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Binding Affinities of Structurally Related Human Rhinovirus Capsid-Binding Compounds Are Related to Their Activities against Human Rhinovirus Type 14

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The binding affinities (K_ds) and the rates of association and dissociation of members of a chemical class of antiviral compounds at their active sites in human rhinovirus type 14 (HRV-14) were determined. On the basis of analysis by LIGAND, a nonlinear curve-fitting program, of saturation binding experiments with HRV-14, the K_ds for Win 52084, Win 56590, disoxaril (Win 51711), and Win 54954 were found to be 0.02, 0.02, 0.08, and 0.22 μM, respectively. The independently determined kinetic rates of association and dissociation resulted in calculated K_d values which were in agreement with the K_d values determined in saturation binding experiments. Scatchard plots of each of four compounds for the binding data indicated that approximately 40 to 60 molecules were bound per HRV-14 virion. Hill plots showed no evidence of cooperativity in binding. Furthermore, the antiviral activities (MICs in plaque reduction assays with HRV-14) for this limited series of compounds (n = 4) correlated well (r = 0.997) with the observed K_ds. Likewise, the absence of detectable binding of Win 54954 to the drug-resistant mutant HRV-14 (Leu-1188) corresponded to a lack of antiviral activity. The positive relationship between the antiviral activities and the K_ds that were determined may have implications for the molecular design of capsid-binding antirhinovirus drugs.

The structure of human rhinovirus type 14 (HRV-14) consists of an icosahedral protein shell with 60 protomer units composed of four nonidentical viral proteins (VP1, VP2, VP3, and VP4) surrounding a positive single-stranded RNA genome (22). A canyon or depression (about 2.5-nm deep) encircles each of the virion's pentamer vertices (22). A number of structurally related antipicornaviral compounds, which were synthesized at the Sterling Research Group, Rensselaer, N.Y. (7, 8), have been instrumental as an aid in exploring the structure of rhinoviruses relative to the early events in the viral replication processes. One of these compounds, disoxaril, was reported to interact directly with the virion capsid and stabilize the capsid conformation against thermal inactivation (10). These compounds were shown by X-ray crystallography studies to bind within a hydrophobic pocket in the viral protein (VP1) beta-barrel structure of the HRV-14 capsid, with the induction of large conformational changes in the canyon floor above the pocket (24). Crystallographic examination of several of these compounds (4), including Win 52084, Win 56590, and disoxaril (Win 51711), showed that all the compounds bind to the same site in the HRV-14 capsid and induce similar conformational changes. However, the orientations and positions of the compounds within the pocket differ (4). Preliminary binding studies with radiolabeled disoxaril (24) estimated that disoxaril is bound to HRV-14 with a stoichiometry of approximately 72 molecules of compound per virion. The importance of compound binding for antiviral activity is also supported by molecular and genetic studies of drug-resistant mutants (11). In several highly drug-resistant mutants with amino acid substitutions which decrease the space within the drug-binding pocket, the entry or binding of compounds within the pocket is blocked.

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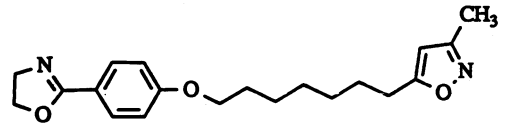
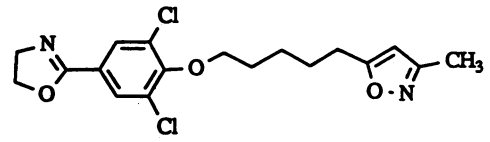
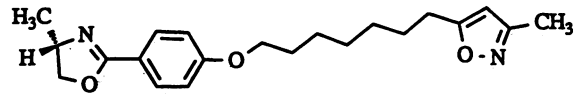
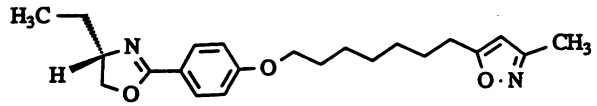
A dual mechanism of action has been suggested for these compounds (20), with the inhibition of adsorption for HRV-14 and the inhibition of uncoating for HRV-2 and poliovirus types 1 and 2. X-ray structural studies of HRV-14 suggested that the canyon serves as the site of viral attachment to its cellular receptor (21, 22). Mechanism of action studies (20) demonstrated that the large conformational changes induced by the compounds in the floor of the canyon in the HRV-14 capsid resulted in an inhibition of the adsorption of the virion to its cellular receptor. In contrast, previous studies demonstrated that the mechanism of action of disoxaril is the inhibition of uncoating for poliovirus types 1 and 2 and HRV-2, while the adsorption of these virions is not inhibited (10, 28).

