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Conformational Change in the Floor of the Human Rhinovirus Canyon Blocks Adsorption to HeLa Cell Receptors

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A series of eight antiviral compounds complexed with human rhinovirus 14 (HRV-14) were previously shown to displace segments of polypeptide chains in the floor of the "canyon" by as much as 0.45 nm in C-alpha positions from the native conformation (J. Badger, I. Minor, M. J. Kremer, M. A. Oliveira, T. J. Smith, J. P. Griffith, D. M. A. Guerin, S. Krishnaswamy, M. Luo, M. G. Rossman, M. A. McKinlay, G. D. Diana, F. J. Dutko, M. Fancher, R. R. Rueckert, and B. A. Heinz, Proc. Natl. Acad. Sci. USA 85:3304–3308, 1988). Because the canyon is thought to serve as the viral receptor-binding site (M. G. Rossmann, E. Arnold, J. W. Erickson, E. A. Frankenberger, J. P. Griffith, H. J. Hecht, J. E. Johnson, G. Kamer, M. Luo, A. G. Mosser, R. R. Rueckert, B. Sherry, and G. Vriend, Nature [London] 317:145–153, 1985; M. G. Rossmann and R. R. Rueckert, Microbiol. Sci. 4:206–214, 1987), these compounds were assessed for their ability to block adsorption of HRV-14 to HeLa cell membrane receptors. In parallel experiments, the compounds were assessed directly for antiviral activity in an in vitro plaque reduction assay in intact HeLa cells. All eight compounds blocked the adsorption of 50% of HRV-14 at approximately the same concentration required to reduce the number of visible plaques by 50% (MIC). A structurally related compound which was inactive in the plaque reduction assay had no effect on HRV-14 binding. A drug-resistant mutant of HRV-14 (Leu-1188), which was less sensitive to the eight compounds in plaque reduction assays was similarly less sensitive in the adsorption assay. We propose that the conformational changes in the floor of the HRV-14 canyon induced by these compounds substantially decrease adsorption of the virion to its receptor. These results provide further evidence for the role of the HRV canyon in receptor binding.

Adsorption of a virus to its specific receptor on a cell represents the earliest stage in the viral life cycle. For the human rhinoviruses (HRVs) which represent the most serologically diverse group of picornaviruses (over 100 serotypes), only two unique receptor groups have been identified (3, 6). The major HRV receptor, which is used by 78 of 88 HRV serotypes and some of the group A coxsackieviruses to initiate infection (3), is a 90-kilodalton protein found on cells of human and chimpanzee origin (12). Thus far, only 10 rhinovirus serotypes have been identified which do not use the major receptor (types 1A, 1B, 2, 29 to 31, 44, 47, 49, and 62) but instead bind to a receptor present on both human and mouse (NIH 3T3) cells (3). Nevertheless, replication of rhinoviruses in murine cells is not usually observed (13).

X-ray crystallographic studies on a major group virus, HRV-14, have suggested that the viral receptor-binding site is a 2.5-nm-deep depression encircling each icosahedral fivefold axis of the virion (the "canyon"; 8). Site-directed mutation of amino acids in the canyon has thus far provided the only direct evidence for the role of the canyon in receptor binding (4). However, X-ray crystallographic data on these mutants is not available, and thus it is not known whether such mutations produce conformational changes at sites other than the canyon. Additional evidence in support of the canyon hypothesis comes from a number of studies (reviewed in reference 9). For example, amino acid residues in the canyon show a much higher degree of conservation throughout the picornavirus family than do other residues on the virion surface (9). Because the antigen-binding domain of an antibody molecule is too large to fit into the narrow canyon, the amino acids which comprise the canyon are not exposed to the immunologic surveillance of the host immune system (8–10). This observation suggests that the immunogenic sites of the virus, including residues on the edges of canyon and on the most external regions of the virus, can undergo mutation in order to evade the host immune response without affecting tissue specificity. Such a strategy would be consistent with the observation that there are only two receptor-binding groups within the more than 100 HRV serotypes.

