N-(3-Cyanophenyl)-2-phenylacetamide, an effective inhibitor of morbillivirus-induced membrane fusion with low cytotoxicity

K. Singethan,1 G. Hiltensperger,2 S. Kendl,1 J. Wohlfahrt,1 P. Plattet,3 U. Holzgrabe2 and J. Schneider-Schaulies1

Correspondence
J. Schneider-Schaulies
jss@vim.uni-wuerzburg.de

1Institut für Virologie und Immunbiologie, University of Würzburg, Germany
2Institut für Pharmazie und Lebensmittelchemie, University of Würzburg, Germany
3Department für klinische Veterinärmedizin, University of Bern, Switzerland

Based on the structural similarity of viral fusion proteins within the family Paramyxoviridae, we tested recently described and newly synthesized acetanilide derivatives for their capacity to inhibit measles virus (MV)-, canine distemper virus (CDV)- and Nipah virus (NiV)-induced membrane fusion. We found that N-(3-cyanophenyl)-2-phenylacetamide (compound 1) has a high capacity to inhibit MV- and CDV-induced (IC50 = 3 μM), but not NiV-induced, membrane fusion. This compound is of outstanding interest because it can be easily synthesized and its cytotoxicity is low [50 % cytotoxic concentration (CC50) ≥ 300 μM], leading to a CC50/IC50 ratio of approximately 100. In addition, primary human peripheral blood lymphocytes and primary dog brain cell cultures (DBC) also tolerate high concentrations of compound 1. Infection of human PBMC with recombinant wild-type MV is inhibited by an IC50 of approximately 20 μM. The cell-to-cell spread of recombinant wild-type CDV in persistently infected DBC can be nearly completely inhibited by compound 1 at 50 μM, indicating that the virus spread between brain cells is dependent on the activity of the viral fusion protein. Our findings demonstrate that this compound is a most applicable inhibitor of morbillivirus-induced membrane fusion in tissue culture experiments including highly sensitive primary cells.

INTRODUCTION

The genus of closely related viruses Morbillivirus, contains measles virus (MV) and canine distemper virus (CDV), which cause devastating diseases in their hosts. MV, one of the most contagious aerosol-transmitted viruses, still causes more than 100 000 deaths each year. After entering the upper respiratory tract, MV exhibits a pronounced tropism for mono- and lymphocytic cells and soon viral replication is detected in draining lymph nodes (Pfeuffer et al., 2003; de Swart et al., 2007; Ferreira et al., 2010). During measles, patients develop a pronounced leukopenia that may be due to enhanced adhesion of lymphocytes in secondary lymphoid organs (Nanan et al., 1999; Dittmar et al., 2008). In immunocompetent patients, MV infection is usually cleared by the virus-specific immune response, while the general immune response to other antigens is suppressed for several weeks after the rash (reviewed by Schneider-Schaulies & Schneider-Schaulies, 2009). Following replication in lymphoid tissues, virus spreads to various organs and can be detected in the skin, the gastrointestinal tract, the lungs and the eyes. It may also enter the brain (reviewed by Ludlow et al., 2009). CNS complications are acute post-infectious measles encephalitis and, based on a persistent infection, subacute sclerosing pan-encephalitis (SSPE), which is always lethal. SSPE does not occur after measles vaccination (Duclos & Ward, 1998).

CDV causes systemic infections similar to but distinct from human measles in carnivores such as canines, felids, ferrets, raccoons and seals, with lethality rates, depending on the host, of up to 100 % (Appel et al., 1972; Harder & Osterhaus, 1997; von Messling et al., 2003). In a high percentage of animals the CNS is infected, causing severe complications with infiltration of inflammatory cells and demyelination (Summers & Appel, 1994; Vandevelde & Zurbriggen, 1995). Encephalomyelitis is the most common cause of death of CDV-infected animals, meaning that CDV is much more neurotropic than MV. Live attenuated vaccines against CDV have existed since the 1950s and their application led to a drastic reduction in CDV epidemics. However, between 1991 and 1995 there were several big outbreaks in France, Japan, Germany and Scandinavia which especially affected dogs with a high vaccination rate (Blixeinkrone-Moller et al., 1993; Gemma et al., 1996). The
reason is probably antigenic drift of the wild-type virus. This generates genetically different strains and makes the vaccine inefficient (Harder & Osterhaus, 1997).

