antibody will only be the starting point for a variety of man-made secondary antibodies, each manufactured to satisfy a special requirement.

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Research Articles

The Site of Attachment in Human Rhinovirus 14 for Antiviral Agents That Inhibit Uncoating

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WIN 51711 and WIN 52084 are structurally related, antiviral compounds that inhibit the replication of rhino (common cold) viruses and related picornaviruses. They prevent the *p*H-mediated uncoating of the viral RNA. The compounds consist of a 3-methylisoxazole group that inserts itself into the hydrophobic interior of the VP1 β-barrel, a connecting seven-membered aliphatic chain, and a 4-oxazolinylphenoxy group (OP) that covers the entrance to an ion channel in the floor of the "canyon." Viral disassembly may be inhibited by preventing the collapse of the VP1 hydrophobic pocket or by blocking the flow of ions into the virus interior.

THE COMMON COLD, POLIOMYELITIS, HEPATITIS A, AND foot-and-mouth disease are among a large group of diseases whose cause can be attributed to various members of the picornavirus family. These viruses are among the smallest RNA-containing animal viruses with a molecular weight of approximately 8.5×10^6, of which roughly 30 percent is RNA. Their external diameter is approximately 300 Å and their protein shells have

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icosahedral symmetry. The single (+)RNA strand sequences have been determined for a large number of picornaviruses, and these have been found generally homologous both in gene order and base sequence. The sequence for human rhinovirus 14 (HRV14, the target of the antiviral compounds discussed in this article) has been determined by Callahan *et al.* (*1*) and Stanway *et al.* (*2*).

The three-dimensional structures of two picornaviruses, HRV14 (*3*) and poliovirus (*4*), have recently been described in atomic detail and that of a third (Mengo virus) is also known (*5*). The three larger structural viral proteins (VP1, VP2, and VP3) form the exterior of the viral capsid, while VP4 is at the interface between the capsid and the RNA. VP1, VP2, and VP3 are each folded into an eight-stranded antiparallel β-barrel and are organized in the capsid with a pseudo $T = 3$ surface lattice (*6*). Four types of neutralizing antibody binding sites were found at insertions in the β-barrel structure, on the extreme surface of the virus, surrounding a 25 Å deep "canyon" on the viral surface. Each canyon encircles one of the 12 vertices of the virus and has been proposed to be the site of receptor binding (*3*). VP1 is the major structural contributor to the walls and floor of the canyon. The canyon floor is formed by relatively conserved sequences of the known picornaviruses, while the neutralizing antibody binding sites are at hypervariable regions of the viral capsid proteins.

Arildone (*7–10*) and WIN 51711 (*11–14*), as well as other chemical classes of compounds (*15*, *16*), inhibit picornavirus replication by preventing viral uncoating without affecting cellular attachment and penetration. These compounds stabilize the virion against alkaline and heat denaturation and loss of VP4. The effect of these substances on the virion properties in some ways resembles that of neutralizing antibodies or of sulfhydryl reagents (*17*, *18*).

WIN 51711 (Fig. 1) represents a class of compounds which inhibits picornavirus replication in tissue culture (*13*) and in animal models of human enterovirus disease (*12*, *19*). A large number of compounds related to WIN 51711 has been synthesized at Sterling-Winthrop Research Institute and tested against several different picornaviruses. The end point used in these studies is the minimal inhibitory concentration (MIC), which is defined as the concentration which reduces plaque numbers by 50 percent in cell culture. Although many of these compounds have a fairly wide spectrum of antipicornavirus activity, their MIC values can vary considerably between different picornaviruses. The MIC values can be dependent on a number of factors: the binding affinity of the compound to the virus, the ability of the bound compound to interfere with the uncoating process, and the incorporation of the compound into the host cell.

We report here an x-ray diffraction investigation of the structure of the antipicornavirus compounds WIN 51711 and WIN 52084 (Fig. 1) while bound to HRV14. WIN 52084 differs from WIN 51711 by only a single methyl group in position 4 on the oxazoline ring. Addition of the methyl group produces an asymmetric carbon atom. We used the racemic mixture for these studies. Attempts were made to correlate MIC values for a representative number of compounds with their probable binding characteristics in HRV14. These observations led to hypotheses on the mechanism of action of the antiviral compounds.

Binding of WIN compounds to crystalline virions. Cubic HRV14 crystals were prepared as described by Arnold *et al.* (*20*). In order to determine whether the antiviral compounds would diffuse into HRV14 crystals and bind to the virions, binding studies were performed with ^{14}C-labeled (27 μCi/μg) WIN 51711 and unlabeled WIN 52084. Crystals (approximately 0.2 mm in diameter) were soaked in 0.5 ml of 10 m*M* tris (*p*H 7.2) buffer containing 0.75 percent polyethylene glycol (PEG 8000) and 0.5 μl of the antiviral reagent solution dimethyl sulfoxide (DMSO) (1 mg/ml). The

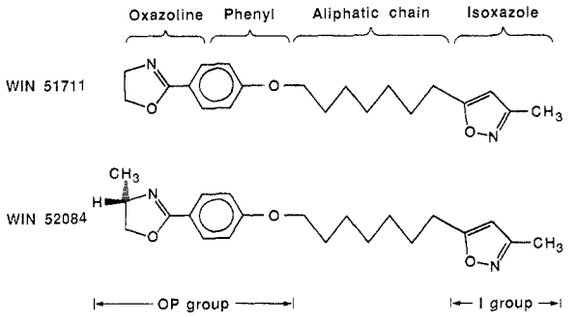


Fig. 1. Formula of the two antiviral compounds used in the studies reported here. They differ only by an additional methyl group on the oxazoline group in WIN 52084 (*S*).

crystals were soaked for 36 hours in solutions of ^{14}C-labeled WIN 51711 with or without WIN 52084. They were then washed extensively by transferring them, via a capillary tube, to three different wells containing 1 ml of 10 m*M* tris, *p*H 7.2, and 0.75 percent polyethylene glycol. The crystals were then transferred to a scintillation vial and disrupted by the addition of one drop of 1*M* HCl. After the crystals were dissolved, 10 ml of scintillation fluid was added and the radioactivity was determined by counting for 15 minutes.

