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Concerted inhibitory activities of *Phyllanthus amarus* on HIV replication in vitro and ex vivo

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Abstract

Phyllanthus amarus derived preparations were previously shown to inhibit RT inhibitor-resistant HIV variants as efficiently as wild-type strains. The drugs target different steps of the HIV life cycle, thereby presenting multiple antiviral activities. Here we show that a water/alcohol extract blocks HIV-1 attachment and the HIV-1 enzymes integrase, reverse transcriptase and protease to different degrees. A gallotannin containing fraction and the isolated ellagitannins geraniin and corilagin were shown to be the most potent mediators of these antiviral activities. The *P. amarus* derived preparations blocked the interaction of HIV-1 gp120 with its primary cellular receptor CD4 at 50% inhibitory concentrations of 2.65 (water/alcohol extract) to 0.48 μg/ml (geraniin). Inhibition was also evident for the HIV-1 enzymes integrase (0.48–0.16 μg/ml), reverse transcriptase (8.17–2.53 μg/ml) and protease (21.80–6.28 μg/ml). In order to prove the in vivo relevance of these biological activities, plant material was administered orally to volunteers and a potent anti-HIV activity in blood could be demonstrated. Sera at a final concentration of 5% reduced HIV replication by more than 30%. These results support the conclusion that *P. amarus* has inhibitory effects on HIV not only in vitro but also in vivo.

Keywords: Phytomedicine; Plant extract; Tannin; Ex vivo blood test

1. Introduction

There is a globally acknowledged demand for a broader, safer and also cheaper repertoire for the treatment of human immunodeficiency virus (HIV) infection. Currently, the approved medication is primarily restricted to target two viral enzymes, the reverse transcriptase (RT) and the protease (PR). Potent combinations comprise at least two RT inhibitors and one PR inhibitor and succeed in maintaining a sustained suppression of virus replication to undetectable levels when measured in the patients′ peripheral blood (Collier et al., 1996; Gulick et al., 1997). Recent evidence, however, indicates that replication-competent virus resists therapy by resting in defined compartments such as lymph nodes (Wong et al., 1997; Furtado et al., 1999). In addition to evidence of continued viral replication in the presence of potent antiretroviral therapy, there are some more practical concerns including difficult adherence of patients to drug combinations, costs, and emergence of viruses resistant to one or more of these agents (Gulick et al., 1997; Yerly et al., 1999), often associated with therapeutic failure (Lucas et al., 1999). These issues highlight the need for anti-HIV agents targeted at additional replicative events.

A variety of natural products have been found to inhibit unique enzymes and proteins crucial to the life cycle of HIV including efficient intervention with the reverse transcription process, but also virus binding, the integrase or protease (for a review see Vlietinck et al. (1998), De Clercq (2000), Jung et al. (2000) and Cos et al. (2004)). Inhibition of HIV has been demonstrated for *P. amarus* among other *Phyllanthus* species (Euphorbiaceae) in vitro (Qian-Cutrone et al., 1996; Ogata et al., 1992), and was previously confirmed also for drug-resistant HIV strains (Notka et al., 2003). It was shown

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that the overall inhibition was composed of antiviral activity targeting different steps of the viral life cycle. Virus internalization was found to be the most relevant target, but it was also evident that intracellular replication events were, to a minor degree, involved in inhibition. As a first mode of action inhibition of the HIV-1 RT was demonstrated in vitro, and kinetic analyses revealed a noncompetitive mechanism of inhibition with respect to the dNTP substrate and a competitive mechanism of inhibition with respect to the poly(rA)-oligo(dT) primer/template, suggesting that *P. amarus* compounds interfere with the primer/template recognition site of the RT. Here we focused on the remaining HIV enzymes integrase and protease, and present new data to clarify the process by which the virus uptake is blocked. Further, an initial experiment to prove that *P. amarus* compounds present in the serum of human volunteers are able to inhibit HIV infection of $CD4^+$ HeLa cells in vitro is described.

2. Materials and methods

2.1. Phyllanthus amarus extracts, fractions and substances

The *P. amarus*-derived test substances were obtained from the Institute of Pharmacy at the Ludwig Maximilians University, Munich, Germany and are listed in Table 1. The W/E extract and the gallotannin fraction were derived from different stages in purification of extracts of *P. amarus* leaves. The gallotannin fraction contains the total of gallotannins, mainly geraniin, corilagin, ellagic acid and brevifolin carboxylic acid and app. 10–15% flavonglycosides; it is approximately six times concentrated. Corilagin and geraniin were purified from *P. amarus* W/E extract. The contents of corilagin and geraniin in the W/E extract were 2.28 and 1.10%, respectively. HPLC analysis demonstrated a >95% purity for corilagin and geraniin. Powdered extracts, fractions and substances were usually reconstituted with phosphate-buffered saline (PBS) to give a final concentration of 1 mg/ml. For a comparative analysis of the different probes, all experiments were performed from one batch.

