Modifications of Nucleic Acid Precursors That Inhibit Plant Virus Multiplication

William O. Dawson and Carol Boyd

Department of Plant Pathology, University of California, Riverside 92521.
This work was supported in part by U.S. Department of Agriculture Grant 84-CTC-1-1402.
Accepted for publication 4 April 1986.

ABSTRACT

Dawson, W. O., and Boyd, C. 1987. Modifications of nucleic acid precursors that inhibit plant virus multiplication. Phytopathology 77:477-480.

The relationship between chemical modifications of normal nucleic acid base or nucleoside precursors and ability to inhibit multiplication of tobacco mosaic virus or cowpea chlorotic mottle virus in disks from mechanically inoculated leaves was tested with 131 analogues. Chemicals tested were selected from 10 general classes of modifications to determine the types of modifications of normal nucleic acid precursors that have greater probabilities of inhibiting virus multiplication. No inhibitory chemicals were found in several classes. Classes of modifications with the highest proportion of antiviral activity were modification of the sugar moiety (five of 13 chemicals were inhibitory) and addition of abnormal side groups (three of seven chemicals were inhibitory). Eight new inhibitors of virus multiplication were identified: 6-aminocytosine; 6-ethylmercaptopurine; isopentenyladenosine; 2-thiopyrimidine; 2,4-dithiopyrimidine; melamine; 5′-iodo-5′-deoxyadenosine; and 5′-methyl-5′-deoxythioadenosine.

Additional key words: antivirals, chemotherapy, control, virus diseases.

The ability to control virus diseases of plants with chemicals would be a valuable addition to existing control strategies. This could be particularly useful in in vitro culture procedures to eliminate viruses from propagation materials. At this time, the major limitation to progress in this field is the difficulty in identifying antiviral chemicals. The small size of viral genomes limits the number of possible virus-specific targets for chemicals. This makes the probability of finding effective antiviral chemicals by traditional methods, which use screens not based on structure-function information, prohibitively small, even though these methods have been effective in identifying antimicrobial chemicals. This situation suggests that some other approach to identifying antiviral chemicals be used. A directed approach, based on an understanding of the structure-function relationship of inhibitors of virus multiplication, is one possibility. Such an approach would enable chemicals with antiviral activity to be designed instead of attempting to identify them from massive screens.

The requirements of a useful antiviral chemical include abilities to inhibit multiplication, spread, or symptom induction of the virus; be selective enough not to harm the host; have broad-spectrum activity against a number of virus diseases; move systemically in the host; and not have harmful effects on the environment. Numerous compounds have been identified that inhibit the multiplication of plant viruses, although none so far identified is selective enough to be useful against virus diseases of crops. Most inhibitory compounds are analogues of normal nucleoside or nucleic acid base precursors of viral RNAs. We need to know what modifications affect activity against virus multiplication and, in general, what types of alteration are likely to be more successful. There are some early reports of tests of limited numbers of compounds for activity against plant viruses (8,9), but it is difficult from these data to relate general modifications to activity.

In this study, we tested 131 representative chemicals in 10 general classes of modified nucleoside and nucleic acid base precursors against the multiplication of two plant viruses from different taxonomic groups, tobacco mosaic virus (TMV) in tobacco and cowpea chlorotic mottle virus (CCMV) in cowpea. Chemicals were chosen to be tested based on two different criteria. The first criterion was that chemicals be of certain general categories of modification of normal nucleoside or nucleic acid base precursors in an attempt to gain some information as to what types of alterations have a higher probability of inhibiting virus multiplication. The second criterion, when possible, was to choose chemical analogues to known inhibitors of plant virus multiplication in an attempt to gain some understanding of structural requirements for inhibition.

MATERIALS AND METHODS

Culture conditions. TMV strain Ul was maintained in Xanthi tobacco plants (*Nicotiana tabacum* L.) and assayed for infectivity on Xanthi nc tobacco or *Phaseolus vulgaris* L. 'Pinto.' CCMV was maintained in cowpea (*Vigna unguiculata* (L.) Walp. subsp. *unguiculata* 'California Blackeye') and infectivity was assayed on soybean (*Glycine max* L. 'Harosoy').

