The plant ribosome inactivating proteins luffin and saporin are potent inhibitors of HIV-1 integrase

T.K. Au, R.A. Collins, T.L. Lam, T.B. Ng, W.P. Fong, D.C.C. Wan*

Department of Biochemistry, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China

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Abstract The ribosome inactivating proteins (RIPs) are a group of proteins that are able to inactivate eukaryotic protein synthesis by attacking the 28S ribosomal RNA. Recent studies have shown that some RIPs possess strong anti-human immunodeficiency virus (HIV) activity. In this study, several common plant RIPs such as agrostin, gelonin, luffin, α-momorcharin, β-momorcharin, saporin and trichosanthin were examined for the ability to interfere with HIV-1 replication in a variety of mechanistic assays in vitro. These assays included the CD4/gp120 interaction assay, HIV-1 reverse transcriptase (RT) assay, HIV-1 protease assay and HIV-1 integrase assay. At the concentration of 100 nM, all RIPs appeared to enhance the CD4/gp120 interaction by about 50%. These RIPs exhibited a very weak suppressive effect on HIV-1 RT and on HIV-1 protease. In contrast, with the exception of agrostin, all the RIPs tested could strongly inhibit HIV-1 integrase, the extent of inhibition ranging from 26.1 to 96.3% in an ELISA-based assay. Two RIPs, saporin and luffin, which elicited over 90% inhibition in the ELISA-based assay, were further characterized in a radiometric assay. Both of these two RIPs evoked a strong dose-dependent inhibition in the 3'end processing and strand-transfer activities of integrase. The results from this study suggest that the anti-HIV property of RIPs may be due to inhibition of HIV-1 integrase.

Key words: HIV-1; Ribosome inactivating protein; Integrase; Protease; Reverse transcriptase; CD4/gp120

1. Introduction

Ribosome inactivating proteins (RIPs) are proteins that specifically interfere with eukaryotic protein translation. Most plant and bacterial RIPs exert their effects through binding to the large 60S ribosomal subunit on which they act as N-glycosidases; specifically cleaving the adenine base A-4324 from its ribose moiety in the 28S ribosomal RNA subunit. This results in the inability of the ribosome to bind elongation factor 2 and thus terminating translation [1]. RIPs are widely distributed in nature but are found predominantly in plants, bacteria and fungi. They vary greatly in their physical properties and cellular effects [2,3]. Many of the plants from which RIPs are isolated are used medicinally in traditional Chinese medicine and the RIPs may account for some of the reported clinical efficacy. These proteins display a myriad of pharmacological properties comprising anti-viral, anti-tumor, immunosuppressive, embryotoxic, anti-fertility [4,5] and enzymatic activities [6].

McGrath et al. [7,8] and Yeung [9] were the first to document that RIPs including trichosanthin, α-momorcharin and β-momorcharin inhibited human immunodeficiency virus (HIV) replication in acutely and chronically infected cells of lymphocyte and mononuclear phagocyte lineage. Ferrari et al. [10] reported that trichosanthin inhibited HIV replication in H9 and CEM-SS cells, and syncytium formation between infected H9 cells and uninfected Sup-T1 cells. Lee-Huang et al. [11,12] detailed the isolation of anti-HIV proteins, which they coined MAP30 and TAP29, respectively, from Momordica charantia seeds and Trichosanthes kirilowii tubers, the same tissues used for preparing momorcharins and trichosanthin. Both MAP30 and TAP29 elicited a dose-dependent inhibition of cell-free HIV-1 infection and replication. Viral-associated reverse transcriptase (RT) activity in HIV-1-infected H9 cells was also inhibited in conjunction with a suppression of syncytium formation in the CD4-positive, syncytium-sensitive, Leu3a-sensitive T cell line CEM-SS and viral core protein p24 expression. Later, they demonstrated that MAP30 and GAP31 (from Gelonium multiflorum) could inhibit HIV-1 integrase with an EC50 of 1 μM [13]. Here, we report our work to determine whether RIPs from certain plants were endowed with the ability to inhibit replication of HIV-1 in a series of in vitro mechanistic assays.

2. Materials and methods

2.1. RIPs

The RIPs agrostin, gelonin, luffin and saporin were purchased from Sigma Chemical Co., St. Louis, MO, USA. α-Momorcharin, β-momorcharin and trichosanthin were purified in-house as detailed in [4]. All RIPs were dissolved in phosphate-buffered saline (PBS) and stored at −20°C until use.

