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# Inhibitory effect of dibenzofuran and dibenzosuberol derivatives on rhinovirus replication in vitro; effective prevention of viral entry by dibenzosuberenone

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**Abstract**

A series of derivatives of dibenzofuran and dibenzosuberol block rhinovirus replication in vitro as judged by their ability to hinder the cytopathic effect in cells infected with HRV14 or HRV16. Both the number and the size of viral plaques were reduced effectively by treatment with these compounds in a dose-dependent fashion, thus affecting viral spread. The compound 2-hydroxy-3-dibenzofuran carboxylic acid was equally effective against HRV16 and HRV14, with $IC_{50}$ values of 25 μM in cytopathy assays. Dibenzosuberenone showed minor differences in selectivity, with $IC_{50}$ values of 10 and 30 μM for HRV16 and HRV14 cytopathy, respectively. Likewise, dibenzosuberenone effectively prevented the production of HRV16 proteins, viral RNA, and infectious virus particles when present at concentrations above 30 μM. Time-of-addition experiments show that compounds must be administered before or during the viral adsorption step in order to be effective antivirals. Dibenzosuberenone can block the adsorption of viral particles on to cells, preventing further steps in the replication cycle, but is not effective as a direct inactivating agent. These compounds likely interact with viral capsid proteins, affecting receptor interactions required for attachment and subsequent entry into cells. 

*Keywords:* Antiviral; Rhinovirus; Virus replication; Virus entry; Dibenzofuran; Dibenzosuberol; Dibenzosuberenone

## 1. Introduction

Rhinoviruses are the predominant causative agents of common colds in humans (Dick and Chesney, 1981; Gwaltney, 1992), and are also major triggers of acute asthma symptoms in susceptible patients (Pattemore et al., 1992; Johnston et al., 1996). Infection of epithelial cells of the upper airway is followed by activation of several inflammatory mechanisms including release of cytokines (Gwaltney, 1995). The search for antiviral agents has lead to the identification of numerous compounds (Carrasco, 1994), but none have shown sufficient efficacy in vivo (Arruda and Hayden, 1996).

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Rhinovirus infections begin with attachment to receptor, entry, uncoating, shutdown of host cell synthesis between 1–2 h post infection (p.i.), viral RNA and protein synthesis (2–3 h p.i.), virus assembly (4–6 h p.i.), and eventual cell lysis to release virus particles (6–10 h p.i.) (Rueckert, 1996). Capsid proteins form a canyon structure that serves to bind receptor (Smith et al., 1986). Numerous compounds have been shown to bind in a nearby hydrophobic pocket, inducing conformational changes in the viral capsid (Rosenwirth et al., 1995; Wang et al., 1998). They inhibit virus attachment to receptor, prevent uncoating or internalization of the virus and in doing so, display antiviral properties (Carrasco, 1994). Compounds such as these include: dichloroflavans (Tisdale and Selway, 1983), WIN compounds (McKinlay, 1985; Diana et al., 1993), phenoxy pyridine carbonitriles (Kenny et al., 1985), and pyridazinamine analogs (Barrow et al., 1990). The pyridazinamine analog pirodavir inhibits uncoating (Andries et al., 1988), but despite a significant antiviral effect in vivo, no clinical benefit occurred against natural infections (Hayden et al., 1995).

The need to identify other chemical entities with efficacy against rhinovirus, still exists. We have identified a series of structurally related compounds that inhibit rhinovirus replication by interfering with an early event in the HRV14 and HRV16 infection cycles. Results are consistent with capsid binding and blocking of virus adsorption. The existence of numerous compounds closely related to dibenzofuran and dibenzosuberenone served as a good platform for our initial structure-related antiviral screens. Further structural modifications of these chemical leads could result in agents with broad-spectrum antirhinovirus activities and enhanced potencies.

