# Antiviral Activity Of Ribosome Inactivating Proteins In Medicine

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**Abstract:** Pokeweed antiviral protein and several other ribosome inactivating proteins are effective against a broad range of viruses. Recent results have shown that their enzymatic activity is not limited to depurination of the large rRNA, they can depurinate other nucleic acids, including viral RNAs. Antiviral activity of RIPs is summarized here in light of their novel activities and recent developments in the field.

## ANTIVIRAL ACTIVITY OF RIBOSOME INACTIVATING PROTEINS

Ribosome Inactivating Proteins (RIPs) have become important agents against viruses mainly by virtue of their broad-spectrum antiviral activity. Although several RIPs are active against viruses that belong to different groups, they maintain specificity against viral infection. Because of their cytocidal properties, RIPs have been used in cancer therapy as the active moiety of immunotoxins that target cancer cells. Their potent toxicity has been exploited as weapons in biological warfare and more recently as instruments of terror [1]. The aim of this current review is to revisit the antiviral activity of RIPs in light of recent advances in the field.

Plants were suggested to contain powerful mediators of viral propagation over 75 years ago. In 1925, extracts from the leaves of several plants, including pokeweed (*Phytolacca americana*), were shown to prevent TMV transmission when mixed in suspension with Tobacco Mosaic Virus (TMV) ([2] and reviewed in [3]). Following isolation and purification of these antiviral agents [4], the same compounds were found to be responsible for the inhibition of protein synthesis as well [5]. These studies led to further characterization of the inhibitory action on protein synthesis with the hope of unraveling the underlying mechanism of antiviral activity. For several years, the mechanism of antiviral activity was thought to be inactivation of the host cell ribosome, leading to inhibition of viral protein translation and host cell death. However, it was not until the advancement of recombinant DNA techniques that researchers were able to generate recombinant wild type and mutant forms of RIPs and express them in heterologous systems. This created a powerful tool, which allowed separation of ribosome inactivation and antiviral activities of these proteins—previously thought to be inseparable.

### Ribosome Depurination as an Antiviral Strategy

RIPs have been shown to be active against viruses that infect animal, plant, and even fungal cells. An attempt to explain the broad-spectrum antiviral activity of RIPs suggests that a common step of the virus-host interaction is targeted. One of the broadest activities of RIPs is to inactivate ribosomes. They act as *N*-glycosidases to remove specific adenine and guanines from the highly conserved sarcin/ricin loop (S/R) in the large rRNA and inhibit protein synthesis (reviewed in [3, 6]). Because all viruses need to utilize the host cell protein synthesis machinery to propagate, host ribosomes would be the obvious targets. The problem, however, arises with specificity. In order to prevent non-specific ribosome inactivation or inactivation of host cells not infected with virus, plants have devised mechanisms to ensure host protection. RIPs are predominantly localized in the cell away from host ribosomes. PAP is extracellular [7] and ricin is localized in protein bodies within the cell [8]. These mechanisms ensure optimal plant growth in the face of individual cell suicide. Tobacco plants that express PAP, but do not properly sequester it are delayed in growth and exhibit lesions characteristic of RIP action [9]. PAP reduces the efficiency of transformation and the transgenic plants that survive the initial transformation with PAP are selected for expressing low levels of the protein [9]. PAP inactivates host ribosomes in these transgenic plants regardless of virus infection [10]. The virus is then at a major disadvantage for replication in the damaged cells, which might explain the antiviral activity. However, the ability to generate variants of RIPs has allowed further refinement of the mechanism of antiviral action. For example, a PAP variant lacking the C-terminus was able to prevent virus infection in tobacco plants [10, 11]. Analysis of the host ribosomes indicated that the antiviral activity could not be attributed to ribosome depurination. This indicated that there was an inherent antiviral activity that did not involve ribosome damage. Furthermore, the majority of studies that described the activity of RIPs against animal viruses demonstrated an antiviral effect that was evident without apparent inhibition of host protein synthesis and cytotoxicity. As shown in Fig. **1**, these findings led us to a model that describes how different domains contribute to cytotoxicity and antiviral activity. For example, the active site domain lies in the region of the overlap; therefore inactivation of the active site will abolish both antiviral activity and ribosome depurination. In contrast, deletion of amino acids at the C-terminus of the protein will eliminate ribosome depurination, but not the antiviral activity. Recent evidence indicates that it is possible to separate ribosome depurination from cytotoxicity [12].

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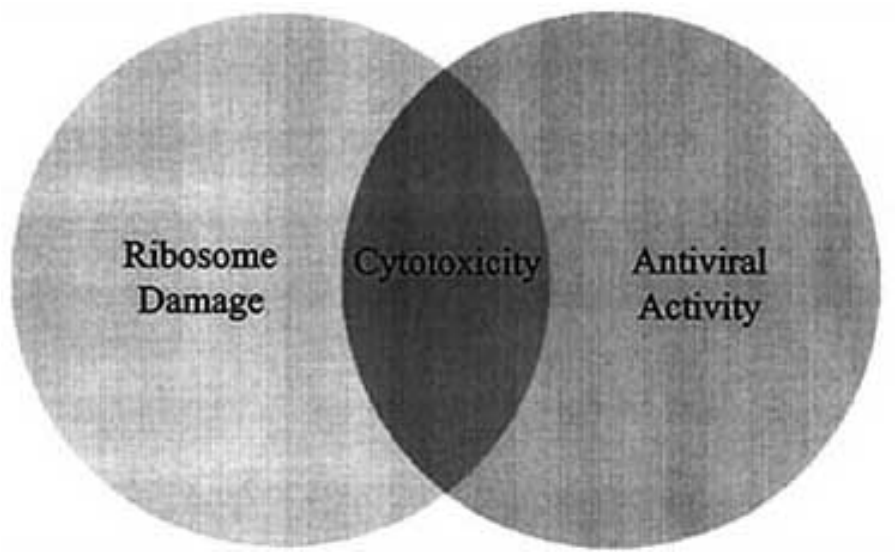


**Fig (1).** A schematic representation of the relationship between various activities of RIPs.

**Plant vs. Animal Viruses**

The activity against plant viruses has been assayed mostly with TMV and Potato Virus X (PVX) infection in tobacco plants (Table I). Because cDNA of PAP and other RIPs have been cloned and transgenic plants have been generated, analysis of mutant forms of RIPs in plants has been a powerful strategy for systematic analysis of the functions of various RIP domains. Mammalian cells differ from plant cells and the mechanism of virus infection and spread similarly varies between plant and animal viruses. The potential to develop pharmaceuticals targeted at preventing disease, especially in human populations, has led to dramatic increases in the knowledge base of how various RIPs function against different animal viruses. Some of the first studies showed that RIPs were effective against viruses as broad as poliovirus, influenza, and herpes simplex virus [13-15]. After the emergence of the AIDS crisis in the mid-1980s, most of the work with RIPs as antivirals shifted towards the elimination or prevention of HIV infection. Trichosanthin was the first RIP to be tested in phase I clinical trials during a time when AZT (zidovudine) was the only drug approved to treat HIV infection [16, 17]. Much of the recent work has focused on the ability of RIPs to act against HIV. The ability to prevent HIV infection can be used as a tool to classify RIPs from different species. While trichosanthin and PAP are very effective anti-HIV agents, ricin is not antiviral. Due to the complexity of its life cycle, HIV provides many steps that can be specifically targeted and many more that can serve as non-specific targets. As an example of non-specific targets, HIV, like other human viral pathogens needs to gain entry into cells, replicate, translate viral proteins, be packaged, and subsequently released. Unlike other non-retroviral pathogens, HIV requires additional specific steps for propagation, namely reverse transcription and host DNA integration. Recent evidence is mounting that RIPs, such as trichosanthin, are able to specifically inhibit the integration step of the HIV replication cycle [18]. This fails to explain how RIPs act against other viruses that lack similar steps in their life cycle. These findings support the hypothesis that RIPs possess both specific and non-specific antiviral activities.

**Nucleic Acid Binding**

RIPs inhibit replication of RNA as well as DNA viruses. RIPs, like PAP, are highly basic and have the ability to bind to nucleic acid substrates. This activity appears to exhibit some specificity, as not all substrates are equally affected. PAP has been shown to inhibit the translation of capped but not uncapped viral RNAs [19]. Recent results have demonstrated that PAP binds to the cap structure and depurinates capped but not uncapped reporter mRNAs *in vitro*, indicating that PAP can act on capped RNAs in addition to rRNA [12]. PAP has been shown to inhibit DNA viruses, such as HSV, by affecting viral protein synthesis without inhibiting host translation [20]. These results indicated that if ribosome inactivation was the source of the antiviral effect, it occurred with a mechanism that exhibited specificity. This specificity suggests that either the viral RNA is targeted prior to translation or that the viral DNA is precluded from efficient transcription, or a combination of the two. These results suggest that PAP has a specific affinity for viral nucleic acids that is in addition to its activity on ribosomes. The affinity for viral nucleic acid might be high due to the increased abundance in either viral DNA or RNA during the replication and/or packaging of viral nucleic acids. As an example, PAP was shown to depurinate TMV and HIV-1 RNA, whereas the ricin toxin A chain (RTA) did not [21, 22].

***In Vivo* vs. *In Vitro***

Both *in vivo* and *in vitro* evidence has been accumulating over the past several years to describe and explain the antiviral activity of RIPs. Many of these specific analyses are difficult to conduct *in vivo*, therefore *in vitro* methods are employed. The caveat with *in vitro* methodology in general, is that enzyme and substrate interactions might be

**Table I. RIP Activity Against Viral Pathogens**

| *RIP* | *Host* | *Immunoconjugate* | *Antiviral activity* | *Target cells* | *$IC_{50}$* | *Ref* |
|---|---|---|---|---|---|---|
| PAP | *Phytolacca americana* | | TMV | | | [9, 24, 33, 55-58, 91] |
| | | | TMV | | <5 nM | [26] |
| | | | SBMV | | | [4] |
| | | | CMV | | | [9, 14, 58] |
| | | | AMV | | | [58] |
| | | | PVX | | | [9, 19, 58] |
| | | | PVY | | | [9, 58] |
| | | | ACMV | | | [58] |
| | | | CaMV | | | [58] |
| | | | PLRV | | | [9] |
| | | | BMV | | | [19] |
| | | | Poliovirus | HeLa | 0.3 μM | [13, 36, 45] |
| | | | Influenza | | | [14] |
| | | | HSV | HeLa, Vero | 0.3 μM | [15, 20, 30] |
| | | | HSV | Vero | 1.2-3.0 μM | [143] |
| | | yes | HCMV | U937, CEM | | [42] |
| | | | Ty*1* yeast retrotransposon | | | [53, 54] |
| PAP-S | *Phytolacca americana* | | HSV | Vero, HEp-2 | 0.3-1.0 μM | [30, 39] |
| | | | Poliovirus | HEp-2 | | [39] |
| PAPII | *Phytolacca americana* | | TMV | | | [61] |
| | | | PVX | | | [61] |
| | | | HSV | HeLa | | [30] |
| | | | TMV | | | [26] |
| Trichosanthin | *Trichosanthes kirilowii* | | TuMV | | | [59] |
| | | | HSV | | 38.4 μg/ml | [74] |
| Dianthin | *Dianthus caryophyllus* | | | | | [60] |
| DAP32 | *Dianthus caryophyllus* | | HSV | HEp-2 | | [39] |
| | | | Poliovirus | HEp-2 | | [39] |
| Gelonin | *Gelonium multiflorum* | yes | HCMV, MCMV | C127I | 35 μg/ml | [127] |
| | | yes | Pichinde virus | Vero | 18 pM | [126] |
| | | | HSV | HEp-2 | | [39] |
| | | | Poliovirus | HEp-2 | | [39] |
| MCI | *Momordica charantia* | | HSV | HEp-2 | | [39] |
| | | | Poliovirus | HEp-2 | | [39] |
| MAP30 | *Momordica charantia* | | HSV | WI-38 | 0.1-0.3 μM | [81] |
| | | | | WI-38 | 0.4 nM | [84] |
| | | | HHV8 | BC-2 | 0.4 nM | [84] |
| GAP31 | *Gelonium multiflorum* | | HSV | WI-38 | 0.2-0.5 μM | [81] |
| | | | | WI-38 | 0.4 nM | [84] |
| | | | HHV8 | BC-2 | 0.4 nM | [84] |

* TMV – tobacco mosaic virus; SBMV – southern bean mosaic virus; CMV – cucumber mosaic virus; AMV – alfalfa mosaic virus; PVX – potato virus X; PVY – potato virus Y; ACMV – african cassava mosaic virus; CaMV – cauliflower mosaic virus; PLRV – potato leaf roll virus; BMV – brome mosaic virus; TuMV – turnip mosaic virus; HSV – herpes simplex virus; HHV8 – human herpes virus 8; HCMV – human cytomegalovirus; MCMV – murine cytomegalovirus

modulated by unknown host factors *in vivo*. The value of the *in vitro* assays, however, is indispensable for characterizing exactly which activities RIPs have and, whether or not they catalyze these reactions *in vivo*. For example, Hudak and colleagues demonstrated *in vivo* that a mutation in the ribosomal protein L3 rendered the ribosome incapable of PAP-mediated depurination [23]. The implication was that an altered ribosomal docking site for the RIP protected against enzymatic cleavage. However, these same ribosomes that could not bind to PAP and were not depurinated *in vivo* were susceptible to depurination by PAP *in vitro*. These results are invaluable for realizing not only the potential ability of RIPs, but understanding the interplay between yet undiscovered factors that modulate the activity of these proteins *in vivo*.

