MAP 30: a new inhibitor of HIV-1 infection and replication

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A new inhibitor of human immunodeficiency virus (HIV) has been isolated and purified to homogeneity from the seeds and fruits of the *Momordica charantia*. This compound, MAP 30 (Momordica Anti-HIV Protein), is a basic protein of about 30 kDa. It exhibits dose-dependent inhibition of cell-free HIV-1 infection and replication as measured by: (i) quantitative focal syncytium formation on CEM-ss monolayers; (ii) viral core protein p24 expression; and (iii) viral-associated reverse transcriptase (RT) activity in HIV-1 infected H9 cells. The doses required for 50% inhibition (ID_{50}) in these assays were 0.83, 0.22 and 0.33 nM, respectively. No cytotoxic or cytostatic effects were found under the assay conditions. These data suggest that MAP 30 may be a useful therapeutic agent in the treatment of HIV-1 infections. The sequence of the N-terminal 44 amino acids of MAP 30 has been determined.

Plant protein; Antiviral agent; AIDS

1. INTRODUCTION

Momordica charantia (MC) is a medicinal plant indigenous to China. The fruit and seed extracts of MC have been used in China for centuries for anti-viral, antitumor and immunopotentiating purposes [1]. In recent years, several proteins have been isolated from the seed extracts of this plant [2]. These proteins belong to the family of single chain ribosome-inactivating proteins. They inhibit in vitro translation of eukaryotic cells by catalytic inactivation of the 60S ribosomal subunit. These proteins were also found to inhibit the multiplication of herpes simplex virus-1 (HSV-1) and of poliovirus I in Hep-2 cells [3]. Fruit extracts of MC have been shown to possess in vivo antitumor activity and immune-enhancement ability. These extracts inhibited the formation of prostate adenocarcinoma in rats [4] and lymphoma in mice [5].

In view of these findings, we were particularly interested to study the effects of MC extracts on human immunodeficiency virus type-1 (HIV-1), the etiologic agent of acquired immunodeficiency syndrome (AIDS).

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In this manuscript, we report the isolation and purification to homogeneity of an anti-HIV protein, MAP 30, from the seeds and fruits of MC. We also report the characterization of the anti-HIV activities and the amino terminal sequence of this plant protein.

2. MATERIALS AND METHODS

2.1. Momordica charantia *plant*

There are many varieties of MC. The content of MAP 30 is most abundant in the medicinal variety, which has not been naturally available in this country. Cultivation has thus been carried out from selected seeds.

2.2. *Cell lines and viruses*

The CD4-positive T cell line CEM-ss (syncytium-sensitive Leu 3a-positive) was used as indicator cell for the microtiter syncytial-forming assay [6]. The H9 cell line was used for p24 expression and viral-RT activity assays. HIV-1 virus stock was obtained from R. Gallo. The virus was prepared and stocked as described previously [7]. The cell lines were maintained in RPMI-1640 with penicillin-streptomycin (100 U/ml) and 10% heat-inactivated fetal calf serum (complete medium).

2.3. *Purification and characterization of MAP 30*

For a routine preparation, 200 g of matured seeds were used. Briefly, the seeds were decorticated and pulverized. It was then extracted with ice-cold 0.15 M NaCl (solution A) by homogenizing for 5 min at a ratio of 6 ml of solution A per gram of seeds. The pH of the extract was adjusted to 3.6 with 1 M HCl. The mixture was stirred gently at 4°C for 15 min. Cell debris were removed by filtration with cheesecloth, followed by centrifugation at 12 000 × *g* for 30 min. The

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cleared supernatant was fractionated by precipitation with ammonium sulfate or chilled acetone (−20°C). The fraction in the 30–60% saturated ammonium sulfate or two volumes of acetone was found to contain anti-HIV activity. The precipitate was dissolved in 50 mM sodium phosphate, pH 6.3 (solution B) and dialyzed against the same solution. This material was referred to as Step 1 sample and was further purified by chromatography on CM-Sepharose CL 6B (CM-S) and Sephadex G75 superfine (Pharmacia-LKB). For the preparations of MAP 30 from ripe fruits, 2–5 kg were used routinely.

