A SENSITIVE ASSAY FOR ANTI-HIV-1 DRUG DISCOVERY IN A BIOLOGICAL SAFETY LEVEL-2 LABORATORY

Chalobon Yoosook[1], Vichai Reutrakul[2], Tawatchai Santisuk[3], Suttiporn Chaichana[2], John M Pezzuto[4], Yumi Dong[4], David J Clanton[5], Rebecca Kiser[5] and Pat Staley[5]

[1]Department of Microbiology and [2]Department of Chemistry, Faculty of Science, Mahidol University; [3]The Royal Forestry Department, Ministry of Agriculture and Co-operatives, Bangkok, Thailand; [4]Program for Collaborative Research in the Pharmaceutical Science, College of Pharmacy, University of Illinois at Chicago; [5]SAIC-Frederick, NCI-FCRDC, Frederick, Maryland, USA

Abstract. Studies involving infectious, wild type HIV-1 must be performed under strict BSL-3 practice. We have employed a defective $^{\Delta Tat/Rev}$MC99 and cloned 1A2 line, *ie,* mutated HIV-1 and Tat/Rev transfected cells to verify anti-HIV-1 activity in a BSL-2 laboratory. A number of extracts from various parts of 11 species of plants were studied. Results were correlated with those of an anti-HIV-1 reverse transcriptase (RT) assay.

INTRODUCTION

Human immunodeficiency virus (HIV) causes a disease characterized by a continuous and high level of viral replication in the plasma and lymphoid tissues (Embretson *et al,* 1993; Pantaleo *et al,* 1993; Piatak *et al,* 1993). The onset of this pandemic has created pressure to search for effective antiviral agents. To date, most of the antivirals in use or in clinical trials are those active against the viral reverse transcriptase (RT), both nucleoside and non-nucleoside RT inhibitors, and the protease inhibitors (Richman *et al,* 1997). Although these drugs are effective, mutated or resistant viral strains have emerged after high doses and prolonged treatment, as observed with other therapeutic regimens (zidovudine or AZT, delavirdine, etc) (Larder *et al,* 1989; Richman *et al,* 1990). Moreover, it was recently shown that AZT treatment, the most commonly used drug in developing countries, might increase the mutation rate of the virus (Julias *et al,* 1997). A combination of regimens has proved effective in the management of these cases (Gulick *et al,* 1997). However, these synthetic drugs and their use for clinical management of the large population of infected individuals in poor countries is not feasible. Thus, an alternative approach is to search for drugs from natural sources as used in medicinal practice.

Correspondence: Dr Chalobon Yoosook, Department of Microbiology, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand.

During the past few years, the Chemistry Department at the Faculty of Science, Mahidol University, Bangkok, Thailand, in collaboration with the College of Pharmacy, University of Illinois at Chicago, USA, has been working for the discovery of HIV-1 RT inhibitors from tropical rain forest plants collected in the Kingdom of Thailand. A number of plant extracts were shown to possess such activities. However, in addition to inhibition of RT, it is realized that anti-HIV-1 activity can be mediated at many other stages in the replication cycle of the virus, such as cell attachment/fusion/entry, viral integration, transcriptional and post-transcriptional processing, and packaging and budding of the virions (Richman *et al,* 1997). Thus, assays which are reflective of all these targets, *eg,* cytoprotection (Weislow *et al,* 1989) and syncytium assays (Nara *et al,* 1987), are required. These types of assay have always employed the use of infectious virions and hence must be performed in a laboratory with biological safety level (BSL)-3 facilities.

Recently, the NCI-AIDS Drug Screening and Development Laboratory, SAIC-Frederick, NCI-FCRDC, Maryland, USA, described a use of Δ Tat/Rev defective HIV-1 ($^{\Delta Tat/Rev}$MC99) and the cloned 1A2 cell line of CEM-SS^{TART}, Tat/Rev transfected, for the discovery of anti-HIV agents in a cytoprotection assay (Kiser *et al,* 1996). This mutated virus has neither been shown to replicate in peripheral blood mononuclear cells nor the integrated genomes reverting to wild type. A comparison of the cytoprotection assay and an alternative assay that inhibits the formation of syncytia was

performed using this defective virus/cell system. In this communication, we report data on both assays using a number of natural product specimens that have been tested for anti-HIV-1 RT activity. These data are relevant for the discovery of anti-HIV-1 agents in a biological safety level-2 facility.

