Coupling the Inhibition of Viral Transduction with a Positive Fluorescence Signal

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Abstract: Cell-based assays for the inhibition of viral infections most commonly couple a positive signal (e.g., an increase in fluorescence) to the infection itself and not to its inhibition. When performing drug screens, compounds decreasing the signal are therefore considered as putative inhibitors. However, this approach can cause the selection of many false positives, since, for example, both killing of the host cell and inhibiting viral cell-entry results in the same signal. Using a model system based on murine leukemia virus (MLV) particles pseudotyped with the G-protein of vesicular stomatitis virus (VSV-G), we have developed generic assays coupling a positive readout to the inhibition of viral transduction. Consequently, the system favors drug candidates (and concentrations thereof) that do not harm human cells and significantly decreases the probability for selecting false positives. The assay allows Z-factors of ~0.9, takes cytotoxic side effects into account and could in theory be adapted for high-throughput screening of inhibitors against further viral species.

Keywords: High throughput screening, antivirals, cell-entry inhibitors, pseudotyped viruses, fluorescence assay.

INTRODUCTION

Viral infections are a continuing threat to health throughout the world. The number of fatalities due to human immunodeficiency virus (HIV) alone was more than two million in 2007 [1]. Furthermore, new viral species such as the "Mexican Flu" or the avian influenza virus (often referred to as "bird flu") continue to be identified and can become extremely dangerous for humans [2-4]. Therefore, the identification of novel antiviral drugs is of high importance and requires tailored screening systems. In general, there are two classes of assays for high throughput screening (HTS) of drug candidates: biochemical assays and cell-based assays. Biochemical assays focus on single purified drug targets. However, this rather artificial environment might result in the selection of compounds having no or adverse effects under physiological conditions. Furthermore, biochemical screens require well-characterized drug targets. In contrast, cell-based assays do not require detailed knowledge about the involved drug targets (potentially several in a single screen) and reflect the clinical situation more closely.

A crucial step in the setup of any cell-based assay is the coupling of the desired effect to an easily detectable signal. In cell-based assays for the inhibition of viral infection, most commonly, a positive readout signal (e.g., an increase in fluorescence) is coupled to the infection itself and not to its inhibition. These systems are based on the expression of a reporter gene (e.g., *GFP, YFP*) within the host cell upon viral cell-entry [5-8]. When screening for potential inhibitors of viral infection, viral particles and host cells are incubated in the presence of the compound to be tested. Subsequently, the reporter signal is determined (e.g., by measuring fluorescence). A decreased signal of a given sample (in comparison to the control sample without any test compound) should hence result from a potent inhibitor of viral cell-entry. However, a drug candidate that inhibits the reporter gene expression (e.g., by killing the host cell) instead of the viral cell-entry will inevitably be selected as a false positive.

Therefore, coupling a positive signal with the inhibition of viral infection seems to be much more desirable. When using viral species mediating severe cytopathic effects (CPEs), this can be achieved by determining cell survival subsequent to the co-incubation of host cells, virus and the drug candidate [9, 10]. However, these approaches are not well suited for high-throughput screening since they usually require microscopic analysis of the samples and the use of replication competent viruses. This not only results in the need for high containment level laboratories but also makes it impossible to distinguish between inhibition of cell-entry and replication. Furthermore, many viruses do not show detectable CPEs in cell culture experiments [11-13].

To circumvent these limitations, we have developed a generic assay system coupling a positive readout signal with the inhibition of viral infection, independently of any CPE. To do so, we have established recombinant indicator cells which generate a strong fluorescence signal unless transduced by non-replication competent pseudotype particles. The system should be adaptable for the screening of inhibitors against a variety of viral species and requires minimal requirements in terms of the containment level.

EXPERIMENTAL

Cells

Puromycin-resistant HEK293T-tPA cells expressing a membrane-bound and HA-tagged form of tissue

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plasminogen activator [14] were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL) supplemented with 10% (v/v) fetal bovine serum (GIBC0-BRL). Cells were incubated at 37°C in an 8% CO_2 atmosphere saturated with water.