2.4. *Microtiter syncytial-forming assay*

Fresh indicator cells in complete medium were plated onto poly(L-lysine)-coated microtiter wells at 50 000 cells in 50 μl/well. The cells were pre-treated with 50 μl of MAP 30 at various concentrations (Table I), for 15 s or 90 min. At the end of these times, 50 μl of a frozen pre-titered HIV stock from H × B3/H9 cells, corresponding to one hundred syncytial forming units (SFU) was added to each well for 60 min. The supernatant containing MAP 30 and the virus was then removed from each well and the cells were washed with complete medium to remove residual free MAP 30 and HIV. The wells were then filled with 200 μl medium (15 s or 90 min preincubation) or re-fed medium containing MAP 30 at the same original concentration (continuous presence of MAP 30 for 120 h). The plates were incubated at 37°C in a humidified incubator at 5% CO_2. Focal syncytium formation representing a single infectious virion unit was scored at day 5 by examination under an inverted microscope.

2.5 *p24 expression and reverse transcriptase assays*

The effect of MAP 30 on HIV-1 replication and transmission in vitro was tested by viral core protein p24 expression [7] and viral-RT activity [8]. H9 cells were inoculated with a titered cryopreserved viral stock of H9/HTLV-IIIB at a multiplicity of infection of 0.005. Cells were incubated at 5×10^7/ml with the inoculum at 37°C for 60 min to allow viral absorption. The cells were then washed to remove unbound virus, and resuspended in complete medium. They were plated at 1×10^5/ml with or without the addition of MAP 30 for the duration of the experiment. In this assay, at the multiplicity of infection used, viral production peaks at day 4. Thus, p24 expression and HIV-associated RT activity were assayed in cell-free supernatants harvested at day 4.

2.6. *Cytotoxicity and cell viability*

Cytotoxicity of MAP 30 was measured by its effects on cellular synthesis of DNA and protein. The synthesis of these macromolecules was measured by pulse labeling the cells with 1 μCi of [^{3}H]thymidine or [^{3}H]leucine 8 h prior to harvesting at day 4. The incorporation of labeled precursor into TCA-insoluble products was measured by scintillation counting. Cell viability was determined by Trypan blue dye exclusion.

2.7. *Sequence analysis*

The N-terminal amino acid sequence of MAP 30 was determined by automated Edman degradation using an Applied Biosystems model 470A protein sequencer, with on-line PTH analyzer.

2.8. *In vitro translation assay*

In vitro translation of eukaryotic cells was measured by the incorporation of ^{3}H-labeled leucine into TCA-insoluble product in a rabbit reticulocyte lysate system [9] (Du Pont-New England Nuclear). The reaction was carried out at 37°C for 30 min in a total volume of 25 μl. The reaction mixture contained 1 μg of globin mRNA, 2 mM $MgAc_2$, 80 mM KAc, translation cocktail (2.5 mM spermidine, 34.5 mg/ml creatine phosphate, 26 mg/ml GTP in 250 mM HEPES buffer) and 1 μCi of [^{3}H]leucine.

3. RESULTS

3.1. *Cultivation of* Momordica charantia

Seeds were planted and germinated after 14 to 20 days. Plants began to bear fruit after 60 to 80 days and the fruit matured to ripeness by 90 to 120 days. Extracts were prepared from various parts of the plant at different stages of maturation for the isolation, purification and identification of antitumor and anti-HIV components. In the course of our work, it became evident that a potent anti-HIV component was present in the fruit and seed, and that its content increased significantly with the maturity of the fruit and seed. Fig. 1A shows a growing MC fruit which is green in color. As the fruit matured, its color turned to light yellow and finally bright orange as it reached full maturity. If the fruits were not harvested at this stage, they split open spontaneously, exposing the matured red seeds as seen in Fig. 1B. Only naturally ripened fruits and seeds were selected for the preparation of MAP 30.

