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A new class of anti-HIV agents: GAP31, DAPs 30 and 32

Sylvia Lee-Huang[1], Hsiang-fu Kung[1], Paul L. Huang[3]*, Philip L. Huang[3]*, Bao-Qun Li[4], Peter Huang[3], Henry I. Huang[3] and Hao-Chia Chen[5]

[1]*Department of Biochemistry, New York University School of Medicine, New York, NY 10016,* [2]*Laboratory of Biochemical Physiology, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD 21701,* [3]*American Biosciences, New York, NY 10021,* [4]*Biological Carcinogenic Development Program, Program Resources Inc., National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD 21701* and [5]*Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, NIH, Bethesda, MD 20892, USA*

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Three inhibitors of human immunodeficiency virus (HIV) have been isolated and purified to homogeneity from Euphorbiaceae himalaya seeds (*Gelonium multiflorum*) and carnation leaves (*Dianthus caryophyllus*). These proteins, GAP 31 (Gelonium Anti-HIV Protein 31 kDa) and DAPs 30 and 32 (dianthus anti-HIV proteins, 30 and 32 kDa), inhibit HIV-1 infection and replication in a dose-dependent manner with little toxicity to target cells. The therapeutic indices of these compounds are in the order 10^4, suggesting that they may be clinically important agents in the treatment of AIDS. The N-terminal amino acid sequences of these proteins show little homology to those of previously described anti-HIV proteins. The structure–function features of these HIV inhibitors, based on the 40–60 amino acid residues of N-terminal sequences, are examined.

AIDS; Antiviral agent; Plant protein; N-terminal sequence

1. INTRODUCTION

We have reported previously the isolation, purification and characterization of two anti-HIV proteins, MAP 30 and TAP 29 [1,2]. These proteins show amino acid sequence homology to the ribosome-inactivating proteins, trichosanthin and ricin A chain [3,4]. Further studies indicate that MAP 30 and TAP 29 also inhibit in vitro eukaryotic translation, and are thus single-chain ribosome-inactivating proteins (SCRIPs). However, in contrast to trichosanthin and ricin A chain, MAP 30 and TAP 29 exhibit little cytotoxicity to target cells.

In this article, we report a new class of anti-HIV SCRIPs from distinct and unrelated plant species, that possess high antiviral potency and low toxicity to normal cells in culture as well as to intact animals. In addition, we also report the N-terminal amino acid sequences of these anti-HIV proteins.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Carboxymethyl cellulose (CM52) was a product of Whatman. Con A Sepharose CL-4B was obtained from Pharmacia-LKB. ^{3}H-labeled thymidine and leucine, and rabbit reticulocyte lysate translation systems were purchased from DuPont-New England Nuclear.

**Present address:* Harvard Medical School and Massachusetts General Hospital, Boston, MA 02114, USA.

Correspondence address: S. Lee-Huang, Department of Biochemistry, New York University Medical School, New York, NY 10016, USA.

2.2. *Anti-HIV compounds: GAP 31, DAP 30 and DAP 32*

All anti-HIV agents used in this study have been purified to homogeneity. GAP 31 was purified from Euphorbiaceae himalaya seeds (*Gelonium multiflorum*). DAPs 30 and 32 were purified from carnation leaves (*Dianthus caryophyllus*). The source materials for these preparations were supplied by American BioSciences, New York.

2.3. *Characterizations*

The homogeneity and size of the purified anti-HIV agents were determined by SDS-PAGE. Protein sequencing was carried out by automated Edman degradation using an Applied Biosystems model 470A protein sequencer, with on-line PTH analyzer.

2.4. *Anti-HIV and other assays*

Anti-HIV activity was measured by microtiter syncytium formation in infectious cell center assay [5], viral core protein p24 expression [6], and viral-associated reverse transcriptase (RT) activity [7].

