

## ***In Vitro* Anti-HIV Activity of a Chinese Fungus Extract<sup>1</sup>**

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**Objective** To investigate the inhibitory effect of the mycelium extract from a Chinese fungus (M1) on HIV-1 and its mode of action. **Methods** Several *in vitro* methods including time of action, time of addition and PCR were used to test the mode of action of M1. **Results** M1 inhibited acute HIV infection *in vitro* and was effective when it was added 12 h after infection. PCR analysis of infected cells demonstrated that M1 delayed the appearance of late product of reverse transcription and HIV was blocked before its RNA expression. **Conclusion** The target of M1 is post-integration of proviral DNA.

**Key words:** Fungus extract; Anti-HIV-1 activity; PCR

### INTRODUCTION

Chemotherapy significantly prolongs the life of AIDS patients, but its drugs are too expensive for most AIDS patients in developing countries. Furthermore, because of their side effects and rapid emergence of drug-resistant strains, there is a continuing need for alternative inhibitors. So traditional drugs and natural products have entered the research area. In recent years some researches indicated that several endophytic fungi of medicinal plants have the same or similar active components to their hosts<sup>[1]</sup>. The products and extracts of fungi exhibit anti-tumour, anti-oxidation, anti-HIV and immunoregulation activities<sup>[2-3]</sup>. *Dendrobium* sp. is a rare Chinese traditional medicine and possesses antiplatelet aggregation, anti-tumour, anti-oxidation and immunomodulatory activities<sup>[4-7]</sup>. The fungi isolated from *Dendrobium* sp. can be used as materials of new drugs, including anti-HIV compounds. In this study, the extract from a Chinese fungus isolated from *Dendrobium* sp., was tested for its inhibitory effect on HIV-1 by a series of *in vitro* experiments.

### MATERIALS AND METHODS

#### *Fungus and Preparation of Extract*

The fungus was isolated and kept in fungi laboratory of our institute under the acquisition number 1, and cultured in wheat bran culture medium (containing dextrose, monopotassium phosphate, magnesium sulfate) on rotary shaker without light for 14 days. The fungus was filtrated to separate mycelium from fermentation liquor. Mycelium was rinsed to eliminate fermentation liquor before desiccation, and dry mycelium was extracted twice with 95% ethanol by ultrasonic for 30 min. The solvents were removed under reduced pressure and evaporated to dryness in a rotary evaporator at 70°C (yield: 32%). The dry extracts were dissolved in RPMI-1640 before use.

#### *Cells and Virus*

The HTLV-1-infected cell line MT-4 cells were maintained at 37°C under 5% CO<sub>2</sub> in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mmol/L L-glutamine, penicillin G

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(50 IU/mL) and streptomycin (50  $\mu\text{g/mL}$ ). HIV-1 (strain HIV-1<sub>SF33</sub>) was obtained from the culture supernatants of MT-4/HIV-1<sub>SF33</sub> cells.

#### Anti-HIV-1 Assay

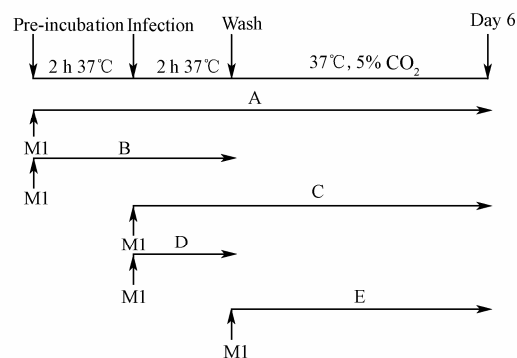
Aliquots of  $6 \times 10^5$  MT-4 cells per mL were infected with HIV-1<sub>SF33</sub> at a multiplicity of infection (MOI) of 0.01. The HIV-infected or mock-infected MT-4 cells were placed in 96-well culture plates (100  $\mu\text{L}$ /well) containing 100  $\mu\text{L}$  of the extract of mycelium (M1) at various concentrations and incubated at 37°C under 5% CO<sub>2</sub>. After 6 days, cell viability was quantified by MTT assay. The p24 core antigen in culture supernatants was assessed with the Vironostika HIV-1 antigen kit (BioMérieux, France).

#### Time of Action of M1

To assess the action time of M1, the compound was added at different time points (Table 1)<sup>[8]</sup>. Treatment A: MT-4 cells ( $1 \times 10^6$  cells/mL) were pre-incubated for 2 h in a 96-well microtiter plate containing 500  $\mu\text{g/mL}$  M1. Cells were infected by adding into wells 100TCID<sub>50</sub> HIV-1 suspension. After two hours' infection, infected cells were washed three times with RPMI and cultured in a 96-well microtiter plate in the presence of M1. Treatment B: The same procedure was employed, but the cells were cultured without the compound after being washed. Treatment C: M1 was added simultaneously with the virus. Treatment D: M1 was present only during infection. Treatment E: M1 was present only after infection.