The quantity of WIN 51711 bound to the crystals was calculated from the activity of labeled soaking solution and the soaked crystals. The activity of the second and third washings was used to estimate the background. The total volume of the crystals was calculated from measurements of the lengths (L) of the crystals from tip to tip and applying these to the formula, $V = L^3/6$, for the volume (V) of an octahedron. The cubic unit cell of HRV14 contains four virions in a volume of $(445)^3$ Å^3 (*20*). The binding stoichiometry could then be estimated from the sum of the crystal volumes used in each experiment, and the number of bound, labeled WIN 51711 molecules.

The results showed that WIN 51711 binds to HRV14 when the virus is in a crystalline form, with a stoichiometry of approximately 1.2 molecules per icosahedral asymmetric unit or 72 molecules per virion. Use of the ^{14}C-labeled WIN 51711 also permitted an investigation of the binding of WIN 52084 in a competition experiment. WIN 52084 (MIC, 0.06 μ*M*) was found to compete for the same site with a 6.4 times greater affinity than WIN 51711 (MIC, 0.4 μ*M*). This is consistent with the ratio of MIC values for HRV14. Thus, for at least these two compounds, the antiviral efficacy is roughly proportional to binding affinity. These results suggest specific binding because the stoichiometry is approximately 60 molecules per virion and the two WIN compounds exhibit different binding affinity while differing only in the substitution of a methyl group on the oxazoline group (Fig. 1).

Measurements of *p*I. Isoelectric focusing measurements were made to determine whether the WIN compounds or $Au(CN)_2$ affected the isoelectric point (*p*I) of the virions. Samples of HRV14 were prepared so that the final virus concentration was 5 mg/ml in 10 m*M* tris, *p*H 7.2, incubated for 1 hour at room temperature in the presence or absence of WIN 51711 at 10 μg/ml or 35 m*M* $Au(CN)_2$. The WIN compound was delivered as a stock solution (1 mg/ml) in DMSO. An agarose gel (10 by 8.5 by 0.33 cm) containing 1 percent agarose (Pharmacia IEF) and ampholytes (Pharmacia) at *p*H 3 to 9 was poured at 75°C on to an agarose gel bond backing, and allowed to harden for 1 hour at 4°C. Wicks soaked in 1*M* NaOH or 0.5*M* H_2SO_4 were placed along the 10-cm length, and the gel was first focused at 5°C for 1¾ hour at a constant amperage of 9 mA under nitrogen. The *p*H of the gels was then

Table 1. X-ray diffraction data processing.

	Native	WIN 51711	WIN 52084	$Au(CN)_2$	Sialic acid
Films used (No.)					
0.3° oscillation	83	21	47	46	0
0.6° oscillation	0	5	12	0	12
Reflections measured					
Number	2,706,020	297,387	591,945	814,535	185,268
Unique, with $F^2 > 1\sigma$	509,915	198,670	335,902	336,319	133,982
R factor*	11.0	14.1	11.0	10.1	13.6

* $R = \dfrac{\sum_h \sum_i |(F_h^2 - F_{hi}^2)|}{\sum_h \sum_i F_h^2} \times 100$, where F_h^2 is the mean of the i observations, F_{hi}^2, for reflection h.

measured every 0.5 cm along the gel with a surface electrode and then stained with Coomassie blue. In order to ensure that the virus migration was not impeded by the 1 percent agarose matrix, the samples were loaded on the gel at four different positions each corresponding to a *p*H value from 6 to 9. In each case, the equilibrium position was identical. To test whether the WIN compound or DMSO affected the gel rather than affecting the *p*I of the capsid, WIN compound was added to protein standards; there was no effect on *p*I.

The WIN 51711 and WIN 52084 compounds caused the *p*I to be increased from 6.9 to 7.2, while the $Au(CN)_2$ compound caused a drop of *p*I to 6.7. The WIN 51711 compound is weakly basic and is uncharged at neutral *p*H. Hence, the change in *p*I should be due to conformational changes that alter the environment of solvent accessible residues. This could be mediated by sterically blocking ion channels leading to the affected side chains, by altering the dynamics of the capsid conformation and hence the accessibility of internal groups, or by changing the pattern of side chain interactions.

The $Au(CN)_2$ compound binds to internal sulfhydryl (SH) groups accessible only through the ion channels (*3*) and produces conformational changes on the capsid similar to the WIN compounds (see below). The dissimilar effects of these compounds on the *p*I may be the result of the binding of charged species to SH groups or additional conformational changes induced by the $Au(CN)_2$.

Diffraction data collection. Crystals, 0.25 to 0.45 mm in diameter, were soaked (as described above) in solutions of WIN 51711 and WIN 52084 for 1 day at room temperature. An $Au(CN)_2$ derivative was also prepared (*21*). In an attempt to determine whether there was a sialic acid binding site, the crystals were soaked in 5 m*M* sialic acid for about 1 day. Data collected from the $Au(CN)_2$ and sialic acid–soaked crystals were subsequently found to be useful controls for the crystals soaked with WIN compounds. The x-ray diffraction data were collected at the Cornell High Energy Synchrotron Source (CHESS) by means of a variation of the "American method" (*21*). Each exposure was 2.25 minutes while the crystal was oscillated 0.3° or 0.6°. The resultant films were processed and scaled (*22, 23*). A summary of the results is shown in Tables 1 and 2.