## 2. Materials and methods

### 2.1. Cell lines and viruses

HeLa-H1 cells were obtained from ATCC and maintained in spinner cultures using DMEM supplemented with 10% fetal calf serum and 0.1% pluronic F-68 (from Gibco/BRL; added to prevent cell clumping). For infection assays, cells were seeded 24 h prior to viral infection unless otherwise noted. Stocks of HRV16 and 14 were graciously provided by Dr Wai-Ming Lee (University of Wisconsin). Virus stocks were prepared in spinner cultures of HeLa-H1 cells grown at 35°C for 8–10 h.

### 2.2. Chemicals and other reagents

All chemicals assayed for antiviral activity were purchased from Aldrich. Compounds were initially dissolved in dimethylsulfoxide (DMSO) to 40 mM and diluted at least 200-fold in cell culture media for assays.

### 2.3. Cytotoxicity assay

The cytotoxicity of compounds was determined using the WST-1 reagent from Boehringer Mannheim, following the manufacturer's instructions. Cells were grown in the presence of drug under the same conditions used for viral infectivity assays.

### 2.4. Cytopathy effect (cpe) inhibition assay

The effect of compounds on virus-induced cell death was determined using a protocol described previously (Merluzzi et al., 1989). Monolayers of HeLa-H1 cells in 96 well plates were infected with HRV14 or 16 at 2–5 M.O.I. (multiplicity of infection) and stained with crystal violet after 48 h. For quantitation, the dye was solubilized in 50% methanol/5% SDS and the absorbance was measured at 560 nm. Inhibitors were added to cell cultures 1 h prior to virus adsorption and were present thereafter. Serum-free DMEM supplemented with $1\times$ non-essential amino acids (NEAA) for all assay steps.

### 2.5. Plaque assay

A plaque formation protocol described previously (Sherry and Rueckert, 1985) was followed with minor changes. HeLa-H1 cells from spinner

flasks were seeded at $1.5 \times 10^6$ cells/60 mm dish. At 24 h, cells were infected with virus diluted in DMEM + 1 × NEAA (1 h adsorption at room temperature). Viral inoculum was retained, and cells were overlayed with Noble agar (Difco Laboratories) and liquid media (Sherry and Rueckert, 1985), for a 48 h incubation at 35°C. Cells were fixed with formaldehyde and stained with crystal violet for plaque counting (Merluzzi et al., 1989). For direct effect of drug on plaque formation, cells were pre-treated with compounds for 1 h prior to virus adsorption and drug was present at all times thereafter. All samples were plated in duplicate.

### 2.6. Virus yield reduction assays

HeLa-H1 cells were treated with drug (1 h pre-treatment) and infected with HRV16 for 1 h at room temperature, followed by incubation at 35°C for 24 h in the presence of drug. Harvested cells by washing once with PBS and collecting in PBS buffer ($Ca^{2+}/Mg^{2+}$ free with 5 mM EDTA) with a scraper. The cells were subjected to one cycle of freeze-thaw followed by centrifugation to extract the virus. For plaque assays, this cell lysate was serially diluted with DMEM + 1 × NEAA and applied to fresh HeLa-H1 cells.

### 2.7. SDS–PAGE and Western blots

The NuPage gel system (Novex, CA) was used to separate proteins under reducing conditions, followed by transfer to nitrocellulose. Primary polyclonal rabbit antibodies specific for the HRV16 3C or 2A proteases (generated to keyhole limpet hemocyanin-conjugated peptides) were used. The antibody to HRV16 3C was raised against two synthetic oligopeptides comprising the N-terminal 11 amino acids and 18 internal amino acids of the protein. The antibody to HRV16 2A was raised against a synthetic oligopeptide comprising the last 19 residues of the protein. Both antibodies were purified using antigen-conjugated columns (prepared using the Sulfolink kit by Pierce Co.). Immunoreactive bands were visualized with HRP-conjugated secondary antibodies and chemiluminescent reagent (ECL, by Pierce Co.).

