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Antiviral Research 61 (2004) 153–159
Antiviral Research
www.elsevier.com/locate/antiviral

# An optical thin film assay incorporating rhinovirus protease inhibitors as detector reagents

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Received 28 May 2003; accepted 9 September 2003

**Abstract**

Human rhinoviruses (HRV) are the main cause of the common cold. Viral replication utilizes the activity of the HRV3C protease (3CP) enzyme [Antimicrob. Agents Chemother. 43 (1999) 2444; Antimicrob. Agents Chemother. 44 (2000) 1236]. Therefore, 3CP is an attractive target for antiviral drug development, and a new class of orally bioavailable irreversible 3CP inhibitors has been designed [J. Med. Chem. 45 (2002) 1607]. We have used related inhibitors to develop a rapid test for rhinovirus. The optical immuno assay (OIA®) thin film detection technology utilizes an optically coated silicon surface to convert specific molecular binding events into visual color changes by altering the reflective properties of light through molecular thin films. The purpose of this study was to develop a rapid assay for the determination of 3CP combining the Thermo Electron Bio Star® OIA technology and the newly designed inhibitor compounds. The advantage of this assay was in its approach, in which therapeutic and diagnostic targets are the same thus allowing patients with detected rhinoviruses to receive optimal treatment.

Three different biotinylated inhibitor compounds were synthesized. The length of the spacer between the inhibitor and biotin core was 5, 10, and 15 atoms. These compounds were incorporated into the OIA format for the HRV assay development. A rapid (20 min) OIA test was developed using a 15 atom spacer biotinylated inhibitor (4). Forty different HRV serotypes were studied and thirty three serotypes of these 40 were detected (80%).


*Keywords:* Human rhinovirus; 3C protease; Serotypes; Inhibitor

## 1. Introduction

Human rhinoviruses (HRV) are the main cause of common colds and can lead to serious secondary illnesses such as otitis, sinusitis, and exacerbation of asthma and bronchitis (Couch, 2001). These diseases affect large populations of people including infants, young children, and older adults. More than 100 different serotypes of HRV are known at this time (Couch, 2001; Rueckert, 2001).

Rhinoviruses belong to the *Picornaviridae* family. All picornaviruses generate a single 250 kDa polyprotein that undergoes proteolytic cleavage resulting in mature capsid and viral proteins that assemble into infectious virions. Polyprotein cleavage is mediated by HRV3C protease (3CP) (Rueckert, 2001).

3CP plays a key role in virus replication by proteolytic cleavage of Gln–Gly bonds present in the viral precursor protein (Rueckert, 2001; Wang, 1999). The key role of 3CP makes it an attractive target for antiviral drug development (Dragovich et al., 1998; Patick and Potts, 1998), and a group of potent, irreversible inhibitors of 3CP has been reported (Wang et al., 1998; Matthews et al., 1999; Patick et al., 1999; Dragovich et al., 1998; Dragovich et al., 1999; Dragovich et al., 2002). Design of these compounds is based on known 3CP structure and its preferred substrate (Patick et al., 1999; Dragovich et al., 1999). These compounds display very rapid irreversible 3CP inhibition with $k_{obs}/[I]$ values up to 1,470,000 $M^{-1}\,s^{-1}$. They also display potent antiviral activity against a diverse set of rhinovirus serotypes

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doi:10.1016/j.antiviral.2003.09.007

