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# Inhibition of wild-type human immunodeficiency virus and reverse transcriptase inhibitor-resistant variants by *Phyllanthus amarus*

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**Abstract**

Substantial progress has been made in research on natural products which effectively inhibit HIV-1 replication. Many active compounds were isolated from traditionally used medicinal plants including *Phyllanthus* species. This study shows that aqueous as well as alcohol-based *Phyllanthus amarus* extracts potently inhibit HIV-1 replication in HeLa $CD4^+$ cells with 50% effective concentration ($EC_{50}$) values ranging from 0.9 to 7.6 μg/ml. A gallotannin enriched fraction showed enhanced activity (0.4 μg/ml), and the purified gallotannins geraniin and corilagin were most active (0.24 μg/ml). HIV-1 replication was also blocked in $CD4^+$ lymphoid cells with comparable $EC_{50}$ values. Applying a cell-based internalization assay, we could demonstrate 70–75% inhibition of virus uptake at concentrations of 2.5 μg/ml for the water/alcohol extract and geraniin. In addition, a concentration-dependent inhibition of HIV-1 reverse transcriptase (RT) could be demonstrated in vitro. The 50% inhibitory concentration ($IC_{50}$) values varied from 1.8 to 14.6 μg/ml. The ability to inhibit replication of a variety of RT inhibitor-resistant HIV-1 strains points to the potential of *P. amarus* extracts, as natural products, in the chemotherapy of HIV infections.


*Keywords:* HIV; Virus entry inhibitor; RT-resistant; *Phyllanthus amarus*; Plant extract; Tannin

## 1. Introduction

The concept of treating HIV infections by antagonizing the viral key enzymes reverse transcriptase and protease in a combination therapy has added considerably to the decline in the morbidity and mortality due to HIV infection in industrialized countries. However, there is a consent that targeting these two enzymes is insufficient to eradicate HIV-1 from infected people (Furtado et al., 1999). Therapeutic failure is a common feature especially in a long-term prospective and can be attributed to several factors. Patients' adherence (Lucas et al., 1999), severe metabolic side-effects and most importantly, rapidly emerging inhibitor-resistant virus variants (Yerly et al., 1999), have been regarded as the major problems. In view of these considerations, new classes of drugs suitable for long-term use that can supplement, or partially substitute, existing drugs and preferably act on new targets engaging novel molecular mechanisms, are definitely needed.

Currently, specific inhibitors of several stages of the viral life cycle, including viral attachment and entry, provirus integration and RNA packaging, are subjected to preclinical investigation or have already entered clinical trials. A substantial number of substances were derived from natural sources (for a review, see Jung et al., 2000 and De Clercq, 2000). The identification of such lead substances of plant origin repeatedly resulted from a broad screening of plants with a long history in folk medicine. Medicinal plants combine the advantages of being relatively non-toxic and hence more tolerable than rationally designed drugs. Furthermore, they represent an affordable and valuable source of pharmacologically active substances with sufficient availability through cultivation.

In this study, we focused on the human immunodeficiency virus (HIV) inhibitory activity of the medicinal plant *Phyllanthus amarus* (euphorbiaceae), originally used for the treatment of liver disease, mainly associated with jaundice, in India. *P. amarus* proved to have anti-HBV

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activities in vitro (Venkateswaran et al., 1987; Lee et al., 1996; Ott et al., 1997; Shead et al., 1992), and was shown to reduce the HBs antigen levels in HBV-infected individuals (Thyagarajan et al., 1988, 1990; Xin-Hua et al., 2001). However, these findings could not be confirmed in other clinical studies (Doshi et al., 1994; Wang et al., 1995; Leelarasamee et al., 1990). Since HBV shares with HIV the need to reversely transcribe genomic RNA into a DNA intermediate, the finding that *P. amarus* among other *Phyllanthus* species blocked the retroviral reverse transcriptase (RT) (el Mekkawy et al., 1995; Suthienkul et al., 1993; Ogata et al., 1992) and inhibited HIV replication (Qian-Cutrone et al., 1996; Ogata et al., 1992) was not too surprising. In view of the demand for new drugs we analyzed the precise anti-HIV potential of *P. amarus* and the molecular target(s) involved in its activity. In addition, we isolated active compounds and determined the resistance profile by testing their effect on a variety of RT inhibitor-resistant HIV strains, in order to evaluate a possible benefit for clinical use in terms of salvage therapy.

## 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. Corilagin and geraniin were purified from *P. amarus* W/E extract, the chemical structures are depicted in Fig. 1. The contents of corilagin and geraniin in the W/E extract were 2.28 and 1.10%, respectively. Powdered extracts, fractions and substances were usually reconstituted with phosphate-buffered saline (PBS) to give a final concentration of 1 mg/ml. The hexane and methanol extracts were first reconstituted in dimethylsulfoxide (DMSO) (Sigma–Aldrich, Deisenhofen, Germany) and diluted in PBS to give a final concentration of 1 mg/ml and 5% DMSO. Removal of gallotannins was performed as described recently using polyamide chromatography columns (Collins et al., 1998).

### 2.2. Reference substances

The approved HIV-1 RT inhibitor AZT (3′-azido-3′-deoxythymidine, FW 267.2) was purchased from Sigma–Aldrich as dry powder and reconstituted in PBS to a concentration of 360 μg/ml. AZT-triphosphate (AZT-TP, FW 507.2) was obtained from RetroTech (Munich, Germany) at a concentration of 1 mM. The HIV-1-neutralizing monoclonal antibody (mab) NEA 9305, which is directed against the HIV-1 V3 loop of the envelope protein, was purchased from NEN (Boston, USA). Heparin sulfate was used as a reference inhibitor of virus uptake. The powder was purchased from Sigma–Aldrich and reconstituted in PBS to a concentration of 1 mg/ml.

### 2.3. HIV virus strains

A selection of RT inhibitor-resistant HIV-1 strains and the HIV-2 strain ROD were kindly provided by the NIBSC (MRC, UK). The resistance profiles and the genetic changes of these strains are listed in Table 2. HIV stocks were generated by infection of $5 \times 10^6$ MT4 cells (in 500 μl medium)

Table 1
Test substances and biological activities

| Test sample | | HIV-1 RT | HIV-1 replication in MAGI | | |
|---|---|---|---|---|---|
| Sample | Description | $IC_{50}$[b] | $EC_{50}$[c] | $CC_{50}$[d] | TI[a] ($CC_{50}/EC_{50}$) |
| W extract | Water extract | 14.6 ± 6.3 μg/ml | 4.5 ± 1.7 μg/ml | >250 μg/ml | >55 |
| M extract | Methanol extract | 12.6 ± 4.0 μg/ml | 7.6 ± 1.1 μg/ml | >250 μg/ml | >32 |
| W/E extract | 50% water/ethanol extract | 8.9 ± 2.2 μg/ml | 0.9 ± 0.4 μg/ml | 466 μg/ml | 517 |
| Gall-free extract | Gallotannins were removed from W/E | n.d.[e] | 143.5 μg/ml | >1000 μg/ml | >6.9 |
| Gall-fraction | Partially purified gallotannins | 5.9 ± 1.6 μg/ml | 0.40 ± 0.05 μg/ml | 407 μg/ml | 1017 |
| Corilagin | Purified gallotannin | 5.9 ± 2.6 μg/ml (9.3 ± 4.1 μM) | 0.24 ± 0.04 μg/ml (0.38 ± 0.06 μM) | 265 μg/ml (417 μM) | 1104 |
| Geraniin | Purified gallotannin | 1.8 ± 0.5 μg/ml (1.9 ± 0.5 μM) | 0.24 ± 0.10 μg/ml (0.25 ± 0.1 μM) | 300 μg/ml (315 μM) | 1250 |
| AZT-TP/AZT | Reference | 1.0 ± 0.3 ng/ml (1.9 ± 0.5 nM) | 3.49 ± 0.19 ng/ml (13.06 ± 4.44 nM) | n.d. | n.d. |

[a] Therapeutic index.
[b] 50% inhibitory concentration.
[c] 50% effective concentration.
[d] 50% cytotoxic concentration.
[e] Not determined.