2.2. Assessment of inhibition of HIV-mediated cell fusion

Fusion of HIV-1-infected cells with uninfected cells in the presence of inhibitors was determined as described recently (Wild et al., 1992). In brief, 10^6 MT4 cells were inoculated with 100 μl of HIV-1 (HX10) (Ratner et al., 1987) stock solution for 2 h at 37 °C. Three milliliters of medium was added and cells were cultured for 3 days at 37 °C. After this cultivation period 10^4 infected MT4 cells were cocultured with 7×10^4 MOLT3 cells with or without drugs for 24 h at 37 °C. As a reference cells were incubated in the presence of a HIV-neutralizing antiserum derived from vaccinated and SHIV-challenged rhesus macaques (Notka et al., 1999). After 24 h cells were examined under the light microscope to determine the number of syncytia formed as a consequence of cell fusion of infected MT4 and uninfected MOLT3 cells.

The lymphoid cell lines (MT4 and MOLT3) were grown in RPMI medium supplemented with 10% fetal bovine serum (FCS) and penicillin/streptomycin.

2.3. HIV-1 gp120-CD4 binding test

The capacity of *P. amarus* compounds to interfere with the binding of HIV-1 gp120 to CD4 was determined using microtiter plates with immobilized recombinant human CD4 kindly provided by the NIBSC (MRC, UK). ELISA plates were coated with 100 ng/well soluble CD4 in 50 μl of 0.1 M carbonate buffer (pH 9.5) for 12 h at 4 °C. As a control, 100 ng/well BSA was also immobilized. Plates were washed five times with 200 μl washing buffer (PBS pH 7.4, 0.05%

Table 1
In vitro activities on HIV-1 functions of *P. amarus* derived samples

P. amarus sample	IC_{50}[a] (μg/ml)			
	CD4-gp120 binding[b]	Reverse transcriptase[c]	Integrase[d]	Protease[e]
W/E extract	2.65 ± 0.44[f] (3)[g]	8.17 ± 1.73 (5)	0.48 ± 0.11 (3)	21.80 ± 3.66 (2)
Gallotannin fraction	1.36 ± 0.57 (3)	5.93 ± 1.89 (4)	0.42 ± 0.15 (3)	12.18 (1)
Geraniin	0.48 ± 0.05 (3)	2.53 ± 1.40 (4)	0.16 ± 0.07 (3)	6.28 ± 2.51 (3)
Corilagin	0.50 ± 0.27 (3)	6.24 ± 2.11 (3)	0.21 ± 0.05 (3)	n.d.

n.d.: not determined.

[a] Fifty percent inhibitory concentration.

[b] CD4-gp120 binding was assessed in an ELISA format using microtiter plates with immobilized recombinant human CD4 and a soluble monoclonal anti-gp120 antibody.

[c] A commercially available RT assay was used to determine the RT activity.

[d] Integrase activity was determined with a rapid microtiter assay using recombinant integrase, substrate oligonucleotides and a streptavidin/anti-digoxigenin ELISA module.

[e] A continuous spectrophotometric assay was used by monitoring the decrease in absorbance at 295 nm associated with the hydrolysis of a chromogenic substrate by a recombinant HIV protease.

[f] 1 S.D.

[g] Number of performed experiments (in duplicate).

Tween 20). Remaining binding capacities were blocked with 200 μl blocking buffer (PBS, 0.1% gelatine) for 1 h at room temperature and plates were washed five times. The coated proteins were incubated with 100 ng recombinant gp120 (MRC) and dilutions of test samples (10% final concentration, in 50 μl blocking buffer) for 1 h and washed five times. Bound gp120 was incubated with 100 μl monoclonal mouse anti-gp120-V3 antibody (diluted 1:2000 in PBS, MRC) for 1 h at 37 °C and washed five times. To each well 100 μl monoclonal anti-mouse HRP-conjugated antibody (diluted 1:2000 in PBS, Dako, Hamburg, Germany) were added and the plates were incubated for 1 h at 37 °C, and unbound antibodies were removed by 10 washes. Finally, the HRP-conjugated antibodies were incubated with 100 μl HRP-substrate (TMB, BD PharMingen, Heidelberg, Germany) for 15 min, the reaction was terminated with 100 μl 1N H_2SO_4 and the plate was read in an ELISA reader (492 nm).

2.4. Quantitative assessment of HIV-1 reverse transcriptase activity in the presence of different Phyllanthus test samples

A commercial RT assay (reverse transcriptase assay, colorimetric by Roche, Mannheim, Germany) was used to determine the inhibitory effect of different extract, fraction and substance concentrations on the HIV-1 reverse transcriptase. Recombinant HIV-1 RT (Roche, 1–2.5 mU) served as RT-reagent. The RT-reagent was incubated with different concentrations of drugs for 2 h and RT activity was measured according to the manufacturer's protocol.