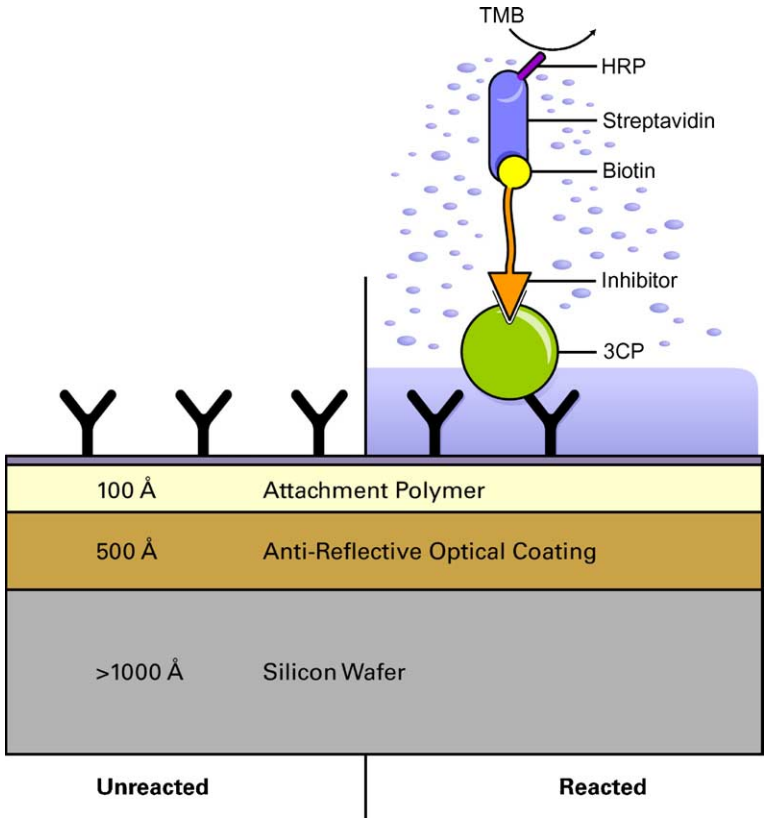



Fig. 1. Optically coated surfaces and biological thin film formation. The unreacted surface is composed of an optically coated silicon wafer plus capture antibody (anti-3CP). 3CP and compound **4** form an irreversible complex in solution. 3CP reacts with the surface antibodies, and the biotin remains accessible to the streptavidin-HRP that reacts with the precipitating TMB substrate to enhance the thin film formation.

(Patick et al., 1999; Zalman et al., 2000; Dragovich et al., 1999).

The purpose of this study was to develop a rapid point of care assay for the measurement of HRV3CP using a combination of the OIA technology and 3CP inhibitors. The OIA principle is based on detection of a physical change in optical thickness of molecular thin films resulting from specific binding events. The OIA assay consists of a silicon wafer to which optical coating and anti-HRV antibodies are attached (Fig. 1). This assay format is advantageous because the diagnostic uses a form of the actual therapeutic in detecting the pathogen. This assures the patient with a positive test result of an appropriate and effective treatment, as the pathogen is now known to be reactive with the therapeutic.

## 2. Materials and methods

### 2.1. Materials

T-polymer (T-structured aminoalkyl-polydimethylsiloxane) was purchased from United Chemical Technologies, Inc. (Bristol, PA). Rabbit sera for IgG purification was obtained from Strategic BioSolutions (Ramona, CA). HiTrap Protein G column, HiTrap NHS activated columns, Sephacryl S-100 and Blue-Sepharose fast flow columns were from Pharmacia (Piscataway, NJ). Tetramethylbenzidine (TMB) was from BioFX Laboratories (Randallstown, MD). Novex 4–20% Tris–Glycine precast gels (1.0 mm × 15 well) and Coomassie Simple Blue were from Invitrogen (Carlsbad, CA). Streptavidin–HRP was from Pierce (Rockford, IL). Minimum Essential Medium with Earle's salts (EMEM) with 10% fetal calf serum (FCS) was from Gibco BRL Products (Rockville, MD). All reagents were analytical grade purity. Additional reagents and methods pertaining to the described chemical syntheses of 3CP inhibitors have been previously described (Dragovich et al., 1998).

### 2.2. Recombinant 3CP purification

HRV2 and HRV16 3CP were purified after induction of expression by *E. coli* strain BL21/DE3 (Webber et al., 1996). The expression plasmid used was designed in-house. Cells were disrupted using a microfluidizer and cleared of cell debris using an ultracentrifuge. After a 40% ammonium sulfate precipitation, the 3CP was resuspended with 25 mM Tris–HCl, pH 7.5, 1 mM EDTA and 1 mM dithiothreitol. It was further purified using chromatography on a Blue-Sepharose fast flow column, followed by gel filtration

chromatography on a 2 l Sephacryl S-100 column. A 50 mM Tris buffer, pH 7.5, containing 1 mM EDTA and 1 mM DTT was used to dilute 3CP (10–100 nM).

