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In vitro pharmacodynamic evaluation of antiviral medicinal plants using a vector-based assay technique

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ABSTRACT

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Aims: Medicinal plants are increasingly being projected as suitable alternative sources of antiviral agents. The development of a suitable *in vitro* pharmacodynamic screening technique could contribute to rapid identification of potential bioactive plants and also to the standardization and/or pharmacokinetic–pharmacodynamic profiling of the bioactive components.

Methods and Results: Recombinant viral vectors (lentiviral, retroviral and adenoviral) transferring the firefly luciferase gene were constructed and the inhibition of viral vector infectivity by various concentrations of plant extracts was evaluated in HeLa or Hep2 cells by measuring the changes in luciferase activity. Cytotoxicity of the extracts was evaluated in parallel on HeLa or Hep2 cells stably expressing luciferase. Amongst the 15 extracts screened, only the methanol (ME) and the ethyl acetate (ET) fractions of the lichen, *Ramalina farinacea* specifically reduced lentiviral and adenoviral infectivity in a dose-dependent manner. Further, chromatographic fractionation of ET into four fractions (ET1–ET4) revealed only ET4 to be selectively antiviral with an IC_{50} in the 20 μg ml^{-1} range. Preliminary mechanistic studies based on the addition of the extracts at different time points in the viral infection cycle (kinetic studies) revealed that the inhibitory activity was highest if extract and vectors were preincubated prior to infection, suggesting that early steps in the lentiviral or adenoviral replication cycle could be the major target of ET4. Inhibition of wild-type HIV-1 was also observed at a 10-fold lower concentration of the extract.

Conclusions: The vector-based assay is a suitable *in vitro* pharmacodynamic evaluation technique for antiviral medicinal plants. The technique has successfully demonstrated the presence of antiviral principles in *R. farinacea*.

Significance and Impact of Study: Potential anti-HIV medicinal plants could rapidly be evaluated with the reported vector-based technique. The lichen, *R. farinacea* could represent a lead source of antiviral substances and is thus worthy of further studies.

Keywords: antiviral, lichen, medicinal plants, pharmacodynamic, *Ramalina farinacea*, vector-based assay.

INTRODUCTION

Medicinal plants, because of their often multiple targets, minor side-effects, low potentials to cause resistance and low costs, are increasingly being projected as suitable alternative sources of antiviral agents (Vlietinck and Vanden Berghe 1991; Cowan 1999; Briskin 2000; Williams 2001; Jassim and Naji 2003). Although several hundreds of plants that have potential as novel antiviral agents have been studied, there still exist innumerable potentially useful medicinal plants waiting to be evaluated and exploited for therapeutic

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applications against genetically and functionally diverse virus families such as Retroviridae, Hepadnaviridae and Herpesviridae (Cowan 1999; Jassim and Naji 2003). Also, methods are needed to link antiviral efficacy/potency and laboratory-based research (Jassim and Naji 2003).

Traditional methods of evaluating antiviral agents have involved one or more of the following *in vitro* techniques: plaque inhibition assay, plaque reduction assay, inhibition of virus-induced cytopathic effect, virus yield reduction assay, end point titre determination assay, reduction or inhibition of the synthesis of virus-specific polypeptides, immunological assays detecting viral antigens and viral enzyme inhibition-based assays (Vlietinck and Vanden Berghe 1991; Cowan 1999). While these methods have successfully been employed to screen a large number of diverse antiviral agents, salient problems related to safety, long assay period, cumbersome and experimental-biased read-out, high cost and inability to distinguish between mere toxic effects of agents on host cells and selective antiviral effects associated with some of these methods has limited their application in high throughput assay of the vast majority of yet un-screened medicinal plants for antiviral activities. Therefore, the development of a suitable *in vitro* pharmacodynamic screening technique that will contribute not only in rapidly screening for potential bioactive plants but also in the standardization and/or pharmacokinetic-pharmacodynamic profiling of the bioactive components is needed. While *in vitro* pharmacodynamic evaluations and consequently, pharmacokinetic-pharmacodynamic (PK-PD) correlations have been well established for most antibacterial and antifungal agents (Dagan 2003; Andes *et al.* 2004; Preston 2004), antiviral agents are yet to be fully amenable to these.

