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An ELISA system for evaluating antiretroviral activity against Rauscher murine leukemia virus

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Summary

A system for evaluating the activity of antiviral agents against Rauscher murine leukemia virus (R-MuLV) has been developed using an enzyme linked immunosorbent assay technique. The activity of various antiviral compounds demonstrated in this assay system has been compared to their activity in the UV-XC plaque reduction assay, which has been used historically for evaluating anti-R-MuLV compounds. The assay is based upon detection of R-MuLV encoded p30 protein production in virus infected murine cells. The assay reagents are readily available and the assay system is amenable to automated data collection systems. Cytotoxicity evaluations are conducted in parallel to the Rauscher MuLV ELISA assay in order to assess drug-induced reductions in cell viability. Cytotoxicity evaluations are important to interpretation of the ELISA results since reductions in cell viability reduce viral protein production which would indicate an antiviral drug effect. This system is less sensitive than the classical UV-XC plaque reduction assay; however, it does offer an alternative to the time-consuming and labor-intensive plaque assay.

Rauscher murine leukemia virus; Retrovirus; ELISA

Introduction

Rauscher murine leukemia virus (R-MuLV) (Rauscher, 1962) infection of

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mice has been used as a model for evaluating anticancer (Chirigos et al., 1963a; Chirigos et al., 1963b) and, more recently, anti-HIV compounds (Ruprecht et al., 1985; Ruprecht et al., 1986). Generally, in vitro assays are conducted prior to in vivo evaluations to assess the potential of the drugs to inhibit virus-induced disease. Historically, these evaluations have been performed using the UV-XC plaque reduction assay (Shannon et al., 1974). This assay system is labor-intensive and requires experienced personnel to quantitate plaque formation. Several workers have reported alternate assay systems for measuring MuLV. Generally these assays have relied upon immunofluorescence (Declève et al., 1974), in situ hybridization (Rein et al., 1982), purothionin A cytotoxicity (Tahara et al., 1979) or radioimmunoassay (Scolnick et al., 1972) for virus quantitations. More recently, focus forming assays have been described which use immunofluorescent (Sitbon et al., 1985) or immunoperoxidase (Nexø, 1977) labelling to aid in detection of MuLV-induced foci. While these systems improve the visual counting efficiency of the assays, they are not amenable to automated data collection and analysis like the newer ELISA methodologies have proven to be. Thus, we have established an assay system which allows automated data collection. This system is similar in concept to assay systems described for quantitating antiviral activity or antiviral antibody responses to herpesviruses (Rabalais et al., 1987), dengue (Fiqueiredo and Shope, 1987), bovine viral diarrhea (Justewicz et al., 1987) and coronaviruses (Smith and Winograd, 1986). For these assays, virus-infected monolayer cultures serve as the antigen source in an indirect ELISA assay.

Materials and Methods

Rauscher MuLV ELISA

Multiwell (96) culture plates were seeded with SC-1 cells (Hartley and Rowe, 1975) in Eagle's minimum essential medium (EMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 100 U penicillin/ml and 100 μg streptomycin/ml [tissue culture medium]. Following overnight incubation (5% CO_2, 37°C) for cell adherence, the medium was removed and DEAE-dextran solution (25 μg/ml in PBS) was added to each well. Following incubation (35 min) and DEAE-dextran removal, the wells were rinsed with phosphate-buffered saline (PBS). Serial dilutions of the test compounds, prepared in tissue culture medium at 1.25 × the desired final concentration, were added to each of 4 replicate wells (80 μl/well). Six virus control wells received 80 μl of tissue culture medium and 6 cell control wells received 100 μl of tissue culture medium. Each of the virus-challenged wells (test and virus controls) then received 20 μl of viral inoculum prepared at 5 × the desired final concentration (222 pfu/well). After 4 days of incubation (37°C, 5% CO_2), the medium was aspirated from the plates and the wells were rinsed with PBS. The cells were fixed with cold ethanol:acetone (2:1) at 4°C for 20 min. The fixative was decanted and the plates were allowed to air-dry overnight at room temperature

(RT). For the ELISA assay, the wells were blocked (1 h, RT) with 2% FBS prepared in PBS. The primary antibody, consisting of a 1:500 dilution of goat anti-Rauscher p30 antibody (NCI/BCB Repository), was added to each well and incubated for 1 h at 37°C. The wells were rinsed 5 times with wash solution (0.05% Tween-20® in PBS) and the second antibody, affinity-purified horseradish peroxidase-labelled anti-goat IgG (Tago Immunologicals, Burlingame, CA) was added. Following incubation for 1 h at 37°C, the wells were rinsed 5 times with wash solution and 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate (6)] ($ABTS/H_2O_2$) substrate (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) was added. The optical density at 405 nm (OD_{405}) was determined spectrophotometrically on a microplate reader following incubation at 37°C for 2 h. The average OD_{405} was calculated for each set of replicates and for the virus and cell controls. The percent reduction in p30 protein was calculated as:

$$\left[1 - \frac{(\text{Drug-Treated } OD_{405} - \text{Cell Control } OD_{405})}{(\text{Virus Control } OD_{405} - \text{Cell Control } OD_{405})}\right] \times 100$$

The percent reduction was plotted and the 50% inhibitory concentration (IC_{50}) was determined.