2.5. Quantitative assessment of HIV-1 integrase activity in the presence of different Phyllanthus drugs

Inhibition of the HIV-1 integrase activity was determined with a rapid microtiter assay according to Hwang et al. (2000) using a streptavidin/anti-digoxigenin ELISA module (Roche). The reaction mix contained 312 nM (400 ng) integrase (kindly provided by the NIBSC (MRC), UK), 7 nM (6 ng) substrate oligonucleotide (5′-ACTGCTAGAG ATTTTCCACA CTGACTAAAA GGGTC (bio)-3′ and 5′-(DIG) GACCCTTTTA GTCAGTGTGG AAAATCTCTA GCA-3′, Geneart, Regensburg, Germany), 20 mM HEPES (pH 7.5), 5% PEG, 10 mM $MgCl_2$, 10 mM DTT, 0.1 mg/ml BSA and 10% DMSO in a final reaction volume of 40 μl. Integrase protein was diluted in 0.1 mg/ml BSA, 0.5 M NaCl, 5 mM EDTA and 20 mM HEPES (pH 7.5) prior to use. To test for inhibition of strand transfer, the standard reaction minus the DMSO was preincubated for 5 min at room temperature. Test chemicals, dissolved in 100% DMSO, were then added to the reaction. The reaction was carried out for 1 h at 37 °C. After the reactions were completed, mixtures were adjusted to 20 mM Tris–HCl (pH 8.0), 400 mM NaCl, 10 mM EDTA and 0.1 mg/ml sonicated salmon sperm DNA in a final volume of 100 μl. The samples were added to streptavidin-coupled microtiter wells, which were then agitated at 37 °C for 1 h. DNA was denatured and unbound DNA was removed by five washes (200 μl/well) with washing buffer (30 mM NaOH, 200 mM NaCl, 1 mM EDTA). The relative activity was determined using an anti-digoxigenin ELISA. Then, 150 μl of anti-digoxigenin-peroxidase (POD) Fab fragments were added to the wells and incubated for 1 h at 37 °C. Unbound anti-dig-POD Fab were removed by five washes with 300 ml/well PBS containing 0.1% Tween-20. Bound DNA was incubated with the peroxidase substrate ABTS until sufficient orange product accumulated. The absorbance of the samples was determined using a microtiter plate reader (405 nm) and was directly correlated to the level of integrase activity in the sample.

2.6. PR inhibition assay

The ability of *P. amarus* derived test samples to inhibit PR activity was determined in a continuous spectrophotometric assay by monitoring the decrease in absorbance at 295 nm associated with the hydrolysis of the chromogenic substrate Lys-Ala-Arg-Val-Nle-Phe(*p*-NO_2)-Glu-Ala-Nle-NH_2, as described recently (Paulus et al., 1999). Measurements were carried out on an Uvikon 930 spectrophotometer (Kontron Instruments, Munich, Germany) equipped with a stirring device and a thermostatted cell holder maintained at 25 °C using a quartz cuvette with 9.5 mm path length and a final volume of 1.8 ml. Assays were conducted at pH 5.0, in 0.1 M sodium acetate, 4 mM EDTA, and 5 mM dithiothreitol. Reactions were initiated by adding enzyme to a final concentration of 22 nM, and progress curves were recorded for 4 min, thereby collecting 600 data points. The initial rate was determined as the slope of the absorbance change during the linear phase of the reaction (up to 2 min) using the software package of the instrument. The concentration that inhibited substrate cleavage by 50% was designated the IC_{50}.

2.7. Infection of multinuclear activation of galactosidase indicator (MAGI) cells

This reporter system is based on a $CD4^+$ HeLa cell line harboring a β-galactosidase gene under the control of the HIV-1 LTR, leading to the expression of β-galactosidase after infection of the cell with HIV, reverse transcription of the RNA genome, integration of the provirus and consecutive production of the HIV Tat protein (*trans*activator of *t*ranscription). It was developed recently to generate an easy-to-use and efficient method for quantitative analyses of HIV infectivity on a cellular level (Kimpton and Emerman, 1992), and was applied with slight modifications. In brief, 1.5×10^4 MAGI cells/well were plated in 48-well culture plates and grown overnight. The next day 70 μl of HIV stock (HX10) or dilutions thereof were preincubated with 15 μl of serum (resulting in 5% final serum concentration) and 15 μl of FCS for 30 min at 37 °C. The medium in the culture plates was replaced by 200 μl of fresh medium, the preincubation mix was added and the cells were cultured

in a humidified atmosphere. Two days after inoculation, infected cells were detected by 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal) staining of cells expressing an endogenous β-galactosidase as a consequence of HIV infection. Cells were fixed by incubation with 0.2% glutaraldehyde and 1% formaldehyde in PBS for 5 min. Fixed cells were washed with PBS twice and overlaid with the staining solution (4 mM K-ferricyanide, 4 mM K-ferrocyanide, 2 mM $MgCl_2$ and 0.4 mg/ml X-gal) for 30 min at 37 °C. The number of blue cells was determined by microscopical observation.

