Antiviral Research, 13 (1990) 219–226
Elsevier

AVR 00411

Antiviral activity of S-adenosylhomocysteine hydrolase inhibitors against plant viruses

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(Received 17 October 1989; accepted 29 January 1990)

Summary

Three SAH hydrolase inhibitors, (*RS*)-3-adenin-9-yl-2-hydroxypropanoic acid (isobutyl ester) [(*RS*)-AHPA]; (*RS*)-9-(2,3-dihydroxypropyl)adenine [(*RS*)-DHPA] and the carbocyclic analog of 3-deazaadenosine (C-c^{3}Ado) were evaluated for their inhibitory activity against tobacco mosaic virus (TMV) and potato virus X (PVX). Using the local lesion assay and ELISA, we demonstrated that all three compounds inhibit the replication of TMV and PVX. Whereas the three compounds proved about equally active against PVX, (*RS*)-AHPA was the most effective against TMV. (*RS*)-AHPA and C-c^{3}Ado induced chlorosis in *Nicotiana tabacum* leaf discs. They also caused a substantial reduction in the growth of the main root of *Phaseolus vulgaris*. (*RS*)-DHPA was less phytotoxic than its two congeners.

SAH hydrolase inhibitor; (*RS*)-AHPA; (*RS*)-DHPA; C-c^{3}Ado; Plant virus; TMV; PVX

Introduction

The use of antiviral chemicals in agriculture has been very restricted so far, although there is a great need of efficient non-toxic antiphytoviral chemicals to control severe viral diseases of important economical crops. With this aim, several compounds, which had already been the subject of investigations with animal viruses, have been recently examined for their inhibitory effects on plant viruses (Hansen, 1988).

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Our own group and R.T. Borchardt's group have recently established a new lead in the search for effective antiviral agents, i.e. carbocyclic and acyclic adenosine analogues, that appear to be targeted at S-adenosylhomocysteine (SAH) hydrolase, a key enzyme in transmethylation reactions (De Clercq, 1987; Keller and Borchardt, 1988). Among the animal viruses, poxviruses (i.e. vaccinia), (−)RNA viruses [i.e. paramyxo (parainfluenza), rhabdo (vesicular stomatitis) and double-stranded (±) RNA viruses [i.e. reo (rota)] are particularly sensitive to this class of compounds (De Clercq, 1987).

We have previously demonstrated that some (+)RNA plant viruses such as tobacco mosaic virus (TMV), potato virus X (PVX), eggplant mosaic virus and a potyvirus isolated from *Solanum palinacanthum* are also sensitive to the inhibitory effect of the SAH hydrolase inhibitor (*RS*)-9-(2,3-dihydroxypropyl)adenine [(*RS*)-DHPA] (De Fazio et al., 1987).

This work has now been extended to other well-known SAH hydrolase inhibitors which were examined by bioassays and indirect ELISA for their inhibitory effects on TMV and PVX, two important plant viruses, that are distributed worldwide and occur in different plant species.

Materials and Methods

Antiviral substances

The acyclic adenosine analogues (*RS*)-3-adenin-9-yl-2-hydroxypropanoic acid (isobutyl ester) [(*RS*)-AHPA] and (*RS*)-9-(2,3-dihydroxypropyl)adenine [(*RS*)-DHPA] were provided by Dr A. Holý (Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague, Czechoslovakia). The carbocyclic analogue of 3-deazaadenosine (C-c^{3}Ado) was a gift from Dr J.A. Montgomery (Southern Research Institute, Birmingham, AL, U.S.A.). Dilutions of drugs were made in distilled water at concentrations of 10, 50, 100, 200, 250 and 500 mg/l. Controls consisted of distilled water only. Of (*RS*)-AHPA fresh solutions had to be prepared at each occasion, because of the instability of this compound in solution (A. Holý, personal communication).

Plant hosts and viruses

Nicotiana tabacum 'White Burley' was chosen as the systemic host for TMV and PVX. As local hosts *Nicotiana glutinosa* was used for TMV and *Gomphrena globosa* for PVX.

Inocula consisted of partially purified virus preparations (1 mg/ml) or crude leaf sap extracts from *N. tabacum* plants.

The leaf disc incubation test was used as the model for assaying the antiviral activity of the chemicals. To this end, two expanded leaves of *N. tabacum* were mechanically inoculated with the virus and approximately 1 h later, 10-mm discs were punched out from the intervenous area of the leaves.