### 2.8. RT-PCR

To compare the amount of viral RNA present in cells infected in the presence or absence of drug, cell lysates were obtained for use in RT/PCR. RNA was extracted using the GlassMAX RNA microisolation system (Gibco/BRL), resuspended in DEPC-treated water, and digested 15 min at 22°C with DNase I digestion to remove any genomic DNA. Dnase I was inactivated with 2.5 mM EDTA for 15 min at 65°C. Reverse transcriptase reactions were carried out using the DNase I digested RNA samples and 3 μM concentration of the downstream oligonucleotide primer OL27 (to be used for subsequent PCR). After heating for 1 min at 90°C, placed reactions on ice and supplemented with the following: 1 × buffer (supplied by enzyme vendor), 10 mM DTT, 0.25 mM dNTPs mix, 5 U RNasin (Gibco/BRL), and 100 U superscript II reverse transcriptase (Gibco/BRL). The samples were subjected to 10 min at 22°C, followed by 45 min at 42°C, 5 min at 45°C, 5 min at 50°C, and 5 min at 95°C. The subsequent PCR reaction was carried out using two primers designated OL26 (upstream, 5′GCACTTCTGTTTCCCC3′) and OL27 (downstream 5′CGGACACCCAAAGTAG3′) that are directed to a highly conserved 5′ non-coding region of the viral genome (Subauste et al., 1995). PCR was conducted in a total volume of 100 μl consisting of: 1 × PCR buffer (from enzyme vendor), 0.25 mM dNTPs mix, 1 μM of each primer, and one AmpliWax PCR Gem (Perkin Elmer, CA). At this point the reaction was heated briefly to 70°C and cooled to room temperature. The reaction was supplemented with 2.5 mM $MgCl_2$ and 5 U of Taq DNA polymerase (Qiagen, CA) for the following PCR regime: one denaturing cycle at 94°C for 2 min, 30 cycles at 94°C for 1 min, 55°C for 2 min, and 72°C for 4 min, followed by a final extension cycle at 72°C for 5 min. PCR products were visualized by electrophoresis on a 6% TBE/acrylamide gels and ethidium bromide staining and quantified by densitometry (Kodak Documentation System 120).

## 3. Results

### 3.1. Reduction of viral cytopathic effect

We selected HRV14 and 16 because they belong to the 'major subclass' of rhinoviruses (Staunton et al., 1989). HRV16 is a good model for clinical studies because it effectively causes cold symptoms in exposed human volunteers (Gwaltney, 1992). HRV14 has been more thoroughly studied at the molecular level, providing valuable insights into viral replication (Rueckert, 1996).

Generally, the compounds to be tested were added to HeLa-H1 cell 1 h prior to addition of virus. They were also present during the 1-h virus adsorption step, and during the remaining 48-h incubation period. The degree of cell death caused by virus-induced cytopathy was quantified by crystal violet stain of remaining cells. Antiviral activity was measured as the concentration resulting in 50% inhibition of cytopathic effect ($IC_{50}$ values) as shown in Table 1. A significant difference in efficacy for closely related compounds was noted. Dibenzofuran (compound 105) was ineffective, while the related compounds 100 and 2096 were clearly inhibitory for both HRV14 and HRV16. Likewise, dibenzosuberol (compound 108) was ineffective against either virus, but the derivative compounds 102 and 104 were inhibitory against both viruses. Cytotoxicity measurements indicated that $LD_{50}$ values (lethal dose 50% for uninfected HeLa-H1 cells) were in the range of 200 μM for these compounds and inhibition of viral cpe was noted at compound concentrations well below the $LD_{50}$ value. Cell morphology was observed also by light microscopy following nuclear and cytoplasmic staining (data not shown). Cell survival was dose-dependent, and the cells appear uninfected in good agreement with the values shown in Table 1.

### 3.2. Direct inhibition of viral infection

Compound 2096, which had shown efficacy in blocking cytopathic effect was tested directly in a plaque assay against HRV14. The compound was again allowed to pre-incubate with HeLa-H1 monolayers 1 h prior to virus infection and was present thereafter. A dose-dependent reduction in initial infection and subsequent spread was observed, since both plaque number and size were reduced markedly (Fig. 1).