3.2. *Purification and characterization of MAP 30*

Step 1 sample (153 mg) was loaded onto a column (1.5 × 34 cm) of CM-S equilibrated with solution B. The column was washed with the same solution to remove unbound impurities. Whereas MAP 30 bound to CM-S and was thus retained on the column. Fractions of 6 ml were collected at a flow rate of 36 ml/h. The elution was monitored by absorbance at 280 nm (A_{280}). Upon reaching the baseline absorbance, the column was then eluted with a linear gradient consisting of 240 ml of solution B and 240 ml of solution B containing 0.2 M NaCl. A typical elution profile is shown in Fig. 2 and five protein peaks were eluted. Fractions in each peak were assayed for anti-HIV activity, inhibition in translation and cytotoxicity. MAP 30 was found in peak 2, which was eluted between 60 to 70 mM NaCl. This material was designated as step 2 sample (14.5 mg). It was further purified by gel filtration on a column (1.5 × 134 cm) of Sephadex G75 in 20 mM sodium phosphate buffer, pH 6.3. The flow rate was at 3 ml/h and 1.5 ml fractions were collected. Homogeneous MAP 30 was eluted as a single peak at about 0.45 column volume.

The size, homogeneity and subunit structure of MAP 30 were determined by SDS-PAGE in the presence and absence of 2-mercaptoethanol. These results are shown in Fig. 3. A single band with a molecular mass corresponding to 30 kDa was obtained for MAP 30 both in the presence and absence of the reducing agent, indicating that this protein consists of a single chain polypeptide.

3.3. *Inhibition of syncytium formation*

This assay quantitates acute cell-free HIV-1 infection and is based on the interaction between fusigenic virus-infected cells expressing the HIV envelope gene products and uninfected adjacent cells bearing CD4 molecules. The results of two independent experiments are summarized in Table I and Fig. 4. A 90 min preincubation of the indicator cells with MAP 30 resulted in dose-dependent inhibition of HIV infection. At 1.67

Fig. 1. (A) Photograph of a MC plant with an immature fruit in its characteristic green color. (B) A ripened fruit with bright orange outer skin and red seed.

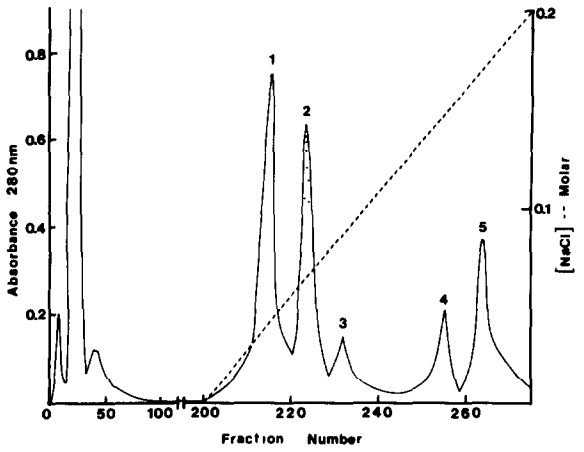

Fig. 2. Purification of MAP 30 by CM-Sepharose CL6B. Step 1 sample (153 mg) was chromatographed on a column of CM-S (1.5 × 34 cm) as described in the text. Absorbance at 280 nm (——), NaCl molar concentration (- -). Fractions of MAP 30 were pooled as marked in peak 2.

and 1670 nM, MAP 30 caused 60% and 86% inhibition on syncytium formation, respectively. An ID_{50} of 0.83 nM was obtained from these results. At the same concentrations, a 15 s pre-treatment caused 23% and 25% of inhibition, respectively. Under none of these conditions was any cytotoxic or cytostatic effect to the indicator cells observed. Continued presence of MAP 30 in the HIV infected cell culture for 120 h produced a higher inhibitory effect at all of the concentrations tested, with complete elimination of syncytium formation at 167 nM (legend of Table I). These results suggest that MAP 30 affects initial HIV infection as well as transmission of viral gene products through cell contact or release of free virions.