The CEM-ss (syncytium sensitive, Leu-3 positive) cell line was used as the indicator cell for the microtiter syncytial-forming assay. The H9 cell line was used for p24 expression and HIV-RT activity assays. HIV-1 virus was prepared and stocked as described previously [6]. Cell lines were cultured in RPMI-1640 medium containing 100 U/ml of penicillin-streptomycin and 10% heat-inactivated fetal calf serum.

The cytotoxicities of the HIV inhibitors were measured by their effects on cellular DNA and protein syntheses in uninfected H9 cells [1]. The toxicity to intact animals was determined by intraperitoneal injection (i.p.) of these inhibitors into adult mice.

The ribosome-inactivating activity was measured by cell-free translation of globin message in a rabbit reticulocyte lysate system as described previously [1].

3. RESULTS

3.1. *Preparation and characterization of GAP 31, DAP 30 and DAP 32*

The anti-HIV compounds were prepared by a three-

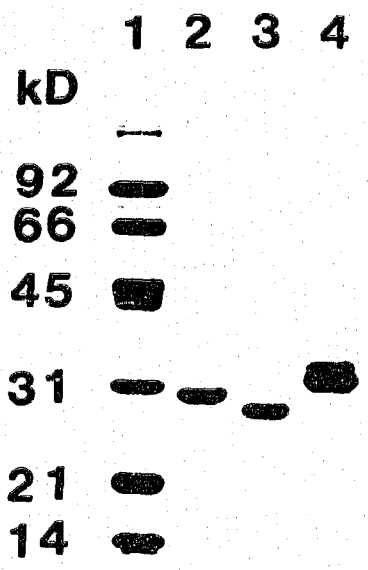

Fig. 1. SDS-PAGE of GAP 31, DAP 30 and DAP 32. Electrophoresis was carried out in the presence of the reducing agent 2-mercaptoethanol in 10% acrylamide at a constant voltage of 90 V for 5 h, until the Bromophenol blue tracking dye reached 1 cm from the lower edge of the gel. The gels were stained with silver stain. Lane 1, molecular weight standards (2 μg each), lanes 2, 3 and 4, GAP 31 (2 μg), DAP 30 (2 μg) and DAP 32 (3 μg).

step procedure consisting of: (a) initial extraction with 10 mM sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl (PBS), (b) cationic chromatography on CM 52, and (c) affinity chromatography on Con A Sepharose CL-4B.

Shelled *Gelonium multiflorum* seeds or cleaned *Dianthus caryophyllus* leaves were extracted with PBS at ratios of 1:5 or 1:2 (w/v), respectively. Extraction was carried out in a tissue blender for 6 min at 4°C. The extract was stirred gently overnight followed by centrifugation at 16 000 × *g* for 30 min to remove cell debris. The supernatant was concentrated by ammonium sulfate precipitation to 90% saturation. The precipitate was dissolved in a minimum volume of 5 mM sodium phosphate, pH 6.4 (buffer A) and dialyzed exhaustively against the same buffer. The dialyzed sample was loaded onto a column of CM 52 (1.5 × 36 cm), which was equilibrated with buffer A. The column was washed with the same buffer A until the baseline of A_{280} was reached. The majority of the impurities were excluded from the column while the anti-HIV proteins were retained. They were eluted with a linear gradient of 0–300 mM NaCl in buffer A (15 × column volume). GAP 31 was eluted at about 0.2–0.26 NaCl. DAP 30 and DAP 32 were eluted at about 0.14 and 0.18 NaCl, respectively. The active fractions were pooled and concentrated by ammonium sulfate precipitation to 85% saturation. The samples were then dialyzed against buffer A and further purified by Con A-Sepharose CL-4B. Impurities were excluded from the column, whereas the anti-HIV activity was selectively bound to Con A and retained. GAP 31 was eluted with 0.1 M α-methylmannoside in buffer A. DAPs 30 and 32 were eluted with 0.25 M α-methylmannoside in buffer A or by 60 mM sodium borate in 10 mM Tris-HCl buffer, pH 7.4. The homogeneity and size of the purified material were determined by SDS-PAGE. Single bands with mol. mass of 31, 30 and 32 kDa were obtained for GAP 31, DAP 30 and DAP 32, respectively (Fig. 1). Identical results were obtained under reducing and non-reducing conditions, indicating that these molecules are single chain polypeptides.