2.2. Assay for cell-free translation inhibitory activity

In the protein synthesis assay [14], the reticulocyte mix and hot mix were prepared as follows. The reticulocyte mix contained rabbit reticulocyte lysate, creatine kinase (5 mg/ml), hemin (0.65 mg/ml) and water in the ratio of 60:4:10:40 (v/v). The hot mix contained creatine phosphate (0.4 M), KC1/MgCl2 (1 M/10 mM) and [1H]leucine (0.25 mcI) in the ratio of 4:7:1. The assay mix was incubated at 30°C for 50 min. The reaction was stopped by the addition of NaOH and H2O2 and incubation at 37°C for 10 min to digest amino-acyl tRNA and decolorize the solution. Trichlooroacetic acid (40%), containing 0.2% casein hydrolysate, was added followed by incubation on ice for 10 min to precipitate [1H]leucine-labelled proteins. The precipitate was filtered through a glass fiber filter, washed with ethanol and dried under vacuum. The filter was submerged in scintillation fluid and radioactivity was counted.

*Corresponding author. Fax: (852)2603-7246.
E-mail: chicheongwan@cuhk.edu.hk

Abbreviations: HIV-1, human immunodeficiency virus type 1; BSA, bovine serum albumin; PBS, phosphate-buffered saline

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2.3. CD4(gp120) interaction assay
Inhibition of the interaction between immobilized recombinant CD4 and recombinant HIV-1 gp120 was determined with a commercially available enzyme immunoassay kit (NEN DuPont, Bedford, MA, USA).

2.4. RT assay
Inhibition of recombinant HIV-1 RT activity was determined with a commercially available ELISA kit (Boehringer Mannheim, Germany).

2.5. Expression and purification of recombinant HIV-1 protease
The expression clone for recombinant human HIV-1 protease was a generous gift from Dr. J.N. Tang (Oklahoma Medical Research Foundation, USA). HIV-1 protease cDNA was cloned in pET3b and transformed into E. coli BL21(DE3)pLysS. HIV-1 protease expression was induced by IPTG. The expressed proteins, which were found predominantly as inclusion bodies, were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The bacterial colony which expressed a high level of the 11 kDa HIV-1 protease was picked and chosen for preparation of large-scale cultures. The procedure for purification of HIV-1 protease from cultures was carried out as described in [15].

2.6. Fluorometric method for HIV-1 protease assay
The activity of HIV-1 protease was assayed by cleavage of a fluorogenic substrate, L-Phe-Glu(EDANS)-Ser-Glu-Asn-Tyr-Pro-Ile-Val-Gln-Lys(DABCYL)-Arg (Molecular Probes, USA), in a microtiter plate format for rapid screening of activity [16]. Briefly, 6.5 μg HIV-1 protease was added to a total of 200 μl reaction buffer (0.1 M sodium acetate, 1 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% dimethyl sulfoxide, 1 mg/ml bovine serum albumin (BSA), pH 4.7) containing 10 μM substrate in the presence or absence of RIPs. After incubation at 37°C for 2 h, the fluorescence intensity in each well was measured using a CytoFluor 2350 fluorescence plate reader (Millipore Corp., USA) with an excitation wavelength at 340 nm and an emission wavelength at 490 nm.