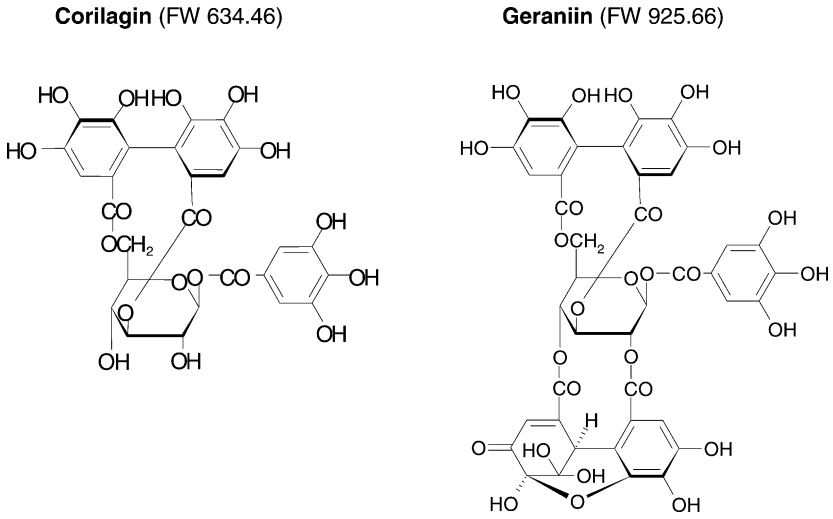


Fig. 1. Chemical structures of corilagin and geraniin.

with 500 μl of the virus strain. Cells were cultured in a volume of 20 ml for 5–7 days and supernatant was cleared of cell debris by low speed centrifugation. An equal volume of fetal calf serum (FCS) was added and 1 ml samples were stored at −80 °C.

### 2.4. Cell culture

MT4 T-lymphoid cells (MRC, ARP016) were grown in RPMI medium (Gibco BRL, Eggenstein, Germany) supplemented with 10% FCS (Gibco BRL) and Penicillin/Streptomycin (Sigma–Aldrich). MAGI cells (MRC, ARP055) (Kimpton and Emerman, 1992) were grown in Dulbecco's modified Eagle medium (Gibco BRL) supplemented with 10% FCS and G418/Hygromycin (Sigma–Aldrich). All cell lines were cultured in a 5% $CO_2$ atmosphere at 37 °C.

### 2.5. Infection of MT4 cells (T-cell assay)

The 50% cell culture infectious dose ($CCID_{50}$) of the reference HIV-1 (HX10) stock (Ratner et al., 1987) was determined to be $10^{-6.1}$/50 μl (about $2 \times 10^7$ infectious virions/ml). Fifty microliter of a $10^4$-fold dilution of this stock correspond to approximately 100 $CCID_{50}$ and is sufficient for infection. The HIV-1 virus stock was diluted $10^4$-fold and 50 μl of this dilution were preincubated with 10 μl of *P. amarus* samples at 37 °C. After 30 min,

Table 2
HIV-1 and -2 strains with different resistance profiles for RT Inhibitors

| | MRC no. | Phenotypic characteristics | Genotype | Reference |
|---|---|---|---|---|
| HIV-1 HX10 | | Wild type HIV-1 | wt | Ratner et al., 1987 |
| HIV-2 rod | EVA121 | Wild type HIV-2 | wt | Clavel et al., 1986 |
| HIV-1 nucleosidic RT inhibitors (NRTI) resistant | ARP141 | AZT resistant (RTMC), $IC_{50}$ for AZT around 100-fold of wt | RT: 67N, 70R, 215F, 219Q | Larder et al., 1989 |
| | ARP145 | 3TC/FTC resistant; $IC_{50}$ for 3TC/FTC around 1000-fold of wt | RT: 184V | Tisdale et al., 1993 |
| | ARP146 | Triple drug resistant (RTMDR1); coresistance to AZT, ddI and Nevirapine | RT: 41I, 74V, 106A, 215V | Larder et al., 1993 |
| | APR1006 | Resistance to carbocyclic nucleoside (1592U89), increased cross resistance to ddI, ddC and 3TC | RT: K65R, M184V | Tisdale et al., 1997 |
| HIV-1 non-nucleosidic RT inhibitors (NNRTI) resistant | ARP1010 | Resistance to a selection of NNRTI | RT: K103N | Balzarini et al., 1993a |
| | ARP1011 | Resistance to a selection of NNRTI | RT: Y188H | Balzarini et al., 1993b |
| | ARP1013 | Resistance to a selection of NNRTI | RT: Y181C | Balzarini et al., 1994 |
| | ARP1014 | Resistance to a selection of NNRTI | RT: G190A | Kleim et al., 1994 |
| | ARP1016 | Resistance to a selection of NNRTI | RT: L100I | Balzarini et al., 1993a |

$5 \times 10^4$ MT4 (40 μl) cells were added. After 2 h of incubation at 37 °C, 300 μl of medium containing each 30 μl of the test sample were added. Cells were cultured for 7 days and fresh medium (containing drugs) was added every other day. After this culture period, the HIV-1 p24 protein content of the supernatant was determined using a commercially available sandwich ELISA assay (NEN). Samples without detectable p24 contents (compared to the reference inhibitors and uninfected negative controls) were regarded as protected from infection. Alternatively, infection was monitored by quantifying the CPE (cytophathic effect) of infected cells using a XTT-based cytotox assay (Sigma–Aldrich).

### 2.6. Infection of multinuclear activation of galactosidase indicator (MAGI) cells

The MAGI reporter system was developed recently in order to generate an easy-to-use and efficient method for quantitative analyses of HIV infectivity on a cellular level (Kimpton and Emerman, 1992). In brief, $1.5 \times 10^4$ MAGI cells per well were plated in 48-well culture plates and grown overnight. The next day 70 μl of HIV stock or dilutions thereof were preincubated with 30 μl of inhibitor or PBS 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 infection, 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.

### 2.7. Quantification of cytotoxic effects

Cytotoxicity of the samples was tested with the XTT-based *In vitro Toxicology Assay Kit* (Sigma). Cells were plated in microtiter plates (tissue culture grade) in a final volume of 400 μl culture medium in a humidified atmosphere (37 °C, 5% $CO_2$). Cell concentrations of $5 \times 10^4$ and incubation times of 7 days were used for MT4 cells and concentrations of $1.5 \times 10^4$ and 2 days of cultivation were used for MAGI cells. After the incubation period, 20% (v/v) of XTT-solution was added to the cells. Cells were incubated for 2–8 h in a humidified atmosphere. The absorbance of the samples at 450 nm was measured against a background control as blank using a microtiter plate reader. Alternatively, cytotoxicity was measured by quantification of viable cells after trypan blue staining (exclusion method).

### 2.8. Virus internalization assay

Virus entry was monitored using an assay described recently (Saphire et al., 1999) with slight modifications. In brief, MAGI cells were preincubated at 4 °C for 60 min (in 100 μl), virus and inhibitor (100 μl) were added, the cells were incubated for 30 min at 4 °C and finally shifted to 37 °C for 2 h. The cells were then washed with 500 μl PBS five times to remove unbound virus, trypsinized for 5 min to remove all attached but not internalized virus, washed again with 500 μl PBS, and finally lysed with 100 μl PBS containing 0.5% NP-40. The HIV-1 p24 content of cell lysates was determined using the p24 sandwich ELISA (NEN). Heparin sulfate at a concentration of 100 μg/ml and an anti-HIV-1 gp120 monoclonal antibody (final dilution 1:40) served as controls.

### 2.9. Quantitative assessment of HIV-1 reverse transcriptase activity in the presence of different Phyllanthus compounds

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 (1–2.5 mU, Roche) served as the 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.

### 2.10. Assessment of the mechanism of RT inhibition

Michaelis-Menten kinetic tests were applied in order to evaluate the mode of RT inhibition. The HIV-1 reverse transcriptase activity at different RT substrate concentrations in the absence and presence of drugs was quantified using the *Screen RTA* (RetroTech; for dNTPs) and the *Reverse Transcriptase Assay, calorimetric* (Roche; for primer/template). From the obtained data, $V_{max}$ and $K_M$ were calculated using a Lineweaver-Burk diagram.