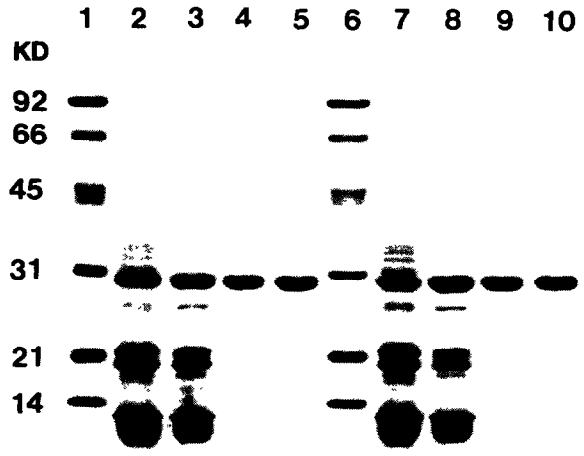

Fig. 3. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of MAP 30 in the presence of absence of 2-mercaptoethanol. Electrophoresis was carried out at a constant voltage of 80 V for 6 h. Lanes 1–5, samples treated with 2-mercaptoethanol: lane 1, molecular weight standards, 2 μg each; lane 2, crude MAP 30, 100 μg (30–60% saturated ammonium sulfate); lane 3, crude MAP 30, 100 μg (2.0 vols acetone); 4 and 5, homogeneous MAP 30, 8 μg each lane, purified from lanes 2 and 3, respectively. Lanes 6–10 are the same corresponding samples as in lanes 1 to 5, but without 2-mercaptoethanol treatment.

3.4. *Inhibition of viral core protein p24 expression*

To assess the antiviral activity of MAP 30 on another human T cell line in a suspension culture, p24 expression and viral-RT activity were examined in HIV-infected H9 cells. The expression of p24 was measured by RIA and expressed in ng/ml. As seen in Table II, in the presence of 0.334 nM MAP 30, the expression of p24 was reduced to 29% of the untreated control. As the concentration of MAP 30 was increased, inhibition of p24 expression also increased. Virtually complete inhibition was observed at 33.4 nM. The ID_{50} was about 0.22 nM. The reduced production of p24 was not due to cytotoxic or cytostatic effects, and no decrease in cellular DNA or protein synthesis was observed at these MAP 30 concentrations.

3.5. *Inhibition of viral associated RT activity*

RT activity was measured using poly(rA)·$p(dT)_{12-18}$ as template-primer and ^3H-labeled dTTP as substrate. The results are expressed as the polynucleotide incorporation of ^3H label in terms of cpm/ml (Table II). HIV-RT activity was reduced to 52, 25, 13 and 6% of control activity, in cells treated with 0.334, 3.34, 33.4 and 334 nM of MAP 30, respectively. The ID_{50} for this assay was about 0.33 nM. The reduction in HIV-RT activity is likely to be due to a decrease in virion production, which is also evidenced by decreased p24 expression.

3.6. *Assays on cytotoxicity*

In order to ascertain that the anti-HIV activity of MAP 30 is virus specific, the effect of MAP 30 on cellular DNA or protein synthesis was determined in uninfected H9 cells. These results are shown in Table II. From 0.334 to 33.4 nM, MAP 30 caused no detectable effect on cellular incorporation of labeled thymidine or leucine, while the majority of p24 and HIV-RT productions were inhibited. Even at 334 nM (in the range of $10^3 \times ID_{50}$), MAP 30 only yielded 25% or 28% reductions in cellular DNA or protein synthesis, respectively, as compared to virtually total inhibition of p24 and HIV-RT production in HIV-infected H9 cells. A therapeutic index of at least 1000 was observed.

3.7. *N-Terminal sequence of MAP 30*

The sequence of the first 44 amino acids from the N-terminus of MAP 30 is shown in Table III. A search in the EMBL protein databank and a structural analysis of MAP 30 sequence reveal homology with the N-terminal

Table I

The effect of MAP 30 on HIV infection as measured by syncytium formation in infectious cell center assay of HIV-infected CEM-SS cells

MAP 30 nM (μg/ml)	Syncytia/well in ICC preincubation		% ICC (V_n/V_o) preincubation		Cytotoxicity preincubation	
	15 s	90 min	15 s	90 min	15 s	90 min
0*	94	94	100	100	-	-
1.67 (0.05)	71,74	35,41	77	40	-	-
16.7 (0.5)	63,75	31,23	73	29	-	-
167 (5.0)	70,74	18,21	71	21	-	-
1670 (50)	62,71	16,10	71	14	-	-

