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Alpha-momorcharin inhibits HIV-1 replication in acutely but not chronically infected T-lymphocytes[1]

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KEY WORDS α-momorcharin; ribosome-inactivating proteins; HIV; antigen p24; giant cells; T-lymphocytes; anti-HIV agents; cytotoxicity

ABSTRACT

AIM: To identify the anti-human immunodeficiency virus type 1 (HIV-1) activities of α-momorcharin (α-MMC) from *Momordica charantia* in acutely and chronically infected T-lymphocytes. **METHODS:** The anti-HIV activities of α-MMC were examined by 1) the inhibition of syncytia formation induced by HIV-1 ⅢB; 2) reduction of p24 core antigen expression level and decrease in numbers of HIV antigen positive cells in acutely and chronically infected cultures. The cytotoxic effects of α-MMC was tested by trypan blue dye exclusion or colorimetric MTT assay. **RESULTS:** α-MMC was found to obviously inhibit HIV-1 ⅢB-inducing C8166 syncytia formation and markedly reduced both expression of p24 core antigen and the numbers of HIV antigen positive cells in acutely but not chronically HIV-1-infected culture. The median effective concentration (EC_{50}) in these assays were 0.016, 0.07, and 0.32 mg $\cdot L^{-1}$, respectively. **CONCLUSION:** α-MMC is a unique component of momorcharin with anti-HIV activity, and markedly inhibited HIV-1 replication in acutely but not chronically HIV-1-infected T-lymphocytes.

INTRODUCTION

A ribosome-inactivating protein (RIP), trichosanthin (TCS, formerly named GLQ223 or compounds Q), purified from the root tubers of *Trichsanthes kirilowii*, was found to preferentially inhibit replication of HIV-1 in acutely infected T-lymphocytes and monocyte-derived macrophages[1]. The results of clinical trial Ⅰ/Ⅱ suggested that the TCS increased $CD4^+$ T cells and improved certain symptoms in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex[2]. From then on, the anti-HIV activities of other RIP, such as TAP29 from *Trichsanthes kirilowii*, MAP30 from *Momordica charantia*, GAP31 from *Gelonium multiflorum*, PAP from *Phytolacca americana*, DAP30 and DAP32 from *Dianthus caryophyllus*, and bryodin from *Bryonia cretica* had been reported[3-7]. RIP-resistant HIV strains have not been found up to now.

A novel RIP, momorcharin (MMC) had been isolated from the seeds of bitter melon (*Momordica charantia*) used in China and Southeast Asia for centuries as a source of medicinal ingredients. MMC consists of three forms designated α, β, and γ momorcharins (α-MMC, β-MMC, γ-MMC) with the molecular weights of 29, 28, and 11.5 kDa, respectively[8]. The crystal structures of α-MMC and β-MMC have been measured. MMC displays multiple pharmacological properties, for in-

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stance, termination of early and mid-term gestation, inhibition of the growth of some transformed trophoblastic cells, and suppression of the immune response. The crude MMC was found to be effective in inhibiting HIV-1 replication in HIV-1-infected T-lymphocytes and monocytes/macrophages[9]. The purpose of this study was to know which form of MMC possessed the anti-HIV activity in either acute or chronic infection of T-lymphocytes by HIV-1 ⅢB.

MATERIALS AND METHODS

Cell lines and virus The $CD4^+$ T cell lines H9 and C8166 were donated by Medical Research Council (MRC), AIDS Reagent Project, UK. All cell lines were maintained in RPMI-1640 (Gibco) supplemented with 10 % (vol/vol) heat-inactivated fetal calf serum, glutamine (Sigma) 2 mmol·L^{-1}, HEPES (Sigma) 10 mmol·L^{-1}, 2-mercapto ethanol (Bio-Rad) 50 mmol·L^{-1}, benzylpenicillin and streptomycin 100 kU·L^{-1}. The virus, H9/HIV-1 ⅢB donated by MRC, was obtained from the supernatant of HIV-1 ⅢB/H9 culture. Virus stocks were stored in small aliquots at −70 ℃.

Reagents and chemicals Anti-HIV-1 p24 monoclonal antibody was kindly donated by Prof K Ikuta (Institute of Immunological Science, Hokkaido University, Japan). An AIDS patient's serum (APS) was kindly provided by Prof H Hoshino (Department of Hygiene and Virology, Gunma University School of Medicine, Japan). Horseradish peroxidase (HRP)-labeled goat anti-human IgG and fluorescein isothiocyanate (FITC)-conjugated sheep anti-human IgG were purchased from Sino-American Biotechnological Corp (Luoyang, China).

Source and purification of α-MMC α, β, γ-MMC were purified from the seeds of *Momordica charantia* using procedures described previously[10].