#### 2.10.1. Statistics and curve fitting

Statistical evaluations (*t*-test) were performed with SigmaBlot Version 5. The 50% inhibitory concentrations were calculated using the CalcuSyn program according to Chou and Talalay (1981, 1984).

## 3. Results

### 3.1. P. amarus extracts inhibit HIV replication in cell culture

For an initial assessment of a potential inhibitory effect on the HIV replication, the *P. amarus* test samples were screened in several dilutions in a HeLa-$CD4^+$-βGal cell

Table 3
Biological activities in MT4 cells

| Test sample | HIV-1 replication in MT4 | | |
|---|---|---|---|
| | $EC_{50}$[b] | $CC_{50}$[c] | TI[a] ($CC_{50}/EC_{50}$) |
| W/E extract | 1.15 ± 0.24 μg/ml | 30.72 ± 5.02 μg/ml | 26.81 |
| Geraniin | 0.46 ± 0.17 μg/ml (0.48 ± 0.18 μM) | 13.53 ± 2.30 μg/ml (14.20 ± 2.41 μM) | 29.27 |

[a] Therapeutic index.
[b] 50% effective concentration (three independent experiments performed in triplicate).
[c] 50% cytotoxic concentration (two independent experiments performed in triplicate).

based bioactivity assay (MAGI assay). In brief, expression of β-galactosidase in the MAGI reporter cell is dependent on infection with HIV strains. Infected cells can in turn be stained using X-gal or adequate substrates. Thus, infection can be quantified by the number of blue cells. Data were collected from four experiments performed in duplicates, fitted into dose–response curves, and the corresponding 50% effective concentration ($EC_{50}$) values were determined for all active samples (Table 1). With the exception of the extreme lipophillic hexane extract turning out to be totally ineffective (not shown), various preparations, extracted by water or alcohol, had effective concentrations consistently in the range of 1–8 μg/ml. Antiviral activity found in several chemical extracts suggested that the plant may contain more than one antiviral compound with different biochemical (solubility) properties. A number of identified substances were purified and subjected to the same test. An anti-HIV activity was so far only shown for geraniin and corilagin, both belonging to the gallotannin class, with an $EC_{50}$ of 0.24 μg/ml (corresponding to 0.25 and 0.38 μM, respectively). The results of the reporter cell assay could be confirmed using the $CD4^+$ lymphoid cells MT4, more closely mimicking natural infection (Table 3). The $EC_{50}$ values obtained for the various solutions in these two assay systems displayed the same tendency and proved to be comparable. However, $EC_{50}$ values determined in the MAGI system were consistently lower than in the MT4 cells, as exemplified for the W/E extract (0.87±0.38 μg/ml in the MAGI and 1.15±0.24 μg/ml in the T cell assay) and geraniin (0.24 ± 0.04 μg/ml in the MAGI and 0.46 ± 0.17 μg/ml in the T cell assay). These examples not only confirm the antiviral activity, but they also prove the reliability of the MAGI reporter assay. The latter is further supported by the results obtained for AZT (13.06±4.44 nM), which were in the range reported recently for inhibition of HIV-1 in HeLa $CD4^+$ cells (10 nM) (Larder et al., 1995).

In order to prove that the observed antiviral effects are due to specific interactions rather than to an overall toxicity, the cytotoxic effects of selected test samples were quantified. The indicator cells tolerated inhibitor quantities exceeding the effective concentrations by more than 500-fold (Table 1). However, cytotoxicity seems to be an obstacle in other cell lines like MT4 cells, since the 50% cytotoxic concentration ($CC_{50}$) values were rather low in these cells (<50 μg/ml for the methanol extract and W/E extract and <25 μg/ml for the water extract and geraniin, respectively). However, the calculated therapeutic indices (TI, ratio of $CC_{50}$ to $EC_{50}$) for W/E extract (30.72/1.15 μg/ml) and geraniin (13.53/0.46 μg/ml) of 26.8 and 29.3, respectively, demonstrated the specificity of inhibition (Table 3).

### 3.2. *P. amarus compounds prevent early replication events of the HIV-1 life cycle*

Inhibition of HIV infection by polyanionic compounds like sulfated polysaccharides was previously shown to involve steps concerning the uptake of virions into the target cells (Mitsuya et al., 1988; Baba et al., 1988), probably by blocking or altering the interaction of the viral envelope protein gp120 with the cellular receptor CD4. The same mechanism of inhibition was suggested for tannins (Ogata et al., 1992; Nonaka et al., 1990; Weaver et al., 1992), which in addition were shown to have anti-RT activity (for a review, see Vlietinck et al., 1998 and Matthee et al., 1999). Therefore, and in order to determine the molecular target of the inhibitors, we concentrated on an early period of the HIV replication. For this purpose the standard MAGI assay was modified as follows. Three experimental settings were performed, i.e. the presence of inhibitors was modified. In one set the inhibitors were present during the whole experiment (infection + cultivation, i.e. standard assay procedure). In the second set the inhibitors were present during infection only, i.e. cells were incubated for 2 h with HIV-1 and inhibitors, washed two times with PBS and fresh medium (without supplements) was added (cultivation without inhibitors). In the third set cells were cultivated with HIV-1 for 2 h (infection without inhibitors), washed two times with PBS and fresh medium including inhibitors was added (cultivation with inhibitors). W/E extract and geraniin were tested at a concentration of 5 μg/ml each, and the reference inhibitor AZT was tested at a concentration of 0.36 μg/ml. Three independent tests were performed in duplicate. The results of one representative experiment are depicted in Fig. 2A. The modified settings were compared to the standard test, and percentage reduction of inhibition was calculated. As can be seen from Fig. 2B, for W/E extract and geraniin removal after the initial infection step resulted in only minor loss of activity (11.7% for W/E extract and 5.2% for geraniin, respectively), while addition after infection substantially decreased inhibition (60.5 and 55.5% loss of activity for W/E extract and geraniin,

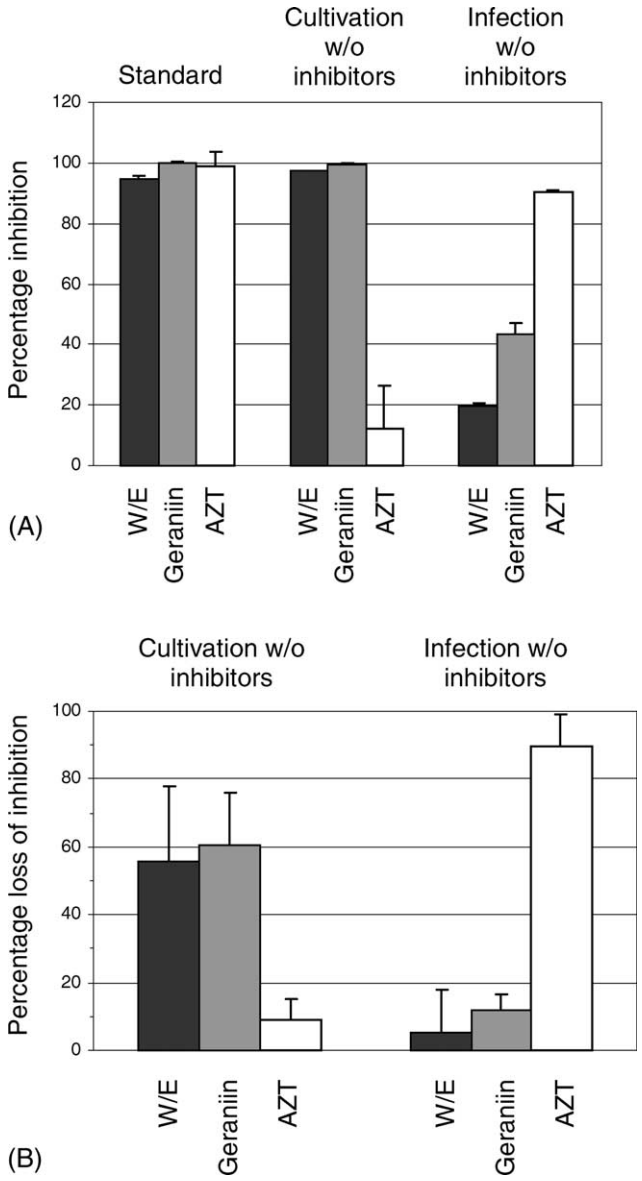


Fig. 2. Comparison of the inhibition of HIV replication in the presence and absence of inhibitor during defined periods. MAGI cells were infected without or in the presence of 5 μg/ml W/E extract (■), 5 μg/ml geraniin (▩) and 0.36 mg/ml AZT (□), either (i) during the whole experiment (standard), (ii) only during the initial infection period of 2 h (cultivation without inhibitors) or (iii) only after the initial infection (infection without inhibitors). (A) A representative experiment, performed in duplicate is shown. Bars represent percentage inhibition of replication given as reduction of the number of blue cells compared to PBS-treated controls. (B) The ratio of (percentage infection standard experiment) to (percentage infection modified experiment) was calculated and is expressed as percentage loss of activity. Error bars represent the standard deviation of three independent experiments performed in duplicate.