A series of eight structurally related compounds synthesized by the Sterling Research Group have been found to block the replication of a number of HRV serotypes. All eight compounds interact with a hydrophobic pocket beneath the canyon floor and induce conformational changes in the neighboring polypeptide chain of up to 0.45 nm in C-alpha positions from the native crystalline structure (2). Since these conformational changes affect polypeptide chains in the floor of the putative receptor-binding site of the virus, we reexamined the mechanism by which these compounds inhibit the replication of HRV-14. The results reported here indicate that conformational changes in the floor of the canyon induced by drug binding interfere with the binding of HRV-14 to its cellular receptor.

MATERIALS AND METHODS

Cells and viruses. Monolayers of HeLa cells were propagated in Hanks minimal essential medium containing 10^5 U of penicillin and 100 μg of streptomycin per liter and either

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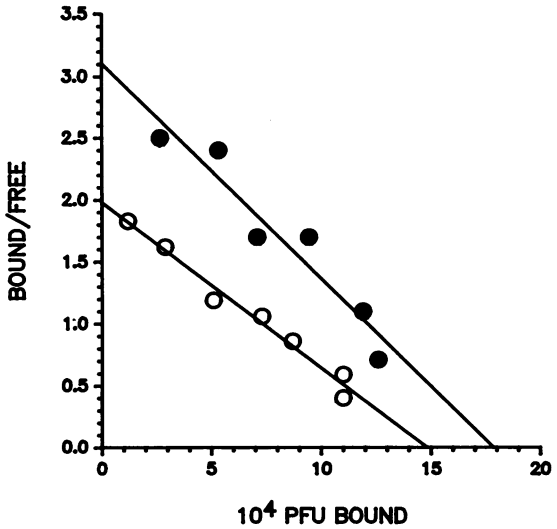


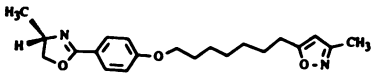
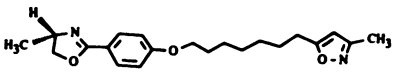
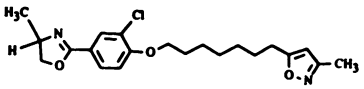
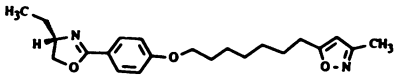
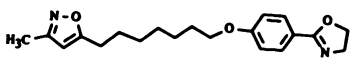
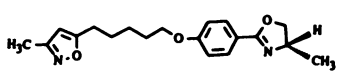
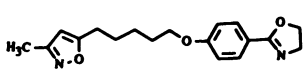
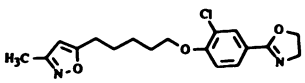
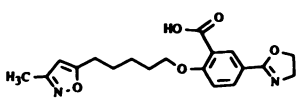
FIG. 1. Scatchard analysis of the binding of HRV-14 (○) and the Leu-1188 mutant (●) as obtained from saturation binding curves. The number of counts per minute per PFU was determined by plaque assay, and the points for this Scatchard analysis were generated with the PFU data.

10% fetal calf serum (GIBCO Laboratories; for 1-day monolayers) or 5% newborn calf serum (for 3-day monolayers).

For preparation of high-titer HRV pools, 3-day monolayers of HeLa cells in 150-cm^2 flasks (Corning Glass Works) were infected at a multiplicity of infection of approximately 0.01 in M199 medium with penicillin and streptomycin (as above). The flasks were incubated for 2 h at 33°C in 2% CO_2, and the inoculum was removed. Fifty milliliters of M199 with 5% fetal calf serum was added, and the flasks were incubated at 33°C until the cytopathic effect was complete. The flasks were then frozen and thawed, and the contents were centrifuged at 25,000 rpm for 90 min in a Beckman SW28 rotor. The virus pellet was suspended in phosphate-buffered saline, frozen at −70°C, thawed, and sonicated, and the titer was determined on HeLa cells.

