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Small-Molecule Screening Using a Whole-Cell Viral Replication Reporter Gene Assay Identifies 2-{[2-(Benzoylamino)Benzoyl]Amino}-Benzoic Acid as a Novel Antiadenoviral Compound▽

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Adenovirus infections are widespread in society and are occasionally associated with severe, but rarely with life-threatening, disease in otherwise healthy individuals. In contrast, adenovirus infections present a real threat to immunocompromised individuals and can result in disseminated and fatal disease. The number of patients undergoing immunosuppressive therapy for solid organ or hematopoietic stem cell transplantation is steadily increasing, as is the number of AIDS patients, and this makes the problem of adenovirus infections even more urgent to solve. There is no formally approved treatment of adenovirus infections today, and existing antiviral agents evaluated for their antiadenoviral effect give inconsistent results. We have developed a whole cell-based assay for high-throughput screening of potential antiadenoviral compounds. The assay is unique in that it is based on a replication-competent adenovirus type 11p green fluorescent protein (GFP)-expressing vector (RCAd11pGFP). This allows measurement of fluorescence changes as a direct result of RCAd11pGFP genome expression. Using this assay, we have screened 9,800 commercially available small organic compounds. Initially, we observed approximately 400 compounds that inhibited adenovirus expression *in vitro* by ≥80%, but only 24 were later confirmed as dose-dependent inhibitors of adenovirus. One compound in particular, 2-{[2-(benzoylamino)benzoyl]amino}-benzoic acid, turned out to be a potent inhibitor of adenovirus replication.

Human adenoviruses (Ads) are very common pathogens and comprise at least 51 different serotypes; together, these form six different species, A to F. Ads are associated with a wide variety of clinical symptoms in humans, such as upper respiratory illness, acute respiratory disease, gastroenteritis, hemorrhagic cystitis, and even keratoconjunctivitis (1, 8, 39, 40). These infections can result in severe disease, although an Ad infection is most commonly self-limited in otherwise healthy individuals. The problem is much more pronounced in immunocompromised individuals. This group is steadily growing as a result of increasing numbers of AIDS patients and patients undergoing immunosuppressive therapy for solid organ or hematopoietic stem cell transplantation and also because of the increased survival times of these patients. Immunocompromised individuals are at high risk of developing disseminated disease and multiple organ failure, and an Ad infection can become a serious life-threatening disease (16, 20, 21). In immunocompromised children, Ads are an important cause of disease, and case fatality rates of above 50% have been reported (16). In pediatric bone marrow transplant (BMT) recipients the incidence of Ad infection is substantially higher than in adult BMT recipients (4).

A number of different Ads have been isolated from immunocompromised patients, most frequently from species A, B, or C (16, 22, 29). Species B serotypes are predominantly associated with renal syndromes, and species C serotypes are usually associated with hepatitis. In recent years, infections with Ad serotype 31 (species A) have been increasingly reported, and they often occur in patients with infections involving multiple Ad serotypes, occasionally with a lethal outcome (16, 23, 26).

There are no approved specific antiviral compounds for treatment of Ad infections available today. Drugs that have been used in clinical settings or in animal models, such as ribavirin, cidofovir, and ganciclovir, have yielded varied results; both successes and failures have been reported. Cidofovir appears to be the most promising antiadenoviral agent of those currently used (5, 12, 17, 32, 33).

Screening of large compound collections with purified protein or whole-cell-based assays, i.e., high-throughput screening, is a common method to identify biologically active compounds. Cell-based approaches are commonly more labor-intensive but have the benefit of a wider screening without the limitation of having a preconceived idea of the mechanism of action. We have developed a unique whole-cell reporter gene assay based on a green fluorescent protein (GFP)-expressing replication-competent Ad vector (35). The assay can identify compounds that directly or indirectly affect adenoviral protein expression. This assay was used to screen approximately 9,800 compounds, resulting in a number of compounds that have an inhibitory effect on Ads without killing the host cells. The inhibitory effect was ascertained at four different stages of the viral replication cycle. Here, we describe the screening method and report on a

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novel inhibitor of Ad replication that is effective on Ad types representing the six species of human Ads.

MATERIALS AND METHODS

Viruses and vector. The RCAd11pGFP vector used in the present study is a replication-competent Ad11 strain carrying a cytomegalovirus-GFP-simian virus 40 insertion in the E1 region of the Ad11p genome (35). The Ads used here were Ad5 (strain F2853-5b), Ad11p (p = prototype, strain Slobitski), Ad4 (strain RI-67), Ad31 (strain 1315/63), Ad37 (strain 1477), and Ad41 (strain Tak). The viruses were propagated in A549 cells and purified on a discontinuous CsCl gradient as described previously (27). The virion band was collected and density was measured on a refractometer. Virions were desalted on a NAP-10 column (GE Healthcare, Buckinghamshire, United Kingdom) and eluted with 1.5 ml of 10 mM phosphate-buffered saline (PBS). The virion concentration was determined by spectrophotometry; 1 optical density unit (i.e., the optical density at 260 nm [OD_{260}] − OD_{330}) corresponds to 280 μg of virions or 10^{12} virus particles/ml. The identity of the adenovirus types was assessed according to their DNA restriction patterns (1).

Cell lines. A549 cells (oat cell carcinoma from the human lung; alveolar basal epithelial cells) were grown in Dulbecco modified Eagle medium (DMEM; Sigma-Aldrich, St. Louis, MO) containing 0.75 g of $NaHCO_3$/liter, 20 mM HEPES (EuroClone, Milan, Italy), penicillin G (100 IU/ml), and streptomycin sulfate (100 μg/ml) combined (1× PEST; Gibco, Carlsbad, CA), and 5% fetal bovine serum (FBS; Gibco) at 37°C. K562 is a nonadherent human erythroleukemia cell line. FSU (Foreskin Umeå) is a diploid fibroblast cell line. K562, and FSU cells were cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 0.75 g of $NaHCO_3$/liter, 20 mM HEPES (EuroClone), 1× PEST (Gibco), and 5% FBS (Gibco) at 37°C.