Each test point was carried out in duplicate. Duplicate wells of indicator cells containing MAP 30 at each concentration without virus exposure were also included for the determination of MAP 30 cytotoxicity. % ICC (infectious cell center) are expressed in terms of V_n/V_o (average number of syncytia in MAP 30 treated samples/average number of syncytia in untreated controls); the values are the averages of two independent experiments. These experiments were carried out by preincubation of the indicator cells with MAP 30 for the specified time prior to the addition of virus. MAP 30 and virus were then removed by washing with complete medium and the cells were incubated with medium alone. In the untreated control experiment (*), the number of SF/ICC per well is the averaged value of 101, 92, 96 and 87. In the continuous presence of MAP 30 for 5 days, the V_n/V_o values are 39, 21, 0 and 0 for 1.67, 16.7, 167 and 1670 nM of MAP 30, respectively.

amino acid sequences of ricin A chain and trichosanthin [10,11]. The sequences of ricin A and trichosanthin are shown underneath the sequence of MAP 30 in Table III. It is interesting to note that most aromatic and hydrophobic amino acids in the predicted B sheet region are identical between these proteins.

3.8. *Inhibition of in vitro translation of eukaryotic cells*

The effect of MAP 30 on in vitro translation of eukaryotic cells is shown in Fig. 5. The results are expressed in terms of [^{3}H]leucine incorporation into TCA-insoluble product. MAP 30 exhibited a dose-dependent inhibition of cell-free translation with an ID_{50} of 3.3 nM.

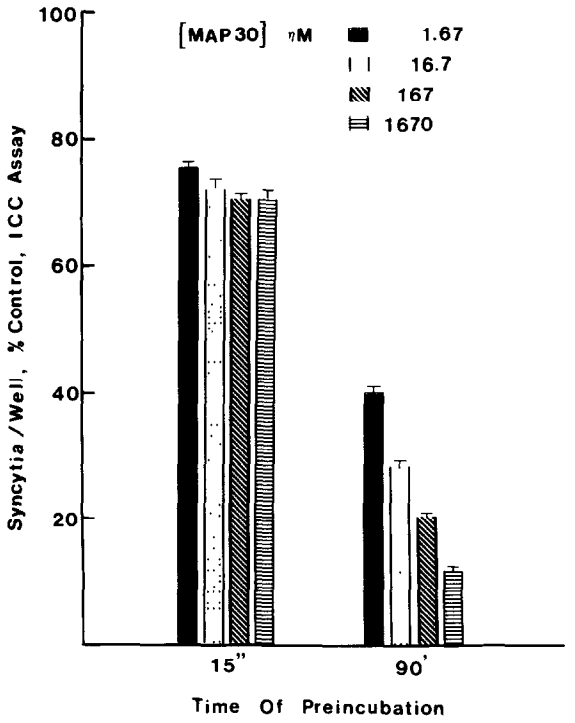

Fig. 4. The effect of MAP 30 on HIV infectivity as measured by syncytium forming unit (SFU) in the infectious cell center (ICC) assay. The results of syncytium per well are expressed in terms of % control. Experimental conditions were described in the legend of Table I. Uncertainties are indicated by error bars.

4. DISCUSSION

In this paper, we have reported the isolation, purification and characterization of an anti-HIV protein, MAP 30, from MC. Our results indicate that MAP 30 mediates potent yet specific interventions of the life cycle of HIV-1. The inhibition of syncytium formation in acutely infected CD4-positive indicator cells suggests that MAP 30 affects initial HIV infection and transmission of viral gene products through cell contact or release of free virions. The inhibition of p24 expression and viral-RT activity indicates that MAP 30 affects virion production and replication. Focal syncytium formation represents single infectious virion units. When MAP 30 is present only during the initial exposure to virus, i.e. 15 s or 90 min preincubation, this assay is specific for the initial phase of viral infection. Inhibition of syncytia formation by itself should result in some decrease in p24 expression and viral associated RT activity when these events are measured in the same assays. In separate studies, we have indeed observed these effects. The effect of MAP 30 on replicative events after internalization of the virus into the target cell can be specifically measured independent of the infection phase. In the in vitro p24 expression and RT activity assays, MAP 30 was added to the infected culture after the initial viral infection. Inhibition of these events may or may not be related to the inhibition on syncytia formation. Further studies are needed to define the molecular mechanism of the anti-HIV activity of MAP 30.