Computation of difference maps. The derivative data were placed on the same scale as the native virus data with the use of local scaling in about 40 resolution shells. A 3 Å resolution difference map with

$$(F_{WIN} - F_{Native}) w_{MR} \exp i\alpha_{MR(N)}$$

coefficients was then computed, where $\alpha_{MR(N)}$ are the phases determined by the molecular replacement real-space averaging procedure of the native data (*3, 21*), F_{WIN} are the structure amplitudes of either of the antiviral compounds complexed with the virus and w_{MR} are weights obtained from the molecular replacement procedure. Terms between 12.0 and 3.08 Å with $w_{MR} > 0.5$ were used in the calculations. Terms with amplitudes larger than 2.5 times the mean native amplitude were rejected because they showed anomalously large differences. The difference maps showed clearly the position of the antiviral compound, as well as other significant positive and negative peaks mostly in the immediate vicinity of the antiviral compound site. These were interpreted in terms of rather large conformational changes associated with binding of the compounds. However, it was difficult to determine the new structural features of the capsid coat from these difference maps. Hence, a map was computed with $F_{WIN} \exp i\alpha_{MR(N)}$ as coefficients. The compound was almost invisible in this map either because of the apparently low stoichiometry of the bound compounds or because of the effect of the bias produced by the $\alpha_{MR(N)}$ phases derived for the native structure. The electron density maps of the WIN compound complexes can be represented as $k\rho_{WIN} + (1 - k)\rho_N$, where ρ_{WIN} is the electron density of the compound-bound virus, while ρ_N is the native structure. The constant k represents the proportion of the density corresponding to the compound-virion complex. Hence, a map with

$$[F_{WIN} - (1 - k)F_N] \exp i\alpha_{MR(N)}$$

coefficients would show the density of the altered virion with the bound compound. The value of k was obtained empirically by computing a number of difference maps and then selecting the map that subjectively gave the most facile interpretation of the density of the compound-virion complex. The best values of k were 0.25 for the two antiviral compounds and 0.03 for the $Au(CN)_2$ compound.

This result showed that, even though the $\alpha_{MR(N)}$ did not include the WIN compound density, the substitution of the two antiviral compounds was about 50 percent. These results were supported by molecular replacement refinement with respect to the F_{52084} amplitudes. They were also consistent with those obtained for the ^{14}C-

Table 2. Percentage of diffraction data measured with $F^2 > 1\sigma$.

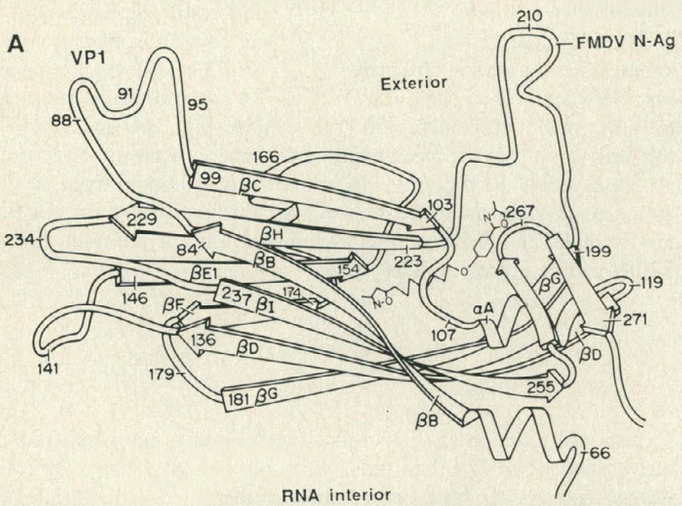
Resolution range (Å)	Native	WIN 51711	WIN 52084	$Au(CN)_2$	Sialic acid
∞ to 30	63	45	46	57	36
30 to 15	86	61	80	70	46
15 to 10	84	60	79	70	48
10 to 7.5	83	58	76	68	45
7.5 to 5.0	81	54	74	65	41
5.0 to 3.5	78	40	63	58	24
3.5 to 3.0	63	18	39	39	11

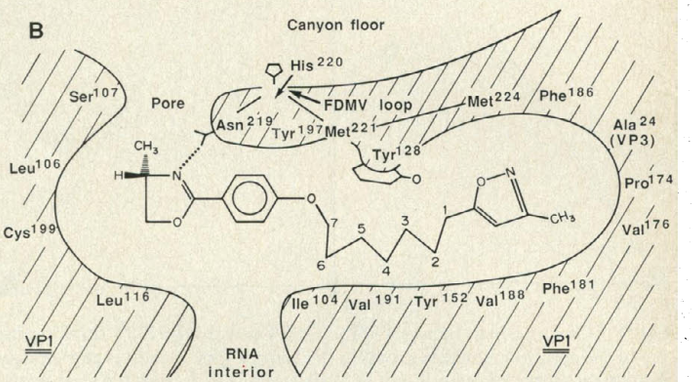
Table 3. HRV14 modifications due to various ligands.

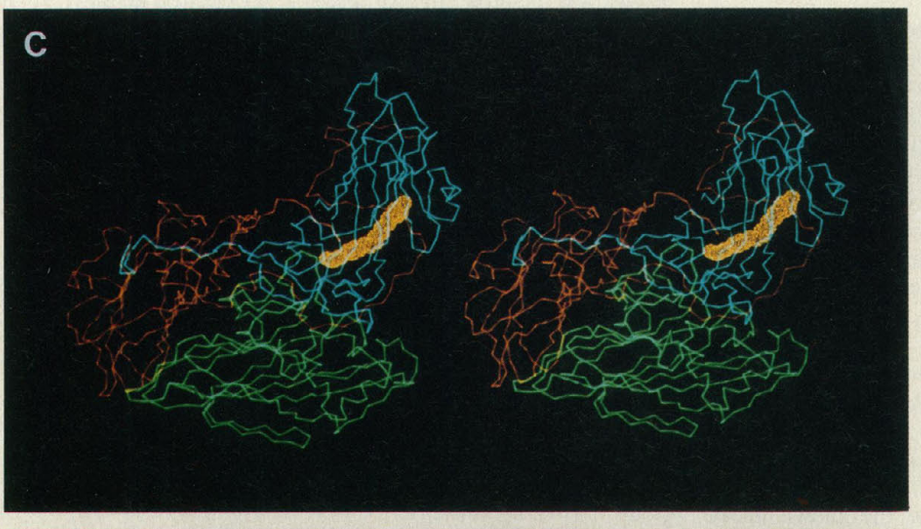
Compound	Date of collection	SH substitution	Putative Ca^{2+} site	WIN compound site	Incidental mutation of residue 170 in VP2
Native HRV14	May 84	No	Yes	No	Leu
$Au(CN)_2$	Nov. 84	Yes	Yes	Low occupancy	Leu
WIN compounds	Dec. 85	No	Increased occupancy	Yes	Val
Sialic acid	Dec. 85	No	Yes	No	Val

labeled compound within the degree of accuracy of the methods and considering the differences of soaking time (24 and 36 hours, respectively). Maps of both WIN compound-HRV14 complexes also showed additional density at a putative Ca^{2+} binding site on the fivefold axis (*3*) and evidence for a single mutational change (*24*).

