The Effect of Neonicotinoid Insecticide Thiacloprid on the Structure and Stability of DNA

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Summary

The application of pesticides and chemical fertilizers constitutes a potential risk to human and animals due to the presence of their residues in the food. Thiacloprid belongs to a group of neonicotinoid insecticides. It shows a cytotoxic/cytostatic effect in human peripheral blood lymphocytes probably due to DNA damage. The use of thiacloprid is increasingly widespread worldwide, therefore is very important the assessment of its possible genotoxic and cytotoxic effects on a living organism. That is the reason why we studied the thiacloprid influence on the structure and stability of DNA in presented work. We have been studied the thiacloprid interaction with calf thymus DNA. Association constant was determined by fluorescence spectroscopy using equilibrium receptor-ligand binding analysis. The thermal denaturation of DNA was used to identify the mode of interaction. Viscosity changes were recorded to confirm/disconfirm the intercalation mode of interaction. Given the results, we can conclude that neonicotinoid pesticide thiacloprid destabilizes DNA. It changes the structure and stability of DNA through binding into the minor groove by hydrophobic or hydrogen interactions.

Key words

Thiacloprid • Interaction • DNA • Spectroscopy • Denaturation of DNA • Viscosity

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Introduction

The introduction of modern inputs, such as pesticides and chemical fertilizers, has substantially increased agricultural productivity, quantity, quality and prolonging the lifetime of food and fodder plants. Pesticides have also revolutionized the fight against endemic diseases in developing countries. Unfortunately, there is a reverse side to this coin: these agents pose a potential risk to humans and animals due to presence of their residues in the food (Elbert *et al.* 2001).

Thiacloprid (TCL, Fig. 1) (3-(6-chloro-3-pyridinylmethyl)-2-thiazolodinylidene)cyanamide) (CAS Number 111988-49-9) is an active component of the pesticide preparation Calypso 480SC.

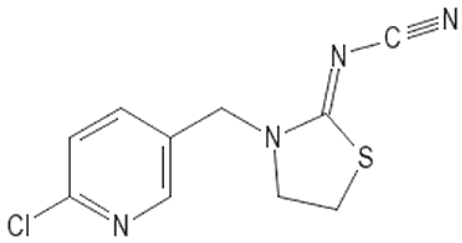

Fig. 1. Chemical structure of thiacloprid

TCL was developed for use on agricultural crops to control insects, especially aphids and little flies. It is protection product used on oilseed rape, fruit trees, vines, vegetables, potatoes and ornamental woods against mammalian and insect pests and flies (Elbert *et al.* 2001, Yu *et al.* 2007, Simon-Delso *et al.* 2015). TCL molecule

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is polar, white powder of TCL is soluble in water, organic solvents such as acetone, dichloromethane, dimethylsulfoxide and ethanol. Solubility is not affected by pH because TCL is stable in pH range 4-9. The low partition coefficient (log P = 1.26) indicates its poor fat solubility and low absorption and distribution in the body (USEPA 2003).

TCL belongs to a group of neonicotinoid insecticides, especially to the chloronicotinyl insecticides (Elbert *et al.* 2001). Neonicotinoid insecticides are systematic insecticides that bind to insect´s nicotinic acetylcholine receptors (nAChR), causing abnormal excitation and leading to the death of insects through convulsive paralysis (Brown *et al.* 2006). Honey bees may also come into contact with these insecticides through the collection and feeding of contaminated nectar, pollen, and water. Many studies have found residues of neonicotinoid insecticides such as imidacloprid, thiamethoxam, and TCL in pollen, honey, and beeswax (Codling *et al.* 2016, Tosi *et al.* 2018). Several studies have investigated the effects of sublethal concentrations of TCL on honey bees, demonstrating negative impacts: on immunocompetence (Brandt *et al.* 2016), learning behaviour (Tison *et al.* 2017, De Lima *et al.* 2017), homing performance (Tison *et al.* 2016), and survival under pathological stress (Doublet *et al.* 2015). On the contrary, to some extent, TCL can be expeditiously detoxified by honey bees, and thus is classified safe for bees (Iwasa *et al.* 2004).