respectively). In contrast, for AZT loss of inhibition was observed when present only during infection (89.4%), and the decrease in activity was negligible when added after the initial infection (9.1%). Thus, W/E extract and geraniin were shown to be most potent during the very early step of infection. At this stage, in contrast to AZT, removal of *P. amarus* compounds after infection had only minor effects on inhibition. Addition of inhibitors after inoculating and carefully washing the cells in order to monitor steps following virus entry, demonstrated that not only entry events are blocked but further, intra-cellular functions are also impaired.

### 3.3. *P. amarus* compounds block virus uptake

The inhibition of HIV-1 uptake by W/E extract and geraniin were tested using a recently described internalization assay, that accounts for optimal conditions of virus uptake and removal of not internalized virus (Saphire et al., 1999). As a measure for internalized virus, total p24 of the infected cell lysates was quantified by a p24 capture ELISA and percentage reduction of p24 content compared to PBS-treated cells was used to monitor inhibition (Fig. 3). Heparin sulfate (93% inhibition in the MAGI assay at the given concentration of 100 μg/ml) and anti-gp120 mab (99% inhibition in the MAGI assay at the given dilution, 1:40) were used as positive controls. We could clearly demonstrate that the *P. amarus* samples block virus entry in MAGI cells in a concentration-dependent fashion (Fig. 3). Concentrations of 10 μg/ml resulted in inhibition of more than 80%. At concentrations of 2.5 μg/ml inhibition was still in the range of 70–75%, and concentrations as low as 0.63 μg/ml were sufficient to reduce virus uptake by 40%. Heparin sulfate was used as a reference inhibitor of virus entry. At a concentration of 100 μg/ml, heparin sulfate blocked virus internalization by 70–75%, comparable to 2.5 μg/ml W/E extract or geraniin. Thus, *P. amarus* compounds were about 40-fold more effective than heparin sulfate.

### 3.4. *P. amarus* compounds inhibit HIV reverse transcriptase

Inhibition of retroviral RTs by plant derived compounds and especially by *P. amarus* has been shown earlier. Accordingly, all *P. amarus* samples which inhibited HIV-1 replication were tested for RT inhibition in vitro, using recombinant HIV-1 RT. Each sample was tested in at least four independent experiments, performed in duplicate. The results demonstrated inhibition of the enzyme in the μg/ml range for all tested samples (Table 1). The 50% inhibitory concentration ($IC_{50}$) values were comparable to a variety of plant-derived extracts and substances which were shown to block retroviral RTs (Kakiuchi et al., 1985; Ogata et al., 1992; Collins et al., 1997; Mlinaric et al., 2000; Hnatyszyn et al., 1999; Chang et al., 1994). A progressive increase of the inhibition potential from W/E extract, to the gallotannin fraction (1.5-fold) and finally geraniin (4.9-fold) illustrates a concentration effect starting from a crude extract via partially purified gallotannins to the purified gallotannin geraniin. This effect was observed for the inhibition of viral replication in a very similar manner (2.25- and 3.75-fold for gallotannin fraction and geraniin, respectively), supporting the important role of gallotannins in the *P. amarus*

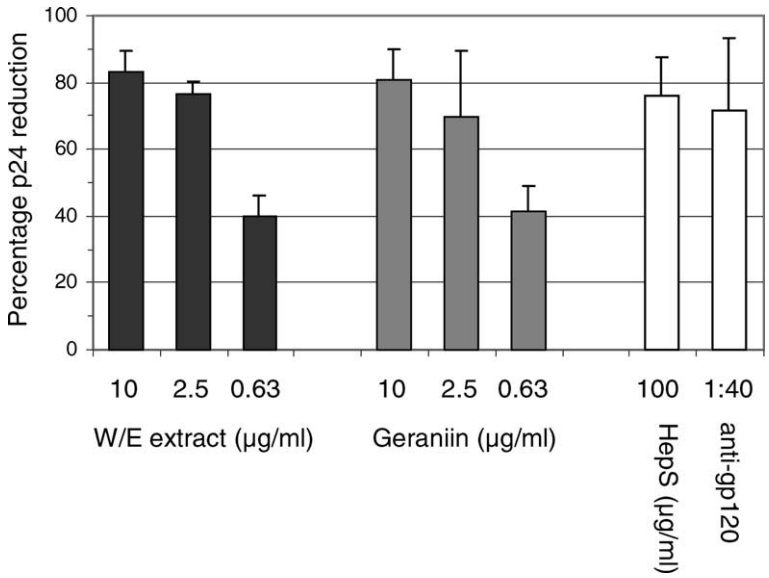


Fig. 3. Prevention of virus entry. MAGI cells were preincubated at 4 °C for 60 min (in 100 µl), virus and W/E extract (■) or geraniin (▩) or references (□) (at the indicated concentration) were added, the cells were incubated for 30 min at 4 °C and finally shifted to 37 °C for 2 h. Cells were then washed with PBS to remove unbound virus, trypsinized for 5 min to remove all attached but not internalized virus, washed again with PBS and finally lysed with PBS containing 0.5% NP-40. The HIV-1 p24 content of cell lysates was determined using the p24 sandwich ELISA. Heparin sulfate at a concentration of 100 µg/ml and an anti-HIV-1 gp120 monoclonal antibody (final dilution 1:40) served as controls. Error bars represent the standard deviation of two independent experiments performed in duplicate. (Bars represent percentage reduction of p24 compared to PBS-treated controls).

antiviral activity. Compared to the inhibition of replication, the RT inhibition by AZT-TP was substantially (6.9-fold) higher (Table 1), as expected for an effective RT inhibitor. In contrast, in vitro inhibition of the HIV-1 RT by the *P. amarus* samples was consistently reduced, resulting in inhibitory concentrations for geraniin (1.9 µM), that were about 1000-fold higher compared to AZT-TP. It is not clear whether the necessary physiological concentrations can be achieved in vivo, because bio-availability data have so far not been collected. Further, the intracellular concentrations of gallotannins have not been determined and accordingly, it is not known to what extent penetration and local concentration play a role.

### 3.5. *P. amarus* RT inhibitors compete with the primer/template substrate

In order to characterize the mode of action by which *P. amarus* inhibitors block the RT, we examined the kinetics (Michaelis-Menten) of the HIV-1 RT activity in the presence and absence of the extract and geraniin. As substrates for the RT, either poly(rA)-oligo(dT) as primer/template or dNTPs were added in increasing concentrations. Double reciprocal plots of the RT activity versus substrate concentration (Linweaver-Burk diagrams) revealed that inhibition of the enzyme is non-competitive with respect to dNTP as the variable substrate (Fig. 3C and E). The apparent $K_M$ is unaffected by the inhibitors and is approximately 26 ± 2 µM under the experimental conditions employed. Conversely, the apparent $V_{max}$ values for dNTP are suppressed as a function of increasing inhibitor concentration. In contrast, AZT-TP, known to compete with dNTP for the active site, was found to inhibit the RT in a competitive mode with respect to the NTPs (Fig. 4A). The kinetics of inhibition were also studied with various concentrations of poly(rA)-oligo(dT) as substrate. A mixed type with a clear tendency to competitive inhibition of primer/template binding was found for W/E extract and geraniin (Fig. 3D and F), while AZT-TP was non-competitive (Fig. 4B), and thus did not influence the primer/template binding. The apparent $V_{max}$ was decreased by the *P. amarus* compounds from 2.43 mU (untreated) to 1.80 mU (W/E extract and geraniin), regardless of the inhibitor, resulting in a 25.9% suppression. Conversely, the apparent $K_M$ values increased from 0.175 nM for the untreated enzyme to 0.605 nM for W/E extract (3.3-fold) and to 1.748 nM for geraniin (10-fold) under the experimental conditions of this representative experiment. These results suggest a dual inhibition mode involving to a smaller amount the independent binding of inhibitor and primer/template and as the superior activity a competition with the primer/template for the respective binding site.