2.7. Expression and purification of recombinant HIV-1 integrase
The plasmid that expressed His-tagged wild-type HIV-1 integrase, pT7-7-His(TX)-HIV-1-IN, was a gift from Dr. S.A. Chow (School of Medicine, UCLA). To express the protein, a 1 l culture of E. coli BL21(DE3) cells containing the expression plasmid was grown at 37°C until OD600 reached 0.7–0.8. Cells were harvested after a 4 h incubation by centrifugation at 40 000 g for 10 min at 4°C. Cells were suspended at a concentration of 10 mg wet cell paste in 20 mM Tris-HCl (pH 8.0), containing 1 mM EDTA, 2 mM β-mercaptoethanol, 0.5 M NaCl and 5 mM imidazole. Lysozyme was added to a concentration of 0.2 mg/ml. After 1 h incubation at 4°C, the lysate was sonicated and centrifuged at 40 000 × g at 4°C for 20 min. The pellet was homogenized in 50 ml buffer A (20 mM Tris-HCl, pH 8.0, 2 M NaCl, 2 mM β-mercaptoethanol) containing 5 mM imidazole. The suspension was rotated at 4°C for 1 h and cleared by centrifugation at 40 000 × g at 4°C for 20 min. The supernatant was loaded onto a 1 ml chelating Sepharose 4 Fast Flow column charged with 50 mM NiSO4 and was equilibrated with buffer A containing 5 mM imidazole. The column was washed with five column volumes of buffer A containing 5 mM imidazole and the protein was eluted with three column volumes of buffer A containing 200 mM and 400 mM imidazole, respectively. Protein-containing fractions were pooled and EDTA was added to a final concentration of 5 mM. The protein was dialyzed against buffer B (20 mM HEPES, pH 7.5, 1 mM EDTA, 1 M NaCl, 20% glycerol) containing 2 mM β-mercaptoethanol and then against buffer B containing 1 mM dithiothreitol. Aliquots of the protein were stored at −70°C.

2.8. HIV-1 integrase assays
A non-radioactive ELISA-based HIV-1 integrase assay was performed according to the DNA-coated plates method as detailed by Chang et al. [17] with some modifications. In this study, 1 μg of Smal-linearized pBluescript SK was coated onto each well in the presence of 200 mM NaCl. The donor DNA was prepared by annealing VU5BR (5′-biotin-GTGGTGAAATCTTCCTACGAT-3′) and VUS (5′-ACTGCTAGATTTTCCACAC-3′) in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and 0.1 M NaCl at 80°C followed by 30 min at room temperature. Integrase reaction was performed in 20 mM HEPES (pH 7.5), containing 10 mM MnCl2, 30 mM NaCl, 10 mM dithiothreitol and 0.05% Nonidet-P40 (Sigma). After the inte- grase reaction, the biotinylated DNA immobilized on the wells was detected by incubation with streptavidin-conjugated alkaline phosphatase (Boehringer Mannheim) followed by colorimetric detection with 1 mg/ml p-nitrophenyl phosphate in 10% diethanolamine buffer, pH 9.8, containing 0.5 mM MgCl2. The absorbance due to the alkaline phosphatase reaction was measured at 415 nm.

The radioactive assays for 3′-end processing and strand-transfer reactions of HIV-1 integrase were performed according to the methods of Chow [18].

3. Results and discussion
The purpose of this work was to determine the suitability of using RIPs as inhibitors of HIV-1 replication. There are many points in the virus life-cycle that can be targeted by potential therapeutic compounds [19]. In the present study, a variety of mechanistic assays based on different therapeutic targets were used to determine the locus of any inhibitory effect produced by the various RIPs. The ribosome inactivating activities of the various RIPs were assessed, prior to assaying their HIV-1 inhibitory properties, by determining their activity in a standard protein synthesis assay using a rabbit reticulocyte lysate. As shown in Table 1, at a concentration of 80 nM, all the RIPs tested could inhibit protein synthesis by 81–100%, indicating that the proteins were biologically active. The RIPs were also analyzed by SDS-PAGE. All proteins appeared to be homogeneous and had the expected molecular weights as estimated from molecular weight markers (data not shown).

The effect of the RIPs on the potential interaction of HIV-1
and its target cell was investigated using an HIV-1 gp120/immobilized CD4 receptor EIA. At a concentration of 100 nM, all RIPs appeared to enhance the interaction by about 50% (Table 2). This enhancement may be attributed to a cross-linking of gp120 molecules due to the lectin-like affinity of the RIPs for carbohydrate groups leading to an enhanced enzyme signal.