TABLE 1

Time of Action of M1: Inhibition of p24 Antigen in Culture Supernatants



Inhibition of p24 Antigen ( $\bar{x} \pm s$ )

Treatment A	85.708 $\pm$ 0.444
Treatment B	0
Treatment C	72.168 $\pm$ 1.823
Treatment D	0
Treatment E	80.704 $\pm$ 6.437

#### Time of Addition Experiment

MT-4 cells were infected with  $1 \times 10^4$  TCID<sub>50</sub> HIV-1 (SF33) at 37°C for 1 h. Then the infected cells were washed three times with RPMI and cultured in a 96-well microtiter plate, and the test compounds were added at different time points (0, 0.5, 1, 2, 4, 6, 12, or 24 h) after infection. Viral p24 antigen in culture supernatants was determined 29 h post-infection<sup>[9]</sup>.

#### DNA-PCR Analysis of Infected Cells

MT-4 cells were infected with  $1 \times 10^4$  TCID<sub>50</sub> HIV-1(SF33) at 37°C for 1 h. Then the infected cells were washed three times with RPMI and cultured in a 96-well microtiter plate, and the test compounds were added immediately after washing. Cells were harvested at different time points (3, 11, or 24 h) after infection. DNA extraction was performed using the QIAamp DNA blood mini kit (QIAGEN, Germany). At 24 h after infection some cell pellets were frozen as dry pellets to quantify HIV RNA.

PCR was performed for 35 cycles at 94°C for 60 s, annealing at 57°C for 60 s, and at 72°C for 60 s. Each sample was amplified with  $\beta$ -actin primers to verify the presence of amplifiable DNA. HIV-1 negative DNA was included in the PCR experiments to monitor cross-contamination. The primer sets for early (long terminal repeat [LTR] U3-R) and late (LTR U3-gag) reverse transcription products were: LTR U3=CAGATATCCACTGACCTTTGG, LTR R=GAGGCTTAAGCAGTGGGTTT, gag=GCTTAA TACTGACGCTCTCGCA<sup>[10]</sup>.

#### Quantification of Infected Cells HIV RNA

HIV RNA of infected cells was determined by COBAS AMPLICOR HIV-1 MONITOR<sup>TM</sup> Test, version 1.5 with some modifications according to the method of Burgard M<sup>[11]</sup>.

#### Statistical Analysis

The data were analyzed by homoscedasticity test and then by Newman-Keuls test to analyze if they had statistical significance.

## RESULTS

#### Anti-HIV-1 Assay

M1 inhibited the p24 core antigen in culture supernatants of HIV-1-infected MT-4 cells whose IC<sub>50</sub> was < 31.25  $\mu\text{g/mL}$ . The CC<sub>50</sub> of M1 was >1000  $\mu\text{g/mL}$  (MTT assay) and selected index (SI) >32 (Fig. 1).

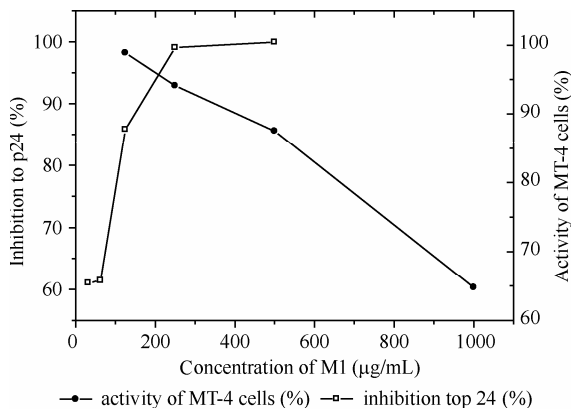


FIG. 1. Toxicity and inhibition to p24 core antigen of M1.

*Time of Action of M1*

The time of action of M1 was studied by adding the compound at different time points of the experiment (Table 1). Treatments B and D were ineffective to block viral infection. To achieve the effect of inhibiting p24 antigen production, the compound must be present during the whole culture (treatments A and C) or after infection (treatment E). The results suggested that M1 began to show its effect after virus entered cells.