The solutions were distributed in 5-cm Petri dishes containing 5 ml of liquid (distilled water for controls). The discs, 10 per plate, were randomly distributed in the plates with the lower surface of the leaf in contact with the liquid. The plates were then incubated for 72 h in a 25°C incubator with a 16 h photoperiod. After incubation the discs were thoroughly rinsed, blotter-dried and ground with 0.01 M pH 7 phosphate buffer (0.1 ml per disc). The sap obtained from the discs was then submitted to the bioassays and indirect ELISA. In preliminary experiments the kinetics of virus replication were measured, and the virus content of the leaf discs was found to reach its maximum after 72 h.

Bioassays

For the bioassays, 35-µl juice from the leaf discs were inoculated in each half leaf (*N. glutinosa*) or opposite leaf (*G. globosa*) of the local hosts. The other half or opposite leaf received the control sap. The inoculated plants were maintained in a greenhouse until local lesions could be counted (usually at 5–7 days after inoculation). *G. globosa* leaves were dusted with carborundum before virus inoculation.

Indirect ELISA

For antibody production, rabbits were injected intramuscularly with purified TMV or PVX preparations (5 mg/ml) emulsified with Freund's complete adjuvant. Thirty days later they received every other day five intravenous injections (5 mg virus/ml). All antisera used in the present work were from bleedings collected 50 days after the start of immunization.

Indirect ELISA of leaf disc extracts was carried out in polystyrene microtitre plates (Nunc-Immuno Plate I) as described by Koenig (1981) and Koenig and Paul (1982). Extracts were centrifuged at low speed and supernatants were diluted 1:100 with 0.05 M sodium carbonate buffer, pH 9.6. Antisera to TMV and PVX were diluted 1:32000 and 1:350000, respectively, with phosphate buffered saline containing 0.05% Tween-20 and 2% polyvinyl pyrrolidone 40000 (PBSTP). Antiviral antibodies were detected by alkaline phosphatase-labelled anti-rabbit IgG (Sigma A-8025) diluted 1:1000 with PBSTP; *p*-nitrophenyl phosphate at 1 mg/ml in 0.1 M diethanolamine buffer, pH 9.8 was used as substrate. After 30 min of hydrolysis, 50 µl of 3 M NaOH was added to each well and the absorbances at A_{405} were read in a photometer (Titertek Uniskam I).

To assess the antiviral effect, the number of local lesions in the treated half or opposite leaf was compared with the number of lesions present in the controls.

To calculate the inhibition percentage (IP), the following formula was used: $IP = 100-[A:B \times 100]$, where A represents the number of local lesions or the absorbance measured at 405 nm for the treated groups, and B represents the corresponding values for the control groups. Nine half leaves or whole leaves (bioassays) or 3 replicates (ELISA) were used for each drug concentration. The experiments were repeated at least five times and Figs. 1, 2 present the average data for all experiments.

Phytotoxic effects

To monitor the phytotoxic effects of the drugs, root inhibition tests were carried out by immersing the intact roots of 3-month-old *N. tabacum* and *N. glutinosa* plants in solutions containing the drugs. Roots of *Phaseolus vulgaris* seedlings 72 h after germination, and *Lycopersicon esculentum* seedlings were also used. In all cases the solutions contained 50 mg/l of the test substances. Distilled water was used as control. After 3 days, roots were transferred to water and on the 7th day the phytotoxic effect was evaluated based on leaf wilting (*N. tabacum* and *N. glutinosa*), or inhibition of adventitious root growth (*L. esculentum*) or main root growth (*P. vulgaris*).

These experiments were repeated at least 3 times.

Results

Inhibition of TMV replication

At low doses (10 and 50 mg/l), the three drugs caused a similar inhibition of TMV replication (IP: 30–50%). At a dose of 100, 200 and 250 mg/l, (*RS*)-AHPA and (*RS*)-DHPA exhibited similar antiviral activity (IP: 60–70%), while C-c^{3}Ado was somewhat less active (IP: 50%) (Fig. 1). At the highest dose tested (500 mg/l), (*RS*)-AHPA achieved the greatest inhibition (IP: 85%).

With ELISA, as with the bioassays, a dose-dependent inhibition of TMV replication was demonstrated for all three drugs. However, the IP values obtained with ELISA were markedly lower than those generated by the bioassays, irrespective of the nature of the compound and the dose applied (Fig. 1).

