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A novel and efficient approach to discriminate between pre- and post-transcription HIV inhibitors

Dirk Daelemans, Christophe Pannecouque, George N Pavlakis, Oriana Tabarrini and Erik De Clercq

[1]Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, 3000 Leuven, Belgium (DD, CP, EDC)

[2]Human Retrovirus Section, National Cancer Institute-Frederick, Maryland 21702-1201, USA (GP)

[3]Dipartimento di Chimica e Tecnologia del Farmaco, Univerista di Perugia, Via del Liceo 1, 06123 Perugia, Italy (OT)

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Running title: Efficient approach to study HIV inhibitors

Dirk Daelemans
Rega Institute for Medical Research
Katholieke Universiteit Leuven
Minderbroedersstraat 10
3000 Leuven, Belgium
phone: ++ 32 16 33 21 60
fax: ++ 32 16 33 21 31
e-mail: dirk.daelemans@rega.kuleuven.ac.be

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Abstract

Established anti-HIV treatments are not always effective or well tolerated, highlighting the need for further refinement of antiviral drug design and development. Given the multitude of molecular targets the anti-HIV agents can interact with, studies on the mechanism of action of newly discovered HIV inhibitors are quite elaborative. Here, we describe the development of an efficient reporter system allowing rapid discrimination between a pre- or post-transcriptional mode of action of anti-HIV compounds based on infection by a replication competent HIV-1 molecular clone expressing the green fluorescent protein as part of the nef multiply spliced RNA. Using fluorescence microscopy and flow cytometry, this system enabled us to differentiate between compounds acting at a pre- or post-transciptional level of the virus life cycle. Antiviral activities were determined for four reference compounds as well as one putative novel HIV inhibitor. The obtained results were in agreement with the known characteristics of the reference compounds and revealed that the novel compound interfered with a target prior to or overlapping with HIV transcription. We showed that, during a single replication cycle, compounds inhibiting a molecular target occurring before or coinciding with HIV transcription suppressed GFP expression, while compounds interfering at a later stage, such as protease inhibitors, which act after transcription, did not inhibit GFP expression. This GFP-based reporter system is adaptable for high-throughput screening.

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Introduction

The introduction of potent combinations of antiviral drugs, referred to as highly active antiretroviral therapy (HAART), is a major breakthrough in the treatment of HIV infections ref AVD en KVV. For the treatment of HIV infections, twenty compounds have been formally approved: (i) the nucleoside reverse transcriptase inhibitors (NRTIs) zidovudine, didanosine, zalcitabine, stavudine, lamivudine, abacavir and emtricitabine; (ii) the nucleotide reverse transcriptase inhibitor (NtRTI) tenofovir disoproxil fumarate; (iii) the non-nucleoside reverse transcriptase inhibitors (NNRTIs) nevirapine, delavirdine and efavirenz; (iv) the protease inhibitors saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir, atazanavir, and (v) the viral entry inhibitor enfuvirtide (see ref (De Clercq, 2002) for review). However, HAART is not able to eradicate HIV from treated patients. Drug resistance is a major cause of concern in patients who do not experience complete shut-down of virus replication under HAART. Because of the continuously increasing number of viruses resistant to therapy (Lazzari et al., 2004), the search for new anti-HIV drugs will remain necessary. The development of anti-HIV compounds continues to be very active, and many lead compounds still emerge from initial antiviral screens. Given the large number of molecular targets with which anti-HIV agents can interfere, the investigation of mechanism of action of newly discovered anti-HIV compounds identified through high throughput screening procedures is often elaborative. We seek to develop rapid and efficient methods that allow the identification of the step in the virus replicative cycle affected by a specific compound. We established a reporter assay using a molecular clone that expresses the GFP reporter protein from multiply spliced RNA.

HIV produces three subsets of mRNA: the small multiply spliced species encoding for regulatory and accessory proteins; the partially spliced mRNAs encoding mainly Vpu and Env; and the unspliced mRNA, which encodes Gag and Gag-Pol polyproteins and also serves

as the viral genome encapsidated in two copies into the viral particle. Control of RNA expression is complex and involves the interplay of cis-acting elements with viral transactivators and several cellular proteins. In a first phase of HIV transcription, only multiply spliced mRNAs are expressed in the cytoplasm to produce Tat, Rev, and Nef proteins. Tat augments levels of viral RNA transcripts by increasing transcriptional elongation (Feinberg et al., 1991) and functions through interaction with a cis-acting RNA sequence, the transactivation responsive element (TAR), located at the 5' end of all viral transcripts (Rosen et al., 1985). Rev is required for efficient transport and expression of the unspliced and partially spliced mRNAs expressing the structural and accessory proteins Gag, Pol, Vif, Vpr, Vpu, and Env (Felber et al., 1989). Rev interacts directly with a distinct RNA element, termed the Rev-responsive element (RRE), within the *env* coding region, which is present in the unspliced and partially spliced mRNAs (Hadzopoulou-Cladaras et al., 1989).

