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Tulipaline A: Structure–activity aspects as a nematicide and V-ATPase inhibitor

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ABSTRACT

Carbonyl groups are known to form covalent adducts with endogenous proteins, but so far, their nematicidal mechanism of action of has been overlooked. The nematicidal activity of ten lactones was tested *in vitro* against the root knot nematodes *Meloidogyne incognita* and *Meloidogyne arenaria*. In particular, the saturated lactones α-methylene-γ-butyrolactone or tulipaline A (1) and γ-butyrolactone (3) were active against *M. incognita* with an $EC_{50/48h}$ of 19.3 ± 10.0 and 40.0 ± 16.2 mg/L respectively. Moreover the α, β-unsaturated lactone 5,6-dihydro-2H-pyran-2-one (2) exhibited the strongest nematicidal activity against the two species with $EC_{50/48h}$ 14.5 ± 5.3 and 21.2 ± 9.7 mg/L respectively. Here we propose that the toxic effects of lactones and aldehydes on *M. incognita* and *M. arenaria* might be a consequence of their vacuolar-type H^+-ATPase (V-ATPase) inhibition activity; in fact α-methylene-γ-butyrolactone (1) and salicylaldehyde (12) produced an increased pH in lysosomal-like organelles on HeLa human cell line and this alteration was most likely related to a V-ATPase impairment.

1. Introduction

Root-knot nematodes are soil worms belonging to the genus *Meloidogyne* that feed on the roots of different crops such as tomato, pepper, watermelons and onions causing swellings, knots or galls and determining a detrimental effect on the harvest [1]. In the past decades, synthetic nematicides have been phased-out from the market because of their indiscriminative use and the toxicity to non-target organisms eventually leading to environmental pollution problems [2,3]. Currently, to treat invasive nematodes two major categories of nematicides are commercially available in Europe (i.e. carbamates and organophosphorus compounds) whose biochemical target is the nematode nervous system enzyme acetylcholinesterase [2]. Recently, the new nematicide fluensulfone, a thiazole derivative supporting a trifluorobut-3-enylsulfonyl group has gained interest with a new and still unknown mode of action. Furthermore, its ability in controlling root-knot nematodes shows a higher reduction of galling index and nematode eggs if compared with the treatment with conventional nematicides fenamiphos or oxamyl [4]. The limited number of categories of nematicidal agents has prompted a search for compounds preferably of plant origin with alternative modes of action [5–7]. Moreover, in order to prevent or delay the rapid development of resistance, one of the major issues in resistance management strategies has been the rotation of compounds with different modes of action [8]. For all these reasons, the future development of new potent tools to control nematode's infection and resistance represents an important challenge for agriculture.

V-ATPases are ATP dependent proton pumps that exploit the ATP hydrolysis to translocate H^+ from the cytoplasm to the lumen of intracellular compartments or, if located within the plasmalemmal boundaries, to the extracellular milieu. Thus V-ATPase activity selectively controls cytosolic and luminal pH as well as membrane potential, eventually driving various physiological process in eukaryotic cells [9]. In nematodes, the V-ATPase seems to have a key role in nematode biology, mainly in nematode cuticle synthesis, nutrition, osmoregulation and reproduction [10,11]. N-ethylmaleimide dicyclohexylcarbodiimide and omeprazole are unspecific inhibitors of V-ATPase, causing molecular interference inhibition at low micromolar concentrations [12–15], while

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antibiotics such us bafilomycin and concanamycins A-F macrocyclic lactones, archazolid and apicularen are potent and specific inhibitors acting at nanomolar levels [16–18].

In the present study, we firstly used a cellular approach to demonstrate a critical requirement for lactones in V-ATPase function known to be involved in different aspects of the homeostasis. Moreover, we tested the *in vitro* toxicity (vitality assay) and the morphological analysis of V-ATPase by treating HeLa cells with α-methylene-γ-butyrolactone or tulipaline A (1), salicylaldehyde (12), and concanamycin A (17).

Then, the *in vitro* nematicidal activity against *Meloidogyne incognita* and *Meloidogyne arenaria* of some selected lactones along with *N*-dodecanoyl-DL-homoserinelactone (9) and *N*-hexanoyl-DL-homoserinelactone (10), which are small diffusible signaling molecules involved in bacteria *quorum sensing*, was evaluated.

2. Materials and methods

2.1. Chemicals

Analytical standards of tulipaline A (1), 5,6-dihydro-2H-pyran-2-one (2), γ-butyrolactone (3), δ-valerolactone (4), γ-nonalactone (5), γ-ethoxycarbonyl-γ-butyrolactone (6), 4,5-dimethyl-3-hydroxy-2,5-dihydrofuran-2-one (7), whiskey lactone (8), *N*-dodecanoyl-DL-homoserinelactone (9), *N*-hexanoyl-DL-homoserinelactone (10), 3-penten-2-one (11), salicylaldehyde (12), furfural (13), damascenone (14), fosthiazate (15), pyocyanin (16), concanamycin A (17), omeprazole (18), abamectin (19), oubain (20), oligomycin (21) and ascorbic acid (22) were purchased from Sigma–Aldrich, Italy. All solvents and reagents were of pesticide grade.