Inhibition of PVX replication

At the lowest dose used (10 mg/l), (*RS*)-AHPA was the most effective of the three drugs in inhibiting PVX replication (IP: 55%) (Fig. 2). At 50 mg/l, (*RS*)-AHPA was about as active as C-c^{3}Ado but still more active than (*RS*)-DHPA. At the higher dose levels (100–500 mg/l), (*RS*)-AHPA and C-c^{3}Ado remained consistently more active than (*RS*)-DHPA in inhibiting PVX replication (Fig. 2). A very high inhibition percentage (about 95%) was accomplished by (*RS*)-AHPA at these dose levels.

ELISA data were similar to the bioassay data (Fig. 2), except for (*RS*)-DHPA, which gave higher IP values with ELISA than with the bioassays.

Phytotoxicity

Chlorosis was observed when tobacco leaf discs were incubated for 72 h in the presence of (*RS*)-AHPA or C-c^{3}Ado, at 400 or 500 mg/l. (*RS*)-DHPA did not show any visible phytotoxicity at doses up to 500 mg/l.

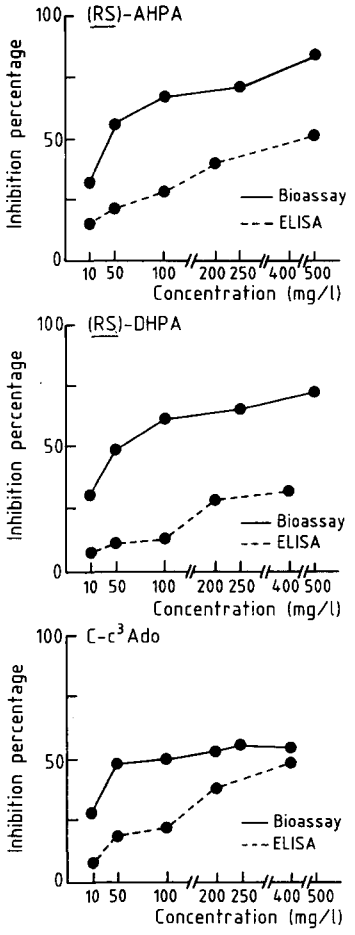

Fig. 1. Inhibitory effects of three SAH hydrolase inhibitors on TMV replication.

When phytotoxicity was assessed following immersing the intact root system of *N. tabacum* or *N. glutinosa* in 50 mg/l solutions of the antiviral substances, a strong burning and irreversible wilting of the leaves were observed for both (*RS*)-AHPA and (*RS*)-DHPA. Examination of the roots of these plants indicated that the old roots were damaged, while new roots had ceased to develop. C-c^{3}Ado did not cause irreversible leaf wilting but inhibited the growth of new roots.

A complete inhibition of adventitious root growth of *L. esculentum* was observed for all three drugs. When *P. vulgaris* main root growth inhibition was measured, the results (Table 1) indicated that the inhibition (IP) caused by (*RS*)-AHPA was 68%, as compared to 47% and 4% for C-c^{3}Ado and (*RS*)-DHPA, respectively. Thus, the latter compound was the least phytotoxic of the three.

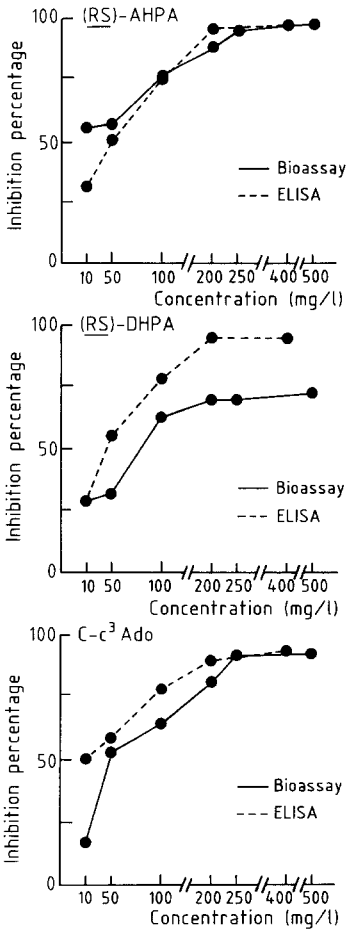

Fig. 2. Inhibitory effects of three SAH hydrolase inhibitors on PVX replication.