The development and characterization of new antivirals depends on appropriate screening assays. A rapid and automated tetrazolium-based colorimetric assay or scoring for cytopathogenicity (CPE) caused by the virus are most frequently used as massive screening tools to identify compounds with anti-HIV activity (Nakashima et al., 1989; Pauwels et al., 1988; Schols et al., 1988). Recently a fluorimetric assay based on the expression of green fluorescent protein (GFP) to analyze HIV reactivation from latency and to screen for inhibitors of the HIV-1 transcription has been described (Kutsch et al., 2002; Kutsch et al., 2004). The development of rapid and efficient screening assays to analyze the mode of function of many lead compounds is important for swift progress in the discovery of better drugs. Here we use a replication-competent recombinant molecular clone of HIV-1 expressing green fluorescent protein (GFP) allowing a contribution to the rapid identification of the viral target of newly discovered inhibitors. The assay was validated with reference compounds and WC-12 (Fig. 1), one of a series of 6-aminoquinolone derivatives recently

shown to exhibit potent anti-HIV activity (Tabarrini et al., 2004). We show that this/our method is able to discriminate between inhibitors acting at the pre- or post transcriptional level. Furthermore, we demonstrate that WC-12 is inhibiting HIV replication by interfering with a transcriptional event. This GFP- based assay to characterize antiviral mechanism of action is rapid, reliable, sensitive, efficient and thus accelerates the evaluation of new drug candidates.

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Materials and Methods

Compounds

Dextran Sulfate [DS, average molecular weight (MW) 5000] was purchased from Sigma (Bornem, Belgium). Nevirapine was obtained from Boehringer Ingelheim (Ridgefield, CN). 3'-Azido-3'-deoxythymidine (AZT) was synthesized according to the method described by Horwitz *et al*. (Horwitz et al., 1964). Ritonavir (ABT538) was obtained from Abbott Laboratories (Abbott Park, IL.). L-708,906 was a kind gift from Dr. D. Hazuda (Merck, NJ) (Hazuda et al., 2000). WC-12 was synthesized and kindly supplied by O. Tabarrini and A. Fravolini.

Cells and Virus

MT-4 (Miyoshi et al., 1981), C8166 (Salahuddin et al., 1983), and CEM (Foley et al., 1965) cells were grown and maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 0.1% sodium bicarbonate and 20 μg gentamicin per ml.

The HIV-1(III_B) strain was provided by R.C. Gallo and M. Popovic (Popovic et al., 1984). The NL4-3.GFP11 strain expressing an enhanced version of GFP (Stauber et al., 1998) instead of Nef has been described previously (Valentin et al., 1998). For all tests described NL4-3.GFP11 virus was obtained from transfection of 293T cells with the molecular clone. Then 1 ml of virus containing supernatant was used to infect 8 x 10^6 MT-4 cells in 40 ml of culture medium. Three days after infection, supernatant was collected and used as viral input in the respective assays.

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In Vitro Antiviral Assays

Evaluation of the antiviral activity of the compounds against HIV-1 strain III_B in MT-4 cells was performed using the MTT assay as previously described (Pauwels et al., 1988). Stock solutions (10 x final concentration) of test compounds were added in 25-µl volumes to two series of triplicate wells so as to allow simultaneous evaluation of their effects on mock-and HIV-infected cells at the beginning of each experiment. Serial 5-fold dilutions of test compounds were made directly in flat-bottomed 96-well microtiter trays using a Biomek 2000 robot (Beckman instruments, Fullerton, CA). Untreated HIV-and mock-infected cell samples were included as controls. HIV-1(III_B) (Popovic et al., 1984) stock (50 µl) at 100-300 $CCID_{50}$ (50 % cell culture infectious doses) or culture medium was added to either the infected or mock-infected wells of the microtiter tray. Mock-infected cells were used to evaluate the effects of test compound on uninfected cells in order to assess the cytotoxicity of the test compounds. Exponentially growing MT-4 cells (Miyoshi et al., 1981) were centrifuged for 5 minutes at 1000 rpm and the supernatant was discarded. The MT-4 cells were resuspended at 6 x 10^5 cells/ml, and 50-µl volumes were transferred to the microtiter tray wells. Five days after infection, the viability of mock-and HIV-infected cells was examined spectrophotometrically using the MTT assay. The MTT assay is based on the reduction of yellow coloured 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Acros Organics, Geel, Belgium) by mitochondrial dehydrogenase of metabolically active cells to a blue-purple formazan that can be measured spectrophotometrically. The absorbances were read in an eight-channel computer-controlled photometer (Multiscan Ascent Reader, Labsystems, Helsinki, Finland), at two wavelengths (540 and 690 nm). All data were calculated using the median OD (optical density) value of three wells. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration of the test compound that reduced the absorbance (OD540) of the mock-infected control sample by 50%. The concentration

achieving 50% protection against the cytopathic effect of the virus in infected cells was defined as the 50% effective concentration (EC_{50}).

Evaluation of the antiviral activity of the compounds against NL4-3.GFP11 in C8166 cells was performed using flow cytometry (see below), HIV-1 core antigen (p24 Ag) in the supernatant was analyzed by the p24 Ag enzyme-linked immunosorbent assay (PE, Brussels, Belgium).