Plasmid Construction

The plasmids pMD-G, encoding the vesicular stomatitis virus G-protein (VSV-G), and pOGP3, encoding MLV gag/pol have been described previously [15]. The MLV-packagable plasmids encoding shRNA targeting either the puromycin *N*-acetyltransferase (pSiren-shRNA-puro) or the HA-tPA fusion protein (pSiren-shRNA-tPA) were generated by direct insertion of double stranded oligonucleotides into the BamHI- and EcoRI-digested vector pSiren-RetroQ (Clontech). Specifically, the following oligonucleotide sequences were used: shPuro(+), 5'-GATCCGCTGCAAGA ACTCTTCCTCATTCAAGAGATGAGGAAGAGTTCTTG CAGTTTTTTACGCGTG-3';shPuro(-),5'-AATTCACGCG TAAAAAACTGCAAGAACTCTTCCTCATCTCTTGAAT GAGGAAGAGTTCTTGCAGCG-3';shtPA(+) 5'-GATCCG GCCACACTCCTTGCCCTTTTTCAAGAGAAAAGGGCA AGGAGTGTGGCTTTTTTACGCGTG-3';shtPA(-)5'-AAT TCACGCGTAAAAAAGCCACACTCCTTGCCCTTTTCT CTTGAAAAAGGGCAAGGAGTGTGGCCG-3'. For the generation of pSIREN-shRNA-puro, the puromycin *N*-acetyltransferase gene in the backbone of the vector was additionally inactivated by digestion with BsiWI, generation of blunt ends using the Klenow polymerase and subsequent religation (out of frame). The plasmid MP71 (encoding a HSV-TK-CD34 fusion protein [16] was a kind gift of Christopher Baum.

Production of Effector Particles

Murine leukemia virus particles pseudotyped with the G-protein of vesicular stomatitis virus were generated by calcium phosphate transfection of HEK293T cells [17]. One day prior to transfection, $1.1x10^7$ cells were seeded in 175-cm^2 culture flasks (Nunc). The next day, triple transfection was performed using a total of 42 µg of plasmid DNA (*env:gag/pol*:transfer vector = 1:2:2). 24 h post transfection, the medium was replaced by 12 mL of fresh DMEM media. 48 h and 72 h post transfection the supernatants were collected, centrifuged at 340 g and filtered through a 0.45 µm filter. The filtered supernatant was subsequently concentrated by ultracentrifugation through a sucrose cushion (25%) for 2 h at 30000 rpm. This procedure resulted in an average viral titer of $2,5x10^7$ i.u/mL as determined by X-Gal assays (using MLV-(VSV-G) particles having packaged a vector encoding lacZ).

Assays

For all assays, HEK293T-tPA cells were seeded in 96 well plates ($2x10^4$ cells/well) pretreated with poly-L-lysine (Becton Dickinson). After 24 h of incubation, at 37°C cultures were pre-incubated for 4 h with AZT at the indicated concentrations. Subsequently, the transduction step was performed using concentrated virus samples (~$2,5x10^7$ i.U./mL). After 2 h of incubation, the medium was replaced by fresh DMEM supplemented with AZT at the indicated concentrations. Compounds inducing cell death (35 µg/mL puromycin (Sigma) for α-Puro effector particles or 40 µM ganciclovir (Sigma) for TK effector particles) were added to the medium on day 1 and on day 3 post transfection. On day 4 post transfection, the fluorescence readout was initiated by addition of 1 mM HDVLK-Amc (Bachem) and 1.67 µM plasminogen (Roche). All measurements were performed with excitation and emission wavelengths of 370 nm and 450 nm, respectively, using a Spectramax M5 microplate reader (Molecular Devices). For assays based on α-tPA effector particles, the readout was performed on day 2 post transfection without prior addition of any toxic compound.

Assays coupling a positive fluorescence signal with the transduction itself (instead of its inhibition) were performed using effector particles having packaged a vector encoding *lacZ*. In this case, the fluorescence readout was initiated on day 4 post transfection by addition of 100 µM Fluorescein di-ß-D-galactopyranoside (FDG; Sigma). These measurements were performed using excitation and emission wavelengths of 571 nm and 590 nm, respectively.