### 3.3. Reduction in viral yield

The effect of various compounds on the number of infectious virions produced by HRV16 infected cells during a 24-h period was measured. Plaque assays were performed on serially diluted cell lysates. As shown in Table 2, we detected a significant dose-dependent effect on plaque forming units. Of the compounds tested, compound 104 was found to be the most potent, reducing viral yield by almost 300-fold when present at 50 μM concentration.

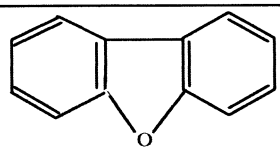
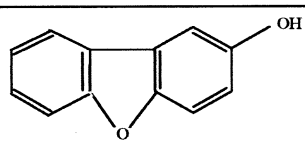
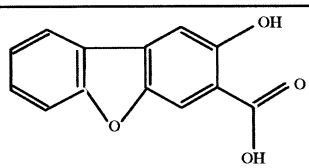
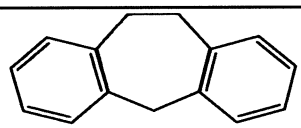
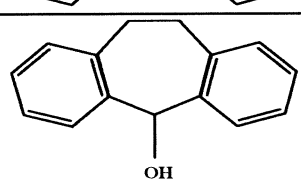
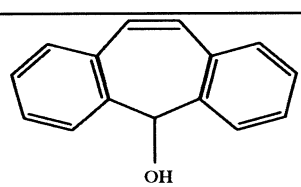
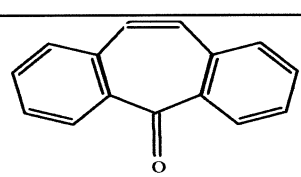
### 3.4. Effect on viral protein production

Hela-H1 cells infected with HRV16 were grown in the absence or presence of compound 104 and analyzed at various times post infection. Cell lysates were probed by Western blot using antibodies raised against the viral 3C protease. Fig. 2 shows the gradual increase in the amount of 3ABC polyprotein precursor and the 3C and 3CD proteins as the infection proceeds in the absence of drug (DMSO only samples). In contrast, the cells grown in the presence of 50 μM of compound 104 contained almost no viral proteins even at 24 h post infection, suggesting an effective suppression of viral protein production (Fig. 2). Similar results were observed using antibodies reactive to HRV16 2A protease and its precursors (data not shown).

### 3.5. Time-of-addition experiment

A time course of drug addition was performed on HRV16 infected cells using compound 104 to determine how early in the infection cycle this drug intervenes. The compound was added 1 h prior to infection, at the same time as the virus, or at 1 h intervals post infection. The infected cells were allowed to grow for 24 h before harvest and the cell lysate was analyzed for infectious virus content. Samples were compared to an infected

Table 1
Antiviral activity against HRV14 and HRV16 and cellular toxicity

| Compound[a] | Structure | $IC_{50}$[b], μM (HRV16) | $IC_{50}$[b], μM (HRV14) | $LD_{50}$[c], μM | chemical name |
|---|---|---|---|---|---|
| 105 |  | >200 | >200 | >200 | dibenzofuran |
| 100 |  | 30 | 70 | 100 | 2-hydroxy-3-dibenzofuran |
| 2096 |  | 25 | 25 | 150 | 2-hydroxy-3-dibenzofuran carboxylic acid |
| 108 |  | >200 | >200 | 150 | dibenzosuberol |
| 107 |  | 200 | 50 | 150 | dibenzosuberane |
| 102 |  | 50 | 15 | 150 | dibenzosuberenol |
| 104 |  | 10 | 30 | >200 | dibenzosuberenone |

[a] Drugs were tested at a concentration range of 5–200 μM in 0.1% DMSO.
[b] Concentration of drug that reduced the viral cytopathic effect by 50%.
[c] Cytotoxicity of compounds against uninfected cells (lethal dose 50%).