### 2.3. Purification of anti-HRV16 3CP antibody

Rabbits were immunized with recombinant HRV16 3CP. A HiTrap Protein G column was used to isolate total IgG from rabbits immunized with HRV16 3CP. Immune serum was loaded on the column and equilibrated with 1× PBS buffer, pH 7.0. The bound antibody was eluted with 0.1 M glycine buffer, pH 2.7 and eluted fractions were neutralized with 1 M Tris buffer, pH 9.0 and dialyzed. The yield was 12 mg of Ab per 1 ml of serum. A specific 3CP antibody was purified using a HiTrap NHS affinity column with HRV2 3CP linked by primary amines as described by manufacturer. Total IgG was diluted 1:5 with 1× PBS, pH 7.0 and applied to the HiTrap 3CP column. Specifically bound antibodies were eluted with 0.1 M glycine buffer, pH 2.7 and the final pH was immediately adjusted to neutral using 1 M Tris buffer, pH 9.0. The eluted fraction was dialyzed against 1× PBS. The yield of affinity purified material was 0.2 mg from 1 mg of total IgG loaded on the column.

### 2.4. Thin film assay surface preparation

Silicon wafers were optically coated with 500 Å of silicon nitride by vapor deposition. The silicon nitride layer participates in the thin film interference effect. T-polymer, applied by spin coating is required for passive adsorption of antibody to the silicon surface. Affinity purified antibody (5 μg ml$^{-1}$) was coated onto the silicon surfaces in 0.1 M HEPES buffer, pH 8.0 for 40 h at 23 °C.

### 2.5. Wash solution

A wash solution containing 10 mM Na-phosphate buffer with 0.5% Proclin® 300 as a preservative, pH 7.0 was used in the assay.

### 2.6. HRV infected cell lysates

HRV serotypes of Rhinovirus were propagated in HeLa cells grown in EMEM medium with 10% fetal bovine serum (Dragovich et al., 1998; Patick et al., 1999; Zalman et al., 2000; Ostroff et al., 2001). The lysates were prepared by three cycles of freeze/thaw (−70 °C) of the cells followed by centrifugation of the lysate at 1200 rpm for 15 min and stored at −70 °C until use. They were titered to determine the $TCID_{50}$ value and diluted with $H_1$-HeLa cell lysates for testing.

### 2.7. SDS-PAGE

SDS-PAGE was performed using 4–20% Tris–Glycine precast gels. Gels were loaded with 3–5 μg of protein per lane, run for 2 h at 125 V and stained with Coomassie Simple Blue.

### 2.8. Compounds **1**–**4**

Three different biotinylated 3CP inhibitors were synthesized for use in this study (compounds **2**, **3**, and **4**; see Fig. 4). The biotin was tethered to the core inhibitor structure at a site known from previous experiments to be tolerant of extensive modification (Dragovich et al., 1998). Tether lengths were defined as the number of atoms connecting the bicyclic biotin moiety and the side-chain nitrogen atom of the $P_3$ inhibitor lysine (5, 10, and 15 atoms; compounds **2**, **3**, and **4**, respectively). All biotin-containing molecules potently and irreversibly inhibited HRV-14 3CP (Table 1). A molecule that lacks the biotin moiety (compound **1**, see Fig. 4) was prepared as a control and also displayed potent anti-3CP properties (Table 1). The 3CP inhibitors described in this study were synthesized using known procedures and chemical intermediates (Dragovich et al., 1999; Tian et al., 2001). Infrared and NMR spectroscopy data as well as elemental analysis of synthesized compounds are included below to confirm the synthesis of the desired compounds.