Infectivity assays. Infectivity was determined by homogenizing the 10 leaf disks from a treatment with a mortar and pestle and dilution in sufficient 10 mM potassium phosphate buffer, pH 7, containing 1% diatomaceous earth to result in approximately 50–150 lesions per half-leaf when assay plants were inoculated with virus from control disks. Each inoculum was applied to half-leaves of assay plants in a random block design with 6–12 replications. Each assay contained at least one water-treated control sample.

Test system. At 24 hr after mechanical inoculation of the upper surface of tobacco leaves 10–15 cm in length with TMV or fully expanded primary cowpea leaves with CCMV, 7-mm-diameter disks were removed from inoculated leaves. Ten disks per treatment were vacuum infiltrated with a solution of one of the chemicals, then allowed to dry on a paper towel on the laboratory bench, after which the disks were floated on the chemical solution for 4 days in 3.5 cm petri dishes in a plant growth chamber at 25 C with a 14-hr photoperiod of approximately 15,000 lx. Control disks were treated similarly with distilled water. Disks then were removed and frozen at −20 C until infectivity was assayed.

Each chemical was tested first at 1 mM (or at saturation if it was not that soluble). If leaf disks treated with a chemical showed evidence of toxicity, the chemical was retested at a series of concentrations each reduced by 1:5. This was continued until there was no evidence of toxicity. This was an attempt not to confuse toxicity with inhibition. When treatments resulted in inhibition,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

the chemicals were retested with a series of concentrations each reduced by 1:5 to determine the lowest concentration that was inhibitory. When 1 mM inhibited less than 90%, the concentration was increased until at least 90% inhibition resulted without visible toxicity. Each experiment was conducted at least three times. Data are reported as the lowest concentration tested that repeatedly resulted in greater than 90% inhibition of virus infectivity. In most cases there was almost total inhibition. The greater than 90% measurement was convenient for what we considered an effective inhibitor.

TABLE 1. Chemicals inhibitory to the multiplication of tobacco mosaic virus (TMV) or cowpea chlorotic mottle virus (CCMV) in leaf disks[a]

		Concentration (mM) required for 90% inhibition	
		TMV	CCMV
Altered positions of normal side groups			
Inhibitory			
6-Aminocytosine		···	0.5
Not inhibitory			
2-Hydroxypyrimidine	5,6-Dihydrouracil		
4,5-Diaminopyrimidine	Pseudouridine		
5,6-Diamino-2,4-dihydroxypyrimidine	2-Methyladenine		
6-Hydroxycytosine	7-Methylguanosine		
5-Aminouracil	1-Methyladenosine		
2-Aminopyrimidine	Xanthine		
4-Hydroxypyrimidine	Hypoxanthine		
5-Methylcytosine	Inosine		
5-Hydroxyuracil	1-Methyladenine		
2-Amino-4-hydroxy-6-methylpyrimidine	2-Hydroxy-6-methylpurine		
Isocytosine	2-Hydroxypurine		
6-Methyluracil	2,8-Dihydroxypurine		
2-Amino-4-methylpyrimidine	Isoguanine		
Alloxan (2,4,5,6-tetraoxypyrimidine)			
Addition of abnormal side groups			
Inhibitory			
5-Diazouracil		1.0	1.0
6-Ethylmercaptopurine		1.0	1.0
N^6-(Δ^2-Isopentenyl)adenosine		1.0	0.1
Not inhibitory			
6-Chloro-2,4-dimethoxypyrimidine	2-Ethylmercapto-4,6-diaminopyrimidine		
5-Carboxy-2-thiouracil	5-Hydroxymethylcytosine		
Addition of halogen			
Inhibitory			
5-Fluorouracil		3.0	3.0
2-Chloroadenosine		0.1	0.33
Not inhibitory			
5-Bromouracil	2-Fluoropyridine		
5-Chlorouracil	5-Chlorocytosine arabinoside		
5-Iodouracil	6-Chloropurine		
5-Bromocytosine	2,6-Dichloropurine		
5-Bromocytidine	6-Chloroguanine		
5-Iodocytidine	8-Chloroxanthine		
2-Chloropyrimidine	2,6-Dichloro-7-methylpurine		
2,4-Dichloropyrimidine	8-Bromoadenosine		
5-Amino-4,6-dichloropyrimidine	2,6-Dibromopurine		
2,4,6-Trichloropyrimidine	8-Bromoguanosine		
2-Chloro-2,4-dimethoxypyrimidine	6-Iodopurine		
2-Chloro-4,5-diaminopyrimidine	5-6-Dichloro-1-β-D-ribofuranosyl benzimidazole		
Addition of sulfur			
Inhibitory			
2-Thiouracil		1.0	···
2-Thiopyrimidine		1.0	···
2,4-Dithiopyrimidine		0.1	0.1
Not inhibitory			
2-Thio-4-hydroxy-6-methylpyrimidine	2-Thio-4-amino-6-hydroxypyrimidine		
2-Thio-6-azauridine	2-Thio-4,6-diaminopyrimidine		
4-Thiouridine	2-Ethylmercapto-4,6-diaminopyrimidine		
2-Thio-6-hydroxyuracil	Trithiocyanuric acid		
2-Thio-5-carboxyuracil	2-Mercaptopyridine		
2-Thiocytosine	6-Mercaptopurine		
2-Thio-5-methylcytosine	6-Mercaptopurine arabinoside		
6-Thio-4,5-diaminopyrimidine	6-Thioxanthine		
2-Thio-4,5-diamino-6-hydroxypyrimidine	2-Thioxanthine		
6-Thio-4,5-diamino-2-hydroxypyrimidine	2,6-Dimercaptopurine		
2-Thio-4,5-diaminopyrimidine	2-Mercaptopurine		
5-Thiouracil	2-Mercapto-6-8-purinediol		
	6-Mercapto-4-aminopyrazolo[3,4-d]pyrimidine		