Reporter gene-based antiviral screening assays quantifying the expression of β-galactosidase (Kimpton and Emerman 1992; Proffitt and Schindler 1995), luciferase (Johansen *et al.* 2004), green fluorescent protein (GFP) (Marschall *et al.* 2000) and secreted alkaline phosphatase (SEAP) (Means *et al.* 1997) upon infection of target cell lines by various viruses offer the advantage of being flexible, rapid, unbiased and amenable to high throughput. Using a SEAP-based reporter gene, Walter *et al.* (1999) developed a rapid phenotypic HIV-1 drug sensitivity assay for protease and reverse transcriptase inhibitors. This study, which revealed good agreement in drug sensitivity pattern between multi-drug-resistant recombinant HIV-1 and the parental viruses, further authenticated the clinical relevance of reporter gene-based antiviral assays. As viral vectors can be designed to exhibit essential properties of the parental virus, viral vector-based assays could provide a mean of rapidly generating clinically transposable data for possible PK-PD profiling. Also, with respect to the current global move towards pilot screening of medicinal plants for anti-HIV activity (Cowan 1999; De Clercq 2000; Williams 2001; Jassim and Naji 2003), lentiviral vector-based assay techniques offer the advantage of safety (nonexposure to the wild-type HIV during the pilot screening process), rapidity (and therefore amenable to high throughput) and flexibility.

We describe here, the applicability of a HIV vector-based assay in the pilot screening of medicinal plant extracts for anti-HIV activity. Bioactive fractions were also screened against an adenoviral vector based on the human adenovirus type 5. Cytotoxicity of the extracts was evaluated in parallel on target cell lines stably expressing the luciferase reporter gene.

MATERIALS AND METHODS

Collection and extraction of plant materials

The list of plant extracts evaluated in this study is presented in Table 1. All the plants were collected in Enugu state, Nigeria, from October 2002 to April 2003. The identity was authenticated by Mr O.A. Ozioko of the Bioresources Development and Conservation Program (BDCP) Centre,

Table 1 Summary of vector-based antiviral screening of plant extracts

Plant part (extract)*	IC_{50}†	TC_{50}‡	SI§
RC root (methanol)	150·0	75·0	0·5
RC leaf (methanol)	11·1	12·5	1·13
RC stem (methanol)	>1000	ND**	ND¶
CC stem (methanol)	>1000	298·6	ND¶
CC leaf (methanol)	49·64	54·31	1·09
CD root (methanol)	152·5	136·96	0·89
PP (water)	389·0	51·0	0·13
PP (methanol)	124·70	152·26	1·22
RF (water)	<100	>1000	ND¶
RF (methanol)	13·5	75·97	5·62
CM stem bark (cold water)	10·5	8·9	0·85
CM stem bark (hot water)	ND**	>3	ND¶
CM root (hot water)	962·17	336·936	0·35
CM root (cold water)	48·96	95·47	1·95
CM leaf (methylene chloride)	262·08	234·24	0·89

The mean values of triplicate experiments are shown.

*Plant parts and the extract: CM, *Combretum micranthum*; CD, *Combretum dolchipeles*; RC, *Richtiae capparoides*; CC, *Cajanus cajan*; PP, *Parmelia perlata*; RF, *Ramalina farinacea*.

†Concentration of extract (μg ml^{-1}) that inhibits viral vector infectivity by 50%.

‡Concentration of extract (μg ml^{-1}) that inhibits reporter gene expression of target cells (HeLa-luc-stable) by 50%. This is a measure of the effect of extracts on target cell metabolism.

§Selectivity index (SI) = TC_{50}/IC_{50}.

¶Not determined (ND), because observed cytotoxic activity was not dose-dependent.

Nsukka. Voucher specimens were deposited at the same centre.

Various parts of the plants were sun-dried, powdered and extracted overnight with either cold water (WA), methanol (ME) or methylene chloride via a cold maceration process. Hot WA extracts were also prepared by boiling plants in distilled WA for 1 h. The aqueous extracts were filtered through gauze, concentrated under vacuum and lyophilized. The ME and methylene chloride extracts were filtered through a Whatman filter paper (no. 1) and concentrated under vacuum.

The bioactive ME fraction from the lichen *Ramalina farinacea* was further subjected to liquid–liquid partitioning to yield n-hexane and ethyl acetate (ET) fractions. By fractionating the ET fraction over a Sephadex (LH20) column (using ME as eluent), four subfractions, ET1–ET4 were obtained.