Compounds. The compound collection screened was purchased from ChemBridge (San Diego, CA) and consisted of 9,800 low-molecular-weight organic compounds. The compounds were dissolved in dimethyl sulfoxide (DMSO) in 5 mM stock solutions and stored in 96-well plates sealed with heat-sealing films at room temperature in the dark in a controlled dry atmosphere. Compounds were analyzed by combined liquid chromatography-mass spectrometry (LC-MS) using a Waters HPLC system equipped with an XTerra MS C_{18} 5-μm, 4.6-mm-by-50-mm column, and an H_2O-acetonitrile-formic acid eluent system using UV analysis was carried out at 212 nm and mass spectra were recorded by detecting negative (ES^-) molecular ions with an electrospray Waters Micromass ZG 2000 instrument. The same LC-MS system was also used for purification with a preparative XTerra Prep MS C_{18} 5-μm, 19-mm-by-50-mm column and an H_2O-acetonitrile eluent system. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded in DMSO-d_6 (with residual DMSO-d_5 [δ_H = 2.50 ppm] and DMSO-d_6 [δ_C = 39.51 ppm] as internal standards) by using a Bruker DRX-400 spectrometer. The data for compound A01 data were in agreement with those published previously (25).

The analytical data for compound A02 were as follows: for ^1H NMR (400 MHz, DMSO-d_6), δ = 7.25 (t, J = 7.4 Hz, 1H), 7.37 (t, J = 7.4 Hz, 1H), 7.60 to 7.71 (m, 5H), 7.97 to 8.01 (m, 3H), 8.07 (dd, J = 1.4 Hz, 7.8 Hz, 1H), 8.53 (d, J = 8.1 Hz, 1H), 8.60 (d, J = 8.3 Hz, 1 H), 11.87 (s, 1H), 12.75 (br s, 1H), and 13.80 (br s, 1H); for ^{13}C (100 MHz, DMSO-d_6), δ = 117.8, 120.8, 121.9, 123.0, 123.5, 123.8, 127.1, 127.9, 128.9, 131.2, 132.0, 132.7, 134.1, 134.5, 139.0, 140.3, 164.8, 167.0, and 169.8; for LC-MS (*m*/*z*), $[M\text{-}H^+]^-$ calculated for $[C_{21}H_{15}N_2O_4]$, 359.10; found, 359.48.

The analytical data for compound A03 were as follows: for ^1H NMR (400 MHz, DMSO-d_6), δ = 7.21 (t, J = 7.5 Hz, 1H), 7.34 to 7.43 (m, 2H), 7.50 to 7.54 (m, 2H), 7.59 to 7.62 (m, 2H), 7.65 to 7.71 (m, 2H), 7.91 to 7.99 (m, 4H), 8.05 (d, J = 7.7 Hz, 1H), 8.29 (d, J = 8.1 Hz, 1H), 8.49 (d, J = 8.2 Hz, 1H), 8.57 (d, J = 8.2 Hz, 1H), 11.66 (s, 1H), 11.76 (s, 1H), 12.62 (br s, 1H), and 13.80 (br s, 1H); and for LC-MS (*m*/*z*), $[M\text{-}H^+]^-$ calculated for $[C_{28}H_{20}N_3O_5]$, 478.14; found, 478.45.

Screening for inhibition of viral replication and toxicity. The screening was performed at the Umeå Small Molecule Screening Facility currently incorporated at the screening platform in Laboratories for Chemical Biology Umeå. A total of 50 μl of RPMI without phenol red (Sigma-Aldrich) supplemented with 0.75 g of $NaHCO_3$/liter, 20 mM HEPES (EuroClone), 1× PEST (Gibco), and 5% FBS (Gibco) was added to each well in a 96-well plate (Multidrop; Thermo Scientific, Waltham, MA). K562 cells (50,000) suspended in 25 μl of RPMI without phenol red, supplemented exactly as described above, were added to the well. RCAd11pGFP vector was added at a concentration of 1 pg per cell in a volume of 25 μl. Then, 1 μl of compound stock solution (5 mM in DMSO) was added with a Robbins Hydra 96 to the wells of the screening plate to give a final compound concentration of 50 μM. Six negative-control wells containing 50,000 K562 cells in 100 μl of RPMI without phenol red and with 0.75 g of $NaHCO_3$/liter, 20 mM HEPES, 1× PEST, 5% FBS, and 1 μl of DMSO were included on each plate. In addition, six positive-control wells containing 50,000 K562 cells in 75 μl of RPMI without phenol red and with 0.75 g of $NaHCO_3$/liter, 20 mM HEPES, 1× PEST, 5% FBS, and 1 μl of DMSO, plus 1 pg of RCAd11pGFP vector/cell in a volume of 25 μl, were included. The plates were incubated at 37°C in an atmosphere of 5% CO_2 for 24 h. GFP expression was assessed as the fluorescence intensity at 485 nm (Wallac 1420 multilabel counter, Perkin-Elmer). After the measurement of fluorescence was completed, the cellular toxicity of the compounds screened was assessed by using an MTT-based in vitro toxicology assay kit (Sigma-Aldrich). This method is based on the principle of conversion by mitochondrial dehydrogenase of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to an insoluble, colored formazan derivative that is then solubilized in acidic isopropanol (6, 9, 31). A total of 10 μl of reconstituted MTT was added per well, and the plate was incubated at 37°C in 5% CO_2 for 2 h. The resulting formazan crystals were dissolved by adding 100 μl of MTT solubilization solution (isopropanol, HCl, and Triton X-100) and shaking the plate for 3 min. The intensity of the dye was measured by absorbance at 570 nm (Wallac 1420 multilabel counter; Perkin-Elmer).

Autofluorescence. Compounds were tested for autofluorescence by measuring the fluorescence emitted from cells and compound when no RCAd11pGFP vector was present. Autofluorescent compounds were not considered for further analysis.

Dose-response analysis. Compounds that met the selection criteria of at least 80% reduction in fluorescence and less than 50% dead cells were further analyzed in a dose-response manner to confirm the hit. The 5 mM compound stocks in DMSO were serially diluted 1:1 in RPMI (Sigma-Aldrich) in 6 steps from 25 μM to 0.78 μM, and assayed in triplicate as previously described for the screening procedure. Toxicity was also assayed in triplicate samples, as described below. The final concentration of DMSO in all assays was <1%.