Several new antiviral agents have been reported as capsid-binding agents or inhibitors of uncoating (25). MDL 20,610 (12); 44 081 R.P. (1); R61837 and 89.365 (2); and Ro 09-0410, RMI-15,731, and 4′,6-dichloroflavan (17, 18) were reported to bind or interact directly with the rhinovirus capsid. MDL 20,610 (12) and Ro 09-0410 (17) bind directly to the rhinovirus capsid, resulting in stabilization of the virion capsid to inactivation by heat and low pH and the inhibition of viral uncoating. Analyses of sedimentation of viral particles in sucrose gradients showed that the binding of [^{3}H]Ro 09-0410 to the capsid of HRV-2 was competitively inhibited by Ro 09-0410, some newer amide analogs of Ro 09-0410, disoxaril, and 4′,6-dichloroflavan (18). Rhinovirus strains resistant to Ro 09-0410, 4′,6-dichloroflavan, and disoxaril showed cross-resistance to all the other agents (18); and rhinovirus strains resistant to R61837 showed cross-resistance to disoxaril, sodium dodecyl sulfate, chalcone, dichloroflavan, and MDL 20,610 (3), suggesting that all of these capsid-binding agents bind to the same site or to sites in close proximity on the capsid. X-ray crystallographic results confirm that R61837 and the Win compounds go to the same binding site (6). Although qualitative evidence for drug binding was provided

TABLE 1. Compound structures, antiviral activities, and specific activities

Compound	Structure	Antiviral activity (MIC [μM][a])	Sp act (Ci/mmol)[b]
Disoxaril		0.88	45.2
Win 54954		2.32	53.4
Win 52084		0.05	65.1
Win 56590		0.04	52.0

[a] MIC, concentration that resulted in a 50% reduction in HRV-14 plaque assays.
[b] Specific activity of the ^{3}H-radiolabeled compound.

for a number of these compounds, quantitative measurements of binding have not yet been published.

The availability of ^{3}H-labeled Win compounds with high specific activities has made possible the determination of the binding affinities (K_ds) and the rates of association (k_{on}) and dissociation (k_{off}) for some members of this chemical class of antiviral compounds at their receptor sites. In addition, a correlation between the K_ds for and the biological activities of the compounds can be demonstrated.

MATERIALS AND METHODS

Cells and viruses. HRV-14, which was originally provided by Roland Rueckert of the Institute for Molecular Virology, University of Wisconsin, Madison, was obtained from Thomas Smith of the Department of Biological Sciences, Purdue University, West Lafayette, Ind. The drug-resistant mutant HRV-14 (Leu-1188), which was highly resistant to disoxaril, was selected in the presence of disoxaril. This virus, referred to as clone SW12 (4) and the Leu-1188 mutant (20), contained a change of the VP1 valine 188 residue to a leucine (in 1188, the 188 refers to the amino acid sequence number and the first number 1 refers to the fact that this amino acid is in the VP1 protomer). The HRV-14 and the Leu-1188 mutant were propagated and titers were determined by plaque assay in HeLa cell monolayers. The HeLa cells, which were originally obtained from Roland Rueckert, were grown in Hanks minimal essential medium with streptomycin and penicillin at 100 μg and 10 U/ml, respectively, and supplemented with 5% fetal bovine serum (GIBCO Laboratories).

HRV-14 and the Leu-1188 mutant were propagated in HeLa cells, using a multiplicity of infection of 3 PFU of virus per cell and following a procedure similar to that described previously for HRV-1A (13). After the 30-min adsorption for HRV-14 and the mutant, the infected cells were incubated for an additional 9 h, until harvest.

Radiolabeled compounds. Four ^{3}H-labeled Win compounds labeled in the aliphatic chain to a high specific activity were obtained from Dupont NEN Research Products (Boston, Mass.). The specific activities of disoxaril {5-[7-[4-(4,5-dihydro-2-oxazolyl)phenoxy]heptyl]-3-methylisoxazole; Win 51711}, Win 54954 {5-[5-[2,6-dichloro-4-(4,5-dihydro-2-oxazolyl)phenoxy]pentyl]-3-methylisoxazole [27]}, Win 52084 {(*S*)-5-[7-[4-(4,5-dihydro-4-methyl-2-oxazolyl) phenoxy]heptyl]-3-methylisoxazole}, and Win 56590 {(*S*)-5-[7-[4-(4-ethyl-4,5-dihydro-2-oxazolyl)phenoxy]heptyl]-3-methylisoxazole} ranged from 45 to 65 Ci/mmol (Table 1).

Virus purification. Virus was purified by a procedure described by Erickson et al. (9), with modifications (omission of EDTA from the buffer and the inclusion of 0.1% 2-mercaptoethanol in the sucrose gradients only, the reduction of the concentration of DNase to 10 μg/ml, and changes in the centrifugation steps). The initial clarification to remove cellular debris was by centrifugation at 12,000 × *g* for 10 min at 10°C in a Sorvall RC5B centrifuge (SS34 rotor), with pH adjustment, as needed, by the dropwise addition of 0.1 N NaOH until a red color was restored to the medium. The virus was initially pelleted during a 2-h run at 85,000 × *g* in a Beckman model ultracentrifuge (SW28 rotor) at 14°C. The sucrose gradients were centrifuged at 165,000 × *g* in a Beckman SW41Ti rotor for 90 min at 10°C. Subsequent steps were carried out on ice, to minimize the loss of virus caused by crystallization.