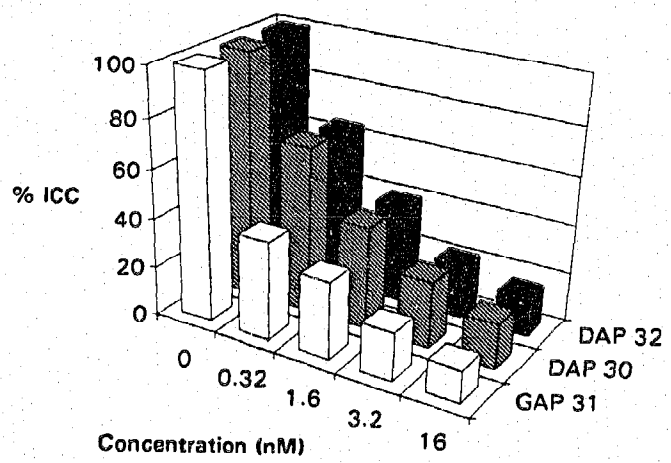

Fig. 2. The effects of GAP 31, DAP 30 and DAP 32 on HIV-1 infection. Results given in this figure represent averages of triplicates from two independent experiments. Triplicate wells of indicator cells containing the testing drugs at each concentration without virus were also included for the determination of the cytotoxic and cytostatic activities of these HIV inhibitors. Infectious cell center (% ICC) are expressed in terms of V_n/V_o, where V_n and V_o are average numbers of syncytia in the drug-treated and untreated samples, respectively.

3.2. *Effects on HIV-1 infection*

The effects of GAP 31, DAP 30 and DAP 32 on HIV-1 infection were measured by their inhibition of syncytium formation in infectious cell center assays. This assay quantitates acute cell-free HIV infection. The results are summarized in Fig. 2. All of the compounds exhibit dose-dependent inhibition of syncytium formation with ID_{50}s of 0.28, 0.83, and 0.76 nM for GAP 31, DAP 30 and DAP 32, respectively. No cytotoxic or cytostatic effects were observed under the assay conditions. These results suggest that GAP 31, DAP 30 and DAP 32 affect the initial HIV-1 infection as well as transmission of viral gene products through cell contact or release of free virions.

3.3. *Effects on HIV-1 replication*

The effects of GAP 31, DAP 30 and DAP 32 on HIV-1 replication were measured in a suspension culture of H9 cells. In this assay, the expression of viral core protein p24 and HIV-RT activity in HIV-infected H9 cells were examined at various concentrations of these agents. The results are shown in Fig. 3. Each of these compounds demonstrates a dose-dependent inhi-