Determination of Z-Factors

Z-Factors were calculated for samples in 96-well plates using the following equation [18]:

$$Z - factor = 1 - \frac{3x(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}$$

σ = standard deviation

μ = mean signal

$x_{(p)}$ = parameter of the positive control

$x_{(n)}$ = parameter of the negative control

RESULTS

General Setup of the Assay

The goal of this work was to set up an assay system coupling a positive signal with the inhibition of viral infection. This can be achieved using engineered host cells (indicator cells) constitutively expressing a reporter gene unless infected by engineered viral particles (effector particles). To implement this idea we used HEK293T cell-derived indicator cells stably expressing a membrane-bound and HA-tagged form of the human tissue plasminogen activator (HA-tPA) [14]. This enzyme converts plasminogen into plasmin which itself allows the conversion of a non-fluororescent substrate (HDVLK-Amc, Bachem, Switzerland) into a fluorescent product (Fig. **1a**). Furthermore, the HA-tag can be used for antibody-based stainings as an alternative readout system. As effector particles, we chose the murine leukemia virus (MLV). This virus seemed to be an ideal model system, since it can be pseudotyped with a variety of heterologous envelope proteins, resulting in particles with the host cell tropism of the corresponding species [19]. Once established, an MLV-based system should therefore be adaptable for the screening of inhibitors against a variety of viral species. Furthermore, the use of non replication-competent pseudotype particles should allow keeping the containment level low. To obtain effector particles capable of entering our HEK293T-derived indicator

