Studies on Anti-HIV Activity of *Indigofera tinctoria*

S. Kavimani*, B. Jaykar*, E. De Clercq, Christophe PannecouQue** and Myrium Witvrouw****

*Department of Pharmacology,
Periyar College of Pharmaceutical Sciences for Girls,
Trichy-620 021, Tamil Nadu, India,
**Rega Institute of Medical Research,
Katholieke University, Leuven, Belgium.

Methanolic extract of whole parts of *Indigofera tinctoria* have been studied against replication of HIV-1 (III B) and HIV-2 (ROD) in MT-4 cells.

Introduction

Acquired immunodeficiency syndrome (AIDS) is a life threatening and debilitating disease state caused by retrovirus infection, and the etiologic agent is now widely known as the human immunodeficiency virus type 1 (HIV-1)[1]. Many compounds of plant origin have been identified that inhibit different stages in the replication cycle of HIV.[2-5] According to De Clercq the replicative cycle of HIV comprises ten steps that could be considered adequate targets for intervention.[6] The present study is designed to find out the effect of methanolic extract of *Indigofera tinctoria*[7] against replication of HIV-1 (III B) and HIV-2 (ROD) in MT-4 cells.

Materials and Methods

Whole parts of *Indigofera tinctoria* (Leguminosae) collected in and around Nilgiri Hills, Tamilnadu, India were used for the extraction. They were dried in shade, pulverised and packed into the Soxhlet apparatus and subjected to hot continuous percolation using methanol. The extract was concentrated by distillation and used for screening (yield 6.1% W/V).

Anti-HIV activity

Cell cultures: The MT-4 cells were grown in RPMI — 1640 DM (Dutch modification) medium (Flow Laboratories, Irvine, Scotland), supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) and 20 µg/ml gentamycin (E. Merck, Darmstadt, FRG). The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Every 3-4 days, cells were spun down and seeded at 3 x 10^5 cells/ml in new cell culture flasks. At regular time intervals, the MT-4 cells were analysed for the presence of mycoplasma and consistently found to be mycoplasma-free.

Virus

HIV-1 (strain HTLV-III_BLAI)[8] and HIV-2 (strain LAV-2ROD)[9] were obtained from the culture supernatant of HIV-1 or HIV-2 infected MT-4 cell lines.[10] The virus titer of the supernatant was determined in MT-4 cells. The virus stocks were stored at –70°C until used.

Anti-HIV assay

Flat bottom, 96-well plastic microtiter plates (Falcon, Becton Dickinson, Mountain View, CA) were filled with 100 µl of complete medium using a Titertek Multidrop dispenser (Flow Laboratories). This eight-channel dispenser could fill a microtiter tray in less than 10 seconds. Subsequently, stock solutions (10X final test concentration) of compounds were added in 25 µl volumes to two series of triplicate wells so as to allow simultaneous evaluation of their effects on HIV- and mock-infected cells. Serial five-fold dilutions were

made directly in the microtiter trays using a Biomek 1000 robot (Beckman). Untreated control HIV-and mock-infected cell samples were included for each compound.

50 µl of HIV at 100 $CCID_{50}$ medium was added to either infected or mock-infected part of a microtiter tray. Exponentially growing MT-4 cells were centrifuged for 5 minutes at 140xg and the supernatants were discarded.

The MT-4 cells were resuspended at 6 x 10^5 cells/ml in a flask which was connected with an autoclavable dispensing cassette of a Titertek Multidrop dispenser. Under slight magnetic stirring 50 µl volumes were then transferred to the microtiter tray wells. The outer row wells were filled with 200 µl of medium. The cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells remained in contact with the test compounds during the whole incubation period. Five days after infection the viability of mock and HIV-infected cells were examined spectrophotometrically by the MTT method.

MTT assay

The MTT assay is based on the reduction of the yellow coloured 3-(4,5-dimethylthiazo 1-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co., St. Louis, MO) by mitochondrial dehydrogenase of metabolically active cells to a blue formazan which can be measured spectrophotometrically. Therefore, to each well of the microtiter plates, 20 µl of a solution of MTT (7.5 mg/ml) in phosphate-buffered saline was added using the Titertek Multidrop dispenser. The trays were further incubated at 37°C in a CO_2 incubator for 1 hour. A fixed volume of medium (150 µl) was then removed from each cup using M96 washer (ICN flow) without disturbing the MT-4 cell clusters containing the formazan crystals.

