Hapivirins and Diprovirins: Novel θ-Defensin Analogs with Potent Activity against Influenza A Virus

Mona Doss, Piotr Ruchala, Tesfaldet Tecle, Donald Gantz, Anamika Verma, Alex Hartshorn, Erika C. Crouch, Hai Luong, Ewa D. Micewicz, Robert I. Lehrer and Kevan L. Hartshorn

*J Immunol* 2012; 188:2759-2768; Prepublished online 15 February 2012;
doi: 10.4049/jimmunol.1101335
http://www.jimmunol.org/content/188/6/2759

**References**
This article cites 35 articles, 13 of which you can access for free at:
http://www.jimmunol.org/content/188/6/2759.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Hapivirins and Diprovirins: Novel θ-Defensin Analogs with Potent Activity against Influenza A Virus

Mona Doss,* Piotr Ruchala,† Tesfaldet Tecle,* Donald Gantz,‡ Anamika Verma,* Alex Hartshorn,* Erika C. Crouch,§ Hai Luong,‡ Ewa D. Micewicz,† Robert I. Lehrer,† and Kevan L. Hartshorn*  

0-Defensins are cyclic octadecapeptides found in nonhuman primates whose broad antiviral spectrum includes HIV-1, HSV-1, and several acute respiratory syndrome coronavirus, and influenza A virus (IAV). We previously reported that synthetic 0-defensins called retrocyclins can neutralize and aggregate various strains of IAV and increase IAV uptake by neutrophils. This study describes two families of peptides, hapivirins and diprovirins, whose design was inspired by retrocyclins. The goal was to develop smaller partially cyclic peptides that retain the antiviral activity of retrocyclins, while being easier to synthesize. The novel peptides also allowed for systemic substitution of key residues to evaluate the role of charge or hydrophobicity on antiviral activity. Seventy-two hapivirin or diprovirin peptides are described in this work, including several whose anti-IAV activity equals or exceeds that of normal θ- or θ-defensins. Some of these also had strong antibacterial and antifungal activity. These new peptides were active against H3N2 and H1N1 strains of IAV. Structural features imparting strong antiviral activity were identified through iterative cycles of synthesis and testing. Our findings show the importance of hydrophobic residues for antiviral activity and suggest that peptides, which often increase a peptide’s serum t1/2 in vivo, can increase the antiviral activity of DpVs. The new peptides acted at an early phase of viral infection, and, when combined with pulmonary surfactant protein D, their antiviral effects were additive. The peptides strongly increased neutrophil and macrophage uptake of IAV, while inhibiting monocyte cytokine generation. Development of modified θ-defensin analogs provides an approach for creating novel antiviral agents for IAV infections. The Journal of Immunology, 2012, 188: 2759–2768.

Influenza virus is a continuing threat to global health, causing ~36,000 deaths annually in the United States alone (1). In nonpandemic years, the virus tends to be seasonal and to affect children and the elderly primarily. The pandemic of 1918, caused by an H1N1 subtype, resulted in millions of deaths, including those of healthy, young adults (2). The first 21st century pandemic occurred in 2009, and again involved an H1N1 variant that caused mortality in young individuals. Because influenza A viruses (IAV) have unstable segmented RNA genomes and multiple wild-animal reservoirs that facilitate genetic reassortment, the threat of future pandemics is likely to remain indefinitely. Influenza viral infections are exemplars of the importance of innate immunity because the mutability of the virus allows it to evade prior adaptive immune responses (3). After initial exposure to a novel influenza viral strain, it takes 5–7 d before specific Abs and T cells arrive in the lung to clear the virus. This delay defines the critical time window in which innate immunity operates to confine influenza viruses to the upper respiratory tract.

Antimicrobial peptides appear to be an important component of innate defense against IAV. Several studies have shown strong anti-influenza activity for defensins (4–7), and a recent study also demonstrated the human cathelicidin, LL-37, inhibits IAV in vitro and in vivo (8). Defensins are cysteine-rich cationic and amphipathic peptides found in plants, insects, mammals, and birds (9, 10). Human neutrophils contain large stores of α-defensins (HNP-1–3) in their primary (azurophilic) granules, and several β-defensins are expressed in human pulmonary (and other) epithelial cells. Collectively, the activity of these α- and β-defensins encompasses Gram-positive and Gram-negative bacteria, fungi, and viruses, whereas α-defensins are cyclic defensins found in certain nonhuman primates, but not in humans, chimpanzees, or gorillas (11). Their disappearance from the Hominidae is attributable to a premature stop codon within the signal sequence. Retrocyclins 1 and 2 can be considered to be humanized 0-defensins because their sequences are encoded within the human genome (12, 13) and because recent experiments showed that human epithelial cells can produce retrocyclin peptides when exposed to an aminoglycoside that allows the stop codon to be bypassed (14).

We have shown that synthetic retrocyclins have stronger IAV-neutralizing activity than α- and β-defensins and that they also induce viral aggregation and promote the uptake of IAV by neutrophils (15). Pulmonary surfactant protein D (SP-D) is an important innate inhibitor of seasonal IAV strains (16). In contrast to α-defensins, HPNP1 and 2, which bind strongly to SP-D and interfere with its antiviral activity (17, 18), retrocyclins and SP-D

Copyright © 2012 by The American Association of Immunologists, Inc. 0022-1767/12/$16.00.
have additive antiviral activity when combined (15). Based upon the favorable antiviral profile of retrocyclins, we developed two series of novel γ-defensin analogs and tested their interactions with IAV.