MAGI cells (MRC, ARP055) (Kimpton and Emerman, 1992) were grown in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 5% FCS, 5% human serum and G418/hygromycin (Sigma-Aldrich) in a 5% CO_2 atmosphere at 37 °C.

2.8. Statistics and curve fitting

Statistical evaluations (Student's paired *t*-test, $n = 6$) were performed with Excel. Fifty percent inhibitory concentrations were calculated using commercially available software: CalcuSyn for Windows (Biosoft, Ferguson, MO, USA) according to Chou and Talalay (1981, 1984).

3. Results

3.1. Inhibition of virus entry is a consequence of compound interfering with the binding of the primary receptor whereas cell fusion is not affected

P. amarus-derived extracts and compounds have been demonstrated to very effectively prevent the internalization of HIV-1 into $CD4^+$ cells at concentrations <2.5 μg/ml (resulting in >60% inhibition) in cell culture (Notka et al., 2003). The exact mode of blocking this crucial event in the viral replication cycle has remained unclear, although an interference with the viral gp120 has been proposed for tannins (Nonaka et al., 1990; Weaver et al., 1992), which were shown to be a major part of the antiviral activity of *P. amarus* (Notka et al., 2003) (the chemical structures of corilagin and geraniin are depicted in Fig. 1). Interference of virus attachment by tannins has also been reported for other viruses, like herpes simplex virus (Fukuchi et al., 1989). To gain additional information on virus uptake, two central steps of these very early events, i.e. the binding to the primary receptor CD4 and the fusion of cell membranes, were analyzed in vitro in detail.

In order to investigate whether fusion is inhibited by *P. amarus* a cell fusion assay was performed. Fusion of HIV-1-infected MT4 cells with uninfected MOLT3 cells is a consequence of HIV-1 gp160 (expressed on infected MT4 cells) binding to the cellular CD4 receptor (on MOLT4 cells) and consecutive conformational changes in gp160. Fusion of the mentioned cells resulted in the formation of syncytia that can readily be detected in light microscopy (Wild et al., 1992). Agents that interfere with one of these very early steps can prevent formation of syncytia (e.g. peptide T-20, neutralizing antibodies, etc.). In this experiment ($n = 3$), 7×10^4 uninfected MOLT3 cells were cocultivated with 1×10^4 infected MT4 cells producing about 200 syncytia/10.000 MT4 cells. Addition of a neutralizing antiserum at a final dilution of 1:100 resulted in complete inhibition of syncytium formation. In contrast, the addition of the W/E extract at concentrations of 10 μg/ml and even 25 μg/ml to the culture, thereby exceeding concentrations already known to efficiently block virus uptake by more than 70%, by a factor of 10, could not prevent syncytium formation or at least reduce the number of syncytia (Fig. 2A). Since cell fusion was apparently not influenced by the compounds, we tested whether the preceding step, the binding of the HIV envelope protein gp120 to its cellular receptor CD4 was impaired.

Using an ELISA assay format, a clear, concentration-dependent inhibition of gp120 binding to CD4 could be shown for the W/E extract, the gallotannin fraction, and the purified gallotannins corilagin and geraniin (Fig. 2B). The BSA coated control reaction was always below background,

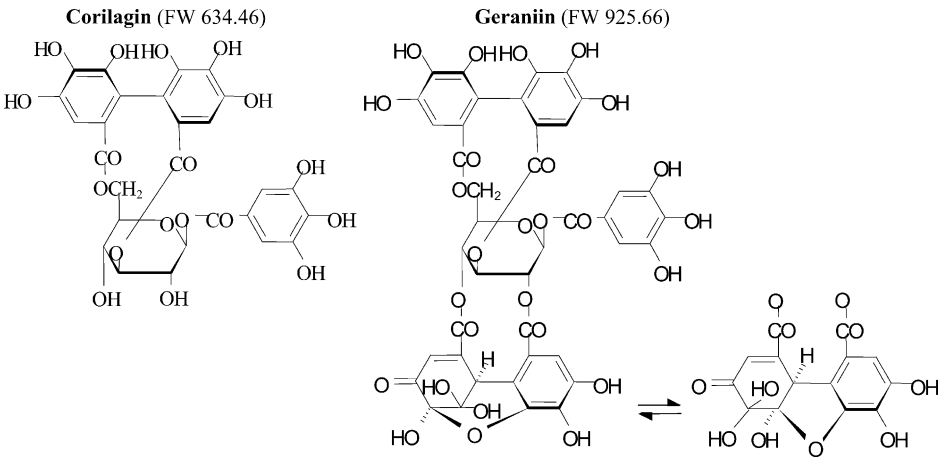

Fig. 1. Chemical structures of corilagin and geraniin.