Enzyme-linked immunosorbent assay (ELISA) for HIV-1 p24 antigen HIV-1 p24 antigen in a cell-free culture medium was determined by an antigen capture ELISA[11]. Briefly, Greiner ELISA plates (USA) were coated with 100 μL per well of capture p24 monoclonal antibody at 10 mg·L^{-1} diluted in $NaHCO_3$ 35 mmol·L^{-1}, and blocked by 5 % milk powder (Japan) for 1.5 h. Diluted sample 100 μL was added and incubated at 37 ℃ for 2 h. After incubation of the well with 1∶500 diluted APS 100 μL for 1 h, HPR-labeled goat anti-human IgG 100 μL was added. Following washed 5 times with PBS, ABTS solution 100 μL was added. After stop of reaction, the plates were read on a Bio-Rad 3550 ELISA reader (test/reference; 405 nm/595 nm). The results were shown by absorbance values.

Determination of HIV-1 antigen expression by indirect immunofluorescence assay (IFA) The HIV-1 ⅢB-infected cells were spun onto the surface of an 8-well multitest slide (ICN Biomedicals, USA) and air-dried at 21 ℃. The cells were fixed for 15 min in cold acetone, air-dried, and used immediately or stored at −70 ℃. For the assay, an APS serum (1∶50) were added and then incubated at 21 ℃ for 15 min. After the slides were washed 5 times, 1∶50 FITC-conjugated sheep anti-human IgG was added, incubated for 15 min, and washed 5 times. The slide was mounted by DABCO (1,4-diazobicyclo-[2,2,2]-octane) solution and examined under the fluorescence microscope. The % decrease of HIV antigen positive cells was calculated: decrease (%) = [1 − (% of IFA-positive cells in α-MMC-treated culture/% of IFA-positive cells in infected control culture)] × 100 %.

Assay for inhibition of syncytium formation α-MMC in PBS was serially diluted with RPMI-1640, and 100 μL of each solution was added to triplicate wells of a 96-well flat-

bottomed microtiter plate before addition of 3×10^4 C8166 cells to each well. Cells were inoculated with 200 $TCID_{50}$ of HIV-1 ⅢB at multiplicity of infection (MOI) of 0.007 for this experiment. The final volume of the culture in each well was 300 μL. After incubation at 37 ℃ for 3 d, syncytia from 5 fields of each well were counted under an inverted microscope (× 100)[12]. The inhibition of syncytium formation was measured by percentage of syncytium number in α-MMC treated culture to that in infected control culture.

Assay for anti-HIV activity of α-MMC in acutely infected cells C8166 cells 5×10^4 in logarithmic-phase growth in complete medium 50 μL were placed onto each well in 96-well plate. The cells were pretreated with 100 μL of α-MMC at various concentration for 60 min. HIV-1 ⅢB stock solution 50 μL containing 200 $TCID_{50}$ was added to each well (MOI was 0.005). The plates were incubated at 37 ℃ in a humidified incubator at 5 % CO_2 for 72 h[13]. Cell viability was determined by trypan blue dye exclusion. The HIV-1 p24 antigen in the supernatant was measured with ELISA assay. HIV antigen positive cells were scored by IFA. AZT was the drug for positive control. All assays were carried out in triplicate.

Assay for anti-HIV activity of α-MMC in chronically infected H9 cells The assay was conducted as described[14]. Briefly, aliquots of 4×10^4 H9 cells chronically infected with HIV-1 ⅢB were washed 3 times to remove extracellular virus, and resuspended in 100 μL of complete medium in 96-well plate. α-MMC was added. After 96-h incubation, the supernatants were tested for HIV p24 antigen by ELISA assay, and compound cytotoxicity was determined by MTT colorimetric assay.

RESULTS

Inhibition of syncytium formation

α-MMC inhibited syncytium formation in a concentration-dependent manner (0.003 – 2.06 $mg \cdot L^{-1}$, Tab 1).

Tab 1. Inhibition of HIV-1 inducing C8166 syncytium formation by α-MMC and its cytotoxicity. $n = 3$ wells. $\bar{x} \pm s$.

α-MMC/ $mg \cdot L^{-1}$	Inhibition/ %*	α-MMC/ $mg \cdot L^{-1}$	Viable cells/ % of control#
2.06	97.6 ± 2.4	30.85	6 ± 3
0.41	81 ± 7	6.17	47 ± 4
0.082	63 ± 5	1.23	80 ± 11
0.0164	50 ± 20	0.247	99 ± 9
0.00328	34 ± 10	0.049	113 ± 18

*The mean number of syncytia in untreated control was 83; #The A value of control was 0.84 ± 0.11.

Its EC_{50} (median effective concentration) and CC_{50} (median cytotoxic concentration) were 16 (95 % confidence limits was 11 – 22) $\mu g \cdot L^{-1}$ and 5 (95 % confidence limits was 1 – 24) $mg \cdot L^{-1}$, respectively (Tab 1). The selective index (SI) of α-MMC was about 328. However, β and γ-MMC had no effects on syncytium formation (data not shown).