The $Au(CN)_2$ difference map showed, in addition to the Au sites, a low level of density in the antiviral compound binding site (mostly but not exclusively in the I site) and the conformationally altered foot-and-mouth disease virus (FMDV) loop (Fig. 2A), corresponding to roughly 10 percent occupancy. There was no increased occupancy of the putative Ca^{2+} site in the $Au(CN)_2$ difference map (Table 3).







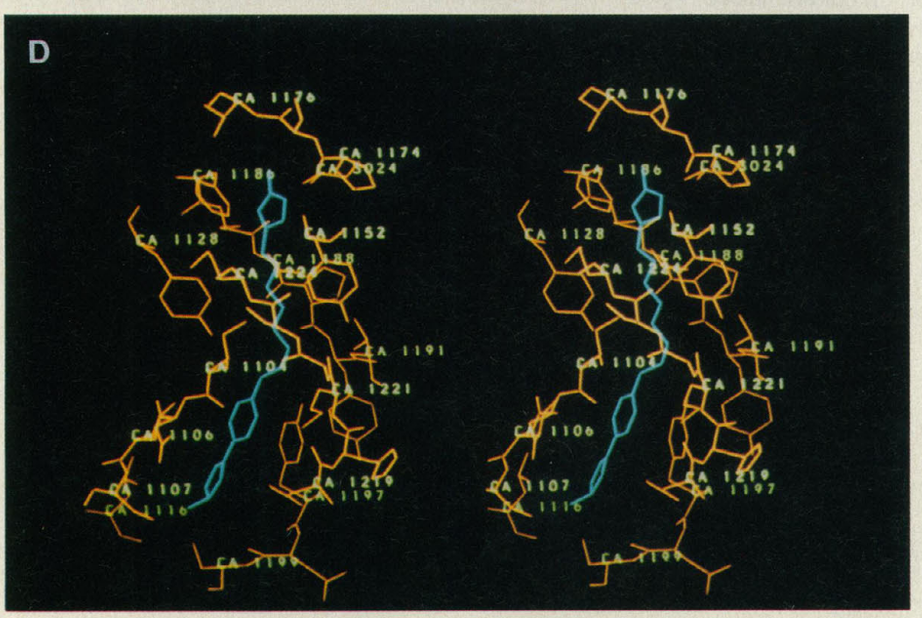


Fig. 2. (A) Ribbon drawing of VP1 with WIN compound binding site. (B) Diagrammatic representation of antiviral compound binding site. (C) Stereoscopic view of a protomer unit (VP1 is blue, VP2 and VP4 are green, VP3 is red) with surface of bound WIN compound in yellow. (D) Stereoscopic view of WIN 52084 in its VP1 environment. The first digit of the numbered residues indicates the VP (1, 2, 3, or 4), while the following three digits give the sequential amino acid number within the protein.

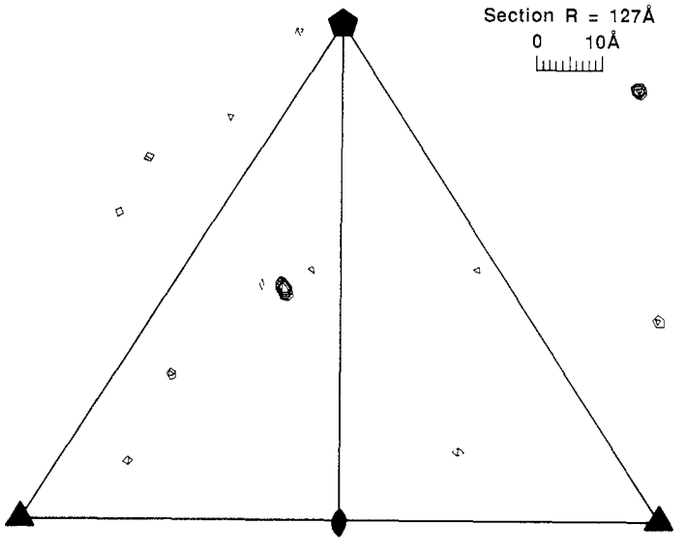


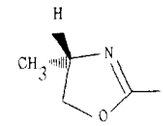
Fig. 3. Difference map between WIN 52084 and WIN 51711 showing the site of the additional methyl group in the former. The section is perpendicular to the icosahedral twofold axis at a distance of 127 Å from the viral center. Fourier coefficients were $(F_{52084} - F_{51711})$ exp $i\alpha_{MR(N)}$.

The sialic acid data were collected in an attempt to discover the site of binding of a possible terminal cellular receptor carbohydrate moiety. However, a difference map showed no significant density apart from a single mutation (*24*). As the crystals used to collect the sialic acid data and those used to collect the antiviral compound data sets were grown at the same time and the data were collected during the same visit to CHESS, the results for the crystals soaked in sialic acid served as an excellent control. Thus, the virus capsid structures for the crystals used for collecting the native data (in May 1984) and for the sialic acid–soaked crystals were identical except for a mutation (*24*) (Table 4). In contrast, the crystals soaked with antiviral compounds showed density at the antiviral compound binding site, conformational changes in VP1, and additional density at the putative Ca^{2+} site, as well as the incidental mutational change (Table 3).

The various electron density maps were examined initially on a mini-map as stacked sections and then on an Evans and Sutherland PS300 computer graphics device, relative to the known native HRV14 structure. A model of the altered portions of VP1 was built in the regions corresponding to the largest changes.