TABLE 1

Phytotoxicity: inhibitory effects of the compounds on the growth of the main root (*Phaseolus vulgaris*)

Treatment[a]	Main root length (mm)		IP[b]
	Treated	Untreated	
(*RS*)-AHPA	45.2	137.1	68
(*RS*)-DHPA	62.3	64.4	4
C-c³Ado	34.3	64.4	47

[a]Treatment consisted of immersing the seedling root in an aqueous solution, containing 50 mg compound per liter, for 72 h.
[b]IP: inhibition percentage.

Discussion

The adenosine analogues (*RS*)-DHPA, (*RS*)-AHPA and C-c^{3}Ado, which have been previously recognized as SAH hydrolase inhibitors (De Clercq, 1987), proved effective in inhibiting the replication of the plant viruses TMV and PVX. SAH hydrolase inhibitors have a unique antiviral activity spectrum, encompassing animal (−)RNA and (±)RNA viruses (De Clercq, 1987) and some (+)RNA plant viruses (Dawson, 1984; De Fazio et al., 1987; Schuster and Holý, 1988).

The mechanism of action of (*RS*)-DHPA, (*RS*)-AHPA and C-c^{3}Ado is based on the inhibition of SAH hydrolase (De Clercq and Holý, 1985). This enzyme participates in transmethylation reactions, using S-adenosylmethionine (SAM) as the methyl donor. Such reactions also occur in plants (Sebestová et al., 1984). When, as a consequence of SAH hydrolase inhibition, SAH accumulates, all processes requiring intensive methylations including the maturation of mRNA (5′ cap formation) are impaired (De Clercq and Montgomery, 1983). According to the present results, this premise also extends to the plant viruses TMV and PVX, whose (+)RNA genome is capped at the 5′-terminus (Matthews, 1982).

The antiviral activity of the three drugs against TMV and PVX was evident not only at the highest doses tested (400 and 500 mg/l) but also at doses as low as 50 mg/l. These data confirm and extend those reported previously by De Fazio et al. (1987) for (*RS*)-DHPA. Recently, Schuster and Holý (1988) also studied the inhibitory effects of DHPA and AHPA on PVX replication. They observed that AHPA was more effective than DHPA at lower doses. Our findings also indicate that (*RS*)-AHPA at 10 mg/l is more active against PVX than either (*RS*)-DHPA or C-c^{3}Ado.

A striking discrepancy was noted between the bioassay IP values and ELISA IP values for TMV (Fig. 1). The bioassay estimates infectivity, whereas ELISA assesses viral antigen content. Gunn and Pares (1988), working with potato leafroll virus, judged the results obtained by ELISA as false positive since it does not take into account the infection-mediated 'stress factor' which leads to the induction of pathogenic proteins. TMV infection is much more stressing than PVX to tobacco plants, and this may explain why bioassays and ELISA tests provided similar IP values for PVX (Fig. 2), but not for TMV (Fig. 1). Because of the 'stress' effect of TMV infection on the plant, bioassays yielded invariably higher IP scores than the ELISA tests (Fig. 1). In striking contrast with the behavior of all three compounds in the TMV tests, (*RS*)-DHPA afforded more effective inhibition of PVX replication in ELISA than in the bioassays (Fig. 2).

(*RS*)-DHPA was the least phytotoxic of the three drugs tested on *Vicia faba* seedling roots (Benés et al., 1986). De Fazio et al. (1987) also ascertained that (*RS*)-DHPA was not phytotoxic in the leaf disc incubation tests or in leaf sprays, even when used at concentrations as high as 500 mg/l. Benés et al. (1984) observed that (*RS*)-DHPA toxicity for *Vicia faba* seedling roots primarily resided in the S-enantiomer rather than the R-enantiomer.

When comparing the findings for the plant and animal systems, we have noted that the concentrations of the SAH hydrolase inhibitors required to inhibit the

replication of plant viruses are higher than those found inhibitory to mammalian viruses (De Clercq, 1987). Also, the inhibitory effects of the drugs on the different mammalian viruses vary considerably depending on the nature of the virus and cell system. These differences, as well as the differential sensitivities of plant and animal viruses to inhibition by SAH hydrolase inhibitors, may be explained by differences between the plant and animal virus-cell systems.

Although the purpose of our present work was to find antiviral compounds with possible use in agriculture, it should be added that, as a note of caution, results obtained in leaf disc assays cannot necessarily be extrapolated to live plants.

Acknowledgements

The authors are indebted to Miss Floriana Plini and Miss Eliana Borges Rivas for helpful technical assistance. The authors are also grateful to Dr A. Holý and Dr J.A. Montgomery for providing the test compounds.

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