Postscreening toxicity tests. (i) XTT. The toxic effect of the compounds on cells was evaluated with an XTT-based in vitro toxicology assay kit (Sigma-Aldrich). This method is based on the principle of conversion by mitochondrial dehydrogenase of 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide (XTT) to a water-soluble formazan derivative. Approximately 50,000 A549 cells were seeded in 96-well plates (Nunc, Roskilde, Denmark) on the day before addition of compounds. The next day, the growth medium was removed, and compound was added to the cells in 100 μl of DMEM (Sigma-Aldrich) with 0.75 g of $NaHCO_3$/liter, 20 mM HEPES, 1× PEST, and 1% FBS. Then, 20 μl of XTT was added per well, and the plate was incubated at 37°C in 5% CO_2 for 2 h. The intensity of the formazan dye was measured spectrophotometrically at a wavelength of 450 nm (34, 36, 41).

(ii) Propidium iodide. Toxicity of the compounds was also assessed by fluorescence-activated cell sorting (FACS) analysis of propidium iodide intercalation of DNA in dead cells. Approximately 200,000 A549 cells were seeded in 12-well plates (Nunc) the day before the addition of compounds. The next day, the growth medium was removed, and compound was added to the cells in DMEM with 0.75 g of $NaHCO_3$/liter, 20 mM HEPES, 1× PEST, and 1% FBS. For the experiments with K562 cells, 200,000 cells in RPMI 1640 with 0.75 g of $NaHCO_3$/liter, 20 mM HEPES, 1× PEST, and 5% FBS were added to 12-well plates just before addition of the compounds. Compounds were added in 5 and 15 μM concentrations. The final concentration of DMSO was <1% in all samples. The plate was incubated at 37°C in 5% CO_2 for 24 h. The cells were harvested, washed, and resuspended in PBS; then, 1 μg of propidium iodide was added to each sample. The cells were analyzed in a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ) using CellQuest software.

FACScan flow cytometry. Approximately 2×10^5 A549 cells were seeded in 12-well plates (Nunc) the day before infection. On the day of infection, the cells in one well were counted to establish the amount of virions to be added. The growth medium was removed, and compound and virus were added simultaneously to the cells in 700 μl of DMEM with 0.75 g of $NaHCO_3$/liter, 20 mM HEPES, 1× PEST, and 1% FBS. For the experiments with K562 cells, 200,000 cells in RPMI 1640 with 0.75 g of $NaHCO_3$/liter, 20 mM HEPES, 1× PEST, and 5% FBS were added to 12-well plates just before infection. Compounds were added in 5 and 15 μM concentrations. The final concentration of DMSO was <1% in all samples. Due to differences in the efficiency of infection, 1 pg of Ad5 or 0.5 pg of Ad11p virions was added per cell. The plate was incubated at 37°C in 5% CO_2 for 24 h. The cells were harvested, washed in PBS, and fixed in 2% paraformaldehyde for 30 min at room temperature. They were then washed in PBS and incubated in PBS containing 2% bovine serum albumin (BSA) and 0.1% saponin (PBS-BSA) for 30 min at room temperature. Thereafter, the cells were incubated for 1 h at room temperature with a mouse monoclonal antibody directed against the Ad hexon protein (MAb 8052; Chemicon International,

Millipore, Billerica, MA) diluted 1:200 (5 μg/ml) in PBS-BSA. After one wash in PBS-BSA, the cells were incubated for 1 h at room temperature with an Alexa Fluor 488-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG (Invitrogen, Carlsbad, CA) diluted 1:500 (4 μg/ml) in PBS-BSA. The cells were then washed in PBS-BSA and analyzed in a FACScan flow cytometer (Becton Dickinson) using CellQuest software.

Quantitative real-time PCR. Approximately 10^5 A549 cells were seeded in 24-well plates (Nunc) on the day before infection. On the day of infection, the cells in one well were counted to establish the amount of virions to be added. The growth medium was removed, and compound and virus were added simultaneously to the cells in 700 μl of DMEM with 0.75 g/liter $NaHCO_3$, 20 mM HEPES, 1× PEST, and 1% FBS. Compounds were added in concentrations ranging from 0.5 to 15 μM. The final concentration of DMSO was <1% in all samples. A 1-pg portion of Ad virions was added per cell. The plate was incubated at 37°C in 5% CO_2 and, 24 h after infection the cells were harvested, washed once and resuspended in PBS. DNA was prepared from the samples by using a QIAamp DNA blood minikit (Qiagen, Solna, Sweden) according to the manufacturer's instructions. The principle of quantitative real-time PCR has been described previously (14), as has the design of primers and probes for analysis of various Ad types representing different adenovirus species with quantitative PCR (QPCR) (2, 15). Briefly, quantitative real-time PCR was carried out using a degenerate primer pair, *Kadgen1* (forward)-*Kadgen2* (reverse) (5′-CWT ACA TGC ACA TCK CSG G-3′ and 5′-CRC GGG CRA AYT GCA CCA G-3′, respectively; DNA Technology A/S, Aarhus, Denmark). This primer pair is specific for the conserved region of the Ad hexon gene and can detect all human Ads. Different FAM-TAMRA probes were used to quantitate Ads from different species: *AdB1B2* (5′-6-FAM-AGG ATG CTT CGG AGT ACC TGA GTC CGG-TAMRA-3′) for Ad11p (15) and *AdC* (5′-6-FAM-AGG ACG CCT CGG AGT ACC TGA GCC CCG-TAMRA-3′) for Ad5 (all from Applied Biosystems, Cheshire, United Kingdom). For Ads from species A, D, E, and F, the probe *AdDF* (5′-6-FAM-CCG GGC TCA GGT ACT CCG AGG CGT CCT-3′) was used (Applied Biosystems). Standard curves ranging from 5 to 5×10^5 genome copies were generated by serial dilution of known amounts of full-length Ad5 or Ad11 DNA. The Ad5 DNA standard was used for the *AdDF* probe system. The amplification was performed in a 25-μl reaction mixture containing the following: 10 μl of Ad5 standard DNA or Ad11 standard DNA or 10 μl of DNA from samples, 2.5 μl of 10× *Taq* buffer, 5 μl of 25 mM $MgCl_2$, 2.0 μl of 2.5 mM deoxynucleoside triphosphates, 1.0 μl of 25 μM *Kadgen1*, 1.0 μl of 25 μM *Kadgen2*, 0.29 μl of 15 μM probe *AdB1B2* or probe *AdDF* or 1.0 μl of 5 μM probe *AdC*, 0.2 μl of AmpliTaq Gold polymerase at 5 U/μl, 0.25 μl of AmpErase uracil *N*-glycosylase (UNG), and 2.76 μl of H_2O for Ad11p and 2.05 μl of H_2O for Ad5 (Applied Biosystems, Roche Molecular Systems, Branchburg, NJ). The program for the real-time PCR was as follows: 2 min at 50°C to activate UNG, followed by amplification and quantitation (10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C). The efficiency of the real-time PCR assay was the same for both probe systems used (data not shown). To standardize the number of adenoviral genome copies per cell, real-time PCR analysis was performed on the same samples using the cellular RNase P as a reference gene. The TaqMan RNase P detection reagents kit (20× mix containing primers and a FAM/TAMRA probe) (Applied Biosystems, Foster City, CA) was used for the analysis. The PCR mixture was otherwise the same as with Ad primers and probes. Real-time PCR was performed in an ABI Prism 7700 sequence detector (Applied Biosystems) and analyzed with sequence detector v1.7a software.