### 3.6. RT inhibitor-resistant HIV-1 and -2 strains are sensitive to *P. amarus* compounds

Geraniin and W/E extract were shown to be active at two distinct sites of the HIV replication, implicating a lower incidence for the emergence of escape mutants. Thus, we were interested to see whether or not HIV strains of the NRTI (nucleoside RT inhibitor) and NNRTI (non-nucleoside RT inhibitor) resistance class were susceptible to geraniin and W/E extract. For this purpose, MAGI cells were infected

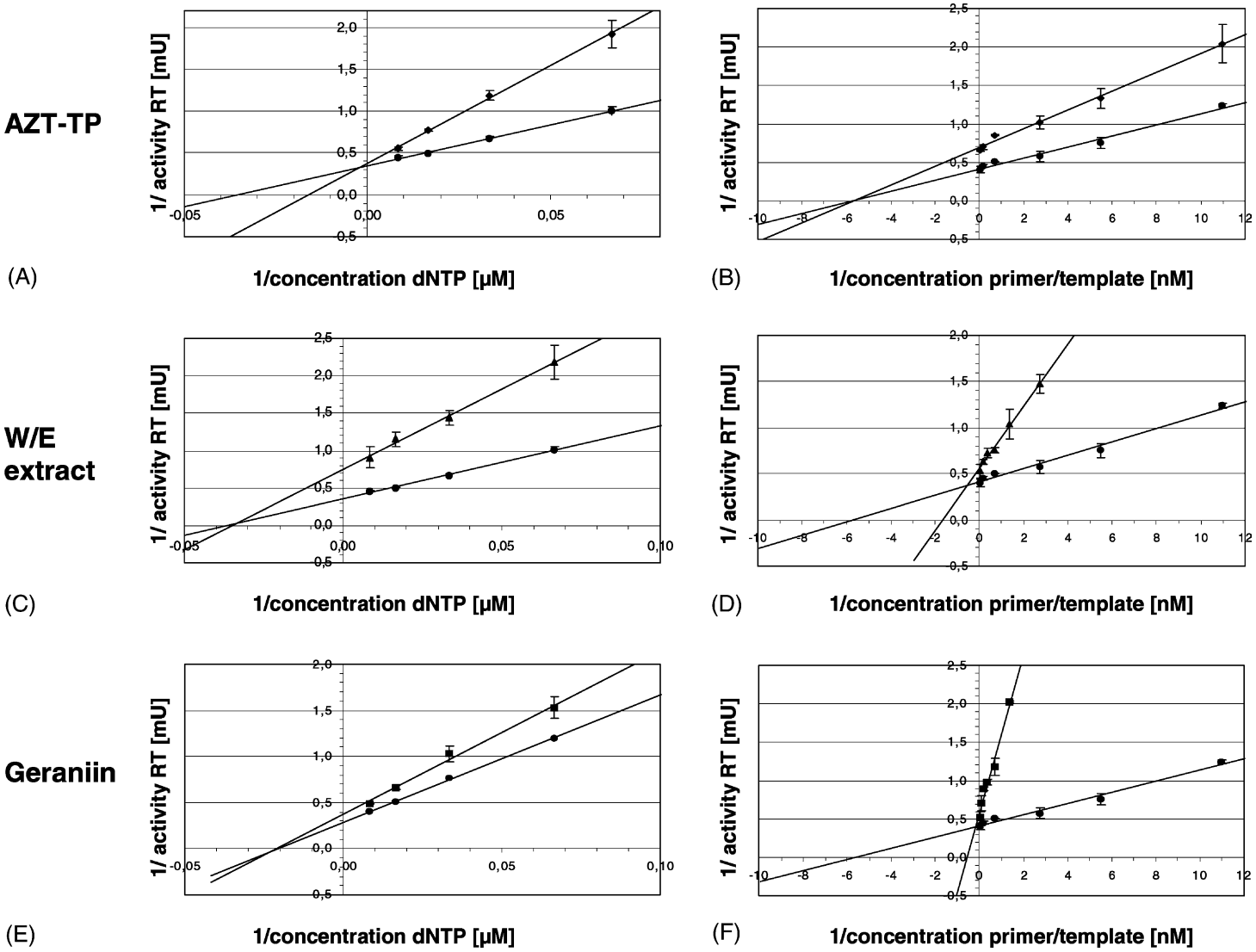


Fig. 4. Kinetic analysis of inhibition of the HIV-1 RT by *P. amarus* compounds. Representative double reciprocal plots of increasing concentrations of either dNTP (panels A, C and E) or poly(rA)-oligo(dT) (panels B, D and E) as a function of the RT activity (mU) by the HIV-1 RT. RT activity at different dNTP concentrations in the absence (●), or in the presence of (A) 2 nM AZT-TP (◆), (C) 2.5 µg/ml W/E extract (▲) or (E) 0.75 µM geraniin (■). RT activity at different primer/template concentrations in the absence (●) or in the presence of (B) 2 nM AZT-TP (◆), (D) 2 µg/ml W/E extract (▲) or (F) 1 µM geraniin (■). Tests were performed in triplicate. The plots were computer-generated by linear regression analyses, and the regression coefficient value (*r*) ranged between 0.968 and 0.998, indicating a high degree of linear relationship between the reciprocal activity of the enzymatic reaction and the reciprocal concentration of the substrate.

Table 4
Inhibition of HIV-2 and -1 wild-type and RT inhibitor-resistant strains

| HIV strain | Phenotype | Inhibition of replication ($EC_{50}$[a] (μg/ml)) | | | |
|---|---|---|---|---|---|
| | | W/E extract | Ratio[e] | Geraniin | Ratio |
| HIV-1 HX10 | wt[b] | 1.92 ± 0.28 | 1.00 | 0.74 ± 0.04 | 1.00 |
| HIV-2 ROD | | 1.38 ± 0.52 | 1.39 | 0.83 ± 0.13 | 0.89 |
| ARP141 | NRTI[c]-resistant | 1.02 ± 0.03 | 1.88 | 0.83 ± 0.24 | 0.89 |
| ARP145 | | 0.94 ± 0.20 | **2.04** | 0.95 ± 0.42 | 0.78 |
| ARP146 | | 1.25 ± 0.22 | 1.54 | 0.71 ± 0.11 | 1.04 |
| ARP1006 | | 2.39 ± 0.16 | 0.80 | 2.40 ± 0.63 | *0.31* |
| ARP1010 | NNRTI[d]-resistant | 1.27 ± 0.11 | 1.51 | 0.83 ± 0.10 | 0.89 |
| ARP1011 | | 1.17 ± 0.27 | 1.64 | 0.89 ± 0.15 | 0.83 |
| ARP1013 | | 1.43 ± 0.25 | 1.35 | 2.10 ± 0.33 | *0.35* |
| ARP1014 | | 1.13 ± 0.18 | 1.70 | 1.10 ± 0.14 | 0.67 |
| ARP1016 | | 2.00 ± 0.07 | 0.96 | 1.62 ± 0.01 | *0.46* |

[a] 50% effective concentration (two independent experiments performed in triplicates).
[b] Wild type.
[c] Nucleoside reverse transcription inhibitor.
[d] Non-nucleoside reverse transcription inhibitor.
[e] Ratio of $EC_{50}$ values HIV-1 HX10: respective strain; ratio > 2 (>2-fold increased effectivity) is indicated in bold; ratio < 0.5 (>2-fold decreased efficacy) is indicated in italics.

with a number of RTI-resistant HIV-1 strains and one HIV-2 strain (not susceptible to NNRTIs), and $EC_{50}$ values for geraniin and W/E extract were determined (Table 4). The HIV-2 strain ROD, the NRTI-resistant strains ARP141, ARP145 and ARP146, as well as the NNRTI-resistant strains ARP1010, ARP1011 and ARP1014 revealed elevated susceptibility to W/E extract when compared to the wt HIV-1 strain (HX10). Strains ARP1006, ARP1013 and ARP1016 demonstrated no significant loss of susceptibility when the $EC_{50}$ values were compared.