## STUDIES WITH SPECIFIC ANTIVIRAL PROTEINS

### PAP: Initial Discovery

We will begin our systematic study of plant RIPs with the earliest and most thoroughly characterized RIP, the pokeweed antiviral protein or PAP from the leaves of *Phytolacca americana*. As discussed above, PAP was amongst the first antiviral agents described in the literature. In 1925, Duggar and Armstrong discovered that extracts from the leaves of several plants, including *P. americana* prevented viral transmission and ultimately infection of other plants when mixed in suspension with TMV [2]. Several years later in 1948, Kassanis and Kleczkowski identified a glycoprotein from the extracts of *Phytolacca esculenta* that could inhibit TMV infection, but whether the protein acted on the virus or the host cell was not conclusively determined [24]. In 1969, Wyatt and Shepard described the isolation and characterization of an inhibitor of Southern Bean Mosaic Virus (SBMV) from *P. americana* and purification of the peptide, referred to as PAP, to 90% homogeneity [4]. They determined that the inhibitor isolated previously [24] was heterogeneous, containing at least three different active peptides. They then demonstrated that the protein inhibitor was not likely a ribonuclease or a glycoprotein. The inhibitor, PAP, was known then as *P*hytolacca *a*mericana peptide and now as the Pokeweed Antiviral Protein.

In 1973, Obrig *et al.* were the first to characterize the protein biochemically [5]. They discovered that PAP could inhibit the translation of globin in a cell-free rabbit reticulocyte system. The large subunit of the ribosome was determined to be catalytically and irreversibly affected by the action of PAP. Additionally, inhibition of both elongation factor 1 (EF1) and elongation factor 2 (EF2)-mediated reactions were described. However, no relationship to the antiviral activity was proposed.

In 1975, Irvin described the purification of PAP to homogeneity [25]. PAP was isolated from young leaves of pokeweed plants grown in the wild during early spring. After ammonium sulfate fractionation, ion exchange chromatography and concentration, PAP was determined to be a monomer with a molecular weight of around 27 kDa. This was later amended to 29 kDa [26] upon MW calculation using the Laemmli method [27]. PAP preparations used prior to these extensive isolation procedures were judged to have a MW of 13kDa, however, the assays were not sensitive enough to be able to accurately determine the true MW. Both PAP-treated reticulocyte and wheat germ ribosomes were previously described to inhibit poly-U translation by only 75% and 60%, respectively. However, Irvin demonstrated that brine shrimp ribosomes could be inactivated nearly 98% by PAP, either with or without preincubation. Moreover, the molar ratio of PAP to ribosomes at 50% inhibition was 1:150, indicating that the inhibition proceeded catalytically. Using purified reticulocyte ribosomes, Irvin showed that complete inhibition was not possible even with a 1:2 molar ratio of PAP to ribosomes (85% inhibition), presumably because of the protective effect of EF2 [28]. With a 1:56 molar ratio, only 30% inhibition was observed. Previously, using natural globin message in reticulocyte lysate, PAP was shown to have a 50% inhibitory effect at a molar ratio of 1:200. These two independent results [5, 25] demonstrated that PAP is at least 4-fold more active on natural mRNA as opposed to poly-U RNA when translated in a rabbit reticulocyte lysate cell free translation system. However, the inhibitor activity in both cases appeared to be enzymatic. The basis of translation inhibition was attributed to altered elongation factor binding and activity [25]. Irvin observed that EF2 GTP hydrolysis was enhanced, suggesting that the modification to the ribosome favored EF2 GTP binding and hydrolysis over EF1 binding. However, the enhanced EF2 GTP hydrolysis was actually detrimental to ribosomal translocation [25]. Several years later, Nygard and Nilsson were able to show two populations of 80S monosomes existed in cells—those that had a high affinity for EF2 GTP, but low GTPase activity and those with a low affinity for EF2 GTP but high GTPase activity [29]. They demonstrated that ricin-mediated damage resulted in a shift in 80S monosomes to increased EF2 GTPase activity. It seems plausible that PAP-mediated damage would have similar effects. Recent results, which demonstrated the specific inhibition of capped mRNA compared to uncapped mRNA by PAP [19], have finally provided an explanation for the apparent discrepancy between PAP's activity on natural, capped versus synthetic, uncapped RNAs. In addition to PAP, several other isoforms of pokeweed antiviral protein were isolated from pokeweed plants and characterized. In 1980, Irvin *et al.* reported the isolation and characterization of the antiviral RIP, PAPII from the summer leaves [26]. PAP expression is continuous throughout spring and summer while PAPII levels increase during the summer, possibly reflecting heightened protection of aging leaves. In 1982, Barbieri *et al.* described the purification and antiviral activity of PAP-S from pokeweed seeds [30]. In 1999, Rajamohan *et al.* reported the isolation of a RIP from the late summer leaves of pokeweed and called it PAPIII [21]. All four proteins from pokeweed, along with still several more [31, 32], see [3] for review) demonstrated both ribosome inactivation and antiviral activity.

Initial reports of the antiviral activity of PAP were attributed solely to translation inhibition. When extracts of PAP combined with purified TMV were inoculated onto leaves of various plant hosts *other than* pokeweed, the virus was inhibited from successfully infecting the host. In fact, in 1954, Gendron and Kassanis reported that antiviral compounds from extracts of many different plants appeared

to inhibit virus infection in diverse hosts with the exception of the host the compound originated from [33]. In order to rationalize why *Phytolacca americana* was not susceptible to ribosome damage, the theory that pokeweed ribosomes were insensitive to RIP activity was proposed. Although this did little to explain why pokeweed itself was inherently resistant to various viruses when encountered in nature, it did provide a testable hypothesis. In 1973, Owens *et al.* demonstrated that pokeweed ribosomes were insensitive to the inhibitory action of PAP as measured by poly-U translation, however, wheat and cowpea ribosomes were still susceptible [34]. It wasn't until 1994, that pokeweed ribosomes were actually demonstrated to be sensitive to ribosome damage [35].

**PAP and Poliovirus**

One of the first methodical approaches in understanding the effect of RIPs on virus propagation was described by Ussery *et al.* in 1974 [36], and published several years later [13]. Specifically, the inhibition of poliovirus infection by PAP was studied in HeLa cells (Table I). Poliovirus is a non-enveloped positive-strand RNA virus that infects animal cells and causes a debilitating neuromuscular disease in humans. The virions do not bud from the cell, instead they accumulate in the cytoplasm and are released when the cell bursts. PAP could inhibit the infectivity of poliovirus by about 96% when applied together with virus to cells. PAP specifically inhibited the level of protein synthesis in virus-infected cells. PAP did not alter the attachment of virus to HeLa cells, nor did it inhibit virus that was pretreated with the RIP upon removal by centrifugation prior to virus inoculation. The RIP was also not capable of inhibiting protein synthesis if added exogenously to HeLa cells in the absence of poliovirus. PAP was thought to enter into the cytosol of HeLa cells during the process of virus infection, either along with the virus, or independently by way of altered membrane permeability induced by virus entry. Poliovirus infection decreases protein synthesis in infected cells by cleaving eIF4GI [37] and eIF4GII [38], resulting in translation initiation arrest. However, 5 μM PAP was able to inhibit protein synthesis an additional 25-30%. These results, along with others [39], suggest that PAP-mediated inhibition of translation was capable of nearly eliminating poliovirus infection. More recent results, indicating that PAP can prevent poliovirus RNA translation independently of ribosome inactivation bring about an alternative explanation for the inhibition of poliovirus infection by PAP [40]. Quite possibly, the decrease in translation rates coupled with or superceded by RNA damage could be responsible for the antiviral activity of PAP on poliovirus.

**PAP and Influenza Virus**

Tomlinson *et al.* [14] initially realized the inhibitory effect of PAP on both animal and plant viruses when they tested the RIP for activity against influenza and cucumber mosaic virus (see below). Influenza is an enveloped segmented negative-strand RNA virus that causes the flu in humans. The life cycle of influenza virus involves transcription of positive-strand RNAs from negative-strand RNA templates as well as "cap-snatching" in which the virus commandeers 5' methylated caps from host mRNAs for its own use [41]. Unlike most other RNA viruses, with the exception of retroviruses like HIV, influenza virus replicates in the cell nucleus. Tomlinson *et al.* took advantage of the hemagglutinin (HA) activity found on the viral envelope to measure infectivity. At a 1:5 dilution of partially purified pokeweed extract, roughly 220 μg/ml, near complete inhibition of influenza HA activity was observed. 47% percent HA activity was shown at an extract dilution of 1:40 or 27.5 μg/ml. Additionally, when the virus was pre-treated with pokeweed extract and subsequently separated from it by centrifugation through a sucrose cushion, full virus infectivity was restored.

**PAP, Herpes Simplex Virus, and Cytomegalovirus**

In 1980, Aron and Irvin described the inhibition of Herpes Simplex Virus type 1 (HSV-1) multiplication and the effect of inhibition of cultured cells with prolonged exposure to PAP [15]. HSV-1 is an enveloped DNA virus and is the causative agent of several types of primary and recurrent diseases including gingivostomatitis, herpes labialis (cold sores), keratoconjunctivitis, and encephalitis. This was the first indication that PAP was effective against DNA viruses. The results confirmed that PAP acted as a general broad-spectrum antiviral as opposed to a selective one. Monkey kidney (Vero) and HeLa cells were used as target cells for HSV-1 inoculation. The virus was mixed with PAP and both agents were simultaneously applied to the cultured cells. The $ID_{50}$ (the concentration at which 50% inhibition is observed) for PAP's effect on HSV-1 in both Vero and HeLa cells was comparable to the $ID_{50}$ on poliovirus in HeLa cells, approximately 0.3 μM (Table I).

In 1982, Barbieri *et al.* purified PAP-S from the seeds of *P. americana* and found its inhibitory activity to be similar to that of PAP and PAPII when tested for virus resistance and protein synthesis [30]. The decrease in HSV-1 infection of Vero cells by PAP-S was also studied. The $ID_{50}$ for virus yield in the presence of the antiviral protein was between 1.0 and 0.3 μM. Foà-Tomasi *et al.* also characterized the antiviral effect of PAP-S against HSV-1 infection of HEp-2 cells [39]. The RIP was able to reduce viral yield, and decrease the number of HSV-1 plaques, while minimally inhibiting protein synthesis in uninfected cells.

In 1983, Teltow *et al.* further characterized the action of PAP against HSV-1 [20]. In particular, the effect of PAP on DNA synthesis was described. This was the first published evidence that the inhibitory effect of PAP could be due to effects independent from ribosome inactivation. Specifically, it was demonstrated that PAP could inhibit HSV-1 DNA replication, as well as virus release. A 12 hour pre-incubation of PAP with cells, followed by washing off unbound PAP subsequently resulted in 91-98% inhibition of viral replication as measured by reduction in plaque-forming units (pfu). Additionally, 3 μM PAP reduced total virus yields by 1 log while reducing virus release from cells by 2 logs at 24 to 48 hours p.i. if pre-incubated and washed as described above.

Gehrz *et al.* [42] described the activity of PAP on another DNA virus, human cytomegalovirus (HCMV). They used antibody-conjugated PAP (see below) to target the RIP to either cells expressing the ubiquitous low density

lipoprotein receptor (LDLr) or infected cells expressing the HCMV viral envelope glycoprotein gp55. The rationale for targeting LDLr is that the receptor-mediated mechanism of internalization would facilitate PAP entry. Virus was incubated with host cells in 96-well flat-bottom microtiter plates for 2 hours to allow adsorption followed by washing cells of free virus. PAP was added either prophylactically at 6 hours pre-infection or therapeutically at 9 hours post-infection and viral plaques were counted 3 to 5 days later. As judged by a $^{51}$Cr-release assay, no cytotoxicity was observed between 1 and 100 ng/ml PAP-anti-LDLr, however, [$^{3}$H]-leucine incorporation was completely inhibited by 100 ng/ml. Although an antiviral effect was seen with as little as 1 ng/ml PAP-anti-LDLr when added before 6 hours or PAP-anti-HCMV when added after 9 hours, the effect was not dose-dependent, calling into question the specificity of the activity. For example, 10 ng/ml of PAP-anti-HCMV inhibited infection by 44% whereas 1 ng/ml of the same immunoconjugate was 52% effective. Unconjugated PAP was able to inhibit infection by 61% at a concentration of 10 ng/ml (344 μM) but only 30% at 100 ng/ml (3440 μM).