Radioactive virus growth. [^{35}S]methionine labeling of HRV-14 and the Leu-1188 mutant were performed essentially as described previously (1). (Amino acid residues are labeled with a four-digit notation. The first digit refers to the viral capsid protein [VP1, VP2, VP3, or VP4], and the last three digits refer to the position from the amino terminus of the protein.) One 150-cm^2 flask of HeLa cells was infected at a multiplicity of infection of 1 in 15 ml of M199 medium at 33°C. At 1 h postinfection, 10 ml of M199 with 1% newborn calf serum was added. At 3.5 h postinfection, the fluid was

TABLE 1. Relationship of MIC and IC_{50} for nine anti-rhinovirus compounds[a]

Compound	Structure	HRV-14 (μM)		LEU-1188 (μM)	
		MIC	IC_{50}	MIC	IC_{50}
I(S)		0.05	0.13	2.61	1.24
I(R)		0.70	0.32	>4.3	16.87
II(R/S)		0.51	0.60	>4.0	5.97
III(S)		0.04	0.18	1.48	0.53
IV		1.02	0.35	>9.1	>26.3
V(S)		0.82	0.66	>4.7	>20.5
VI		1.08	0.88	4.13	>37.2
VII		3.44	1.21	>4.4	>20.6
VIII		>279.0	>75.3	>279.0	>75.3

[a] All assays were performed at least twice, and the results varied by less than twofold.