The N-terminal 44 amino acid sequence of MAP 30 has been determined. This information represents the first protein sequence data from the plant MC. Com-

Table II

The effect of MAP 30 on HIV replication as measured by p24 expression and viral-RT activity in HIV-infected H9 cells

MAP 30	p24 Expression		RT activity		% Cellular, [^{3}H]-	
nM (μg/ml)	ng/ml	% control	cpm $\times 10^3$/ml	% control	thymidine	leucine
0	2106	100	806	100	100	100
0.334 (0.01)	610	29	422	52	102	101
3.34 (0.1)	189	9	202	25	99	102
33.40 (1.0)	42	2	108	13	101	98
334.0 (10)	0	0	52	6	75	72

The amount of p24 viral antigen produced was measured by RIA and expressed in ng/ml. Viral-RT activity was assayed by the incorporation of [^{3}H]dTTP into acid-insoluble products and expressed in terms of cpm $\times 10^3$/ml. Cytotoxicity was measured by cellular uptake of [^{3}H]thymidine or [^{3}H]leucine into TCA-precipitable products in pulse labeling experiments as described in Methods. Results are normalized to values obtained for control cultures without MAP 30. Control cpm for [^{3}H]thymidine was 206 217, for [^{3}H]leucine was 62 439. Values shown are averages of duplicates in two independent experiments. Uncertainties are within 6%.

Table III

N-terminal amino acid sequence of MAP 30 and its comparison with trichosanthin and ricin A chain

	1									10
MAP 30	**Asp -**	**Val -**	**Asn -**	**Phe -**	**Asp -**	**Leu -**	**Ser -**	**Thr -**	**Ala -**	**Thr -**
Tri 1	Asp -	Val -	Ser -	Phe -	Arg -	Leu -	Ser -	Gly -	Ala -	Thr -
Ric A	Pro - Ile -	Ile -	Asn -	Phe -	Thr -	Thr -	Ala -	Gly -	Ala -	Thr
	11									20
MAP 30	**Ala -**	**Lys -**	**Thr -**	**Thr -**	**Thr -**	**Lys -**	**Phe -**	**Ile -**	**Glu -**	**Asp -**
Tri 1	Ser -	Ser -	Ser -	Tyr -	Gly -	Val -	Phe -	Ile -	Ser -	Asn -
Ric A	Val -	Gln -	Ser -	Tyr -	Thr -	Asn -	Phe -	Ile -	Arg -	Ala -
	21									30
MAP 30	**Phe -**	**Arg -**	**Ala -**	**Thr -**	**Leu -**	**Pro -**	**Phe -**	**Ser -**	**His -**	**Lys -**
Tri 1	Leu -	Arg -	Lys -	Ala -	Leu -	Pro -	Asn -	Glu -	Arg -	Lys -
Ric A	Val -	Arg -	Gly -	Arg -	Leu -	Thr -	Thr -	Gly -	Ala -	Asp -
	31									40
MAP 30	**Val -**	**Tyr -**	**Asp -**	**Ile -**	**Pro -**	**Leu -**	**Leu -**	**Tyr -**	**Ser -**	**Thr -**
Tri 1	Leu -	Tyr -	Asp -	Leu -	Pro -	Leu -	Ile -	Arg -	Ser -	Ser -
Ric A	Val -	Arg -	His -	Glu -	Ile -	Pro -	Val -	Arg -	Leu -	Pro -
	41			44						
MAP 30	**Ile -**	**Ser -**	**Asp -**	**Pro -**						
Tri 1	Leu -	Pro -	Gly -	Ser -						
Ric A	Leu -	Pro -	Ile -	Asn -						

Comparison of the N-terminal 44 residues of MAP 30 to the N-terminal sequences of trichosanthin (Tri 1) and ricin A chain (Ric A) residues 7–51 as reported earlier [10]. Boxed regions are identical or conserved amino acids of these plant proteins. Substitutions of amino acid with similar physio-chemical properties of the side chains are taken as conservative as with Ser and Thr; Val, Leu and Ile; Asp and Glu.

parison of this sequence to the EMBL data bank reveals 34% or 25% homology to ricin A chain and 57% or 43% homology to trichosanthin when conserved or identical residues are considered. Like ricin and trichosanthin, MAP 30 also inhibits in vitro translation of eukaryotic cells. Distinct from these compounds, little cytotoxicity has been observed for MAP 30.

