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Anti-HIV-1 activity of trichobitacin, a novel ribosome-inactivating protein[1]

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KEY WORDS trichobitacin; protein synthesis inhibitors; HIV-1; anti-HIV agents; plant proteins; giant cells; HIV antigens

ABSTRACT

AIM: To determine whether trichobitacin, a novel ribosome-inactivating protein purified from the root tubers of *Trichosanthes kirilowii*, possesses the anti-HIV activity. METHODS: The inhibition of syncytial cell formation induced by human immunodeficiency virus type 1 (HIV-1) was determined under microscope, reduction of HIV-1 p24 antigen expression level was measured by ELISA, and decrease in numbers of HIV-1 antigen positive cells in acutely and chronically infected cultures were detected by indirect immunofluorescence assay. RESULTS: Trichobitacin was found to greatly suppress syncytial cell formation induced by HIV-1 and to markedly reduce both expression of HIV-1 p24 antigen and the number of HIV antigen positive cells in acutely but not chronically HIV-1 infected culture. The median inhibitory concentration (IC_{50}) in inhibition of syncytial cell formation and HIV antigen positive cells were 5 $\mu g \cdot L^{-1}$ (95 % confidence limits: 1.3－20 $\mu g \cdot L^{-1}$) and 0.09 $mg \cdot L^{-1}$ (95 % confidence limits: 0.011－0.755 $mg \cdot L^{-1}$), respectively. CONCLUSION: Trichobitacin is a novel ribosome-inactivating protein with anti-HIV-1 activity.

INTRODUCTION

Trichosanthin (TCS), TAP29, and karasurin are single-chain ribosome-inactivating proteins (RIP) purified from the root tubers of Chinese medicinal herb *Trichosanthes kirilowii* Maxim[1－3]. TCS and TAP29 had been found to preferentially inhibit replication of human immunodeficiency virus type 1 (HIV-1) *in vitro*[1,2]. The clinical trials demonstrated that TCS had the ability to decrease serum HIV-1 p24 antigen level and to increase percentage of $CD4^+$ T cells in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex[4]. In addition, seven other RIP (MAP30, GAP31, PAP, DAP30, DAP32, bryodin, and α-MMC) had been reported to inhibit HIV-1 replication *in vitro*[5].

Recently, a novel single-chain RIP, named trichobitacin, from the press-residue of the root tuber of *T kirilowii* Maxim was purified and characterized[6,7]. In this study, the anti-HIV activities of trichobitacin were explored.

MATERIALS AND METHODS

Purification and characterization of trichobitacin

Trichobitacin was isolated and purified from the root tubers of *T kirilowii* (Pinghu, Zhejiang province, China), and characterized as described previously[6,7].

Reagents and chemicals

Dextran sulfate (MW 500 000, Pharmacia), skim milk powder and human polyclonal anti-HIV serum were kindly donated by Dr HOSHINO Hiroo (Department of Hygiene and Virology, Gunma University School of Medicine, Japan). Monoclonal antibody to HIV-1 p24 was kindly donated by Dr IKUTA Kazuyoshi (Institute of Immunological Science, Hokkaido University, Japan). Horseradish peroxidase (HRP)-labeled goat anti-human IgG and fluorescein isothiocyanate (FITC)-conjugated sheep anti-human IgG were purchased from Sino-American Biotech Corp.

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Cell lines and virus H9 and C8166 cells were donated by Medical Research Council (MRC), AIDS Reagent Project, UK. The H9/HIV-1 ⅢB cell line, which had been persistently infected with HIV-1 ⅢB, was also donated by MRC. All cell lines were maintained in RPMI-1640 (Gibco) supplemented with 10 % (vol/vol) heat-inactivated fetal calf serum. HIV-1 ⅢB was obtained from the culture supernatant of H9/HIV-1 ⅢB cells. Virus stocks were stored in small aliquots at −70 ℃.

Measurement of HIV-1 p24 antigen HIV-1 p24 antigen was measured using an antigen capture ELISA as described previously[5].

Determination of HIV-1 antigen expression HIV-1 antigen expression was determined by indirect immunofluorescence assay (IFA)[5].

Inhibition assay for the cytopathic effects of HIV-1 Trichobitacin serially diluted with RPMI-1640 was added to triplicate wells of a 96-well flat-bottomed microtiter plate, then 3×10^4 C8166 cells and 200 $TCID_{50}$ of HIV-1 ⅢB stock solution were added immediately to each well. After incubation at 37 ℃ for 72 h without changing medium, syncytial cells from five different fields of each well were examined and counted under an inverted microscope (100×)[8]. The cytotoxic effect of trichobitacin on C8166 was measured by MTT assay as described previously[9], and the results were shown by absorbance (A). 3′-Azido-3′-deoxythymidine (AZT, Sigma) and dextran sulfate were the drugs for positive control in each experiment. The % inhibition of syncytial cell formation was calculated by percentage of syncytial cell number in drugs treated culture to that in infected control culture.