Binding experiments. A549 cells were washed twice and detached from the culture flask with 0.05% EDTA in PBS, resuspended in culture medium, and allowed to recover for 1 h at 37°C. The cell suspension was centrifuged at room temperature at 450 × *g* for 5 min and resuspended in PBS containing 1% FBS and 0.01% NaN_3 (PBS-FBS-NaN_3); 200,000 cells per well were dispensed in a 96-well microtiter plate (Nunc). The plate was placed on ice, and the compound was added to final concentrations of 5 and 15 μM. The final concentration of DMSO was <1% in all samples. Portions (5 pg) of ^{35}S-labeled Ad5 or Ad11p virions (with labeling done as described previously by Segerman et al. [37]) were added per cell, and the plate was incubated on ice on a rocking platform for 1 h. After incubation, the cells were washed three times with PBS-FBS-NaN_3, pelleted by centrifugation at 800 *g* for 5 min at 4°C and resuspended in 100 μl of PBS. The suspension was transferred to scintillation tubes containing 2 ml of scintillation liquid (Wallac OptiPhase HiSafe 3; Perkin-Elmer), and the cell-associated radioactivity was measured as counts per minute by using a liquid scintillation counter (Wallac 1409).

Statistical analysis. Statistical analyses (*t* tests) were performed with GraphPad Prism software version 4.03 (GraphPad Software, San Diego, CA).

TABLE 1. Inhibitory effect and toxicity observed in screening 24 compounds that were later verified as inhibitors

Compound[a]	% Inhibition of GFP[b]	% Viable cells[c]
A02	100	100
A04	92	43
A05	99	51
A06	82	71
A07	99	63
A08	89	86
A09	96	57
A10	81	51
A11	99	51
A12	82	67
A13	92	49
A14	90	55
A15	88	56
A16	87	59
A17	91	43
A18	92	66
A19	99	100
A20	97	53
A21	80	84
A22	96	60
A23	81	52
A24	93	90
A25	78	60
A26	91	45

[a] The screening was performed once in K562 cells, and the compound concentrations were 50 μM.
[b] Inhibition was assayed by fluorometric readout of GFP expression from the Ad11p vector.
[c] Toxicity was determined by the MTT toxicity test.

RESULTS

Screening. As mentioned above, our screening assay is based on GFP expression from the RCAd11pGFP vector in a K562 cell system. The 9,800 compounds were screened for their ability to inhibit emitted fluorescence and hence expression of the adenoviral genome. To be considered as a potential hit, the compound had to decrease the intensity of fluorescence by more than 80% and kill no more than 50% of the cells. The primary hits of the screening procedure were 408 distinct compounds that showed properties of inhibition of RCAd11pGFP expression in K562 cells, representing a hit rate of ca. 4%. None of the compounds selected for further study were autofluorescent.

Validation of hits. To verify the hits and to exclude false positives, the compounds were serially diluted in seven steps for dose-response analysis using a screening assay. Twenty-four compounds that had the highest level of inhibition without parallel cytotoxicity were selected. The fluorescence inhibition and cellular toxicity detected in the screening process of these 24 hits are summarized in Table 1. One of the most efficient and least toxic compounds is A02, 2-{[2-(benzoylamino)benzoyl]amino}-benzoic acid. This compound was evaluated further as a potential drug candidate. Serial dilution of compound A02 in the screening setup with RCAd11pGFP in K562 cells showed a clear dose response, with a 50% effective concentration (EC_{50}) of 28.6 μM (Fig. 1a). A similar inhibition profile was obtained when A02 was evaluated by FACS analysis of RCAd11pGFP in A549 cells (Fig. 1b).

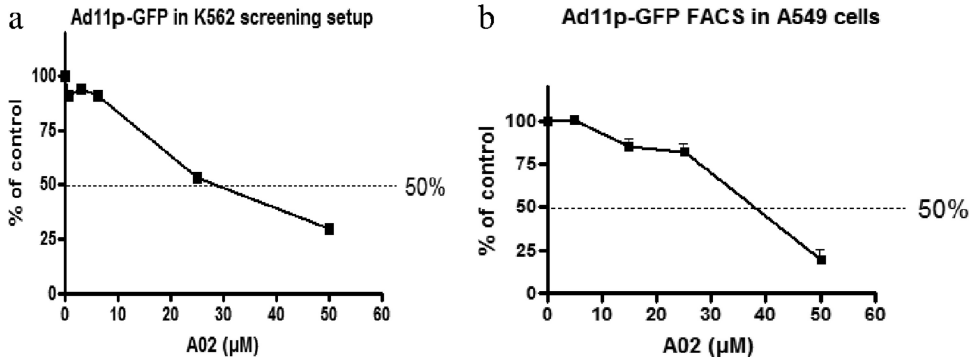

FIG. 1. (a) Dose response for A02 inhibition of GFP expression from the RCAd11pGFP vector in K562 cells. The fluorescence intensity was measured after 24 h of incubation with compound A02 and vector. (b) Inhibition of GFP expression from the RCAd11pGFP vector in A549 cells. The fluorescence intensity assayed by FACS analysis after 24 h of incubation with compound A02 and vector.