Flow cytometry

Flow cytometric analysis was performed on a FACSCalibur flow cytometer equipped with a 488 nm argon-ion laser and a 530/30 nm bandpass filter (FL1: detection of GFP associated fluorescence) (Becton Dickinson, San Jose, CA). Before acquisition, cells were pelleted at 1000 rpm for 10 min and fixed in 3% paraformaldehyde solution. Acquisition was stopped when 10,000 events were counted. Data analysis was carried out with Cell Quest software (BD Biosciences). Cell debris was excluded from the analysis by gating on forward versus side scatter dot plots.

Microscopy

Cells were imaged in culture medium through a Zeiss Axiovert 40 CFL inverted microscope through a LD A-Plan 20x/0.30 objective using a Canon Powershot G5 camera. GFP fluorescence was detected using a filter set with band-pass 450-490 nm for excitation and long pass 515 nm for emission.

Time-of-addition experiments

Time-of-addition experiments were adapted from ref (Pauwels et al., 1990). Briefly, C8166 cells were infected with NL4-3.GFP11 (222,612 pg/ml). Following a 1 hour adsorption

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period cells were distributed in a 96-well tray at 45,000 cells/well and incubated at 37°C. Test compounds were added at different times (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 24, and 25h) after infection. Dextran sulfate was used at 100 µg/ml, and nevirapine at 2 µg/ml. The number of GFP-expressing cells was monitored by FACS analysis at 30h after infection.

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Results

Growth kinetics of the recombinant NL4-3.GFP11 in different cell lines

To analyze the mode of function of potential anti-HIV drugs, we used a reporter virus based on a replication-competent HIV-1 molecular clone (NL4-3) tagged with an enhanced mutant form of the green fluorescent protein (GFP11) (Palm et al., 1997; Stauber et al., 1998). In this clone the *nef* gene in the proviral plasmid NL4-3 (Adachi et al., 1986) was replaced with the GFP11 coding sequence (Fig. 2). Therefore, GFP is expressed from the nef multiply spliced viral mRNAs. Recombinant virus derived from this molecular clone was produced in 293T cells. It replicated well in cell culture and transduced the GFP gene to the host cells. Infected cells fluoresced brightly when measured by flow cytometry or fluorescence microscopy (Fig. 3) providing a direct and quantitative marker for HIV-1 infection in individual live cells.

The recombinant virus NL4-3.GFP11 was propagated in MT-4 cells and was subsequently tested for growth characteristics in different cell lines. MT-4, C8166, and CEM cells were infected with different amounts of virus and the ratio of infected cells expressing GFP was determined over a period of 5 days (Fig. 4). Different growth kinetics were measured in different cell lines. The recombinant NL4-3.GFP11 replicated best in C8166 cells followed by replication in MT-4 cells, whereas the number of GFP-expressing CEM cells was much lower. The reason for the lower replication efficiency of NL4-3.GFP11 in CEM cells is yet unknown. We therefore decided to use C8166 for further mechanism of action studies.

NL4-3.GFP11 for the detection of novel anti-HIV compounds

On the basis of quantitative assessment of the GFP expression of the recombinant virus, we established a novel detection system for the evaluation of HIV antiviral activities. The GFP-based antiviral assay was compared to the conventional tetrazolium-based colorimetric assay

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(Pauwels et al., 1988). To this end, a number of anti-HIV inhibitors were tested for their antiviral effects against NL4-3.GFP11. The human T cells C8166 were infected with NL4-3.GFP11 in the presence of inhibitors of HIV replication and 3 days after infection both p24 production in the supernatant and the number of GFP expressing cells were assessed. The well-characterized HIV inhibitors nevirapine (NNRTI), L-708,906 (IN) (Hazuda et al., 2000) and ritonavir (PI) were used in these model studies. Each of the compounds caused a strong reduction in the number of GFP expressing cells as compared to the untreated infected controls (Fig 5). This reduction correlated well with the decrease in soluble p24 production. Furthermore, the calculated IC_{50}'s for the reference compounds in this GFP-based assay were similar to the IC_{50}'s obtained by a previously established tetrazolium-based colorimetric assay (Pauwels et al., 1988) (Table I). Therefore, this GFP-based assay is a reliable assay for the detection of anti-HIV drugs.

We next evaluated the anti-HIV activity of WC-12 (Tabarrini et al., 2004). WC-12 is one of a series of 6-aminoquinolone derivatives that has been recently shown to inhibit HIV replication in MT-4, CEM cells and PBMCs. However its exact antiviral mechanism of action has not been fully elucidated. Treatment of NL4-3.GFP11-infected C8166 cells with WC-12 resulted in a decrease in the level of GFP expression (Fig. 5C). The observed inhibitory effect was concentration-dependent. At 0.4 µg/ml WC-12 decreased the number of GFP-expressing cells by 56% and at 2 µg/ml the level of GFP-expressing cells was decreased by 96%, however, at this concentration only 40% of the cells were viable. The number of GFP-expressing cells correlated well with the amount of soluble p24 in the cell supernatant indicating that GFP-expression is a good measure of virus production.