TCL is insecticide that enters the body through intimate contact or ingestion. Its mechanism of action is based on disruption of controlled synaptic transmission by irreversible binding to the nicotine acetylcholine receptor and attacks the nervous system of insects (Yu *et al.* 2007). Neonicotinoids have low affinity for nAChR of vertebrates at physiological pH, because they are not protonated. As a result, they were supposed to show low acute and chronic toxicity in mammals, fish and birds, predominantly singers and poultry (Singla and Sandhu 2015). Nevertheless, it has been shown that TCL exhibits a relatively high level of acute toxicity for fish (Osterauer and Köhler 2008). After ingestion, TCL is rapidly absorbed, widely distributed inside the body, extensively metabolized and secreted in the urine. The target organ is the liver (Barden 2001).

Significant DNA damage was observed for *Eisenia fetida*, we can predict that TCL may have a harmful effect on earthworms (Feng *et al.* 2015). Some research groups reported that TCL showed a cytotoxic/cytostatic effect in human peripheral blood lymphocytes (Kocaman *et al.* 2014) and bovine peripheral lymphocytes (Galdiková *et al.* 2015) most probably due to the increased levels of DNA damage and that this effect resulted probably from the inhibition of DNA synthesis and cell proliferation. Because the use of TCL has become increasingly widespread throughout the world, the assessment of its possible genotoxic and cytotoxic effects on living organisms is very important. For this reason, we have aimed at TCL influence on the structure and stability of DNA. This interaction was studied by using fluorescence spectroscopy, absorption spectroscopy with Peltier module and viscosity measurements.

Methods

Materials and solution preparation

TCL and calf thymus DNA were obtained from Sigma-Aldrich (Germany). Stock solution of TCL ($5 \cdot 10^{-3}$ mol/l) was prepared in 100 % ethanol, which was purchased from Sigma-Aldrich (Germany). DNA ($3 \cdot 10^{-3}$ mol/l) was dissolved in Tris-EDTA buffer (TE, 10 mmol/l Tris and 1 mmol/l EDTA), pH = 7.4 by stirring and held at 4 °C. Ethidium bromide (EtBr) basic solution in water was purchased at concentration $1.3 \cdot 10^{-2}$ mol/l from Sigma-Aldrich (Germany).

UV-Vis studies

UV-Vis measurements were made on absorption spectrophotometer Cary 60 UV-Vis Agilent Technologies using quartz cuvettes of 1-cm path length at room temperature. UV-Vis titration were performed by constant concentration of TCL ($2 \cdot 10^{-5}$ mol/l), concentration of DNA was changed in ratio $2 \cdot 10^{-5} – 10^{-4}$ mol/l.

Fluorescence measurements

Fluorescence experiments were carried out at room temperature on spectrofluorimeter RF – 5301 PC Shimadzu. The bandwidth of excitation slit was set at 5 nm and emission slit at 3 nm. The emission spectra at different DNA concentration ($3 \cdot 10^{-5}$- $4.8 \cdot 10^{-4}$ mol/l) and fixed concentration of TCL ($3 \cdot 10^{-5}$ mol/l) were recorded from 480 to 510 nm at room temperature and the excitation wavelength of 245 nm in Tris-EDTA buffer, pH 7.4. The fluorescence intensity was monitored at 493 nm. The fluorescence quenching data were plotted according to the Stern-Volmer equation

$$\frac{F_0}{F} = 1 + K_{SV}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and in the presence of the quencher (Q) (Turro 1978). Then the binding parameters such as association constant (K_a) and the number of binding sites (n) were calculated according to Hill equation (Min *et al.* 2004, Tian *et al.* 2004)

$$\frac{\log(F_0 - F)}{F} = \log K_a + n \log[Q] \quad (2)$$

where F_0 and F are the fluorescence intensities of TCL in the absence and presence of quencher (DNA), $[Q]$ is the total DNA concentration. By the plot of $\log(F_0 - F)/F$ versus $\log[Q]$, the number of binding sites n and association constant can be obtained.