In the process of verifying the identity of the inhibitory compounds, combined analysis by LC-MS of the purchased compounds was performed. It turned out that the A02 solution contained three different molecules. The three components were separated by LC, and their structures (Fig. 2) were confirmed by MS and NMR spectroscopy. For compound A01, the data were in agreement with those published (25). The effect of the three molecules on the replication of Ad5 in A549 cells was assessed in a QPCR assay. A significant inhibitory effect on Ad5 replication could only be observed for the original compound, A02. Neither the smaller (A01) nor the larger (A03) molecule showed any antiadenoviral effect (Fig. 3). Experimental data presented in all figures were obtained with pure compounds.

Inhibition of wild-type Ad5 and Ad11p. The antiviral potency of compound A02 in the A549 cell system was assessed by measuring the effect on newly synthesized viral genomes of Ad5 and Ad11p by the QPCR assay. Titration resulted in comparable EC_{50}s of 3.7 and 2.9 μM for Ad5 and Ad11p, respectively (Fig. 4). Detection of inhibition of DNA replication by QPCR for wild-type Ad5 and Ad11p is substantially more efficient than detection of inhibition of GFP expression from the viral vector in K562 and A549 (compare Fig. 1a, 1b, and 4). A binding assay using isotope-labeled virions was used to address whether the compound would prevent viral adhesion to host cells. At 15 μM, compound A02 has no effect on Ad5 or Ad11p binding to the surface of A549 cells (Fig. 5a). To further verify inhibition of viral replication, the effect on the expression of the most abundant viral structural protein (hexon) in A549 cells was studied by FACS analysis. The results showed that expression of Ad5 and Ad11p hexon protein is inhibited by compound A02 in a dose-dependent manner (Fig. 5b).

Toxicity. Toxicity of A02 in the A549 cell system was also analyzed by titration and XTT detection, giving a 50% cytotoxic concentration (CC_{50}) of 199 μM (Fig. 4). This can then be combined to give selectivity index values (SI = CC_{50}/EC_{50}) of 54 and 68 for Ad5 and Ad11p, respectively. The toxicity in cell systems, including K562, A549, and the fibroblast cell line FSU, was also evaluated with propidium iodide using FACS. The toxicity of A02 at 15 μM is low, with <2% dead cells after 24 h of incubation with the compound in all three cell lines tested by exposure to propidium iodide, followed by FACS analysis (Fig. 5c).

Effect on different adenovirus species. With the clear-cut effect of A02 on both Ad5 (species C) and Ad11p (species B) verified at several levels of the infection cycle, we performed an analysis to ascertain whether A02 could also affect Ads of other species (4). The results are summarized in Table 2. DNA

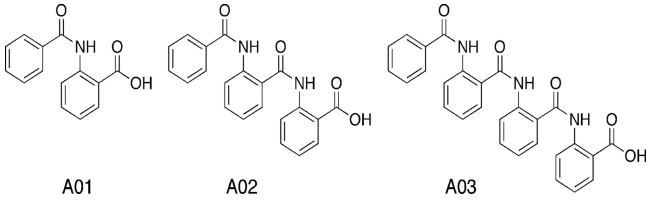

FIG. 2. Chemical structures of compound A02 and its analogues separated from the purchased sample. A02, 2-{[2-(benzoylamino)benzoyl]amino}-benzoic acid; A01, 2-(benzoylamino)-benzoic acid; A03, 2-{[2-[[2-(benzoylamino)benzoyl]amino]benzoyl]amino}-benzoic acid.

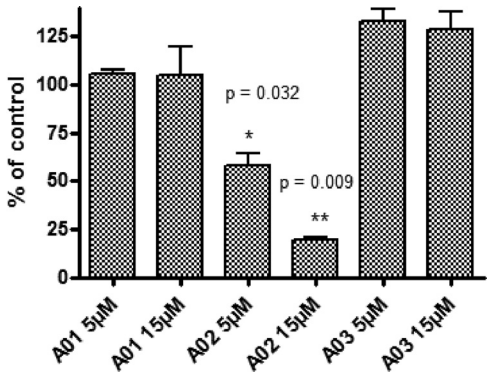

FIG. 3. Effect of A02, A01, and A03 on Ad replication. The separated compounds were tested for inhibitory effect on Ad replication by QPCR. Ad5 was allowed to infect A549 cells with or without compound. After 24 h of incubation DNA was prepared from cells and virus and analyzed by QPCR. As an internal control, the cellular gene RNase P was included in the assay. All values are normalized to RNase P. Error bars represent the standard deviation of the means from three independent experiments run in duplicates. The statistical significance was determined by unpaired *t* test, and a *P* value of < 0.05 was considered significant. Statistical analyses were performed by using GraphPad Prism.

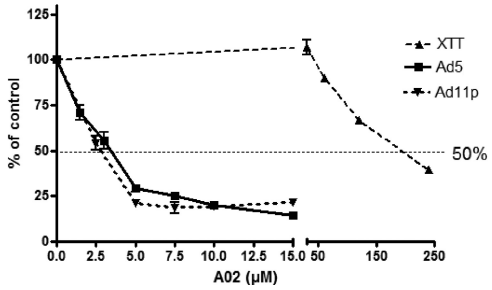

FIG. 4. Titration of the effect of A02 on Ad5, Ad11p, and the toxic effect in A549 cells after 24 h of incubation with virus and/or compound A02. The EC_{50}s for Ad5 and Ad11p were 3.7 and 2.9 μM, respectively. The CC_{50} for compound A02 in A549 cells was 199 μM. The EC_{50} is the concentration at which the Ad replication is inhibited by 50% as determined by QPCR, and CC_{50} is the concentration at which the cytotoxicity is 50%, i.e., 50% of the cells are viable, as determined by the XTT assay. Error bars represent the standard deviation of the means from three independent duplicate experiments.

replication of all Ads tested by the QPCR assay is inhibited by compound A02 in a dose-dependent way. A02 appears to have a general effect on Ads from all species. The antiviral drugs ribavirin and cidofovir have previously been evaluated as antiadenoviral agents. The results for ribavirin are not conclusive (24, 30), and we thus tested the effect of ribavirin on replication of the Ad5 and Ad11p genome. We found that ribavirin had no significant effect on Ad5 or Ad11p DNA replication (data not shown). Cidofovir is more established as an antiadenoviral drug (3, 10, 11, 13). To verify the functionality of the QPCR assay the effect of cidofovir on Ad5 and Ad11p after 24 h was evaluated. It appears that A02 inhibits Ad5 and Ad11p DNA replication about five times more efficiently than cidofovir (Table 2).