## PAP and Human Immunodeficiency Virus

PAP and other RIPs have shown a dramatic effect on the inhibition of HIV-1 virus activity (Table II). In 1990, the first reported activity of PAP on HIV-1 replication was described by Zarling *et al.* [43]. Initially, CD4+ T-cells and macrophages were isolated from peripheral blood mononuclear cells and treated with HIV-1 (LAV-1$_{BRU}$ isolate) for 90 minutes at a multiplicity of infection (MOI) of 0.5. Unbound virus was then washed off and purified PAP was added for 5 days, after which reverse transcriptase (RT) activity and viral protein p24 production were assayed. The $ID_{50}$ for p24 production in infected cells was 5 nM whereas no cytotoxicity was evidenced in uninfected cells at a concentration of 50 nM. This concentration was more than 10 times less potent than MAP30 (see below), however, different viral strains and methodologies were utilized in those experiments and a therapeutic index was not precisely determined in this study. Zarling *et al.* further increased the potency of PAP by conjugation of the toxin to antibodies for CD4, CD5, and CD7. The PAP-immunoconjugate apparently entered cells via receptor-mediated endocytosis, thus increasing specificity. The CD4 antigens present on T-cells can combine specifically with gp120 on the HIV-1 envelope. As a result, the $ID_{50}$ values ranged between 1 and 50 pM for inhibition of p24 production, with the highest specificity by PAP-anti-CD4. As a control, PAP-anti-CD19 which reacts with B-cells only, caused only 20% inhibition at 1 nM. PAP-anti-CD4 was able to affect uninfected T-cells with an $ID_{50}$ value of 1 nM. Similar results were seen by Uckun *et al.* in 1998 [44]. In that study, a side-by-side comparison of the CD7 conjugate and the CD4 conjugate was conducted. The $ID_{50}$ for inhibition of HIV-1 p24 production by PAP-anti-CD4 was 30 pM, while that of PAP-anti-CD7 was 20 pM. The unconjugated RIP demonstrated an $ID_{50}$ of about 8 nM (Table II). RT activity was commensurately inhibited and T-cell toxicity was not witnessed with either immunotoxin at doses up to 4.5 nM.

At 50 pM, the immunoconjugate only occupied 3% of the available CD4 antigen sites and did not prevent normal CD4-dependent cellular processes [43]. According to the various analyses, no potency was lost if the incubation of PAP-anti-CD4 with infected cells was delayed by 24 hours, indicating no effect on early stages of infection. Additionally, release of HIV-1 was not inhibited. These results contradict the earlier findings of the temporal dependence of PAP's antiviral activity with both poliovirus [45] and HSV [20] infection. This could indicate that PAP can target HIV-1 infection at a different stage of the life cycle. Quite possibly, the membrane permeability changes in HIV-1-infected cell membranes lasts longer than that observed with poliovirus infection. Alternatively, the antiviral effect of PAP can still be attributed to a general mechanism of selective RNA damage that is manifest differently in the various virus classes and/or is dose-dependent. PAP was also tested *in vitro* in peripheral blood lymphocytes (PBL) isolated from HIV-1 seropositive donors [43, 46]. Complete inhibition was seen with 0.05 to 0.5 pM of PAP-anti-CD4. Additionally, with 5 pM immunotoxin, complete inhibition was observed at 22 days even if PAP-anti-CD4 was removed at day 5.

The efficacy of PAP immunoconjugates against 22 different clinical isolates of HIV-1 was studied by Erice *et al.* in 1993 [47]. PAP-anti-CD4 was compared to AZT in virus strains from both AZT-treated and untreated patients. Peripheral blood mononuclear cells were infected *in vitro* with serum-purified virus followed by 5 to 10 day incubations with either PAP-anti-CD4 or AZT, respectively. The average $ID_{50}$ values (measuring RT activity) with AZT treatment was 126 nM and 2498 nM for virus strains from previously AZT-treated and untreated patients, respectively. In contrast, the average $ID_{50}$ values for PAP-anti-CD4 were 48 pM and 16 pM, respectively. The data indicates that not only was the PAP immunotoxin more effective than AZT in inhibiting HIV-1, it can be used with AZT-resistant strains.

In 1998, Uckun *et al.* also determined the efficacy of PAP-anti-CD4 and PAP-anti-CD7 in a mouse model of AIDS [44]. The researchers showed that PAP-anti-CD7 was more effective in eliciting virus remission as determined both by PCR and virus culture than by CD4 conjugate. Additionally, both immunotoxins were superior to AZT and d4T nucleoside analogs. Cynomolgus monkeys were also treated with PAP-anti-CD7 for one-hour followed by serum collection and determination of antiviral activity *in vitro*. Even at a 1:100 dilution of the serum, anti-HIV activity was still evident. As a result of the work, a phase I trial with PAP-anti-CD7 was launched in July 1997. The limiting factor in clinical use appeared to be the generation of antibodies against both the toxin and the mouse monoclonal used for conjugation; however, the toxin could be slightly altered and the MAb could be humanized to prevent rejection. Two years later [48], further studies of PAP-anti-CD7 in HIV-1 infected monkeys indicated virus levels could fall below detection even two months after the infusion was given. Additionally, studies in human HIV-1 patients with sub-optimal doses of the immunoconjugate demonstrated a reduction in viral p24 levels for up to 7 weeks. It was concluded that a higher dose might have increased the duration of remission.

**Table II. RIP activity against HIV-1**

| *RIP* | *Host* | *Immunoconjugate* | *$IC_{50}$* | *Ref* |
|---|---|---|---|---|
| PAP | *Phytolacca americana* | CD4 | 16-48 pM | [47] |
| | | | 0.5 nM | [47] |
| | | CD7 | 20 pM | [44] |
| | | CD4 | 30 pM | [44] |
| | | | 8 nM | [44] |
| | | | 0.4-0.5 μg/ml | [51] |
| | | | 89-130 ng/ml | [52] |
| | | | 14 nM | [21] |
| | | | 5 nM | [43, 46] |
| | | CD4 | 1 pM | [43, 46] |
| | | CD5 | 50 pM | [43, 46] |
| | | CD7 | 6 pM | [43, 46] |
| PAP-S | *Phytolacca americana* | CD4-binding region on gp120 | 0.14-1.7 nM | [49] |
| | | Variable region on gp120 | 43-470 pM | [49] |
| | | | <30 nM | [50] |
| PAPII | *Phytolacca americana* | | 26 nM | [21] |
| PAPIII | *Phytolacca americana* | | 17 nM | [21] |
| Trichosanthin | *Trichosanthes kirilowii* | | >340 nM | [71] |
| | | | | [16, 18, 62, 65, 68, 69, 72, 73] |
| TAP29 | *Trichosanthes kirilowii* | | 0.34-0.46 nM | [71] |
| GLQ223 | *Trichosanthes kirilowii* | | 30 ng/ml | [65] |
| | | | | [67, 70] |
| Trichoanguin | *Trichosanthes anguina* | | | [99] |
| MAP30 | *Momordica charantia* | | 0.22-0.83 nM | [76, 84] |
| | | | 1.5 nM | [77] |
| | | | 0.2-0.3 nM | [78] |
| | | | | [78-80, 82] |
| MRK29 | *Momordica charantia* | | | [97] |
| GAP31 | *Gelonium multiflorum* | | 0.20-0.31 nM | [84, 92] |
| | | | | [78-80, 82] |
| DAP30 | *Dianthus caryophyllus* | | 0.7-0.9 nM | [92] |
| DAP32 | *Dianthus caryophyllus* | | 0.7-0.9 nM | [92] |
| Bryodin | *Bryonia dioica* | | | [93] |
| Luffin | *Luffa cylindrica* | | 5 nM | [50] |
| | | | | [18, 98] |
| Saporin | *Saponaria officinalis* | | | [18] |

An alternate approach, which is the conjugation of PAP-S to anti-gp120 antibodies, was studied by Kim *et al.* in 1990 [49]. Anti-gp120 was previously shown to neutralize HIV-1 without conjugation to a RIP. After conjugation, it was hoped that internalization of the cytotoxic agent would not only neutralize, but also destroy HIV-1 within cells prior to budding. PAP-S was used in this case and the activity of the immunotoxin against three diverse strains of HIV-1 was tested. The experiments indicated $ID_{50}$ values for cytotoxicity to the various HIV-1 infected cells to be between 0.14 and 1.7 nM. Killing of uninfected cells was not evident at concentrations up to 53 nM. One disadvantage of this approach compared with that of Zarling *et al.* [43] relates to the antibody conjugate. Although the viral gp120 can easily mutate in infected patients due to selective pressure, the CD4 receptor site on cells cannot so readily alter their affinity for anti-CD4 molecules. A second

disadvantage is competition of the gp120 immunotoxin with circulating, humorally-derived anti-gp120.

The activity of unconjugated PAP-S on HIV-1 infected cells was analyzed by Olson *et al.* in 1991 [50]. PAP-S was determined to inhibit HIV-1 RT activity by 50-75% at 0.03 μM and completely at 0.3 μM. PAP-S was also more effective at inhibition of both RT activity and p24 production compared with AZT, a nucleoside inhibitor used as the standard therapy for HIV-1 infection at the time the report was published. No differences were observed if PAP-S was added simultaneously with virus or three days post-infection. This again corroborates the findings by Zarling *et al.* [43] that initial events in HIV-1 infection are not targeted by the RIP. Cytotoxicity to uninfected cells was not evident at concentrations of PAP below 1 μM by both trypan blue exclusion and *in vivo* methionine labeling.

In 1999, Rajamohan *et al.* [21] described the effects of PAP on HIV-1 replication and provided a biochemical basis for the inhibition observed (see also [51, 52]). Three isoforms of PAP were assayed (PAP, PAPII, and PAPIII) along with ricin toxin A-chain (RTA). All RIPs were shown to inhibit protein synthesis in a cell-free translation extract between 3 to 6 pM. The $ID_{50}$ values for inhibition of HIV-1 by PAP in human peripheral blood mononuclear cells were between 14 to 26 nM, whereas RTA had no effect. Initially, depurination of the viral genome was examined by aniline treatment. Aniline cleaves the phosphodiester bonds at abasic sites on the RNA molecule. Treatment with aniline suggested that all PAP isoforms were able to depurinate HIV-1 RNA, as well as genomic RNA of TMV and the M2 bacteriophage. RTA, on the other hand, did not possess such activity. The release of adenine from 1 μg of HIV-1 RNA by 5 μM of the RIP was quantified to be roughly 168, 105, and 63 pmol for PAPIII, PAPII, and PAP, respectively. Again, RTA did not cause any release of adenine when tested on all viral RNAs. In all cases, RIPs were incubated with viral RNA for 4 hours at 37°C. The specificity of PAP for depurination of both adenines and guanines from viral and ribosomal RNA was supported by these results [22]. The damage to the viral nucleic acid, and not the ribosome, may actually represent an alternate basis for the antiviral activity of PAP.

## PAP and Fungal Viruses

The antiviral action of PAP was shown to be effective against fungal viruses [53]. Specifically, PAP could inhibit the Ty*1* yeast retrotransposon without causing inhibition of the L-A and $M_1$ yeast killer virus system (Table I). Both elements replicate utilizing slightly different mechanisms that are intimately linked to translational fidelity. The yeast Ty*1* element requires a +1 ribosomal frameshift, while the L-A virus requires a -1 frameshift. Additionally, unlike traditional plant and animal viruses, their fungal counterparts are transmitted only vertically instead of horizontally from cell to cell, thus allowing characterization of antiviral activity separate from viral release. It was shown that the differences in activity against the viruses was in fact due to the ability of PAP to specifically prevent +1 programmed ribosomal frameshifting. A follow-up study using non-toxic PAP variants demonstrated that ribosome depurination could be separated from the effects on +1 ribosomal frameshifting [54]. Specifically, PAPc (a C-terminal 25 amino acid truncated form of PAP) was shown to bind tightly to ribosomes without depurinating the rRNA, yet it inhibited +1 frameshifting. Frameshifting by HIV-1, which utilizes a -1 signal, was not affected by PAP or PAPc, indicating that the antiviral activity against HIV-1 is not due to inhibition of viral frameshifting. The mechanism of either +1 or -1 frameshifting requires the ribosome to be in slightly different conformations, thus the inhibition of frameshifting by PAP is likely dependent on altered translational kinetics. For example, +1 frameshifting requires an unoccupied A site while the substrate for -1 frameshifting is an occupied A and P site. The -1 frameshift occurs immediately prior to translocation. Inhibition of translocation by PAP may lock the ribosomes in a conformation unfavorable for +1, but not for -1 frameshifting. These results provided evidence that PAP had viral RNA-specific effects *in vivo*, which would partially explain its multi-faceted antiviral activity [53, 54].

## PAP and Plant Viruses

The initial discovery of PAP was through the observation that pokeweed extracts could inhibit viral infection (see above). The activity of PAP on plant viruses was more precisely characterized following purification of the RIP. Tomlinson *et al.* initially tested the RIP for activity against Cucumber Mosaic Virus (CMV) [14]. CMV, a cucumovirus of the family Bromoviridae, is encoded by positive-strand RNA. This virus produces lesions that mottle, twist and curl leaves while fruit develop yellow shrunken areas. They demonstrated that extracts from pokeweed were only inhibitory to virus infection when applied simultaneously with the viral inoculum. Application of pokeweed extract, either crude or partially purified, three hours prior to virus inoculation did not inhibit the virus or result in cell toxicity, unlike the observation in animal tissue culture. Most likely, the plant cell wall precludes RIP attachment and/or entry. Furthermore, when the virus was pre-treated with pokeweed extract and subsequently separated from it by centrifugation, full virus infectivity was restored. The antiviral protein(s) from pokeweed most likely must enter into the cell simultaneously with the virus in order to be inhibitory.