#### 2.8.1. Inhibitor **1**

IR (cm$^{-1}$) 3273, 1852, 1540, 1508; $^1$H NMR (DMSO-$d_6$) $\delta$ 1.16–1.33 (m, 2H), 1.21 (t, 3H, $J$ = 7.1 Hz), 1.36–1.69 (m, 6H), 1.71–1.83 (m, 1H), 1.97–2.08 (m, 1H), 2.15–2.28 (m, 1H), 2.45 (s, 3H), 2.65–2.76 (m, 2H), 2.78–3.16 (m, 4H), 4.09 (q, 2H, $J$ = 7.1 Hz), 4.34–4.53 (m, 3H), 5.46 (dd, 1H, $J$ = 15.6, 1.1 Hz), 6.56 (s, 1H), 6.71 (dd, 1H, $J$ = 15.6, 4.7 Hz), 6.97–7.06 (m, 2H), 7.18–7.26 (m, 2H), 7.83 (s, 3H), 8.22 (d, 1H, $J$ = 8.7), 8.34 (d, 1H, $J$ = 8.1 Hz), 8.55 (d, 1H, $J$ = 8.1 Hz); Anal. ($C_{31}H_{41}FN_6O_7 \cdot 1.5H_2O$) C, H, N.

#### 2.8.2. Inhibitor **2**

$^1$H NMR (DMSO-$d_6$) $\delta$ 1.15–1.85 (m, 19H), 1.98–2.10 (m, 2H), 2.16–2.30 (m, 1H), 2.47 (s, 3H), 2.78–3.17 (m, 9H), 4.07–4.18 (m, 4H), 4.27–4.56 (m, 5H), 5.45 (dd, 1H, $J$ = 15.7, 1.2 Hz), 6.57 (s, 1H), 6.72 (dd, 1H, $J$ = 15.7, 4.7 Hz), 6.99–7.08 (m, 2H), 7.20–7.27 (m, 2H), 7.59 (s, 1H), 7.72–7.78 (m, 1H), 8.20 (d, 1H, $J$ = 8.7 Hz), 8.33 (d, 1H, $J$ = 8.1 Hz), 8.51 (d, 1H, $J$ = 7.8 Hz); Anal. ($C_{41}H_{55}FN_8O_9S \cdot 1.5H_2O$) C, H, N.

Table 1
HRV-14 3CP inhibition properties of compounds **1**–**4**

| Compound no. | Number of spacer atoms | $k_{obs}$/[I] (M$^{-1}$ s$^{-1}$) |
|---|---|---|
| 1 | NA | 866000 |
| 2 | 5 | 1300000 |
| 3 | 10 | 2600000[a] |
| 4 | 15 | 840000 |

[a] Single point assay.

*2.8.3. Inhibitor **3***

$^1$H NMR ($CD_3OD$) $\delta$ 1.28 (t, 3H, $J = 7.0$ Hz), 1.32–1.98 (m, 18H), 2.15–2.29 (m, 5H), 2.44–2.56 (m, 4H), 2.69 (d, 1H, $J = 12.8$ Hz), 2.91 (dd, 1H, $J = 12.8$, 4.7 Hz), 2.76–3.08 (m, 2H), 3.12–3.32 (m, 6H), 4.17 (q, 2H, $J = 7.0$ Hz), 4.26–4.31 (m, 1H), 4.45–4.59 (m, 4H), 4.87–4.91 (m, 7H), 5.45 (dd, 1H, $J = 15.7$, 1.7 Hz), 6.46–6.49 (m, 1H), 6.70 (dd, 1H, $J = 15.7$, 5.1 Hz), 6.93–7.01 (m, 2H), 7.20–7.26 (m, 2H), 8.43 (d, 1H, $J = 6.8$); HRMS calcd for $C_{45}H_{62}FN_9O_{10}S$ [$MCs^+$] 1072.3378, found 1072.3420.

*2.8.4. Inhibitor **4***

IR ($cm^{-1}$) 3295, 1666, 1561, 1543, 1202; $^1$H NMR ($CD_3OD$) $\delta$ 1.25–1.98 (m, 19H), 1.28 (t, 3H, $J = 7.1$ Hz), 2.16–2.29 (m, 7H), 2.47–2.53 (m, 1H), 2.48 (s, 3H), 2.66–2.75 (m, 1H), 2.88–3.10 (m, 4H), 3.13–3.29 (m, 7H), 4.17 (q, 2H, $J = 7.1$ Hz), 4.26–4.36 (m, 1H), 4.44–4.62 (m, 4H), 4.84–4.97 (m, 9H), 5.46 (d, 1H, $J = 15.7$ Hz), 6.47 (s, 1H), 6.70 (dd, 1H, $J = 15.7$, 5.1 Hz), 6.92–7.04 (m, 2H), 7.19–7.30 (m, 2H), 8.40–8.49 (m, 1H); HRMS calcd for $C_{49}H_{69}FN_{10}O_{11}S$ [$MCs^+$] 1157.3906, found 1157.3948.