Trichosanthin is a 26 kDa protein isolated from the root tuber of Trichosanthes [12,13]. This protein has been used for inducing abortions and for treating trophoblastic tumors [14–16]. It has also been shown to inhibit protein synthesis in vitro [17]. Recently, trichosanthin was reported to have anti-HIV activity and re-named GLQ 223 [18]. It is important to note that under identical assay conditions, MAP 30 is much less cytotoxic as compared to GLQ 223. For example, at ID_{90} (inhibitory dose at 90% inhibition) for HIV-RT activity, GLQ 223 caused about 35% and 40% inhibition on cellular synthesis of DNA and protein, respectively ([18] and our unpublished observations), whereas at the same inhibitory dose, MAP 30 showed no detectable inhibition on the synthesis of these macromolecules (Table II). Even at $10 \times ID_{90}$, MAP 30 caused only about 25% and 28% inhibition on cellular incorporation of ^{3}H-labeled thymidine and leucine, respectively. MAP 30 is thus at least one order of magnitude lower in cytotoxicity than GLQ 223. The lower in vitro cytotoxicity of MAP 30 suggests that it may have a much better therapeutic index.

The isolation of several bioactive proteins from MC has been reported [2–5]. Some of these proteins are active in ribosome inactivation while others have been

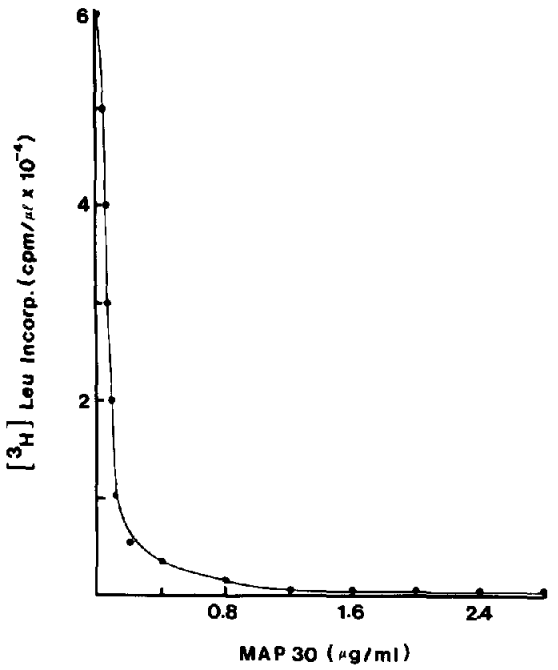

Fig. 5. Effect of MAP 30 on the in vitro translation in a reticulocyte lysate system. The extent of protein biosynthesis was measured by the incorporation of [^{3}H]leucine (cpm $\times 10^4/\mu$l) into TCA-insoluble product as a function of MAP 30 concentration.

reported to be active in inhibiting the development of tumors in animals [4,5] as well as the multiplication of viruses in Hep-2 cells [3]. These proteins are known as MC inhibitors (23–24 kDa) and Momorcharins alpha and beta (32 and 28 kDa). No amino acid sequence data have been reported on these proteins; thus, no comparison can be made with our sequence data on MAP 30.

Our results demonstrate that MAP 30 inhibits both infection and replication of HIV-1. Treatment of HIV-infected individuals with MAP 30 may block further depletions of CD4-T cells and inhibit viral replication. Such effects may be important in maintaining latency for asymptomatic patients. MAP 30 may prove to be synergistic in conjunction with other drugs such as AZT. The question of how effective MAP 30 is, alone or in combination with other anti-HIV drugs, in the treatment of AIDS can only be answered by appropriate clinical trials. In view of the magnitude of the AIDS pandemic, the absence of a protective vaccine, and the paucity of nontoxic therapy, the potential application of MAP 30 should be considered.

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