Cell lines

HeLa (ATCC CCL-2), Hep2, P4CCR5 and 293T cells were propagated in D-10, consisting of Dulbecco's modified Eagle's medium (DMEM) with high glucose, 2 mmol l^{-1} L-glutamine and supplemented with 10% heat-inactivated foetal bovine serum (FBS), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Tissue culture medium and supplements were purchased from Invitrogen (Karlsruhe, Germany). The cell cultures were maintained in a humidified 5% CO_2 atmosphere at 37°C.

Generation of lentiviral, retroviral and adenoviral vectors transferring the luciferase gene

The lentiviral vector expressing the luciferase gene (ViCL2ΔSB) was constructed by ligating the firefly luciferase cDNA subcloned from pGEM-Luc (Promega, Madison, WI, USA) into the *Sna*BI-*Spe*I site of VCGΔSB-SIN. VCGΔSB-SIN was derived from VGΔBH-SIN (Grunwald *et al.* 2004) by replacing the murine leukaemia virus (MLV) promoter of VGΔBH-SIN with a human cytomegalovirus (CMV) promoter and by deletion of the *Spe*I-*Bsp*EI fragment spanning exon 1 of *tat* and *rev*. The luciferase gene in ViCL2ΔSB is driven by an internal CMV promoter situated immediately downstream. Lentiviral vector particles were generated by transient cotransfection of ViCL2ΔSB (5 μg) with a codon-optimized HIV-1 *gag-pol* expression plasmid (Hgp^{syn}, 5 μg) (Wagner *et al.* 2000), *tat* expression plasmid (pctat, 2 μg) (Malim *et al.* 1988), *rev* expression plasmid (pcrev, 2 μg) (Malim *et al.* 1988), a SEAP expression plasmid (pSEAP, 0·1 μg) and a vesicular stomatitis virus (VSV-G) expression plasmid (pHIT-G, 2 μg) (Fouchier *et al.* 1997) using the calcium phosphate coprecipitation method into 293T cells as previously described by Schnell *et al.* (2000).

The Moloney MLV-based retroviral vector transferring the luciferase gene (GC Luc3 EN) was prepared thus: luciferase cDNA was PCR amplified from pGL3 basic (Promega) and subcloned as *Not*I-*Xho*I fragment into the retroviral plasmid pSAM-EN (Morgan *et al.* 1992), upstream of the encephalomyocarditis virus (EMCV) IRES and neo^r. Luciferase encoding retroviral vectors were produced by cotransfection of pGC Luc3 EN, the Moloney MLV *gag-pol* expression plasmid (pHIT 60) (Soneoka *et al.* 1995) and VSV-G expression plasmid (pHIT-G) in 293T cells.

The adenoviral vector encoding the luciferase gene has been described previously by Steinstraesser *et al.* (2005). Briefly, beginning with a first generation E1- and E3-deleted adenoviral vector, a replication-competent adenoviral vector (Ad.OW126) was generated, which harbours in the E1 region the firefly luciferase cDNA subcloned from pGEM-Luc (Promega), an IRES element, and an Ad5 E1A ΔE1B-55K gene. The entire expression cassette is driven by the human CMV-IE promoter in parallel to the transcriptional orientation of the adenovirus E1 gene products and terminated by the bovine growth hormone polyadenylation site.

Generation of stable cell line

Polyclonal HeLa and Hep2 cell lines stably expressing the luciferase gene (HeLa-luc-stable and Hep2-luc-stable respectively) were generated by transducing cells plated in 24-well plates (60 000 cells per well) with 200 μl of lentiviral vector particles or the MLV-based vector particle both transferring the luciferase gene for 2 h followed by addition of 1 ml D-10 medium. Two days later, cells were transferred to 1 of 6-well plate and transduced again with 1 ml of the vector particles. Cells were subsequently expanded resulting in HeLa-luc-stable or Hep2-luc-stable cells.