The inhibitors were dissolved in methanol, stored at −20 °C at a concentration of 4 mM and used in the assay at a concentration of 20 μM in 50 mM Tris buffer, pH 7.5, containing 1 mM EDTA.

*2.9. Optical immuno assay (OIA) test*

The OIA surfaces were coated with affinity purified anti-HRV3CP antibodies. All incubations were performed at room temperature. Fifteen microliters of a 20 μM solution of inhibitor was mixed with 15 μl of purified 3CP or infected lysates and incubated for 10 min to form an enzyme-inhibitor complex that was placed on the OIA surface. After 10 min of incubation, the surface was rinsed with wash solution and 30 μl of diluted streptavidin-HRP was added. After 10 min of incubation the surface was washed and 30 μl of the TMB substrate was added (Fig. 1).

Non-instrumented, qualitative results are easily determined visually with this thin film technology. Quantitative results can be obtained by measuring color contrast with a CCD camera and image processing (Ostroff et al., 1999).

## 3. Results

*3.1. Testing inhibitors with different linker length*

We tested three biotinylated inhibitors with different linker lengths for their ability to detect purified 3CP in the OIA assay (Fig. 1). The inhibitors were compound **2** (5 carbon linker), compound **3** (10 carbon linker), and compound **4** (15 carbon linker). Control experiments consisted of assays run with no inhibitor or a non-biotinylated inhibitor (compound **1**). When equimolar amounts of the three biotinylated inhibitors were compared, the strength of the assay signal was generally stronger when a longer spacer was used (Table 2) and more serotypes were detected when compound **4** was used (data not shown) indicating that spacer length was an important factor to improve the number of serotypes detected. A dose titration of compound **4** (200 nm to 20 μm) was also performed. To obtain maximum signal, an excess of biotinylated inhibitor over 3CP (1 μm) was required. No signal was obtained with negative controls (data not shown).

The three biotinylated inhibitors were also tested for their ability to detect seven serotypes of rhinovirus: 2, 14, 16, 21, 23, 39, and Hanks. Extracted HeLa cells and influenza virus were used as negative controls. The biotinylated inhibitor with the longest linker (compound **4**) was again the most effective reagent for detecting rhinovirus, and no signal was obtained using the negative controls. The qualitative level of detection using compound **4** for several different rhinoviruses in cell lysates was measured and is shown in Table 3.

*3.2. Detection of different HRV serotypes*

In another experiment, detection of 40 different rhinovirus serotypes was tested using the OIA assay and compound **4**.

Table 2
Relative (qualitative) OIA signal strength for different lysates with different inhibitors on the OIA surfaces

| Inhibitor | HRV14 | HRV2 | HRV16 | HRV21 | HRV23 | HRV39 | HANKS | HELA | FLU |
|---|---|---|---|---|---|---|---|---|---|
| **4**: 15-atom spacer | 2 | 4 | 2 | 3 | 3 | 1 | 1 | 0 | 0 |
| **3**: 10-atom spacer | 2 | 4 | 2 | 2 | 2 | 1 | 1 | 0 | 0 |
| **2**: 5-atom spacer | 0 | 3 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |

*Visual signal key*: 0, negative; 1, weak; 2, moderate; 3, strong; 4, very strong.

Table 3
Limit of detection for HRV infected cell lysates

| Lysate | HRV2 | HRV14 | HRV16 | HRV21 | HRV23 | HRV39 | HANKS |
|---|---|---|---|---|---|---|---|
| Limit of detection | $10^5$ | $10^7$ | $10^7$ | $10^5$ | $10^6$ | $10^6$ | $10^4$ |

Surface: affinity purified anti-HRV16 antibody. Detection: compound **4** + streptavidin-HRP. Last dilution detected in pfu $ml^{-1}$.