Effects of α-MMC on HIV-1 antigen expression in acutely infected cells In the presence of α-MMC 0.04 $mg \cdot L^{-1}$, the expression of p24 antigen was reduced to 42.5 % of the untreated control and the HIV antigen positive cells was decreased to 31.7 % of the control. The EC_{50} was 0.1 (95 % confidence limits: 0.01 – 0.5) $mg \cdot L^{-1}$ and 0.32 (95 % confidence limits: 0.04 – 2.45) $mg \cdot L^{-1}$, respectively (Tab 2). Cytotoxic or cytostatic effects were not found under these concentration.

Effects of α-MMC on expression of HIV-1 p24 antigen in chronically infected H9 cells α-MMC did not affect the HIV-1 ⅢB replication in the chronically infected cells and

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Tab 2. Effects of α-MMC in HIV-1 ⅢB-acutely infected C8166 cells. $n = 3$ wells. $\bar{x} \pm s$.

α-MMC/ mg·L^{-1}	Percent decrease of HIV antigen positive cells*	Inhibition of p24 antigen expression/ %#
5.0	83 ± 8	85.8 ± 0.1
1.0	64 ± 11	74.3 ± 0.3
0.2	44 ± 4	64.0 ± 1.5
0.04	31 ± 7	42.5 ± 0.4

*The ratio of IFA$^+$ cells in infected control culture was 19.8 ± 1.3; #Inhibition (%) of p24 antigen expression = [1 − (A value of α-MMC treated culture/A value of control culture)] × 100 %. A value of control was 0.593 ± 0.012.

p24 antigen expression was not changed even in the presence of the cytotoxic concentration (Tab 3).

Tab 3. Effects of α-MMC on p24 antigen expression in H9 cells chronically infected with HIV-1 ⅢB. $n = 3$ wells. $\bar{x} \pm s$.

α-MMC/ mg·L^{-1}	Viable cells/ % of control*	p24 antigen level/ % of control#
5.0	8 ± 1	86 ± 3
1.0	24 ± 2	108 ± 9
0.2	69 ± 3	110 ± 3
0.04	57 ± 6	90 ± 3

*The absorbance value of control was 1.27 ± 0.15;
#The absorbance value of control was 0.253 ± 0.006.

DISCUSSION

Four RIP (α, β, γ-MMC and MAP30) had been purified from the seeds of bitter gourd (*Momordica charantia*), also called "Kuguazi" in Chinese. Among them, MAP30 was shown to be effective in inhibition of the HIV-1 infection by topological inactivation of viral DNA, inhibition of viral integrase. MAP30 also possesses anti-tumor activity and cell-free ribosome-inactivating activities[15]. Our study indicated that β- and γ-MMC were not able to inhibit HIV-1 ⅢB inducing C8166 syncytia formation.

The α-MMC is a glucoprotein consisting of 250 amino acids. The amino acid sequence of α-MMC has been determined, and is 54 % identical with β-MMC. α-MMC shares about 48 % amino acids homology to MAP30[15]. In the present investigation, we found that α-MMC obviously inhibited syncytium formation induced by HIV-1 ⅢB, markedly reduced expression of p24 antigen, and decreased the numbers of HIV antigen positive cells in acutely HIV-1-infected culture. α-MMC was not able to block HIV infection cell fusion in co-culture and did not protect HIV-1-infected host cells (MT-4 or C8166) from dying. α-MMC did not inhibit expression of p24 antigen in chronically HIV-1-infected H9 cells.

These results showed that α-MMC did not interfere with adsorption, mature, infectivity, release and replication of virus. α-MMC is a unique component with anti-HIV activity in momorcharins.

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α-苦瓜子蛋白抑制急性感染而不抑制慢性感染 T 淋巴细胞中 HIV-1 的复制[1]

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关键词 α-苦瓜子蛋白；核糖体失活蛋白；HIV；抗原 p24；巨细胞；T 淋巴细胞；抗 HIV 剂；细胞毒性

目的：研究 α-苦瓜子蛋白(α-MMC)在 HIV-1 急性和慢性感染 T 淋巴细胞中的抗 HIV-1 活性. **方法**：以合胞体抑制实验，HIV-1 p24 核心抗原表达水平和 HIV 抗原阳性细胞的百分率检测了 α-MMC 的抗 HIV 活性. **结果**：α-MMC 对 HIV-1 ⅢB 诱导 C8166 细胞形成合胞体有显著的抑制作用. α-MMC 显著地抑制了 HIV-1 急性感染 T 细胞中 p24 抗原的表达水平和减少了 HIV 抗原阳性细胞的百分率. 在上述三种测定中，α-MMC 的 EC_{50} 分别是 0.016，0.07 和 0.32 mg·L^{-1}. 但是，α-MMC 不影响慢性感染 HIV-1 ⅢB/H9 中 p24 抗原表达水平. **结论**：α-MMC 是苦瓜子蛋白中唯一的具有抗 HIV-1 活性的成份. α-MMC 抑制急性感染中 HIV-1 的复制而对慢性感染 T 细胞中的 HIV-1 复制无影响.

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