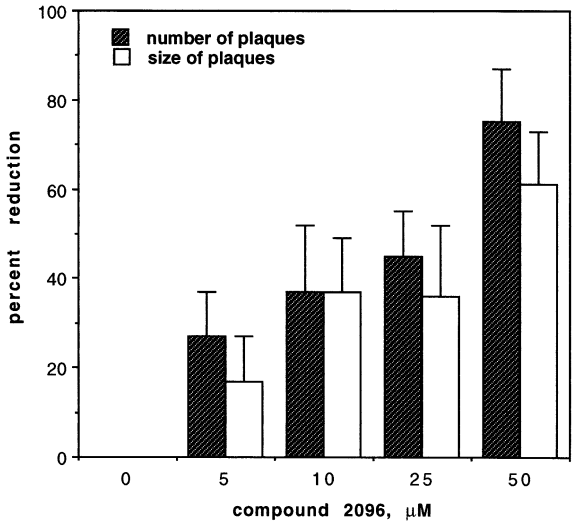



Fig. 1. Reduction in initial infection and subsequent spread of HRV14 virus in HeLa-H1 infected cells. The number and size of viral plaques was determined after a 48-h period in the presence of varying concentrations of compound 2096. Results represent the average values for plaque assays conducted in triplicate.

Table 2
Direct inhibition of viral infection by representative compounds [a]

| Compound | Concentration (μM) | PFU/ml | Fold change |
|---|---|---|---|
| DMSO | 0.01% | $2\times10^6$ | 0 |
| 100 | 20 | $4\times10^5$ | 5 |
| 100 | 50 | $1.5\times10^5$ | 13 |
| 2096 | 20 | $3\times10^5$ | 7 |
| 2096 | 50 | $1\times10^5$ | 20 |
| 2096 | 100 | $1\times10^4$ | 200 |
| 102 | 20 | $1\times10^6$ | 2 |
| 102 | 50 | $4\times10^5$ | 5 |
| 104 | 20 | $2\times10^5$ | 10 |
| 104 | 50 | $7\times10^3$ | 285 |

[a] Reduction in infectious virus yield was determined by plaque assay using a serial dilution of cell lysates from HRV16 infected HeLa-H1 cells. Cells were grown in the presence of drug for 24 h before harvesting. Cell lysates were diluted serially and applied to fresh HeLa-H1 cell monolayers for a 48-h plaque assay using agar overlays. The results represent average values from plaque assays conducted in triplicate in which plaques could be unambiguously counted.

culture that received only DMSO. The results shown in Fig. 3 indicate that the drug is mosteffective when given either at the time of infection, or 1 h prior to infection. If added 1 h p.i., less than 30% inhibition is observed, and by 5 h p.i. no inhibition occurs. This again suggests thatthe compound works during an early stage of the viral infection cycle, probably on adsorption or uncoating. The partial inhibition seen between 1 and 4 h p.i. suggests that the infection was not fully synchronized and that some viruses were slower in initiating infection.

### 3.6. Block in viral adsorption

We wanted to probe the interaction of our most potent compound with virus, looking for interference in virus adsorption to cells, or direct viral inactivation in the absence of cells (Fig. 4). To test for direct effect on virus, 50 μM compound 104 was incubated for 1 h at room temperature with HRV16 alone. These samples lacking cells were diluted 20-fold to reduce drug concentration below the efficacious dose, and used to infect cells in the standard 1 h adsorption and infection protocol. To test for inhibition during adsorption, 50 μM of compound 104 was added to a suspension of HRV16 and HeLa-1 cells. The cells were collected by centrifugation, resuspended in serum-free media without any drug and plated. In the case of samples treated during adsorption, a dramatic block in the production of viral proteins 3C and 2A was observed (Fig. 4). To confirm that viral protein detection correlates with virus production, we isolated RNA from cell extracts and used HRV16 specific primers (Subauste et al., 1995) for the detection of viral genomic RNA via RT-PCR. The results, as shown in Fig. 4b, closely parallel those obtained for viral protein detection, thus confirming the ability of compound 104 to prevent virus adsorption to HeLa-H1 cells. In contrast, virus samples pre-treated with drug prior to adsorption were not affected suggesting that compound 104 do not directly inactivate the virus.