Anti-HIV activity assay in acutely infected cells Before 200 $TCID_{50}$ HIV-1 ⅢB diluted solution was inoculated with 5×10^4 C8166 cells, these cells were pretreated with trichobitacin at various concentration at 37 ℃ for 60 min. The plates were incubated in a humidified incubator at 5 % CO_2 for 3 d[10]. AZT was the drug for positive control. All assays were carried out in triplicate. The total number of viability cells in each well was counted on d 3 by trypan blue dye exclusion and relative amount of viable cells was calculated. Terminated cell culture was centrifuged, and cell-free supernatant from each well was used to measure the HIV-1 p24 antigen level with ELISA assay. The cell pellet was resuspended and applied to determine viral antigen expression by IFA.

Anti-HIV activity assay in chronically infected H9 cells The assay was conducted as described elsewhere[5, 11]. H9/HIV-1 ⅢB cells were washed three times to remove extracellular virus. Trichobitacin diluted at various concentrations was added. After 4-day incubation at 37 ℃ in a humidified incubator, the supernatant from each well was used to measure the HIV-1 p24 antigen with ELISA assay, and compound cytotoxicity was determined by MTT assay.

RESULTS

Suppression of syncytial cell formation Trichobitacin exhibited concentration-dependent suppression of giant cell formation. Trichobitacin at 0.116 $mg\cdot L^{-1}$ and 0.001 $mg\cdot L^{-1}$ caused 96 % and 27 % inhibition of syncytial cell formation, respectively. The IC_{50} was 5 $\mu g\cdot L^{-1}$ (95 % confidence limits：1.3 − 20 $\mu g\cdot L^{-1}$), whereas the CC_{50} (median cytotoxic concentration) was 2.0 $mg\cdot L^{-1}$ (95 % confidence limits：0.54 − 7.46 $mg\cdot L^{-1}$). The selective index (SI) of trichobitacin is 400. The IC_{50} and CC_{50} of AZT were 0.132 $mg\cdot L^{-1}$ (95 % confidence limits：0.02 − 0.9 $mg\cdot L^{-1}$) and 793.1 $mg\cdot L^{-1}$ (95 % confidence limits：183.7 − 3419.8 $mg\cdot L^{-1}$) respectively. Its SI is about 6008. (Tab 1)

Tab 1. Suppression of HIV-1 inducing C8166 syncytia formation by trichobitacin and its cytotoxicity. $n=6$ wells from 2 experiments, 3 wells for each experiment. $\bar{x}\pm s$.

Agents	Concentration /$mg\cdot L^{-1}$	Inhibition of syncytia formation/% *	Concentration /$mg\cdot L^{-1}$	Viable cells/% of control†
AZT	6.7	88 ± 3	2500	16 ± 5
	1.34	81 ± 2	500	64 ± 4
	0.268	56 ± 9	100	94 ± 9
	0.0536	42 ± 8	20	99 ± 3
Trichobitacin	0.116	96 ± 2	4.35	28 ± 2
	0.0232	84 ± 11	0.87	74 ± 1
	0.00464	55 ± 19	0.174	97 ± 2
	0.00093	27 ± 24	0.0348	107 ± 3

*The mean number of sycytial cells in untreated control was 45；†The cell viability was measured by MTT assay, and A value of control was 1.11 ± 0.03.

Effects of trichobitacin on HIV-1 replication in acutely infected cells In presence of 0.04 $mg\cdot L^{-1}$, trichobitacin exhibited 53 % inhibition of the expression

of p24 antigen, and the HIV antigen positive cells were decreased by 44 % (Tab 2). The IC_{50} of 0.09 mg·L^{-1} (95 % confidence limits：0.011－0.755 mg·L^{-1}) was obtained from the results of percent decrease of HIV antigen expression. Since 88.4 % of viable cells were observed at 0.04 mg·L^{-1}, the reduced expression of antigen was not related to cytotoxic or cytostatic effects of trichobitacin.

Tab 2. Effects of trichobitacin on HIV-1 replication in acutely infected C8166 cells. $n = 6$ wells from 2 experiments, 3 wells for each experiment. $\bar{x} \pm s$.

Compounds	Concentration /mg·L^{-1}	Viable cells /% of control*	Decrease of IFA^+ cell/%#	Inhibition of p24 expression /%†
AZT	0.08	118±3	71±3	85.7±1.2
Trichobitacin	5.0	47±7	76±8	84.2±1.2
	1.0	78±5	63±5	80.2±2.1
	0.2	90±18	55±5	61.8±0.8
	0.04	88±13	44±5	52.6±2.2

*The amounts of viable cells were determined by trypan blue dye exclusion；#The ratio of IFA^+ cells in infected control culture was 20±1；† p24 antigen was measured by ELISA assay, A of the untreated control was 0.593±0.012.