For geraniin the same tendency was observed. However, in contrast to W/E extract no enhanced susceptibility compared to the wild-type strain was seen. The HIV-2 strain ROD, the NRTI-resistant strains ARP141, ARP145 and ARP146, and the NNRTI-resistant strains ARP1010, ARP1011 and ARP1014 demonstrated no or only a minor decrease in the susceptibility to geraniin compared to the wt HIV-1 strain (see Table 4), with the lowest susceptibility for ARP145 and ARP1014. Strains ARP1006, ARP1013 and ARP1016 demonstrated minor resistance with more than two-fold increased $EC_{50}$ for ARP1006, ARP1016 and ARP1013. Taken together, most of the tested strains showed no signs of resistance to *P. amarus* samples. To the contrary, a number of strains were inhibited more strongly than the wild-type strain by the W/E extract. However, loss of susceptibility was apparent for three strains, irrespective of the inhibitor tested suggesting that changes in the RT at positions K65, M184 (ARP1006), Y181 (ARP1013) and L100 (ARP1016) to some degree influenced inhibition by *P. amarus* compounds.

## 4. Discussion

Although the antiviral activities of different *Phyllanthus* species have been reported earlier (Ogata et al., 1992; el Mekkawy et al., 1995; Suthienkul et al., 1993; Qian-Cutrone et al., 1996), potential therapeutic use in view of emerging resistant HIV virus variants has so far not been considered. Accordingly, we examined the antiviral activities of *P. amarus* with a major emphasis on the inhibition of RT-resistant HIV-1 strains. On the basis of $EC_{50}$ values, determined by means of a generally accepted cell-based reporter assay, we have shown that a *P. amarus* water/alcohol extract was capable to restrict replication of the majority of RT inhibitor-resistant viruses as good or even better than the HIV-1 and -2 wild-type reference strains. In a second approach we focused on the molecular details of the antiviral activity of *P. amarus*. Although it was shown previously that certain plant extracts or related components like tannins interfere with two critical steps of the viral life cycle such as virus attachment and reverse transcription, the exact contributions of these activities to the overall inhibition has not yet been determined. Herein we compared the obtained inhibition data on the basis of their individual $EC_{50}$ values for replication and $IC_{50}$ values for RT inhibition in combination with observations during early replication steps in order to evaluate their specific role in controlling HIV replication.

In spite of a large variety of potent HIV inhibitors, a number of severe problems are still associated with HIV infection. Two major concerns are the emergence of resistant or even multiresistant viruses during combination therapy and noxious side effects associated with some inhibitors. In consequence, as long as the eradication of the virus is impossible and a protective vaccine is not available, effective inhibitors capable of controlling resistant virus variants with a more favorable tolerance profile are urgently needed. In addition, since the most afflicted portion of HIV-infected individuals resides in poor countries, new therapeutics must be affordable. *P. amarus* has been used for centuries and has proven

to be well tolerated in many human trials (for a review, see Liu et al., 2001). The plant can readily be grown in large quantities and antiviral compounds derived from *P. amarus* can be prepared by general extraction methods. Accordingly, *P. amarus* combines good tolerability, sufficient supply and low costs. Here we demonstrated that a defined extract and an individual compound are capable of controlling the replication of HIV-1 as well as HIV-2. The inhibition of four NRTI- and five NNRTI-resistant HIV-1 strains was directly compared to the inhibition of HIV-1 wild type (see Table 4). Only one strain from each resistance group (ARP1006 and ARP1016) had a negligible elevated $EC_{50}$ value compared to the wild type strain in case of the W/E extract. Geraniin showed a comparable pattern with respect to the distribution of $EC_{50}$ values. However, the difference between the $EC_{50}$ of the wild-type HX10 and the two less controlled strains ARP1006 and ARP1016 was increased, and an additional strain in the NNRTI group, ARP1013, displayed an increased $EC_{50}$ (see Table 4). Geraniin seems to be one potent mediator of the overall antiviral activity; it interferes, as shown in Fig. 3, with the virus uptake, but obviously its inhibitory effect on the replication is also influenced by the RT of the respective strain. Hence, the RT is to a certain degree a relevant target. It has been shown previously that different plant extracts display a mode of action that is completely different from the approved RT inhibitors by being competitive with respect to the primer/template (Pengsuparp et al., 1995; Goldman et al., 1990; el Mekkawy et al., 1995). Here we revealed the same mechanism of RT inhibition for the gallotannin geraniin. Since defined mutations in the RT gene associated with resistance to either NRTIs or NNRTIs confer a reduced susceptibility to HIV infection, the respective amino acids are most likely also involved in the binding of the primer/template being the primary target of RT inhibition by *P. amarus*. On the other side, the W/E extract is effective irrespective of the RT variations tested. This could be due to additional active principles included in the extract, all contributing to inhibition of HIV replication.

Although the contribution of RT inhibition by tannins is ambiguous, early data proposed that antiviral activity is closely related to an early block in virus–cell interaction (Nonaka et al., 1990; Kilkuskie et al., 1992; Ogata et al., 1992). In order to address this issue by quantitative means we opposed the inhibition of virus entry and post-entry steps in a series of defined experimental settings. The use of a cell-based reporter assay allowed to monitor and distinguish events occurring very early in the viral life cycle. The entry of virions into the cell was blocked by W/E extract and geraniin. The loss of inhibitory potential was minimal when the inhibitors were removed during the period of virus attachment and internalization (2 h after inoculation) (see Fig. 2), suggesting that the block at this stage is the major part of the antiviral activity. Hence, in accordance with earlier investigations and the results of the internalization assay, the predominant antiviral effect is based on cell–virus interaction.

On the other hand, for geraniin and partially also for W/E extract a very effective inhibition of the HIV-1 RT was demonstrated in vitro, suggesting a close connection of RT inactivation and HIV inhibition. Two observations support this idea. First, in the cell-based assay, addition of inhibitors after virus entry is most likely completed (removing excess virus after 2 h and adding inhibitors) results in a reproducible inhibition of virus replication. Second, inhibition of RT inhibitor-resistant strains and, more precisely, a decreased susceptibility of defined strains for both, W/E extract and geraniin, implies a role of the anti-RT activity in the overall inhibition. From the data obtained it can be inferred that both activities contribute to the overall inhibition, albeit to a different extent.

It has been speculated, that the antiviral activity of gallotannins is due to their observed cell pathogenic effect rather than to a specific activity (Kilkuskie et al., 1992). However, the calculated TI for the inhibition of MAGI cells was constantly greater than 500, indicating a highly specific activity. We have observed that the cytotoxic effects of *P. amarus* extracts could be attributed to the gallotannins. Extraction of the gallotannins from the W/E extract resulted in loss of cytotoxicity at concentrations >1 mg/ml, while the antiviral effect declined. In contrast, the W/E extract caused complete cell death at a concentration of 1 mg/ml. In addition, primary hepatocytes and various cell lines (HepG2-2.2.15, HuH-7) tolerate concentrations of W/E extract >1 mg/ml (data not shown). On the other hand, the TI values determined in MT4 cells confirm earlier observations of highly cytotoxic activities of gallotannins (Kilkuskie et al., 1992). However, most approved anti-HIV-1 agents (especially RT inhibitors) were shown to have decreased TIs, when determined in MT4 cells as compared to primary cells e.g. PBMC. The TI values were in most cases higher in PBMC (more than 50-fold, e.g. stavudine (Balzarini et al., 1989; Chu et al., 1988) or zalcitabine (Mitsuya and Broder, 1986; Chu et al., 1988). Therefore, it seems likely that the cytotoxic effects are a matter of these specific, very sensitive cell types and allow no prediction of the in vivo situation. Beside cytotoxic effects in cell culture, tannins have a very ambiguous reputation in respect to effects in humans. Reported disadvantages of tannins include the induction of liver damage, anti-nutritional activity resulting from complex formation with different proteins and carcinogenic potential (for a review, see Chung et al., 1998). On the other hand, the high potential to bind proteins has been demonstrated to result in specific in vitro activities like antiviral, antimutagenic, anticarcinogenic, and immunmodulatory, arguing for sufficient selectivity for a potential therapeutic use (Chung et al., 1998). Regarding the *P. amarus* W/E extract, comparable extracts were proven to be well tolerated in clinical studies, and, in view of the antiviral activities in particular against RT inhibitor resistant strains, the gallotannins included in this extract have the potential for HIV therapy.