TABLE 2. DNA replication inhibition in A549 cells for representative Ads from all species

Ad type (species)	Mean EC_{50} (μM) ± SD[a]	
	A02	Cidofovir
Ad31 (A)	3.9 ± 0.8	ND
Ad11p (B2)	2.9 ± 1.3	16.5 ± 4.6
Ad5 (C)	3.7 ± 0.9	19.9 ± 5.8
Ad37 (D)	4.7 ± 1.4	ND
Ad4 (E)	3.6 ± 0.6	ND
Ad41 (F)	2.4 ± 0.1	ND

[a] Values are means of at least two independent duplicate QPCR experiments. ND, not determined.

DISCUSSION

Adenovirus infections are a common cause of morbidity and mortality in immunocompromised individuals in general, and in pediatric patients in particular (4, 16, 18). Established anti-

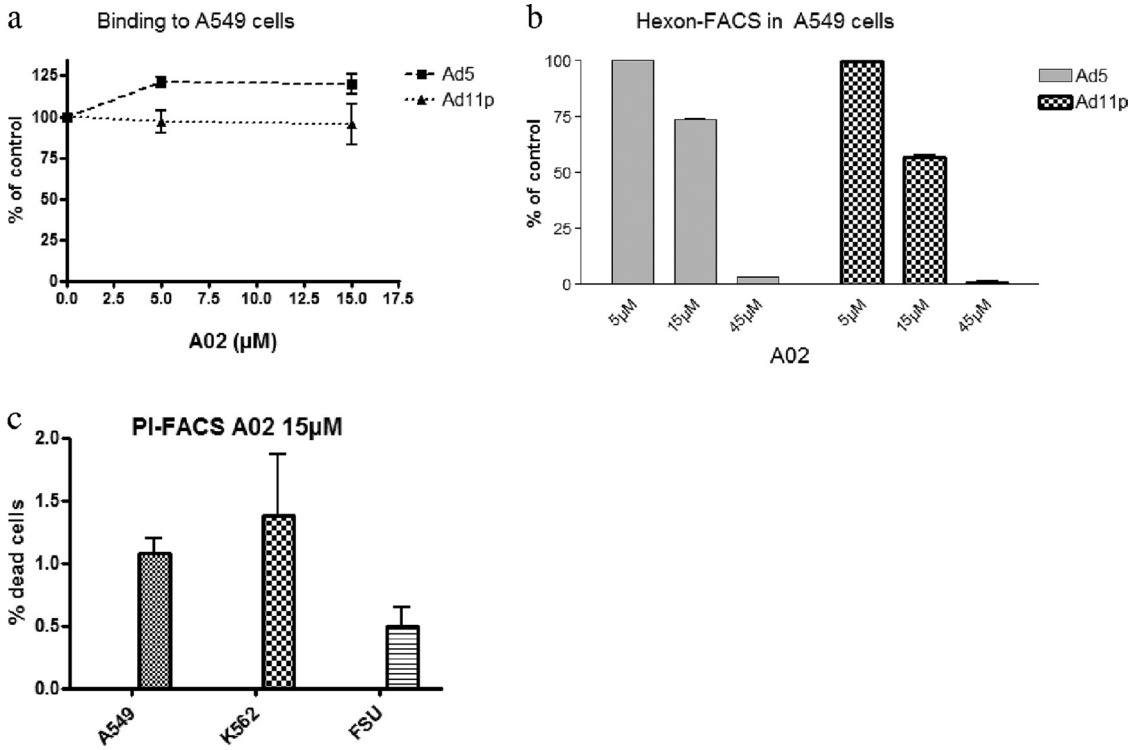

FIG. 5. (a) Binding of ^{35}S-labeled Ad5 or Ad11p to A549 cells in the presence of A02. Error bars represent the standard deviation of the means from three independent duplicate experiments. (b) Flow cytometry assay detecting Ad hexon protein after 24 h of incubation with virus and compound A02. Error bars represent the standard deviation of the means from two independent duplicate experiments. (c) Flow cytometry assay detecting dead cells where propidium iodide has intercalated the DNA. FACS analysis was performed after 24 h of incubation with a 15 μM concentration of compound A02. K562 cells were used mainly in the screening assay; A549 cells were used for most verification assays. Error bars represent the standard deviation of the means from two independent duplicate experiments.

viral drugs including cidofovir, ribavirin, and ganciclovir have been tested for antiadenoviral activity both in *in vitro* experiments and in the clinical setting. The clinical efficacy is inconclusive, since varying results have been reported for the drugs. Of the approved drugs, cidofovir appears to be most effective against Ads (7, 24, 30, 33). However, cidofovir is associated with nephrotoxicity and acute renal failure (19, 28, 42). Most *in vitro* experiments of the antiadenoviral effect of cidofovir address the outcome of the drug after longer times than 24 h, which was the time point evaluated here (3, 13). This could explain why we observed a slightly higher EC_{50} than seen in other studies. The need for new antiadenoviral substances is clearly increasing due to the large number of immunocompromised patients undergoing transplantations and also patients suffering from AIDS or with genetic immunodeficiencies.

Screening-based strategies are well suited for identification of compounds with potential antiadenoviral activity. Our unique assay is based on a replication-competent Ad11p vector. The GFP gene is located in the E1 region of the Ad11p genome, and detection of fluorescence by GFP expression is directly correlated to Ad11p genome expression. This assay, developed for antiadenoviral screening, is versatile due to its robustness, its simplicity, and the direct measurement of inhibition of Ad genome expression. K562 cells were used in the screening assay, since they are suspension cells that are permissive for Ad11p infection. Any hits found in a screening campaign must, however, be thoroughly verified since screening can be imprecise in many respects. We decided to concentrate the verification on Ad5 inhibition since other potential antiadenoviral drugs have been evaluated on the basis of their effects on species C adenovirus types (24, 38). There is no replication-competent Ad5 vector available; thus, Ad5 could not be used for screening. K562 cells are not permissive for Ad5 infection, and the cell line of choice for verification was A549.