Determination of the step in the virus cycle affected by the action of new anti-HIV drugs

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In NL4-3.GFP11, the *GFP* gene is expressed from the multiply-spliced nef mRNAs. Therefore, GFP expression from this clone enabled us to determine whether an inhibitor interferes with a target before or after the expression of multiply spliced mRNA. To study the effect of the well-characterized HIV inhibitors on the GFP expression during a single round of infection, C8166 cells were infected with NL4-3.GFP11 in the presence of inhibitors of the viral entry (DS5000), reverse transcription (nevirapine), integration (L-708,906), or protease cleavage (ritonavir). Cells were harvested 24h after infection (time required for a single round of replication) and the number of GFP-expressing cells was monitored by flow cytometry (Fig. 6). Toxicity of the compounds was assessed from the forward versus side scatter dot plots. Cell debris was excluded from the analysis by gating on forward versus side scatter dot plots (R1). As expected, DS5000 (0.57%), nevirapine (1.70%) and L-708,906 (3.00%) caused a dramatic decrease in GFP-expressing cells compared to the untreated control (9.30 % GFP expressing cells). In contrast, in cultures treated with ritonavir the number of GFP-expressing cells (9.19%) was comparable to the untreated control. These results are consistent with the conclusion that, in a single infection round inhibitors interfering with a viral target occurring before the expression of multiply-spliced mRNA inhibit the expression of GFP, while drugs interfering with a target functioning after the expression of multiply-spliced RNA (e.g. protease) do not inhibit GFP expression. Therefore, this assay should be suitable for the rapid determination of mechanism of action of new anti-HIV inhibitors. In fact, when WC-12 was evaluated in the assay (Fig. 6), it behaved like DS5000, nevirapine and L-708,906 in that it inhibited the expression of GFP, thus pointing to a target situated before the expression of multiply-spliced RNA.

Determination of target of action of new anti-HIV drugs

To more accurately pinpoint which target along the HIV replicative cycle is affected by candidate drugs, a time-of-addition experiment was set up (Fig. 7). The cells were infected at high multiplicity of infection and the compounds were added at 1, 2, 3,.., 9 hours after infection as indicated. GFP expression was measured by flow cytometry at 30 hours after infection. Depending on the target of drug action, addition of the compounds could be delayed for a certain number of hours characteristic for each compound without loss of antiviral activity. Dextran sulfate, which interacts with the virus adsorption (Baba et al., 1988; Mitsuya et al., 1988), must be added together with the virus (= 0 hours) to be active; addition at 1 hr or later post infection did not block viral replication because adsorption had already occurred at this time. For AZT and nevirapine the addition could be delayed for about 5 hours. Addition of L-708,906 could be postponed for 6 to 8 hours before an increase of the number of infected cells could be detected (Fig. 7). This time is accepted to coincide with the last step of the HIV integration event, namely strand transfer (Pannecouque et al., 2002). Addition of WC-12 can be postponed even longer suggesting a target of interaction happening later than the integration process. We show that the developed assay can be easily adapted to accurately pinpoint the site of interaction of candidate HIV inhibitors.

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Discussion

The increasing number of HIV-1 strains resistant to the current antiretroviral drugs necessitates the development of new drugs and hence new and rapid evaluation assays. Here we show the successful use of a recombinant HIV molecular clone expressing GFP for the discrimination between a pre- or post-transcriptional antiviral mechanism of action. We showed that GFP production was strictly proportional to virus replication and could be successfully used as a measure for virus production in antiviral screening assays. Viral replication was detected by fluorescence microscopy and quantified by flow cytometry. Advantages of the GFP-based antiviral assay are the simplicity of handling, the ease and reliability of quantification, and the rapid testing, *in casu* 3-day measurements. The well-characterized HIV inhibitor, dextran sulfate, nevirapine, L-708,906 (Hazuda et al., 2000) and ritonavir efficiently inhibited viral replication from NL4-3.GFP11-infected C8166 cells as measured by GFP expression and p24 Gag ELISA. The 50% effective concentration (IC_{50}) data resulting from GFP expression analyses were similar to IC_{50} values from a previously established tetrazolium-based colorimetric assay (Pauwels et al., 1988). In addition, GFP expression correlated well with p24 production suggesting that GFP expression is a reliable measure for HIV replication (Table I).

In NL4-3.GFP11 the *nef* gene is replaced by *gfp*, which is consequently expressed form multiply spliced RNA. Similar constructs have been described earlier (Lee et al., 1997; Page et al., 1997). Therefore, in a single round of infection, inhibitors of the viral replication acting at a stage occurring before the expression of multiply spliced viral RNA (e.g. virus binding, reverse transcription, integration or transcription) will actively suppress GFP expression. Inhibitors interfering with a target acting after the expression of multiply spliced viral RNA will not inhibit GFP expression. This was evident from our experiments with well-established HIV inhibitors. Dextran sulfate, nevirapine, L-708,906 (Hazuda et al., 2000)

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inhibited GFP expression from cells infected with NL4-3.GFP11 and analyzed for GFP expression one day after infection. The protease inhibitor, ritonavir, did not inhibit GFP expression as compared to the untreated control. This is consistent with the fact that protease inhibitors act after viral RNA expression.