Viscosity tests

The viscosity of DNA solution was measured at 298.1 ± 0.2 K using Ostwald viscometer and thermostatic water bath. DNA/EtBr, DNA/TCL solutions were prepared in the Tris-EDTA buffer (pH = 7.4) as the solvent and the flow times of pure DNA ($5 \cdot 10^{-5}$ mol/l) and their mixtures at various ratios ($2 \cdot 10^{-5}$ – $1.3 \cdot 10^{-4}$ mol/l) through a capillary were read by use a digital stopwatch with an accuracy of ± 0.02 s. To accurately evaluate the average relative viscosity of the samples, the mean values of ten replicate measurements were used. The data were presented as (t/t_0) versus concentration of DNA and ligand (TCL or EtBr) ratio (Sahoo *et al.* 2008).

DNA melting studies

Thermal stability of DNA was recorded on absorption spectrophotometer Cary 60 UV-Vis Agilent Technologies connected with Peltier module. The melting temperatures (T_m) of pure DNA ($3 \cdot 10^{-5}$ mol/l) and DNA/TCL complexes (1/1, 1/2 and 1/3) were measured by monitoring the 260 nm absorption intensities of samples at a variety of temperatures. Concentration of TCL were changed from $3 \cdot 10^{-5}$ to $9 \cdot 10^{-5}$ mol/l. Temperature was increased with step 5 °C by this experimental arrangement. Temperature range of measurement was 25-95 °C. The samples were filtrated on MillexGV (0.22 μm) before measurement. Melting curves were fitted by Van´t Hoff equation (Pace 1990)

$$A = A_{min} + \frac{A_{max} - A_{min}}{1 + e^{\left[\frac{\Delta H}{R} x \left(\frac{1}{T} - \frac{1}{T_m}\right)\right]}} \quad (3)$$

where A is absorbance, A_{min}, A_{max} are minimal and maximal measured absorbance, respectively, H is enthalpy of transition, R is gas constant, T, T_m are actual temperature and melting temperature.

All spectra and graphs were evaluated in Origin 6.0 and 8.0 and Grafit programs.

Results

UV-Vis studies

Fig. 2 presents the UV-Vis absorption spectra. The absorption band of TCL was observed in the UV region with maximum at approximately 245 nm.

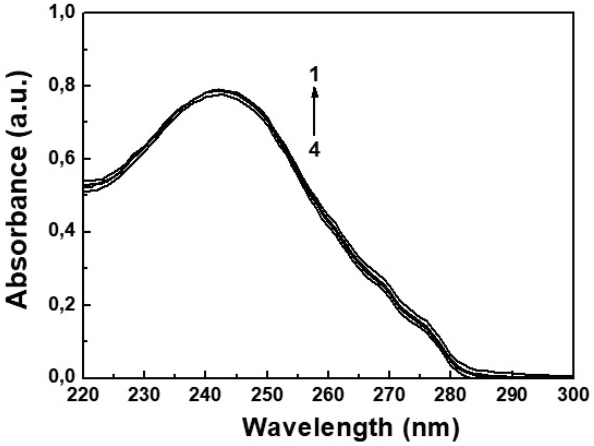

Fig. 2. Absorption spectra of TCL ($2 \cdot 10^{-5}$ mol/l) after addition of DNA ($2 \cdot 10^{-5}$ – 10^{-4} mol/l) in Tris-EDTA buffer pH 7.4, lines 1 – 4: c (TCL/DNA) = 1/0, 1/1, 1/2 1/5, respectively. The arrow indicates the absorbance changes with increasing of DNA concentration.

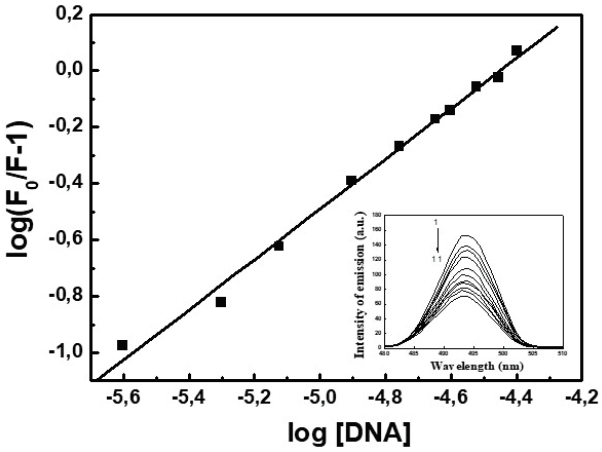

Fig. 3. Hill plot of DNA quenching effect on TCL fluorescence. Insert: Fluorescence spectra of TCL in the absence and presence of DNA; c (TCL) = $3 \cdot 10^{-5}$ mol/l and c (DNA) = $3 \cdot 10^{-5}$ – $4.8 \cdot 10^{-4}$ mol/l, lines 1 – 11: c (TCL/DNA) = 1/0, 1/1, 1/2, 1/3, 1/5, 1/7, 1/9, 1/10, 1/12, 1/14, 1/16, respectively. λ_{exc} = 245 nm, λ_{em} = 480 – 510 nm

