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Short Communication

Antiviral activity of tenofovir against *Cauliflower mosaic virus* and its metabolism in *Brassica pekinensis* plants

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ABSTRACT

The antiviral effect of the acyclic nucleoside phosphonate tenofovir (*R*)-PMPA on double-stranded DNA *Cauliflower mosaic virus* (CaMV) in *Brassica pekinensis* plants grown *in vitro* on liquid medium was evaluated. Double antibody sandwich ELISA and PCR were used for relative quantification of viral protein and detecting nucleic acid in plants. (*R*)-PMPA at concentrations of 25 and 50 mg/l significantly reduced CaMV titers in plants within 6–9 weeks to levels detectable neither by ELISA nor by PCR. Virus-free plants were obtained after 3-month cultivation of meristem tips on semisolid medium containing 50 mg/l (*R*)-PMPA and their regeneration to whole plants in the greenhouse. Studying the metabolism of (*R*)-PMPA in *B. pekinensis* revealed that mono- and diphosphate, structural analogues of NDP and/or NTP, are the only metabolites formed. The data indicate very low substrate activity of the enzymes toward (*R*)-PMPA as substrate. The extent of phosphorylation in the plant's leaves represents only 4.5% of applied labelled (*R*)-PMPA. In roots, we detected no radioactive peaks of phosphorylated metabolites of (*R*)-PMPAp or (*R*)-PMPApp.

In contrast to that for RNA viruses, the literature on the use of antiviral chemicals against plant DNA viruses is extremely sparse. While testing of newly synthesised compounds or those found effective in human or animal medicine could bring advances, their effects on DNA plant viruses may be different. Among possibly effective compounds might be acyclic nucleoside phosphonates (ANPs), which have been found to exhibit antiviral activity spectra (De Clercq, 2007). However, there is no information about the metabolism of ANPs in plants.

Cauliflower mosaic virus (CaMV; genus *Caulimovirus*, family *Caulimoviridae*) is a mechanically and aphid transmissible virus reducing yield and quality of brassica crops worldwide. Recently, we developed an *in vitro* method for testing antiviral compounds against plant viruses based on growing *Brassica pekinensis* on liquid medium. In an earlier study, (*R*)-PMPA revealed low phytotoxicity to *B. pekinensis* (Špak et al., 2010). Therefore, we selected *B. pekinensis* and CaMV for the present study with the aim to evaluate the antiviral activity of (*R*)-PMPA on CaMV and its potential for eliminating the virus from plants. We also studied uptake and metabolism of (*R*)-PMPA in *B. pekinensis* to better understand tenofovir's mode of action in plants.

Isolate CaMV 1 (Špak, 1989) was used for this study. Cultivation and inoculation of plants and the experimental scheme for assessing virus inhibition were as described by Špak et al. (2010). Virus inhibition expressed as relative virus concentration in plants was estimated by absorbances at 405 nm in ELISA with CaMV 1 antibodies (Bioreba AG, Switzerland) and statistically evaluated as described by Špak et al. (2010).

Three independent experiments were conducted. The first experiment was repeated twice with 26 plants in total for each (*R*)-PMPA treated (50 mg/l), infected control, and healthy control. Twelve and 14 plants were used for the first and second replicate, respectively. ELISA was conducted in 2-week intervals up to 6 weeks. The second experiment was conducted with 11 plants per (*R*)-PMPA (50 mg/l), infected control, and healthy control. ELISA was conducted in 3-week intervals up to 9 weeks in order to observe the effect of prolonged application of (*R*)-PMPA and regeneration of plants. In the third experiment (*R*)-PMPA was applied at concentrations of 12.5, 25 and 50 mg/l. Groups of 21 plants were used for each concentration, infected control, and healthy control. ELISA was conducted in 3-week intervals up to 9 weeks. All plants in the experiments were treated in the same way, except that infected and uninfected controls were not treated with (*R*)-PMPA.