Even though relatively small (18 Xaa), γ-defensins are difficult to synthesize. They belong to cysteine-rich peptides containing a circular backbone and characteristic ladder of three disulfide bridges (see Fig. 1) forming a β-hairpin structure. Such compounds require two postsynthetic steps, as follows: oxidation (disulfide bonds’ formation) and circularization via amide bond, resulting in a low final yield of the desired product. Therefore, our current studies focused on the modification of γ-defensins with the general goal of simplifying their structure and preserving and/or possibly enhancing their antiviral properties. Hapivirins (HpVs) had the trisulfide ladder of γ-defensins, but did not have a fully circular backbone (see Fig. 1). The HpVs had various hydrophobic substitutions at a single amino acid site (position X1). In case of diprovirins (DpVs), their β-hairpin structure was imposed by structural element, –(D)Pro–(L)Pro– moiety, making positions previously occupied by four cysteines (positions X2 and X3) available for probing. In turn, substitutions in positions X4, X5, and X6 investigated the role of hydrophobicity, the presence of a “clipping” disulfide bridge, and the feasibility of N-terminal substitutions, respectively.

Materials and Methods

Preparation of IAV, bacteria, and fungi

IAV was grown in the chorioallantoic fluid of 10-d-old chicken eggs and purified on a discontinuous sucrose gradient, as previously described (19).

The virus was dialyzed against PBS to remove sucrose, aliquoted, and stored at ~80°C until needed. A/Philippines/82 (H3N2) (Phil82) was provided by E. Anders (Department of Microbiology, University of Melbourne, Melbourne, Australia). The A/PR/8/34/H1N1 (PR-8) strain was a gift of J. Abramson (Bowman Gray School of Medicine, Winston-Salem, NC). The hemagglutinin titer of each virus preparation was determined by titration of virus samples in PBS with thoroughly washed human type O, Rh(–) RBC, as described (19). Postthawing the viral stocks contained ∼5 × 10^8 PFU/ml.

Staphylococcus aureus (Wood 46 strain; ATCC 10832) and Candida albicans (Berkhout anamorph; ATCC MYA-3573) were obtained from American Type Culture Collection.

S. aureus was grown overnight by inoculating the lyophilized bacteria in LB broth and used at a dilution that yielded ∼100 colonies on agar culture dishes after 24 h. To test for inhibition of bacterial growth, the diluted bacteria were incubated for 30 min at 37°C with peptides in PBS and then inoculated on agar plates using sterile glass beads to spread bacteria evenly over the surface. Results were...
expressed as percentage of control colonies in peptide-treated samples. Inhibition of growth of \textit{C. albicans} was tested in a similar manner. Fluorescent bioparticles of \textit{S. aureus} and zymosan were obtained from Molecular Probes for use in studies of particle aggregation using fluorescent microscopy, as described (20).

\textbf{HpV/DpV preparation}

All HpV and DpV peptides were synthesized by the solid-phase method using CEM Liberty automatic microwave peptide synthesizer (CEM, Matthews, NC), applying 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry (21) and standard, commercially available amino acid derivatives and reagents (EMD Biosciences, San Diego, CA; Chem-Impex International, Wood Dale, IL). Rink amide MBHA resin (EMD Biosciences, San Diego, CA) was used as a solid support. Peptides were cleaved from resin using modified reagent K (trifluoroacetic acid [TFA], 94% [v/v]; phenol, 2% [w/v]; water, 2% [v/v]; triisopropylsilane, 1% [v/v]; 1,2-ethanedithiol, 1% [v/v]; 2 h) and precipitated by addition of ice-cold diethyl ether. Reduced peptides were purified by preparative reverse-phase HPLC (RP-HPLC) to 90% homogeneity, and their purity was evaluated by MALDI spectrometry as well as analytical RP-HPLC.

\textbf{Disulfide bond formation.} Peptides were dissolved at a final concentration 0.25 mg/ml in 50% solution of DMSO in H$_2$O and stirred overnight at room temperature. Subsequently, peptides were lyophilized and repurified on a preparative C18 SymmetryShield RP-HPLC column to $>95\%$ homogeneity, and their purity was evaluated by MALDI spectrometry as well as analytical RP-HPLC.

\textbf{Analytical HPLC.} Analytical RP-HPLC was performed on a Varian ProStar 210 HPLC system equipped with ProStar 325 Dual Wavelength UV-Vis detector with the wavelengths set at 220 and 280 nm (Varian, Palo Alto, CA). Mobile phases consisted of solvent A, 0.1% TFA in water, and solvent B, 0.1% TFA in acetonitrile. Analyses of peptides were performed with an analytical reversed-phase C18 SymmetryShield column, 4.6 $\times$ 250 mm, 5 $\mu$m (Waters, Milford, MA) or analytical RP C18 Vydis 218TP54 column, 4.6 $\times$ 250 mm, 5 $\mu$m (Grace, Deerfield, IL), applying a linear gradient of solvent B from 0 to 100% over 100 min (flow rate: 1 ml/min).