We observed a very slight increase in the absorbance intensity with growing of DNA concentration.

Fluorescence measurements

For this study, the fluorescence behaviour of TCL has been monitored. Fig. 3 (insert) displays the fluorescence emission spectra of TCL in presence of increasing concentration of DNA. The emission maximum of TCL at 493 nm gradually decreased. The quenching of fluorescence intensity of TCL on addition of DNA is indicative of an interaction. A quantitative estimation of the quenching in terms of the fluorescence-quenching constant was analysed using Stern-Volmer equation (1). The representative plot of F_0/F versus [Q] (not shown) of the present system shows a linear growth of the curve with addition of DNA. The quenching constant (K_{SV}) was found to be $2.8 \cdot 10^4$ l/mol and correlation coefficient for the K_{SV} value was R = 0.998.

Subsequently, the values of K_a and number of binding sites n were determined by linear regression of $\log(F_0/F-1)$ versus $\log[Q]$. This dependence represents Hill plot (Fig. 3) expressed by equation (2). The calculated value of association constant K_a was $9.3 \cdot 10^3$ l/mol, correlation coefficient for the K_a value was R = 0.996 and number of binding sites was set at approximately 1 (n = 0.89). Results obtained from fluorescence measurements are summarized in Table 1.

Viscosity tests

Further information on the nature of the interaction can be obtained through hydrodynamic studies. This includes viscometric measurements.

We have been measured the variation in relative viscosity of DNA/EtBr and DNA/TCL complexes. In the viscosity curve in Fig. 4, we detected a significant increase in viscosity for EtBr and not considerably change upon adding TCL into DNA.

DNA melting studies

When DNA solutions are exposed to extremes of pH or heat, the double helical structure of DNA undergoes a transition into a random single-stranded form at the melting temperature. The melting temperature can be also altered by the interaction of DNA with small molecules (Ma *et al.* 2012). We have proved our results by denaturation of pure DNA and DNA/TCL complexes in ratios 1/1, 1/2, 1/3 by absorption spectroscopy connected with a Peltier module. Melting curves that can be seen in Fig. 5 show a destabilizing effect of the TCL on DNA. They were fitted using Van´t Hoff equation (3) to obtain main thermodynamic characteristics (T_m, T, ΔT, ΔH).

Melting temperature T_m is shifted about 2.6 °C from pure DNA (64.5 °C) to the complex DNA/TCL in 1/2 concentration ratio (61.9 °C). Also Van´t Hoff enthalpy ΔH is decreasing from 413.6 kJ/mol (pure DNA) to 390.6 kJ/mol (DNA/TCL = 1/2) (Table 2). Measurements for complex DNA/TCL in 1/3 (Table 2) shows a two-phase character.

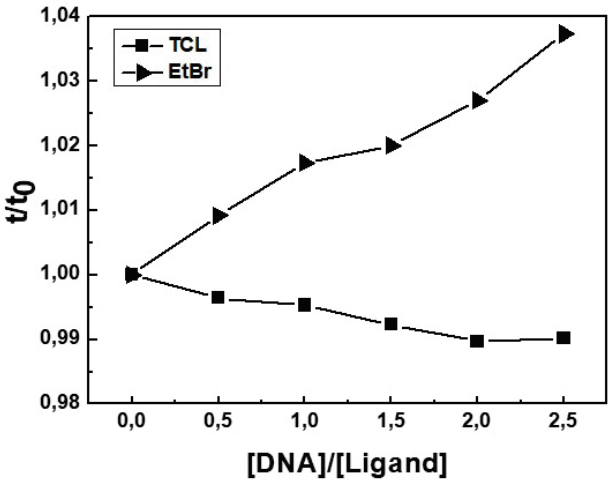

Fig. 4. Plot of flow time t/t_0 versus [*DNA*]/[*ligand*], where t is the flow time of the complex DNA/ligand (TCL or EtBr, respectively) and t_0 is the flow time of DNA. c (DNA) = $5 \cdot 10^{-5}$ mol/l, c (ligand) = $2 \cdot 10^{-5}$ – $1.3 \cdot 10^{-4}$ mol/l; [*DNA*]/[*ligand*] = 1/0, 1/0.5, 1/1, 1/2, 1/2.5