Zoonotic outbreaks of respiratory diseases and encephalitis affecting humans, horses and pigs in Australia, Malaysia and Singapore have led to the isolation of two novel paramyxoviruses, Hendra and Nipah (NiV) viruses, which have been assigned to the genus *Henipavirus* in the family *Paramyxoviridae* (Murray *et al.*, 1995; Chua, 2003). The natural hosts of both viruses are fruit bats of the genus *Pteropus*. While causing a respiratory disease with low mortality rates in pigs, a severe febrile encephalitis with high mortality rates was observed in NiV-infected humans (estimated case fatality rate, 40–50%). Recent outbreaks of NiV infection in Bangladesh and India have led to further human deaths and may have been a result of both direct bat–human and human–human transmissions (Butler, 2004; Chadha *et al.*, 2006). EphrinB2-positive cells, including endothelial cells, are the major cellular targets for NiV and syncytia of endothelial cells in blood vessels are recognized as a characteristic feature of NiV infection (Wong *et al.*, 2002; Negrete *et al.*, 2005). A late-onset encephalitis may follow months after the acute infection, indicating virus persistence in the brain (Tan *et al.*, 2002).

Membrane fusion induced by paramyxoviruses is an essential step in the viral infection cycle that is mediated by cooperation of both viral envelope glycoproteins, the attachment protein (H or G) and the fusion protein (F). After cleavage of the precursor F protein into F1 and F2 subunits, these subunits form an active H–F1/2 complex mediating pH independent virus–cell and cell–cell fusion. Small-molecule inhibitors, fitting into a pocket of the MV F protein and preventing membrane fusion by blocking the natural interaction between the two essential heptad-repeat regions within the F protein, have been designed. They interfere with the conformational changes of the F protein at the beginning of the fusion process (Plemper *et al.*, 2004, 2005; Sun *et al.*, 2006). Given the structural similarity of paramyxovirus F proteins, we tested some of these small-molecule inhibitors and several derivatives in a NiV F protein-based fusion assay (Niedermeier *et al.*, 2009). Molecular modelling indicated that one compound fitted well into a cavity present in the NiV F protein. This compound inhibited NiV-induced cell fusion but not MV-induced cell fusion, thus underlining specific differences between morbillivirus and henipavirus F proteins (Niedermeier *et al.*, 2009).

We investigated other small-molecule fusion inhibitors, including N-(3-cyanophenyl)-2-phenylacetamide (compound 1; Sun *et al.*, 2006), and describe its high capacity to inhibit MV- and CDV-induced, but not NiV-induced, membrane fusion. Compound 1 is of outstanding interest because it can be synthesized easily and its cytotoxicity is low. Even primary human peripheral blood lymphocytes (PBL) and primary dog brain cells (DBC) tolerated virus-inhibitory concentrations of this compound without signs of cytotoxicity.

## RESULTS

### Compounds 1 and 5 block morbillivirus envelope protein-induced, but not NiV-induced, cell fusion

We synthesized five acetanilide derivatives (Fig. 1), including the MV-induced fusion inhibitor AS-48 (compound 5;
compound 11f in Sun et al., 2006), using a modified method as described by Sun et al. (2006) (see Methods). These compounds were tested in a cell–cell fusion assay based on the transfection of viral envelope proteins in the absence of other viral genes. The envelope proteins of MV (strain Edmonston), CDV (strain Onderstepoort) and NiV (Malaysia brain isolate) were used. In this fusion assay the mean sizes of the syncytia (number of nuclei in a syncytium) are presented as a percentage of the control versus increasing concentrations of the compounds (Fig. 2). In this assay, MV-induced cell fusion was most efficiently inhibited with compound 1 (IC50 = 3 μM; identical to compound 3g in Sun et al., 2006) followed by compound 5 (IC50 = 8 μM; identical to compound AS-48), and less efficiently by compounds 3, 2 and 4. CDV-induced cell fusion was also efficiently inhibited by compounds 1 and 5 (IC50 = 6 and 4 μM, respectively), whereas NiV-induced cell fusion was not inhibited in this concentration range. Thus in our hands, compound 1 was slightly better at inhibiting MV envelope protein-induced cell fusion than compound 5. Both substances were similarly active for CDV envelope protein-induced fusion. Since the synthesis of compound 1 is straightforward and easy, this compound was an interesting candidate for further investigation.