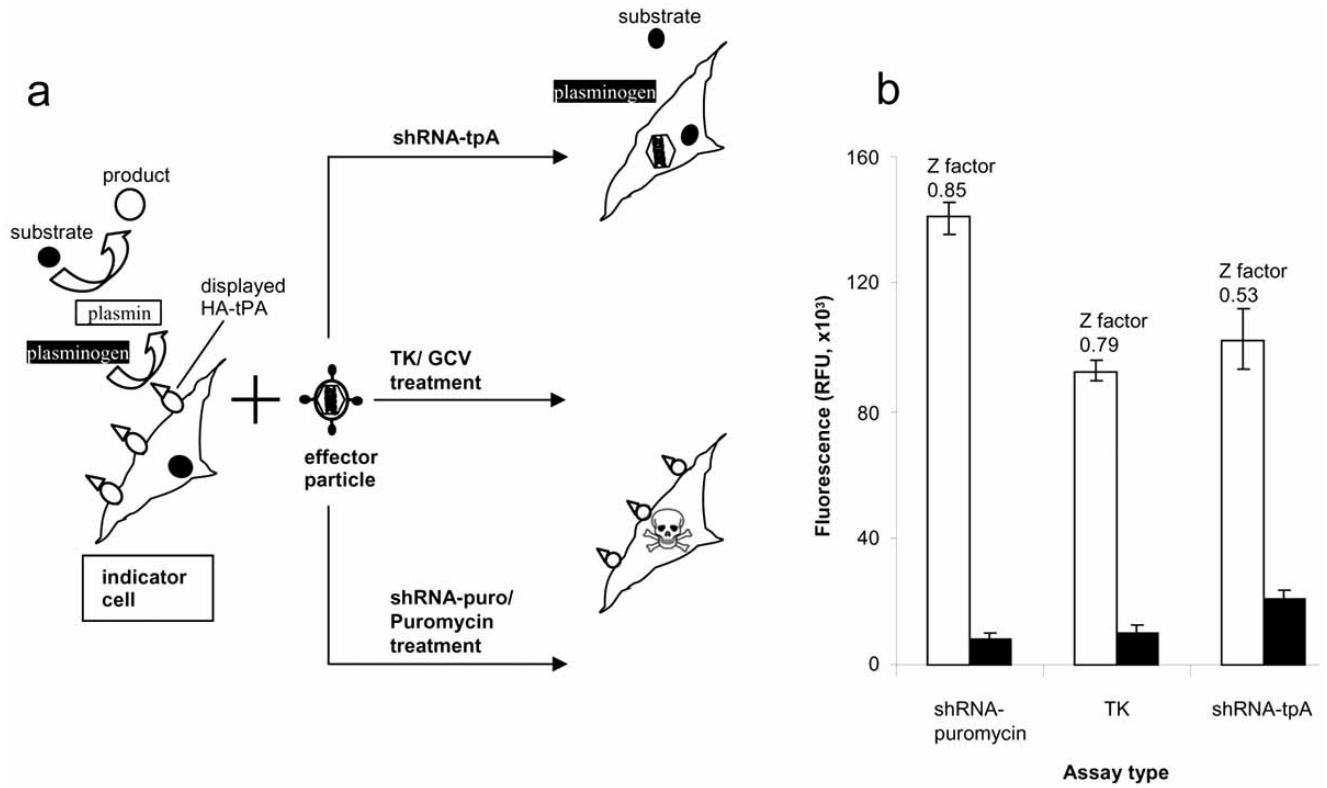

Fig. (1). Coupling a positive fluorescence signal to the inhibition of viral cell entry. (**a**) Human indicator cells displaying a membrane-bound and HA-tagged form of the tissue plasminogen activator (HA-tPA) convert plasminogen into plasmin, which in turn converts a non-fluorescent substrate into a fluorescent product. This reporter gene signal can be shut down upon viral cell-entry using three different types of effector particles. Particles having packaged a vector encoding shRNA mediating the degradation of HA-tPA mRNA (α-tPA) enter the indicator cells and decrease the expression of the reporter gene. Particles having packaged a vector encoding herpes Simplex virus thymidine kinase (HSV-TK) enter the indicator cells and mediate cell death upon the addition of Ganciclovir (GCV). Particles having packaged a vector encoding shRNA targeting the puromycin resistance of the indicator cells (α-Puro) enter the indicator cells and mediate cell death upon the addition of puromycin. (**b**) Fluorescence signals for the three different particle types. Indicator cells were incubated with the corresponding particles in the presence (white) and absence (black) of 25 μM AZT. Subsequently, the fluorescence signals (y-axis) were determined using a plate reader.

cells [14], we pseudotyped MLV particles with the G-protein of vesicular stomatitis virus (MLV(VSV-G Env)). Furthermore, we compared three different approaches based on RNA interference (RNAi [20]) and suicide genes for the efficient shut down of the reporter gene upon cell-entry (Fig. **1a**): First, we generated effector particles having packaged a vector encoding short hairpin RNA (shRNA) mediating the degradation of HA-tPA mRNA (α-tPA particles). This way, the reporter enzyme should be downregulated upon viral entry into the indicator cells. Second, we produced effector particles transducing a suicide gene (herpes-Simplex virus thymidine kinase; TK particles) which, upon addition of the corresponding substrate (ganciclovir), mediates cell death of the indicator cells. Consequently, the reporter gene activity should be eliminated as well as unspecific conversion of the fluorogenic substrate due to other cellular enzymes. Finally, we also generated effector particles having packaged a vector encoding shRNA abolishing the puromycin resistance of the indicator cells (α-Puro particles). Hence, in the presence of puromycin the indicator cells should be killed efficiently. In summary, all three types of effector particles should mediate a strongly decreased reporter gene signal upon cell-entry, whereas non-transduced cells should show the maximum signal intensity.

To prove this hypothesis, we incubated indicator cells with the three types of effector particles in the presence and absence of 25 μM AZT. This well-characterized inhibitor of reverse transcriptase should efficiently inhibit transduction in the corresponding samples. During the following days we then performed the fluorescence readout. For each type of effector particle, we determined the optimal time point (resulting in the highest difference of the fluorescence signal for samples with and without AZT) for the fluorescence readout and the addition of compounds (ganciclovir or puromycin) mediating cell death (for HSV-TK particles and α-Puro particles). Subsequently the best results for each kind of effector particles were compared to determine the most powerful assay system (Fig. **1b**). While all types of particles mediated a high fluorescence signal in the presence of AZT and a low fluorescence signal in absence of AZT, the signal-to-background ratio (the quotient of those two values) differed significantly: Using α-tPA particles, the ratio was just 4.9, whereas for HSV-TK particles and α-Puro particles values of 9.3 and 16.5 were obtained, respectively. To analyse the power of each assay system we also determined the Z-factor, a statistical parameter [18] characterizing the power of a high throughput assay. Assays having a Z-factor between 0.5 - 1 are considered to be excellent assays (with 1

being the theoretical optimum). For the three different particle types we obtained Z-factors of 0.53 (α-tPA), 0.79 (HSV-TK) and 0.85 (α-Puro). Taken together this clearly showed that α-Puro particles were most suitable for the novel assay system for which reason all further experiments were performed with this particle type.