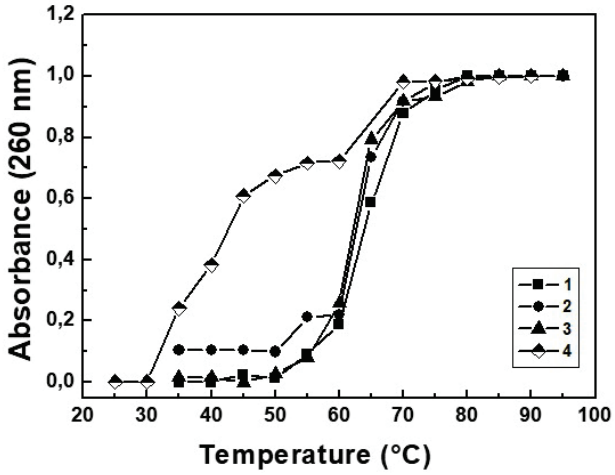

Fig. 5. Normalized melting curves of pure DNA (1) and complexes TCL/DNA in ratio 1/1 (2), 1/2 (3), 1/3 (4). c (DNA) = $3 \cdot 10^{-5}$ mol/l and c (TCL) = $3 \cdot 10^{-5}$ – $9 \cdot 10^{-5}$ mol/l

Table 1. Quenching constant (K_{SV}), association constant (K_a) and number of binding sites (n) for the interaction of TCL with DNA at room temperature

T (K)	K_{SV} (l/mol)	R[a]	K_a (l/mol)	R[b]	n
298	2.8·104 ± 0.0467	0.998	9.3·103 ± 0.1268	0.996	0.89 ± 0.0261

[a] Correlation coefficient for the K_{SV} value, [b] Correlation coefficient for the K_a value

Table 2. Thermodynamic characteristics determined from the melting curves of pure DNA and complexes DNA/TCL

	1st phase					2nd phase				
	T_m (°C)	T_1 (°C)	T_2 (°C)	ΔT (°C)	ΔH (kJ/mol)	T_m (°C)	T_1 (°C)	T_2 (°C)	ΔT (°C)	ΔH (kJ/mol)
DNA	64.5	56.2	73.6	17.4	413.6					
DNA/TCL 1/1	63.4	56.2	71.7	15.5	396.1					
DNA/TCL 1/2	61.9	56.4	68.0	11.6	390.6					
DNA/TCL 1/3	36.6^{AT}	25.2	50.1	24.9	200.4	63.2^{GC}	58.9	69.9	11	315.4

[AT] T_m for AT pairs, [GC] T_m for GC pairs

Discussion

Small molecules, such as pesticides, bind to the double helix of DNA by two dominant modes, referred to as groove binding and intercalation. Groove binding involves docking the thin ribbon-like molecules in the DNA minor groove, in close proximity to the sugar-phosphate backbone. In contrast, intercalation of small molecules into the helix involves the insertion of the drug – usually a planar aromatic cation – into the base stack of the helix (Kumar *et al.* 1993). When a molecule is intercalated between base pairs of nucleic acids, usually a marked substantial hypochromicity of the absorption maximum is observed (Sahoo *et al.* 2008). We recorded although slight but still an increasing TCL absorption spectra when DNA was added. The method of absorption spectroscopy is a bulk method and the changes in our experimental absorption spectra were very weak. It was unclear to conclude if TCL interacts with DNA. Therefore, we have used the fluorescence spectroscopy to study the effect of TCL on DNA molecule.