Treatment of CaMV-infected plants with (*R*)-PMPA at a concentration of 50 mg/l in the first experiment led to significant decrease

Abbreviations: (*R*)-PMPAp, N^6-methyl-(*R*)-9-[2-(phosphonomethoxy)propyl]adenine monophosphate; (*R*)-mePMPA, N^6-methyl-(*R*)-9-[2-(phosphonomethoxy)propyl]adenine; (*R*)-me_2PMPA, N^6-dimethyl-(*R*)-9-[2-(phosphonomethoxy)propyl]adenine; (*R*)-PMPHx, (*R*)-9-[2-(phosphonomethoxy)propyl]hypoxanthine.

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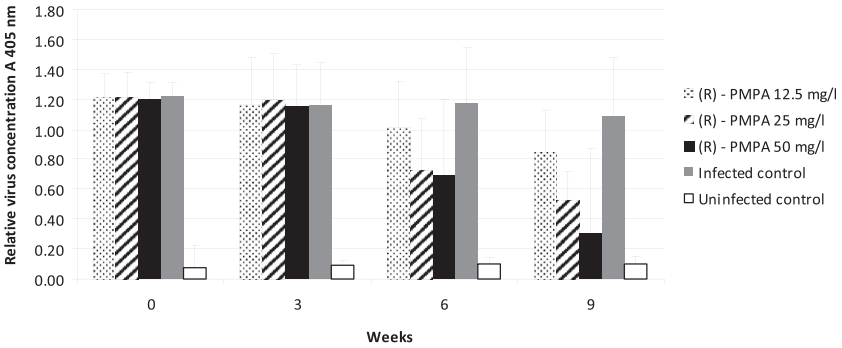

Fig. 1. Statistical evaluation of relative *Cauliflower mosaic virus* concentration in *Brassica pekinensis* cv. Manoko plants. Plants were cultivated for 9 weeks on MS liquid medium supplemented with tenofovir (*R*)-PMPA at concentrations of 12.5, 25 and 50 mg/l. Values represent mean plus SD absorbance at 405 nm in ELISA.

of virus concentration ($P < 0.05$) within 6 weeks compared to the infected control plants. After 6 weeks of treatment, we obtained only 11 plants negative by ELISA from 26 tested plants. Therefore, we decided to prolong the exposure of plants to (*R*)-PMPA in the following experiments.

Prolonging (*R*)-PMPA plant treatment to 9 weeks in the second experiment led to lower mean final virus concentration in plants but just 7 of 11 treated plants negative by ELISA were obtained. There was no statistical difference in mean absorbance between (*R*)-PMPA-treated and healthy control plants at the 9th week.

The data from the third experiment (Fig. 1) demonstrate a progressive virustatic effect of increasing (*R*)-PMPA concentration. Statistical analysis at the 9th week after (*R*)-PMPA application revealed significant decrease in virus concentration ($P < 0.05$) between infected control plants and plants treated with 25 and 50 mg/l (*R*)-PMPA. The difference between groups of plants treated with 25 versus 50 mg/l was not significant. Six and seven ELISA negative plants were obtained with 25 and 50 mg/l, respectively. However, when using PCR, which has a lower virus detection limit than does ELISA, only 6 of the 7 ELISA negative plants treated with 50 mg/l were negative. The DNA for PCR was isolated from about 0.1 g of infected leaves using a NucleoSpin Plant II kit (Macherey Nagel, Germany) according to the Manufacturer's recommendations and eluted with 30 μl of water. CaMV-specific primers 5′-AAGCTTACAGTCTCAGAAGACCAAAG (Rommens et al., 1991) and 5′-TAGAGGAAGGGTCTTGCGAAGG were used to amplify a 346 bp segment from the end of ORF VI (35S promoter region of the sequence NC_001497). The 20 μl PCR reaction mixture contained 10 mM Tris–HCl, pH 8.8; 50 mM KCl; 0.1% Triton X-100; 1.5 mM $MgCl_2$; 200 μM each of dNTPs; 1 U Taq DNA polymerase; 1 μl of DNA; and 20 pmol of CaMV primers. The amplifications were performed using an amplification program of 35 cycles with 20 s denaturation at 95 °C, 30 s annealing at 58 °C, and 30 s synthesis at 72 °C. All samples were analyzed using 1.5% agarose gel electrophoresis.