The effects of the RIPs on HIV-1 RT, protease and integrase were studied. As shown in Table 2, the RIPs tested were only weak inhibitors of RT even when present at 133 nM. The most potent was lu\textsuperscript{u}n which inhibited RT by 27%. Lee-Huang et al. [11] reported that, at the concentration of 0.33 nM, MAP30 could decrease virus-associated RT activity by 50% in HIV-1-infected H9 cells. However, the inhibition was most likely due to a decrease in HIV number rather than a direct inhibition of HIV-1 RT. Similar to RT, the RIPs tested were only weak inhibitors of HIV-1 protease; only about 10% of inhibition could be attained at a concentration of 2 \mu M. Huang et al. [20] have recently shown that two RIPs, MAP30 and GAP31, were resistant to proteolytic digestion. At 10% (w/w) protease, only the N- and C-termini were accessible. The purified proteolytic fragments of these two RIPs remained fully active in the anti-HIV assay. Therefore, the lack of inhibition in this study was unlikely due to the digestion of RIPs by HIV-1 protease in the reaction mixtures. For HIV-1 integrase, with the exception of agrostin, which did not show any inhibition, all RIPs tested at 5 \mu M could inhibit HIV-1 integrase by 26-96%. The most potent were lu\textsuperscript{u}n and saporin, which inhibited integrase by more than 90% in the ELISA-based assay. Gelonin, \alpha-momorcharin and trichosanthin inhibited integrase by 50-68% while \beta-momorcharin elicited only 26% inhibition. The response curves for lu\textsuperscript{u}n and saporin on integrase inhibition are shown in Fig. 1. Both RIPs were nearly equipotent in inhibiting HIV-1 integrase, the concentrations required for 50% inhibition being

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Effects of RIPs on protein synthesis in a rabbit reticulocyte lysate system</th>
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<tbody>
<tr>
<td>RIP</td>
<td>Inhibition (%)</td>
</tr>
<tr>
<td>Agrostin</td>
<td>81.6 ± 1.0</td>
</tr>
<tr>
<td>Gelonin</td>
<td>N.D.</td>
</tr>
<tr>
<td>Luffin</td>
<td>93.0 ± 2.8</td>
</tr>
<tr>
<td>\alpha-Momorcharin</td>
<td>95.8 ± 5.4</td>
</tr>
<tr>
<td>\beta-Momorcharin</td>
<td>100 ± 0.0</td>
</tr>
<tr>
<td>Saporin</td>
<td>91.5 ± 2.5</td>
</tr>
<tr>
<td>Trichosanthin</td>
<td>81.3 ± 4.3</td>
</tr>
</tbody>
</table>

The concentration of RIPs used in each case was 80 nM. The results shown were mean ± S.D. (n = 3). N.D.: not determined.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Effects of RIPs on CD4/gp120 interaction, HIV-1 RT, protease and integrase</th>
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</thead>
<tbody>
<tr>
<td>RIP</td>
<td>CD4/gp120</td>
</tr>
<tr>
<td>Agrostin</td>
<td>47.5 ± 4.0</td>
</tr>
<tr>
<td>Gelonin</td>
<td>N.D.</td>
</tr>
<tr>
<td>Luffin</td>
<td>56.0 ± 1.2</td>
</tr>
<tr>
<td>\alpha-Momorcharin</td>
<td>48.8 ± 5.0</td>
</tr>
<tr>
<td>\beta-Momorcharin</td>
<td>58.6 ± 5.5</td>
</tr>
<tr>
<td>Saporin</td>
<td>59.7 ± 0.7</td>
</tr>
<tr>
<td>Trichosanthin</td>
<td>51.4 ± 3.7</td>
</tr>
</tbody>
</table>

The concentrations of RIPs used in CD4/gp120 interaction assay, RT assay, protease assay and integrase assay were 100 nM, 133 nM, 2 \mu M and 5 \mu M, respectively. The results shown were mean ± S.D. (n = 3). N.D.: not determined. Data in parentheses represent enhancement while data without parentheses represent inhibition. RT, PR and IN stand for reverse transcriptase, protease and integrase, respectively.