Dose-Response Experiments

In a next step, we incubated indicator cells and effector particles in the presence of different concentrations of AZT (10^{-8} M to 10^{-3} M). Subsequently, we determined the fluorescence signals relative to those of control cells which had been incubated in absence of AZT and any viral particles (the fluorescence of these cells was considered as 1; Fig. **2a**). As expected, increasing concentrations of the drug correlated with increasing levels of inhibition. However, for concentrations above 25 μM, the fluorescence signal was decreasing again, most likely due to cytotoxic effects. It is well known that AZT has adverse effects on a variety of human tissues [21, 22]. Hence the strongest fluorescence signal should not be obtained at maximum inhibitor concentrations but rather at concentrations exhibiting an optimal balance between viral inhibition and cytotoxicity.

To further prove that the novel assay allows cytotoxic effects to be monitored, we performed the same experiments in the presence of different concentrations of sodium azide (1.5×10^{-7} M to 1.5×10^{-2} M), a highly cytotoxic compound without specific inhibitory properties. In this case, the highest relative fluorescence signal was obtained in the complete absence of the sodium azide (with an intensity indicating the lack of inhibitory properties), while increasing concentrations correlated with even lower fluorescence signals (Fig. **2b**). This clearly demonstrates that the novel assay system enables simultaneous monitoring of inhibitory and cytotoxic effects.