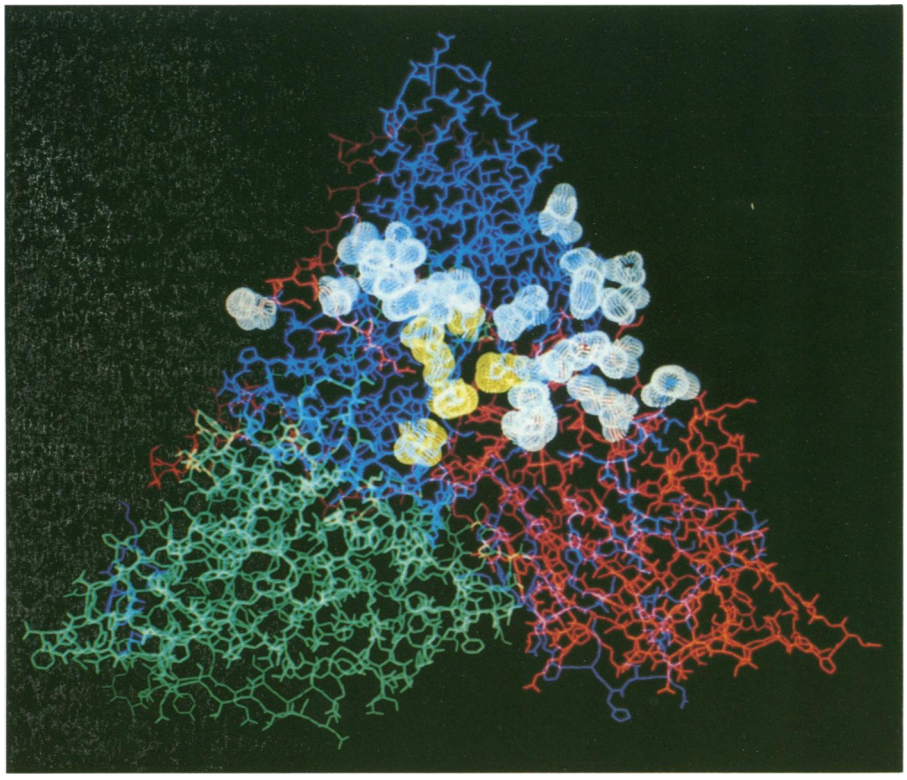


FIG. 2. Canyon residues of HRV-14 that move upon binding of antiviral compounds. The triangle (generated in Chem-X, Chemical Design Inc., Mahwah, N.J.) represents one icosahedral asymmetric unit (60 units per virion) with viral protein 1 (VP1) in blue, VP2 and VP4 in green, and VP3 in red. The Van der Waals dot surfaces (generated in Chem-X) of the HRV-14 surface residues that line the putative virion receptor canyon (9) are color coded according to the magnitude of the movement induced by the binding of antiviral compounds (2): white dot surfaces, canyon residues which move 0.04 nm or less; yellow dot surfaces, canyon residues with 0.05 nm or more movement. The highlighted dot surfaces do not correspond exactly with those shown in Fig. 3 due to slight differences in the trimming of the neighboring icosahedral asymmetric units (9). Canyon residues which do not move more than 0.04 nm when the drugs bind are His-1078, Val-1079, Thr-1080, Asp-1101, Trp-1102, Gly-1156, Asn-1159, Asp-1164, Tyr-1166, Val-1278, Lys-3093, Asp-3177, Pro-3178, Gln-3226, Thr-3227, and Ser-3229. Canyon residues which move more than 0.04 nm when the drugs bind are Lys-1103, Pro-1155, Val-1217, His-1220, and Ser-1223 (2, 9).

removed and 15 ml of L-methionine-free Dulbecco medium was added which contained 100 μCi of [^{35}S]methionine (DuPont, New England Nuclear) per ml. The flask was incubated until the cells detached and was then frozen at −70°C. After it was thawed, the suspension was spun at 5,800 rpm in a Sorvall SS34 rotor for 5 min at 10°C. The supernatant was put into a flask, and polyethylene glycol 8000 and NaCl were added to final concentrations of 7 and 2.2%, respectively. The mixture was stirred overnight at 4°C.

The precipitated virus was pelleted by centrifugation at 9,500 rpm for 15 min in the Sorvall SS34 rotor, and the supernatant was discarded. The pellet was suspended in 1 ml of R buffer (10 mM Tris hydrochloride [pH 7.5], 0.2 M NaCl, 50 mM $MgCl_2$, 10% [wt/vol] glycerol), and sodium deoxycholate and Nonidet P40 were added to 0.3 and 0.6%, respectively. The mixture was held at 4°C for 30 min and clarified by centrifugation at 5,800 rpm in a Sorvall SS34 rotor at 10°C for 5 min. The resulting supernatant was layered onto a continuous 5 to 20% sucrose gradient in R buffer and spun at 34,900 rpm for 2 h in a Beckman SW41 rotor. The gradients were fractionated with an ISCO fractionator in 10-drop fractions (about 0.5 ml) into tubes containing 0.2 ml of 0.09% bovine serum albumin in R buffer. The fractions were frozen at −70°C. Samples (10 μl) of each fraction were precipitated with trichloroacetic acid, and the radioactivity was determined.

Preparation of membranes. Cell-free membrane extracts were prepared as described previously (12). HeLa cell monolayers (20 roller bottles) were dislodged with 50 mM EDTA (10 ml per roller bottle for 10 to 15 min at 37°C), and the cells were pooled and washed three times in cold phosphate-buffered saline (1,000 rpm for 10 min in an IEC centrifuge). The cells were counted after the second wash and were suspended at 6×10^7 cells per ml in 10 mM phosphate buffer (pH 7.0). The cells were swollen on ice for 15 min and then were disrupted by Dounce homogenization (15 to 20 strokes, B pestle). Cell debris and nuclei were pelleted at 3,000 rpm in an SS34 rotor, and the supernatant was transferred to a heat-sealable tube. The supernatant was spun at 46,600 rpm for 1 h in a Beckman Ti50 rotor at 5°C. The resulting supernatant was discarded, and the pellet was suspended in phosphate-buffered saline to 6 mg/ml and stored frozen at −70°C.