Vector-based antiviral assay

To determine the effect of the plant extracts on lentiviral, retroviral or adenoviral vector infectivity, HeLa cells (lentiviral and adenoviral vectors) or Hep2 cells (retroviral vector) were plated in triplicates into 96-well plates at 6000 cells per well and incubated overnight. Various concentrations of the extracts (at twice the final concentrations indicated) were prepared in DMEM (without FBS) such that the final DMSO concentration did not exceed 0·5%. These various concentrations were preincubated with equal volumes of the vector supernatant (100 μl) for 30 min at 37°C. Culture medium from the

cell monolayers was replaced with 50 μl of the above vector/extract mixtures in triplicates and incubated for 2 h at 37°C + 5% CO_2. Fresh D-10 medium (50 μl) containing the drugs at the final concentrations stated were then added and the culture incubated for 1 (adenoviral vector) or 2 (lentiviral and retroviral vectors) days. Control wells (containing vector alone but without extracts) received D-10 medium containing 0·5% DMSO (0·5% DMSO D-10) instead. The supernatant of the HeLa or Hep2 cells were removed and cells washed with 200 μl of 1X PBS. Cells were lysed in 50 μl of 1X Cell Culture Lysis Reagent (Promega Corporations) and 20 μl of the cell lysates were used in the firefly luciferase assay as described by the manufacturer using a microtiter plate luminometer (Orion, Berthold Detection Systems, Pforzheim, Germany). Each single value of the triplicates was expressed as percentage of the mean of triplicates of control cultures (infected with same vector in the absence of the extracts or 0·5% DMSO D-10 without extracts) and the mean and standard deviation of the percentage values was calculated for each triplicate.

Cytotoxicity determination

The effect of the extracts on cell metabolism was determined in parallel by plating HeLa-luc-stable or Hep2-luc-stable cells exactly the same way as HeLa/Hep2 cells (as described above for the vector-based assay) and introducing exactly the same concentrations of the extracts. The control wells (without extract) contained 0·5% DMSO in D-10. Two days after, the supernatant of the stable cells were removed and cells processed as described above for the estimation of luciferase activity. Each single value of the triplicates was expressed as percentage of the mean values of triplicates of control cultures (containing 0·5% DMSO D-10 without extracts) and the mean and standard deviation of the percentage values was calculated for each triplicate.

For the screening of ET4 against the wild-type HIV-1, cytotoxicity of the fraction on P4CCR5 indicator cells was evaluated by estimating the effect of the fraction on cell proliferation using the 5-bromo-2′-deoxyuridine (BrdU) Cell Proliferation ELISA with chemiluminescent detection (Roche Diagnostics GmbH, Mannheim, Germany) as previously described by Steinstraesser *et al.* (2005). Briefly, 1 day after P4CCR5 cells were plated in 96-well plates at a density of 6×10^3 cells per well, BrdU was added to the cells with a resulting concentration of 10 μmol l^{-1} for the last 22 h of the incubation period. After removing the culture medium, the cells were fixed and DNA was denatured in one step with FixDenat. Thereafter the cells were incubated with anti-BrdU-peroxidase (POD) for 1 h at room temperature. The chemiluminescent detection was measured after automatic injection of substrate solution with a microtiter plate luminometer (Orion, Berthold Detection Systems).

Screening of ET4 against wild-type HIV-1

HIV-1 (molecular clone NL4-3) was generated by cotransfecting 2·5 μg of DNA plasmid (pNL4-3) with 2·5 μg calf thymus carrier DNA (Boehringer, Mannheim, Germany) into 293T cells using the calcium phosphate coprecipitation method. Viruses were harvested 2 days later and aliquots were stored at −80°C after determination of their titre. Inhibitory effect of ET4 against HIV-1 was evaluated in P4CCR5 cells plated in 96-well plates (6000 cells per well) a day prior to the experiment. Frozen virus stocks were thawed immediately before use, mixed with various concentrations of ET4 and preincubated at 37°C for 30 min as described above for the vector-based assay. Triplicate aliquots (50 μl) of the virus/ET4 mixtures were used to replace culture supernatant of the P4CCR5 cell monolayers to yield an MOI of 0·05. Control wells contain the virus alone but without ET4. After 2 h incubation at 37°C + 5% CO_2, fresh D-10 medium, with or without drugs was added to the appropriate wells. Cultures were incubated for 2 days and HIV-1 infectivity determined by microscopic enumeration of blue-stained cells following a modification of the β-galactosidase assay previously described by Kimpton and Emerman (1992).