Binding affinity studies. Binding of ^{3}H-labeled Win compounds to HRV-14 and the Leu-1188 mutant was measured by a modification of a previously described procedure (19) in which samples were centrifuged through small columns of Sephadex G-50 to separate free radiolabeled compound from that bound to virus. Sephadex G-50 (fine), which was obtained from Pharmacia, Piscataway, N.J., was equilibrated overnight or longer in 10 mM Tris buffer (pH 7.5). After first

prewetting the columns with 0.5 ml of Tris buffer (5 ml polypropylene Select-S columns; 5 Prime → 3 Prime, Inc., Paoli, Pa.), the columns were packed with 3 ml of Sephadex by using three successive centrifugations at 1,000 × *g* for 4 min in an IEC PR7000 centrifuge at 23°C. Columns were then washed once with 2 ml of buffer and then twice with 0.3 ml of buffer, using the same centrifugation speed.

To determine nonspecific binding, increasing concentrations of radiolabeled compound were incubated with purified virus in the presence or absence of excess unlabeled compound. Incubation mixtures of 0.66 ml, containing either 2.2 μg (for Win 54954 and disoxaril) or 0.44 μg (for Win 52084 and Win 56590) of purified virus in 10 mM Tris buffer (pH 7.5), were used. Additional reactions containing bovine serum albumin at amounts equivalent to the virus concentration used were included as another check for nonspecific binding. Radiolabeled compound was added to the incubation mixtures by 100-fold dilutions from 100× concentrated solutions in dimethyl sulfoxide. The specific activities of these radiolabeled compounds were diluted with unlabeled compound 10-fold for Win 54954 and disoxaril and 2- and 4-fold for Win 52084 and Win 56590, respectively. The virus and compounds were incubated in buffer until they reached equilibrium (4 h) at 23°C. Two 0.3-ml samples were removed from each incubation mixture. The first sample was placed on top of a Sephadex column, which was then centrifuged to separate the compound bound to virus from unbound compound by passage through the Sephadex columns during centrifugation, as described above. The amount of bound radioactivity was determined for the entire column eluate, which was collected into a 15-ml Corning polypropylene centrifuge tube and then transferred into a scintillation vial. The second sample was placed directly into a vial for the determination of the amount of total radioactivity in the assay. For each concentration of drug, the amount bound in the presence of excess unlabeled compound (nonspecific) was subtracted from the amount bound in the absence of unlabeled compound (total bound) to determine the amount of specific binding. Then, to determine the amount of free compound, the disintegrations per minute of total bound compound was subtracted from the total disintegrations per minute. The molar concentrations of specifically bound and free compounds were determined from the disintegrations per minute of radioactivity by using the specific activities and molecular weights of the compounds. The amount of virions per unit volume was determined from the concentration of virus present in the samples (either 1 or 0.2 μg of virus) by using the molecular weight of rhinovirus (8.2×10^6 [23]) and Avogadro's number. The data from the saturation binding studies were analyzed by the method of Scatchard by using linear regression (5). The K_d was determined from the negative reciprocal of the slope of the line, and the apparent maximum number of binding sites (B_{max} or n value for these data) was estimated from the extrapolation of the line to the abscissa intercept. Transformation of the saturation data for these compounds into Hill plots, in which log $[B/(B_{max}-B)]$ was plotted versus log [free compound], was also done to determine whether the binding showed any cooperativity (5, 14). In addition, the binding data were analyzed by using the EBDA computer program (15), which processes raw data from radioligand binding experiments into a form suitable for use by a nonlinear curve-fitting program, such as LIGAND (16). The EBDA program gives preliminary estimates of binding constants and generates Scatchard and Hill plots. These data were then analyzed by the LIGAND program to obtain K_d determinations.

Determination of $t_{1/2}$ and k_{off}. To determine the half-life ($t_{1/2}$) and k_{off} (5), the virus-compound mixture was diluted 100-fold into Tris buffer containing excess unlabeled compound (to prevent the reassociation of labeled compound and virus) after the 4-h incubation period at 23°C was completed. Immediately (at zero time) and at various times thereafter, aliquots were removed for total and bound radioactivity determinations, as described above. The results were plotted as the ln (percent bound/percent bound at zero time) versus the time, and the $t_{1/2}$ was determined. The time at which one-half of the compound that was bound at zero time remained was the $t_{1/2}$. The k_{off} was calculated from the following equation: $k_{off} = 0.693/t_{1/2}$.

Determination of k_{on}. For the determination of k_{on}, tubes containing radiolabeled compound and virus in Tris buffer were set up as described above for the binding experiments. However, instead of an incubation for 4 h to reach equilibrium, samples were incubated for various times after the addition of compound, and then the bound and total radioactivities were determined. The data were plotted as disintegrations per minute of radiolabeled compound bound versus time, and the k_{on} was calculated from the linear portion of the curve by using the following formula (5): $k_{on} = 2.303/[t(L-R)] \times \{\log [R(L-x)]/[L(R-x)]\}$, where L is the concentration of radiolabeled compound, R is the receptor concentration (determined in saturation experiments), t is time, and x is the amount of compound bound at t.