Binding assay. An adaptation of the membrane-binding assay described by others was used (3). Ten microliters of [^{35}S]methionine-labeled HRV-14 or the Leu-1188 mutant was mixed with 5 μl of 3× drug (in 2% dimethylsulfoxide [DMSO]) or 2% DMSO alone in a 1.5-ml microcentrifuge tube and incubated for 1 h at room temperature. The membrane preparation (20 μl) was added with 5 μl of 5× drug (or 2% DMSO) to the virus-drug mixture and incubated for 2 h at room temperature. The membranes were pelleted at 13,000 × *g* in a Fisher microcentrifuge for 2 min. The

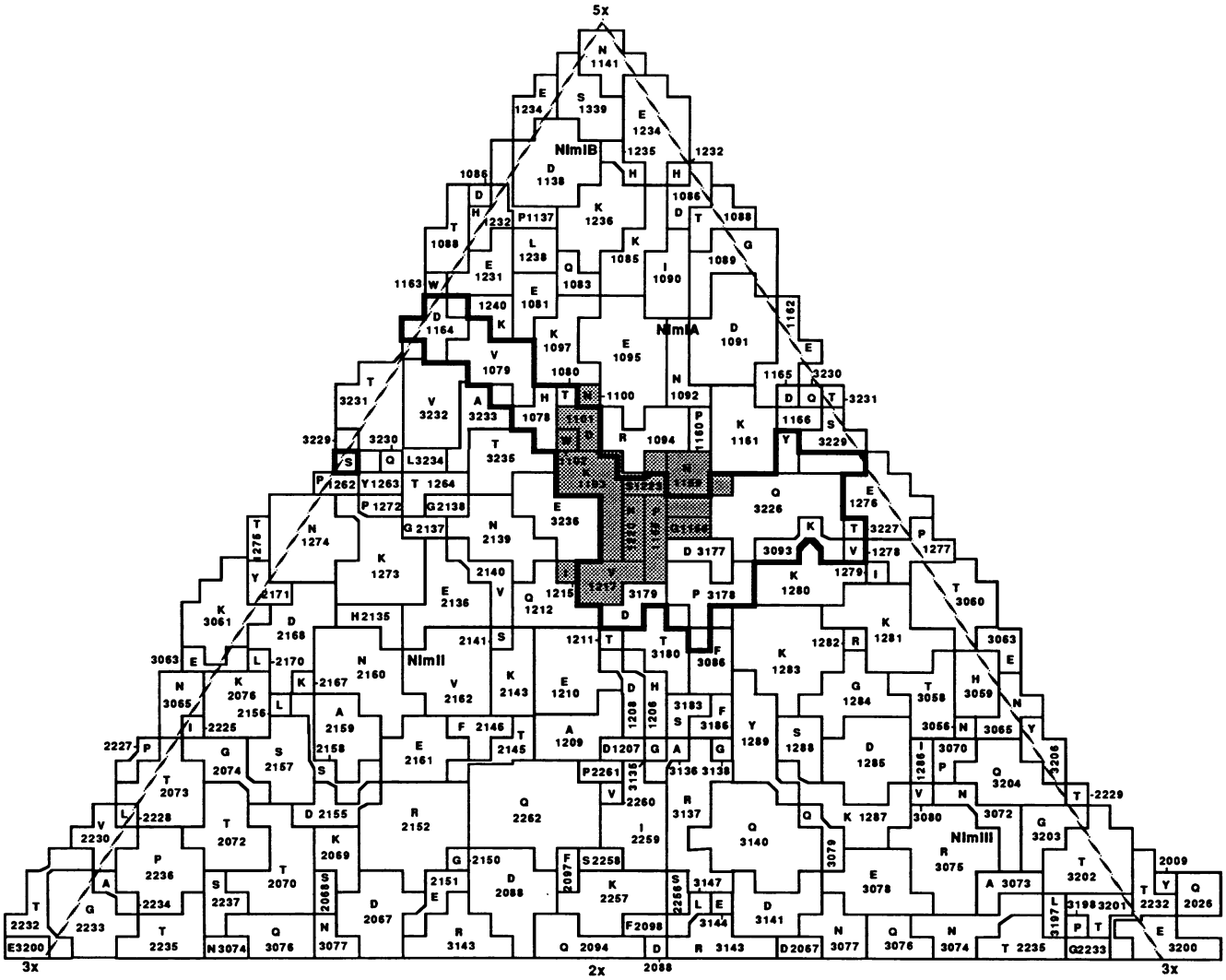


FIG. 3. Plot of surface residues for one icosahedral asymmetric unit of HRV-14 (9). The position of icosahedral five-, three-, and twofold axes are marked. The area exposed to solvent is shown on a raster. Residues are labeled with the single-letter amino acid code. Their sequence numbers are given plus 1000, 2000, or 3000 according to whether they are in VP1, VP2, or VP3, respectively. The canyon, identified by atoms with a viral radius of less than 13.8 nm, is outlined with a thick black line. The surface residues that undergo a conformational change on binding of a compound to HRV-14 are shaded (2). These residues are almost completely confined to the canyon region.

