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Short Communication

Analogues of pyrimidine base precursors as antiphytoviral agents

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Summary

Dihydroorotic acid hydrazide (DHOH) did not inhibit the replication of potatovirus X (PVX) in leaf disks of *Nicotiana tabacum* 'Samsun'. In contrast, 5-fluoroorotic acid (5-FOA) completely inhibited the replication of PVX, as demonstrated by a serological virus assay as well as a local lesion bioassay using *Gomphrena globosa* as the test plant. The corresponding base analogue 5-fluorouracil (5-FU) had only a weak inhibitory effect. Time-course inhibition experiments in synchronized virus-infected leaf disks led us to conclude that 5-FOA, as well as 5-azadihydrouracil (5-ADHU), an uracil catabolite analogue, inhibit the same early event in virus infection. Neither 5-FOA nor 5-ADHU had a direct inactivating effect on free PVX virions.

Antiphytoviral substances; Pyrimidine base precursors; 5-Azadihydrouracil; 5-Fluoroorotic acid; 5-Fluorouracil; Potato virus X

Introduction

In the search for agents capable of inhibiting plant virus multiplication, similarly to the search for inhibitors of animal and human viruses, particular attention has been paid to antimetabolites interfering with nucleic acid biosynthesis. In particular purine and pyrimidine base nucleoside and nucleotide analogues have been pursued as antiphytoviral agents (Hecht and Diercks, 1978; Ralph and Wojcik, 1976; Schuster, 1983; Schuster and Byhan, 1980). None of the reported substances possesses the characteristics of an ideal antiviral agent. Some of the analogues, i.e. 2-thiouracil, inhibit the replication of some plant viruses, but stimulate the repli-

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cation of others. Quite often, the (inhibitory or stimulatory) effects depend on the concentration and time of application. Another troublesome property of these substances is in many cases phytotoxicity or toxicity for animals and humans.

A successful approach to overcome some of these difficulties has been the use of pyrimidine base catabolite analogues, especially 5-azadihydrouracil (5-ADHU), also referred to as 2,4-dioxohexahydro-1,3,5-triazine (DHT). This substance is able to reduce, but not completely arrest, the replication of various plant viruses (Schuster, 1982; Schuster et al., 1979a, b), thereby increasing the plant or crop yield, i.e. the tuber masses of virus-infected potato stocks (Schuster et al., 1979_c). Plants tolerate high doses of 5-ADHU. Moreover, the toxicological data, such as LD_{50}, for mammals are excellent (Schuster, 1982). As 5-ADHU is much more effective against PVX than 5-azauracil (Huber, 1986), it is plausible that 5-ADHU is more easily converted to an active virus inhibitor (Schuster, 1982) than 5-azauracil. The same may be true for analogues of pyrimidine base precursors. Based on these considerations, we started an investigation on pyrimidine base precursor analogues as antiphytoviral agents. Dihydroorotic acid hydrazide (DHOH) (Golovinsky et al., 1981), 5-fluoroorotic acid (FOA) (Golovinsky and Spassova, 1981) and 5-fluorouracil (5-FU) were included in the assays.

Materials and Methods

Chemicals

D,L-Dihydroorotic acid hydrazide (DHOH) and 5-fluoroorotic acid (5-FOA) were synthesized in the laboratory of E. Golovinsky; 5-fluorouracil (5-FU) was purchased from Boehringer (Mannheim); 2-thiouracil (2-TU) was a gift from the Research Institute Manfred von Ardenne (Dresden); 5-azadihydrouracil (5-ADHU = 2,4-dioxohexahydro-1,3,5-triazine = DHT) was synthesized at Leuna-Werke (Leuna); cycloheximide (CX) was purchased from the Upjohn Company (Kalamazoo, Mich.).

Virus and virus host; inoculation procedure and application of substances

Potato virus X (PVX), ringspot strain H 19, and *Nicotiana tabacum* L. 'Samsun' were used in the experiments. The plants were cultivated in an air-conditioned room at a temperature of 25 ± 2°C during a 16-h light period and at a temperature of 20 ± 2°C during the 8-h period of darkness. When the plants had developed 7–8 leaves, fully expanded leaves were cut off and inoculated with pre-clarified virus-containing juices obtained from infected *N. glutinosa* L. As abrasive, 600 mesh carborundum was used. After inoculation, the leaves were rinsed with water and thereafter 10-mm diameter tissue disks were punched out using a cork borer and sampled. Each sample contained 40 disks. Three dilutions were used for each chemical and the experiments were performed in triplicate or quadruplicate. The disks were vacuum-infiltrated with the inhibitors or with water, and then laid on top of inhibitor solutions (or water) in 10-cm diameter petri dishes.

Production of synchronous virus synthesis and estimation of the time course of inhibition

Synchronous virus synthesis was achieved by arresting virus multiplication at a very early stage at low temperatures (5 ± 2°C) and shifting to permissive room temperature (22°C) after 5 days. This differential temperature inoculation (DTI)procedure has been originally described by Dawson et al., 1973, 1975), and slightly modified by Schuster and Arenhövel (1984). The time course of virus inhibition by the test substances was followed by using the DTI procedure.

Virus assays

Four days after inoculation, or when using the DTI procedure, 72 h after the shift to the permissive room temperature, PVX yield (concentration) was assayed serologically, based on 4–8 replicative determinations, as described previously (Schuster et al., 1979b). The highest dilution step at which a precipitate no longer occurred served as the index. The average indexes were calculated for each series. The percentage of inhibition (I%) was determined as follows:

$$I\% = 100 - \frac{AVC_{treatment}}{AVC_{control}} \times 100$$

$AVC_{Treatment}$ being the average virus concentration in the treated leaf disks and $AVC_{Control}$ the average virus concentration in the corresponding control disks.