Fig. 2. Inhibition of HIV-1 integrase-catalyzed 3’-end processing (A) and strand-transfer (B) by lu\textsuperscript{u}n and saporin. The reaction products were separated by denaturing gel and visualized by autoradiography. Lane 1, positive control with integrase. Lane 2, unreacted substrate. Lanes 3-7, reactions in the presence of indicated concentrations of lu\textsuperscript{u}n. Lanes 8-12, reactions in the presence of indicated concentrations of saporin. Reaction was performed in a volume of 20 \mu l containing 20 mM HEPES, pH 7.5, 10 mM MnCl\textsubscript{2}, 30 mM NaCl, 10 mM dithiothreitol, 0.05% Nonidet-P40, 10% DMSO, 0.3 \mu M integrase and 50 nM 5’-\textsuperscript{32}P-labelled oligonucleotide substrates. The reaction mixtures were incubated at 37°C for 1 h and stopped by the addition of 10 \mu l of loading buffer (98% deionized formamide, 10 mM EDTA, pH 8.0, and 0.05% bromophenol blue, 0.05% xylene cyanol) followed by heating at 80°C for 5 min. 5 \mu l from each of the reaction mixtures was used for denaturing gel electrophoresis. S indicates unreacted substrate, TC indicates terminal processed products and ST indicates strand-transfer products.
0.25 μM for saporin and 0.53 μM for lufern. These figures were comparable to the value of 1 μM reported for MAP30 and GAP31 [13]. Integrase acts in two steps: the first step processes the linear viral DNA by removing two nucleotides from each 3'-end, leaving recessed 3'-OH termini. This step is known as 3'-end processing. The second step, strand-transfer, is a trans-esterification of phosphodiester bonds in which a host DNA strand is cut, and the 5'-end of the cut is joined to a processed viral 3'-terminus. In order to further characterize the mode of inhibition by lufern and saporin, these two steps were assayed separately using 32P-labelled oligonucleotides of viral terminal sequence in a cell-free system. PAGE in a 20% denaturing gel allows the separation of reaction products from the substrate [18]. Fig. 2 shows the effects of lufern and saporin on the 3'-end processing and strand-transfer activities of HIV-1 integrase. It was found that both activities could be inhibited by lufern and saporin in a dose-dependent manner. The 3'-end processing was assayed using a 21-mer double-stranded oligonucleotide that mimics the 5' end of the HIV LTR as substrate. 3'-End processing by integrase would liberate a GT dinucleotide from the 3'-end, resulting in the formation of a 19-mer oligonucleotide. As shown in Fig. 2A, the accumulation of the 19-mer oligonucleotide cleavage product decreased as the concentrations of RIPs increased. To assay strand-transfer, a 19-mer oligonucleotide was annealed to a 21-mer oligonucleotide, this 3'-end recessed substrate mimics the 3'-end processed product and undergoes strand-transfer reaction only. The strand-transfer reaction products accumulated as oligonucleotides with slower mobilities in denaturing gel. Lufern and saporin could also inhibit the strand-transfer activity of HIV-1 integrase in a dose-dependent manner (Fig. 2B). It can be estimated that the concentration for 50% inhibition was ~30–60 nM for lufern and ~60–130 nM for saporin in both assays as the accumulations of reaction products were nearly decreased by half in these concentration ranges.

RIPs are a diverse group of proteins with a wide range of physical properties and cellular effects. Recent studies have identified a number of RIPs with significant anti-HIV-1 effects at nanomolar concentrations [11,12,21]. However, the ribosome inactivating activity of RIPs may not account for their anti-HIV activities. For instance, Barbieri et al. [3] showed that trichosanthin could selectively inhibit HIV RNA and protein accumulation without affecting host cell gene expression. Recently, Huang et al. [20] demonstrated that proteolytic fragments of MAP30 and GAP31 were fully active against HIV-1 but not in ribosome inactivation. These results suggested that RIPs' anti-HIV and ribosome inactivating activities are entirely different. In this study, the lack of a striking effect of RIPs on CD4/gp120 interaction, HIV-1 RT and also HIV-1 protease suggests that the mechanism of anti-HIV action of the RIPs does not involve CD4/gp120, HIV RT and protease. The HIV-1 integrase represents an attractive possibility for the development of anti-HIV drugs because the host cells do not make or require integrase.

Lee-Huang et al. first demonstrated that two RIPs, MAP30 and GAP31, possess inhibitory activity on HIV-1 integrase [13]. More recently, the solution structure of MAP30 was resolved [22]. The DNA glycosylase/apurinic lyase activity of MAP30 was identified and related to its HIV-1 integrase inhibitory action. The differential HIV-1 integrase inhibitory activities of RIPs in this study may be due to their different DNA glycosylase/apurinic lyase activities. Whether similar glycosylase/apurinic lyase activity could be observed in lufern- and saporin-associated HIV-1 integrase inhibition requires further investigation. Taken these together, it suggests that certain members of the RIP family are potent integrase inhibitors.

In conclusion, we found two other members of RIPs, lufern and saporin, that are potent HIV-1 integrase inhibitors. These findings confirm and further extend the notion that the anti-HIV effects of these RIPs may be attributed to their potent HIV-1 integrase inhibition.

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References