*Time of Addition Experiment*

To understand at which stage M1 interacted with HIV replication cycle, compounds were added at different time points after the adsorption of HIV-1 to the MT-4 cells (Fig. 2). Dextran sulfate (DS) and AZT were run in parallel as reference compounds. Dextran sulfate interfering with virus binding, was no longer able to inhibit HIV-1 replication if added 0 h (or later) after the adsorption. Reverse transcriptase

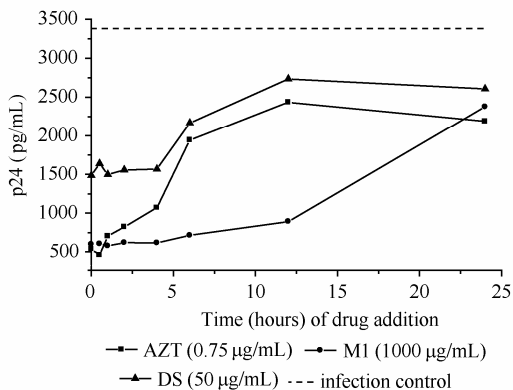


FIG. 2. Time of addition experiment. MT-4 cells were infected with  $1 \times 10^4$  TCID<sub>50</sub> HIV-1 (SF33). The test compounds were added at different time points after infection. Viral p24 antigen in culture supernatants was determined 29 h post-infection.

inhibitor, AZT began to lose its inhibitory effect if the treatment was delayed for 4 h. However M1 could decrease the p24 production even if it was added 12 h after the infection. These results suggested that M1 inhibited late stage of HIV life cycle.

*DNA-PCR Analysis of Infected Cells*

In a single round of infection, products related to various stages of reverse transcription process could be detected in time-dependent studies (Fig. 3). To detect early steps in reverse transcription, LTR specific primer pair was used. This primer pair flanked on the first region of DNA synthesized from RNA of the virus. To detect integrated proviral HIV DNA in infected cells, primer pair specific for HIV LTR and gag was used.

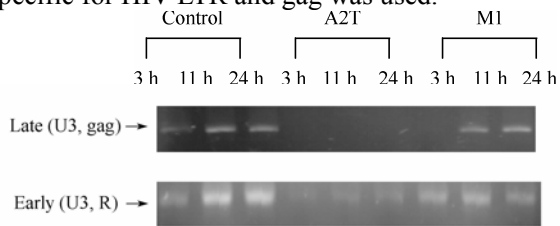


FIG. 3. PCR analysis of early (U3, R) and late (U3, gag) MT-4 cells infected with HIV-1 (SF33).

Early product was detected in all samples. In M1-treated cells, there was less cDNA synthesis and the least in AZT-treated cells. Late product was detected in untreated cells after 3 h of infection. But in M1-treated cells it did not appear until 11 h, suggesting that there was a delay in progression from early to late product. In AZT-treated cells, late products were not detectable.

*Quantification of HIV RNA of Infected Cells*

To detect if HIV was blocked before its RNA expression, HIV RNA of infected cells was quantified (Fig. 4). The results were expressed as the number of copies of HIV RNA per million cells. The HIV RNA level was significantly lower in M1-and AZT-treated cells than in control cells, and it was the lowest in AZT-treated cells. The results of this experiment were consistent with DNA-PCR analysis.

DISCUSSION

The replication cycle of HIV comprises ten steps: adsorption, fusion, uncoating, reverse transcription, integration, DNA replication, transcription, translation, maturation and budding (assembly/release)<sup>[12]</sup>. Time of action experiment suggested that M1 took effect

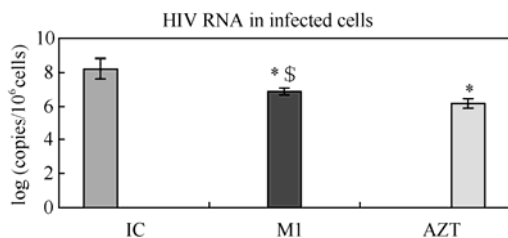


FIG. 4. Quantification of HIV RNA of infected cells at 24 h after infection. Compared to infected control (IC) group \* $P < 0.05$ ; Compared to AZT group  $^{\$}P < 0.05$ .

after virus entered cells. The time of addition studies indicated that M1 inhibited late steps of HIV life cycle. Analyses of cDNA products demonstrated that M1 delayed the appearance of late products, but did not inhibit the post-binding HIV replicative processes leading to the integration of proviral DNA. These events would include the formation and transport of the pre-integration complex to the nuclei, and its integration into host genome. Quantification of HIV RNA of infected cells indicated that HIV was blocked before its RNA expression. So it can be inferred that the target of M1 is post-integration of proviral DNA.

Since crude extracts of the fungus were used, the effective concentration of M1 to suppress HIV replication in cell culture was considerably higher than that of the anti-HIV compounds used in clinical practice. But the toxicity of M1 was very low. The toxicity and inhibition to p24 core antigen of M1 were concentration-dependent, suggesting that the extract was stable and only one component of the extract was activated. Further studies to determine the active components are now in progress.

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