\textbf{SP-D preparation}

Recombinant human SP-D was produced in stably transfected CHO-K1 cells, as previously described (22). For these studies, the dodecameric fraction of recombinant human SP-D was used, unless otherwise specified. The collectin preparations used in this study were tested for endotoxin using a quantitative endotoxin assay (\textit{Limulus} amebocyte lysate; BioWhittaker). The final concentrations of endotoxin in protein samples containing the highest concentrations of collectins were $\sim$20–100 pg/ml (or 6–12 endotoxin U/ml using an internal assay standard).

\textbf{Fluorescent focus assay of IAV infectivity}

Viral neutralization was measured using a fluoresce Per the Editorial nt focus reduction assay. In brief, A549 (respiratory epithelial cell line) or Madin–Darby canine kidney (MDCK) cell monolayers were prepared in 96-well plates and grown to confluency. These layers were then infected with diluted IAV preparations at a multiplicity of infection of $\sim$1:100. The infection was conducted for 45 min at 37˚C in PBS and tested for presence of IAV-infected cells after 7 h using a mAb directed against the influenza A viral nucleoprotein, provided by N. Cox (Centers for Disease Control, Atlanta, GA), as previously described (23). IAV was preincubated for 30 min at 37˚C with defensins, SP-D, or control buffer, followed by addition of these viral samples to the A549 or MDCK cells. The viral incubation with defensins was conducted in PBS with added calcium and magnesium (Dulbecco’s PBS; Life Technologies) with no serum added during the incubation.

\textbf{FIGURE 3.} IAV-neutralizing activity of HpVs. Aliquots of the Phil82 strain of IAV were preincubated with 1 or 2 $\mu$g/ml indicated HpVs. Results are mean ± SEM of three experiments using the fluorescent focus assay on MDCK cells. All of the tested HpVs significantly reduced infectious foci as compared with virus alone ($p < 0.05$).

\textbf{FIGURE 4.} Dose response for viral neutralization by HpV 11, HpV 17, HpV 18, and HpV 19. Neutralizing activity of the most potent HpVs was compared using A549 and MDCK cells. (A–C) Used the Phil82 viral strains; (D) used the PR-8 strain. The results shown are mean ± SEM of four experiments. All concentrations of the peptides tested significantly inhibited IAV replication as compared with control ($p < 0.05$).
Table I. Neutralizing activity of DpVs

<table>
<thead>
<tr>
<th>Peptide</th>
<th>% Infected Foci</th>
<th>Peptide</th>
<th>% Infected Foci</th>
</tr>
</thead>
<tbody>
<tr>
<td>DpV 1</td>
<td>99 ± 5%</td>
<td>DpV 1601</td>
<td>99 ± 10</td>
</tr>
<tr>
<td>DpV 2</td>
<td>71 ± 7%</td>
<td>DpV 1602</td>
<td>35 ± 10</td>
</tr>
<tr>
<td>DpV 3</td>
<td>95 ± 9%</td>
<td>DpV 1603</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>DpV 4</td>
<td>70 ± 7%</td>
<td>DpV 1604</td>
<td>66 ± 13</td>
</tr>
<tr>
<td>DpV 5</td>
<td>87 ± 7%</td>
<td>DpV 1605</td>
<td>46 ± 13</td>
</tr>
<tr>
<td>DpV 6</td>
<td>91 ± 1%</td>
<td>DpV 1606</td>
<td>41 ± 14</td>
</tr>
<tr>
<td>DpV 7</td>
<td>83 ± 11%</td>
<td>DpV 1607</td>
<td>3 ± 2.6</td>
</tr>
<tr>
<td>DpV 8</td>
<td>87 ± 13%</td>
<td>DpV 1608</td>
<td>62 ± 11</td>
</tr>
<tr>
<td>DpV 9</td>
<td>32 ± 10%</td>
<td>DpV 1609</td>
<td>9 ± 9</td>
</tr>
<tr>
<td>DpV 10</td>
<td>83 ± 13%</td>
<td>DpV 1610</td>
<td>16 ± 9</td>
</tr>
<tr>
<td>DpV 11</td>
<td>98 ± 12%</td>
<td>DpV 1611</td>
<td>21 ± 10</td>
</tr>
<tr>
<td>DpV 12</td>
<td>76 ± 7%</td>
<td>DpV 1612</td>
<td>37 ± 14</td>
</tr>
<tr>
<td>DpV 13</td>
<td>0 ± 0%</td>
<td>DpV 1613</td>
<td>80 ± 8.6</td>
</tr>
<tr>
<td>DpV 14</td>
<td>64 ± 6%</td>
<td>DpV 1614</td>
<td>42 ± 18</td>
</tr>
<tr>
<td>DpV 15</td>
<td>52 ± 6%</td>
<td>DpV 1615</td>
<td>5 ± 5</td>
</tr>
<tr>
<td>DpV 16</td>
<td>15 ± 7%</td>
<td>DpV 1616</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>DpV 17</td>
<td>63 ± 6%</td>
<td>DpV 1617</td>
<td>36 ± 13</td>
</tr>
<tr>
<td>DpV 18</td>
<td>101 ± 9%</td>
<td>DpV 1618</td>
<td>52 ± 15</td>
</tr>
<tr>
<td>DpV 19</td>
<td>81 ± 16%</td>
<td>DpV 1619</td>
<td>40 ± 16</td>
</tr>
<tr>
<td>DpV 20</td>
<td>Not done</td>
<td>DpV 1620</td>
<td>11 ± 4</td>
</tr>
<tr>
<td>DpV 21</td>
<td>16 ± 8%</td>
<td>DpV 1621</td>
<td>25 ± 8</td>
</tr>
<tr>
<td>DpV 22</td>
<td>19 ± 7.5%</td>
<td>DpV 1622</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>DpV 23</td>
<td>3 ± 2</td>
<td>DpV 1623</td>
<td>83 ± 9</td>
</tr>
<tr>
<td>DpV 24</td>
<td>83 ± 9%</td>
<td>DpV 1624</td>
<td>43 ± 10</td>
</tr>
<tr>
<td>DpV 25</td>
<td>101 ± 17%</td>
<td>DpV 1625</td>
<td>97 ± 23</td>
</tr>
<tr>
<td>DpV 26</td>
<td>61 ± 20</td>
<td>DpV 1626</td>
<td>101 ± 17</td>
</tr>
<tr>
<td>DpV 27</td>
<td>36 ± 10</td>
<td>DpV 1627</td>
<td>97 ± 23</td>
</tr>
<tr>
<td>DpV 28</td>
<td>61 ± 20</td>
<td>DpV 1628</td>
<td>101 ± 17</td>
</tr>
<tr>
<td>DpV 29</td>
<td>36 ± 10</td>
<td>DpV 1629</td>
<td>97 ± 23</td>
</tr>
<tr>
<td>DpV 30</td>
<td>1 ± 1</td>
<td>DpV 1630</td>
<td>101 ± 17</td>
</tr>
<tr>
<td>DpV 31</td>
<td>2 ± 2</td>
<td>DpV 1631</td>
<td>36 ± 10</td>
</tr>
<tr>
<td>DpV 32</td>
<td>0 ± 0</td>
<td>DpV 1632</td>
<td>101 ± 17</td>
</tr>
<tr>
<td>DpV 33</td>
<td>2 ± 2</td>
<td>DpV 1633</td>
<td>36 ± 10</td>
</tr>
</tbody>
</table>