To determine more specifically at which stage of the HIV replication cycle a compound interferes we performed time-of-addition experiments. Cells were infected at high virus multiplicity of infection to ensure that the virus replicative steps would be synchronized in the whole cell population, and the compounds were added with time lags of up to 9 hours after infection. Depending on the stage of interaction, addition of compounds could be delayed for a specific number of hours without loss of antiviral activity. For example, addition of the NNRTI nevirapine could be delayed until 5 hours after infection. We clearly show that this assay is useful for the rapid and efficient determination of mechanism of action of new anti-HIV drugs. Therefore, using this simple and rapid GFP-based assay we are not only able to rapidly determine whether the candidate HIV inhibitor acts pre- or post-transciptionally. Using the time of addition method, this assay allows a more precise identification of the step in virus cycle affected by a drug. Therefore, we propose that this assay can streamline the characterization of the mode of action of many new candidate compounds against HIV.

In C8166 cells, 0.4 µg/ml WC-12 down regulated HIV expression by more than 60%, as measured by GFP fluorescence levels and p24 Gag ELISA, without any apparent toxicity (Fig. 5). WC-12 is one out of a series of 6-aminoquinolone derivatives that has been recently shown to inhibit HIV replication in MT-4, CEM cells and PBMCs (Tabarrini et al., 2004). Time-of-addition experiments suggest that these molecules inhibit the HIV long terminal repeat-driven transcription. In fact, other quinolone derivatives have been demonstrated to inhibit the HIV transcription process (Baba et al., 1998; Baba et al., 1997; Okamoto et al.,

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2000; Parolin et al., 2003; Richter et al., 2004a; Richter et al., 2004b; Witvrouw et al., 1998). Using our newly developed assay, we could establish that WC-12 inhibits the HIV replication by interfering with a target coinciding with the HIV transcription process (Fig. 6 and 7).

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Footnotes

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Figure legends

Figure 1

Structure of WC-12

Figure 2

Schematic diagram showing the recombinant NL4.3 genome with an insertion of the *gfp* gene in the *nef* open reading frame.

Figure 3

Infection of MT-4 and C8166 cells by NL4-3.GFP11 as visualized by light microscopy (upper panels) and fluorescence microscopy (lower panels).

Figure 4

Kinetics of viral replication in different cell lines. MT-4, C8166, and CEM cells were infected with different amounts of NL4-.3.GFP11 and GFP expressing cells were monitored by flow cytometry at different times after infection.

Figure 5

Concentration-dependent HIV-inhibitory effects of reference compounds and WC-12 and determination of cytotoxicity. C8166 cells were infected with NL4-3.GFP11 (8904 pg/ml) and treated with known and putative antivirals at a range of concentrations as indicated. Virus replication was assayed for both p24 production and GFP expression at 3 days post infection. One hundred percent of GFP expression or p24 levels were defined for the

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infection control (no compound). For the determination of cytotoxicity , uninfected controls were analyzed by MTT.

Figure 6

Determination of pre- or post-transcriptional mechanism of action of new anti-HIV drugs. Cells were infected with NL4-3.GFP11 (222,612 pg/ml). Twenty four hours after infection, cells were analyzed for GFP expression as marker for infection by flow cytometry. Levels of GFP expression were then quantified by flow-cytometric analysis.

Figure 7

Time-of-addition experiment. C8166 cells were infected with NL4-3.GFP11 (222,612 pg/ml), and test compounds were added at different times after infection. Virus-associated GFP-expression was measured by flow cytometry at 30 h post infection. The graph is representative of two different experiments

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Table I. Comparison of activities of reference compounds in the different assays

Compound	EC_{50} (µg/ml) Assay			CC_{50} (µg/ml)[a]
	MTT	GFP expression	p24 production	
Nevirapine	0.014 ± 0.006	0.028 ± 0.005	0.008 ± 0.002	> 4
L-708,906	2.01 ± 1.63	0.98 ± 0.08	0.93 ± 0.24	≥27.7
WC-12	0.27 ± 0.14	0.27 ± 0.04	0.11 ± 0.06	3.83 ± 0.98
Ritonavir	0.04 ± 0.02	0.02 ± 0.01	0.015 ± 0.004	> 20