Compound 1 efficiently inhibits MV and CDV uptake and virus-induced cell fusion

In order to assess the ability of compound 1 to inhibit syncytium formation induced by infectious wild-type MV and CDV, we used the recombinant wild-type viruses rMV–IC323–eGFP and rCDV–A75/17red, which express eGFP and tomato red, respectively. As target cells in this infection–inhibition assay we used Vero cells expressing the appropriate receptors, human and canine CD150 (Vero–hSLAM and Vero–dogSLAM). In the first series of experiments virus and inhibitors were mixed, added to the cells and the mixture incubated for 48 h at 37 °C. Live cells were analysed using a UV microscope and photomicrographs were taken (Fig. 3). Syncytium formation was inhibited in a dose-dependent manner by compound 1 and only few single infected cells were observed in the presence of >10 μM compound 1. Similar results were observed using compound 5 (data not shown).

To investigate the effect of the inhibitors separately on virus–cell and cell–cell fusion, we either pre-incubated virus with the inhibitors or added the inhibitors after infection of the cells (Fig. 4). In Fig. 4(a), rMV–IC323–eGFP and rCDV–A75/17red were mixed with compound 1 and incubated for 15 min at room temperature prior to infection of Vero–hSLAM and Vero–dogSLAM cells for 16 h. Under these conditions the number of infected cells decreased with increasing concentrations of the inhibitors. In Fig. 4(b), the cells were first infected, then washed and further incubated in the presence of compound 1 for 24 h at 37 °C. This treatment reduced the size of syncitia in an inhibitor concentration-dependent manner, but did not reduce the number of infected single cells (foci of infection). Similar results as are shown for compound 1 were obtained with compound 5 (data not shown). In addition, pre-incubation of cells with compounds 1 or 5, and subsequent infection in the absence of these compounds did not affect infection or syncytium formation (data not shown). Thus both virus–cell and cell–cell fusions were inhibited by compounds 1 or 5.

![Graphs](https://example.com/graphs)

**Fig. 2.** Dose-dependent inhibition of envelope protein-induced cell fusion. Vero cells were transfected with plasmids expressing the F and H proteins of MV strain Edmonston (a), F and H proteins of CDV strain Onderstepoort (b) and F and G proteins of NiV (c). Compounds were added after transfection at the concentrations indicated and the cells were incubated for 24 h at 37 °C. Mean numbers of nuclei in at least 50 syncytia per concentration per compound were quantified by microscopy. Relative fusion is presented as a percentage relative to the untreated control cells (100%). Mean values and SD of three independent experiments are presented. SD for compounds 1 and 5 are also shown.
Fig. 3. Inhibition of syncytium formation by compound 1 in recombinant wild-type MV- and CDV-infected Vero–hSLAM and Vero–dogSLAM cells. (a) Vero–hSLAM cells were infected with rMV–IC323–eGFP. (b) Vero–dogSLAM cells were infected with rCDV–A75/17red at an m.o.i. of 0.1. Compound 1 was mixed with virus prior to infection in increasing concentrations (0–30 μM). Virus and inhibitor were left in the cell culture and incubated for 48 h at 37 °C. Fluorescence and bright-field images were taken using a 20× objective lens.
As a consequence of the inhibition of virus-induced membrane fusion, the titres of infectious viral particles were reduced by compounds 1 and 5 in a concentration-dependent manner (Fig. 5). Viral titres of rMV–IC323–eGFP and rCDV–A75/17red were determined using Vero–hSLAM and Vero–dogSLAM cells for virus production and titration, respectively. Inhibition of viral titres of more than 2 logs (>99.5%) was obtained with concentrations of 30 μM for compounds 1 and 5.

Fig. 4. Virus–cell fusion and cell–cell fusion are inhibited by compound 1. (a) The viruses (rMV–IC323–eGFP and rCDV–A75/17red) were mixed with compound 1 and incubated for 15 min at room temperature prior to infection of cells. Vero–hSLAM and Vero–dogSLAM cells, upper and lower panels respectively, were incubated at 37 °C for 16 h. (b) The cells were first infected with the viruses for 1 h at 37 °C, then washed with PBS and further incubated in medium in the presence of compound 1 for 24 h at 37 °C. Images were taken using a 20× objective lens.
Cytotoxicity of the compounds