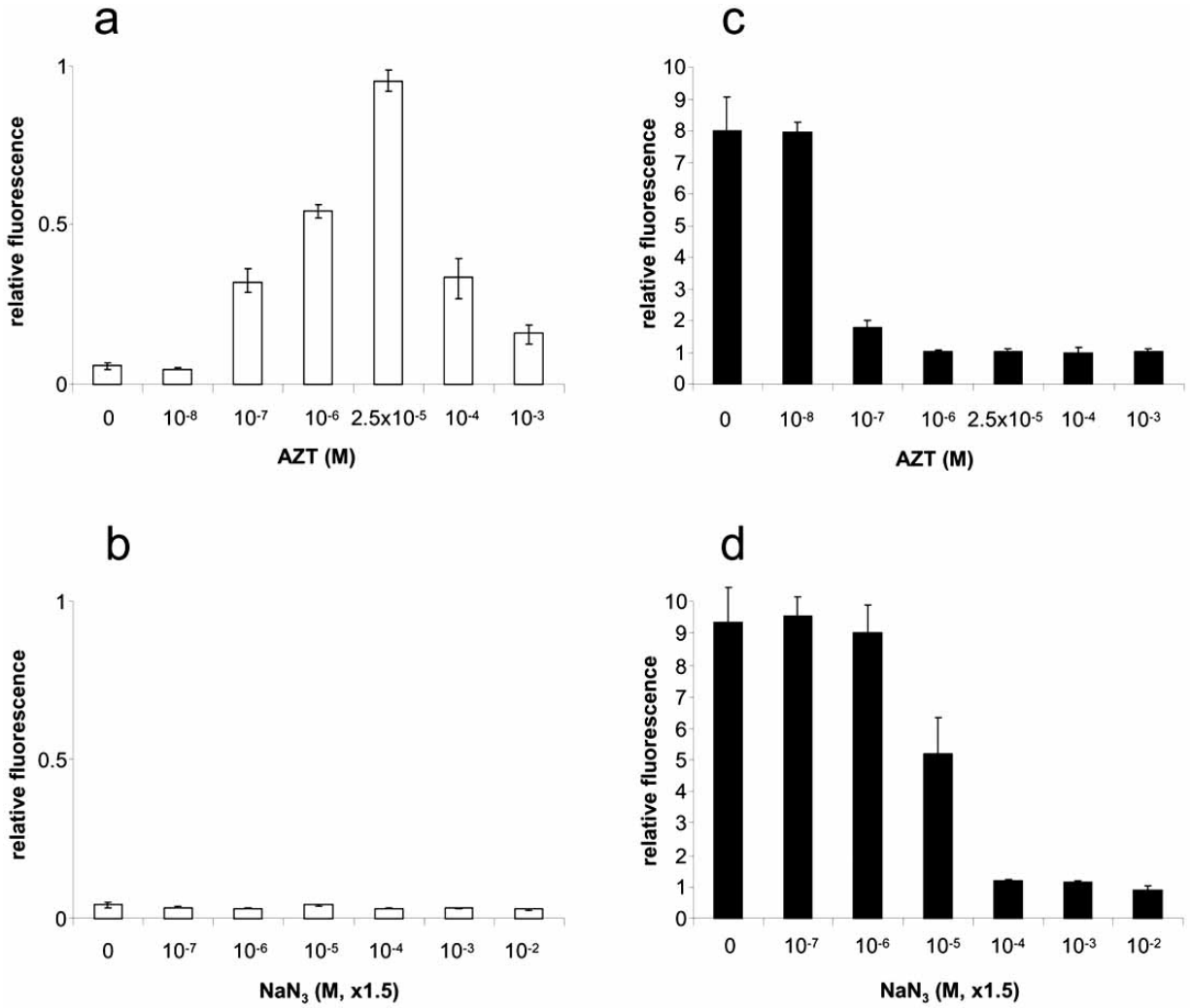

Fig. (2). Coupling a positive fluorescence signal with the inhibition of viral transduction (left) or with the transduction itself (right). Indicator cells were transduced with α-Puro effector particles in the presence of different concentrations of the specific inhibitor AZT (**a**) or the non-specific, toxic compound NaN_3 (**b**). The fluorescence signals (y-axis) were determined after the addition of plasminogen and the fluorogenic (plasmin) substrate HDVLK-Amc and are shown relative to those of control cells which had been incubated in absence of drugs and any viral particles (fluorescence = 1). For comparison with conventional assay systems, HEK293T cells were transduced with particles having packaged a β-galactosidase encoding gene in the presence of different concentrations of the specific inhibitor AZT (**c**) or the non-specific, toxic compound NaN_3 (**d**). The fluorescence signals (relative to the control samples without drugs and virus) were determined after the addition of the fluorogenic β-galactosidase substrate FDG.

Comparison with Conventional Assay Systems

For a comparison with conventional assay systems, we repeated the dose-response experiments using a setup coupling a positive fluorescence signal with the transduction step itself rather than its inhibition. To do this, we incubated the indicator cells with effector particles having packaged a vector encoding β-galactosidase (instead of α-Puro shRNA). This allowed a positive fluorescence signal to be obtained upon transduction and subsequent addition of the fluorogenic β-galactosidase substrate Fluorescein di-ß-D-galactopyranoside (FDG). Using this conventional setup, increasing concentrations of AZT correlated with decreasing fluorescence signals (indicating less transduction events) and does not take into account the cytotoxic effect of high concentrations of AZT (Fig. **2c**). When using sodium azide as a model inhibitor, this systematic error became even more obvious: Even though this compound does not mediate any specific inhibition of viral cell-entry, increasing concentrations correlated with decreasing fluorescence intensities (the readout for efficient viral inhibition; Fig. **2d**). Hence a system coupling a positive signal with the transduction step itself (rather than its inhibition) is not suitable for the selection of specific inhibitors. In contrast, the novel assay system allowed inhibitory and cytotoxic effects to be monitored simultaneously, thus enabling the identification of highly specific compounds that do not harm human cells in the effective concentration range.

Reliability of the Assay

In further experiments, we addressed the reproducibility of the novel assay system. For this purpose, we performed two completely independent sets of experiments (Run 1 and Run 2) on two different days and compared the results. Each experiment contained triplicates of all samples for which the fluorescence was determined (using AZT concentrations of 10^{-8} M to 10^{-3} M; 21 samples per run). Subsequently, the values obtained for Run 1 were plotted against the corresponding values for Run 2 (Fig. **3**). The resulting data points in the dot plot show a linear correlation, demonstrating a high degree of reproducibility for the assay. The fitted trend line has a coefficient of determination (R^2) of 0.95. In parallel, we determined the (mean) relative standard deviation (RSD) between the replicates in one run (intra-comparison) and between the two runs (inter-comparison). The values of 11% (intra-comparison) and 15% (inter-comparison) obtained demonstrate the reliability and robustness of the assay.