[a] Toxicity was determined in parallel using the MTT-assay

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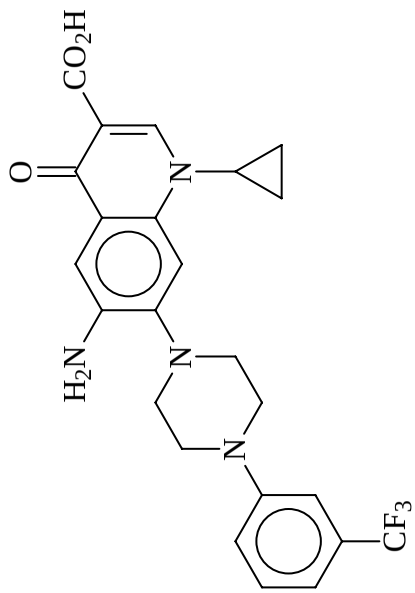
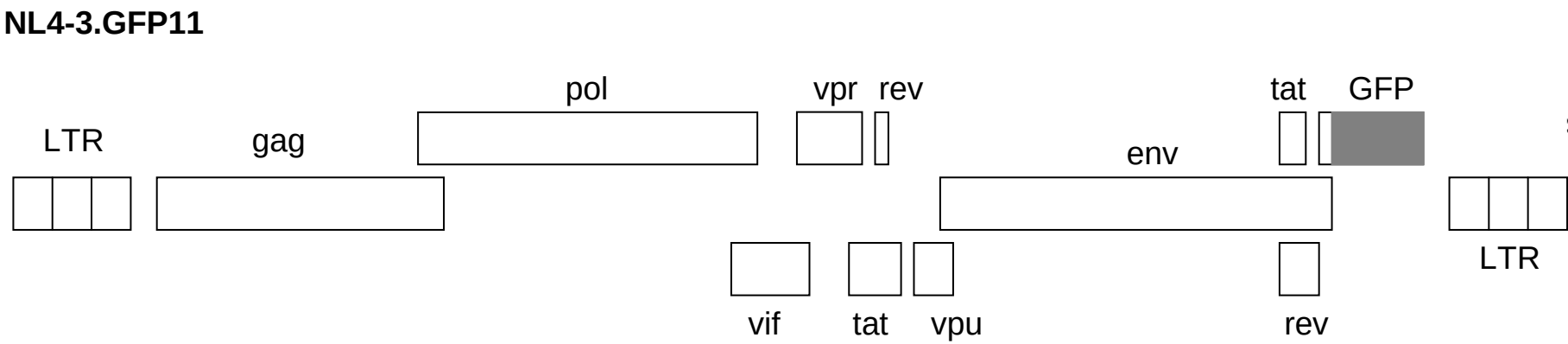


Figure 1

Figure 2



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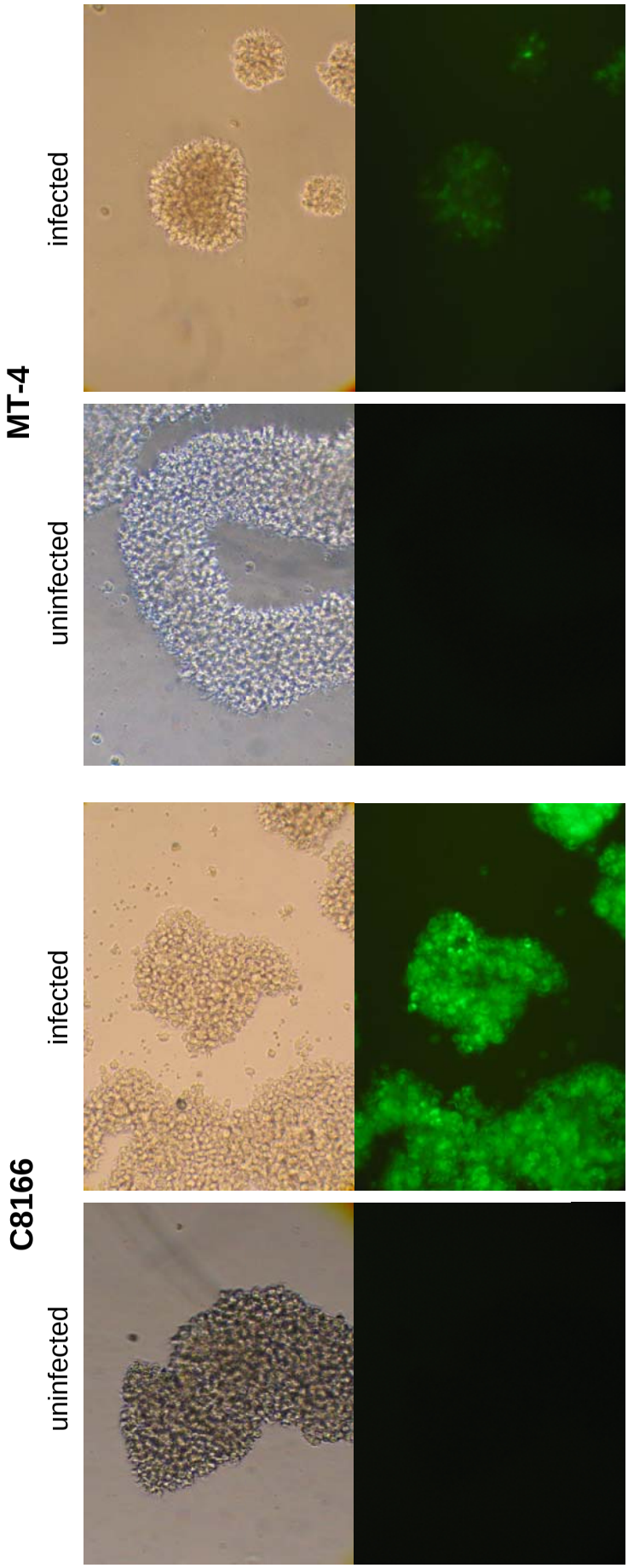