Cytotoxicity in Vero cells was determined using the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), which is incorporated in living cells and converted into a violet formazan product by mitochondrial dehydrogenases. After treatment of Vero cells, no considerable cytotoxic effects of compounds 1 or 5 were observed at concentrations ≤100 μM (Fig. 6a), and at concentrations of 300 μM 46 and 28% of cells remained vital, respectively. Compound 5 was always slightly more toxic than compound 1. Since Vero cells are quite robust and may not reflect the sensitivity of primary cells, we also assessed the toxicity of compound 1 using phytohaemagglutinin (PHA)-stimulated primary human peripheral blood lymphocytes (PBL). In this case we used the DNA-intercalating substance 7-amino-actinomycin D conjugated to APC-Alexa Fluor 750 (7AAD), which is incorporated into damaged cells, and evaluated the result by flow cytometry. Compound 1 was non-toxic in PBL at concentrations ≤100 μM, whereas compound 5 was non-toxic only at concentrations ≤30 μM (Fig. 6b). The CC50 values of compounds 1 and 5 in PBL were approximately 300 and 100 μM, respectively.

Fig. 5. Reduction of virus titres by treatment with compounds 1 and 5 in MV- and CDV-infected Vero cells. Vero–hSLAM and Vero–dogSLAM cells (1×10⁵), were infected with rMV–IC323-eGFP (a) and rCDV–A75/17red (b), respectively, at an m.o.i. of 0.1 in the presence of increasing concentrations of compounds 1 or 5, as indicated, and incubated for 3 days. As in Fig. 4, the compounds were mixed with virus prior to infection and left in the culture. Total virus (cell-bound and released) was prepared and titrated using Vero–hSLAM and Vero–dogSLAM cells (results with SD of three independent experiments are presented).

Fig. 6. Determination of cell viability in the presence of compounds using Vero cells and PBL. (a) Vero cells (1×10⁵ per well) were incubated for 48 h with increasing concentrations of compounds 1 or 5. After removal of the medium, cells were incubated for 2 h at 37 °C with MTT solution, then for 45 min at room temperature with extraction solution; finally, aliquots were evaluated using an ELISA reader. Results are presented as percentage viability relative to DMSO-treated control cells. (b) PBL (2×10⁵) were treated with the compounds 1 or 5 for 48 h and processed for flow cytometry. Before measurement, the cells were incubated with 7AAD for 10 min, then washed with FACS buffer and the incorporation of 7AAD measured using the far-red channel of the flow cytometer. Results are the mean values from three independent experiments and are presented as the percentage of viable cells relative to untreated healthy cells.
Compound 1 can be used in sensitive primary cultures of lymphocytes and brain cells

The above findings encouraged us to test the capacity of compound 1 to inhibit the infection of primary human PBL with recombinant wild-type MV and DBC with recombinant wild-type CDV. PBL were stimulated with PHA for 48 h prior to infection to stimulate the expression of the MV receptor CD150 (not shown; Tatsuo et al., 2000; Erlenhoefer et al., 2001). The cells were then infected at an m.o.i. of 0.5 with rMV–IC323–eGFP in the absence or presence of increasing concentrations of compound 1 and 48 h later the infection of CD3+ T and CD19+ B lymphocytes was analysed by flow cytometry (Fig. 7). A higher percentage of B than T lymphocytes was infected (approximately 50 and 30%, respectively) with the recombinant wild-type MV (Fig. 7b). In addition, the mean fluorescence intensity (MFI) of eGFP, which directly reflects the level of virus replication, was three- to fourfold higher in B than in T lymphocytes, and was 50% inhibited by compound 1 (and compound 5, data not shown) at concentrations of approximately 20 μM in both cell types (Fig. 7c).

DBC were infected with rCDV–A75/17red at an m.o.i. of 0.01 and cultivated for 12 days at 37 °C in the presence or absence of concentrations of 50 μM compound 1 (Fig. 8). The number of foci and the cell-to-cell spread were drastically reduced in the presence of compound 1, indicating that the activity of the F protein is required for cell-to-cell spread of CDV in persistently infected DBC. Morphologically, there was no sign of toxicity detectable in compound 1-treated DBC cultures, thus demonstrating that this concentration is well tolerated by these sensitive primary brain cells.