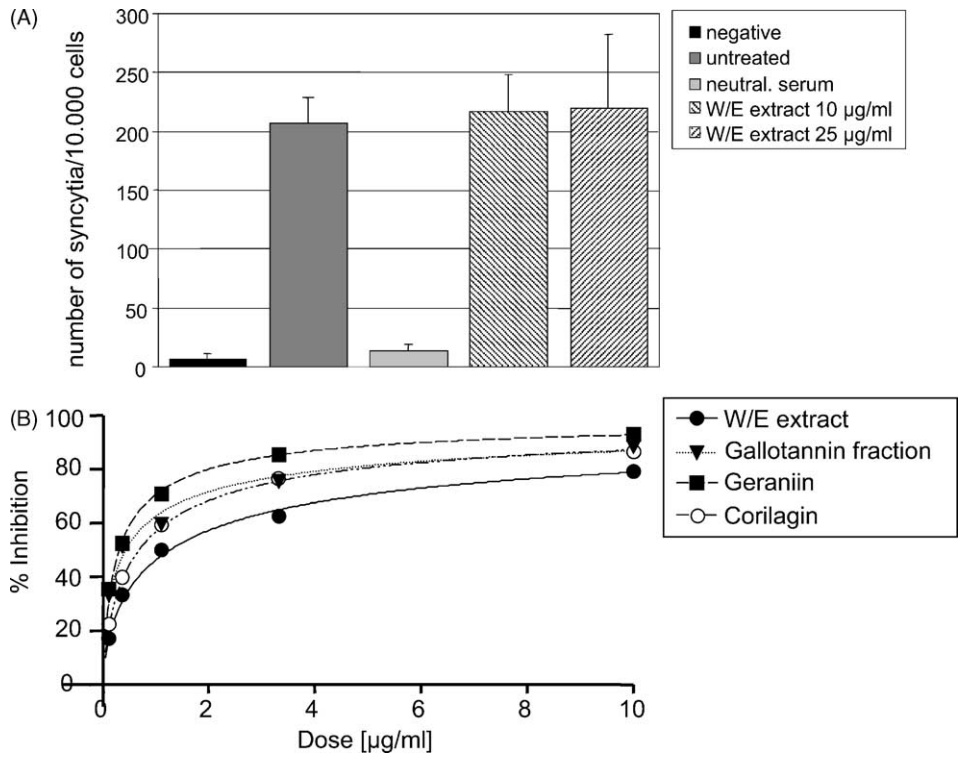

Fig. 2. Interference with virus entry. (A) Inhibition of HIV-1 gp160 mediated cell fusion. 7×10^4 MOLT3 cells were cocultivated with 10^4 uninfected (negative) or HIV-1-infected MT4 cells in the absence (untreated) and presence of inhibitors (as indicated) and the number of induced syncytia was determined for each sample after 24 h. A neutralizing serum (1:100 diluted) served as positive control and the W/E extract was used at concentrations of 10 and 25 μg/ml. Error bars represent 1 S.D. for triplicate samples. (B) Representative dose response curves for the inhibition of HIV-1 gp120 binding to immobilized CD4 by *P. amarus* samples. The inhibitory effects of W/E extract, gallotannin fraction, geraniin and corilagin are illustrated. Curves were generated from duplicate samples using the CalcuSyn software.

i.e. CD4 coated and no gp120 added, proving the specificity of the envelope protein–receptor binding (not shown). The calculated IC_{50} values of three independent tests, performed in duplicate are shown in Table 1. The IC_{50}'s were in the range of 2.65 (W/E extract) to 0.48 and 0.50 μg/ml for the purified gallotannins, with the gallotannin fraction having an intermediate value of 1.36 μg/ml. The order of potency (gallotannins > gallotannin fraction > W/E extract) matches exactly the concentration process with respect to the gallotannins starting from the W/E extract to the gallotannin fraction and the purified substances, and was constantly found in each performed test (see below).

3.2. The HIV-1 key enzymes RT, IN and PR were inhibited by P. amarus derivatives to different degrees

The inhibition of HIV RT by different *Phyllanthus* species and a variety of gallotannins has been reported earlier (el Mekkawy et al., 1995; Suthienkul et al., 1993; Nonaka et al., 1990; Kilkuskie et al., 1992; Ogata et al., 1992). We have recently provided evidence that *P. amarus* and gallotannins not only block virus uptake into target cells, but also exert antiviral activity during post-entry events (Notka et al., 2003). Accordingly, inhibition of RT was demonstrated in vitro by means of biochemical assays. However, due to the potent block of virus uptake, it has not been possible to specifically prove inhibition of the RT process intra-cellularly. Consequently, because the post-entry effects could not be correlated unequivocally with RT inhibition, the effects of the *P. amarus* derived test samples on the remaining HIV-1 enzymes integrase and protease were also determined in biochemical test systems. The comparison of the obtained inhibition strength (on the basis of calculated IC_{50} values) should add to the understanding of the antiviral capacity of *P. amarus*. In addition, in order to investigate the role of gallotannins, the inhibition of RT, IN and PR were investigated in parallel with the test samples W/E extract, gallotannin fraction, geraniin and corilagin. The resulting IC_{50}'s are listed in Table 1, and Fig. 3 graphically shows representative inhibition curves. The different HIV-1 enzymes as well as the HIV-1 gp120 were all inhibited by each of the test samples. However, a very clear tendency to differentiated inhibition potential became obvious. The IN was inhibited most efficiently, followed by gp120 binding (factor 2.5–5 less), RT (factor 15–30 less) and finally PR (factor 30–100 less). The above mentioned concentration effect was also confirmed, suggesting that the gallotannins account for most of the antiviral activity.