The WIN 51711 and WIN 52084 binding site. The electron density for both the WIN 51711 and WIN 52084 compounds was unequivocal and could readily be fitted with the corresponding chemical structure (see cover photo). Two flattened bulges at one end were interpreted as the oxazoline and phenoxy groups (the OP site), while a single, slightly flattened, bulge at the other end was interpreted to be the isoxazole group (the I site). The two ends were connected by a thinner density corresponding to the aliphatic chain.

The oxazoline and phenoxy groups were roughly coplanar, consistent with the crystal structure of WIN 51711 (*25*). A difference map between WIN 52084 and WIN 51711 showed the position of the additional methyl group on WIN 52084 (Fig. 3). The center of the peak was above the mean plane of the oxazoline group. Since the absolute "hand" of the virus electron density is known from laevo amino acids, right-handed α-helices, and left-handed β-sheet twists, for example, the hand of the asymmetric carbon atom in WIN 52084 can be represented as



corresponding to an (*S*) structure. This isomer is ten times more active against HRV14 than the (*R*) isomer. The more active optical isomer appears to bind with much higher affinity to HRV14 because it is this structure that is seen in the electron density map of the WIN 52084 complex with HRV14.

The OP group is located in a hydrophilic region where there is an opening (pore) in the base of the canyon (Fig. 2). The pore opens into a large channel leading to the viral RNA interior ending in the vicinity of the icosahedral threefold and fivefold axes. These channels are the likely pathways for diffusion of $Au(CN)_2$ to the internal binding sites (*3*). The proximity of the nitrogen in the oxazoline ring to the hydrogen of the Asn^{219} amide group suggests the presence of a hydrogen bond that may orient the OP end of the WIN compounds. Tyrosine-197 is roughly coplanar with the phenoxy group of the WIN compounds. The phenolic oxygens of Tyr^{197} and Tyr^{128} are both within 3.6 Å of the ether oxygen.

The I group binds into a hydrophobic pocket (Fig. 2 and Table 5) in the interior of the VP1 β-barrel, formed in part by the displacement of Tyr^{152} and Met^{221} by the WIN compounds. The greater hydrophobicity of the I binding site, with respect to the OP binding site, is consistent with the greater hydrophobicity of the isoxazole group.

The electron density corresponding to the aliphatic chain is slightly bowed where it skirts Tyr^{128} of VP1. The seven-membered chain was modeled to the density by introducing a slight dihedral twist about each single bond while maintaining essentially *trans* conformations of the dihedral angles along the aliphatic chain.

When Met^{221} is displaced by the phenoxy group of the WIN compounds, large conformational changes occur in polypeptide strand βH leading from the FMDV loop (Fig. 2A), across the canyon floor (Fig. 4 and Table 6). In the native virus this methionine blocks the entrance to the hydrophobic pocket which forms the I site. The main chain between residues 213 and 224 is displaced (the conserved Gly^{214} and Gly^{222} residues are the pivot points of this change) as much as 3 Å in places and some of the side chains are displaced considerably larger distances. In particular His^{220}, which forms part of the canyon lining but is not in the immediate neighborhood of the WIN compound binding site, undergoes the largest conformational change. Smaller conformational changes occur between residues 150 and 157 including the 2 Å shift of Tyr^{152} (Fig. 4).

Effectiveness of WIN 51711 and WIN 52084 on different picornaviruses. Several compounds that have been synthesized and

Table 4. Changes in RNA sequence corresponding to residue 170 of VP1 in HRV14.

Isolate	Date	RNA sequence	Amino acid
Original (*2, 3*)	Before November 1983	**AUA**	Ile
		↓	
KUO_2F_5 derivative	November 1983	CUU	
Native	May 1984	CUC	
$Au(CN)_2$ derivative	November 1984	**CUA**	Leu
		CUG	
		↓	
WIN 51711	December 1985	GUU	
WIN 52084	December 1985	GUC	
Sialic acid	December 1985	**GUA**	→ Val
		GUG	

Table 5. Residues within 3.6 Å of the bound WIN compound. The numbering of the aliphatic chain carbons is given in Fig. 2B. Abbreviations for viruses: HRV, human rhinovirus; PV, poliovirus; FMDV (A10), foot-and-mouth disease virus strain A10 (*32*).

Viral protein	Residue number	HRV14 amino acid type	Interaction	HRV2, HRV39, HRV49	PV1 Mahoney	PV1 Sabin	PV2 Lansing	PV3 Leon	Mengo	FMDV (A10)
1	104	Ile	Phenolic oxygen	Ile	Ile	Ile	Ile	Ile	Leu	Leu
1	106	Leu	Phenyl	Leu	Tyr	Tyr	Tyr	Tyr		
1	107	Ser	Methyl on oxazoline	Gln	Lys	Lys	Lys	Lys		
1	116	Leu	Oxazoline	Phe	Leu	Leu	Leu	Leu	Ser	Ala
1	128	Tyr	Phenolic oxygen, C-3 in aliphatic chain	Ile	Leu	Phe	Phe	Phe	Val	Ile
1	152	Tyr	C-4, C-2, C-1 of aliphatic chain	Tyr	Tyr	Tyr	Tyr	Tyr	Cys	Val
1	174	Pro	Isoxazole	Ala	Pro	Pro	Pro	Pro	Pro	Pro
1	176	Val	Methyl of isoxazole	Val	Ile	Ile	Val	Ile	Val	Ala
1	186	Phe	Isoxazole	Phe	Ile	Ile	Ile	Ile	Phe	Leu
1	188	Val	C-2 and C-3 of aliphatic chain	Leu	Val	Val	Val	Val	Val	Leu
1	191	Val	C-6 of aliphatic chain	Leu	Val	Val	Val	Val	Asn	Thr
1	197	Tyr	Phenol C-7 and C-5 of aliphatic chain	Tyr	Tyr	Tyr	Tyr	Tyr	Leu	Leu
1	199	Cys	Oxazoline	Met	His	His	His	His	Ala	Thr
1	219	Asn	Phenyl; N of oxazoline	Asn	Asn	Asn	Asn	Asp	Ser	Gly
1	221	Met	Phenyl	Met	Phe	Phe	Phe	Phe	Phe	Met
1	224	Met	C-1 of aliphatic chain	Leu	Leu	Leu	Leu	Leu	Leu	Ala
3	24	Ala	Isoxazole	Ala	Ala	Ala	Ala	Ala	Ile	Val

Compound	MIC (μM)	HRV2	HRV39	HRV49					
WIN 51711	0.4	3.8	9.3	4.7	8.7	1.1	0.8	1.1	Inactive	Inactive
WIN 52084	0.06	0.1	2.2	0.8	>9.3	6.9	5.7	1.2	Inactive	No data

tested for activities against a number of different picornaviruses are shown in Table 6. These data represent only a small subset of the available information, but are sufficient to demonstrate some of the features required to obtain an effective compound that inhibits viral uncoating.