DISCUSSION

We investigated the capacity of two previously described MV inhibitors and three new derivatives to inhibit MV-, CDV- and NiV-induced membrane fusion. MV and CDV envelope protein-mediated membrane fusion was selectively inhibited by compounds 1 and 5, whereas NiV F/G protein-mediated cell fusion was not affected. Compound 1 [N-(3-cyanophenyl)-2-phenylacetamide] was found to be of special interest because it can be synthesized easily and its cytotoxicity is low. Even primary human PBL and primary DBC tolerated virus-inhibitory concentrations of compound 1 without signs of cytotoxicity. The finding that a higher percentage of B than T lymphocytes is infected by wild-type MV and that B cells express more viral protein, as indicated by eGFP, reflects a finding in lymphatic tissue by Condack et al. (2007). The infection of both B and T cells was inhibited by compounds 1 or 5 by 50% at
concentrations of approximately 20 μM. The spread of recombinant wild-type CDV in DBC was nearly completely inhibited by concentrations of 50 μM compound 1, suggesting that infection and virus spread in these persistently infected cultures are dependent on the activity of the viral fusion protein. Thus, compound 1 is a most applicable inhibitor for tissue culture experiments with highly sensitive primary cells and may be a good candidate for application in an animal model of MV or CDV infections.

Compound 1 was assessed earlier as a derivative of an effective inhibitor of MV-induced membrane fusion (AS-48, compound 5) (Plemper et al., 2004, 2005; Sun et al., 2006). Sun et al. (2006) tested the activity of compound 1 (compound 3g in Sun et al., 2006) using Vero cells and an attenuated vaccine-like MV strain (Edmonston), which uses the receptor CD46 on Vero cells (Erlenhofer et al., 2002). In this publication the most efficient compound was compound 5, which carries nitro and amido groups meta and ortho to the anilide nitrogen, respectively. This compound was also tested and found to be active against various wild-type MV strains with IC₅₀ values of 0.6–3 μM (Plemper et al., 2005). Importantly, compounds 1 and 5 interact, presumably, with the same region in the viral F protein, since compound 5-resistant strains, with a mutated aa 462 in the C-terminal heptad-repeat domain, were also much less effectively inhibited by compound 1 (compare IC₅₀ of 6.5 μM with IC₅₀ of >600 μM for resistant strains) (Doyle et al., 2006; Sun et al., 2006). The IC₅₀ concentration of compound 1, obtained in our fusion-inhibition assay using transfection of the viral envelope proteins of MV strain Edmonston (IC₅₀ of approximately 3 μM), coincided quite well with the described value for MV Edmonston (6.5 μM). In addition, we obtained similar results for the CDV envelope proteins of strain Onderstepoort (IC₅₀ of approximately 6 μM). These data suggest that compound 1 interacts at a similar site for the CDV-F protein and for the MV-F protein site determined earlier.

In comparison with certain peptides derived from heptad-repeat regions (HR) of the F protein, which imitate the natural interaction between HR-A and HR-B necessary for the conformational change of the F protein, small-molecule inhibitors are not immunogenic, are more soluble, are easier to synthesize and show better gastrointestinal adsorption (Meanwell & Krystal, 2000). In tissue culture experiments compound 1 may replace the fusion-inhibiting peptide (FIP; Z-D-Phe-Phe-Gly where Z is a benzoyloxycarbonyl protection group; Richardson & Choppin, 1983). FIP is widely used for inhibition of morbillivirus-induced cell fusion but its mechanism of action is not clear. In contrast to compound 1, FIP is supposed to interact with the cell membrane and to inhibit the conversion of phospholipid bilayers to the hexagonal phase (Epand, 1986).