*aAll peptides were tested at 40 µg/ml.

Measurement of IAV aggregation

Aggregation of IAV particles was assessed following addition of various concentrations of antimicrobial peptides by monitoring increases in light absorbance by viral suspensions at 350 nm. This was done using a Perkin-Elmer Lambda 35 UV/Vis spectrophotometer. Confirmation of IAV aggregation was obtained by electron microscopy, as described below. Aggregation of fluorescent bacteria and zymosan was assessed by fluorescence microscopy.

Electron microscopy

Antimicrobial peptides were incubated with Phil82 IAV at 37°C for 30 min, and a 4-µl sample was placed on each copper grid. After the unbound virus was blotted off, the grid was fixed with 4 µl 2.5% glutaraldehyde for 5 min. Samples were stained with 1% sodium phosphotungstate (pH 7.3) (Sigma-Aldrich) for 10 s, and excess stain was blotted off. The grids were then air dried and stored in a grid box until examined with a Phillips 300 electron microscope.

Human neutrophil preparation

Neutrophils from healthy volunteers were isolated to >95% purity by dextran sedimentation, followed by Ficoll-Paque gradient separation for the removal of mononuclear cells, and then hypotonic lysis to eliminate any contaminating erythrocytes, as previously described (19). Cell viability was determined to be >98% by trypan blue staining. The isolated neutrophils were resuspended at the appropriate concentrations in control buffer (PBS) and used within 2 h. Neutrophil collection was done with informed consent, as approved by the Institutional Review Board of Boston University School of Medicine.

Measurement of IAV uptake by neutrophils and RAW cells

FITC-labeled IAV (Phil82 strain) was prepared, and uptake of virus by neutrophils or RAW 264.7 cells was measured, as previously described (23). Briefly, IAV was incubated with neutrophils or RAW cells for 30 min at 37°C in the presence of control buffer or retrocyclins (RCs). Extracellular fluorescence was quenched by addition of trypan blue (0.2 mg/ml) to the samples. After they were washed, the neutrophils were fixed with 1% paraformaldehyde, and neutrophil/RAW cell-associated fluorescence was measured using flow cytometry. The mean intracellular fluorescence (2000 cells/sample) was measured (24).

Measurement of human monocyte TNF-a production in response to IAV

Human peripheral blood monocytes were isolated by magnetic bead separation, as described above, and infected with a multiplicity of infection of ~50 of Phil82 IAV for 45 min at 37°C. The virus was either used alone or after 45-min incubation with various concentrations of LL-37. After this, the cells were pelleted, washed with PBS, and then cultured for 24 h at 37°C in RPMI 1640 with 10% autologous serum in a CO2 incubator.
defensins at inhibiting this viral strain on a weight basis were RC2 and HpVs 11–15. In our prior experiments, the most effective infectivity at a concentration of 1 μg/ml dose of DpV 13, in which only one experiment was done. All results shown were using 20 μg/ml, except with HpV 17, in which 70 μg/ml was used.