The inhibitory effect of a particular compound on the replication of a given virus serotype will depend on (i) the interactions the compound makes within the virion (Table 5), (ii) the residues affected by conformational changes induced by the binding of the compound (Table 6), and (iii) the residues that govern the particular properties in the virus which the compound alters. The latter category might include residues around the pore leading into the ion channels, at the putative Ca^{2+} site, at subunit interfaces, or residues at subunit interfaces. Amino acid sequence alignments predict no differences between HRV2, 39, and 49 for category (i) (Table 5) and little differences in category (ii). Nevertheless, Table 5 shows significant differences, of up to a factor of 22, in MIC values for these viruses. Thus caution must be exercised in the interpretation of MIC values (which depend not only on the affinity of binding but also the biological effect of the binding, intracellular concentrations and perhaps other unknown factors) merely in terms of binding when comparing the effect of a given compound with different viruses. Conversely, it is reasonable to compare MIC values for different compounds on a given virus primarily in terms of binding affinity both because (iii) above does not apply and because of the supportive results reported here on competition experiments with both WIN 51711 and WIN 52084 on HRV14.

The only polar interaction that the WIN compounds presumably make with HRV14 (at Asn^{219}) is conserved in rhino- and polioviruses. However, in Mengo virus and FMDV, Asn^{219} is replaced by serine and glycine, respectively. The structure of Mengo virus (*5*) has a VP1 binding pocket similar to that observed in HRV14, but access to the pocket is more restricted. The lack of activity of WIN 51711 in Mengo virus, FMDV, and hepatitis A virus may thus be related to either the inability to orient the oxazoline group in the absence of a polar interaction or to inaccessibility of the corresponding binding site.

The most significant difference in the WIN binding pocket between HRV14 and HRV2 is that Val^{188} and Val^{191} have both been changed to leucine. This is likely to cause severe steric hindrance to the insertion of the isoxazole group and aliphatic chain into the hydrophobic pocket. The increase of the MIC value for WIN 51711 in HRV2 with respect to HRV14 is thus not surprising. That this is not the case for WIN 52084 could be

Table 6. Residues that undergo conformational changes due to binding of WIN compounds.

	Residues in chain leading from FMDV loop across canyon floor at HRV14 VP1 residue No.													Residues in highly conserved section at carboxyl end of βE corner at HRV14 VP1 residue No.							
Virus	213	214	215	216	217	218	219	220	221	222	223	224	225	150	151	152	153	154	155	156	157
HRV14	Tyr	Gly	Ile	Thr	Val	Leu	Asn	His	Met	Gly	Ser	Met	Ala	Ala	Met	Tyr	Val	Pro	Pro	Gly	Ala
HRV2	Tyr	Gly	Thr	Ala	Asn	Thr	Asn	Asn	Met	Gly	Ser	Leu	Cys	Tyr	Met	Tyr	Val	Pro	Pro	Gly	Ala
HRV39	Tyr	Gly	Val	Ser	Val	Thr	Asn	Asp	Met	Gly	Thr	Leu	Cys	Tyr	Met	Tyr	Val	Pro	Pro	Gly	Ala
HRV49	Tyr	Gly	Tyr	Val	Ser	Thr	Asn	Asn	Met	Gly	Ser	Leu	Cys	Tyr	Met	Tyr	Val	Pro	Pro	Gly	Ala
PV1 (Mahoney)	Tyr	Gly	Ala	Ala	Ser	Leu	Asn	Asp	Phe	Gly	Ile	Leu	Ala	Ile	Met	Tyr	Val	Pro	Pro	Gly	Ala
PV2 (Lansing)	Tyr	Gly	Ala	Ala	Ser	Leu	Asn	Asp	Phe	Gly	Ser	Leu	Ala	Ile	Met	Tyr	Val	Pro	Pro	Gly	Ala
PV3 (Leon)	Tyr	Ser	Ala	Met	Thr	Val	Asp	Asp	Phe	Gly	Val	Leu	Ala	Ile	Met	Tyr	Val	Pro	Pro	Gly	Ala

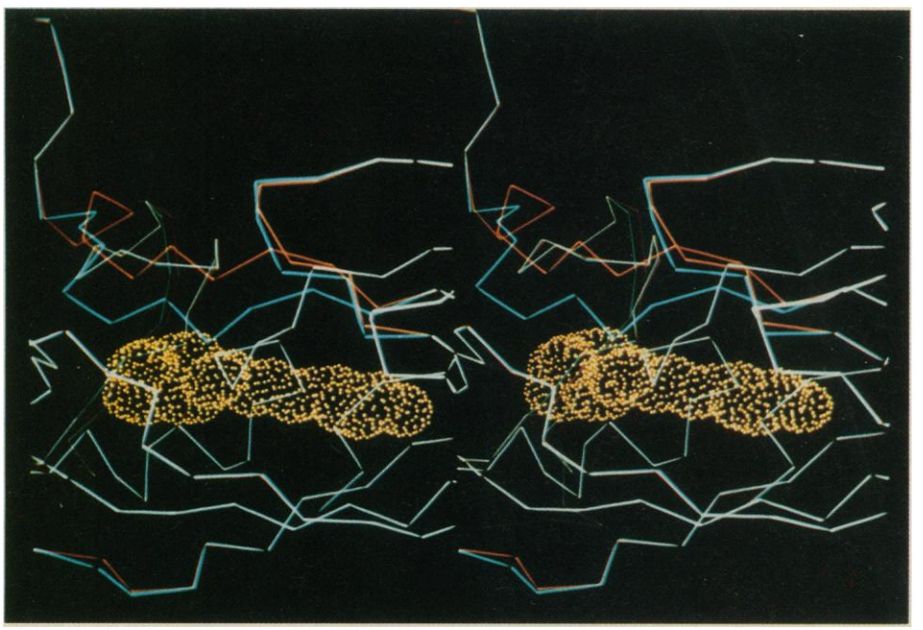


Fig. 4. Superposition of the native structure (light blue) of HRV14 VP1 (red) with the structure after binding a WIN compound. The canyon floor is at the top.

interpreted in terms of the overriding effect of the improved binding of the oxazoline ring.