supernatant was removed and counted in liquid scintillation fluid, and the pellet was suspended in 0.2 ml of 1% sodium dodecyl sulfate and counted. The concentration of compound which reduced the counts per minute in the pellet by 50% over that of the DMSO-treated controls was determined and recorded as the 50% inhibitory concentration (IC_{50}). Murine L-cell (American Type Culture Collection, Rockville, Md.) membranes, prepared in a manner identical to that for the HeLa cells, and bovine serum albumin without any membranes were used as negative controls for membrane binding.

Plaque reduction assay. The plaque reduction assay has been described previously (7). Briefly, medium was aspirated from 1-day HeLa cell monolayers, and the cells were infected with 1.0 ml of virus in M199 medium containing 80 to 100 PFU of virus. The plates were incubated for 1 h at 33°C in 2% CO_2, after which the inoculum was replaced with 3 ml per well of overlay medium containing twofold dilutions of compound in 0.5% DMSO or DMSO alone. The plates were incubated at 33°C for 2 to 4 days until plaques developed. The cells were fixed with 5% glutaraldehyde and stained with 0.25% (wt/vol) crystal violet. The concentration of compound that reduced the plaque counts to 50% of the number on control plates was determined and recorded as the MIC.

RESULTS

Preliminary experiments were conducted to optimize conditions in the membrane-binding assay. Time course analysis of the binding of HRV-14 to the membranes indicated that the total counts of virus bound increased roughly linearly through 2 h at which time a plateau was attained at about 65% of input radioactive counts bound. Further incubation for 2 h resulted in only an additional 5% of input radioactive counts bound (data not shown). The optimal protein concentration of the HeLa cell membrane preparation for virion binding was determined to be 6 mg/ml (data not shown).

The kinetics of binding of both [^{35}S]methionine-labeled HRV-14 and the Leu-1188 drug-resistant mutant with the

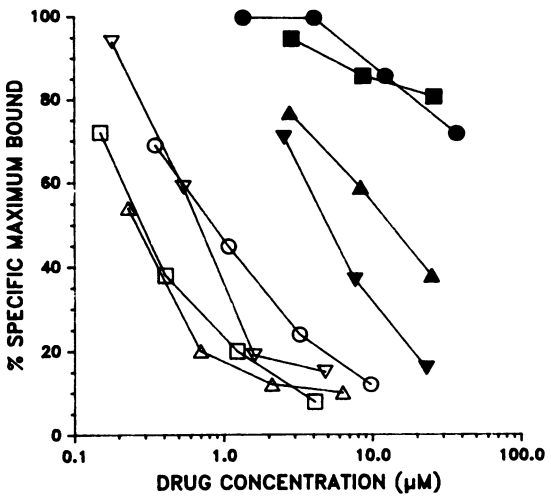


FIG. 4. Dose-response curves of adsorption of HRV-14 (open figures) and the Leu-1188 mutant (solid figures) in the presence of compounds I(R) (△, ▲), II (▽, ▼), IV (□, ■), and VI (○, ●).

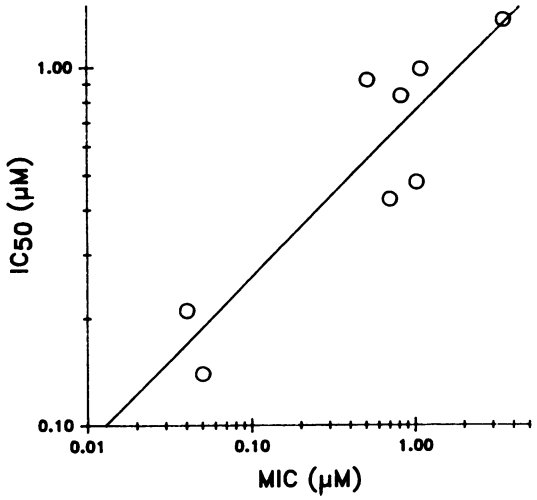


FIG. 5. Correlation of MIC and IC_{50} data for the eight compounds with activity against HRV-14 ($r^2 = 0.81$).