RESULTS

Initial binding studies demonstrated that the majority (90%) of the infectious virus was recovered in the Sephadex G-50 column eluates and that >99% of the unbound radioactive compound remained in the column during the separation by centrifugation. In addition, no significant binding of compound to bovine serum albumin occurred at concentrations equivalent to the virus concentrations examined (data not shown).

Time course studies, in which radiolabeled compounds were incubated with purified HRV-14 for various lengths of time at 23°C before samples were taken for the determination of bound and free compound, showed that binding reached equilibrium at 4 h (data not shown). Radiolabeled compounds and virus were thus incubated for 4 h in the binding studies.

Specific binding, which was total binding minus nonspecific binding, was plotted as the concentration of compound versus the disintegrations per minute of radiolabeled compound bound (Fig. 1). In all cases, specific binding was observed, and the nonspecific binding at the K_d ranged from 3 to 28%.

The Scatchard plots of the specific binding data were analyzed by linear regression, and from this analysis the equilibrium K_ds for these compounds and HRV-14 were calculated (Fig. 2). The K_ds for Win 52084, Win 56590, disoxaril, and Win 54954 from the Scatchard plots were 0.02, 0.02, 0.09, and 0.26 μM, respectively. The correlation coefficients for the Scatchard plot data were −0.828, −0.858, −0.967, and −0.874 for Win 52084, Win 56590, disoxaril, and Win 54954, respectively. The ordinate scales for the disoxaril and Win 54954 plots and the Win 52084 and Win 56590 plots were an order of magnitude different, which reflects the lower ratio values of bound/free compound for the former two compounds versus those for the latter two. The n values (B_{max}), or apparent maximum number of binding sites for all four compounds, were approximately 40 to 60 molecules of