Effectiveness of modifications to WIN 51711 and WIN 52084. The optimal length of the aliphatic chain $(CH_2)_n$ for HRV14 inhibition is when n is either 5 or 7 (Table 7) (*10*). Chains where n is less than 5 would probably reduce the hydrophobic binding within the pocket and cause steric hindrance to the I group in the more restricted portions of the binding site. Chains where n is greater than 7 would cause severe steric hindrance as the isoxazole group is pushed further into the hydrophobic pocket and the aliphatic chain is severely buckled. Chains with n even would require *cis* bonds in the aliphatic chain to keep the isoxazole group (assuming the same binding of the OP group) out of sterically hindered positions.

The hand of the asymmetric carbon atom in the oxazoline ring of WIN 52084 is clearly significant because the *S* isomer is ten times more active than the *R* isomer (Table 7). This result can be explained by improved hydrophobic interactions of the *S* methyl group with a hydrophobic pocket formed by Leu^{106} and Ser^{107} (Fig. 2D). This interaction would stabilize the conformation of the oxazoline ring thereby allowing for a favorable orientation for hydrogen bonding to Asn^{219}. The *R* methyl group would be expected to have no positive effect on hydrophobic interactions, consistent with the similar MIC values obtained for the *R* methyl compound and the desmethyl compound WIN 51711.

The antiviral activity can be improved by introducing suitable additional hydrophobic groups onto the phenyl group (Table 7). Larger hydrophobic groups in position 2 improve the efficacy in HRV2 possibly because of an improved interaction with a phenylalanine in place of Leu^{116} in HRV14. These groups have little effect when the compounds are tested on HRV14 where these aromatic groups are absent. The comparative observations are available only when the aliphatic chain is of length $n = 5$.

The above explanation of the MIC observations is entirely qualitative and regards only the binding component represented in the MIC values. It may be possible to quantitate such results with the use of energy calculations of various compounds bound to the conformationally changed virion and by checking the resultant predicted binding mode with x-ray diffraction observations.

Function of the hydrophobic pocket which binds the WIN compounds. The general effect of the antiviral compounds discussed above is to increase viral stability, prevent uncoating, and increase the *p*I from 6.9 to 7.2. The function of the antiviral compounds might be compared to that of a nicotinamide adenine dinucleotide (NAD) cofactor in dehydrogenases (*26, 27*). The adenine end of NAD binds into a hydrophobic pocket and enhances protein stability just as the I group of the WIN compounds might increase viral stability by filling the empty hydrophobic pocket within the VP1 β-barrel (*28*). The nicotinamide of NAD is functionally necessary for enzymic catalysis whereas the OP group of the WIN compounds perhaps functions to stop ions entering the virion through the pore on the floor of the canyon (Fig. 2B), thus possibly decreasing the permeability of the virion to cesium ions. Alternatively, the WIN compounds might lock the conformation of VP1 such that the virus cannot disassemble by collapsing the hydrophobic pocket.

The observed increase in occupancy of the putative Ca^{2+} ion on the fivefold axis may also contribute to or be the result of increased

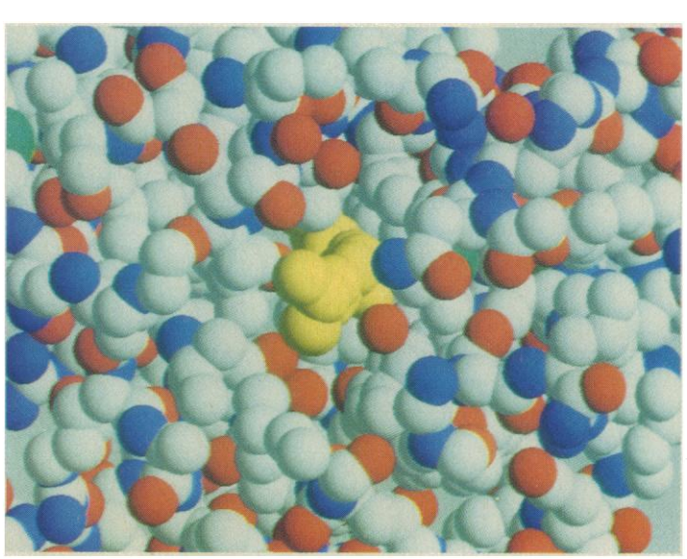
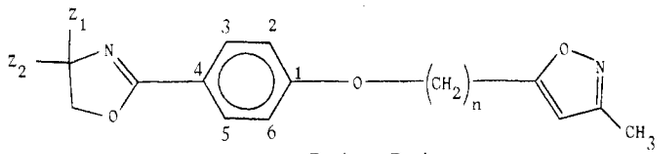


Fig. 5. Space filling view of WIN compound binding site viewed from the OP end when compound fills cavity. The atomic positions correspond to those when an antiviral agent is bound. In the absence of the compounds, Met^{221} of VP1 occupies a part of the cavity. The atomic radii are 1.5 Å.

Table 7. Effectiveness of a variety of compounds: MIC values (μM).