Continued on next page

TABLE I, continued

		Concentration (mM) required for 90% inhibition	
		TMV	CCMV
Addition of N in pyrimidine ring			
Inhibitory			
6-Azauracil		2.0	2.0
5-Azacytidine		···	0.1
2,4,6-Triamino-*s*-triazine:melamine		···	0.1
Not inhibitory			
5-Azacytosine	Cyanuric acid (2,4,6-trihydroxy-1,3,5-triazine)		
2-Thio-6-azauridine	Trithiocyanuric acid		
6-Azacytidine	*s*-Triazine		
5-Azauracil	3-Amino-1,2,4-triazine		
Removal of N from pyrimidine ring			
Inhibitory			
3-Deazauridine (2,4-dihydroxypyridine riboside)		1.0	0.1
2,3-Diaminopyridine		1.0	1.0
Not inhibitory			
3-Deazauracil	Pyridine		
2,4-Dihydroxypyridine	2-Mercaptopyridine		
2-Hydroxypyridine	2-Fluoropyridine		
3,4-Diaminopyridine	8-Valerolactam		
Removal of N from purine ring			
Inhibitory			
Tubercidin (7-deazaadenosine)		0.01	0.01
Not inhibitory			
5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole	2-Benzimidazolemethanol		
Benzimidazole			
Shift of N in pyrimidine ring			
Not inhibitory			
3,6-Dihydropyridazine	Glycine anhydride		
Pyrazine			
Shift of N in purine ring			
Not inhibitory			
Formycin A	4-Amino-6-mercaptopyrazolo[3,4-d]pyrimidine		
Formycin B	4-Amino-6-hydroxypyrazolo[3,4-d]pyrimidine		
4-Hydroxypyrazolo-[3,4-d]pyrimidine	4-Aminopyrazolo[3,4-d]pyrimidine		
Alteration of sugar moiety			
Inhibitory			
5′-Iodo-5′-deoxyadenosine		1.0	1.0
5′-Methyl-5′-deoxythioadenosine		0.1	···
Adenine 9(2,3-dihydroxypropyl)monohydrate		···	0.1
Cordycepin (3′-deoxyadenosine)		0.2	0.2
Adenine arabinoside		0.6	0.6
Not inhibitory			
2′,3′,5′-Tri-*o*-acetyl guanosine			
2′,3′,5′-Tri-*o*-acetyl-6-azauridine			
Acycloguanosine: 9-(2-hydroxyethoxymethyl)-guanine			
Cyclocytidine: 2,2′-anhydro-(1-β-D-arabinofuranosyl)-cytosine			
Uracil arabinoside			
Hypoxanthine arabinoside			
6-Mercaptopurine arabinoside			
5-Chlorocytosine arabinoside			

[a] Chemicals were obtained from one of the following sources: Sigma Chemical Co., St. Louis, MO; Aldrich Chemical Co., Milwaukee, WI; Calbiochem-Behring Corp., La Jolla, CA; and Boehringer, Mannheim, West Germany.