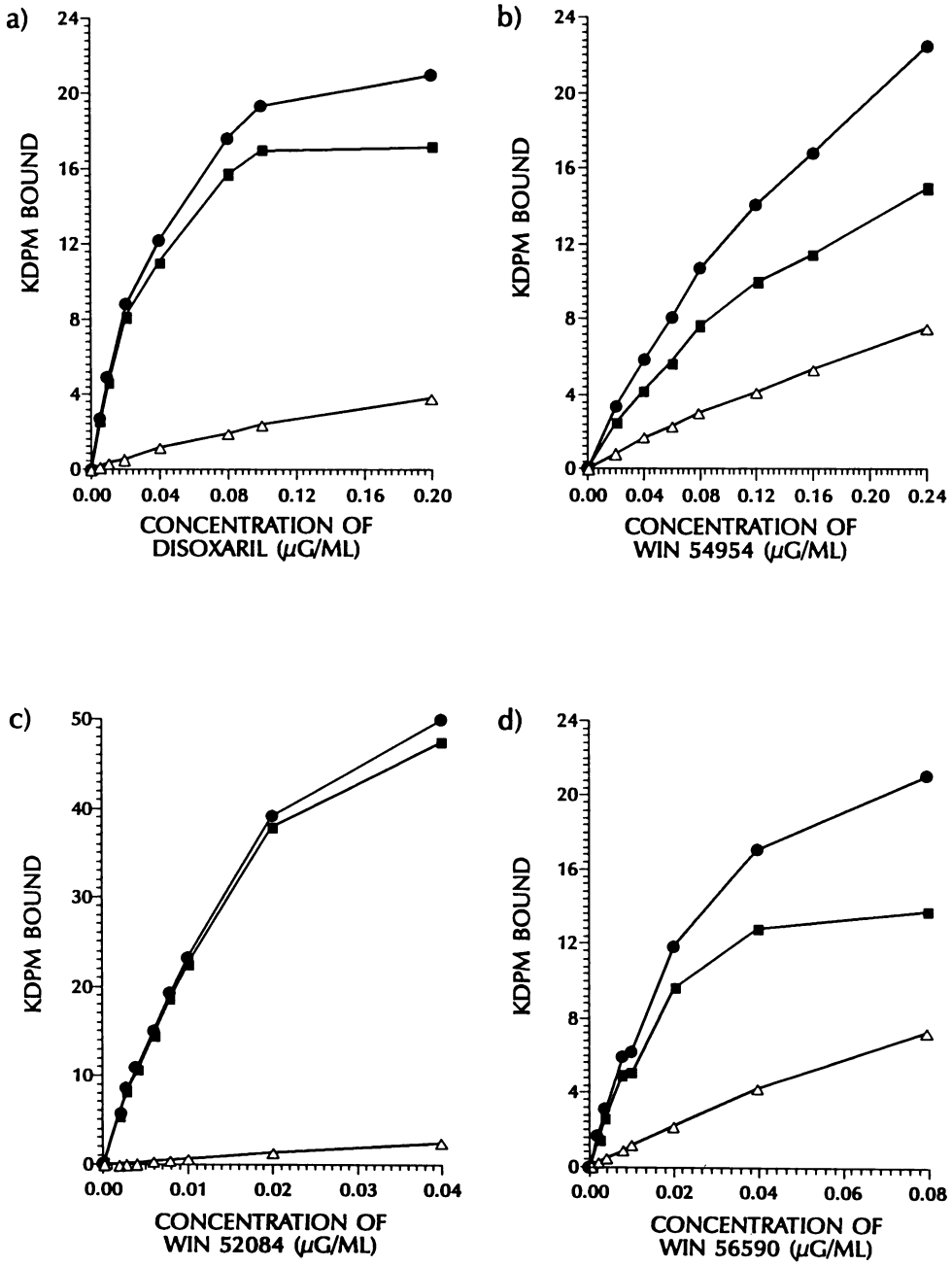


FIG. 1. Determination of specific binding of disoxaril (a), Win 54954 (b), Win 52084 (c), and Win 56590 (d) to HRV-14. Data were plotted as the disintegrations per minute bound as total bound (●), specifically bound (■), and nonspecifically bound (△) versus the concentration of compound used. To determine that which was nonspecifically bound, the virus was incubated with ^{3}H-labeled compounds and unlabeled compound (1 μg/ml for disoxaril, Win 54954, and Win 52084; 0.2 μg/ml for Win 56590). Two experiments were done with each compound, and the means of duplicate samples from one experiment are plotted.

compound bound per virion (Table 2). The K_ds determined by using the nonlinear curve-fitting program of LIGAND to analyze the binding data are shown in Table 2.

To determine whether there was any cooperativity of binding (i.e., whether the binding of one molecule of drug to virus influences the subsequent binding of other drug molecules), the saturation data were transformed to give Hill plots (data not shown). The Hill plots for Win 52084, Win 56590, disoxaril, and Win 54954 gave slopes of 0.954, 0.953, 0.974, and 0.931, respectively, which demonstrates no cooperativity of binding. The Hill coefficients (slopes) were also estimated by the EBDA program for Win 52084, Win 56590, disoxaril, and Win 54954 as 0.956, 0.974, 0.978 and 0.952, respectively. Also, the Hill binding constants or K_d's (5, 14) were calculated from the abscissa value, where log $[B/(B_{max}-B)]$ is 0. The Hill binding constants for the Win 52084, Win 56590, disoxaril, and Win 54954 experiments were 0.03, 0.03, 0.10, and 0.32 μM, respectively, which were similar to the K_ds determined by saturation binding.

The k_{off}s for compounds and virus were determined. In the

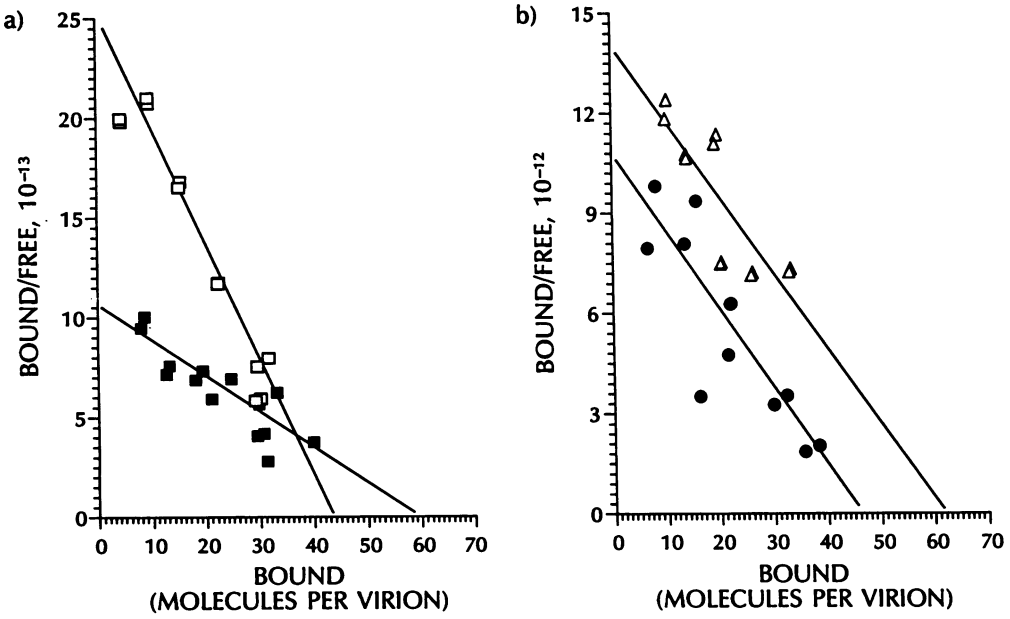


FIG. 2. Scatchard plots of HRV-14 and Win 54954 (■) and disoxaril (□) (a) and Win 52084 (△) and Win 56590 (●) (b). The ratio of bound/free compound was plotted versus the number of molecules of drug bound per virion. To calculate the ratio of bound compound to free compound, the molar concentrations of bound and free compound were converted to molecules of compound for determination of number of molecules of compound per virion, resulting in the numbers (10^{-12} or 10^{-13}) on the y axis. Two experiments were done with each compound, and duplicate points from one experiment were plotted.