Local lesion assays in *Gomphrena globosa* L. were performed in some cases to provide supplementary information to the serological determination of the virus content. For these bioassays juice pools were prepared by mixing all 4–8 preparations. If required, the pooled juices were diluted to the point where lesions could be readily scored (Schuster, 1977).

Estimation of inactivation of the free virions by the substances

To estimate the direct effect of 5-FOA on free PVX virions, compound solutions at twice the concentration of the vacuum-infiltrated solutions were mixed with juices of virus-infected plants at a ratio of 1:1. Controls were made with water. One hour later, the juices were diluted 1:10 and inoculated on *Gomphrena globosa* leaves, where local lesions were scored a few days later.

Statistical significance

The significance of the observed differences was determined by Student's *t*-test. The results are marked by the following symbols: +, $P \leq 0.05$; ++, $P \leq 0.01$; +++, $P \leq 0.001$ or n.s., not significant.

Results and Discussion

As shown in Table 1, the dihydroorotic acid analogue DHOH did not produce any inhibition of PVX at a concentration of 0.5 or 1 mM. At 5 mM DHOH was

TABLE 1

Inhibition of potato virus X by pyrimidine base precursor analogues

Compound	Test[a]	Inhibition (%) at a compound concentration of		
		0.5 mM	1 mM	5 mM
D,L-Dihydroorotic acid hycrazide (DHOH)	S	0	0	(Tox.)[b]
5-Fluoroorotic acid (5-FOA)	S	100 (+++)[c]	100 (+++)	–[d]
	L	100 (+++)	–	–
5-Fluorouracil (5-FU)	S	10.6 (n.s.)	0	10.6 (n.s.)
	L	–	–	35.3 (++)

[a] S: Results of serology tests. L: Results of local lesion tests in *Gomphrena globosa*.
[b] Toxicity.
[c] Levels of significance are indicated in parentheses: +++, $P \leq 0.001$; ++, $P \leq 0.01$; +, $P \leq 0.05$; n.s., not significant.
[d] Not tested.

highly phytotoxic. Thus, DHOH does not act as a specific virus inhibitor. On the other hand, the orotic acid analogue 5-FOA completely inhibited the replication of PVX, even at the lowest concentration tested (0.5 mM). These results were confirmed by the local lesion bioassay. No damage was observed in the host tissue disks. Thus, 5-FOA may be considered as a potent and selective PVX inhibitor. In contrast with 5-FOA, 5-FU caused only a very small inhibitory effect. Using the serological precipitation test, which is based on the viral coat protein reaction, nearly no inhibition was observed. Only the local lesion bioassay, which is based on virus RNA infectivity, showed a significant inhibition (about 35%). 5-FOA did not inhibit the activity of free PVX virions. After having added 5-FOA to PVX-containing juices, an average of 27 local lesions formed on the leaves of *Gomphrena globosa*, whereas 32 lesions were noted for the corresponding controls, the difference (16%) being non-significant. Thus, 5-FOA must achieve its inhibitory activity on PVX by interfering with the viral replication cycle.

To obtain more information on the mode of action of 5-FOA the time course of inhibition was determined without (Fig. 1A) or with (Fig. 1B) preincubation of the virus-infected leave disks for 5 days at 5°C.

In contrast with cycloheximide, which acts at a very late event, 5-FOA inhibits a very early event of the PVX replication cycle. The time course of inhibition by 5-FOA was almost identical to that of 5-ADHU and 2-TU, whether the leaves were immediately incubated at the permissive temperature (Fig. 1A) or preincubated at 5°C for 5 days (Fig. 1B). 5-FOA, 5-ADHU and 2-TU achieved approximately 60–70% inhibition of PVX synthesis after the 5 daypreincubation period (Fig. 1B). Apparently, the pyrimidine base precursor analogue 5-FOA inhibits the same event in the replication cycle of PVX as the uracil analogues 5-ADHU and 2-TU.

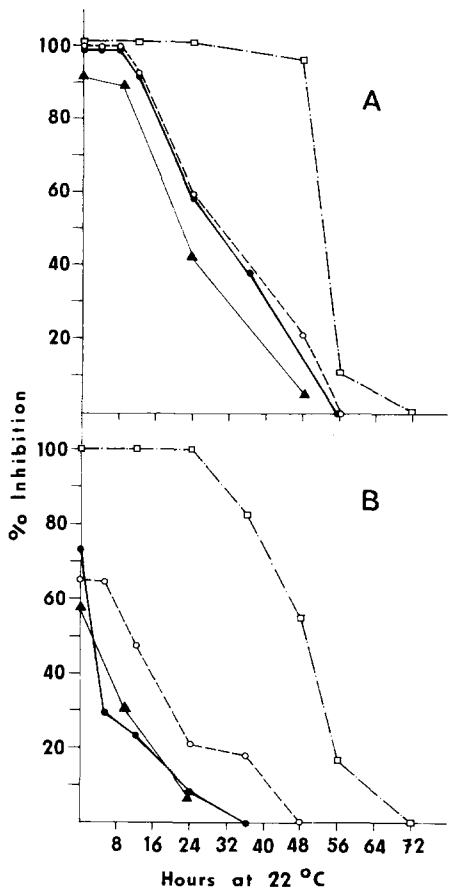

Fig. 1. Time course of inhibition of PVX synthesis by 0.5 mM 5-FOA (▲—▲), 1 mM 5-ADHU (●—●), 5 mM 2-TU (○---○) or CX (5 μg/ml) (□···□) in disks of tobacco leaves inoculated with PVX and maintained at the permissive (room) temperatures after inoculation (A) or preincubated for 5 days at 5°C (B). Average values for 4–8 separate determinations.

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