Taken together, the presented data on the antiviral activities of *P. amarus* led to the conclusion that the inhibition of

RT inhibitor-resistant HIV strains is based on the presence of multiple active principles targeting at least two central events in the replication cycle. Interference with virus entry seems to be the main target. However, intracellular replication events are also targeted. The primer/template binding site of the HIV-1 RT and viral surface components may prove to represent new targets for a therapeutic use, especially in the case of multi-resistant HIV variants in terms of a salvage therapy. W/E extract may be of special benefit, since the resistant strains were in general better inhibited than the wild-type strain. This can rely on disadvantages in the replication of these strains as a consequence of a reduced fitness mediated by the resistance-associated mutations in the RT gene, as demonstrated for foscarnet-resistant variants (Tachedjian et al., 1998).

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## References

Baba, M., Nakajima, M., Schols, D., Pauwels, R., Balzarini, J., De Clercq, E., 1988. Pentosan polysulfate, a sulfated oligosaccharide, is a potent and selective anti-HIV agent in vitro. Antiviral Res. 9, 335–343.

Balzarini, J., Van Aerschot, A., Herdewijn, P., De Clercq, E., 1989. 5-Chloro-substituted derivatives of 2′,3′-didehydro-2′,3′-dideoxyuridine, 3′-fluoro-2′,3′-dideoxyuridine and 3′-azido-2′,3′-dideoxyuridine as anti-HIV agents. Biochem. Pharmacol. 38, 869–874.

Balzarini, J., Karlsson, A., Perez-Perez, M.J., Camarasa, M.J., Tarpley, W.G., De Clercq, E., 1993a. Treatment of human immunodeficiency virus type 1 (HIV-1)-infected cells with combinations of HIV-1-specific inhibitors results in a different resistance pattern than does treatment with single-drug therapy. J. Virol. 67, 5353–5359.

Balzarini, J., Karlsson, A., De Clercq, E., 1993b. Human immunodeficiency virus type 1 drug-resistance patterns with different 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine derivatives. Mol. Pharmacol. 44, 694–701.

Balzarini, J., Karlsson, A., Sardana, V.V., Emini, E.A., Camarasa, M.-J., De Clercq, E., 1994. Human immunodeficiency virus 1 (HIV-1)-specific reverse transcriptase (RT) inhibitors may suppress the replication of specific drug-resistant (E138K)RT HIV-1 mutants or select for highly resistant (Y181C → C181I)RT HIV-1 mutants. Proc. Natl. Acad. Sci. U.S.A. 91, 6599–6603.

Chang, C.W., Hsu, F.L., Lin, J.Y., 1994. Inhibitory effects of polyphenolic catechins from Chinese green tea on HIV reverse transcriptase activity. J. Biomed. Sci. 1, 163–166.

Chou, T.C., Talalay, P., 1981. Generalized equations for the analysis of inhibitions of Michaelis-Menten and higher-order kinetic systems with two or more mutually exclusive and nonexclusive inhibitors. Eur. J. Biochem. 115, 207–216.

Chou, T.C., Talalay, P., 1984. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv. Enzyme Regul. 22, 27–55.

Chu, C.K., Schinazi, R.F., Arnold, B.H., Cannon, D.L., Doboszewski, B., Bhadti, V.B., Gu, Z.P., 1988. Comparative activity of 2′,3′-saturated and unsaturated pyrimidine and purine nucleosides against human immunodeficiency virus type 1 in peripheral blood mononuclear cells. Biochem. Pharmacol. 37, 3543–3548.

Chung, K.-T., Wei, C.-I., Johnson, M.G., 1998. Are tannins a double-edged sword in biology and health? Trends Food Sci. Technol. 9, 168–175.

Clavel, F., Guetard, D., Brun-Vézinet, F., Chamaret, S., Rey, M.A., Santos-Ferreira, M.O., Laurent, A.G., Dauguet, C., Katlama, C., Rouzioux, C., 1986. Isolation of a new human retrovirus from West African patients with AIDS. Science 233, 343–346.

Collins, R.A., Ng, T.B., Fong, W.P., Wan, C.C., Yeung, H.W., 1997. A comparison of human immunodeficiency virus type 1 inhibition by partially purified aqueous extracts of Chinese medicinal herbs. Life Sci. 60, PL345–PL351.

Collins, R.A., Ng, T.B., Fong, W.P., Wan, C.C., Yeung, H.W., 1998. Removal of polyphenolic compounds from aqueous plant extracts using polyamide minicolumns. Biochem. Mol. Biol. Int. 45, 791–796.

De Clercq, E., 2000. Current lead natural products for the chemotherapy of human immunodeficiency virus (HIV) infection. Med. Res. Rev. 20, 323–349.

Doshi, J.C., Vaidya, A.B., Antarkar, D.S., Deolalikar, R., Antani, D.H., 1994. A two-stage clinical trial of *Phyllanthus amarus* in hepatitis B carriers: failure to eradicate the surface antigen. Indian J. Gastroenterol. 13, 7–8.

el Mekkawy, S., Meselhy, M.R., Kusumoto, I.T., Kadota, S., Hattori, M., Namba, T., 1995. Inhibitory effects of Egyptian folk medicines on human immunodeficiency virus (HIV) reverse transcriptase. Chem. Pharm. Bull. (Tokyo) 43, 641–648.

Furtado, M.R., Callaway, D.S., Phair, J.P., Kunstman, K.J., Stanton, J.L., Macken, C.A., Perelson, A.S., Wolinsky, S.M., 1999. Persistence of HIV-1 transcription in peripheral-blood mononuclear cells in patients receiving potent antiretroviral therapy. N. Engl. J. Med. 340, 1614–1622.

Goldman, M.E., Salituro, G.S., Bowen, J.A., Williamson, J.M., Zink, D.L., Schleif, W.A., Emini, E.A., 1990. Inhibition of human immunodeficiency virus-1 reverse transcriptase activity by rubromycins: competitive interaction at the template.primer site. Mol. Pharmacol. 38, 20–25.

Hnatyszyn, O., Broussalis, A., Herrera, G., Muschietti, L., Coussio, J., Martino, V., Ferraro, G., Font, M., Monge, A., Martinez-Irujo, J.J., Sanroman, M., Cuevas, M.T., Santiago, E., Lasarte, J.J., 1999. Argentine plant extracts active against polymerase and ribonuclease H activities of HIV-1 reverse transcriptase. Phytother. Res. 13, 206–209.

Jung, M., Lee, S., Kim, H., 2000. Recent studies on natural products as anti-HIV agents. Curr. Med. Chem. 7, 649–661.

Kakiuchi, N., Hattori, M., Namba, T., Nishizawa, M., Yamagishi, T., Okuda, T., 1985. Inhibitory effect of tannins on reverse transcriptase from RNA tumor virus. J. Nat. Prod. 48, 614–621.

Kilkuskie, R.E., Kashiwada, Y., Nonaka, G.I., Nishioka, I., Bodner, A.J., Cheng, Y.C., Kuo, H., 1992. HIV and reverse transcriptase inhibition by tannins. Bioorg. Med. Chem. Lett. 2, 1529–1534.

Kimpton, J., Emerman, M., 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated beta-galactosidase gene. J. Virol. 66, 2232–2239.

Kleim, J.P., Bender, R., Kirsch, R., Meichsner, C., Paessens, A., Riess, G., 1994. Mutational analysis of residue 190 of human immunodeficiency virus type 1 reverse transcriptase. Virology 200, 696–701.