RESULTS

One hundred thirty-one chemicals, representing 10 general classes of nucleic acid precursor analogues, were tested for ability to inhibit the multiplication of TMV and CCMV by greater than 90% without visible toxicity to the leaf disks. Compounds that inhibited virus multiplication less than 90% were arbitrarily considered not to be sufficient inhibitors to be useful to define a structure-function relationship. They are listed in Table 1 according to general types of modifications. A few chemicals have more than one type of modification and are listed twice.

Base analogues with alterations of normal side groups. This group includes purines and pyrimidines with alterations consisting of additions or deletions of amino, hydroxyl, and methyl side groups. We found no chemical of this type to test that was known previously to inhibit multiplication of a plant virus. Of 17 pyrimidine analogues and 11 purine analogues tested, only one compound, 6-aminocytosine, was inhibitory, and this compound inhibited only CCMV (Table 1). Several compounds closely related to 6-aminocytosine were not inhibitory. Because the percentage of active chemicals in this category was low, it appears that alteration of positions of normal side groups of nucleic acid bases has a low probability of producing a chemical inhibitory to the multiplication of these viruses.

Nucleic acid bases with abnormal side groups. 5-Diazouracil, which was reported to inhibit multiplication of TMV (8), also inhibited CCMV (Table 1). Of six other compounds that were tested, two new inhibitors were found. 6-Ethylmercaptopurine inhibited both viruses. Interestingly, the other inhibitory compound was isopentenyladenosine, the nucleoside of a plant hormone.

Three of seven chemicals tested in this general category were inhibitory, suggesting that it would be profitable to examine more chemicals with this type of alteration.

Halogenated nucleic acid bases. 2-Chloroadenine already has been reported to inhibit TMV multiplication (9). The nucleoside 2-chloroadenosine effectively inhibited both TMV and CCMV, being slightly more active toward TMV. None of several analogues similar to 2-chloroadenosine that were tested were active against either virus.

5-Fluorouracil, which replaces uracil in TMV RNA and inhibits multiplication at higher concentrations (5), also inhibited CCMV. Bases with other halogens at this position were not inhibitory. Overall, only two of 26 halogenated bases were inhibitory.

Nucleic acid bases with sulfur groups. 2-Thiouracil was one of the first inhibitors of plant virus multiplication to be identified (1). It effectively inhibits multiplication of TMV but does not inhibit CCMV (4). 2-Thiouracil, which is incorporated into TMV RNA (6), was thought to inhibit multiplication by interrupting viral RNA synthesis. However, we recently have shown that 2-thiouracil does not inhibit polymerization of TMV RNA, but instead inhibits some earlier function (3). Two other pyrimidines with sulfur groups were found to be inhibitory, 2-thiopyrimidine and 2,4-dithiopyrimidine. All three inhibitory compounds have thio groups at the 2′ position. However, other 2-thiopyrimidines with additional modifications were not inhibitory. Interestingly, 2,4-dithiopyrimidine also inhibited multiplication of CCMV, whereas 2-thiouracil did not.

None of the sulfur-modified purines were inhibitory. Overall, only a small percentage of sulfur-modified bases were active (three of 28 tested).

Addition of nitrogen in the pyrimidine ring. 6-Azauracil (7) and 5-azacytidine (2) are known to inhibit multiplication of some plant viruses. Both were inhibitory to CCMV and 6-azauracil inhibited TMV (Table 1). The analogue of 6-azauracil with the nitrogen in the 5′ position was not inhibitory nor was 2-thio-6-azauracil. The base of the nucleoside, 5-azacytidine, was not inhibitory, nor was 6-azacytidine.

An effective new inhibitor of CCMV is melamine (2,4,6-triamino-*s*-triazine). It is interesting in that it is a relatively inexpensive synthetic compound that has less resemblence to normal nucleic acid bases because it is a regular triazine. However, other triazines and other similar compounds were not inhibitory.

