_2$O was observed. The hyphal growth unit (total length of a hyphae or mycelium to the number of tips) characterizing the branching was 3.7 times lower compared to control (unpublished results). Branching of *Botrytis cinerea* hyphae is stimulated by compounds active at the membrane (Betina, 1981).

The permeabilization of the membrane can occur by different mechanisms. One of them is the oxidative damage. Copper ions can enter the cyclic oxidation-reduction reactions under reducing conditions and there are generated free radicals under these conditions (Ďuračková, 1998). Ascorbic acid (used as reducing element) has intensified the anti-yeasts activity of the tested complexes (Tab. 3.). If *Candida albicans* cells have been cultivated with ascorbic acid (the final concentration of 15 mmol · L^{-1}) and [Cu(2-MeSNic)$_2$(MeNia)$_2$(H$_2$O)$_2$] · H$_2$O simultaneously, then IC$_{50}$ value of this copper complex has 7-fold declined to the point of 0.25 mmol · L^{-1}.

As a result of the induced oxidative stress, the reactive oxygen species can be formed in the extracellular space, on surface membrane di-

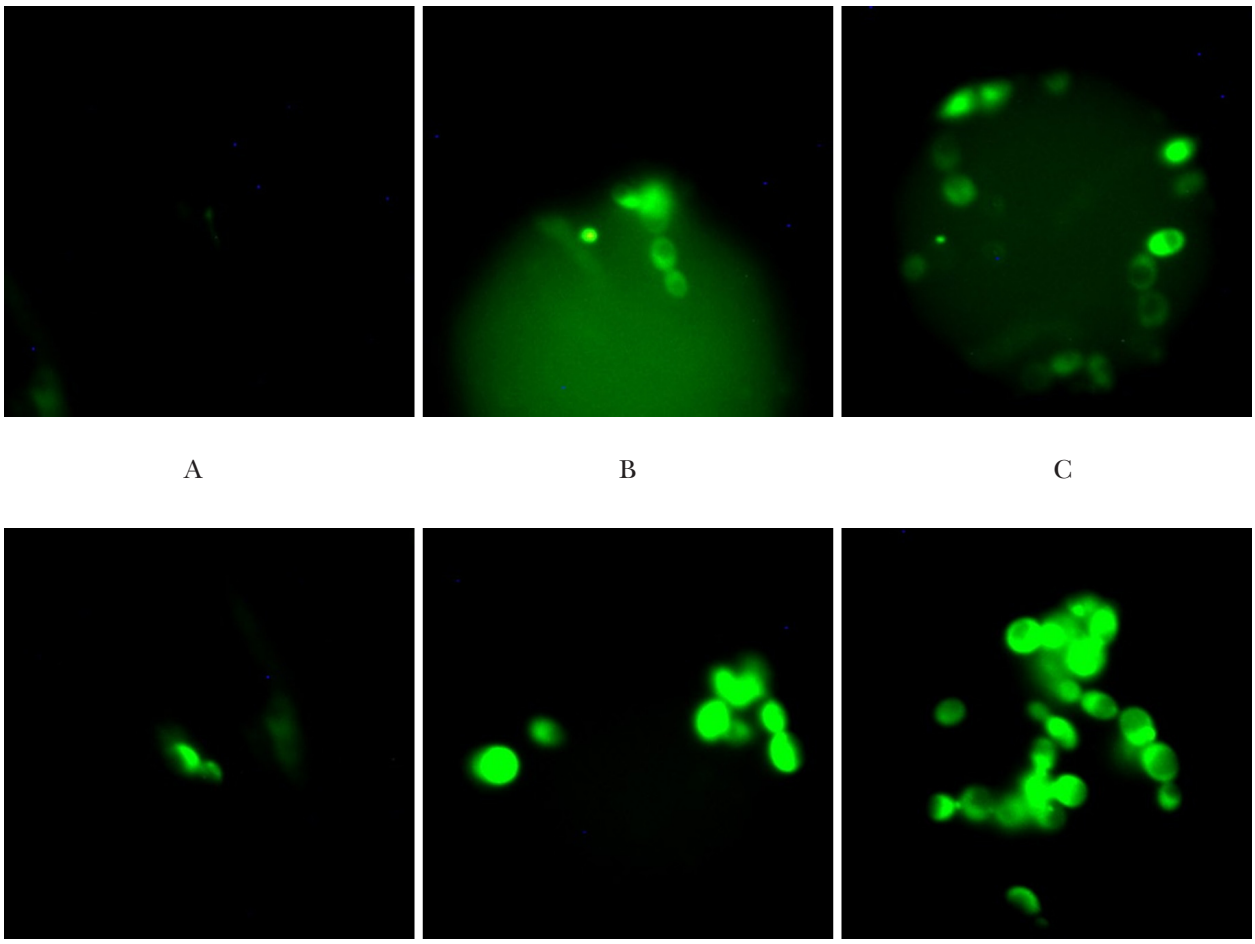

Fig. 2. Comparing of intracellular ROS production (visualization by DFC) in *Candida albicans* cells in the absence (A, B, C) and in the presence (D, E, F) of AA (15 mmol · L^{-1}) and compounds (0.1 mmol · L^{-1}): $[Cu(2\text{-}MeSNic)_2(H_2O)]_2$ (B, E), $[Cu(2\text{-}MeSNic)_2(MeNia)_2(H_2O)_2] \cdot H_2O$ (C, F) and control (1 % DMSO) (A, D).

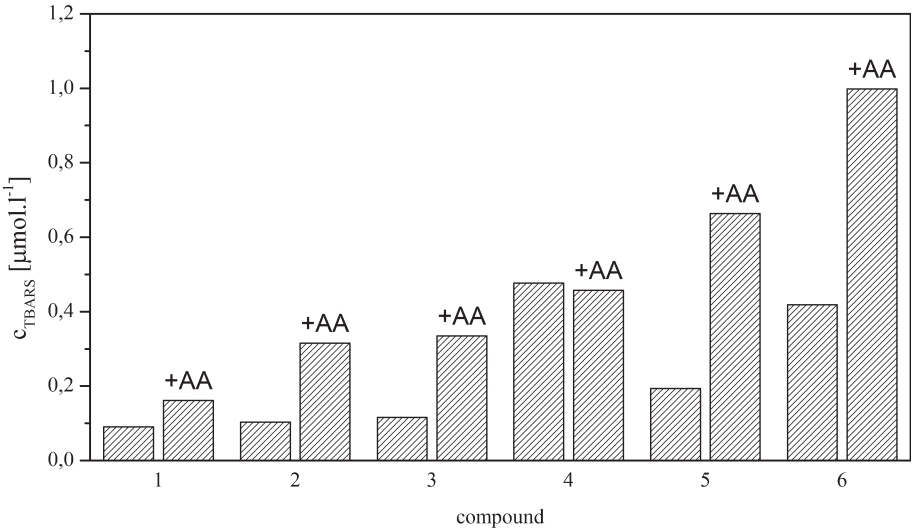

Fig. 3. Increase of TBARS (MDA) concentration after 3 hours incubation *Candida albicans* cells in the presence of the tested compounds (final concentration 0.1 mmol · L^{-1}) and AA (final concentration 15 mmol · L^{-1}); 1 — control (1 % DMSO), 2 —2-MeSNicH, 3 — MeNia, 4 — $CuSO_4$, 5 — $[Cu(2\text{-}MeSNic)_2(H_2O)]_2$, 6 — $[Cu(2\text{-}MeSNic)_2(MeNia)_2(H_2O)_2] \cdot H_2O$.