Effects of trichobitacin on HIV-1 p24 antigen expression in chronically infected H9 cells Trichobitacin did not obviously affect the HIV-1 III_B replication in the chronically infected H9 cells *in vitro* (Tab 3). The level of p24 antigen was slightly reduced by trichobitacin at the highly cytotoxic concentration.

Tab 3. Effects of trichobitacin on p24 antigen expression in H9 cells chronically infected with HIV-1. $n = 6$ wells from 2 experiments, 3 wells for each experiment. $\bar{x} \pm s$.

Compounds	Concentration /mg·L^{-1}	Viable cells /% of control*	p24 antigen level /% of control†
AZT	0.08	97±0.1	102±11
Trichobitacin	5.0	0.3±0.2	58±1
	1.0	12±3	52±9
	0.2	35±10	98±6
	0.04	79±13	95±1

*The viable cells was measured by MTT assay (the A of control was 1.27±0.15)；† The A of control was 0.253±0.01.

DISCUSSION

Seven RIP have been discoveried from *T kirilowii*, those are trichobitacin, TCS, TAP29 and karasurin from the root tubers, trichokirin[12], α-kirilowin[13] and β-kirilowin[14] from the seeds. They have similar biological activity and physicochemical properties. All of them are single-chain RIP, and both trichokirin and probably trichobitacin are glycoprotein[6]. High homology in amino acid sequence among them was found. RNA N-glycosidase activity of TCS and trichokirin was found, and abortion in mice was induced by TCS, α-kirilowin, β-kirilowin and karasurin[1,13,14]. Trichobitacin also possesses RNA N-glycosidase activity and inhibits the growth of human placental trophoblastic cells at the same grade as that of TCS[6]. In comparison with TCS, trichobitacin exhibited more strongly anti-HIV activity, and the CC_{50} of trichobitacin was equivalent to TCS (SI：400 *vs* 200 or less)[8].

The most intriguing biological property is the anti-HIV activity of those tested proteins. Trichobitacin, similar to TCS and TAP29, obviously inhibited syncytium formation induced by HIV-1 III_B, markedly reduced expression of p24 antigen, and decreased in the numbers of HIV antigen positive cells in acutely HIV-1 infected culture, but did not protect HIV-1 infected host cells (MT-4 or C8166) from dying. The exact mechanism of RIP on anti-HIV activities remains to be determined. Compounds disturbing virus maturity, infectivity and release, and replication of viral genome may inhibit HIV production in chronically infected H9 cells. AZT and other nucleoside analogues do not exhibit anti-HIV activity in these cells[15]. Present study showed that trichobitacin did not inhibit expression of p24 antigen in chronically HIV-1 infected H9 cells. In addition, trichobitacin was unable to block fusion between the chronically HIV infected H9 cells and uninfected C8166 cells in 24 h coculture. This result suggested that trichobitacin did not interfere with the adsorption and binding between host cells and viruses.

Although all RIP with anti-HIV activity have RNA *N*-glycosidase activity and all RIP tested so far have abortifacient activity, β and γ-momorcharins and some other RIP with RNA *N*-glycosidase and/or abortifacient activities do not possess anti-HIV activity[5,8]. Therefore, the direct relationship between RNA *N*-glycosidase activity, abortifacient activity and anti-HIV activity of the RIP remains to be ex-

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具有抗 HIV-1 活性的新核糖体失活蛋白-栝楼蛋白[1]

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关键词 栝楼蛋白；蛋白质合成抑制剂；HIV-1；抗 HIV 剂；植物蛋白；巨细胞；HIV 抗原

目的：研究新核糖体失活蛋白-栝楼蛋白是否具有抗 HIV-1 活性. **方法**：合胞体抑制实验以光镜检查，以 ELISA 测定 HIV-1 p24 抗原表达水平，用间接免疫荧光技术测定 HIV 抗原阳性细胞的百分率. **结果**：栝楼蛋白对 HIV-1 诱导 C8166 细胞形成合胞体有显著的抑制作用. 栝楼蛋白显著地抑制了 HIV-1 急性感染 T 细胞中 p24 核心抗原的表达水平和减少了 HIV 抗原阳性细胞的百分率. 栝楼蛋白抑制合胞体形成和 HIV 抗原阳性细胞的 IC_{50} 分别是 5 $\mu g \cdot L^{-1}$ 和 0.09 mg $\cdot L^{-1}$. 栝楼蛋白不影响慢性感染 T 细胞中 HIV-1 p24 抗原表达水平. **结论**：栝楼蛋白是具有抗 HIV-1 活性的新的核糖体失活蛋白.

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