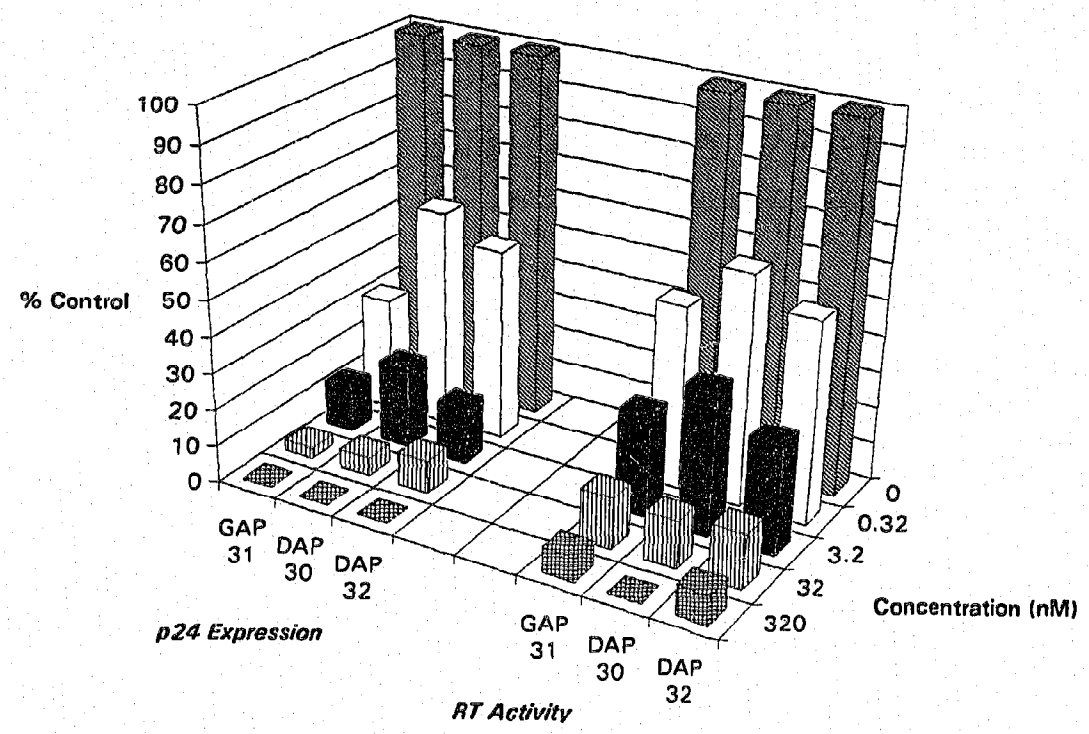

Fig. 3. The effects of GAP 31, DAP 30 and DAP 32 on HIV-1 replication. p24 production was determined by RIA and expressed in ng/ml. Viral-RT activity was determined by the incorporation of [^{3}H]dTTP into TCA-precipitable products and expressed in terms of cpm × 10^3/ml. These values in control culture (without the addition of the inhibitor) were 2219 ng/ml and 846 × 10^3 cpm/ml, respectively, as determined in triplicates in two independent experiments.

bition of HIV-1 replication. The ID_{50}s for GAP 31, DAP 30 and DAP 32 were 0.23, 0.86 and 0.71 nM for p24 expression, and 0.32, 0.88 and 0.76 nM for HIV-RT activity. The reduction in p24 expression was not due