Binding of morbillivirus attachment protein H to its host cell receptor simultaneously activates protein F, which will then undergo a structural rearrangement leading to virus–cell or cell–cell membrane fusion. These processes are not only important during acute infections, but may also influence viral spread during persistent infections in the brain. It has been demonstrated that the F protein of wild-type CDV is a major determinant of persistent infection (Plattet et al., 2005, 2009). CDV-A75/17, a virulent demyelinating viral strain isolated from the lymphoid tissues of an experimentally infected dog, spreads efficiently in DBC cultures with extremely limited cell–cell fusion and a low level of cytolysis, whereas the attenuated laboratory strain Onderstepoort induces ample syncytium formation and cell lysis. However, non-cytolytic cell-to-cell spread promotes viral persistence and the development of chronic disease (Vandevelde & Zurbriggen, 2005). In line with these results, it has been documented that haemagglutinin with IC₅₀ of 600 M, which is less effective against CDV-A75/17, coincided quite well with the described value for MV Edmonston (6.5 μM), which is much less effective against CDV-A75/17, coincided quite well with the described value for MV Edmonston (6.5 μM), which was strictly required to allow efficient CDV-A75/17 spread in DBC (Wyss-Fluehmann et al., 2010). However, in the latter study the absence of protein H may have influenced protein F transport competence and/or proper folding of protein F. Here we now extend these results further using one of the fusion inhibitors (compound 1) binding to a cavity in protein F by clearly demonstrating that, in the presence of protein H, protein F is also essential for controlling CDV cell-to-cell lateral spread in brain cell cultures. These data support the notion that cell-to-cell transmission of wild-type CDV in brain cells lacking signalling lymphocyte activation molecule (SLAM) expres-
sion is driven by a classical mechanism of fusion, thereby presumably implicating an as yet unidentified cellular receptor, protein H attachment and subsequent triggering of protein F.

Ribavirin (RBV) is the only approved antiviral that is in use against MV infections of the CNS (SSPE) and NiV infections. It is a water-soluble synthetic nucleoside with broad-spectrum antiviral properties that has been shown to be active in vitro against many viruses (Huffman et al., 1973; Gururangan et al., 1990; Honda et al., 1994; Ishii et al., 1996; Chong et al., 2001; Graci & Cameron, 2006; Elia et al., 2008). Although RBV was used as an antiviral agent during the first NiV outbreak in 1999 in Malaysia, its antiviral properties could not be confirmed in the generally accepted hamster model (Georges-Courbot et al., 2006). In the case of SSPE the treatment with inosiplex (isoprinosine), intraventricular alpha interferon and RBV, which is applied in many cases, may at best prolong the disease course (Weissbrich et al., 2003). Unfortunately, the high concentrations of RBV required have undesirable side effects, e.g. haemolytic anaemia (De Franceschi et al., 2000). Future work will show whether compounds with lower toxicity based on phenyl-2-phenylacetamide may be suitable for application in vivo.

METHODS

Cells, viruses and antibodies. Vero cells (African green monkey; ATCC CRL 6318), Vero cells expressing human CD150 (Vero-hSLAM) and Vero cells expressing canine CD150 (Vero-dogSLAM; Vero-hSLAM and Vero-dogSLAM were gifts of Dr Y. Yanagi, Kyushu University, Fukuoka, Japan) were cultured in Eagle’s minimal essential medium (MEM) containing 5% FCS, 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹. Recombinant MV wild-type strain IC323-eGFP (pMV–IC323-eGFP (Hashimoto et al., 2002)) was propagated using Vero-hSLAM. CDV strain Onderstepoort large plaque was propagated in Vero cells and recombinant CDV wild-type strain A75/17, expressing tomato red (pczCF5–NiVFtag2 and pczCF5–NiVG (Moll et al., 2004)). The CDV strain OND-LP protein H and protein F expressing plasmids used for transfection were pCG–CDV–H and pCG–CDV–F. The MV strain Edmonston protein H- and protein F-expressing plasmids were pCG–H5 and pCG–F1. For transient expression cells were transfected using polyethyleneimine (PEI 25 kDa; Polysciences). Briefly, a monolayer of 4 x 10⁵ Vero cells per well of a six-well plate were washed with pre-warmed medium. Mixtures of 2 µg DNA in 50 µl serum-free medium and 3 µl PEI (1 mg PEI ml⁻¹ in serum-free medium) in 50 µl serum-free medium (incubated for 30 min at room temperature) were added to 1 ml medium per well. Syncytia were quantified after 24 or 48 h of incubation at 37 °C. Compounds were added to the media prior to infection at the indicated concentrations. Phase-contrast photomicrographs were taken of random regions by using a 20 x objective lens with a digital camera (Leica) and the mean number of nuclei per syncytium in each well was calculated from several experiments.