In 1980, Irvin *et al.* described the purification of a second antiviral protein from pokeweed (PAPII) that was very distinct from PAP and expressed only during the summer [26]. The properties of PAPII were characterized and compared against PAP, which is also found in summer leaves but is the major antiviral expressed in spring leaves. Specifically, they found that PAPII was an equally potent antiviral protein, achieving complete inhibition of TMV infection of *Phaseolus vulgaris* at levels above 50 nM for either PAP or PAPII when mixed with 165 μg/ml of virus. Additionally, at 5 nM of antiviral protein, an 89% and 73% inhibition of TMV infection was observed with PAP and PAPII, respectively. Both proteins inhibited *in vitro* translation of poly-U RNA with an $ID_{50}$ between 0.1 pM and 0.3 pM, though PAPII seemed to be more potent. Taylor *et al.* [55] found that a concentration of 30 nM PAP was sufficient inhibit TMV infection of tobacco leaves, a concentration in agreement with the earlier findings.

Additionally, tobacco protoplasts were protected from TMV infection by 300-330 nM PAP [56, 57].

In 1991, Chen *et al.* described the antiviral activity of PAP when applied exogenously to tobacco leaves [58]. They showed that the application of 25 ng/ml of PAP could inhibit TMV local lesion formation by 68%. At higher concentrations, the inhibition was even greater. 800 ng/ml PAP was able to inhibit TMV infection, regardless of the concentration of viral inoculum (from 0.5 to 40 μg/ml) indicating that PAP concentration was the determinant of antiviral activity. PAP was shown to inhibit five different RNA and two different DNA viruses, truly earning the title of a broad-spectrum antiviral. These viruses included TMV, Alfalfa Mosaic Virus (AMV), Cucumber Mosaic Virus (CMV), Potato Virus X (PVX), Potato Virus Y (PVY), African Cassava Mosaic Virus (ACMV), and Cauliflower Mosaic Virus (CaMV). The method of application was also critical to antiviral activity. The best activity was seen when PAP and the virus were added simultaneously. Less activity was seen when the application of PAP was delayed even by 5 minutes, and at 50 minutes was completely ineffective. When PAP was injected into leaf intercellular spaces, the antiviral activity was retained for as long as 48 hours. Aphid-mediated infection of PVY was not affected by exogenous PAP application, nor was the ability of aphids to acquire PVY from infected leaves treated with PAP. The ability of PAP to affect a wide range of plant viruses with different replication strategies and genome organization indicated that host cell targeting by PAP was responsible for the antiviral effects. PAP did not seem to target a physical feature of virus prior to entry. However, the possibility still remained that PAP affected virus-specific reactions occurring within the host cell, such as viral RNA replication and translation.

In 1993, Lodge *et al.* described the antiviral activity of PAP against a variety of plant viruses in transgenic plants [9]. PAP was applied exogenously to tobacco and potato plants and the inhibition of PVX (a potexvirus) and PVY (a potyvirus) infection was assessed. Mechanical inoculation of virus was inhibited completely by 15 μg PAP per leaf, however, aphid-mediated inoculation was not inhibited by PAP with either PVY or potato leafroll virus (PLRV), a luteovirus. Wild type PAP and a PAP variant were introduced into transgenic tobacco plants. The transgenic plants that expressed high levels of protein (>10 ng/mg protein) exhibited stunted growth and mottled lesions characteristic of PAP expression. Plants that expressed moderate levels of PAP (1-5 ng/mg), did not exhibit lesions. Extracts from the plants expressing recombinant PAP were able to inhibit cell-fee translation with the identical potency as the purified protein. The transgenics were challenged with PVX, PVY and CMV, and the results indicated significant protection against the three different viruses. The best protection, which lasted up to 8 weeks, was against PVY, followed by PVX and finally CMV. Interestingly, both mechanical and aphid inoculation of PVY were equally inhibited. These results suggested that the previous inability of exogenously applied PAP to inhibit aphid-mediated virus inoculation was due to the insufficient entry of the RIP into the plant cell. A good correlation between the level of PAP expression and the extent of virus resistance was not observed, suggesting that unknown factors, in addition to level of expression were responsible. Finally, PAP was shown to be present in the intercellular fluid of the transgenic tobacco leaves. During the following years, various other RIPs, such as trichosanthin [59] and dianthin [60], were also shown to have antiviral activities in transgenic plants.

Using transgenic tobacco, Wang *et al.* [61] described the activity of PAPII against plant viruses. PAPII accumulated to at least 10-fold higher levels than wild-type PAP, indicating that PAPII had reduced toxicity compared with PAP. Resistance to both TMV and PVX was observed in transgenic tobacco plants expressing PAPII and the level of resistance correlated with the amount of PAPII protein expressed.

## Trichosanthin and TAP29

Following the early studies on the antiviral activity of PAP, other RIPs were more frequently being characterized for antiviral activity. In 1989, McGrath *et al.* described the anti-HIV activity of GLQ223, a purified and formulated preparation of trichosanthin, a 26 kDa type I RIP isolated from the root tubers of *Trichosanthes kirilowii* [62]. Trichosanthin had been used for years in China as an abortifacient ([63], and [64] for review). The RIP was tested for activity against chronic and acute anti-HIV infection of primary cells, as well as transformed cell lines. Effects on chronic HIV infection were also tested using primary cells from infected seropositive donors. The T-lymphoblastoid cell line (VB) was initially inoculated with GLQ223 and virus ($HIV\text{-}1_{DV}$). Infectivity was measured by both cytopathic changes as well as cell-free viral p24 antigen. Cells were preincubated with HIV-1 at an MOI of 0.005 in 24-well culture plates. Unbound virus was washed off and the RIP was added. Upon 4 days of RIP incubation with infected cells, a dramatic dose-response inhibition of p24 production was observed. At 16 ng/ml (about 0.615 nM), a 73% decrease in p24 levels was noted. At this concentration, cellular DNA synthesis was inhibited by roughly 10% and protein synthesis was not noticeably repressed, providing evidence for a virus-specific effect in host cells. This was in contrast to uninfected cells where protein synthesis was inhibited by about 20%. HIV-1 infection somehow seemed to protect against RIP activity on the ribosome, possibly by offering the RIP a higher affinity substrate than the ribosome. Increasing the RIP concentration above 393 ng/ml (15 nM) appeared to abolish the protective effect, supporting the notion of a substrate switch. A 2-day exposure of the virus-infected cells to 3.14 μg/ml (120 nM) of the RIP resulted in a decrease of greater than 95% in p24 levels, yet no measurable effect on DNA or protein synthesis. Viral HIV-1 RNA levels decreased remarkably in cells infected with HIV-1 and treated with 3.14 μg/ml GLQ223, yet no effect was seen on cellular γ-actin mRNA. Cells of the macrophage/monocyte lineage are presumed to be the reservoirs of HIV-1 and the source of latent reactivation of the virus. Effects of the RIP on chronic HIV-1 infection were studied in macrophage cells inoculated 20 days earlier with the virus. At 4 days after treatment, GLQ223 was able to completely inhibit p24 expression in the cytoplasm of these cells if applied for 3 hours at a concentration of 500 ng/ml, compared to no significant inhibition by a continuous

exposure to 40 μM of the nucleoside inhibitor AZT (3'-azido-3'-deoxythymidine). However, the brief treatment of GLQ223 resulted in a 40% loss of macrophage viability when assayed at 14 days after treatment. When monocytes and macrophages were extracted from HIV-seropositive donors and treated under the same conditions, the cells showed either complete or nearly complete loss of p24 expression at 5 days after treatment.

McGrath *et al.* further characterized the effect of GLQ223 on chronically infected macrophages and compared toxicity to uninfected controls [65]. Again, GLQ223 was shown to have an inhibitory effect on viral p24 production with an $ID_{50}$ value of near 30 ng/ml (Table II). The application of GLQ223 for 3 hours followed by incubation for 5 days without the RIP was inhibitory to protein synthesis in a concentration-dependent manner only when applied to infected cells. The inhibitory effect on protein synthesis in uninfected macrophages was only 10-20%, but not apparently dependent on concentration. The inhibition of p24 production was determined for at least one month after the initial 3 hour pulse when used at a concentration of 50 ng/ml or higher. The levels of HIV-1 RNA were also not detectable in these macrophages at 5 days after the pulse. During this phase I trial, some toxic effects were noted with 36 μg/kg of GLQ223, however, no clinical effects on HIV-1 antigen levels were noted. The studies were conducted following only one dose of the RIP.

These initial studies led to increased interest in GLQ223 being a potentially curative drug for those living with HIV. As a result of the successes reported by researchers, underground and unofficial clinical trials were conducted in the US and monitored by physicians around the country. The drug was imported illegally from China and the clinical trial was overseen by the AIDS advocacy group called Project Inform (www.projinf.org) without the authorization of the FDA [66]. Early studies reported lethal anaphylactic shock as a major adverse effect, even after the drug was diluted from the body. Byers *et al.* reported on one of these studies in 1990 [16]. Specifically, multi-center phase I and II trials with trichosanthin obtained from China were conducted. Neurotoxicity was associated with a low number ($<50/mm^3$) of CD4+ cells. In 18 of the patients with a detectable level of p24 at the start of the study, the average decrease in p24 levels was 42% of baseline. The method of trichosanthin administration was either intramuscularly or intravenously, 2 to 3 times, either weekly or every 3 days. Doses ranged from 10-30 μg/kg. The findings indicated a modest improvement for patients who entered the trial with higher numbers of CD4+ cells.

Adverse neurological reactions in association with GLQ223 administration were further probed [67]. The researchers used an *in vitro* human brain cell aggregate culture model. Trichosanthin itself was not capable of causing neurologic injury. However, supernatants from GLQ223-treated HIV-infected macrophages caused significant aberrations to human brain cells. There was also some degree of damage caused by supernatants from trichosanthin-treated uninfected macrophages as well as untreated HIV-infected macrophages. These results would explain why administration of trichosanthin to uninfected patients did not normally result in neurotoxicity.

In 1992, Mayer *et al.* [68] conducted an open label pilot study of trichosanthin. 20 seropositive patients were given 20 μg/kg trichosanthin once every four weeks for up to 12 weeks. Four subjects showed progressive but transient reductions in p24 levels. Ten patients demonstrated significant increases in CD4+ cells. The authors concluded that, in the short term, trichosanthin seemed to reduce viral activity and improve certain symptoms in healthy asymptomatic HIV+ patients, but not in those with full-blown AIDS.

In the 1994, phase II clinical trial reported by Byers *et al.* [69], 93 patients were given 1.2 mg of trichosanthin (Chinese formulation) first weekly then monthly while CD4+ cell levels were monitored. The dose was equivalent to 17 μg/kg in a 70 kg person. The dosing was based on mimicking the 3 hour *in vitro* exposure of trichosanthin to macrophages [65]. The results showed an overall decrease in the rate of loss of CD4+ cells by the end of the trial.

Kahn *et al*, in 1994 [70], evaluated the safety, activity, and pharmacokinetics of multiple doses of GLQ223 in 22 patients with AIDS or AIDS-related Complex (ARC). GLQ223 was administered intravenously at doses of 8, 16, 24, 36, and 50 μg/kg by constant infusion over 3 hours to achieve a concentration in serum of 50 ng/ml, again similar to anti-HIV concentrations reported by McGrath *et al.* [65]. For patients who received 36 and 50 μg/kg, target concentrations in serum were achieved and sustained. A concomitant increase in CD4+ and CD8+ T cells was also observed in these same patients.

In 1991, the reduced toxicity of TAP29 (*Trichosanthes* Anti-HIV Protein of 29 kDa) compared to trichosanthin was analyzed by Lee-Huang *et al.* [71]. TAP29 was the second RIP isolated from root tubers of from *T. kirilowii*. N-terminal sequencing of TAP29 revealed several differences from trichosanthin. Syncytium formation was assayed by first adding TAP29 or trichosanthin for 90 minutes, followed by HIV-1 for 60 minutes, and washing cells free of both unbound virus and RIP. Syncytium formation quantitates acute cell-free HIV-1 infection. The inhibition of syncytia indicates a repression of the interaction between fusigenic virus-infected cells expressing HIV-1 envelope proteins and uninfected adjacent cells bearing CD4. TAP29 was determined to have an $ID_{50}$ of 0.34 nM for syncytium formation and 100% inhibition at 34.4 nM (Table II). Trichosanthin was found to have significant toxicity, as measured by inhibition of protein synthesis, when given at concentrations above 0.344 nM. Additionally, at this concentration, only 21% inhibition of syncytium formation was observed. The differences between toxicity profiles reported previously for trichosanthin [65] are due to shorter incubation times of RIP with cells in these later studies (90 minutes *vs*. 4 days). When HIV-1 propagation was assessed by p24 levels and RT activity, $ID_{50}$ values of 0.37 nM and 0.46 nM, respectively, were obtained for TAP29 (Table II). At 100 times these $ID_{50}$ values, little to no cytotoxicity was observed as measured by both inhibition of protein and DNA synthesis. In contrast, trichosanthin exhibited 26-31% more cytotoxicity at 3.4 nM, 10 times the $ID_{50}$ value for TAP29. It was determined that the therapeutic index of TAP29 was at least 1000 times higher than trichosanthin, the active ingredient in GLQ223. The differences most likely

arose from increased entry of GLQ223 into non-infected cells. This was supported by the finding that both TAP29 and trichosanthin inhibited cell-free translation equally with an $ID_{50}$ value of 3.7 nM.