MATERIALS AND METHODS

Viruses and cell lines

The laboratory RF strain of HIV-1 was used; CEM-SS (human T-cell lymphoblastic leukemia) cell line was from P. Nara (Nara and Fischinger, 1988). The ΔTat/Rev defective HIV-1 ($^{\Delta Tat/Rev}$MC99) and the cloned 1A2 cells derived from CEM-SS^{TART} cells were described previously (Chen *et al,* 1992; Kiser *et al,* 1996). Cells were grown in RPMI 1640 plus 10% fetal calf serum and 1 μg/ ml gentamicin and maintained in log phase of growth; virus stocks from the appropriate cell lines were prepared according to the method described by Kiser *et al* (1996). Virus stocks were titrated using the syncytium assay (Nara *et al,* 1987) in 96-well tissue culture plates. Titers of the virus were recorded as syncytium forming unit (SFU)/ml.

Plant extracts

The following plants were used: *Anogeissus acuminata* Wall, leaves, twigs, stems and bark; *Barleria lupulina* Lindl, leaves and twigs; *Bischofia javanica* Bl, bark; *Calophyllum teysmannii,* bark; *Clinacanthus nuthans* Lindau, leaves and stems; *Combretum* sp, above ground part; *Cyathocalyx* sp, bark; *Glochidion* sp, leaves and twigs; *Goniothalamus* sp, leaves and twigs; *Mammea* sp, bark; *Ventiligo* sp, hardwood. All voucher specimens are kept at the Royal Foresty Department, Ministry of Agriculture and Co-operatives, Bangkok, Thailand.

The plant parts were air-dried at room temperature. Ground plant materials were then percolated in methanol (4x3 liters). The extracts were filtered through Whatman filter no. 1 and evaporated using a rotary evaporator at below 40°C and freeze-dried.

Inhibition of HIV-1 reverse transcriptase assay

Heterodimeric HIV-1 (p66/p51) was supplied by Dr SH Hughes (ABL, Frederick Cancer Research and Development Center, Frederick , MD, USA). Plant extracts were dissolved in dimethyl sulfoxide (DMSO) to obtain a concentration of 2 mg/ml, treated with polyvinylpyrrolidone (Sigma) to remove tannins, and then analyzed for potential to inhibit HIV-1 RT using the method and reaction mixture described by Tan *et al* (1991). The reaction was carried out in 96-well microtiter plates, in duplicate. The treated plant extract (10 μl) was added into 80 μl of the reaction mixture. The reaction was started by the addition of 0.8 μg/10 μl/well of RT, followed by incubation at 37°C for 1 hour and terminated by heating the mixture to 70°C for 5 minutes and then chilling on ice for 15 minutes. Aliquots (90 μl) of each reaction mixture were spotted uniformly onto 2.5 cm diameter DE-81 filters, kept at an ambient temperature for 1 hour, followed by 4 washings in 5% aqueous Na_2HPO_4. $7H_2O$, and 2 additional washings in distilled water. The filters were then thoroughly dried and subjected to scintillation counting in a nonaqueous scintillation fluid. Fagaronine chloride and DMSO (10% v/v) were used as positive and negative controls, respectively. Percentage inhibition was calculated as (1-test/DMSO) x 100. Results of the assay were recorded as follow: very active, inhibition >70%; moderately active, inhibition >50% to 70%; weakly active, inhibition >30% to 50%; inactive, inhibition equal <30%.