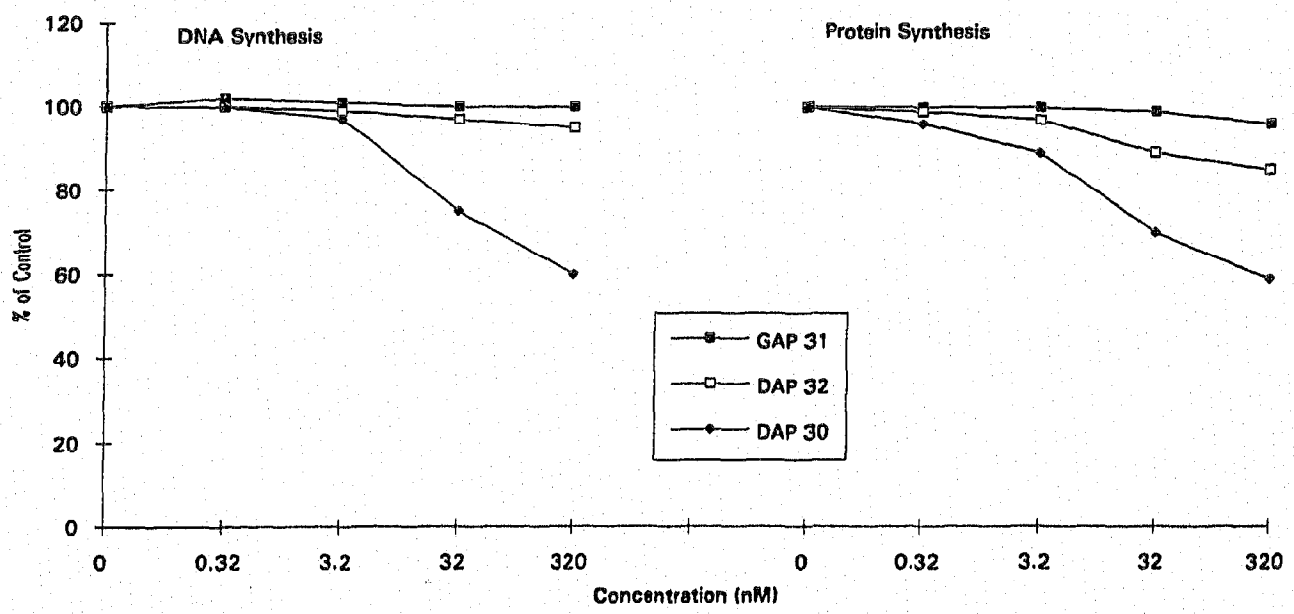

Fig. 4. Cytotoxicities of GAP 31, DAP 30 and DAP 32. Cytotoxicities were measured by cellular incorporation of ^{3}H-labeled thymidine or leucine into TCA-precipitable products in pulse-labeling experiments. H9 cells were seeded into 96-well plates at 2 × 10^4/well. The culture was pulsed with 1 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine or [^{3}H]leucine 8 h prior to harvesting at day 4. Cellular synthesis of DNA and protein were determined by scintillation counting of the incorporation of labeled precursors Results are normalized to values obtained for control cultures without drug. Control cpm for ^{3}H-labeled thymidine and leucine were 201 × 10^3 and 61 × 10^3, respectively. Results shown are averages of triplicates in two independent experiments.

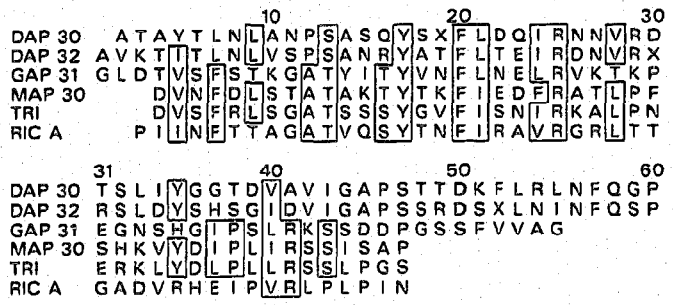

Fig. 5. Comparison of the N-terminal amino acid sequences of GAP 31, DAP 30 and DAP 32 to the N-terminal sequences of MAP 30, trichosanthin (TRI) and ricin A chain (RIC A) residues 7–51 as reported earlier [1,3,4]. Sequences are aligned to maximize similarities between the proteins. Boxed regions are identical or conserved residues.

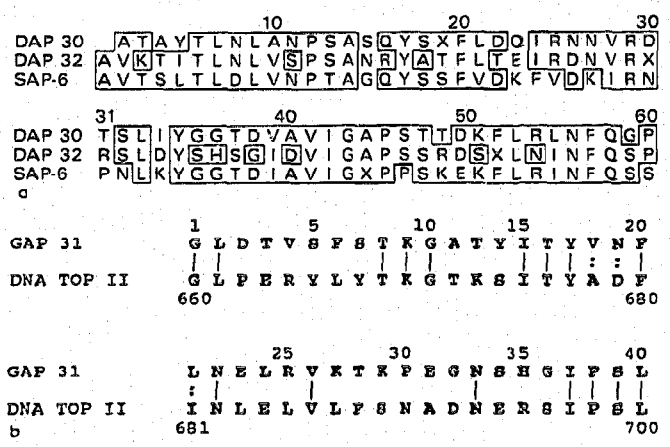

Fig. 6. (a) Comparison of the N-terminal sequences of DAP 30 and DAP 32 to that of saporin-6 (SAP-6) from *Saponaria officinalis*, residues 25–70 [10]. Boxed regions are identical or conserved residues. (b) Homology of the N-terminal amino acid sequence of GAP 31 with DNA topoisomerase II (DNA TOP), residues 660–699, from *Drosophila melanogaster* [11]. Sequences are aligned to show maximum similarity. Solid lines indicate identical amino acids. Dotted lines indicate conserved amino acids.

to cytotoxic or cytostatic effects; no inhibition of DNA or protein synthesis was detected at the dose level of the assay. The decrease in HIV-RT activity is likely to be due to an inhibition in virion production, which is also evidenced by the reduced p24 expression.