*p < 0.05 compared with control colony counts (in which no peptide was added).

### Results

#### Viral neutralization by HpV

Fig. 1 shows the amino acid sequences of the HpVs and DpVs that were synthesized for these studies. The HpVs all have a relatively rigid hairpin structure due to the presence of three-disulfide bond ladder with alternating hydrophobic and basic amino acids adjacent to the cysteines. Variants were made simply by changing one amino acid in the hairpin loop (X1, the red residue in Fig. 1). Several of the unusual amino acids used to substitute for X1 are shown in Fig. 2. Fig. 3 shows results of screening assays for the antiviral activity of the HpV peptides 1–16. Neutralization was assessed using a fluorescent focus assay and the seasonal H3N2 strain, Phil82. Obvious differences in activity were found among the HpV variants, and several caused significantly inhibited IA V replication as compared with control (Fig. 5, group 1), or added to the MDCK or A549 cells 15 (group 1) or 45 (group 3) min after viral infection of the cells. In group 1, all defensins significantly inhibited viral nucleoprotein expression 7 h postinfection (with the single exception of DpV 21).

#### Effect of delaying addition of defensins on neutralizing activity

All the peptides were used at concentrations of 1 μg/ml. The defensins were either preincubated with IAV (Phil82 strain) as in Fig. 5 (group 1), or added to the MDCK or A549 cells 15 (group 1) or 45 (group 3) min after viral infection of the cells. In group 1, all defensins significantly inhibited viral nucleoprotein expression 7 h postinfection (with the single exception of DpV 21 in A549 cells). In contrast, no defensin inhibited IAV in group 2 or 3. All of the peptides tested in group 1 significantly inhibited IAV replication as compared with control (p < 0.05), with the exception of DpV 21.
these analogs are shown schematically in Fig. 1. DpVs 1–21 are variants in which amino acids 4, 6, 9, and 11 (positions occupied by cysteines in HpVs) are substituted simultaneously with various amino acids, as follows: glycine (DpV 1), alanine (DpV 2), \(\beta\)-alanine (DpV 3), and so on. Of special note are DpVs 13 and 16, in which these four amino acids are isoleucine or leucine, respectively. A large set of variants of DpV 16 (DpVs 1601–1622) retains these leucines, but varies amino acids 1, 5, and 12. DpVs 1623–1629 are identical to DpV 16 except for modification or substitutions of cysteines 2 and 13. Finally DpV 1630–32 are identical to DpV 16, except for having methylsulfonyl, polyethylene glycol (PEG5), or amino-oxyacetic acid (Aoa)-PEG5 attachments. As shown in Table I, several of these compounds, including DpVs 13, 16, 1607, 1609, 1615, 1616, 1623, and 1630–1632, had strong neutralizing activity in initial screening assays.

In follow-up dose-response experiments (Fig. 5), we found that

![Table III](Image)

<table>
<thead>
<tr>
<th>Control</th>
<th>DpV 1607 1.5 (\mu)g/ml</th>
<th>DpV 1632 0.4 (\mu)g/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100%</td>
<td>28 ± 2</td>
</tr>
<tr>
<td>SP-D 5 ng/ml</td>
<td>74 ± 10</td>
<td>34 ± 14</td>
</tr>
<tr>
<td>SP-D 10 ng/ml</td>
<td>50 ± 6</td>
<td>32 ± 13</td>
</tr>
</tbody>
</table>

Mean ± SEM of four experiments using A549 cells in the fluorescent focus assay. \(^*p < 0.05\) compared with SP-D alone.

![FIGURE 7](Image)

**FIGURE 7.** Viral-aggregating activity of selected HpVs, DpVs, and RCs. (A–C) Show results of viral aggregation assays using light scattering, in which control buffer (IAV alone) or HpVs, DpVs, or RCs were added at time 0. The results are expressed as percentage of light absorption at time 0 and are mean ± SEM of three or more experiments in each case. In (A) and (B), 8 \(\mu\)g/ml peptides were used, and in (C) 10 \(\mu\)g/ml was used. Aggregation induced by HpV 11 and 17 was highly significant compared with control \((p < 0.001)\) and was significantly greater than aggregation induced by RC2 by ANOVA. Aggregation induced by HpV 15 and RC2 was also significant compared with IAV alone \((p < 0.05)\). Aggregation induced by DpV 13 was significantly greater than that induced by RC1, as assessed by ANOVA (C). The lower panels show results of electron micrographs of untreated virus, or virus treated with the indicated HpVs or DpVs demonstrating virus aggregation. The electron microscopy results were representative of at least three similar experiments using 40 \(\mu\)g/ml peptides.
DpVs 13, 16, 1623, and 1632 had particularly strong activity. These results are of interest because DpVs 13 and 16 have the hydrophobic isoleucine and leucine substitutions in positions 4, 6, 9, and 11 (although note that tert-Leu substitutions as in DpVs 14 and 15 did not confer as much activity). DpV 1623 is identical to DpV 16 apart from having no disulfide bond, because cysteines in positions 2 and 13 possess free sulphydryl groups. DpVs 1630–32 are identical to DpV 16 apart from having PEG₅ (21-amino-4,7,10,13,16,19-hexaoxaheneicosanoic acid) or methylsulfonyl attachments, indicating that such attachments may increase activity. This was particularly evident in the case of DpV 1632, in which the PEG₅ substituent was additionally modified with Aoa.