RESULTS

A schematic representation of the vector-based assay is given in Fig. 1. For anti-HIV vector assays, a lentiviral vector plasmid (ViCL2ΔSB) containing luciferase as a reporter gene was constructed. The infectious vector particles were generated by transient cotransfection of the vector plasmid (ViCL2ΔSB) with packaging plasmids encoding *tat*, *rev*,

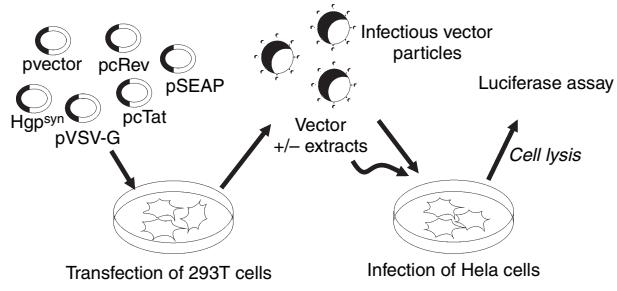

Fig. 1 Vector-based assay technique including basic production scheme for the lentiviral vector. The vector plasmid containing the luciferase reporter gene is cotransfected with packaging plasmids into 293T cells to yield the lentiviral vector particles. The resulting vector supernatant is incubated with various concentrations of plant extracts and extract/vector mixture is used to infect HeLa target cells. After 2 days, cells are lysed and the luciferase activity is determined as a measure of vector infectivity

gag-pol and VSV-G all necessary for viral infection. Transient supply of the packaging plasmids and the self-inactivation of the vector by deletion of the U3 region in the 3′-LTR ensure that the resulting lentiviral vector is only capable of a single round of replication, which makes such viral vectors comparatively safer than their parental viruses. The infectious vector particles additionally differ from the wild-type HIV-1 virus in that they lacked some of the HIV-1 accessory genes such as *nef*, *vif*, *vpu* and *vpr*. Because the transgene (in this case, luciferase) is integrated into the genome of target cells in the process of infection, vector infectivity in the presence or absence of various concentrations of the extracts is easily determined luminometrically as a function of the amount of luciferase expression. In this system, luciferase expression is driven by an internal CMV promoter and occurs after integration. This implies that inhibitors of late stages of the viral replication cycle such as assembly and budding are not detected by the screening assay.

The adenoviral vector, on the other hand, is replication-competent and the luciferase expression is driven by an early internal CMV promoter. Depending on the time point of analysis, inhibition of both early and late stages of the adenoviral replication cycle can be detected using the Ad.OW126 adenoviral vector.

Amongst the 15 extracts screened, only the ME and WA extract of the lichen, *R. farinacea*, specifically reduced HIV-1 vector infectivity (Table 1). However, because the activity of WA was not dose-dependent (Fig. 2a), definite EC_{50} and TC_{50} values could not be estimated. Further antiviral screenings were therefore limited to ME. ME reduced both lentiviral and adenoviral vector infectivity in a dose-dependent manner (Fig. 2b,c). Cell metabolism (as monitored by the effect of extracts on the cell line stably expressing the luciferase gene), was also affected but to a considerably lower extent. We used the effect of extracts on cellular metabolism (as determined by extent of luciferase expression of stable cell lines) as an estimate of cytotoxicity because of the recent observation by Steinstraesser *et al.* (2005) that some defensins could modulate host cell metabolism without affecting cell viability as assayed, for example, by the standard MTT method. They demonstrated that the standard MTT viability assay was less sensitive than the luciferase-based cell metabolism assay in delineating cytotoxicity, especially at lower concentrations of their test substances. We were therefore interested in using a more stringent method to discriminate between cytotoxicity and specific antiviral activity. As only living/viable cells would stably express the integrated luciferase, cytotoxic extracts would be expected to reduce the luciferase expression of the stable cell lines in a dose-dependent manner. Besides, using the luciferase-based assay to screen for cytotoxicity allows for side-by-side