Fluorescence spectroscopy can be used to clarify the binding characteristics of the chromophore with other molecules (Modukuru *et al.* 2005). An enhanced fluorescence is believed to be one of the criteria for intercalative binding (Sehlstedt *et al.* 1994, Zupan *et al.* 2004). The classical intercalator EtBr is known to exhibit significant fluorescence enhancement on binding to double-stranded DNA (dsDNA) (Bresloff and Crothers 1981, Olmsted and Kearns 1977). On the other hand, the interaction between agent naproxen and dsDNA in a groove-binding mode causes a strong fluorescence quenching (Ye *et al.* 2005). Thus it is apparent that further experiments were required to determine the mode of binding since no conclusion may be drawn based on fluorescence spectral behaviour alone.

Comparing the TCL association constant with typical DNA intercalators such as EtBr (K_a = $1.4 \cdot 10^6$ l/mol) (Chen *et al.* 2012) or danthron (K_a = $7.2 \cdot 10^6$ l/mol) (Gholivand *et al.* 2011) and drugs binding by weak electrostatic forces to the surface of DNA as they are naringin (K_a = $3.1 \cdot 10^3$ l/mol) (Jourdan *et al.* 1985) and vincristine ((K_a = $1.0 \cdot 10^3$ l/mol) (Tyagi *et al.* 2010) we can claim that association constant of our pesticide does not point an intercalation mode and binding via electrostatic forces as well. We can suppose that TCL binds to the DNA groove, since the value of the association constant corresponds to this type of binding (Gholivand *et al.* 2011).

The hydrodynamic method, which is rather sensitive to variations in the length of the DNA molecule, can be the most effective method for studying the DNA-

complex binding modes without requiring X-ray crystallography or structural NMR data (Li *et al.* 2006). In classical intercalators the length of DNA helix is increased, given that base pairs are distanced to accommodate the interacting species. This increases the viscosity (Kashanian *et al.* 2010). On the other hand, the partial and non-classical intercalations may bend the DNA helix, and hence lower its length and consequently the viscosity. Further, the molecular binding in the DNA grooves causes less marked positive or negative changes in the viscosity of the solution (Kelly *et al.* 1985, Sahoo *et al. 2008*). Result of viscometric measurement of DNA/TCL complexes is the stagnancy/slight decrease in viscosity. We recorded a significant change in viscosity behavior as a result of TCL addition to the DNA solution. Viscometric measurements confirm the observation we have given from previous measurements and finally we can claim that TCL does not interact with DNA through intercalation mode but by binding into the groove.

Usually, classic intercalation of small molecules into the double helix causes stabilization of base stacking and leads to a significant increase of T_m (approximately 5-8 °C), whereas non-intercalation (e.g. groove or electrostatic) binding induces no obvious increase in T_m (Sun *et al.* 2011). From our measurements it is obvious that melting temperature increment was smaller than 5 °C (2.6 °C), and therefore it can be deduced that TCL interacted with DNA via non-classic intercalation. This interaction can be realized via groove or electrostatic binding mode. Decrease of Van´t Hoff enthalpy means that lower energy is needed for denaturation of 50 % base pairs of DNA in the complex with TCL. The changes in all thermodynamic parameters lead us to a claim that TCL destabilizes the DNA molecule. This destabilization is associated with the groove binding interaction mode (Bi *et al.* 2008). In our experiments the melting curve of the complex DNA/TCL in 1/3 indicates a two-phase character with an expression destabilization of AT regions in DNA. It is generally supposed that small molecules which consist of at least two aromatic rings coupled by non-rigid bond enabling their torsional flexibility, like pesticide TCL bind preferentially into the minor groove of DNA, especially to regions which are rich in AT-base pairs (Lavery and Pullman 1981, Ihmels *et al.* 2005).

In conclusion, the association constant value of TCL leads us to assume that this neonicotinoid pesticide does not bind into the DNA structure by an intercalative mode of interaction. TCL influence on length and denaturation of the DNA macromolecule points to the groove binding mode of interaction. Incorporation of this insecticide occurs probably into DNA minor groove by hydrophobic or hydrogen interactions. The fact that the intercalative mode of interaction represents in higher value of association constant, increase of DNA length, slight decrease in viscosity and thermal stabilization of DNA due to ligand binding (Bi *et al.* 2008) proves our conclusions.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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