Recently, Wang *et al.* [72] were able to demonstrate that the anti-HIV activity of trichosanthin was independent of ribosome inactivation. Previously, the researchers found that a correlation had existed between the two activities [73], however, in light of recent mutational analyses, the conclusions were reversed. By replacing the C-terminal extension of the preproprotein trichosanthin, almost all anti-HIV activity was lost, whereas the ribosome depurination ability remained unaffected.

In addition to HIV-1, trichosanthin has also been shown to be effective against HSV-1 [74]. In these studies, trichosanthin exhibited an $ID_{50}$ of 38.4 μg/ml with regards to inhibition of HSV antigen production. Acyclovir and interferon-α2a treatments, which have previously been shown to be effective against HSV-1 infection, were potentiated by addition of trichosanthin. Individually, 1.0 ng/ml of acyclovir or 100 units/ml of interferon-α2a are ineffective in preventing viral antigen production. However, when combined with trichosanthin, an $ID_{50}$ was achieved with 100 to 125-fold lower amounts.

## MAP30

In addition to *Phytolacca sp.* and *Trichosanthes sp*., RIPs have been isolated from various other sources. One such RIP is momordin from *Momordica charantia*. The amino acid sequence was initially characterized in 1982 [75]. Several years later, the same RIP was studied for its antiviral potential. Due to the increased therapeutic index of newly discovered RIPs, such as TAP29, compared to trichosanthin, much of the recent research has focused on understanding their more specific antiviral activity. In 1990, Lee-Huang *et al.* [76] reported the isolation and purification of MAP30 (*Momordica* Anti-HIV Protein of 30 kDa), a protein that is identical to momordin[1]. They also characterized the anti-HIV activity of the RIP. Briefly, cells were pre-incubated with MAP30 for 90 minutes prior to addition of virus. After virus adsorption during the first 60 minutes, cells were washed free of both MAP30 and HIV-1. An $ID_{50}$ of 0.83 nM was achieved against syncytium formation (Table II). A very short pre-incubation (15 sec) at 0.83 nM resulted in 25% inhibition, indicating that a short pre-incubation could inhibit the initial phase of virus infection. Additionally, no cytotoxicity was observed at these concentrations. Continual presence of MAP30 to cells after virus adsorption enhanced the anti-HIV activity at higher MAP30 concentrations (167-1670 nM). An $ID_{50}$ of 0.22 nM and 0.33 nM was achieved against inhibition of p24 production and RT activity, respectively. At 33.4 nM MAP30, no inhibition of cellular DNA synthesis or protein production was observed, yet 98% and 87% inhibition of p24 and RT activity, respectively was shown. At 334 nM, only 25% inhibition of cellular processes was demonstrated suggesting a very high therapeutic index (~1000). The $ID_{50}$ for cell-free translation inhibition was 3.3 nM.

In comparison, the $ID_{90}$ (inhibitory dose at 90% inhibition) for trichosanthin (GLQ223) on HIV-RT activity caused about 35% and 40% inhibition of cellular synthesis of DNA and protein, respectively [62]. In contrast at the same dose, MAP30 showed no comparable inhibition. At 10 x $ID_{90}$, MAP30 caused only about 25% inhibition on cellular DNA and protein synthesis. MAP30, thus, had a much better therapeutic index than GLQ223.

One small observational study in 1992, on the use of *M. charantia* extract, showed a marked increase in T- helper cells[2]. The patient who had the largest increase per $mm^3$ of blood went from 480 T-helper cells to 1370 after treatment for three years. The patient with the smallest increase went from 336 T-helper cells to 446 over a period of eleven months. However, data was only collected from 6 patients and the study was not controlled or blinded, so it was difficult to draw meaningful conclusions.

In 1995, Bourinbaiar *et al.* [77] described how MAP30 could potentiate the anti-HIV effects of dexamethasone and indomethacine. In these studies, an $ID_{50}$ of 1.5 nM was obtained for preventing syncytium formation in acutely infected MT-4 cells. This value is slightly higher than previous reports but the differences were probably due to lack of pre-incubation and differences in cell types (MT-4 *vs*. H9 [75]). MAP30 was able to reduce the $ID_{50}$ of the steroid dexamethasone or indomethacin by about 1000-fold. Other Non-steroidal anti-inflammatory drug (NSAIDs) had no anti-HIV activity and MAP30 did not alter the $ID_{50}$ of AZT.

In 1995, Lee-Huang *et al.* [78] described the anti-HIV properties of recombinant MAP30. The authors described the cloning of the RIP from bitter melon leaves. MAP30 and α-momorcharin (a RIP previously isolated from the same plant) share only 48% amino acid homology. MAP30 has an N-terminal leader of 23 amino acids but no C-terminal extension and is glycosylated, unlike PAP. The $ID_{50}$ of both recombinant and natural MAP30 were nearly identical at between 0.2 nM and 0.3 nM for syncytium formation, p24 inhibition, and RT inactivation. Additionally, the $ID_{50}$ for cytotoxicity was greater than 3 μM while that for cell-free translation was about 3.2 nM. It was shown that MAP30 could convert supercoiled HIV-1 DNA in to a relaxed form that could not be converted back into the supercoiled form by DNA gyrase. The conclusion was that MAP30 topologically inactivated HIV-1 DNA. This has implications for various biological roles of the DNA including integration.

Lee-Huang *et al.* looked specifically at the integration step of HIV-1 replication [79]. The indication for this was the previous reports indicating that MAP30 and GAP31 (*Gelonium* Anti-HIV Protein of 31 kDa) could bind to supercoiled DNA and relax it, rendering it topologically inactive [78, 80]. HIV-1 integrase binds to HIV-1 DNA and catalyzes the insertion of the viral genome into the host. The first activity studied was integrase-mediated 3'-processing of the viral LTRs. Both MAP30 and GAP31, when added simultaneously to the *in vitro* reaction, were able to completely inhibit the processing event at equimolar

[1]Fiorenzo Stirpe, personal communication.

[2]Zhang, Q. and Khanyile, C. *VIII Int Conf AIDS*, Amsterdam **1992**, *8*, pp. 148 (abs pub 7597).

concentrations. Using pUC18 as a target for insertion, the integration reaction (strand transfer) was monitored in the presence of the RIPs. Oligonucleotides that mimic the ends of HIV-1 DNA were inhibited from integrating if MAP30 or GAP31 were present at molar ratios around 40:1 and 2:1, respectively, with reference to HIV-1 integrase. The disintegration reaction, where the inserted genome is excised from the target, was also studied. It appeared that disintegration was prevented if MAP30 or GAP31 were present at 3:1 molar ratios with reference to HIV-1 integrase. These results were also duplicated with various other RIPs, including trichosanthin ([18], see below).

In 1996, Bourinbaiar and Lee-Huang [81] examined the inhibition of HSV infection by MAP30 and GAP31. No loss of viability was observed in uninfected WI-38 cells (embryonic lung fibroblast line) using concentrations of MAP30 and GAP31 up to 334 nM. This was similar to the toxicity profile of acyclovir (ACV), an HSV specific nucleoside analog. At 24 hours after infection with HSV, the cells displayed cytopathic changes, unless either MAP30 or GAP31 was added during the incubation with virus. This was quantified by measuring viral antigen after 1 to 2 day incubation. The $ID_{50}$ for MAP30 was between 0.1 to 0.3 μM and that for GAP31 was between 0.2 to 0.5 μM in both ACV-sensitive and resistant strains of HSV-1 and HSV-2. This represents an increase of almost 1000-fold compared with anti-HIV concentrations, however, at these levels, neither RIP showed evidence of toxicity in WI-38 cells. At these concentrations (0.3 μM), 25% inhibition of protein and DNA synthesis was observed in H9 cells. The differences might be due decreased ability of the RIP to enter the WI-38 cells. Previous reports also support the idea that RIPs have different inhibitory effects on different cells [3]. In all cases, MAP30 was more effective than either GAP31 or ACV in HSV inhibition. The efficacy of RIPs against ACV-resistant virus suggests a mechanism of HSV inhibition that is unlike that of nucleoside inhibitors.

In 1999, Huang *et al.* [82] reported that proteolytic fragments of both MAP30 and GAP31 were biologically active. Limited proteolysis allowed for description of the structure-function correlation in both RIPs. The central fragments of the proteins were protected from protease digestion, presumably by the compact and rigid nature of the RIPs. The C-terminus however was more susceptible to cleavage. Three major proteolysis products were generated, of which the two largest retained anti-HIV activity ($MW_r$ of 21-22 kDa and 25-26 kDa). The $ID_{50}$ values for anti-HIV activity were similar to the parent RIPs, namely between 0.2 nM and 0.4 nM. The central proteolytic fragments of MAP30 and GAP31 were able to inhibit HIV-1 p24 expression, prevent HIV-1 integrase, and topologically relax supercoiled DNA. However, unlike the full length RIPs, these proteolytic fragments were neither cytotoxic nor inhibitory to cell-free translation. Interestingly, the C-terminal 76 amino acids and the N-terminal 10 amino acids of MAP30 were not necessary for activity against HIV-1. When aligned against PAP, the non-essential C-terminal residues of MAP30 begin at glutamine 205 in the PAP sequence. Positioning a stop codon in place of tryptophan 238 results in similar effects: antiviral activity without inhibition of translation *in vivo*. These independent results would indicate the importance of the C-terminus in regulating ribosome inactivation without affecting antiviral activity. The immediate thought would be that the C-terminus is required for binding to the ribosome. In fact, deletion of the C-terminus in PAP prevents ribosome dissociation [54]. These results imply that release of RIPs from the ribosome is a co-requisite for *N*-glycosidase activity or that the C-terminus is required for proper folding and positioning of the protein on the ribosome. In the crystal structure, it was noted that the C-terminal 20 residues of PAP are hyper-flexible [83]

In 2002, Arazi *et al.* reported the expression of MAP30 and GAP31 in cucurbit plants (squash, cucumber, melon, pumpkin) [84]. This approach was intended to provide an alternative source of edible antiviral protein. Zucchini Yellow Mosaic Virus-AGII (ZYMV-AGII) was used as a plant virus vector to direct expression of the RIPs to the fruit. Squash leaves inoculated with viral vector containing either MAP30 (MAP30-SQ) or GAP31 (GAP31-SQ) developed lesions on their leaves indicative of RIP expression. Unfortunately, information regarding the yield of protein and whether the fruits expressed the RIPs was not provided. Upon purification of the RIPs from the squash leaves, antiviral activity was determined. The $ID_{50}$ value for HIV-1 syncytia formation and p24 production were around 0.24 to 0.31 nM. Toxicity to uninfected cells, as measured by inhibition of either protein or DNA synthesis, was not evident even at 3.3 μM. MAP30-SQ and GAP31-SQ exhibited $ID_{50}$ values near 0.4 nM for both HSV and Human Herpes Virus 8 (HHV8) in assays described previously. In all cases, the squash-derived RIPs were identical in inhibiting viral propagation to the naturally derived RIPs.

## Other Antiviral RIPs

In 1962, Ragetli and Weintraub [85, 86] described two inhibitors of TMV infection from carnation leaves (*Dianthus caryophyllus*), which were later characterized by Stirpe *et al.* [87]. In 1978, Grasso and Shepherd isolated a basic antiviral RIP from *Chenopodium amaranticolor*, and partially characterized similar antiviral proteins from 13 other plant species [88]. Seed extracts were also screened for antiviral proteins [89, 90]. In 1981, Stevens *et al.* reported on the correlation between the inhibition of TMV infection with the reduction in protein synthesis by purified plant extracts [91]. The eight inhibitors tested included peptides such as PAP, ricin, abrin, modeccin, gelonin from *Gelonium multiflorum* and *Momordica charantia* inhibitor (MCI) as well as crude extracts from *Bryonia dioica* seeds and carnation leaves. Of the purified proteins, PAP was found to be the only inhibitor of TMV capable of retaining activity after reduction by β-mecaptoethanol. Presumably A-chain and β-chain dissociation occurs for ricin, abrin and modeccin and instability might be the problem for gelonin and MCI. Although not purified to homogeneity, the RIPs from *Bryonia dioica* and *Dianthus caryophyllus* were able to both inhibit translation as well as TMV infection. The researchers proposed that many plants contained RIPs, yet their abundance might vary and their levels may be too low to be easily detected. Additionally, in 1982, Foà-Tomasi *et al.* [39] reported on the inhibition of both poliovirus and herpes simplex virus infection inhibition by RIPs from various plants, including gelonin, dianthin 32 from carnation, and

MCI. For a complete list of plant antiviral proteins, the reader is directed to a comprehensive review published by Barbieri *et al.* [3].