3.4. *Cytotoxicity and toxicity*

The cytotoxicities of GAP 31, DAPs 30 and 32 were determined by their effects on cellular DNA and protein syntheses in uninfected H9 cells. The cells were grown in the presence and absence of the anti-HIV agent. Eight hours prior to harvest on day 4, the cells were pulse-labeled with [^{3}H]thymidine or [^{3}H]leucine. Cellular incorporation of [^{3}H]thymidine or [^{3}H]leucine into TCA-precipitable DNA or protein was measured. The results are shown in Fig. 4. From 0.32 to 32 nM, GAP 31 and DAP 32 caused no detectable effect on cellular incorporation of labeled thymidine or leucine, while p24 production and HIV-RT activity were inhibited more than 90% (Fig. 3). At 32 nM however, DAP 30 showed 25 and 30% inhibition of the incorporation of ^{3}H-labeled thymidine and leucine. At 320 nM, or 1000 × ID_{50}, still no inhibition of cellular DNA or protein synthesis was observed for GAP 31. At this concentration, DAP 32 demonstrated 5 and 15% reduction on the incorporation of ^{3}H-labeled thymidine and leucine, respectively, and DAP 30 exhibited 40% inhibition of the incorporation of these precursors.

Cytotoxicity to uninfected cells in culture may be expressed as toxic dose 50 (TC_{50}), the dose at which cellular protein and DNA synthesis is inhibited by 50%. As summarized in Table I, the therapeutic index of GAP 31, defined as the TD_{50} divided by the ID_{50}, exceeds 10 000, using any of the three assays of antiviral activity. The therapeutic indices for DAP 32 and DAP30 are in the range of 10 000 and 1000, respectively.

The toxicity of GAP 31, DAP 30 and DAP 32 to intact animals was studied on 6- to 8-weeks-old CF1 mice. Filter-sterilized (0.2 μm) anti-HIV protein in PBS was injected i.p. at doses of 0.1, 1.0, 10 and 100 mg per 100 g body weight every three days. Control animals received similar injections of sterile PBS. The experiments were carried out using 4 mice in each group. The results are summarized in Table I. GAP 31 showed the least toxicity to intact mice, with an LD_{50} of 59–64 mg/kg. The LD_{50}s for DAP 32 and DAP 30 are 42–46 mg/kg and 12–16 mg/kg, respectively. In comparison, the LD_{50}s for trichosanthin and ricin are 5–7 mg/kg and 2–3 μg/kg.

3.5. *Ribosome-inactivating activity*

Ribosome-inactivation was determined by the incorporation of [^{3}H]leucine into TCA-insoluble material as a function of the concentration of the anti-HIV proteins. All three HIV inhibitors exhibit dose-dependent ribosome inactivation. As seen in Table I, ID_{50s} of 4.1, 3.2 and 2.3 nM were obtained for GAP 31, DAP 30 and DAP 32, respectively. Thus these anti-HIV agents are also single-chain ribosome-inactivating proteins (SCRIPs). They inhibit eukaryotic translation in cell-

Table I

The comparison of ribosome-inactivating activity, anti-HIV activity, cytotoxicity and toxicity of various anti-HIV SCRIPs

SCRIP	Ribosome inactivation	Anti-HIV ID_{50} (nM)			Cyto-toxicity	Toxicity
	ID_{50} (nM)	Syncytia	P24	RT	TD_{50} (nM)	LD_{50} (mg/kg)
GAP 31	4.1	0.28	0.23	0.32	>3200	59–64
DAP 30	3.4	0.76	0.85	0.88	910	12–16
DAP 32	2.3	0.76	0.71	0.76	>3200	42–46
TRI	3.7	0.34	0.37	0.46	340	5–7
RIC						$2–3\times10^{-3}$

TRI, trichosanthin; RIC, ricin

free systems. However, they are not toxic to intact cells because they do not enter intact normal cells.