**Antibacterial and antifungal activity of HpVs and DpVs**

An important feature of defensins is their broad spectrum activity, killing not only viruses, but also bacteria and fungi. Therefore, we tested the antibacterial and antifungal activity of HpVs and DpVs that had strong antiviral activity. As shown in Table II, DpVs 13 and 16 had strong activity against both *S. aureus* and *C. albicans*. DpV1632 had less activity than DpVs 13 and 16. HpV17 was also tested, but this peptide had only limited activity, despite its strong antiviral activity. The antibacterial activity of DpVs 13 and 16 was greater than that of HNP1, HNP2, or RC1. Their antifungal activity was also greater than the HNPs, and very similar to that of RC1.

**Effect of delayed addition of potent HpVs or DpVs on viral-neutralizing activity**

Prior reports have indicated that some antimicrobial peptides inhibit IAV principally through interacting with epithelial cells rather than with the virus (5). To test whether this is true for the HpVs or DpVs, we compared the effects of varying the timing of addition of HpV 11, HpV 17, and DpV 1632 with respect to the addition of virus to either MDCK cells or A549 cells. We also used RC2 for comparison. As shown in Fig. 6, allowing the virus to infect the cells for 15 or 45 min prior to adding the peptides caused a marked diminution of their viral-neutralizing activity.

**Interactions of HpVs and DpVs with SP-D**

HNPs 1–3 and retrocyclins bind to pulmonary SP-D (15, 18). In the case of the HNPs (but not of the retrocyclins), this leads to competitive effects such that the IAV-neutralizing activity of combinations of HNPs and SP-D is less than occurred with SP-D alone. As shown in Table III, DpVs 1607 and 1632 had additive activity when combined with SP-D. Similar results were obtained with HpVs 6 and 11 (data not shown).

**Viral aggregation by HpVs and DpVs**

As we have reported for HNPs and retrocyclins, some of the active HpVs and DpVs were also able to induce viral aggregation, as assessed using the Phil82 viral strain (Fig. 7). Certain HpVs were particularly effective in this regard, including HpV 11, HpV 15, and HpV 17 (Fig. 7A, 7B), all of which had strong neutralizing activity as well. Note that these were more potent in this assay than RC2, which was the most active retrocyclin in this assay in former studies. Several DpVs were also tested for viral-aggregating activity. DpV 13 had the strongest aggregating activity of the DpVs tested (Fig. 7C), exceeding that of RC1 and RC2. Note that DpV 2 did not cause viral aggregation (Fig. 7B), which is consistent with its lack of viral-neutralizing activity. Electron micrographs confirmed the presence of viral aggregates as shown in the lower panels of Fig. 7. Of interest, HpVs and DpVs also caused aggregation of fluorescently labeled *S. aureus* and zymosan particles (Fig. 8), indicating that this aggregating property applies to other microorganisms besides IAV.

**HpVs and DpVs increase neutrophil and monocyte uptake of IAV**

Preincubation of IAV with several of the most highly neutralizing HpVs and DpVs also resulted in increased neutralophil uptake of the virus (Fig. 9A, 9B), including HpVs 17 and 18, and DpVs 13, 1631, and 1632. In further studies, HpV 14 and DpV 1622 also increased neutralophil uptake of IAV significantly compared with control buffer, whereas peptides that had weaker antiviral activity (e.g., HpV 6 and DpVs 2 and 1617) did not (Table IV). Hence, the ability of HpVs and DpVs to promote neutralophil uptake of IAV correlated overall with their antiviral activity. Note that both HpV 17 and HpV 18 increased viral uptake by RAW cells as well (Fig. 9C).

**HpVs and DpVs reduce human monocyte generation of TNF-α in response to IAV**

Some of the peptides were also tested for their ability to inhibit IAV-induced TNF-α production by human monocytes. DpVs 16 and 1630 and HpVs 11 and 19 inhibited the TNF response to varying degrees (Fig. 10).

**Discussion**

The key finding in this work is the feasibility of creating synthetic 6-defensin analogs with equal or greater activity against IAV than their natural counterparts. The analogs comprised two distinct subgroups, HpVs and DpVs. Some members of each group had neutralizing activity comparable to that of wild-type defensins. The HpVs resembled primate retrocyclins in having an intramolecular ladder of three evenly spaced disulfide bonds. Strikingly, changing a single amino acid in the hairpin loop of HpVs resulted in marked changes in antiviral activity. Several HpVs had 50% neutralizing activity at concentrations of ~500 ng/ml. Several DpVs had similar activity because they reduced viral infectivity to <20% of control at 3 µg/ml and DpV 1632 had a 50% neutralizing dose <0.4 µg/ml (Table III). In our prior studies, the most active 6-defensins RC2 and RC101 showed 50% neutralizing activity of ~1.25 µg/ml. Hence, based on neutralizing activity alone, the HpVs and DpVs are worthy of further investigation.