UV-XC plaque reduction assay

The UV-XC plaque reduction assay has been described previously (Shannon et al., 1974). Briefly, 6-well tissue culture plates were seeded with SC-1 cells in tissue culture medium. After overnight incubation (37°C, 5% CO_2), the medium was aspirated from the wells and 2 ml of the drug solution (1.25 ×) and 0.5 ml of the virus inoculum were added to replicate (3) wells. Controls included cell controls (tissue culture medium only, no drug or virus) and virus controls (virus in medium, no drug). On day 3 post-inoculation, the medium was decanted and the cultures were irradiated with ultraviolet light. Each well then received XC cells (Svoboda, 1961). On day 3 post-irradiation, the cultures were rinsed with PBS, fixed with 10% buffered formalin and stained with 0.1% crystal violet. After air-drying, the plaques were enumerated with the aid of a dissection microscope. Antiviral activity was expressed as the reduction in the mean number of plaques counted in the drug-treated, virus-infected cultures compared with the mean number of plaques in the untreated, virus-infected control cultures. The drug concentration which reduced the mean number of plaques by 50% (IC_{50}) was calculated using regression analysis for semi-log curve fitting. Cytotoxicity was determined in parallel by the MTT assay performed in 96 well plates.

Cytotoxicity assay

Cytotoxicity determinations were performed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay (Mosmann,

1983). For this, SC-1 cell monolayers were prepared in multiwell (96) tissue culture plates in parallel to the ELISA or UV-XC plates. For cytotoxicity assays performed in parallel to the ELISA assay, the cells were pretreated with DEAE-dextran. The test compounds were added to each well at the desired final concentration in a volume of 100 μl. Controls included cells incubated in tissue culture medium alone (cell controls) and color controls which contain no cells but received all the remaining reagents. The plates were incubated in parallel with the ELISA (4 days) or UV-XC (3 days) plates. Following incubation, each well received 50 μl of a 2 mg/ml solution of MTT prepared in tissue culture medium. After a 7-h incubation at 37°C, 100 μl of a 10% sodium dodecyl sulfate (SDS): 0.01 N HCl solution were added to each well and the plates were incubated overnight at 37°C. The optical density at 570 nm was determined spectrophotometrically on a microplate reader. The average OD_{570} was calculated for each set of replicates and the percent of cell control was calculated for each drug dilution as:

$$\left[\frac{(\text{mean drug treated } OD_{570} - \text{color control } OD_{570})}{(\text{mean cell control } OD_{570} - \text{medium control } OD_{570})}\right] \times 100$$

These data were plotted in conjunction with the percent reduction in virus production. The minimum toxic concentration was defined as that concentration which reduced the cell viability to less than 70% of the cell control.

Results and Discussion

A series of compounds have been assayed in the ELISA and UV-XC systems. The IC_{50} and MTC results of 12 compounds are presented in Table 1. The results obtained with the two systems correlate well although the drug concentrations required to suppress p30 protein production, as measured in the ELISA system, are generally higher than those required to suppress UV-XC plaque formation. One compound, diarylsulfone 277, demonstrated antiviral activity in the UV-XC plaque reduction assay; however, no activity was detected by the p30 protein reduction assay (ELISA). This compound was inactive in a virus yield reduction assay (data not shown) thus, the ELISA assay may be more indicative of the activity for some compounds. In the case of this compound, the activity detected by the UV-XC plaque reduction assay was believed to result from cytotoxicity to the XC cells used as an overlay.

Graphic presentation of the UV-XC and ELISA data for 2′3′ dideoxythymidine (AZT) is shown in Fig. 1. Reductions in virus production, either as percent plaque reduction or as percent reduction in p30 protein, are graphed on the left Y-axis as the virus-infected cells. Cytotoxicity, presented as the percent of cell control, is graphed on the right Y-axis as the toxicity control cells. Data presented in this form is readily interpreted visually without the need for

TABLE 1

The IC_{50} and MTC for selected antiviral compounds assayed by the ELISA assay and by the UV-XC plaque reduction assay

Compound	NSC no.	ELISA		UV-XC	
		IC_{50} (μg/ml)	MTC (μg/ml)	IC_{50} (μg/ml)	MTC (μg/ml)
Castanospermine	614552	7.5	18.9	0.813	18.9
Rhodium complex	619179	7.5	<32	9.06	10
Terbium polyoxometalate	622102	5.0	>100	1.83	100
Diarylsulfone 263	624231	–*	263	–	<1000
ATA polymer (unfractionated)	624540	3.0	<100	0.19	>100
27-mer antisense oligodeoxynucleotide	624958	4.2	>32	0.18	>16
Diarylsulfone 277	627708	–	>277	10.46	277
ATA polymer (high molecular wt)	629465	1.3	<100	0.11	100
ATA polymer (intermediate mol wt)	629467	2.5	<100	0.33	100
ATA polymer (low molecular wt)	629469	17.5	<100	1.98	>100
AZT	602670	0.001	>2.26	0.0004	>2.26
ddC	606170	4.5	>100	1.0	>100

*– = no IC_{50} was reached.

extensive analysis of the numerical values.