3.6. *N-Terminal amino acid sequence*

The N-terminal amino acid sequences of GAP 31, DAP 30 and DAP 32 are shown in Fig. 5. Comparison of the N-terminal sequence of these proteins with those of MAP 30 [1], trichosanthin [3] and ricin A chain [4] reveals little homology. When considering identical residues, GAP 31 shows only 0.2, 6.8, and 11% homology to ricin A chain, MAP 30, and trichosanthin, respectively. When both identical and conserved residues are considered, GAP 31 shows 22.7% homology to each of the three proteins. Similarly, DAP 30 and DAP 32 show 8% homology for identical residues and 14% homology for conserved residues with MAP 30, trichosanthin, and ricin A chain. Most homology was found in aromatic (tyrosine and phenylalanine), hydrophobic, and hydroxyl-containing amino acids. Furthermore, GAP 31 shows little homology with DAP 30 and DAP 32 (7% identical and 12% conserved). Although the sequences of the two DAPs differ, 54.5% identity was found in the N-terminal 55 amino acid overlap.

Multiple forms of ribosome-inactivating proteins have been isolated from *Gelonium multiflorum* and *Dianthus caryophyllus* [8,9]. However, no amino acid sequence data have been reported. Thus no comparison can be made with the sequence of our HIV inhibitors reported here.

Comparison of the GAP 31, DAP 30 and DAP 32 sequences to the EMBL data bank reveals some interesting findings. As shown in Fig. 6a, both DAP 30 and DAP 32 show significant homology to saporin-6 (SAP 6), a SCRIP isolated from *Saponaria officinalis* [10]. In the N-terminal 60 amino acid overlap, 65% identity was found between dianthin 30 and saporin-6, and 48.3% identity was found between dianthin 32 and saporin-6. The finding of this homology, led us to assay SAP-6 for antiviral activity and cytotoxicity. As expected, it exhibits the same level of anti-HIV activity and cytotoxicity as DAP 30.

As seen in Fig. 6b, 48% homology was found between GAP 31 (residues 1–40) and a DNA topoisomerase II, residues 660–699 [11]. In the 40 amino acid overlap, 40% identity is accountable. The significance of this homology between an anti-HIV protein and a DNA specific endonuclease is intriguing. Although these proteins have distinct specificities, they may share a common strategy of action. Whether this is so warrants further study.

Table II

Comparison of the N-terminal sequence 10–16 of some non-toxic and toxic anti-HIV proteins

SCRIP	Amino acid sequence 10–16	Cyto-toxicity	Refer-ences
TAP 29	-Thr-Ser-**Lys**-**Lys**-**Lys**-Val-Tyr-	–	[2]
MAP 30	-Thr-Ile-**Lys**-Thr-Tyr-Thr-**Lys**-	–	[1]
GAP 31	-**Lys**-Gly-Ala-Thr-Tyr-Ile-Thr-	–	
DAP 32	-Val-Ser-Pro-Ser-Ala-Asn-**Arg**-	–	
DAP 30	-Asn-Pro-Ser-Ala-Ser-Gln-Tyr-	+	
SAP-6	-Asn-Pro-Thr-Ala-Gly-Gln-Tyr-	+	[10]
TRI	-Thr-Ser-Ser-Ser-Tyr-Gly-Val-	++	[3,14,15]
RIC A	-Thr-Val-Gln-Ser-Tyr-Thr-Asn-	+	[4]

Basic amino acids, lysine (Lys) and arginine (Arg), are bold faced. SAP-6, saporin-6; TRI, trichosanthin; RIC A, ricin A chain.