The discovery of more than one molecule in the most promising hit illustrates the necessity for quality control and thorough validation to verify that hits found in a screening campaign represent homogenous preparations of the correct molecule, with the desired biological activity. In this particular case, the finding provided an opportunity for a preliminary analysis of the structure-activity relationship. The antiadenoviral effect of A02 only, but not the analogs, has been verified in a number of assays. There appears to be a size restriction for the compound to exert its inhibitory effect. Since neither the smaller analog A01 nor the larger A03 analog had inhibitory effects, it is tempting to speculate that there may be a pocket in the target protein into which A02 fits, where A01 is too small to cover the required site and A03 is too bulky to fit.

Considering the fact that DNA replication of all Ad types tested was inhibited by A02, although not with the same efficiency, inhibition by this compound appears to be general for human Ads (Table 2). Inhibition of Ad31 is especially important, since this is one of the most threatening adenovirus types, which can infect immunocompromised individuals in general and pediatric transplant recipients in particular (16). The sensitivity of the four assays used for characterization of A02 varied; the QPCR assay appears to be the most sensitive, followed by hexon FACS, Ad11pGFP FACS, and Ad11pGFP in the screening setup (Fig. 1a and b, Fig. 4, and Fig. 5b).

In conclusion, the screening assay presented here is a very simple and useful approach to discover novel compounds that inhibit Ad infection. Based on this assay, we have described and in various ways verified the inhibitory and toxic properties of compound A02, which appears to be a promising candidate for further development as a functional all-purpose antiadenoviral drug.

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REFERENCES

1. **Adrian, T. H., G. Wadell, J. C. Hierholzer, and R. Wigand.** 1986. DNA restriction patterns of adenovirus prototypes 1 to 41. Arch. Virol. **91:**277–299.
2. **Allard, A., B. Albinsson, and G. Wadell.** 2001. Rapid typing of human adenoviruses by a general PCR combined with restriction endonuclease analysis. J. Clin. Microbiol. **39:**498–505.
3. **Baba, M., S. Mori, S. Shigeta, and E. De Clercq.** 1987. Selective inhibitory effect of (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine and 2′-norcyclic GMP on adenovirus replication in vitro. Antimicrob. Agents Chemother. **31:**337–339.
4. **Baldwin, A., H. Kingman, M. Darville, A. B. M. Foot, D. Grier, J. M. Cornish, N. Goulden, A. Oakhill, D. H. Pamphilon, C. G. Steward, and D. I. Marks.** 2000. Outcome and clinical course of 100 patients with adenovirus infection following bone marrow transplantation. Bone Marrow Transplant. **26:**1333–1338.
5. **Bordigoni, P., A.-S. Carret, V. Venard, F. Witz, and A. Le Faou.** 2001. Treatment of adenovirus infections in patients undergoing allogeneic hematopoietic stem cell transplantation. Clin. Infect. Dis. **32:**1290–1297.
6. **Carmichael, J., W. G. DeGraff, A. F. Gazdar, J. D. Minna, and J. B. Mitchell.** 1987. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Res. **47:**936–942.
7. **Chen, F. E., R. H. S. Liang, J. Y. Lo, K. Y. Yuen, T. K. Chan, and M. Peiris.** 1997. Treatment of adenovirus-associated haemorrhagic cystitis with ganciclovir. Bone Marrow Transplant. **20:**997–999.
8. **De Jong, J. C., A. G. Wermenbol, M. W. Verweij-Uijterwaal, K. W. Slaterus, P. Wertheim-Van Dillen, G. J. Van Doornum, S. H. Khoo, and J. C. Hierholzer.** 1999. Adenoviruses from human immunodeficiency virus-infected individuals, including two strains that represent new candidate serotypes Ad50 and Ad51 of species B1 and D, respectively. J. Clin. Microbiol. **37:**3940–3945.
9. **Denizot, F., and R. Lang.** 1986. Rapid colorimetric assay for cell growth and survival: modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J. Immunol. Methods **89:**271–277.
10. **de Oliveira, C. B. R., D. Stevenson, L. LaBree, P. J. McDonnell, and M. D. Trousdale.** 1996. Evaluation of cidofovir (HPMPC, GS-504) against adenovirus type 5 infection in vitro and in a New Zealand rabbit ocular model. Antivir. Res. **31:**165–172.
11. **Diaconu, I., V. Cerullo, S. Escutenaire, A. Kanerva, G. J. Bauerschmitz, R. Hernandez-Alcoceba, S. Pesonen, and A. Hemminki.** 2010. Human adenovirus replication in immunocompetent Syrian hamsters can be attenuated with chlorpromazine or cidofovir. J. Gene Med. **12:**435–445.
12. **Gavin, P. J., and B. Z. Katz.** 2002. Intravenous ribavirin treatment for severe adenovirus disease in immunocompromised children. Pediatrics **110:**e9.
13. **Hartline, C. B., K. M. Gustin, W. B. Wan, S. L. Ciesla, J. R. Beadle, K. Y. Hostetler, and E. R. Kern.** 2005. Ether lipid ester prodrugs of acyclic nucleoside phosphonates: activity against adenovirus replication in vitro. J. Infect. Dis. **191:**396–399.
14. **Heid, C. A., J. Stevens, K. J. Livak, and P. M. Williams.** 1996. Real-time quantitative PCR. Genome Res. **6:**986–994.
15. **Hernroth, B. E., A. C. Conden-Hansson, A. S. Rehnstam-Holm, R. Girones, and A. K. Allard.** 2002. Environmental factors influencing human viral pathogens and their potential indicator organisms in the blue mussel, *Mytilus edulis*: the first Scandinavian report. Appl. Environ. Microbiol. **68:**4523–4533.
16. **Hierholzer, J. C.** 1992. Adenoviruses in the immunocompromised host. Clin. Microbiol. Rev. **5:**262–274.
17. **Hoffman, J. A., A. J. Shah, L. A. Ross, and N. Kapoor.** 2001. Adenoviral infections and a prospective trial of cidofovir in pediatric hematopoietic stem cell transplantation. Biol. Blood Marrow Transplant. **7:**388–394.