2.2. V-ATPase assay

HeLa cells used throughout the study were grown in Dulbecco's Modified Eagles's medium (DMEM, D5796, Sigma–Aldrich) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma–Aldrich). Cell samples were allowed to reach 80% confluence and then adherent cells were detached by means of trypsin activity and again seeded, at least in triplicate, for being subjected to the following chemical treatment: salicylaldehyde (12), tulipaline A (1) (both dissolved in sterile water) and concanamycin A (17) (dissolved in sterile DMSO). Experiments were performed both for toxicity evaluation (vitality assay) and for morphological *in vivo* analysis of V-ATPase. In order to assess alive cells, resuspended cells were stained by Trypan Blu and manually counted using a hemocytometer, while any perturbation of the ATPase proton pump was detected by the use of the LysoTracker Yellow HCK-123 in DMSO (Molecular Probes), a selective fluorescent acidotropic for labeling and tracking acidic organelles in live cells [19]. The retention of the fluorescent dye inside spherical organelles include the lysosomal pathway making it as a favorite choice compared to traditionally used neutral red and acridine orange that lack specificity and photostability. Briefly, HeLa cells grown in coverslips were washed several times and incubated at 37 °C for at least 30 min in serum free DMEM medium containing LysoTracker probe at 1 μM concentration. Finally, cells were washed with normal DMEM and observed using a fluorescent microscope.

2.3. Nematode population

A population of *M. incognita* (Kofoid et White) Chitw. and *M. arenaria* were reared on tomato (*Solanum lycopersicum* L.). All plants were maintained in a growth chamber at 25–28 °C, 60% relative humidity and 16 h photoperiod, in plastic pots (18 cm diameter) containing sandy soil (pH 7.2; sand >99%; silt <1%; clay <1% and organic matter = 0.75%). Plants used for inoculations were 7 weeks old, at the five-leaf stage. After 40 days, the plants were uprooted and the roots were washed free of soil and cut into 2 cm pieces. Eggs were extracted according to the sodium hypochlorite procedure [20] and second-stage juveniles (J2) were allowed to hatch in modified Baermann funnels at 28 °C. All J2 hatching in the first 3 days were discarded and thereafter J2 collected, after 24 h were used in the experiments.

2.4. Nematicidal assay

The nematicidal activity of pure compounds, in terms of nematode juveniles' motility suppression, was tested and the EC_{50} values were calculated. Stock solutions of pure compounds were prepared by dilution with methanol or water, whereas working solutions were obtained by dilution with distilled water containing the polysorbate surfactant 20 (Tween-20). Final concentrations of methanol and Tween-20 in each well never exceeded 1 and 0.3% v/v, respectively, because preliminary trials showed that motility of nematodes exposed at those concentration levels was similar to motility of nematodes maintained in distilled water [21]. Distilled water, as well as a mixture of water with methanol and Tween-20 at concentrations equivalent to those in the treatment wells, served as control. Water extracts were diluted with water. Twenty juveniles were used per treatment well in flat bottom 96-well plates (Greiner bio-one). The plates were covered in order to prevent evaporation and were maintained in the dark at 28 °C. Border wells containing plain water with nematodes were placed around the wells of each treatment to check the vapor drift among wells that could possibly interfere with the efficacy results. Juveniles were observed with the aid of an inverted microscope (Euromex, The Netherlands) at 40× magnification after 24, 48 and 96 h and were ranked into two distinct categories: motile or immotile. After the last assessment (48 h), the nematodes were transferred into plain water, after washing in tap water through a 20 μm pore screen to remove the excess of test compounds and they were assessed again after 24 h for the re-obtainment of motility.

2.5. Statistical analysis

Treatments of motility experiments were replicated six times and each experiment was performed twice. The percentages of immotile J2 in the microwell assays were corrected by elimination of the natural death/immotility in the water control according to the formula corrected% = [(mortality% in treatment − mortality% in control)/(100 − mortality% in control)] × 100 and they were statistically analyzed by ANOVA test combined over time. Because ANOVA analysis indicated no significant treatment by time interaction, means were averaged over experiments. Corrected percentages of immotile J2 treated with test compounds were subjected to nonlinear regression analysis using the log–logistic equation proposed by Seefeldt et al.: $Y = C + (D - C)/\{1 + \exp[b(\log(x) - \log(EC_{50}))]\}$, where C = the lower limit, D = the upper limit, b = the slope at the EC_{50}, and EC_{50} = the test compounds concentration required for 50% death/immotility of nematodes after elimination of the control (natural death/immotility) [22]. In the regression equation, the test compounds concentration (%w/v) was the independent variable (x) and the immotile J2 (percentage increase over water control) was the dependent variable (y). The mean value of the six replicates per essential oil and compound concentration and immersion period was used to calculate the EC_{50} value.