4. DISCUSSION

The anti-HIV activities in GAP 31, DAP 30 and DAP 32 described here suggest that these compounds may be useful therapeutic agents in the treatment of HIV-1 infection. Our results indicate that GAP 31 has the highest therapeutic index based on both TD_{50}/ID_{50} and LD_{50}/ID_{50} ratios. The high anti-HIV activity and low cytotoxicity of GAP 31 have also been independently confirmed in human peripheral blood monocytes. These results will be reported separately.

While the anti-HIV activity of these compounds are similar, their cytotoxicities differ considerably. Little is known about the bases of such difference. Recently, we reported that two highly related anti-HIV proteins from trichosanthes displayed significant difference in cytotoxicity [2]. Although their amino acid sequences at the N-terminal portion of 44 residues are highly homologous, a stretch of five residues at position 12–16 namely **-Lys-Lys-Lys-Val-Tyr-** for the non-toxic TAP 29 [2] and **-Ser-Ser-Tyr-Gly-Val-** for the toxic trichosanthin differ completely [3,12–15]. Examination of other non-toxic anti-HIV proteins, such as MAP 30 previously identified [1] as well as GAP 31 and DAP 32 reported here as compared with relatively toxic DAP 30, SAP-6 [9] and ricin A chain [4], the absence of basic amino acids is noted in all toxic anti-HIV proteins. Table II indicates their difference. Interestingly, all of the non-toxic compounds have one or more Lys or Arg residues in this region; GAP 31 has a Lys at position 10, DAP 32 has an Arg at position 16, and MAP 30 has two Lys residues at positions 12 and 16. These Lys and Arg residues offer potential tryptic cleavage sites. These residues are absent in the corresponding regions of the toxic SCRIPs, DAP 30, SAP-6, trichosanthin and ricin A chain. These results raise the possibility that the absence of basic residues in this unique region may play a role in the cytotoxicity of these anti-HIV proteins.

The mechanism of anti-HIV action of these compounds remains to be elucidated. Our results indicate that they seem to exert their effects at multiples stages of the viral life cycle, affecting both viral infection and replication. The basis of their specificity may lie in the selective binding or uptake of these agents by viral infected cells; alternatively, they may be acting on the

biochemical differences of the viral life cycle, or infected-cell metabolism as opposed to normal cell metabolism. These mechanisms of action are not mutually exclusive, and some combination of them may account for the anti-HIV action of these compounds. Perhaps as striking as the specificity of action these agents display, is the observation that all of the plant anti-HIV proteins we have examined so far possess SCRIP activity as well. SCRIPs inactivate ribosomes by hydrolytically cleaving a glycosidic linkage between the adenine and the ribose at a highly specific site on the 28S rRNA. The co-localization of an anti-HIV activity with this N-glycosidase activity suggests that these two activities may be related. Considering that HIV is a retrovirus and that SCRIPs are, in essence, highly specific RNA active reagents, raises the possibility that SCRIPs may exert their anti-HIV activity on the virus or viral specific metabolism directly.

The extensive sequence homology between GAP 31 and DNA topoisomerase II [11] is worth noting. A stretch of 8 residues, ITYVNFLN of position 15–22 in GAP 31, is homologous to ITYADFIN at position 674–681 of DNA topoisomerase II. The complete homology over an octapeptide may imply a functional similarity between these proteins. GAP 31 is an anti-HIV SCRIP, capable of hydrolytic cleavage of a glycosidic linkage between the adenine and the ribose at A_{4324} on the 28S rRNA [16,17]. This function requires that it recognizes a specific base sequence in the rRNA. Topoisomerase II, on the other hand, is a specific endonuclease which makes double-stranded breaks at hypersensitive sites of DNA. This action is essential for the topological conversions required during replication, transcription and chromatin assembly [18]. These recognition sites are commonly conserved and located in the loop regions of the nucleic acids [19]. Consequently, they may be the preferred targets for the integration of retroviruses as well [20]. Studies on the structural-functional relationships of these proteins are being pursued and may provide useful insight into their anti-HIV action.

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