k_{off} determinations, a large dilution into buffer containing excess unlabeled compound was done to eliminate possible reassociation of the compound with the virus binding site (5). The slowest k_{off} was estimated to be that for Win 56590, followed by those for Win 52084, Win 54954, and then disoxaril (Fig. 3 and Table 2). For Win 52084 and Win 56590, an extrapolation of the line to determine the $t_{1/2}$ was required, which might have resulted in a more imprecise estimation of the $t_{1/2}$s for these compounds. One possible explanation for this extrapolation was the occurrence of retention effects. The high affinities of these compounds and their low solubilities in aqueous media resulted in experimental conditions in which low concentrations of compound had to be used. In addition, the concentration of the binding site compared with the K_d was still relatively high, which might have caused retention effects because of a reduced ability of bound compound to diffuse away from the virus.

In addition, the rates of association of compounds and virus were determined. The slowest rate was found for Win 54954, followed by those for Win 56590, Win 52084, and then disoxaril (Fig. 4 and Table 2).

The independently determined k_{on}s and k_{off}s were used to calculate the K_d in the equation $K_d = k_{off}/k_{on}$. In all cases, the calculated K_d was within a factor of three- or fourfold of the K_d determined from the saturation experiments. However, some degree of imprecision was possibly introduced when an extrapolation was made to estimate the k_{off}s for Win 52084 and, especially, for Win 56590. This might have resulted in some bias to the low side in determining the calculated K_d.

The relationship of K_d and antiviral activity for this limited series of compounds was determined by plotting the K_ds versus the MICs in a plaque assay. Given the limited data ($n = 4$), a straight linear regression line (with the linear equation $y = 0.01288 + 0.08769x$) fit the data well ($r = 0.997$), indicating a positive relationship between binding and MIC (Fig. 5). In addition, experiments showed no detectable specific binding of Win 54954 to the resistant Leu-1188 mutant compared with specific binding of approximately 70,000 dpm of [^{3}H]Win 54954 to HRV-14 under the same experimental conditions. Thus, the absence of binding of Win 54954 to the resistant Leu-1188 mutant correlated with the absence of antiviral activity of this compound for this mutant. The structures of the four compounds that were investigated in the binding studies, along with their MICs for HRV-14, are given in Table 1.

TABLE 2. Binding parameters of ^{3}H-radiolabeled Win compounds and HRV-14

Compound	K_d (μM)[a]	n[b]	$t_{1/2}$ (min)	k_{off} (min^{-1})	k_{on} (M^{-1} min^{-1} [10^5])	Calculated K_d (μM)[c]
Disoxaril	0.08	39	32	0.022	7.23	0.03
Win 54954	0.22	48	99	0.007	1.06	0.07
Win 52084	0.02	63	295	0.0025	3.45	0.007
Win 56590	0.02	43	480	0.0014	2.65	0.005

[a] K_d obtained by the LIGAND program.
[b] n is the number of molecules of compound bound per virion.
[c] k_{off}/k_{on} = calculated K_d.

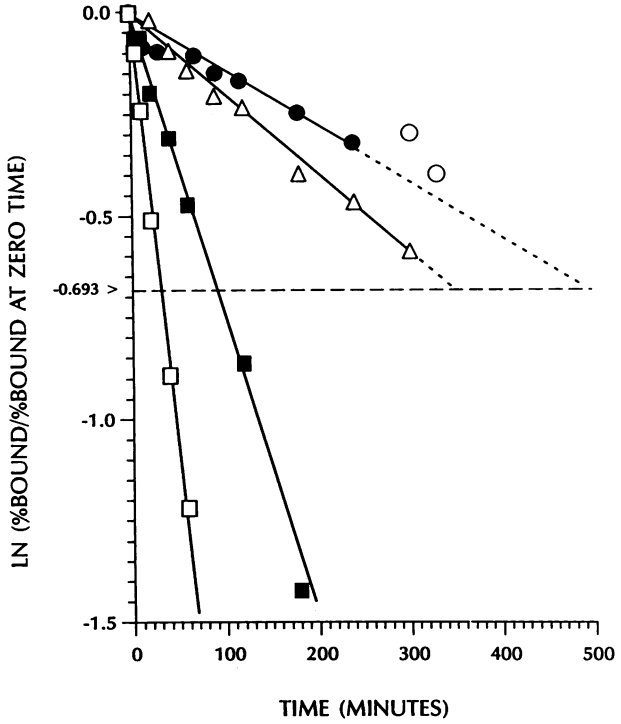


FIG. 3. Determination of the $t_{1/2}$s for HRV-14 and disoxaril (□), Win 54954 (■), Win 52084 (△), and Win 56590 (●). The data were plotted as ln (percent bound at time t/percent bound at zero time) with $t_{1/2}$ = the time where y = ln (0.5) or −0.693 (reference line given) versus time. For Win 52084 and Win 56590, an extrapolation was made (indicated by broken lines) to the reference line. Also, for Win 56590, the last two time points (○) were not included in the line determination because they were not linear with the other points, possibly because of retention effects. Single datum points from one experiment are presented for all four compounds. Experiments were repeated twice for disoxaril and Win 54954 and three times for Win 52084 and Win 56590.