![FIGURE 8. Aggregation of *S. aureus* and zymosan particles by HpV 11 and DpV 1607. Fluorescently labeled *S. aureus* and zymosan particles were obtained from Molecular Probes and incubated with various HpVs and DpVs, followed by examination using a fluorescent microscope. Representative photomicrographs (from three or more experiments) displaying aggregation of *S. aureus* by HpV 11 (24 µg/ml) and DpV 1607 (60 µg/ml) are shown.](http://www.jimmunol.org/content/journals supplemental/jimmunol_supplemental_images_4.png)
We also tested a limited set of the more potent antiviral peptides for antibacterial and antifungal activity. Of interest, DpVs 13 and 16 also caused strong inhibition of growth of *S. aureus* and *C. albicans*, greatly exceeding the activity of HNPs. Both peptides also showed similar inhibition of *C. albicans*, and greater inhibition of *S. aureus*, than RC1. In contrast, HpV 17 had modest antibacterial and antifungal activity indicating that antiviral and antibacterial (or antifungal) activity are not consistently correlated. The broad spectrum antimicrobial activity of the DpVs is another attractive feature of this group of peptides.

*S. aureus* superinfection is a catastrophic consequence of IAV infection, and hence, a peptide capable of inhibiting both IAV and *S. aureus* could be valuable (26–28).

Structure-function analysis of the HpV and DpV variants tested provides insights into the molecular features that are most important for antiviral activity. Whereas cationic charge has been considered the most important determinant of defensin-mediated antimicrobial activity, our findings suggest that increasing hydrophobicity can have a marked impact on antiviral activity. The feature of HpVs conferring the best antiviral activity was increased nonaromatic-type hydrophobicity in the loop of the molecule (position 7) delivered by L-cyclohexylalanine or (S)-octahydroindole-2-carboxylic acid, which can be considered a constrained analog of Cha. Because HpV 11 and HpV 17 were among the most active HpVs, their side chains may represent optimal hydrophobicity as well as spatial arrangements for maximal antiviral effect, especially considering that fairly similar substitutions did not confer similar effects (see Fig. 2).

DpVs 1–21 were designed to simplify the β-hairpin structure of HpVs, which is imparted and rigidified by a tridisulfide ladder. The β-hairpin structure of DpVs was imparted by incorporating the –(D)Pro–(L)Pro– moiety described by Robinson (25). In this group of analogs, the most effective substitutions were L-isoleucine (DpV 13) and L-leucine (DpV 16), with isoleucine being superior to leucine. Substitution with L-valine also led to increased activity compared with alanine or polar amino acids, although this increase was modest compared with the L-isoleucine or L-leucine substitutions. Of interest, the D-isomers of leucine, valine, or alanine all had lower activity than the L-isomers.

Given the strong activity of the L-leucine variant (DpV 16), this peptide became the starting point for a series of additional peptides having substitutions at positions 1, 5, and 12. In this case, hydrophobic substitutions were not clearly advantageous, although Tle, Cpg, cyclohexylglycine, and Tyr analogs showed

![FIGURE 9](http://www.jimmunol.org/)

**FIGURE 9.** Comparison of the ability of HpVs or DpVs to increase viral uptake by neutrophils or RAW cells. FITC-labeled Phil 82 virus was used to detect viral uptake by flow cytometry. (A) Shows uptake of virus that was treated with various concentrations of HpV 17 and 18. (B) Shows similar experiments using RAW cells. Results are mean ± SEM of seven experiments for (A) and (B) using separate neutrophil donors or RAW cells harvested on different days. (C) Shows neutrophil uptake of virus after preincubation with the indicated DpVs. Results are mean ± SEM of three experiments using separate neutrophil donors. All of the tested DpVs caused significant increases in viral uptake by neutrophils at the 30 μg/ml concentration (p < 0.03), but only DpV 1632 caused a significant increase in uptake at 15 μg/ml. All of the tested peptides caused significant increases in neutrophil or RAW cell uptake of IAV (p < 0.05).

![TABLE IV](http://www.jimmunol.org/)

**Table IV.** Ability of selected peptides to increase viral uptake by neutrophils

<table>
<thead>
<tr>
<th>Defensin</th>
<th>% of Control Neutrophil Uptake of IAV</th>
</tr>
</thead>
<tbody>
<tr>
<td>HpV 6</td>
<td>131 ± 14</td>
</tr>
<tr>
<td>HpV 14</td>
<td>201 ± 41</td>
</tr>
<tr>
<td>DpV 2</td>
<td>99 ± 15</td>
</tr>
<tr>
<td>DpV 1617</td>
<td>142 ± 75</td>
</tr>
<tr>
<td>DpV 1622</td>
<td>206 ± 41</td>
</tr>
</tbody>
</table>

Mean ± SEM of 4–7 experiments, each involving a different neutrophil donor. The defensin concentration used for these experiments was 60 μg/ml.

*Significant increase compared to control (p < 0.05).