Larder, B.A., Darby, G., Richman, D.D., 1989. HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. Science 243, 1731–1734.

Larder, B.A., Kellam, P., Kemp, S.D., 1993. Convergent combination therapy can select viable multidrug-resistant HIV-1 in vitro. Nature 365, 451–453.

Larder, B.A., Kemp, S.D., Harrigan, P.R., 1995. Potential mechanism for sustained antiretroviral efficacy of AZT-3TC combination therapy. Science 269, 696–699.

Lee, C.D., Ott, M., Thyagarajan, S.P., Shafritz, D.A., Burk, R.D., Gupta, S., 1996. *Phyllanthus amarus* down-regulates hepatitis B virus mRNA transcription and replication. Eur. J. Clin. Invest. 26, 1069–1076.

Leelarasamee, A., Trakulsomboon, S., Maunwongyathi, P., Somanabandhu, A., Pidetcha, P., Matrakool, B., Lebnak, T., Ridthimat, W., Chandanayingyong, D., 1990. Failure of *Phyllanthus amarus* to eradicate hepatitis B surface antigen from symptomless carriers. Lancet 335, 1600–1601.

Liu, J., McIntosh, H., Lin, H., 2001. Chinese medicinal herbs for chronic hepatitis B: a systematic review. Liver 21, 280–286.

Lucas, G.M., Chaisson, R.E., Moore, R.D., 1999. Highly active antiretroviral therapy in a large urban clinic: risk factors for virologic failure and adverse drug reactions. Ann. Intern. Med. 131, 81–87.

Matthee, G., Wright, A.D., Konig, G.M., 1999. HIV reverse transcriptase inhibitors of natural origin. Planta Med. 65, 493–506.

Mitsuya, H., Broder, S., 1986. Inhibition of the in vitro infectivity and cytopathic effect of human T-lymphotrophic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) by 2′,3′-dideoxynucleosides. Proc. Natl. Acad. Sci. U.S.A. 83, 1911–1915.

Mitsuya, H., Looney, D.J., Kuno, S., Ueno, R., Wong-Staal, F., Broder, S., 1988. Dextran sulfate suppression of viruses in the HIV family: inhibition of virion binding to CD4+ cells. Science 240, 646–649.

Mlinaric, A., Kreft, S., Umek, A., Strukelj, B., 2000. Screening of selected plant extracts for in vitro inhibitory activity on HIV-1 reverse transcriptase (HIV-1 RT). Pharmazie 55, 75–77.

Nonaka, G., Nishioka, I., Nishizawa, M., Yamagishi, T., Kashiwada, Y., Dutschman, G.E., Bodner, A.J., Kilkuskie, R.E., Cheng, Y.C., Lee, K.H., 1990. Anti-AIDS agents, 2: inhibitory effects of tannins on HIV reverse transcriptase and HIV replication in H9 lymphocyte cells. J. Nat. Prod. 53, 587–595.

Ogata, T., Higuchi, H., Mochida, S., Matsumoto, H., Kato, A., Endo, T., Kaji, A., Kaji, H., 1992. HIV-1 reverse transcriptase inhibitor from *Phyllanthus niruri*. AIDS Res. Hum. Retroviruses 8, 1937–1944.

Ott, M., Thyagarajan, S.P., Gupta, S., 1997. *Phyllanthus amarus* suppresses hepatitis B virus by interrupting interactions between HBV enhancer I and cellular transcription factors. Eur. J. Clin. Invest. 27, 908–915.

Pengsuparp, T., Cai, L., Constant, H., Fong, H.H., Lin, L.Z., Kinghorn, A.D., Pezzuto, J.M., Cordell, G.A., Ingolfsdottir, K., Wagner, H., 1995. Mechanistic evaluation of new plant-derived compounds that inhibit HIV-1 reverse transcriptase. J. Nat. Prod. 58, 1024–1031.

Qian-Cutrone, J., Huang, S., Trimble, J., Li, H., Lin, P.F., Alam, M., Klohr, S.E., Kadow, K.F., 1996. Niruriside, a new HIV REV/RRE binding inhibitor from *Phyllanthus niruri*. J. Nat. Prod. 59, 196–199.

Ratner, L., Fisher, A., Jagodzinski, L.L., Mitsuya, H., Liou, R.S., Gallo, R.C., Wong Staal, F., 1987. Complete nucleotide sequences of functional clones of the AIDS virus. AIDS Res. Hum. Retroviruses 3, 57–69.

Saphire, A.C., Bobardt, M.D., Gallay, P.A., 1999. Host cyclophilin A mediates HIV-1 attachment to target cells via heparans. EMBO J. 18, 6771–6785.

Shead, A., Vickery, K., Pajkos, A., Medhurst, R., Freiman, J., Dixon, R., Cossart, Y., 1992. Effects of *Phyllanthus* plant extracts on duck hepatitis B virus in vitro and in vivo. Antiviral Res. 18, 127–138.

Suthienkul, O., Miyazaki, O., Chulasiri, M., Kositanont, U., Oishi, K., 1993. Retroviral reverse transcriptase inhibitory activity in Thai herbs and spices: screening with Moloney murine leukemia viral enzyme. Southeast Asian J. Trop. Med. Public Health 24, 751–755.

Tachedjian, G., Mellors, J.W., Bazmi, H., Mills, J., 1998. Impaired fitness of foscarnet-resistant strains of human immunodeficiency virus type 1. AIDS Res. Hum. Retroviruses 14, 1059–1064.

Thyagarajan, S.P., Subramanian, S., Thirunalasundari, T., Venkateswaran, P.S., Blumberg, B.S., 1988. Effect of *Phyllanthus amarus* on chronic carriers of hepatitis B virus. Lancet 2, 764–766.

Thyagarajan, S.P., Jayaram, S., Valliammai, T., Madanagopalan, N., Pal, V.G., Jayaraman, K., 1990. *Phyllanthus amarus* and hepatitis B. Lancet 336, 949–950.

Tisdale, M., Kemp, S.D., Parry, N.R., Larder, B.A., 1993. Rapid in vitro selection of human immunodeficiency virus type 1 resistant to 3′-thiacytidine inhibitors due to a mutation in the YMDD region of reverse transcriptase. Proc. Natl. Acad. Sci. U.S.A. 90, 5653–5656.

Tisdale, M., Alnadaf, T., Cousens, D., 1997. Combination of mutations in human immunodeficiency virus type 1 reverse transcriptase required for resistance to the carbocyclic nucleoside 1592U89. Antimicrob. Agents Chemother. 41, 1094–1098.

Venkateswaran, P.S., Millman, I., Blumberg, B.S., 1987. Effects of an extract from *Phyllanthus niruri* on hepatitis B and woodchuck hepatitis viruses: in vitro and in vivo studies. Proc. Natl. Acad. Sci. U.S.A. 84, 274–278.

Vlietinck, A.J., De Bruyne, T., Apers, S., Pieters, L.A., 1998. Plant-derived leading compounds for chemotherapy of human immunodeficiency virus (HIV) infection. Planta Med. 64, 97–109.

Wang, M., Cheng, H., Li, Y., Meng, L., Zhao, G., Mai, K., 1995. Herbs of the genus *Phyllanthus* in the treatment of chronic hepatitis B: observations with three preparations from different geographic sites. J. Lab. Clin. Med. 126, 350–352.

Weaver, J.L., Pine, P.S., Dutschman, G., Cheng, Y.C., Lee, K.H., Aszalos, A., 1992. Prevention of binding of rgp120 by anti-HIV active tannins. Biochem. Pharmacol. 43, 2479–2480.

Xin-Hua, W., Chang-Qing, L., Xing-Bo, G., Lin-Chun, F., 2001. A comparative study of *Phyllanthus amarus* compound and interferon in the treatment of chronic viral hepatitis B. Southeast Asian J. Trop. Med. Public Health 32, 140–142.

Yerly, S., Kaiser, L., Race, E., Bru, J.P., Clavel, F., Perrin, L., 1999. Transmission of antiretroviral-drug-resistant HIV-1 variants. Lancet 354, 729–733.