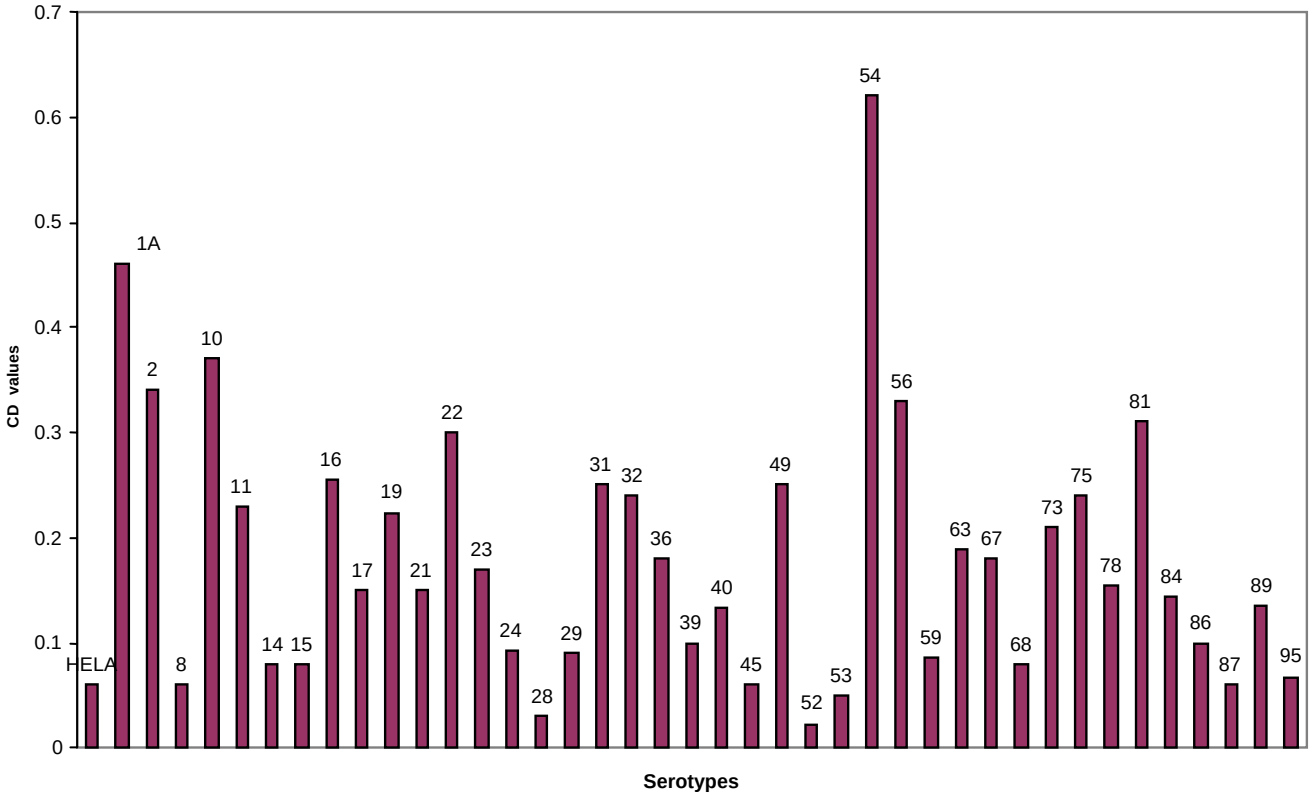



Fig. 2. HRV serotypes reactivity in rhinovirus inhibitor assay.

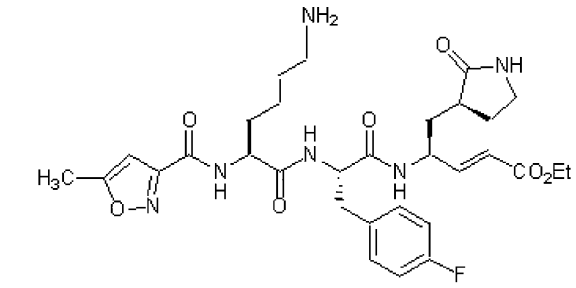

Fig. 3. Visual depiction of OIA/inhibitor assay with selected HRV serotypes.