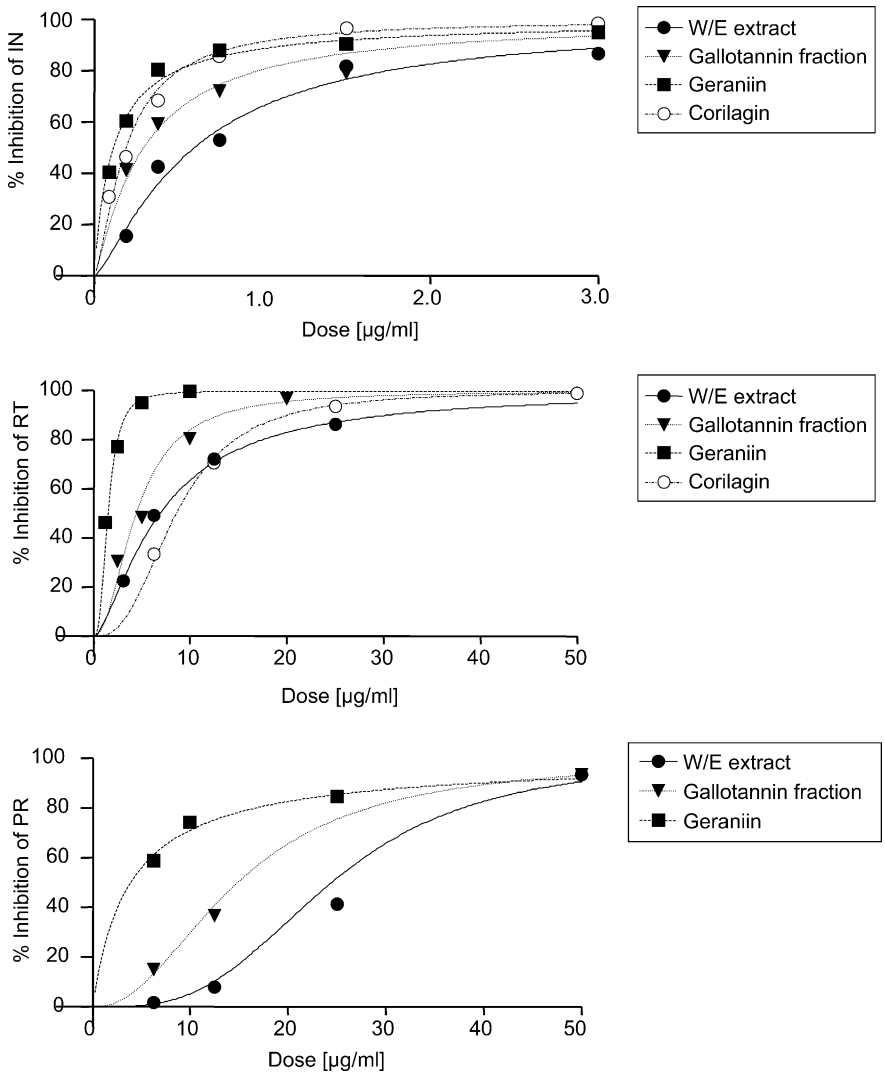

Fig. 3. Representative dose response curves for inhibition of HIV-1 enzymes by *P. amarus* derived test samples. The inhibitory effects of W/E extract, gallotannin fraction, geraniin and corilagin on HIV-1 IN (A), RT (B) and PR (C) are illustrated. Curves were generated from duplicate samples using the CalcuSyn software. Symbols are defined for each figure.

3.3. P. amarus induces antiviral effects in the serum of individuals after oral application

The presented data confirmed earlier findings by us and others showing that *P. amarus* contains compounds or complexes with strong potential to control HIV replication in cell culture and interfere with proteins of HIV-1 origin when tested in vitro. However, so far evidence for an effective in vivo activity is missing. In order to generate an affordable and fast preclinical model to evaluate potential anti-HIV effects in the blood of individuals, we applied (in a preliminary experimental setting) a method to test serum samples after oral application of *P. amarus* material for inhibition of virus replication using a $CD4^+$ reporter cell line (MAGI cells) (Kimpton and Emerman, 1992). MAGI cells were adapted to 5% human serum and inhibition of HIV replication by serum was determined accordingly. Three test persons received a single dose of either 450 mg lamivudine or 1200 mg W/E extract. Blood samples were collected prior to application and at 1 h intervals thereafter for 3 h. Serum was prepared from the samples and subjected to the MAGI assay in triplicate, respectively. The final serum concentration was 5%, because higher concentrations were not tolerated by the cells. The *P. amarus* treatment samples readily showed significant virus inhibition by 25% as early as 1 h post-application in one test person and reduction of virus replication by more than 30% in both persons 3 h after application. The lamivudine samples on the other hand reduced infection significantly only after 3 h and to a much lesser extent (17%). Fig. 4 graphically represents the results of two independent MAGI tests performed in triplicates. Significant inhibition with respect to the pre-application sample is indicated by an asterisk ($P < 0.005$).