Cytoprotection assay for anti-HIV

The cytoprotection assay used in this study was as described in the NCI's AIDS Virus Drug Screening Laboratory (AVDSL) (Weislow *et al,* 1989; Kiser *et al,* 1996). In brief, 5,000 CEM-SS cells per well were plated into 96-well tissue culture plates followed by an addition of one-half log dilutions of the extract or drug into 2 rows of cells. The RF strain of HIV-1 was then added into the appropriate wells at a multiplicity of infection of 0.25 SFU/ cell. Control wells contained cells alone (uninfected control), cells with virus only (infected control) or a series of extracts or drugs with uninfected cells (toxicity control). The final volume was 200 μl/ well. The assay was performed in duplicate, *ie,* master and sister plates. Appropriate half-log concentrations of AZT were included as controls. The plates were incubated at 37°C in an atmosphere of 5% CO_2 for 6 days and stained with 50 μl of a solution of 1 mg/ml XTT tetrazolium salt (Boehringer Mannheim) and 1% phenazine methosulfate (Sigma) per well. After incubation

for 4 hours, microscopic appearances of the cells were observed and recorded as toxic or cytostatic, if any. Optical densities at $A_{450-650}$ were measured and analyzed by computer software monitored by the VAX 9210 Mainframe computer system of the AVDSL, NCI, USA. Activities were expressed as 50% effective concentration of the drug exhibiting protection toward 50% of the cells (EC_{50}) and 50% inhibitory concentration of the drug exhibiting toxicity towards 50% of the cells (IC_{50}).

Syncytium assay for anti-HIV

The effect of drug or extract which could inhibit syncytium formation by HIV-1 in the syncytium assay was determined in 96-well tissue culture plates, using the protocol described previously (Nara *et al,* 1987). 1A2 cell line and $^{\Delta Tat/Rev}$MC99 were used. Each plant extract was prepared at a concentration of 20 mg/ml in DMSO. The assay protocol was similar to that of the virus assay except one half-log dilutions of the extracts were added in triplicate into test wells and allowed to incubate for 1 hour before adding 100-200 SFU/50 µl of virus into the test and control wells. 200 SFU/50 µl of the virus were added in triplicate into the test and control wells and allowed to adsorb for 1 hour before adding one half-log dilutions of the extracts. Other controls included wells containing cells with extracts only and cells without the extract. AZT was used as a positive control. Both uninfected and infected cells were treated with the extracts, observed in parallel, and recorded for the appearance of cytotoxicity or cytostasis. After 3 days of incubation, syncytia were counted. Results were expressed as the concentration of extract that inhibited 50% syncytium formation by the virus (EC_{50}) as estimated from a linear plot. This was recorded as follows: active, EC_{50} can be obtained at a non-toxic or non-cytostatic concentration; inactive, number of SFU is more than 50% of control at the highest non-toxic or non-cytostatic concentration tested. The IC_{50} value was determined as described for that of the cytoprotection assay.

RESULTS

Our preliminary studies of organic extracts of various plants by the cytoprotection assay employing either the wild type or $^{\Delta Tat/Rev}$MC99 did not reveal anti-HIV-1 activity in a number of preparations that possessed anti-HIV-1 RT activity. Furthermore, the mutated virus was shown to be less sensitive than the wild type virus in the cytoprotection assay for AZT (Kiser *et al,* 1996). Thus, an alternative method was to use the syncytium assay. The efficiency of the syncytium assay for the discovery of anti-HIV-1 compounds was then compared to the cytoprotection assay using the mutated and wild type viruses, respectively, and the appropriate cell lines as described in Materials and Methods. The average EC_{50} value of AZT for the syncytium assay was 2.0×10^{-9} M and the average EC_{50} for the cytoprotection assay was 1.3×10^{-8} M (Table 1). These results demonstrate that the syncytium assay was approximately 15-fold more sensitive than the cytoprotection assay as used in this study, at least for detecting the anti-HIV-1 activity of AZT.

The syncytium assay using $^{\Delta Tat/Rev}$MC99 and 1A2 was then applied to verify anti-HIV-1 activity of 15 plant extract preparations (11 species). All speci-

Table 1

Anti-HIV-1 activity of AZT as determined by cytoprotection and syncytium assays.[a]

Assay (virus, cell line)	Experiment	EC_{50} (M)
Cytoprotection (RF, CEM-SS)	Master plate	1.3×10^{-8}
	Sister plate	1.3×10^{-8}
Syncytium ($^{\Delta Tat/Rev}$MC99, 1A2)	1	2.9×10^{-9}
	2	1.1×10^{-9}

[a]IC_{50} of A2t was>10^{-6}M.

Table 2

Anti-HIV-1 activity of plant extracts as determined by reverse trancriptase and syncytium assays.