Compound	Z_1	Z_2	Position 2	Position 3	n	HRV14	HRV2
			Optimum length of aliphatic chain				
WIN 53670	H	H	H	H	4	Inactive	7.3
WIN 52035	H	H	H	H	5	0.7	1.1
WIN 53337	H	H	H	H	6	2.9	2.1
WIN 51711	H	H	H	H	7	0.4	3.5
WIN 53455	H	H	H	H	8	3.9	5.9
			Substitution on the oxazoline group				
WIN 51711	H	H	H	H	7	0.4	3.8
WIN 52084 *S/R*	H	CH_3	H	H	7	0.06	0.11
WIN 52084 *S*	H	CH_3	H	H	7	0.04	0.08
WIN 52084 *R*	H	CH_3	H	H	7	0.4	1.7
WIN 52193	CH_3	CH_3	H	H	7	0.1	1.5
			Substitutions on the phenolic group				
WIN 52035	H	H	H	H	5	1.2	1.0
WIN 54089	H	H	CH_3	H	5	0.8	0.09
WIN 53338	H	H	Cl	H	5	2.5	0.06
WIN 54274	H	H	H	CH_3	5	1.6	1.2
WIN 54090	H	H	H	Cl	5	0.3	0.9

stability of VP1 when the WIN compound binding site is occupied. Thus the channel down the fivefold axis is also blocked when WIN compounds are bound. The closing of pores on the viral surface will exclude solvent from charged groups within the virus and is presumably responsible for the change of the measured *p*I. The altered environment of His^{220} may also contribute to the *p*I change, although this residue is not conserved in other picornaviruses. The presence of the compounds may thus prevent ions from entering the large channels in the viral coat protein leading to the RNA, thereby preventing viral swelling and disassembly such as occurs in plant viruses (*29, 30*).

The true function, if any, of the hydrophobic pocket into which the antiviral compounds fit so snugly is not clear (Fig. 5). A similar hydrophobic pocket occurs in VP1 of Mengo virus, although it is inaccessible from the viral exterior and the WIN compounds do not bind to that virus (*5*). A possible suggestion is provided by the results of $Au(CN)_2$ binding to HRV14 and the structure of poliovirus Mahoney type 1 (*4*). $Au(CN)_2$ reacts with sulfhydryl groups (residues 69 on VP1 and 7 and 248 on VP2) (*3*), which are accessible only by virtue of the channels within the virus. An electron density map of the $Au(CN)_2$ data at 3 Å resolution shows a partial occupancy of the antiviral compound binding site by a foreign molecule as well as conformational changes in VP1 similar to those produced by the binding of the WIN compounds. The electron density of poliovirus 1 Mahoney reported by Hogle *et al.* (*4*) also shows weak density at precisely the position occupied by the WIN compounds in HRV14. The surrounding chains in the poliovirus map appear to have taken on a roughly similar conformation as in the HRV14 complexes with the WIN compounds. Although there are no bound ligands at the antiviral compound binding site in native HRV14, in HRV14 treated with sialic acid, or in native Mengo virus, two instances exist where an unknown molecular species has found its way into this site. The $Au(CN)_2$ has induced the binding of a molecule fortuitously available within the virus or in the external solvent. For poliovirus, such a molecule may have bound during the virus purification process. This molecule may affect the virion permeability to cesium ions (*31*) or lock the virion into a conformation that would normally be changed during uncoating. The proximity of the hydrophobic WIN compound binding pocket to the base of the canyon suggests it may be involved with membrane interactions after receptor binding. The antiviral compounds might compete with the unknown molecule, and thereby interfere with the normal uncoating process.

This is the first description of an antiviral drug interaction with a virion at atomic resolution. The results offer plausible mechanisms for the biological activity of this class of compounds and raise the possibility of antiviral drug design for rhinoviruses as well as other picornaviruses.

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24. In the difference maps of WIN 51711 and 52084, there was one additional negative peak. The depth of the negative peak and the height of the largest positive WIN compound related peaks were about equal. The additional peak was far removed from the WIN compound binding site. A peak at the same site was also present in a difference map of the sialic acid–soaked crystals with respect to native HRV14. This map showed no other significant features. In contrast, this peak was not present among data collected in August 1984 on an abortive uranyl derivative, nor was it in the November 1984 $Au(CN)_2$ data. Inspection of the electron density at this position in either one of the WIN compound electron density maps suggested that residue Leu^{170} in VP2 was, in fact, a valine. Reexamination of the native HRV14 map, based on May 1984 data, showed clearly that this residue was a leucine, inconsistent with the published isoleucine sequence (*1, 2*). It has, therefore, tentatively been concluded that a mutation of leucine to valine occurred between June 1984 and December 1985. In addition, there was an earlier mutation, prior to the May 1984 data collection, from isoleucine to leucine. These changes can all be explained in terms of a hypervariable single base (Table 4) permitting a sequence change of an external amino acid.
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32. The sequence alignments are based on unpublished work by A. Palmenberg.

33. We thank the staff of the Cornell High Energy Synchrotron Source (CHESS) for their assistance in our data collection; J. Griffith, J. Erickson, E. Frankenberger for participation in the native and heavy atom derivative data collection; R. Rueckert (University of Wisconsin) for stimulating discussions; R. Colonno (Merck Sharp & Dohme) for the enthusiastic initial analysis of $Au(CN)_2$-treated HRV14; S. Lemon (University of North Carolina) for MIC measurements with respect to hepatitis A virus; R. K. Kullnig (Sterling-Winthrop Research Institute) for providing the crystal structure of WIN 51711; A. Palmenberg (University of Wisconsin) for the alignment of picornavirus amino acid sequences and G. Cordova (Du Pont de Nemours and Company) for permission to compare the HRV39 and HRV49 amino acid sequences with HRV14. Figure 5 was generated with the use of the program ANIMOL kindly provided by R. M. Himer (Du Pont de Nemours and Company) and implemented by M. G. Webb and R. P. Millane on a lexidata graphics system. Supported by NIH and NSF grants and by a gift from Sterling-Winthrop Research Institute (M.G.R); by an NSF grant for the use of the Purdue University Computer Center; and an NIH postdoctoral fellowship during part of this work (E.A.). Atomic coordinates of the native virus are deposited at the Brookhaven Protein Data Bank.

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