In 1991, Lee-Huang *et al.* described the antiviral properties of GAP31, an RIP from *Gelonium multiflorum* [92]. Additionally, two RIPs from carnation were discussed, DAP30 and DAP32, which were both previously isolated by Stirpe and colleagues [75, 87] and referred to as dianthin 30 and 32, respectively. HIV-1 syncytium formation was inhibited in a dose-dependant manner with all three RIPs. The $ID_{50}$ values were 0.28, 0.83, and 0.76 nM for GAP31, DAP30, and DAP32, respectively, and no toxicity was observed at the doses assayed (Table II). The $ID_{50}$ values for inhibition of p24 expression and RT activity for GAP31 were 0.23 and 0.32 nM, respectively. The $ID_{50}$ values for the DAPs were between 0.7 and 0.9 nM for both HIV-specific activities. The p24 expression of cellular proteins other than the virally-encoded p24 was not affected. The toxicity profile of GAP31 was far better than that of the DAPs. For instance, at 320 nM, GAP31 showed no inhibition of either protein or DNA synthesis in the uninfected cell, whereas DAP32 demonstrated a 5% and 15% reduction in both of these activities. Additionally, DAP30 exhibited more than a 40% reduction in both cellular processes, indicating a lower therapeutic index. Ribosome inactivation demonstrated $ID_{50}$ values of about 2 to 4 nM for all three compounds. The researchers also described the anti-HIV activity and toxicity profile of saporin-6 (SAP-6), another RIP, to be very similar to DAP30. N-terminal amino acid sequencing was thought to provide some clue into determining whether a RIP would have a high Therapeutic Index (TI) in the treatment of HIV-1. For example, the researchers grouped MAP30, GAP31, and TAP29 as RIPs with the highest TI. Next, trichosanthin (or GLQ223), DAP30, and SAP-6 had a very low TI, but still inhibited HIV-1, nonetheless. DAP32 was ordered in between the two groups while the RIP ricin had no effect against HIV-1 infection. A comparison of the first 10-16 N-terminal residues of the RIPs correlated the presence of basic amino acids (K and R) to a higher TI. It is interesting to note that PAP, like GAP31, contains a single lysine in this region. Interestingly, gelonin, a RIP related to but distinct from GAP31 from *G. multiflorum*, shows no anti-HIV activity [18, 93] but has effects that inhibit intracellular parasites such as malaria [94].

In 1994, Lee-Huang *et al.* described the minimal region of GAP31 required for anti-HIV activity [95]. Several synthetic peptides displayed a dose-dependent inhibition of syncytium formation, namely V5-K42, K10-K42, and dimeric V5-K42 with $ID_{50}$ values near 36 μM. This compared with over a 100000-fold increase in the $ID_{50}$ value of full-length GAP31 (0.3 nM). All three peptides also inhibited p24 production and RT activity with slightly lower $ID_{50}$ values (19-23 μM) without inhibition of cellular DNA and protein synthesis. Studies of these peptides on uninfected cells indicated that concentrations up to 300 mM did not cause any detectable cytotoxicity. Ribosome inactivation, as measured by inhibition of cell-free globin translation, was complete at 20 μM. However, ribosome depurination was not directly determined. The researchers also showed that the peptides could bind to HIV-1 LTR DNA as assayed by a gel shift and that this binding could be reversed by 0.5% SDS. These peptides also bound rRNA (both 28S and 18S) and globin mRNA. This binding was also reversed by 0.5% SDS, yielding intact RNA. Immunofluorescence showed GAP31 inside HIV-1 infected cells but not uninfected cells. Activity was shown also against cytomegalovirus and hepatitis B virus with similar $ID_{50}$ values as obtained for HIV-1. Considering the lack of differences in activity among the three peptides, K10-K42 was deemed to be the minimal 33 amino acid sequence required for antiviral activity. In 1999, Fink *et al.* [96] refuted the clinical utility of the GAP31-derived peptide K10-K42. The group aimed to determine whether intracellular expression of the K10-K42 peptide would potentiate its antiviral efficacy *in vitro*. Unexpectedly, the antiviral activity appeared to be the result of precipitation of viral and non-viral proteins *in vitro*. This precipitating ability was specific to the sequence of the K10-K42 peptide and seemed to negate the potential for therapeutic applications.

Several other anti-HIV RIPs have also been characterized. For example, in 2001, a group from Thailand isolated an inhibitor from *Momordica charantia*, MRK29 and characterized it as a peptide of MW 28.6 kDa [97]. About 630 nM of crude MRK29 could inhibit HIV-1 RT by 50%. Around 6 nM of partially purified MRK29 was able to cause 82% inhibition of HIV-1 p24 production in infected cells. Bryodin, a single chain RIP from *Bryonia dioica* was demonstrated to also show anti-HIV activity with efficacy similar to trichosanthin [93]. Bryodin exists in plants in two isoforms, namely bryodin-1 and bryodin-2. Both isoforms have different effects on virus activity. Whereas bryodin-1 is slightly more selective for entering infected *vs*. uninfected cells, bryodin-2 is more effective against HIV-1 infection in persistently infected macrophages. As with many other RIP studies, they found that bryodin could inhibit viral translation more specifically than host translation, indicating a virus-specific effect. Several other anti-HIV RIPs include mostly single chain inhibitors such as luffin [98] and trichoanguin [99].

## Proposed Activities

Since the earliest studies on RIPs, the connection between ribosome inactivation and antiviral activity has been utilized to explain the most likely mechanism of antiviral activity, namely inhibition of protein synthesis. However, recent results indicate that the two activities can be separated and that the effect against viruses might be mediated via specific nucleic acid interactions. RIPs seem to have different ribosome specificities. For example, some RIPs act only on non-self ribosomes, whereas others act on all ribosomes. Several RIPs seem to inactivate extensive classes of viruses whereas other antiviral RIPs are not so broad-spectrum. Some RIPs bind to and alter RNAs other than rRNA and some RIPs can prevent very specific stages of virus infection, *e.g.* HIV-1 integration. We will use the well-characterized PAP as the model RIP to illustrate these seemingly diverse mechanisms. Specifically, we will discuss the entry of RIPs into virally infected host cells, the correlation between antiviral activity and ribosome inactivation, the effect of RIPs on various host factors, and

the consequence of RIP action on nucleic acid substrates other than rRNA.

**RIP Entry into Cells**

Numerous studies have indicated that the antiviral activity of PAP was lost if the virus was separated from the RIP prior to host cell inoculation [13, 14, 20]. Additionally, PAP was found not to bind to or covalently modify the virus particle itself [45, 100]. The evidence, therefore, suggests that host-cell modifications allow for both RIP entry and antiviral effect, yet how exogenously applied RIP managed to enter into the cytosol to inhibit virus production still needed to be characterized. Understanding the entry of antiviral RIPs into cells is a critical factor in developing effective therapeutics.

Studies on trichosanthin and MAP30 (see above) indicated that only virus infected cells were highly permissive to the RIP. Similarly, PAP has also been shown to preferentially enter virus infected cells. Most of the evidence accumulated to date points to entry of the RIP upon changes in membrane permeability induced by virus entry [101, 102]. As changes in membrane permeability are subtler in plant cells due to the presence of a cell wall, the entry of exogenous PAP must be tightly associated with virus entry. Reports have confirmed that PAP can penetrate virus-infected plant cells if the cell walls have been removed [56, 57]. Pokeweed plants, and more recently transgenic plants expressing recombinant PAP may have prevented the entry of RIPs into the cytosol, by sequestering the protein in the intracellular spaces [7, 9].

In 1980, HSV infection demonstrated that prolonged exposure of cells to PAP during the incubation with virus was required for inhibition of replication [15]. For example, 70% inhibition was seen when PAP was left in the presence of inoculated Vero cells for 24 hours; however, only 40% inhibition was seen when left for 6 hours. Additionally, if PAP was left for 23 hours, but not during the first hour of viral adsorption, the inhibition was only 30%. These results indicate that 40% of PAP's inhibitory effect was due to the action of the RIP during the first hour of virus adsorption. PAP did not bind to virus to prevent adsorption and so the change in membrane permeability might have allowed PAP to gain access to host cellular machinery. This data also suggested that 30% of PAP's inhibitory activity occurred as a result of its entry between 6 and 24 hours after virus inoculation. These results indicate that PAP continues to enter the cell during the later phases of virus replication and would probably continue to do so until the virus was destroyed, as determined by changes in membrane permeability. Recently, it has been demonstrated that HSV can alter membrane permeability differentially throughout its replication cycle in HeLa cells. Specifically, at 8 hours after HSV inoculation, a temporally regulated membrane depolarization occurs, as indicated by a small change in the transmembrane potential [15]. Aron and Irvin also described that only after prolonged incubation with PAP (36 hours) were uninfected cells susceptible to PAP cytotoxicity. Considering that the doubling time for cultured HeLa cells is around 24 hours [103], there might be a small window of time during cell division that the membranes are more susceptible to PAP entry. In fact, the permeability was shown to be highest during early G1 phase in these cultured HeLa cells [104].

In 1983 [20], Teltow and colleagues demonstrated that pre-treatment of cells with PAP added an extra level of antiviral ability. As a result, a 12 hour pre-incubation of PAP with cells, followed by washing off unbound PAP resulted in 91-98% inhibition of viral replication, thus suggesting that PAP has some affinity to the cell membrane that could not be washed away with culture media (Earle's balanced salt solution, EBSS). The small amount of PAP that was left bound to the cell, either specifically or non-specifically was sufficient to cause a devastating effect on virus replication upon virus entry and/or increases in viral-induced plasma membrane permeability.

In 1988, Aron and Irvin clarified the cytotoxicity of PAP on cultured Vero and HeLa cells [105]. These cell types were used previously in viral assays and were inhibited in translation only when either PAP was present along with virus or prolonged exposure to PAP was allowed. Both HeLa and Vero cells were killed by a 48-hour exposure to 3.0 μM PAP. Higher inhibition of protein synthesis was observed, the longer the cells were exposed to PAP. Further analyses demonstrated that as PAP became cell-associated during an initial 24 - hour exposure, removal of PAP did not completely reverse effects on protein synthesis. Most likely, as was observed with poliovirus-mediated entry of PAP [13, 45], the RIP can attach to cells during the initial incubation, however, the rate-limiting step is actually transport across the membrane. Unlabeled PAP could compete with radiolabeled PAP for nonspecific sites on the plasma membrane. These results indicated that a specific receptor is most likely not required for PAP entry. Because the protein synthesis inhibition was not observed for at least 24 hours after the addition of PAP, cellular events could possibly be required for PAP entry, for example membrane turnover and/or cell division. It has been noted that trichosanthin could more easily enter cells with high pinocytotic activities such as trophoblasts, macrophages, and virus-infected cells. Trophoblasts have been demonstrated to allow RIP entry [63] and as a consequence trichosanthin is used to induce abortions.

In 1990, the entry of PAP into the cell during poliovirus infection was studied in more depth [45]. Specifically, it was shown that virus adsorption was sufficient to increase the permeability of the cell membrane enough to allow the RIP to enter. At 1 hour p.i., poliovirus caused very little translation arrest in infected cells up to an MOI of 100 pfu/cell. However, in the presence of increasing amounts of PAP, increased translation inhibition was observed, suggesting that a virus-mediated event allowed PAP to enter. An MOI of 20 pfu/cell and 0.4 μg of cycloheximide (CHX) with or without 3 μM PAP were incubated and the inhibition of protein synthesis by PAP at 9 hours p.i. was used as a marker for membrane permeability as was described for alpha-sarcin [102]. It was shown that PAP could inhibit protein synthesis by 86% compared to control cells. The consequence of CHX was to inhibit macromolecular synthesis immediately after virus entry but would effectively be diluted out by 9 hours p.i., thus providing evidence that an early gene product of either the virus or the cell was not responsible for increased membrane

permeability. To confirm these findings, UV-inactivated poliovirus was inoculated onto HeLa cells in the presence of PAP. Low-dose UV-inactivated virus was capable of entry into the cytoplasm but not of viral replication and translation. High-dose UV-inactivated virus suffered sufficient damage to the capsid protein and was rendered incapable of adsorption. It was shown that PAP was able to enter the cell and shut down host protein synthesis by 95% with unirradiated virus and 84% with low-dose irradiated virus. This compares with 82% and 0%, respectively, for control cell translation inhibition in the absence of PAP. Remarkably, only 13% translation inhibition was observed with high-dose irradiated virus plus PAP. These results confirmed that not only was viral entry sufficient for PAP entry, but that a viral gene product was not required. Additionally, the integrity of the capsid was required for effective PAP entry, either through a direct interaction with PAP or a change in permeability caused by viral adsorption. Previously, it was demonstrated that the association of PAP with the virus did not critically reduce the infectivity of the virion. In this study, PAP was chemically cross-linked to the capsid. This forced association resulted in only a 17% loss of virus yield, reinforcing the notion that PAP association with the virus is not a critical determinant of its ability to enter cells. Therefore, the importance of capsid integrity for PAP entry can be attributed to efficient viral adsorption. Furthermore, inhibition of virus penetration and decapsidation by sodium azide and methylamine, respectively, did not alter either membrane permeability to PAP or PAP entry and translation inhibition. Therefore, PAP need not enter the cell physically attached to the virus. The kinetics of PAP entry into cells induced by virus adsorption was also characterized. Specifically, when PAP is incubated with cells, it is not able to gain entry and inhibit protein synthesis. However, virus adsorption allows PAP to enter more freely. A 10 minute incubation prior to virus adsorption provides a 10% increase in translation inhibition. A 20 minute preincubation results in a 50% block of translation. And a 40 minute preincubation results in a 75% block. In contrast, 90% inhibition is observed when PAP and virus are added simultaneously. These results suggest that membrane association by PAP is not rate limiting but dramatically hastened by virus adsorption. In contrast, traversal of the plasma membrane is rate limiting and occurs appreciably only in the presence of virus (or excess PAP). The virus-induced permeabilization of the membrane is similar to the observation with dimeric RIPs. The viral capsid may play a similar role to that of the β-chains of dimeric RIPs [106].