Removal of nitrogen from pyrimidine and purine rings. Previously we reported that 3-deazauridine, 2,3-diaminopyridine, and tubercidin (a purine analogue) inhibited the multiplication of both TMV and CCMV (2). Here we tested these compounds with 11 other chemicals with these types of modifications. None of the other pyrimidines or purine analogues tested were inhibitory. Even the base (3-deazauracil) of the inhibitory nucleoside, 3-deazauridine, was not inhibitory.

Shift of nitrogen in pyrimidine and purine rings. Three pyrimidine and six purine analogues were obtained and tested that had this type of alteration, but none were inhibitory.

Alteration of the sugar moiety. Previously, we reported that adenine-9(2,3-dihydroxypropyl)monohydrate, cordycepin, and adenine arabinoside inhibited multiplication of TMV or CCMV (2). Ten additional nucleosides with modifications of the sugar were tested. Two additional compounds that are altered at the 5′ position, 5′-iodo-5′-deoxyadenosine and 5′-methyl-5′-deoxythioadenosine, were found to be inhibitory, although the latter compound did not inhibit CCMV.

Tri-*o*-acetyl modification of the sugar was not active. Several other arabinoside nucleosides also were not active. However, five of 13 compounds with modifications of the sugar moiety were inhibitory to at least one of the viruses.

DISCUSSION

The principal objective of this work was to gain some information on the general types of alteration of nucleic acid precursors that would have the greatest probabilities of activity against virus multiplication. With a better understanding of structure-function relationships of inhibitory compounds, we hope we can design chemicals with greater efficacy against virus diseases. A relatively high percentage of nucleosides with sugar modifications were active. This corresponds to findings that animal antiviral chemicals with this type of modification appear to be relatively selective. Also, the addition of novel side groups to bases or nucleosides resulted in a relatively high proportion of inhibition. Therefore, nucleosides with these types of modification should be examined for antiviral activity.

One observation that emerged from our examination of a large number of analogues of normal nucleoside and nucleic acid bases for ability to inhibit the multiplication of TMV or CCMV was the preciseness of the structural requirements for antiviral activity. Only a few modifications were inhibitory. Most analogues had no activity. Most alterations of active compounds resulted in loss of activity.

It should be emphasized that in this work chemicals were tested only for ability to inhibit virus multiplication, which is only one step toward developing an effective antiviral chemical. However, eight new inhibitors of virus multiplication were identified and several of these chemicals have unusual properties. Understanding the mode of action of these new inhibitors should be valuable in identifying chemical-susceptible targets that could lead to the design of a next generation of antiviral chemicals and perhaps, one step closer to using chemicals to help control virus diseases.

LITERATURE CITED

1. Commoner, B., and Mercer, F. 1951. Inhibition of the biosynthesis of tobacco mosaic virus by thiouracil. Nature (London) 168:113-114.
2. Dawson, W. O. 1984. Effects of animal antiviral chemicals on plant viruses. Phytopathology 74:211-213.
3. Dawson, W. O., and Grantham, G. L. 1983. Effect of 2-thiouracil on RNA and protein synthesis in synchronous and asynchronous infections of tobacco mosaic virus. Intervirology 19:155-161.
4. Dawson, W. O., and Kuhn, C. W. 1972. Enhancement of cowpea chlorotic mottle virus biosynthesis and *in vivo* infectivity by 2-thiouracil. Virology 47:21-29.
5. Gordon, M. P., and Staehelin, M. 1959. Studies on the incorporation of 5-fluorouracil into a virus nucleic acid. Biochim. Biophys. Acta 36:351-361.
6. Jeener, R., and Rosseels, J. 1953. Incorporation of 2-thiouracil-^{35}S in the ribose nucleic acid of tobacco mosaic virus. Biochim. Biophys. Acta 11:438.
7. Ralph, R. K., and Wojcik, S. J. 1976. Inhibition of turnip yellow mosaic virus by pyrimidine analogue. Biochim. Biophys. Acta 444: 266-268.
8. Schlegel, D. E., and Rawlins, T. E. 1954. A screening test of the effect of organic compounds on production of tobacco mosaic virus. J. Bacterol. 67:103-109.
9. Schneider, I. R. 1954. The effect of purines, purine analogs and related compounds on the multiplication of tobacco mosaic virus. Phytopathology 44:243-247.