Solubilization of the formazan crystals was achieved by adding 100 µl 10% (v/v) Triton X-100 in acidified isopropanol (2 ml concentrated HCI per 500 ml solvent) using the M96 washer. Complete dissolution of the formazan crystals could be obtained after the trays had been placed on a plate shaker for 10 min. Finally, the absorbances were read in a eight-channel computer-controlled photometer (Multiscan MCC, ICN Flow) at two wavelengths (540 and 690 nm). The absorbance measured at 690 nm was automatically subtracted from the absorbance at 540 nm, so as to eliminate the effects of non-specific absorption. Blanking was carried out directly on the microtiter plates with the first column wells which contained all reagents except the MT-4 cells.

All data represent the average values for a minimum of three wells. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration of compound that reduced the absorbance (OD_{540}) of the mock-infected control sample by 50%. The percent protection achieved by the compounds in HIV-infected cells was calculated by the following formula:

$$\frac{(OD_T)\ \text{HIV} - (OD_c)\ \text{HIV}}{(OD_c)\ \text{mock} - (OD_c)\ \text{mock}} \text{ expressed in \%}$$

Whereby (OD_T) HIV is the optical density measured with a given concentration of the test compound in HIV infected cells; (OD_c) mock is the optical density measured for the control untreated mock infected cells; all OD values determined at 540 nm. The dose achieving 50% protection according to the above formula was defined as EC_{50}.

Results and Discussion

The methanolic extract of *Indigofera tinctoria* have been screened for anti-HIV activity and cytotoxicity (Table 1) against HIV-1 (III B) and HIV-2 (ROD) replicating in acutely infected MT-4 cells. From the results, selectivity indices (SI) ratio of 50% cytotoxicity concentration (CC_{50}) to 50% effective concentration (EC_{50}) were low (approximately 1) against HIV-1 (III B) strain and HIV-2 (ROD) strain.

The extract exhibited an average EC_{50} (µg/ml) of 113 and 125, maximum protection of 7.5 and 9 respectively against HIV-1 (III B) and HIV-2 (ROD) strain.

TABLE 1
Anti-HIV activity of *I. tinctoria*

Compound	Strain	EC_{50}	EC_{90}	CC_{50}	Maximum Protection
Methanolic	III B	>113	>113	=113	7.5
extract of	III B	>125	>125	>125	9
I. tinctoria	R O D	>125	>125	>125	9

EC_{50}, EC_{90} and CC_{50} expressed in µg/ml

Acknowledgement

The authors thank the AICTE, New Delhi for financial support under TAPTEC and Prof. E. De Clercq, Rega Institute of medical research, Belgium for anti-HIV screening.

REFERENCES

1. Verma, S., Recent advances in the chemotherapy of AIDS, *Ind. J. Physio-Pharmacol.*, 1994, 38 (1), 5.
2. Vlietinick, A.J., De Bruyne, T., Apers, S. and Pieters, L.A., *Planta Medica*, 1998, 64, 97.
3. Otake, T., Mori, H., Morimoto, M. and Namba, T., *Phytother. Res.*, 1995, 9 (1), 6.
4. Kusumoto, I.T., Shimada, I., Kakiuchi, N., Hattori, M. and Namba, T., *Phytother. Res.*, 1992, 6 (5), 241.
5. Kusumoto, I.T., Kakiuchi, N., Hattori, M., Namba, T., Sutardjo, S. and Shimotohno, K., *Shoyakugaku Zasshi*, 1992, 46 (2), 190.
6. De Clercq, E., *Clin. Microbiol. Rev.*, 1995, 8, 200.
7. *The Wealth of India, Raw Materials*, Council of Scientific and Industrial Research, New Delhi, Vol. V, 1991, 183.
8. Popovic, M., Sarangadharan, M.G., Read, E. and Gallo, R.C., *Science*, 1984, 224, 497.
9. Clavel, F., Guyader, M., Guetard, D., Salle, M., Montagrier, L. and Alizon, H., *Nature*, 1986, 324, 691.
10. Pauwels, R., De Clercq, E., Desmyter, J., Balzarini, P., Gouban, P., Herdesijn, P., Vanderhaughe, H. and Vandeputle, M., *J. Virol. Methods*, 1987, 16, 171.