![FIGURE 10](http://www.jimmunol.org/)

**FIGURE 10.** Ability of HpVs or DpVs to inhibit IAV-induced TNF generation by human monocytes. Human peripheral blood monocytes were infected with IAV (Phil82 strain), followed by culture in vitro for 18 h. TNF was measured by ELISA on culture supernatants, as described. Pretreatment of IAV with the indicated peptides reduced cytokine production as compared with virus alone. Results are mean ± SEM of four to five experiments using separate monocyte donors. *Significant reduction, p < 0.05.
considerable activity. Increased activity was seen with more polar substitutions: that is, threonine, serine, and arginine. Because the parental compound (DpV 16) has limited solubility in the aqueous solutions, further increase in hydrophobicity may be detrimental, whereas polar residues are beneficial.

Retaining the intact sequence of DpV 16, but reducing cysteines 2 and 13 or adding PEG5, methylsulfonyl attachments, or Aoa-PEG5 to the molecule gave the greatest increase in activity. The fact that addition of PEG5 or methylsulfonyl group to the peptide significantly increased activity may be useful, because these attachments may also prolong the T1/2 of the peptide in physiological conditions. The presence of the Aoa is particularly beneficial. Its effects may be partially explained by the presence of modified hydroxyamine group. Hydroxyamine was shown to inactivate IAV (29, 30) by cleaving fatty acids from viral hemagglutinin, lowering fusogenic and hemolytic activity. Although the concentration of hydroxyamine in the aforementioned experiments was much higher (1 M), perhaps DpV 1632 augments its intrinsic antiviral activity by delivering the Aoa moiety to sites that are conducive to allow fatty acid cleavage. Replacing the cysteines with various polar or charged amino acids generally resulted in loss of activity.

HNPs were reported to exert effects on epithelial cells that inhibit replication of IAV (5). In the present experiments, IAV was generally preincubated with defensins before infection of epithelial cells. To ascertain whether the activity of HNP and DpV resulted from events before or after viral internalization, we compared preincubating IAV with the peptide to introducing the peptides after the virus had interacted with cells for 15 or 45 min. Surprisingly, delaying peptide addition for as little as 15 min markedly diminished neutralizing activity. This suggests that under our experimental conditions the HpVs and DpVs exerted their antiviral effects by interacting with the virus itself. We believe that the ability of collectins or antimicrobial peptides to induce viral aggregation is an important correlate of antiviral activity. Viral aggregation can reduce particle numbers and promote clearance of virus from the airway through mucociliary action or uptake by phagocytes. We studied IAV aggregation mainly with peptides that showed strong activity in the neutralization assays. In general, highly neutralizing HpVs and DpVs also had strong viral-aggregating activity, so these properties may be closely related. Note that the aggregating activity of the most potent peptides tested in this paper (e.g., HpV 11 or HpV 17) greatly exceeded that of retrocyclins RC2, which had the strongest aggregating activity among the 0-defensins tested in our prior studies. Some of the HpVs and DpVs also caused aggregation of bacteria or zymosan. The ability of the peptides to induce aggregation suggests that they may oligomerize, as has been reported for some defensins and retrocyclins (31). We have found that oligomerization is important for the antiviral activity of collectins (32).

HNPs and retrocyclins have been reported to have pro- and anti-inflammatory effects in the lung and to promote viral or bacterial uptake by phagocytes (4, 15). The opsonizing activity of defensins may relate to the ability of these defensins to induce viral or bacterial aggregation. HpVs and DpVs showed opsonizing activity for IAV comparable to that shown by HNPs and RCs. Significantly, peptides that displayed strong aggregating activity, such as HpV 17, also had the strongest opsonizing activity. It is notable that the HpVs and DpVs were also capable of inhibiting IAV-induced TNF-α responses in monocytes. Production of TNF-α and other strong proinflammatory cytokines may be deleterious during IAV infection (33, 34), so this effect could be beneficial during severe IAV infection in vivo.

Overall, our findings suggest that developing synthetic 0-defensin analogs as potential therapeutics for IAV is promising and should be pursued further by in vivo studies. One concern might be that local inflammatory effects of the peptides are administered through the respiratory tract; however, recent reports indicate that retrocyclins applied in this manner have significant protective activity against serious respiratory pathogens, including severe acute respiratory syndrome coronavirus and avian influenza (H5N1) in mice (35, 36). Because retrocyclins are also highly effective against other viruses, including HIV and HSV, it will be important to determine whether the HpVs and DpVs are similarly active against these other viruses. If so, the small size and simple structures of DpVs and HpVs could make them excellent candidates for use as topical microbicides as well as potential systemic therapeutics. Of note, HpVs and DpVs appeared to have additive neutralizing activity when combined with SP-D, quite unlike the competitive effects seen when HNP1 and SP-D were combined. These findings suggest that HpVs or DpVs would not interfere with functional activities of SP-D if used to enhance lung immunity in vivo.

Acknowledgments
We thank Mitchell R. White for invaluable advice and assistance in viral preparation and various experimental methods.

Disclosures
Hapivirins and diprovirins are protected by patent rights (provisional application filed, UC case 2009-055-2; US 61/228,301; P.R. and R.I.L. are coinventors). The other authors have no financial conflicts of interest.

References