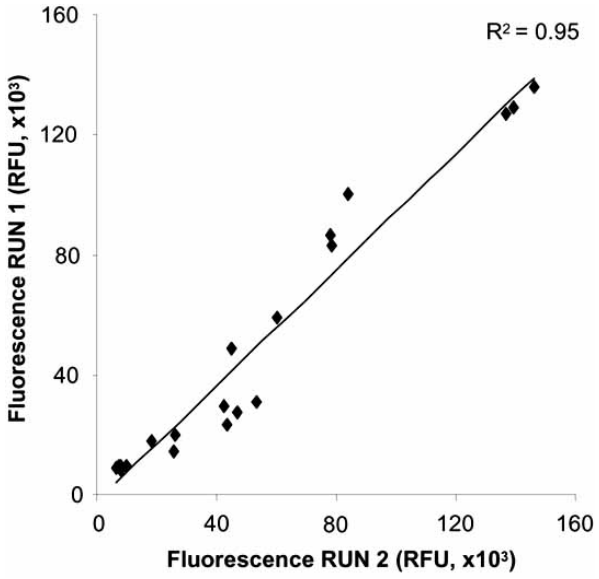

Fig. (3). Reproducibility of the viral inhibition assay. Two completely independent sets of experiments were performed on two different days (using the same sample compositions). The fluorescence signals for all samples of Run 1 were plotted against the corresponding fluorescence signals of Run 2. R^2 = determination coefficient.

DISCUSSION

We have developed a generic fluorescence assay coupling a positive fluorescence signal with the inhibition of viral transduction. This allows screens for viral inhibitors to be performed in which cytotoxic compounds (or concentrations thereof) are excluded, thus significantly decreasing the number of false positives. Using MLV effector particles and AZT as a model drug, we observed a 50% reduction of the relative fluorescence signals for a concentration between 0.1 μM and 1 μM. This is in good agreement with other studies determining an IC_{50} value of 0.1 μM for amphotropic MLV on HEK293T cells [23]. Our assay turned out to be highly reliable (Z-factor of 0.85) and offers further advantages due to the fact that it is based on non replication-competent pseudotype particles. First of all, the application of pseudotype particles allows the containment level to be kept low. Second, it offers high flexibility in terms of the viral species against which inhibitors can be selected. MLV particles can be efficiently pseudotyped with a variety of heterologous envelope proteins, resulting in the host cell tropism of the corresponding species such as HIV [7], influenza [24] and hepatitis C [25]. Therefore, exchanging the envelope proteins of the effector particles described here (and expressing our reporter system in cells permissive for the corresponding species) should enable the selection of inhibitors against clinically relevant viruses. This should be feasible as long as high-titer pseudotyped viral particles can be generated and sufficient expression levels of the reporter enzyme can be obtained in the corresponding host cells.

We found that the most efficient effector particles (α-Puro particles) were made by packaging a vector encoding shRNA which abolished the puromycin resistance of the indicator cells. Hence, after transduction, the indicator cells die in the presence of puromycin. A further application of these α-Puro effector particles is the controlled killing of cells. In our hands the expression of shRNA targeting the Puromycin resistance mediated cell death much more efficient than the well-established suicide gene Thymidine Kinase (TK). This was not only reflected by the signal to noise ratio of the two different assays (using either TK-particles or α-Puro particles), but was also confirmed by microscopical analysis of the corresponding samples. While at the day of the assay readout, TK-particles/ganciclovir treated samples still contained a significant number of living

cells, hardly any viable cells were observed in the α-Puro particles/puromycin treated samples (data not shown). Hence the α-Puro effector particles can be used for the specific selection of non-transduced cells without the need for fluorescence activated cell sorting (FACS). This could be of special interest when transducing a cell library expressing genetically-encoded inhibitors (such as peptides, antibodies, shRNAs and ribozymes). In this case, the elimination of all permissive cells should result in the specific selection of potent inhibitors.

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