Data from the three experiments demonstrate that already during 6–9 weeks after application (*R*)-PMPA reduced the virus concentration in part of the plants to below the ELISA and PCR detection limits.

At the end of the third experiment, 6 plants negative by PCR were selected for initiation of meristem tip cultures together with positive and negative controls. The apical dome of about 3 mm was excised and cultivated on semisolid Murashige and Skoog's (MS) medium with 50 mg/l of (*R*)-PMPA. After 3 months, 5 regenerated plants were transferred to MS medium without (*R*)-PMPA but with 1 mg/l of indole-3-acetic acid (IAA) to obtain rooted plants. After 6 weeks, 5 rooted plants were planted in soil in the greenhouse and repeatedly tested negative by PCR at intervals of 1 month up to the 7th month from excision. Five infected control plants treated in the same way on media without (*R*)-PMPA remained infected.

The CaMV genome is replicated by reverse transcription of an RNA intermediate (Haas et al., 2002). (*R*)-PMPApp is a potent inhibitor of the reverse transcriptase activity (Suo and Johnson, 1998) but not of DNA polymerases (Birkuš et al., 2002). Helliot et al. (2003) had supposed that the mechanism of tenofovir's selective antiviral activity on replication can most likely be ascribed to inhibition of the reverse transcriptase activity, but formal proof was needed to demonstrate an inhibitory effect of the (*R*)-PMPApp.

For the study of (*R*)-PMPA's metabolism in *B. pekinensis*, tenofovir and its putative metabolites (*R*)-PMPAp, (*R*)-PMPApp, (*R*)-mePMPA, (*R*)-me_2PMPA and (*R*)-PMPHx were synthesized as previously described by Holý et al. (1989, 2001) and Votruba et al. (2010). [^{14}C-U]-AMP was obtained from NEN (Boston, MA) and [8-^{3}H]-(*R*)-PMPA was prepared according to Elbert et al. (2010). Alumina A305 and polyvinylpyrrolidone were purchased from Sigma–Aldrich (Prague) and HiTrap™ DEAE FF 5 ml from Amersham Biosciences (AP Czech, Prague). *B. pekinensis* plants were grown in liquid MS medium without sucrose as described by Špak et al. (2010). [8-^{3}H]-(*R*)-PMPA (0.74 MBq/plant and 74 MBq/μmol) was added to the 14-day-old plants at final concentration 10 μmol l^{-1}, and incubation in the presence of the compound then followed for 24, 48, 72, 96, 144 and 168 h. Cultivation was at 25 °C in the laboratory. At the indicated time intervals, the leaves of plants and roots were four times washed with 2 ml of phosphate-buffered saline at 4 °C by centrifuging at 5300*g* for 1 min. The sediment was resuspended in 1 ml of 5% TCA. After 10 min of vigorous stirring, the precipitate was sedimented for 5 min at 11,000*g*. An aliquot of TCA extract was then mixed with the same volume of 1,1,2-trichloro-1,2,2-trifluoroethane-trioctylamine (4:1, v/v). The aqueous phase was separated by centrifugation at 11,000*g* for 5 min and an appropriate aliquot was analyzed in a Waters HPLC system as described by Špak et al. (2010). Fifteen-second fractions were collected and radioactivity was counted in an Aquasafe-500 scintillator from 4 ml volume per sample. Peaks of (*R*)-PMPA, (*R*)-PMPAp, and (*R*)-PMPApp were identified with the aid of external standards.