DISCUSSION

The data presented here represent the first quantitative studies of K_d and of k_{on} and k_{off} for a class of picornaviral capsid-binding compounds. In these studies, the binding of the ^{3}H-labeled Win compounds was specific and saturable and resulted in data which could be interpreted by Scatchard analysis to obtain K_d values. The binding data were also analyzed by a nonlinear curve-fitting program, LIGAND, and the K_ds determined by this computer program were similar to those obtained by Scatchard analysis. The identified values for the slope in the Hill plots and their 95% confidence interval included 1, indicating that there was no positive or negative cooperativity of binding. The Hill binding constants or K_d's were found to be similar to the Scatchard values for the K_ds. For all four compounds, it was estimated that approximately 40 to 60 molecules of compound were bound per virion, a result consistent with 60 protomers per virion (22) and one binding site per protomer.

The observed K_ds were shown to have a positive relationship with their biological activity, as represented by antiviral activities (MICs) (Fig. 5). In addition, experiments with a drug-resistant variant, HRV-14 (Leu-1188), which has a greatly reduced susceptibility to antiviral compounds, demonstrated no detectable binding to Win 54954. Previously, X-ray crystallographic studies of this drug-resistant mutant indicated that the leucine side chain would sterically hinder drug binding (4). The K_d for a compound, however, is not the only determinant of the antiviral activity in the plaque reduction assay. As suggested previously (24), the antiviral activities of these compounds are a consequence not only of their K_ds to virus but also of their incorporation into host cells and, possibly, other factors, such as their susceptibilities to metabolic degradation in tissue culture.

The correlation of the K_d with the biological activity has relevance to rational drug design, because the design of compounds with greater K_ds should result in corresponding greater biological activities. Since this has only been deter-

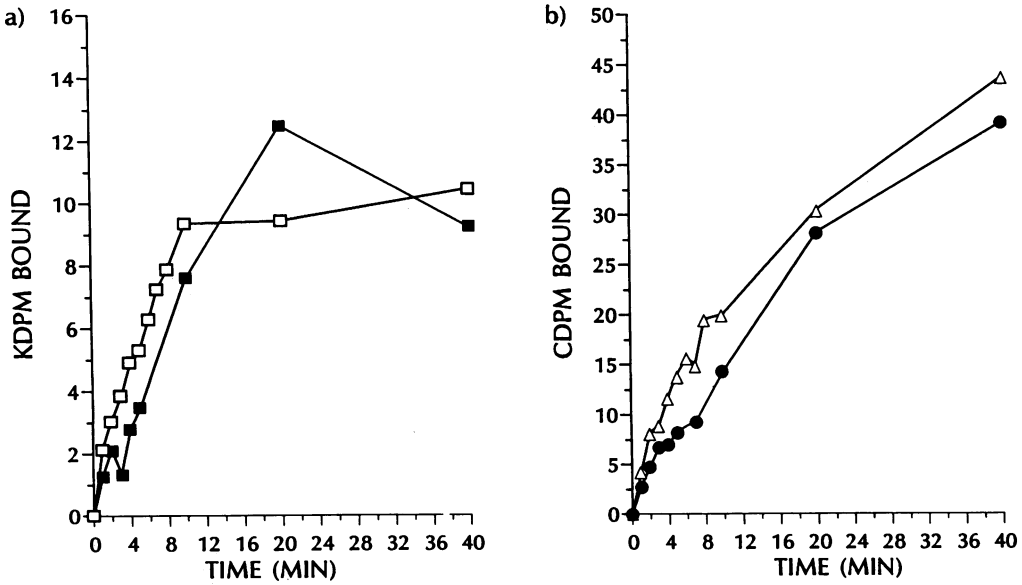


FIG. 4. Determination of the k_{on} of disoxaril (□) and Win 54954 (■) (a) and Win 52084 (△) and Win 56590 (●) (b). The data were plotted as disintegrations per minute bound versus time. Two experiments were done with disoxaril, Win 52084, and Win 56590; and four experiments were done with Win 54954. Single datum points from one experiment are presented for disoxaril, Win 54954, and Win 52084. The mean of triplicate datum points from one experiment with Win 56590 is presented.