18. **Howard, D. S., G. L. Phillips Ii, D. E. Reece, R. K. Munn, J. Henslee-Downey, M. Pittard, M. Barker, and C. Pomeroy.** 1999. Adenovirus infections in hematopoietic stem cell transplant recipients. Clin. Infect. Dis. **29:**1494–1501.
19. **Izzedine, H., V. Launay-Vacher, and G. Deray.** 2005. Antiviral drug-induced nephrotoxicity. Am. J. Kidney Dis. **45:**804–817.
20. **Janner, D., A. M. Petru, D. Belchis, and P. H. Azimi.** 1990. Fatal adenovirus infection in a child with acquired immunodeficiency syndrome. Pediatr. Infect. Dis. J. **9:**434–436.
21. **Klinger, J. R., M. P. Sanchez, L. A. Curtin, M. Durkin, and B. Matyas.** 1998. Multiple cases of life-threatening adenovirus pneumonia in a mental health care center. Am. J. Respir. Crit. Care Med. **157:**645–649.
22. **Kojaoghlanian, T., P. Flomenberg, and M. S. Horwitz.** 2003. The impact of adenovirus infection on the immunocompromised host. Rev. Med. Virol. **13:**155–171.
23. **Kroes, A. C. M., E. P. A. de Klerk, A. C. Lankester, C. Malipaard, C. S. de Brouwer, E. C. J. Claas, E. C. Jol-van der Zijde, and M. J. D. van Tol.** 2007. Sequential emergence of multiple adenovirus serotypes after pediatric stem cell transplantation. J. Clin. Virol. **38:**341–347.
24. **Lankester, A. C., B. Heemskerk, E. C. J. Claas, M. W. Schilham, M. F. C. Beersma, R. G. M. Bredius, M. J. D. van Tol, and A. C. M. Kroes.** 2004. Effect of ribavirin on the plasma viral DNA load in patients with disseminating adenovirus infection. Clin. Infect. Dis. **38:**1521–1525.
25. **Lee, C. K., and Y. M. Ahn.** 1989. Reactions of amides with potassium permanganate in neutral aqueous solution. J. Organic Chem. **54:**3744–3747.
26. **Leruez-Ville, M., M. Chardin-Ouachee, B. Neven, C. Picard, I. Le Guinche, A. Fischer, C. Rouzioux, and S. Blanche.** 2006. Description of an adenovirus A31 outbreak in a paediatric haematology unit. Bone Marrow Transplant. **38:**23–28.
27. **Mei, Y.-F., K. Lindman, and G. Wadell.** 1998. Two closely related adenovirus genome types with kidney or respiratory tract tropism differ in their binding to epithelial cells of various origins. Virology **240:**254–266.
28. **Meier, P., S. Dautheville-Guibal, P. M. Ronco, and J. Rossert.** 2002. Cidofovir-induced end-stage renal failure. Nephrol. Dial. Transplant. **17:**148–149.
29. **Michaels, M. G., M. Green, E. R. Wald, and T. E. Starzl.** 1992. Adenovirus infection in pediatric liver transplant recipients. J. Infect. Dis. **165:**170–172.
30. **Morfin, F., S. Dupuis-Girod, S. Mundweiler, D. Falcon, D. Carrington, P. Sedlacek, M. Bierings, P. Cetkovsky, A. C. M. Kroes, M. J. D. van Tol, and D. Thouvenot.** 2005. In vitro susceptibility of adenovirus to antiviral drugs is species-dependent. Antiviral Ther. **10:**225–229.
31. **Mosmann, T.** 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods **65:**55–63.
32. **Muller, W. J., M. J. Levin, Y. K. Shin, C. Robinson, R. Quinones, J. Malcolm, E. Hild, D. Gao, and R. Giller.** 2005. Clinical and in vitro evaluation of cidofovir for treatment of adenovirus infection in pediatric hematopoietic stem cell transplant recipients. Clin. Infect. Dis. **41:**1812–1816.
33. **Naesens, L., L. Lenaerts, G. Andrei, R. Snoeck, D. Van Beers, A. Holy, J. Balzarini, and E. De Clercq.** 2005. Antiadenovirus activities of several classes of nucleoside and nucleotide analogues. Antimicrob. Agents Chemother. **49:**1010–1016.
34. **Roehm, N. W., G. H. Rodgers, S. M. Hatfield, and A. L. Glasebrook.** 1991. An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. J. Immunol. Methods **142:**257–265.
35. **Sandberg, L., P. Papareddy, J. Silver, A. Bergh, and Y.-F. Mei.** 2009. Replication-competent Ad11p vector (RCAd11p) efficiently transduces and replicates in hormone-refractory metastatic prostate cancer cells. Hum. Gene Ther. **20:**361–373.
36. **Scudiero, D. A., R. H. Shoemaker, K. D. Paull, A. Monks, S. Tierney, T. H. Nofziger, M. J. Currens, D. Seniff, and M. R. Boyd.** 1988. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. Cancer Res. **48:**4827–4833.
37. **Segerman, A., N. Arnberg, A. Erikson, K. Lindman, and G. Wadell.** 2003. There are two different species B adenovirus receptors: sBAR, common to species B1 and B2 adenoviruses, and sB2AR, exclusively used by species B2 adenoviruses. J. Virol. **77:**1157–1162.
38. **Toth, K., J. F. Spencer, D. Dhar, J. E. Sagartz, R. M. L. Buller, G. R. Painter, and W. S. M. Wold.** 2008. Hexadecyloxypropyl-cidofovir, CMX001, prevents adenovirus-induced mortality in a permissive, immunosuppressed animal model. Proc. Natl. Acad. Sci. **105:**7293–7297.
39. **Wadell, G.** 1984. Molecular epidemiology of human adenoviruses. Curr. Topics Microbiol. Immunol. **110:**191–220.
40. **Wadell, G., A. Allard, and J. C. Hierholzer.** 1999. Adenoviruses, p. 970–982. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology, 7th ed. ASM Press, Washington, DC.
41. **Weislow, O. S., R. Kiser, D. L. Fine, J. Bader, R. H. Shoemaker, and M. R. Boyd.** 1989. New soluble-formazan assay for HIV-1 cytopathic effects: application to high-flux screening of synthetic and natural products for AIDS-antiviral activity. J. Natl. Cancer Inst. **81:**577–586.
42. **Zedtwitz-Liebenstein, K., E. Presterl, E. Deviatko, and W. Graninger.** 2001. Acute renal failure in a lung transplant patient after therapy with cidofovir. Transplant Int. **14:**445–446.