Concentration of 10 μmol l^{-1} and 0.74 MBq/plant of [8-^{3}H]-(*R*)-PMPA was chosen as optimal. As external standards, we selected in addition to the two (*R*)-PMPA phosphates also (*R*)-PMPHx and N^6-substituted derivatives (*R*)-N^6mePMPA and (*R*)-N^6me_2PMPA. Data from HPLC analysis of individual extracts from whole *B. pekinensis* plants at different time intervals had shown that the highest intracellular level of (*R*)-PMPA metabolites appeared after 96 h of incubation with the drug (Fig. 2). The peaks found at the retention times 8.7, 14.5 and 23.5 min correspond to the (*R*)-PMPA and metabolites formed (i.e. (*R*)-PMPAp and (*R*)-PMPApp, respectively). Two peaks with relatively short retention time (2.5 and 4 min) do not correspond to any PMPA derivative. Most probably, they display instability of [^{3}H]-(*R*)-PMPA during the long-time incubation of plants at 25 °C (Elbert et al., 2010). This result shows that

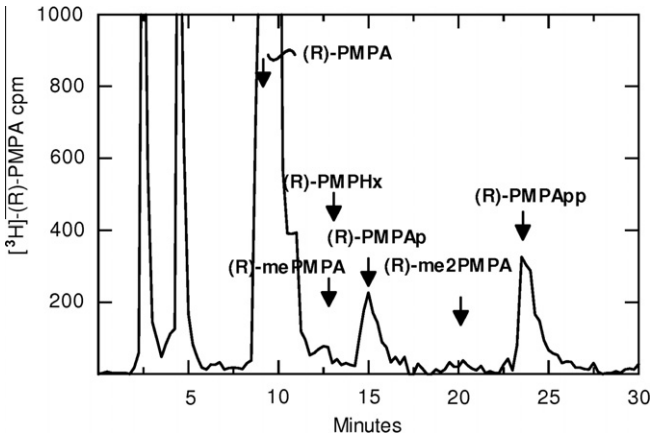

Fig. 2. HPLC analysis of acid-soluble pool from leaves of *Brassica pekinensis* after 96 h growth in the presence of 10 μM [^{3}H]-(*R*)-PMPA.

mono- and diphosphate, structural analogues of NDP and/or NTP, are the only metabolites formed in *B. pekinensis* plants. This means that the compound does not underlie deamination or modification at the 6-amino group of adenine moiety. The extent of phosphorylation represents in the leaves of the plant only 4.5% of applied labelled (*R*)-PMPA. In roots, we detected no radioactive peaks of phosphorylated metabolites (*R*)-PMPAp or (*R*)-PMPApp.

In experiments with phosphorylation of (*R*)-PMPA catalyzed by pre-purified extract from *B. pekinensis*, 60 g of leaves harvested from 6-week-old plants were frozen in liquid nitrogen and disintegrated to a powder in the presence of Alumina A 305. The powder was then suspended in 200 ml of 50 mM Tris–HCl, pH 7.8; 4 mM $MgCl_2$; 1 mM EDTA; 1 mM DTT (buffer A) containing polyvinylpyrrolidone (0.7 mg/ml); and 2 ml of protease inhibitor cocktail for plant cell extracts (Sigma–Aldrich, P 9599). The resulting extract was clarified by centrifugation (35 min, 30,000*g*) and fractionated by ammonium sulfate precipitation (33–70%). The precipitated proteins were dissolved in buffer A and desalted on Sephadex PD-10 columns in the same buffer. The eluate was then applied onto a HiTrap™ DEAE FF 5 ml column equilibrated with buffer A and proteins were eluted by discontinuous gradient of NaCl from 0 to 0.5 mol l^{-1} (stepwise with 2 column volumes of each 0, 0.1,. . .0.5 M NaCl). Aliquots of individual fractions were then tested for nucleotide kinase activities in a reaction mixture containing 50 mM Tris–HCl; 50 mM KCl; 2 mM $MgCl_2$; 1 mM ATP; 1 mM DTT; 50 μM [^{14}C]-AMP; and various concentrations of [^{3}H]-(*R*)-PMPA. The reaction was carried out at 25 °C and stopped by spotting a 2 μl aliquot onto a PEI cellulose plate (pre-spotted with 0.01 μmol of each AMP, ADP, ATP and/or PMPA, PMPAp and PMPApp). The plate was developed in the solvent system 4 M LiCl in 1 M acetic acid (1:4). The spots were visualized under UV light (254 nm) and cut out for radioactivity determination in the toluene-based scintillation cocktail.