HeLa cell receptor were determined in saturation binding experiments. The amount of labeled virus was increased while holding both the specific activity of the virus and the membrane concentration steady. Figure 1 shows the results of a representative saturation binding analysis as fitted into the Scatchard equation. The K_d for the viruses is estimated as the negative reciprocal of the slope of the line as determined by linear regression. For HRV-14 and the Leu-1188 mutant, 1 K_d in this experiment was estimated to be 1.87×10^6 and 1.4×10^6 PFU/ml, respectively. Neither virus bound significantly to L-cell membranes or bovine serum albumin (less than 10% bound in each case; data not shown). Thus, it appears that the mutation at position Val-1188 to Leu-1188 does not adversely affect the interaction of the Leu-1188 mutant with the receptor. In all of the binding assays with compounds, the virus concentration was equal to 1 K_d.

X-ray crystallographic analyses have shown that compounds I(S), I(R), and II through VII (Table 1) bind in a hydrophobic pocket within the viral protein VP1 beta barrel below the canyon floor (2, 11). Compound VIII was included as a negative control in our studies because it lacked activity against both HRV-14 and the Leu-1188 mutant in the in vitro plaque reduction assay. The nine drugs differ only by the length of the hydrocarbon chain (either five or seven methylene units) and by the substitutions on the oxazoline and phenyl groups. Nevertheless, their MICs in an in vitro plaque reduction assay span more than 3.8 orders of magnitude from 0.04 to >279 μM. All of the compounds were substantially less active against the Leu-1188 mutant (which was selected against compound IV), and only three compounds were able to inhibit replication of the Leu-1188 mutant at concentrations below levels which are toxic to the cells.

Crystallographic analyses of HRV-14 native and drug-bound virions have shown that binding of any of the eight active compounds induced similar, dramatic conformational changes confined to the canyon floor (Fig. 2 and 3; 11). The movement in the beta-barrel structure below the canyon floor is largely independent of which compound is bound and involves a concerted displacement of three regions in VP1, leading to a systematic movement of a beta sheet, i.e., residues 1213 to 1224, 1151 to 1159, and 1100 to 1110 (2). If the canyon hypothesis is indeed valid, one would expect that virus containing any of the active compounds would not be able to interact with its receptor as well as the native virus. Shown in Fig. 4 are representative curves of virus adsorption on membranes in the presence of different concentrations of compounds I(R), II(R/S), IV, or VI. These compounds (as well as the other four active drugs) inhibited adsorption of HRV-14 on membranes as a function of the concentration of drug. The IC_{50} was approximately the same as the concentration of compound required to reduce plaque titers by 50% in vitro (Table 1, MIC). All of the compounds with activity against HRV-14 were substantially less active against the Leu-1188 mutant, although in two instances an IC_{50} could be measured at subtoxic levels [compounds I(S) and III(S)]. Even at the highest drug concentrations noted in Table 1, compound VIII had no effect on the adsorption or replication of either virus.

Seven of the eight active compounds had an IC_{50} for HRV-14 within a threefold concentration (i.e., one dilution) of their MIC (Table 1, Fig. 5). The only exception was compound III(S), with the lowest MIC for HRV-14 (0.04 μM) but an IC_{50} more than fourfold higher (0.18 μM). Interestingly, this compound was also the most active against the Leu-1188 mutant by plaque reduction assay. For this mutant, however, the IC_{50} was nearly threefold lower than the MIC. If one compares the wild-type and mutant viruses directly, the MIC of compound III increased nearly 40-fold for the mutant while the IC_{50} increased by only a factor of three. Five of the remaining eight compounds that were active against HRV-14 were inactive against the Leu-1188 mutant by plaque reduction assay. In each case, the IC_{50}, if obtainable experimentally, was above the maximum testable level in the plaque reduction assay. Overall, the correlation of MIC to IC_{50} for the eight compounds which were active against HRV-14 was very high ($r^2 = 0.81$; Fig. 5), strongly indicating a cause and effect relationship between these two measures of activity.