## Correlation of RIP Activity on Ribosomes to Antiviral Activity

Ribosome depurination was thought for a very long time to be linked inextricably with antiviral activity. Most mutations or treatments that inactivated ribosome depurination concomitantly prevented antiviral effects. However, several clues through the years led researchers to consider whether or not the two were actually inseparable. Recently, it has been shown that the trichosanthin anti-HIV activity could be abolished in a mutant form of the protein that could still depurinate ribosomes [72].

In 1983, Teltow *et al.* characterized the action of PAP against HSV-1 and published the first evidence that the inhibitory effect of PAP could be due to effects independent from ribosome inactivation [20]. Even though HSV infection caused a decrease in host protein synthesis, the effect of pre-incubation with PAP resulted in a further 30% decrease in protein synthesis between 1 and 20 hours p.i. There also seemed to be a differential effect on HSV-Infected Cell Polypeptides (ICPs). Specifically, γ ICPs involved in virus release were most affected at 18 hours p.i, which could explain the 2 log reduction in virus release.

In 1994, Taylor *et al.* attempted to correlate ribosome depurination of RIPs to antiviral activity [55]. They initially observed the differences in specificity between depurination of tobacco and yeast ribosomes to various RIPs. PAP was found to be twice as effective in damaging tobacco ribosomes, whereas, DAP32 (dianthin 32) was over 30 times as effective in depurinating yeast ribosomes compared to tobacco. Ricin toxin A-chain, tritin, and barley RIP showed considerable effectiveness in depurinating yeast ribosomes preferentially. Only PAP and DAP32 were equally effective at inhibition of TMV infection in tobacco leaves. Also, only PAP and DAP32 were able to enter cells in the absence of virus and cause depurination *in situ* (see also [58]) indicating that the two events were linked.

In 1995, Hur *et al.* described the isolation of PAP mutants involved in mediating toxicity [107]. One of these mutants, PAPc (W237Stop) was truncated at the C-terminus and as a result, lost ribosome depurinating activity [10, 19]. However, the antiviral activity was still intact, suggesting that virus resistance was not dependent on ribosome inactivation. Initially, it was shown that expression of the PAPc, in contrast to wild-type PAP, in yeast did not result in depurination of host ribosomes. This was not due to reduced ribosome association or stability of the mutant RIP [23, 54]. When expressed in transgenic tobacco plants, PAPc again did not depurinate host ribosomes. The transgenic plants appeared phenotypically normal with no evidence of toxicity and did produce the truncated RIP, albeit at fairly low concentration. 5 ng of either PAPc purified from transgenic plants or wild-type PAP was applied exogenously to tobacco leaves. A similar reduction in PVX virus-related lesions was observed in both cases. In contrast the application of a non-transgenic extract or extract containing $PAP_{E176V}$ (active site mutant) resulted in no inhibition of viral symptoms. PVX symptoms and antigen levels were markedly reduced in PAPc expressing transgenic plants up to 21 days post infection. *In vitro* studies demonstrated that both Brome Mosaic Virus (BMV) RNAs and PVX RNA were prevented from being translated after treatment with PAP and PAPc [19]. These results demonstrated that the C-terminal sequences of PAP were critical for cytotoxicity, but not antiviral activity indicating that they can be dissociated [10].

## RIP-Mediated Modulation of Host Factors

There is growing evidence that related RIPs, such as PAP, can cause global mRNA changes that might relate to virus resistance. Two studies in transgenic plants expressing PAP described the up-regulation of certain pathogenesis-related proteins [108, 109]. In 1997, Smirnov *et al.*

determined that the signal for antiviral activity could be spread systemically in the plant [108]. They studied the mechanism of virus resistance by grafting wild-type tobacco plants on transgenic tobacco plants expressing PAP. The results indicated that PAP expression in transgenic rootstocks of grafted plants promoted viral resistance to infection in wild-type scions. In the rootstocks, PAP expression resulted in a slight increase in salicylic acid (SA) accumulation and pathogenesis-related (PR) protein synthesis. Interestingly, virus resistance was observed in the grafted scions in the absence of detectable levels of PAP, SA accumulation, and PR-protein synthesis. The enzymatic activity of PAP was required to promote virus resistance in systemic tissues, as transgenic plants expressing an active-site variant of PAP did not demonstrate antiviral activity [108]. In contrast to viral resistance, fungal resistance required PAP-mediated induction of PR proteins, [109]. These differences demonstrate the alternate endogenous pathways involved in pathogen defense. Similarly in plants expressing PAPII, the PR-protein, PR1 was constitutively expressed, yet no increase in salicylic acid levels was detected [61].

In 1999, Hudak *et al.* demonstrated that the binding of PAP to ribosomal protein L3 (RPL3) in yeast was required for the toxicity of PAP *in vivo* [23]. The ribosomes in cells expressing the *mak8-1* mutant allele of *RPL3* were neither depurinated nor were able to bind the RIP. However, co-immunoprecipitation studies indicated that PAP bound directly to L3 or Mak8-1p *in vitro* that was not associated with the ribosome. These results demonstrated for the first time that a ribosomal protein could provide a receptor site for a RIP and allow subsequent rRNA depurination. The resulting association with host factors, such as ribosomal proteins, might allow for global changes in ribosome metabolism.

In 2001, Sun *et al.* conducted microarray analysis to determine the genes affected by treatment with MAP30 in cells chronically infected with Kaposi's Sarcoma-Associated Virus (KSAV or HHV8) [110]. Human Herpes Virus 8 (HHV8) is etiologically linked to Kaposi's sarcoma (KS), a malignant condition most prevalent in AIDS patients. The researchers report that MAP30 inhibited malignant growth of HHV8 infected cells (BC-2) by affecting expression of viral and cellular genes required for their expansion and proliferation. Initially, they demonstrated an $ID_{50}$ value of 0.3 to 0.6 nM for viability of BC-2 cells with up to an 18-hour incubation with MAP30. They also demonstrated that the expression of viral-specific genes encoding v-cyclin D, v-interleukin-6 and vFLIP was inhibited. In addition to belonging to the only group of viral genes expressed during the latent phase of the HHV8 life cycle, these proteins are thought to be important for tumorigenesis. A pathway-specific cDNA microarray analysis of BC-2 cells indicated effects on several genes required for survival of virus infected cells, including those in the NFκβ and p53 signaling pathways. MAP30 was also suggested to promote cell death in virally infected cells by increasing the abundance of the pro-apoptotic genes caspase-3 and CRADD. Additionally, a decrease in several immunomodulatory cytokines was noted.

RIPs appear to require various host cofactors to achieve maximal activity. For example, gelonin was among the first RIPs shown to require a specific tRNA to enhance its translation inhibition activity [111]. Additionally, agrostin, barley RIP and PAP-S seemed to also require host tRNAs to upregulate ribosome depurination activity [112]. However, the impact of tRNA modulators on antiviral activity still needs to be carefully investigated. Recent evidence suggests that the expression of certain RIPs might actually be induced upon virus challenge. Girbes *et al.* demonstrated that beetin 27 and beetin 29, two single chain RIPs from sugarbeet, were only expressed in plants that were exposed to either of three different viruses or mediators of virus resistance (SA or $H_2O_2$) [113].

## RIP Activity on Nucleic Acid

The highly basic nature of plant RIPs [3] is thought to confer nucleic acid binding ability. In fact, PAP has an isoelectric point of around 9.5 [114], which actually facilitates its purification [25]. The difference between RIPs and other basic proteins, like histones, is the specificity with which the plant protein acts. Not all nucleic acids are targeted *in vivo*, and the site of action on various nucleic acid substrates is highly specific. An important caveat is in ascribing antiviral activity to a peptide without determining the nature of the physical interaction. This was evident in the studies described by Fink *et al.* [96] whereby the previously characterized anti-HIV activity of a RIP fragment was attributed to simply precipitation of the viral RNA by a highly basic protein.

Teltow *et al.* observed that HSV infection reduced host cell DNA synthesis, but not immediately [20]. From 5-8 hours p.i. in untreated cells, host cell DNA synthesis is reduced from greater than 90% to less than 60%. During this period, the effect of pre-incubation with PAP was further reduction of DNA synthesis by an additional 40% to 25%. PAP's effect of reducing DNA synthesis was targeted specifically to HSV DNA. CsCl density centrifugation was used to separate viral from host DNA labeled with [*methyl*-$^3$H]thymidine. Compared to infected cells not treated with PAP, cells pretreated with PAP showed a 90% reduction in viral DNA synthesis. In contrast a nominal reduction in host DNA synthesis was demonstrated.

Studies on other RIPs by Lee-Huang *et al.* [79] demonstrated that both MAP30 and GAP31 were able to convert supercoiled plasmid DNA into relaxed forms. Additionally, the integrase-mediated nicking of supercoiled DNA was repairable by DNA gyrase while the RIP-mediated damage was not. In 2000, Au *et al.* [18], published evidence that luffin, saporin, trichosanthin, β-momorcharin and gelonin were able to specifically inhibit the integration step of HIV-1 infection without significant inhibition of the RT activity. In 1999, Wang *et al.* published the solution structure of MAP30 and showed that it folds very similarly to ricin and other RIPs [115]. It was also shown that the HIV-1 LTR DNA binds directly into the active-site cleft in a much the same way that HIV-1 integrase binds the viral DNA. It was demonstrated that the RIP was a DNA glycosylase/apurinic lyase (DGAL). MAP30 covalently binds to either supercoiled DNA or HIV-1 LTR upon removal of an adenine. They demonstrated that $Zn^{2+}$ and $Mn^{2+}$ aid in binding of negatively charged nucleic acids into

the active-site cleft. They also determined that the solution structure requires depurination of rRNA and DNA to occur in the same active-site pocket. However, the apurinic lyase activity on depurinated dsDNA occurs via nucleophilic attack by a neighboring lysine side chain: K195 for MAP30, which is homologous to K232 for PAP, and K200 for gelonin.

More recently, other RIPs have been shown to depurinate not only the S/R loop of rRNA but also deadenylate adenine-containing polynucleotides [116, 117], single-stranded DNA [118], and double-stranded DNA [119]. In 1994, saporins, from the common weed Soapwort, was the first RIP described to remove adenines from RNA and tRNA substrates [116]. Saporin-L1, -L2, and -R2 were characterized as "polynucleotide:adenosine glycosidases" or PNAG. The results indicated for the first time a possible action of RIPs on the viral genome during infection of cells. Forty-nine other type I RIPs were shown to not possess PNAG activity. In 2000 [120], PNAG was reported to be the *only* activity of gelonin, momordin-I, PAP-S, and saporin-S6 on DNA. Therefore, the activities of linearization, topological changes, and guanosine removal were discounted for at least these four RIPs. The researchers used scanning force microscopy to demonstrate that saporin-S6 bound extensively yet variably to plasmid DNA without evidence of fragmentation.

Rajamohan *et al.* [21] described the depurination of HIV-1 and TMV RNA by PAP. Specifically, they demonstrated that PAP, PAPII, and PAPIII could cause concentration-dependent depurination of RNA from HIV-1, TMV, and bacteriophage MS 2. They showed that although PAPII and PAPIII were more effective than PAP in depurinating viral RNA, all three PAP species could depurinate non-viral mouse RNA and inhibit cell-free protein synthesis. The depurination of viral RNA was not detected with the translation inhibitor RTA. These studies indicated that the antiviral activity of PAP might be related to the depurination of viral RNA.