Figure 3

Figure 4

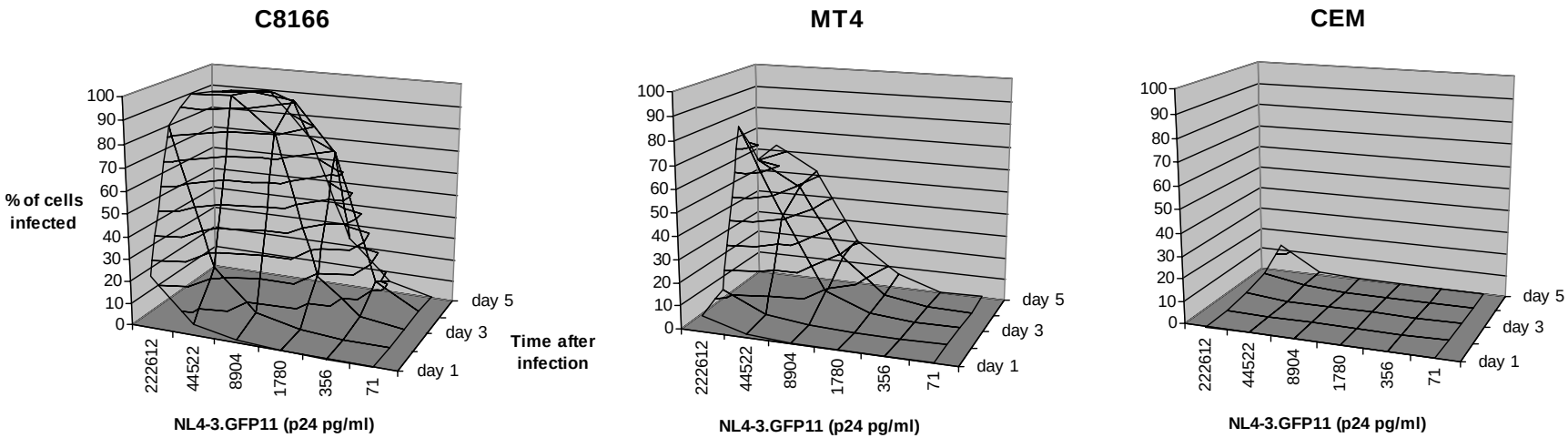
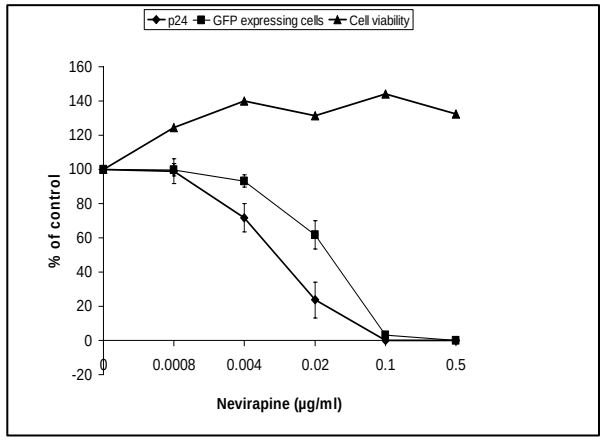
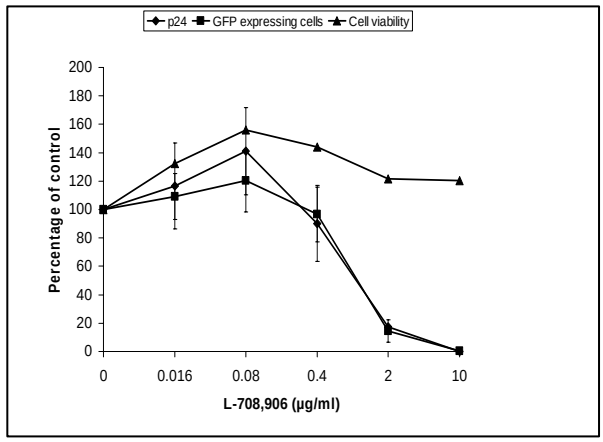
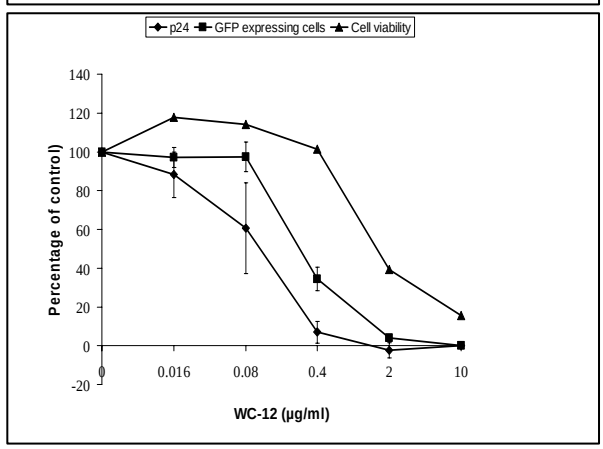