The data are shown in Fig. 2. Thirty three of the 40 serotypes (80%) were detected with a detection limit which varied from $10^8$ to $10^4$ pfu ml$^{-1}$, depending on the strain tested. These conclusions were based on visual results with comparisons to the negative controls, and examples of such visual signals observed with several HRV serotypes are shown in Fig. 3.

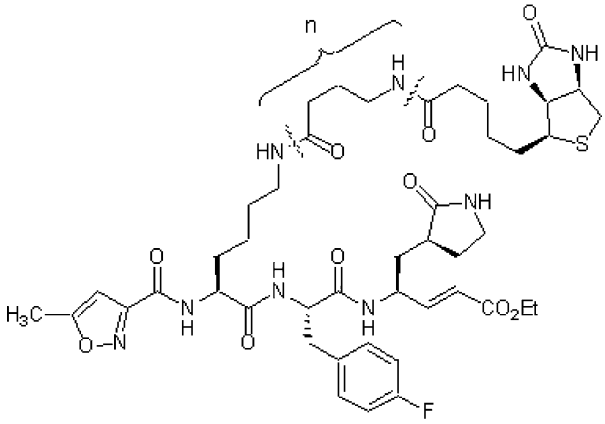
## 4. Discussion

Using the 3CP inhibitors as HRV detection reagents combines the principles of enzymology and the OIA technology to produce a valuable diagnostic tool. The HRV3C protease and the described biotinylated inhibitors form an irreversible complex during their interaction in solution. Using different spacer lengths between the inhibitor and the biotin core it is possible to create a complex where the biotin moiety is not obstructed by the active site of the 3C enzyme. The resulting complex reacts with the OIA surface antibodies in a manner which allows the biotin to remain accessible to the Streptavidin-HRP (Fig. 1). Our results also show some dependence of signal strength on spacer length. Compound **2**, with only five atoms between the inhibitor and biotin core, gives the weakest signal. Compound **3**, with a 10-atom spacer generally gives better signal, and compound **4** (Fig. 4), with 15-atom spacer generally gives the best signal. We also examined a non-biotin-containing inhibitor (compound **1**) as a control, and did not observe any signal (data not shown). The analyzed samples containing influenza virus were also negative (Table 2).

The optimal assay format involved conducting the reaction between the 3CP inhibitor and enzyme in solution prior to complexation with the OIA surface. In assays where the enzyme interacted with the surface antibodies first and then inhibitor was added, the signal was very weak (data not shown). One potential reason for this observation is that the active site of the capture antibody-bound 3C enzyme is not available to bind the inhibitor as well as it is in



**Compound 1**



| | |
|---|---|
| **Compound 2** | **n = 0** |
| **Compound 3** | **n = 1** |
| **Compound 4** | **n = 2** |

Fig. 4. Chemical structures of modified inhibitors **1**–**4**.

solution. Similarly, if the inhibitor and streptavidin were reacted first, there was no observed signal at all. Some possible explanations for this result are that the inhibitor–streptavidin complex does not fit into the active site of the surface bound enzyme or its conformation is different in solution.

One of the most important issues for the assay is the stability of the 3C enzyme. The experiments described above clearly demonstrate the possibility of using small molecule enzyme inhibitors as detection reagents in the OIA format. However, the stability of the targeted infectious agents has not been evaluated in any detail and should be a primary focus of subsequent assay development. While the overall analytical assay sensitivity, $10^4$ to $10^6$ pfu ml$^{-1}$ for the tested serotypes would appear to provide adequate sensitivity for these serotypes, the clinical sensitivity of the assay remains unresolved pending actual clinical data. Analytical sensitivity data for more serotypes would be required prior to the initiation of any clinical study. Synthesis of longer spacers between the inhibitor and biotin molecules in the future may open the opportunity to create highly sensitive detection molecules for different serotypes of rhinovirus.

## Acknowledgements

We wish to thank Drs. Diana Maul and Catherine McCall for critical reading of the manuscript and Mr. Christopher High for help in preparation of the figures.

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