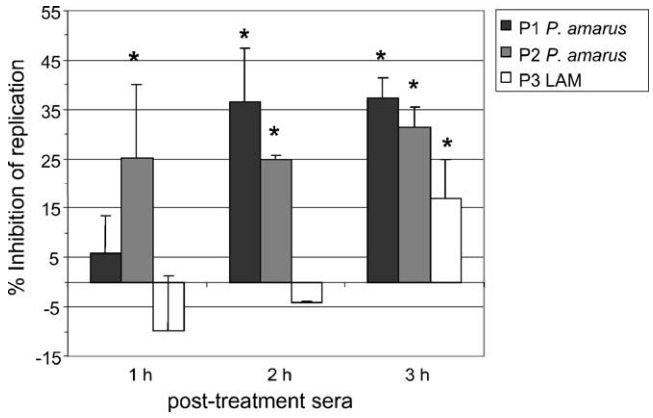

Fig. 4. Inhibition of HIV-1 replication in the presence of serum derived from volunteers after single dose oral drug application. Volunteers received either 1200 mg *P. amarus* material (P1 and P2) or 450 mg lamivudine (P3), and blood was collected before, and 1, 2 and 3 h after treatment. MAGI cells were inoculated with HIV-1 in the presence of pre- and post-treatment serum (1, 2 and 3 h post-administration, as indicated). Percent inhibition was calculated with respect to the value of the pre-treatment serum of each person. Error bars represent 1 S.D. of two independent experiments performed in triplicates. *: $P < 0.005$ by Student's paired *t*-test.

4. Discussion

The medicinal plant *P. amarus* has not only a recorded use in folk medicine for centuries, but has been shown to have multiple beneficial biological effects on a scientific level. Antioxidant, antitumor, anticarcinogenic and antiinflammatory potential have been attributed to *P. amarus* (Sripanidkulchai et al., 2002; Raphael et al., 2002; Rajeshkumar et al., 2002; Kiemer et al., 2003). A more relevant antiviral activity concerns the reduction of viral load in woodchuck hepatitis B virus (WBV)-infected woodchucks and in individuals suffering from viral hepatitis (Venkateswaran et al., 1987; Thyagarajan et al., 1988, 1990). At least two targets, the viral polymerase and the HBV enhancer I element, have been identified in vitro (Lee et al., 1996; Ott et al., 1997), although, neither the relevant target for the in vivo activity nor the effective compound(s) were defined. Nevertheless, the therapeutic benefit confirmed the inhibitory potential found in preclinical investigations. We have presented a detailed analysis of in vitro activities of *P. amarus* on defined targets representing the key steps of the HIV-1 replication cycle and first evidence for a potential anti-HIV activity in individuals.

The antiviral activities of different *Phyllanthus* species has been reported before, and *P. amarus* among other *Phyllanthus* species, was shown to inhibit HIV replication (Qian-Cutrone et al., 1996; Ogata et al., 1992) and to block retroviral RTs (el Mekkawy et al., 1995; Suthienkul et al., 1993; Ogata et al., 1992). Other HIV enzymes have so far not been subjected to a screening for inhibition by *P. amarus*. This encouraged us to evaluate additional targets and compare the obtained inhibition potentials on the basis of 50% inhibitory concentrations (IC_{50}'s). Since all of the presented data were obtained from artificial test systems, we cannot expect that the in vivo situation is pictured accurately; however, a comparison of the antiviral effects on the basis of a defined variable should give a reasonable rating. It turned out that the known target, the RT, was not the most efficient one. Instead the IN and the gp120/CD4 binding were inhibited at substantially lower concentrations (see Table 1). Inhibition of the protease could be demonstrated, albeit at very high concentrations suggesting that it has no in vivo relevance as a potential target.

Recently, we argued that the prevention of virus entry has the highest in vivo relevance, based on cell culture experiments, showing that virus entry is efficiently blocked (Notka et al., 2003). Here we further specified the underlying mechanism by demonstrating that inhibition of virus entry is specifically associated with an interference of virus envelope protein gp120 binding to the cellular receptor CD4 (although we do not know whether the co-receptors or the HIV gp41 are also involved). Fusion is not impaired. In contrast to neutralizing antibodies or soluble forms of CD4 which were shown to inhibit cell fusion in this assay (Wild et al., 1994), *P. amarus* antiviral compounds interfere with virus attachment, but do not prevent virus–cell fusion. Similar observations have been reported by others (Salzwedel et al., 1999), showing a nearly complete inhibition of virus entry while having only a limited effect on syncytium formation. The concentration of Env protein on the surface of cells and of virus is different and might be responsible for the observed discrepancy. The reduced concentration of Env protein on the virus surface is most likely sufficient for inhibition of virus entry by blocking Env binding.