rectly, or within the cells. After the exposure of yeast cells to $[Cu(2\text{-}MeSNic)_2(H_2O)_2]$ and $[Cu(2\text{-}MeSNic)_2(MeNia)_2(H_2O)_2] \cdot H_2O$ (final concentration of 0.1 $mmol \cdot L^{-1}$) in the presence or absence of ascorbic acid for 6 hours, cells were stained with 2´,7´-dichlorofluorescein diacetate. This probe was converted by reactive oxygen species in the cells to highly fluorescent 2´,7´-dichlorofluorescein (Fig. 2.). Production of endogenous reactive oxygen species in the presence of copper complexes was also observed in other types of cells, such as cell lines (Paulíková et al., 2008) or algae (Morelli and Scarano, 2004).

Lipid peroxidation of *Candida albicans* cells membrane was quantified by determination of TBARS. Data showed that the presence of ascorbic acid caused an increase of the MDA concentration in the case of all tested compounds (except $CuSO_4$) (Fig. 3). The highest level of lipid peroxidation according to production of intercellular reactive oxygen species was found in the presence of $[Cu(2\text{-}MeSNic)_2(H_2O)_2]$ and $[Cu(2\text{-}MeSNic)_2(MeNia)_2(H_2O)_2] \cdot H_2O$ (Fig. 2).

Conclusion

The anti-yeast activity of 2-MeSNicH is increased by preparing of copper complex and by adding bioactive ligand MeNia in to the molecule. Copper complexes$[Cu(2\text{-}MeSNic)_2(H_2O)_2]$ and $[Cu(2\text{-}MeSNic)_2(MeNia)_2(H_2O)_2] \cdot H_2O$ interact and affect the function of yeast cell membrane. Induction of oxidative stress potentiates the inhibitory effect of copper complexes via formation of intracellular reactive oxygen species and lipid peroxidation.

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