Plant	Part	HIV-1 RT assay		IC_{50} (µg/ml)	Syncytium assay		
		Inhibition (%)	Activity		EC_{50} (µg/ml)	Activity	TI (IC_{50}/EC_{50})
Anogeissus acuminata Wall	Leaves and twigs	98.3	Very active	50.8	0.99	Active	51.3
Anogeissus acuminata Wall	Stems	86.4	Very active	51.8	1.98	Active	26.2
Anogeissua acuminata Wall	Bark	87.4	Very active	42.5	< 0.8	Active	> 53.1
Barleria lupulina Lindl	Leaves and twigs	0	Inactive	166.7		Inactive	
Bischofia javanica Bl	Bark	79.8	Very active	60.2	7	Active	8.6
Calophyllum teysmannii	Bark	50.5	Moderately active	98.7	1.3	Active	75.9
Clinacanthus nutans Lindau	Leaves	12.2	Inactive	> 250		Inactive	
Clinacanthus nutans Lindau	Stems	0	Inactive	> 250		Inactive	
Combretum sp	Above ground	76.5	Very active	72.3	6.5	Active	11.1
Cyathocalyx sp	Bark	40.8	Weakly active	168.9	71.3	Active	2.4
Glochidion sp	Leaves and twigs	91.5	Very active	44.6	1.3	Active	34.3
Goniothalamus sp	Leaves and twigs	43.3	Weakly active	114.3	42.2	Active	2.7
Mammea sp	Bark	91.5	Very active	16.4	0.7	Active	23.4
Ventilago sp	Hardwood	97.1	Very active	54.1	17	Active	21.6
Ventilago sp (acetone extract)	Hardwood	98.5	Very active	90.9	24.2	Active	22.7

mens were coded and anti-HIV-1 RT activity of each was pre-determined. However, different codes were blindly assigned to the plant extracts for the syncytium assay which were later decoded for comparison of results. Different parts of plants were indicated; results of anti-HIV-1 RT activity, cytotoxicity of the extracts, anti-syncytium forming activity as well as therapeutic indices (TI; IC_{50}/EC_{50}) are shown in Table 2. Of 11 plant species, two were inactive for anti-HIV-1 RT activity. Since cells which show signs of toxicity or cytostatic in the presence of extract may not be able to support viral replication, only the numbers of syncytia appeared in the test wells which did not exhibit such signs was used for estimation of the EC_{50} values. Results of anti-HIV activity from the syncytium assay were comparable to the HIV-1 RT assay. Although the $^{\Delta Tat/Rev}$MC99 used in the syncytium assay is a defective virus, it acquires HIV-1 Tat/Rev products from the transfected host cells and thus reflects activities against the wild type virus. It is therefore concluded that the syncytium assay using the mutated or non-infectious virus, and appropriate cell line in a BSL-2 laboratory, is suitable for investigating or screening anti-HIV activity of plant extracts.

DISCUSSION

Studies on HIV-1 which can cause a devastating disease must be carried out in a BSL-3 laboratory, especially when mass screening of antivirals is required. The cytoprotection assay is suitable for such a purpose since the results can be obtained by colorimetry and the investigator can handle up to 20 specimens at one time. As it is necessary for us to perform HIV experiments in a BSL-2 laboratory, a mutated HIV-1 was selected for antiviral screening. When the resulting AZT activities obtained by the cytoprotection assay was compared to the syncytium assay, it was found that the latter was far more sensitive than the former (Table 1). Similar results have been previously observed (Weislow *et al,* 1989).

The results of our syncytium assays are in agreement with those of HIV-1 RT assays (Table 2). This syncytium assay is therefore suitable for the screening of antivirals, at least those with anti-HIV-1 RT activity. However, there are many drawbacks as the procedure is labor intensive and very tedious, thus

requiring an experienced person to perform the assay. Furthermore, since the syncytium assay is cell-based, activities of drugs or extracts may not be observed if they are very toxic to the cells used in the system. This might mask the antiviral activity of test substances. However, this assay does not require radioactive materials or expensive equipment and should identify many viral targets, making it very useful for the discovery of anti-HIV drugs in a BSL-2 laboratory.

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