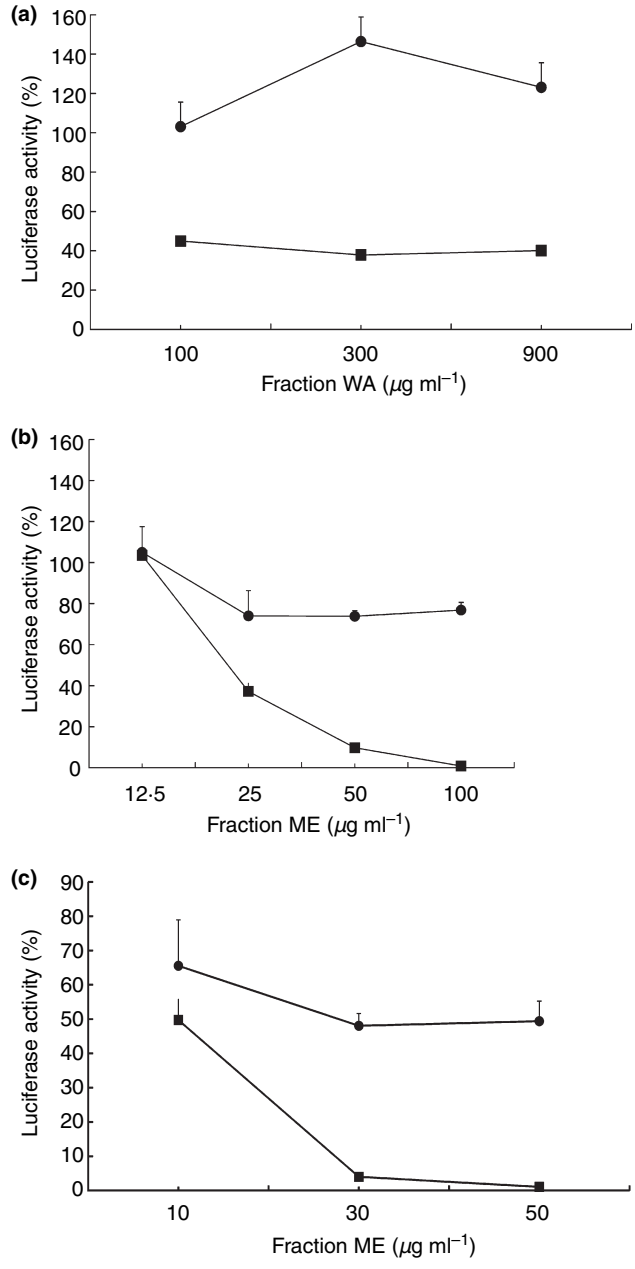

Fig. 2 Inhibitory effect of fractions water (WA) and methanol (ME) of *Ramalina farinacea* against viral vectors. (a) Inhibition of lentiviral vector infectivity by fraction WA. Inhibitory effects of fraction ME for lentiviral (b) and adenoviral vector (c). Vector infectivity (■) in the presence of the indicated amounts of the fractions is expressed as percentage luciferase activity of HeLa target cells transduced with the same vectors in the absence of the fractions. Cytotoxicity (●) of the extracts was evaluated in parallel by incubating HeLa cells stably expressing the luciferase gene with the same amount of fractions. The luciferase activity is expressed as percentage of the luciferase activity of cells cultured in the absence of the fractions. The mean values and the standard deviation of triplicates is given

evaluation of cytotoxic and antiviral effects of extracts with the same read-out.

The selectivity index of 5·62 for the anti-HIV vector and 10·4 for the antiadenoviral vector assay suggests the antiviral activity of ME against these vectors is really specific and not just a consequence of its influence on cell metabolism and/or toxicity. ME was further fractionated by liquid–liquid partition chromatography into two fractions namely: n-hexane (NH) and ET. Vector-based screening revealed that while ET was selectively inhibitory against both the HIV-1 vector and the adenoviral vector, NH exhibited mostly cytotoxicity, with very slight antiviral activity (Fig. 3a–d). Further chromatographic fractionation of ET into four fractions (ET1–ET4) revealed only ET4 to be selectively antiviral against both HIV (IC_{50}, 22 μg ml^{-1}) and adenoviral (IC_{50}, 25 μg ml^{-1}) vector (Fig. 4a,b). In order to enhance vector titre and cell tropism, the HIV-1 vector used in this study was pseudotyped with the G-protein of the VSV-G. To evaluate whether ET4 selectively targets VSV-G or a lentiviral protein, the inhibitory effect against a MLV vector was determined. Strong inhibition of the MLV vector was similarly observed, suggesting that a common component is targeted by ET4 (Fig. 4c).