We could show that post-entry events were to a smaller degree involved in the overall inhibition by *P. amarus* (Notka et al., 2003). In view of the herein obtained data, we speculate that the IN is a more relevant target than the RT. Considering that the observed activities, although demonstrated in highly artificial systems, do have in vivo relevance, *P. amarus* extracts may represent a well balanced combination of antiviral activities with recorded tolerance in human trials (Liu et al., 2001). Supportive data were obtained from studies for selection of drug-resistant HIV variants. After 12 weeks of virus replication using MT4 cells and sub-optimal inhibitor concentrations (W/E extract at a concentration of 1 μg/ml), we were not able to rescue a drug-resistant virus variant (data not shown).

A series of extracts, fractions and substances derived from *P. amarus* were tested so far. Repeatedly it was observed that gallotannins, especially corilagin and geraniin, are very potent inhibitors in all test systems performed, suggesting that gallotannins participate to a high degree in *P. amarus*' antiviral activity. However, the role of gallotannins in bioactivity testing has been the subject of conjecture (for a review, see Chung et al. (1998)). Tannins are a group of secondary metabolites ubiquitous in plants (Haslam, 1996), that have been shown to inhibit reverse transcriptase (Tan et al., 1991; Kakiuchi et al., 1985), DNA polymerases of cellular and viral (Epstein-Barr virus) origin (Nakane et al.,

1990; Xu et al., 2000; Liu et al., 1999), DNA topoisomerase I and II (Kashiwada et al., 1993), and the binding of HIV-1 gp120/CD4 T-cell receptor (Collins et al., 1997; Tan et al., 1991; Houghton, 1996; Weaver et al., 1992). Further, it was repeatedly shown that most polyphenolics are inhibitory to HIV-1 IN (Fesen et al., 1994; Eich et al., 1996). Removal of tannins from crude herb extracts by passing through a polyamide resin mini-column resulted in an extreme reduction of enzyme inhibition and indicated that the anti-HIV-1 IN and RT activity in most of the crude herb extracts was largely due to tannins or other polyphenolics (Au et al., 2001; Notka et al., 2003). Since these substances showed high affinity for proteins and affected a number of biochemical reactions, tannins are generally considered as unselective inhibitors. On the other side, a number of bioactivities have been attributed to tannins. And although considered rather unspecific, the high potential to bind proteins has been demonstrated to result in specific in vitro activities, suggesting that there is some degree of selective binding sufficient for potential therapeutic use of tannins (Chung et al., 1998). In fact, a very differentiated interference of 16 receptor/radioligand interactions by 20 plant derived phenolic compounds has been demonstrated (Zhu et al., 1997). Some of the components tested, including geraniin, were selective for one or two receptors, leading the authors to the conclusion that inhibition of those receptor/ligand binding by phenolic compounds cannot be explained solely in terms of phenolic–protein binding, but involves some specificity, as has been shown earlier (Hagerman and Butler, 1981). These results represent a convincing example for a rather selective mode of protein binding, as we suggest it for the inhibition of HIV by tannins. It further supports our reasoning for a specific inhibition of HIV replication in cell culture merely on the basis of a specific interaction with the HIV envelope protein.

In order to support the relevance of in vitro tests for the clinical development of natural products, we tested whether *P. amarus* can induce a detectable anti-HIV activity in humans. To our knowledge, the only comparable ex vivo blood test was performed in order to investigate different applications of artemisinin derivatives, approved anti-malaria therapeutics, for their schizontocidal activity against *Plasmodium falsiparum* (Ubalee et al., 1999). Distinct degrees of ex vivo blood activities were reported demonstrating the close correlation of these blood tests and the clinical outcome. The results of the present study suggest that *P. amarus* after oral application indeed induced a time-dependent effect that was measurable in a reporter cell. An effect of more than 30% reduction of replication is rather high considering that the final concentration of the sera was limited to 5%. Up to now it is not clear, whether *P. amarus* constituents or possible metabolites account for this inhibition. The hypothesis of active metabolites gets support from two findings: First, the observed inhibition by serum is unexpectedly high compared to the inhibition of replication by the W/E extract. Second, HPLC analyses of sera after application of *P. amarus* material revealed a time-dependent appearance of an additional, yet unidentified peak in the gallotannin elution region, overlapping with the time-dependent inhibition profile of the herein tested sera (unpublished data). However, until this peak substance is identified and tested, the exact mechanism of this in vivo activity cannot be completely clarified.

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