Cell viability assays. MTT (Sigma) is incorporated into living cells and converted into a violet formazan product by mitochondrial dehydrogenases. Only living vital cells and cells in the very early phase of apoptosis can transform MTT to crystals. Vero cells (2.5 x 10⁵) in a six-well plate were pre-incubated for 2 h with the chemical compounds. Medium was removed and the cells incubated for 2 h with 1 ml fresh medium per well (MEM with 5% FCS). After removal of the medium, cells were incubated for 2 h at 37 °C with 750 µl MTT solution per well. The solution was removed and the cells incubated for 45 min at room temperature with 750 µl extraction solution. Twelve 50 µl aliquots were transferred into a 96-well plate and A570 evaluated using an ELISA plate reader. Results (mean values) were presented as percentage viability relative to control cells that were treated with amounts of DMSO equivalent to those used for the compounds.

Expression plasmids, transfection and quantification of cell–cell fusion. NiV glycoprotein genes (F and G; GenBank accession no. AF212302; Malaysian isolate from human brain tissue kindly provided by Pierre Rollin and Tom Ksiazek, Centers for Disease Control and Prevention, Atlanta, GA, USA) were cloned into vector pczCFG, a derivative of the replication-deficient murine leukemia virus, yielding pczCF5–NiFtag2 and pczCF5–NiVG (Moll et al., 2004). The CDV strain OND-LP protein H and protein F expressing plasmids used for transfection were pCG–CDV–H and pCG–CDV–F. The MV strain Edmonston protein H- and protein F-expressing plasmids were pCG–H5 and pCG–F1. For transient expression cells were transfected using polyethyleneimine (PEI 25 kDa; Polysciences). Briefly, a monolayer of 4 x 10⁵ Vero cells per well of a six-well plate were washed with pre-warmed medium. Mixtures of 2 µg DNA in 50 µl serum-free medium and 3 µl PEI (1 mg PEI ml⁻¹ in serum-free medium) in 50 µl serum-free medium (incubated for 30 min at room temperature) were added to 1 ml medium per well. Syncytia were quantified after 24 or 48 h of incubation at 37 °C. Compounds were added to the media prior to infection at the indicated concentrations. Phase-contrast photomicrographs were taken of random regions by using a 20 x objective lens with a digital camera (Leica) and the mean number of nuclei per syncytium in each well was calculated from several experiments.

Synthesis of small-molecule inhibitors. Compounds 1–5 (Fig. 1) were synthesized in a similar way to that which has been described for compound 5, a lead structure of the third generation of the substances oxazole 1 and acetamide 4 (Plomper et al., 2004, 2005; Sun et al., 2006). Compound 1 was synthesized according to Sun et al. (2006), using equimolar amounts of 3-aminobenzonitrile and phenylacetyl chloride in the presence of sodium carbonate. The resulting product was suspended in an aqueous solution of sodium hydroxide (2 M) and refluxed for 30 h. Compound 2 was precipitated by the addition of concentrated hydrochloric acid. For esterification, 1 equiv. of compound 2 and 10 equiv. of thionyl chloride were dissolved in methanol and refluxed for 17 h. The solvent was removed in vacuo and the residue was recrystallized from methanol to yield product 3 (Fig. 1b). Compounds 4 and 5 were synthesized by a two-step procedure, starting off with the amidation of the corresponding benzoic acids. Therefore, 1 equiv. of the corresponding benzoic acid, 5 equiv. of ethyl chloroformate and 7 equiv. of triethylamine were dissolved in absolute acetone under argon atmosphere. The in situ-generated anhydrides were converted into the corresponding primary amides using ammonium carbonate. The final amidation was carried out in tetrahydrofuran (THF), using equimolar amounts of the corresponding amide and phenylacetyl chloride in the presence of 3 equiv. of pyridine to yield compounds 4 and 5. The crude products were purified and isolated by column chromatography (Fig. 1c).

Compounds 1–5 (Fig. 1) were purified and isolated by column chromatography (Fig. 1c).

Phycoerythrin PE-labelled antibodies to CD5, CD14, CD19 and CD69 were obtained from Becton Dickinson.

Phycoerythrin PE-labelled antibodies to CD3, CD14, CD19 and CD69 were obtained from Becton Dickinson.
To determine the toxicity of the compounds in cells growing in suspension (PBMC), incorporation of 7AAD (7AAD staining solution; ebioscience) into damaged cells was measured by flow cytometry. Fluorescence was detected in the far-red range of the spectrum (650 nm long-pass filter).

ACKNOWLEDGEMENTS

We thank the Deutsche Forschungsgemeinschaft and the Elite Netzwerk Bayern for financial support.

REFERENCES