Figure 5


p24
GFP expressing cells
Cell viability
% of control
160
140
120
100
80
60
40
20
0
-20
0
0.0008
0.004
0.02
0.1
0.5
Nevirapine (µg/ml)


p24
GFP expressing cells
Cell viability
Percentage of control
200
180
160
140
120
100
80
60
40
20
0
0
0.016
0.08
0.4
2
10
L-708,906 (µg/ml)


p24
GFP expressing cells
Cell viability
Percentage of control)
140
120
100
80
60
40
20
0
-20
0
0.016
0.08
0.4
2
10
WC-12 (µg/ml)

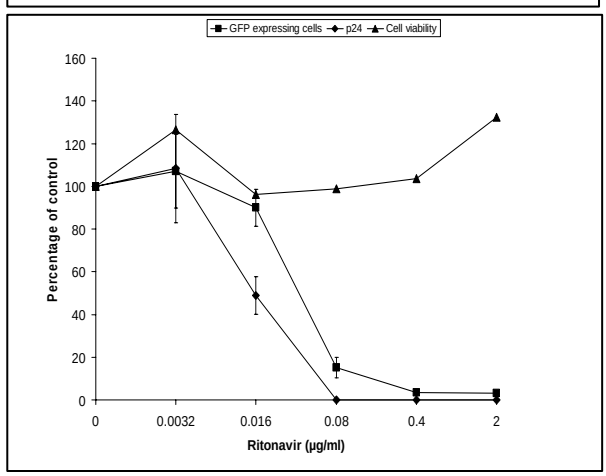

GFP expressing cells
p24
Cell viability
Percentage of control
160
140
120
100
80
60
40
20
0
0
0.0032
0.016
0.08
0.4
2
Ritonavir (µg/ml)

Figure 6

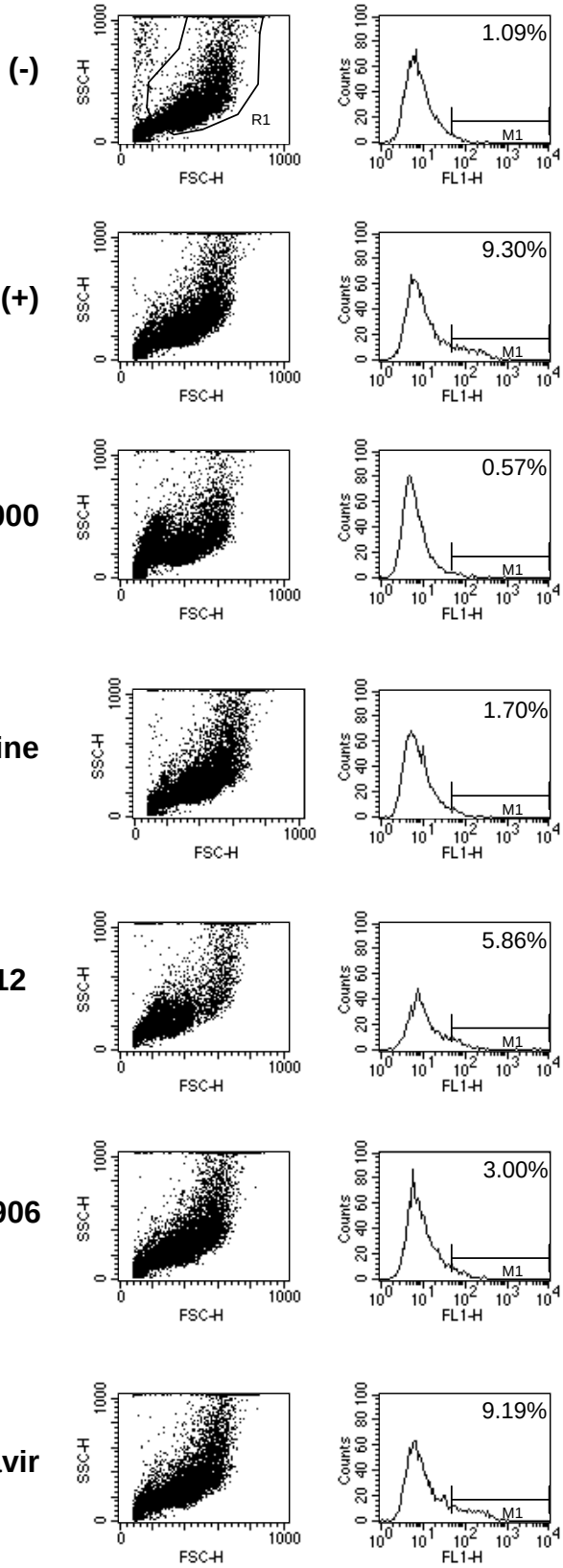

(-)
(+)
DS5000
Nevirapine
WC-12
L-708,906
Ritonavir
SSC-H
FSC-H
R1
Counts
FL1-H
M1
1.09%
9.30%
0.57%
1.70%
5.86%
3.00%
9.19%

Figure 7