## 4. Discussion

In this study we sought to investigate and characterize the mode of action of several anti-picor-

navirus agents. Initial screen of several derivatives of dibenzofuran and dibenzosuberol measured their inhibitory effect on virus growth at increasing doses. Viral inhibition was found to be dose dependent for a number of compounds. We also observed a direct effect on virus yield and reduction in both number and size of virus plaques. Note that a series of bis-basic derivatives of dibenzofuran had been shown previously to inhibit the replication of another picornavirus, encephalomyocarditis virus (Albrecht et al., 1977).

Titration by plaque assay showed compound 104 (dibenzosuberenone) was the most potent inhibitor, and was equally effective against HRV14 and 16. The decrease in viral titer correlated also with the elimination of viral protein production and viral genomic RNA. As a means of determining how early in the infection cycle dibenzosuberenone intervenes, we conducted a time course of drug addition on HRV16 infected cells. This compound proved most effective when added 1 h prior to or concurrent with the virus suggesting interference with virus adsorption by either inactivating virus or preventing binding to cellular receptor. Testing the effects of pre-treatment of virus suggests that direct viral inactivation is not the mechanism of action.

However, treatment during the adsorption phase was very efficacious, suggesting that the drug must be present during the virus–cell attachment period and not essential thereafter. This is consistent with a compound which binds to the capsid and blocks adsorption (Pevear et al., 1989; Carrasco, 1994). Because the hydrophobic pocket-formed by the capsid proteins of icosahedral viruses tend to be highly conserved (Rossmann, 1988), compounds that bind in this region could be effective against numerous serotypes. Having found simple tricyclic compounds with anti-rhinoviral properties offers new scaffolds for further drug development, ideally selective to rhinovirus, but also potentially effective against numerous serotypes.

It is worth noting that symptoms of rhinovirus colds may not result from direct virus-induced tissue damage, but rather from release of inflammatory mediators (Gwaltney, 1995). HRV infections enhance allergic airway inflammation by yet unknown mechanisms, but local cytokine release

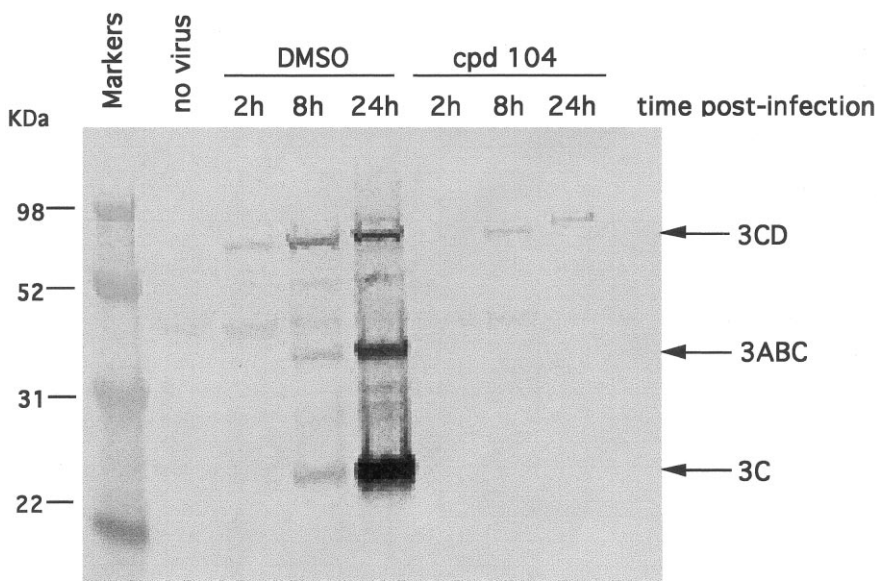



Fig. 2. Effect of compound 104 on viral protein yield. HeLa-H1 cells infected with HRV16 were grown in the presence of 50 μM compound 104 or DMSO alone for 2, 8 and 24 h. Cell lysates were run on SDS–PAGE and immunoblotted using a polyclonal antibody raised against the 3C protease domain. As indicated three reactive polypeptides were observed corresponding to precursors 3ABC, 3CD and mature 3C proteins.