DISCUSSION

Because of their vast serotypic diversity, the HRVs have presented a particularly difficult target for classical approaches to immunization against viral infections. Consequently, treatment with anti-HRV compounds offers the

greatest hope for the eventual treatment of the common cold in humans. In this report, we describe the structure-function relationship of eight antiviral compounds which inhibit the replication of HRV-14. All eight of these structurally related compounds have been shown in previous reports to bind in a hydrophobic pocket in the VP1 beta barrel and to induce conformational changes restricted to the neighboring polypeptide chain. These conformational changes impinge on the putative viral receptor-binding site (Fig. 2 and 3) and, as shown in this report (Fig. 4, Table 1), inhibit adsorption of the virus to HeLa cells. Thus, adsorption of a virus to its specific receptor can be blocked not only by agents which directly interfere with the receptor-ligand complex (3) but also by those which, by indirect actions, alter the conformation of the viral receptor-binding site. These results give further confirmation of the involvement of the HRV canyon in virion adsorption to host cells.

Previously, one of our compounds (compound IV, disoxaril), was reported to block the uncoating of poliovirus types 1 and 2 (5, 14) and HRV-2 (5). In the latter example, [^{3}H]uridine-labeled HRV-2 and poliovirus type 2 (MEF strain) adsorbed to intact HeLa cells at the same rate whether in the presence or absence of disoxaril. Furthermore, as assessed by the development of resistance to neutralizing antibodies, disoxaril did not affect the ability of the viruses to penetrate into the cell. However, neutral red-encapsidated virus assays, electron microscopy, and measurements of RNA synthesis all indicated that disoxaril inhibited uncoating of poliovirus types 1 and 2 and HRV-2. We have recently confirmed the lack of adsorption blockade by disoxaril and related compounds on HRV-2 in a whole cell binding assay (unpublished data). In these experiments, [^{3}H]uridine-labeled HRV-14 was again blocked by disoxaril from adsorbing to HeLa cells at its MIC, whereas identically prepared HRV-2 was not affected by even nine times its MIC (unpublished data). Thus, the mode of action of the drugs varies with the particular HRV serotype and possibly with the receptor groupings.

Since there is no X-ray crystallographic data of HRV-2 complexed with disoxaril, one can only speculate as to why its adsorption is not blocked by this compound. Crystallographic studies on another minor binding group serotype, HRV-1A, indicate that the native conformation of the drug-binding pocket resembles the open conformation of drug-bound HRV-14 (S. Kim, T. Smith, M. Chapman, M. Rossmann, M. McKinlay, F. Dutko, P. Felock, and D. Pevear, manuscript in preparation), and the three-dimensional structure of compound VII complexed with HRV-1A suggests that no significant movement occurs in the canyon floor upon drug binding. In the absence of a drug-induced conformational change, inhibition of binding to the minor group receptor would, therefore, not be expected.

Compound III(S) provides the only exception to the direct relationship (i.e., less than a threefold difference) between MIC and IC_{50} for HRV-14. In this instance, the concentration of drug required to block the replication of HRV-14 is more than fourfold less than the amount required to reach an IC_{50}, indicating that a second mechanism of action may exist for this compound. It appears that most of the resistance of the Leu-1188 mutant to compound III(S) could be explained by elimination of this second mechanism of action, since the MIC increases by a factor of 37 while the IC_{50} increases less than threefold. Resolution of the three-dimensional structure of the Leu-1188 mutant complexed with compound III(S) may provide valuable insight into selective effects of mutations on compound activity. Such observations may contribute to our understanding of the dynamics of the uncoating process.

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