In 2003, Uckun *et al.* used computer-aided molecular modeling to determine that the binding of viral RNA occurred differently than that of rRNA [121]. The active site cleft was differently affected in either case, thereby providing the ability to generate mutations of PAP that favor viral RNA binding over rRNA depurination. One of the double-alanine substitution mutants, termed FLP-102 (K151A and I152A) was 331-fold less toxic for inhibition of cell-free translation than wild-type PAP with an $ID_{50}$ value around 30 nM. Although FLP-102 bound to ribosomes through association with L3, the mutant RIP did not cause appreciable depurination and purine release. FLP-102 was 10-fold less active in removal of adenines and guanines from *E. coli* rRNA compared with wild-type PAP, yet 3 to 4 times more active on HIV-1 RNA. Compared to wild-type PAP, FLP-102 was almost 15 times more potent on inhibition of HIV-1 replication. In contrast, the antiviral activity was specific for HIV-1 and not effective against ECHO virus, CMV, or Respiratory Syncytial Virus (RSV). The toxicity of the mutant PAP in mice at doses as high as 8.2 mg/kg was also dramatically diminished. FLP-102 was as effective as a combination of AZT and 3TC (Nucleoside Reverse Transcriptase Inhibitors (NRTI)), in a mouse model of NRTI-resistant, HIV-1 infection, however, wild-type PAP was not.

Evidence is currently mounting on the ability of PAP to specifically bind to and destabilize mRNA. In 2000, Hudak *et al.* demonstrated that PAP could specifically depurinate capped mRNA *in vitro* [19]. Capped mRNA that was treated with PAP was unable to be translated after separation from the RIP. PAP-treated uncapped mRNA, in contrast, was not depurinated and could be translated indicating that PAP recognized the appropriate mRNA substrate through the 5' cap structure. Both PAP and PAPc inhibited translation of capped BMV and PVX viral RNAs. The translation inhibition of both capped luciferase mRNA and BMV vRNAs were overcome by addition of increasing concentrations of the cap analog, $m^7$GpppG. Translation of the uncapped luciferase mRNA, however, was not affected by the cap analog [19]. In 2002, Hudak *et al.* demonstrated that PAP specifically bound to the $m^7$Gppp cap structure of mRNA but did not remove it [12]. PAP bound very tightly to $m^7$Gppp-Sepharose resin with an affinity that appeared to exceed that of eIF4E, the eukaryotic initiation factor 4E responsible for binding to cap. Modeling of $m^7$Gppp cap structure into the active site of PAP indicated that the cap structure would bind to the active site in a similar manner to guanine. Insertion of $m^7$GMP into the active site of PAP resulted in a minor shift in the molecule compared with GMP, not expected to change the interaction of the guanine ring with the active site residues Tyr 72 and Tyr 123 (Fig. **2**). Because the same residues bind the adenine of the S/R loop of rRNA, increasing concentrations of the cap analog successfully prevented ribosome depurination in a dose-dependent manner. The affinity of PAP for capped mRNA compared to rRNA was shown to be nearly similar, with only a four-fold higher affinity for the rRNA, indicating that the capped RNA would compete with the S/R loop of rRNA for binding to PAP [12].

These results were extended to RIPs other than PAP [40]. Saporin, and a RIP from *Mirabilis expansa* (ME1) were able to inhibit luciferase mRNA, TMV, and BMV viral RNA via cap-mediated binding and subsequent RNA depurination. Interestingly, no depurination of capped alfalfa mosaic virus was observed, indicating that the possession of a 5' cap structure was not sufficient for depurination by these RIPs. Additionally, uncapped tomato bushy stunt virus, satellite panicum mosaic virus, and uncapped RNA containing the poliovirus internal ribosome entry site (IRES) were not depurinated at multiple sites throughout their sequence. However, the translation of these RNAs was inhibited after they are separated from the RIPs, indicating that damage other than depurination might contribute to antiviral activity.

In 2002, Parikh *et al.* demonstrated that PAP could specifically destabilize its own mRNA *in vivo* [122]. Yeast expressing PAP showed a reduction in translation and a subsequent decrease in PAP mRNA abundance. These studies complemented the *in vitro* findings that PAP can target and depurinate capped mRNA. However, the activity on PAP mRNA appeared to be highly specific as various endogenous yeast mRNAs were not destabilized. An increase in PAP mRNA abundance prior to its destabilization was attributed to the inhibition of translation elongation, which

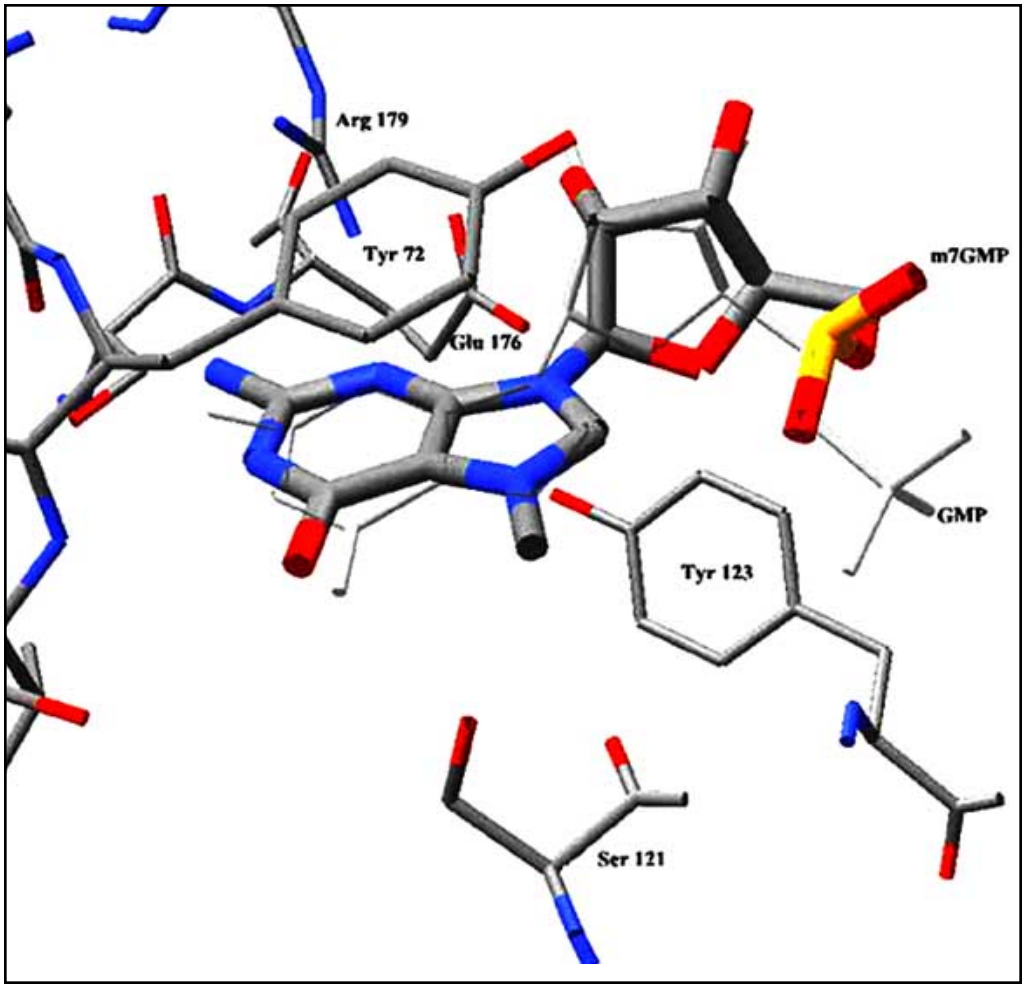


**Fig (2).** Structural model of $m^7GMP$ binding to PAP. Crystallographically determined PAP active site is shown with energetically minimized $m^7GMP$ (colored) and GMP (light gray) modeled. Red = Oxygen, Blue = Nitrogen, and Gray = Carbon. Adapted from Hudak *et al., RNA* **8**:1148-1159, 2002, with permission.

is known to stabilize mRNAs. One clue into the *in vivo* function of the RIP comes from the correlation between mRNA destabilization and ribosome depurination. A novel dual-primer extension assay to accurately measure ribosome depurination *in vivo* was developed [122]. The results indicated that PAP could depurinate ribosomes *in trans* and that maximum depurination coincided with maximal mRNA abundance. In combination with the previous studies indicating that PAP has similar affinity for rRNA and mRNA, a model on substrate selection was presented. Specifically, at low concentrations of PAP and mRNA substrate, the ribosome is primarily targeted by PAP. However, as PAP production increases, coupled with increases in local mRNA concentration on ribosomes as a result of elongation inhibition, PAP switches from predominantly targeting ribosomes to targeting mRNA. As a result, translation continues to remain inhibited, yet the extent of rRNA depurination decreases. PAP mRNA levels decrease because PAP mRNA, rather than the rRNA is targeted for degradation under these conditions. A similar mechanism may be responsible for PAP-mediated inhibition of virus infection. For example, during TMV infection in tobacco plants, viral RNA can represent up to 75% of total nucleic acid in the cell without significantly affecting the rRNA pool [123]. During heightened vRNA production, PAP can target the vRNA without affecting host protein synthesis. These results help explain how RIPs such as PAP can inhibit virus replication and infection in cells without causing obvious cytotoxicity.

## USES OF RIPS IN MEDICINE

### RIPs as Immunotoxins

The application of antiviral RIPs to benefit mankind stands as the common theme of the research reviewed above. As viral pathogens affect both our health as well as the health of our food crops, engineering RIPs with minimal toxicity and maximal therapeutic effect would be an elegant solution. Some of the drawbacks of using antiviral proteins include modulating their toxicity so that only the infected cells are targeted by the RIP. One solution is to use RIPs with very high therapeutic indexes. Another effective solution, as explained above for PAP, has been conjugation of the RIP to monoclonal antibodies (see [124] for a review on how various immunotoxins might be used in the treatment of HIV-1). The localization of the RIP is then controlled by an epitope of choice. Targets can include viral

envelope glycoproteins or host cell antigens. The caveat in using virus-specific epitopes involves the high mutation rates generally observed by viral pathogens. Conserved regions are preferable for targeting over highly variable domains. Additionally, if the virus is not released from cells via budding and subsequent acquisition of an envelope (*e.g.* poliovirus), this strategy cannot be employed. An alternate method of targeting host cell antigens has been shown to be particularly effective against HIV-1. Drawing from these studies, it is important to determine the cell tropism of the particular virus, such as CD4+ T-cells. It is also critical that the natural function of a cellular receptor not be hindered. This can be accomplished by applying a minimal concentration of immunotoxin that would occupy very few available sites on the cell surface. The method of internalization is not well understood for receptors involved in signal transduction cascades, however, the close proximity of the RIP to infected cells combined with altered membrane permeability presumably aids in entry (see above), while concomitantly preventing toxicity to uninfected cells. Eventually, the efficacy of immunotoxin may be limited by the generation of neutralizing antibodies against the RIP moiety. Fortunately, many (if not all) plants that produce antiviral RIPs seem to express various isoforms of the proteins that differ greatly in immunogenicity. Another drawback that immunotoxins can prevent is the rapid elimination of circulating antiviral proteins. PAP immunoconjugates, for example, have a serum half-life at least 30 times longer than unconjugated PAP [125].

Besides PAP, both gelonin and ricin have effectively been conjugated to antibodies. Gelonin was found to be effective against pichinde virus, an arenavirus, when conjugated to hyperimmune polyclonal sera from rabbits challenged with pichinde virus [126]. Against the human pathogen HCMV, gelonin was conjugated to purified polyclonal human IgG and the immunotoxin was also effective in reducing translation rates specifically in HCMV-infected cells [127]. As these conclusions were drawn from *in vitro* testing, more work needs to be conducted in properly designed animal trials [128]. Ricin was conjugated to antibodies for several years [129-132]. The major limitation of ricin immunotoxins (as well as other RIPs) has been a pathology known as "vascular leak syndrome" where fluid fills the lungs and causes extreme morbidity in the patient [133-135]. Recently, Smallshaw and colleagues have shown that a single point mutation in ricin toxin can eliminate vascular leak syndrome without compromising the toxin's enzymatic action [136]. They identified ricin amino acids 74-76 (LDV) that act alone to induce caspase activation and apoptosis. This motif is shared by viral disintegrins, which disrupt the function of integrins [137]. These results contradicted the prevailing view that the toxin kills endothelial cells, as it kills target cells. Vitetta's group proposed that the mechanism of action might involve disrupting integrins. They tried to block the disintegrin function of ricin by making mutations in the LDV sequence and the neighboring amino acids and showed that a five-fold higher dose of one of these mutants (Asn 97 to Ala) could be administered to mice without killing them [136]. Even though ricin itself is not inherently antiviral, a similar strategy can be employed to reduce the toxicity of antiviral RIPs to endothelial cells.

RIP containing immunotoxins, once described as "magic bullets", have not yet reached their full potential due to their side effects. In spite of recent progress in defining various activities of RIPs, the relationship between ribosome depurination, cytotoxicity and antiviral activity is still not clear for many toxins and immunotoxins. Recent evidence indicates that different portions of the molecules may be responsible for these activities. RIPs have been shown to depurinate rRNA, as well as other nucleic acid substrates. However, details of their interactions with the host translation apparatus and the basis for their selectivity are not well understood. Further studies that address how these proteins enter cells, how they inhibit protein synthesis and how they exert their cytotoxic and antiviral effects will lead to the generation of immunotoxins that are enhanced in targeted activity and yet greatly reduced in cytotoxicity. Furthermore, unraveling the mechanisms of antiviral activity and cytotoxicity of RIPs will allow researchers to extract the full therapeutic potential of these cytotoxic agents and develop more effective treatment protocols against viral infection and cancer.

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