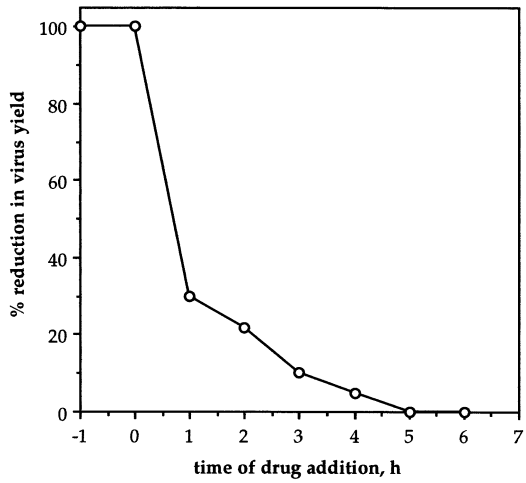



Fig. 3. Inhibitory effects of compound 104 added at hourly intervals during the infection process. To determine the stage of antiviral intervention, HeLaH1 cells infected with HRV16 (M.O.I. ~5) were incubated in medium containing either 50 μM of compound 104 or DMSO. The additive was included at the times noted and retained in the media until cells were harvested 24 h p.i. Cell lysates were prepared and virus yield was determined by cpe assay of serially diluted samples. The data are presented as percent inhibition as compared with cpe measured for samples that received no drug.

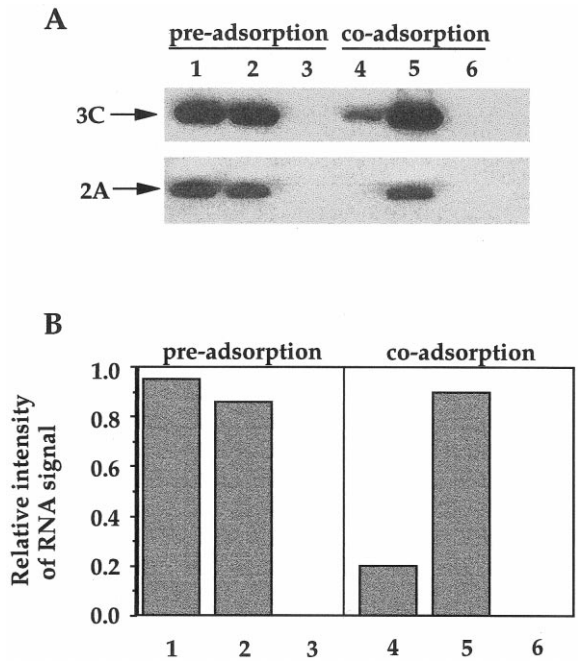



Fig. 4. Effect of compound 104 on initial viral adsorption. For pre-adsorption experiment, HRV16 was incubated for 1 h with 50 μM of compound 104. The samples was diluted 20-fold in serum-free media and added to cells for infection. For co-adsorption experiment, HeLa-H1 cells were infected with HRV16 in the presence or absence of 50 μM of compound 104 (for the adsorption period only). In both cases, total cell lysates were prepared 24 h p.i. and Western blots carried out using either antibodies against HRV16 3C or 2A proteins. Arrows indicate the mature viral proteins identified in the samples (panel A). The same cellular extracts were used to isolate viral RNA for quantitation by RT-PCR. The DNA fragments observed were the expected size of 380 bp (Subauste et al., 1995), and were quantified by densitometry (panel B). Samples are as follows: (1) virus and drug; (2) virus and vehicle; (3) no virus or drug.

and eosinophil recruitment are potential causes. It has been suggested that beneficial cold therapy will require combinations of antiviral and anti-inflammatory agents (Arruda and Hayden, 1996), especially in the case of asthmatic subjects. Some of the compounds analyzed in this study, such as dibenzosuberone derivatives (Smerberck and Pittz, 1986) and dibenzofuran (Summers and Moore, 1988) have anti-inflammatory properties. The potential therapeutic value of compounds with both anti-inflammatory and antiviral activities is worth considering in the treatment of the common cold.

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