We have assayed the positive control drug, AZT, multiple times. The average ID_{50} for AZT in these assays (12) was 0.002 μM with a median IC_{50} of 0.002 μM and a standard deviation of 0.0010. We feel that this indicates that the results are reproducible from assay to assay.

The primary difference between the ELISA assay and the UV-XC plaque reduction assay is in the drug concentrations required to suppress the virus production endpoint. The UV-XC plaque reduction assay measures the capacity of an antiviral compound to reduce production of infectious virus. When rat XC cells are placed into contact with MuLV-infected cells, syncytia are formed. When these XC cells are overlaid onto MuLV-infected SC-1 cell monolayers, they fail to infiltrate the SC-1 cell sheet in the MuLV-infected areas resulting in lightly stained areas (plaques). MuLV infected SC-1 cells that are killed by exposure to UV light are still capable of inducing syncytia. The XC cell overlay grows on the dying SC-1 cells as a uniform monolayer except where MuLV infection is present. In these areas, there are 'holes' in the XC cell monolayer which contain multiple giant cells or focal masses of giant cells. Production of these syncytia is presumably a function of the gp70 glycoprotein as typified by other retroviruses (i.e. gp 120 in HIV). In contrast, the ELISA system measures the capacity of a compound to reduce production of the virus protein, p30, which forms part of the virion core similar to the p24 protein in HIV. This protein is encoded by the viral nucleic acid. Reductions in p30 protein production will occur if a compound interferes with translation of the viral nucleic acid. Thus, compounds which interfere with virus attachment, penetration, integration, transcription and translation will reduce p30 protein production. A compound which interferes with late stages in the virus

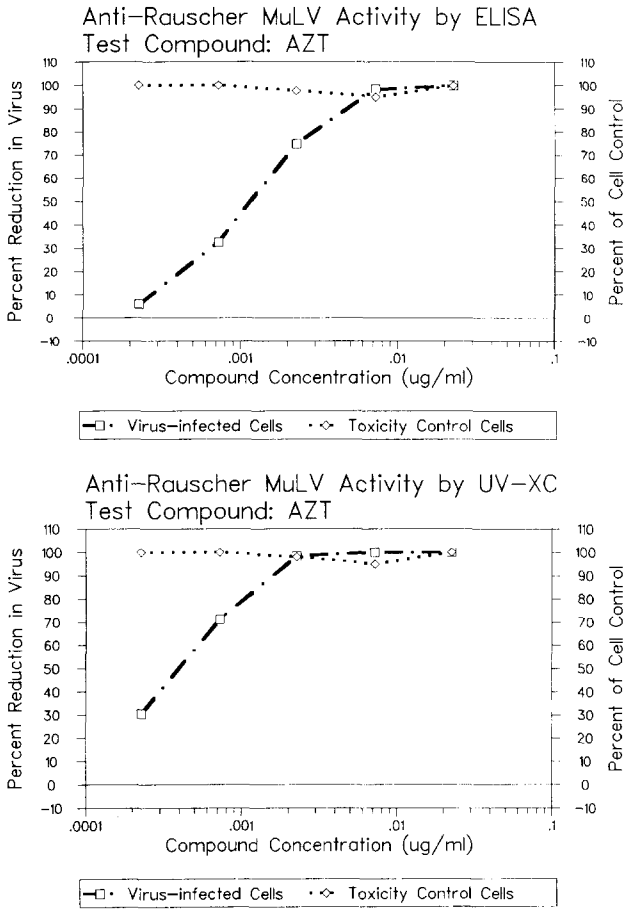

Fig. 1. Graphic presentation of the antiviral activity and cytotoxicity of AZT evaluated by UV-XC plaque reduction assay and by ELISA p30 protein assay.

production cycle (i.e. virus budding) would not necessarily reduce p30 protein levels. The only cases in which the ELISA assay does not give results compatible with those found in the UV-XC assay are: (1) assays of compounds which have toxic effects on the XC cells and (2) assays of compounds which are active late in the virus replication cycle or which act by interfering with the infectivity of the progeny virus so that p30 protein is produced but the progeny virus is unable to infect new target cells.

While the system described here is not as sensitive to antiviral drugs as the UV-XC plaque reduction assay, it does offer an alternative method for antiviral drug evaluations. We have found compounds that produce toxicity to the XC cell overlay which could be evaluated in this test system. Also, smaller quantities of compound are required for the ELISA since it is performed in 96-well plates rather than the 6-well plates used in the UV-XC plaque reduction

assay. We have used this system to evaluate analogs of known positive compounds for selecting the optimal drug for in vivo testing. Further, the system can be modified at various steps including (1) increasing the virus replication phase (i.e., 6-day incubation), (2) altering the virus inoculum dose, (3) altering the ELISA reagent concentrations and incubation times and (4) collecting supernatants for yield reduction assays. Thus, this system can be adjusted to meet the desired goals.

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