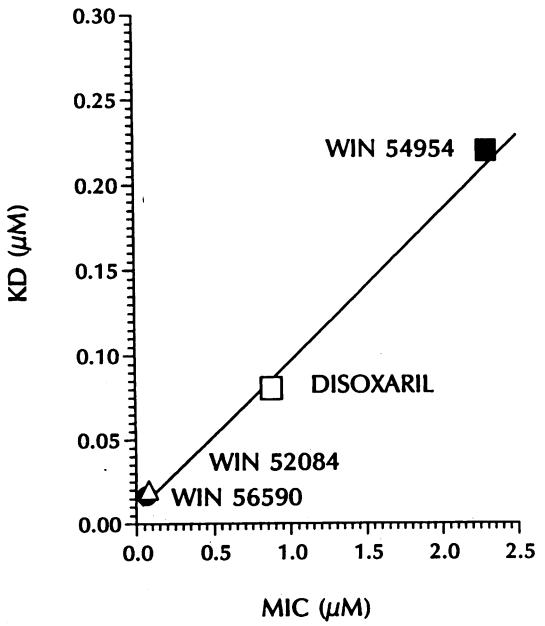


FIG. 5. Relationship of the MIC and the K_d for antiviral compounds and HRV-14 ($r = 0.997$). The K_d (in micromolar) was plotted versus the MIC (in micromolar) for disoxaril (□), Win 54954 (■), Win 52084 (△), and Win 56590 (●). The K_ds were the means from two separate experiments with duplicate determinations.

mined to be the case for the limited series of compounds examined in the study described here, the correlation of the K_d to the MIC must still be investigated for other chemical classes of compounds to determine whether this is a general rule. It is conceivable that a more metabolically stable compound with a greater ability to penetrate cells would have a K_d which is lower than expected on the basis of its in vitro antiviral activity.

Detailed analysis of k_{on}s and k_{off}s revealed that the rank order of compounds, determined by k_{on}s, did not agree with the rank order of compounds by binding affinity. For example, disoxaril has the fastest k_{on} (723,000 M^{-1} min^{-1}), but it also has a fast k_{off} (0.022 min^{-1}), resulting in a K_d of 0.08 μM. In contrast, Win 52084 and Win 56590 had slower k_{on}s (345,000 and 265,000 M^{-1} min^{-1}, respectively) but had extremely slow k_{off}s (0.0025 and 0.0014 min^{-1}, respectively), which resulted in greater K_ds (0.02 μM) for both compounds. The relatively slow k_{on}s for these compounds compared with those for other ligand-receptor interactions, for example, [^{3}H]doxepin (tricyclic antidepressant) binding to histamine H_1 receptors in rat brain with a k_{on} of 1.38×10^9 M^{-1} min^{-1} (26), most likely reflect the energy of desolvation; hydrophobic binding energy; the energy required to induce the conformational changes which take place within the hydrophobic pocket of the virus (4); and the energy required for the drug to adapt the conformation necessary for binding.

The rank order of the k_{off}s also did not agree with the rank order of the K_ds. For example, disoxaril has a faster k_{off} (0.022 min^{-1}) than that of Win 54954 (0.007 min^{-1}) but had a K_d of 0.08 μM compared with a K_d of 0.22 μM for Win 54954. This occurs because disoxaril has a faster k_{on} (723,000 M^{-1} min^{-1}) relative to that for Win 54954 (106,000 M^{-1} min^{-1}). One determinant of the k_{off} is the energy of binding within the hydrophobic pocket. A higher binding energy may result in a more strongly bound compound and, consequently, a slower k_{off}. Disoxaril had the fastest k_{off}, which might predict that it would have the lowest K_d. In fact, disoxaril had a threefold higher K_d than Win 54954 because of a faster k_{on}. Win 54954 had a slower k_{off} than disoxaril, presumably because the addition of the chlorine substituents to Win 54954 increases the hydrophobic interactions upon binding of this compound, and consequently, the K_d was increased. In comparison with disoxaril and Win 54954, the higher K_ds of Win 52084 and Win 56590, which are the *S* enantiomers, were the result of their slower k_{off}s. Previously, more potent antiviral activity was reported for these *S* enantiomers, and it was proposed (8) that the *S*-methyl and *S*-ethyl substituents of Win 52084 and Win 56590, respectively, undergo favorable hydrophobic interactions with leucine 1106-serine 1107 in the hydrophobic pocket of HRV-14. These interactions are important determinants of the binding of these compounds to HRV-14 (8). The higher relative K_ds for these compounds is consistent with the favorable hydrophobic interactions and binding energy in the pocket with these *S* enantiomers, which has resulted in approximately 10-fold higher antiviral activities.

The X-ray crystal structure of a minor receptor group rhinovirus, HRV-1A, has been determined, and it has been shown that the binding of Win compounds did not induce extensive conformational changes similar to those seen in HRV-14 (13). The mode of action of these compounds against HRV-1A and other rhinovirus serotypes is being investigated. Examination of the binding parameters of these compounds to HRV-1A is also planned. The results of those studies will be interesting, since X-ray crystallography has shown (13) that the binding of these compounds did not induce large conformational changes in HRV-1A like they did with HRV-14. Binding studies with other rhinovirus serotypes and other drug-resistant mutants are in progress and will contribute information that is useful for the molecular design of antiviral agents, as well as for an increased understanding of the replication processes of these viruses.

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