Fractions eluted from the HiTrap™ DEAE FF column within the range 0.1–0.3 M NaCl contained nucleotide kinases able to transfer phosphate from ATP and creating [^{14}C]-ADP and [^{14}C]-ATP with up to 36% conversion. We found a substantially different phosphorylation pattern for [^{3}H]-(*R*)-PMPA as a substrate. Only the second 0.2 M NaCl eluate catalyzed formation of [^{3}H]-(*R*)-PMPAp and [^{3}H]-(*R*)-PMPApp (Fig. 3). These findings indicate inefficient but significant phosphorylation of (*R*)-PMPA, which proceeds with relatively low affinity because the reaction does not reach a plateau even up to a concentration of 500 μmol l^{-1}. For example, at 50 μM concentration, the efficiency of (*R*)-PMPA phosphorylation is about one order of magnitude lower than that of natural substrate AMP.

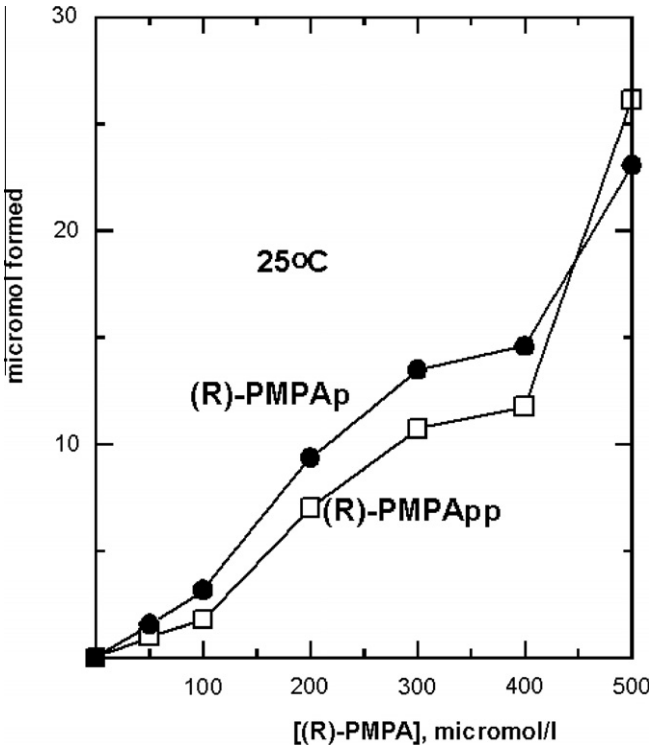

Fig. 3. Phosphorylation of (R)-PMPA catalyzed by pre-purified extract from *Brassica pekinensis*. Formation of [^{3}H]-(*R*)-PMPAp and [^{3}H]-(*R*)-PMPApp catalyzed by 0.2 M NaCl eluate from the HiTrap™ DEAE FF column.

Enzymes of bacterial and animal origin reported to be involved in the phosphorylation of tenofovir include 5-phosphoribosyl -1-pyrophosphate (PRPP) synthetase (Balzarini et al., 1991), AMP (dAMP) kinase (Merta et al., 1992; Krejčová et al., 2000), and nucleoside diphosphate kinase (Horská et al., 2006). Plants encode enzymes that are their homologues. We suppose that the formation of (*R*)-PMPAp is crucial, and that it most probably is catalyzed by an isoform of adenylate kinase (Schlattner et al., 1996).

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