To get a preliminary insight into the step in the lentiviral or adenoviral vector replication cycle that is inhibited by ET4, kinetic studies were carried out. Preincubating ET4 and the viral vectors for 30 min prior to infection significantly inhibited vector infectivity more than when vectors and extract are added at the same time (Fig. 5a,b). The lowest activity was obtained when ET4 was added 2 h (for lentiviral vector) or 4 h postinfection (for adenoviral vector). For the lentiviral vector, this kinetic pattern strongly supports the inhibition of an entry step, although slight inhibition of a postentry step could also be implicated. In contrast for the adenoviral vector, it appears only early infection steps are inhibited.

To confirm the inhibitory activity of ET4 for wild-type HIV-1, P4CCR5 indicator cells were infected with the HIV-1 molecular clone NL4-3 in the presence or absence of graded concentrations of ET4. P4CCR5 cells express CD4 and CCR5 coreceptors and produce β-galactosidase upon expression of the HIV-1 viral *tat* gene after infection. Therefore, reduction of viral infectivity could be determined by counting β-galactosidase-positive cells. ET4 clearly reduced HIV-1 infectivity (IC_{50}, 2·01 μg ml^{-1}) in a dose-dependent manner (Fig. 6), while inhibition of cell proliferation was only observed at concentrations above 20 μg ml^{-1}. Thus, the vector-based assay technique described here could effectively identify an extract containing one or more compounds with anti-HIV activity.

DISCUSSION

The present study was primarily designed to evaluate the antiviral efficacy of selected Nigerian medicinal plants against HIV-1 using a viral vector-based assay that essentially screens for inhibitors of early steps in the HIV-1 replication cycle. As HIV-1 is an enveloped RNA virus, we decided as a matter of contrast, to also evaluate the activity of the bioactive extracts against a nonenveloped DNA virus (in this case, adenovirus-based vector). Therefore, the pilot screening of all the plant extracts was basically against the lentiviral (HIV-based) vector while the antiadenoviral screening was evaluated only with extracts that demonstra-

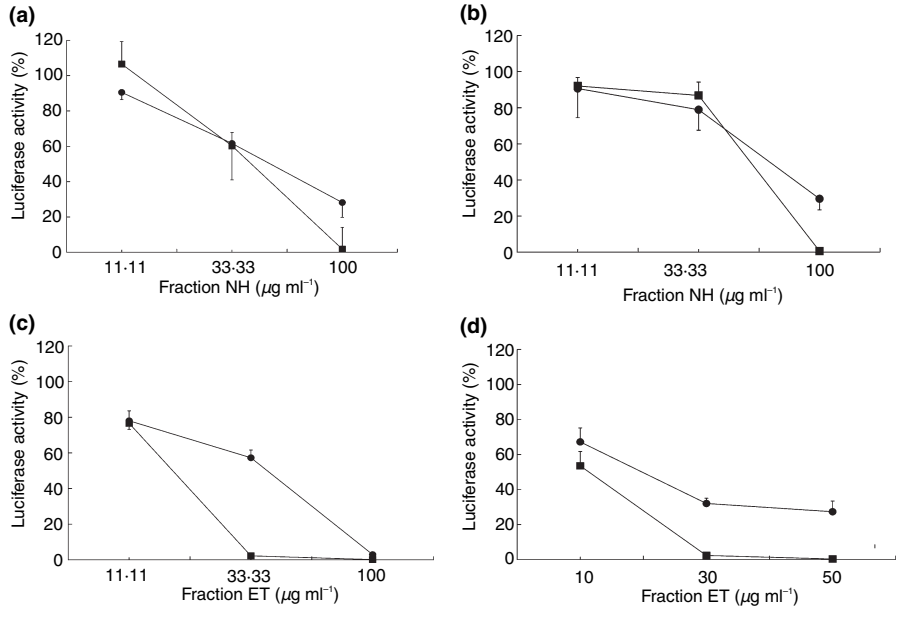

Fig. 3 Inhibitory effect of fractions h-hexane (NH) and ethyl acetate (ET) of *Ramalina farinacea* against viral vectors. Vector infectivity (■) in the presence of the indicated amounts of fraction NH with the lentiviral vector (a) or the adenoviral vector (b) or of fraction ET with the lentiviral vector (c) or the adenoviral vector (d). Cytotoxicity (●) of the extracts was evaluated in parallel as described in Fig. 2

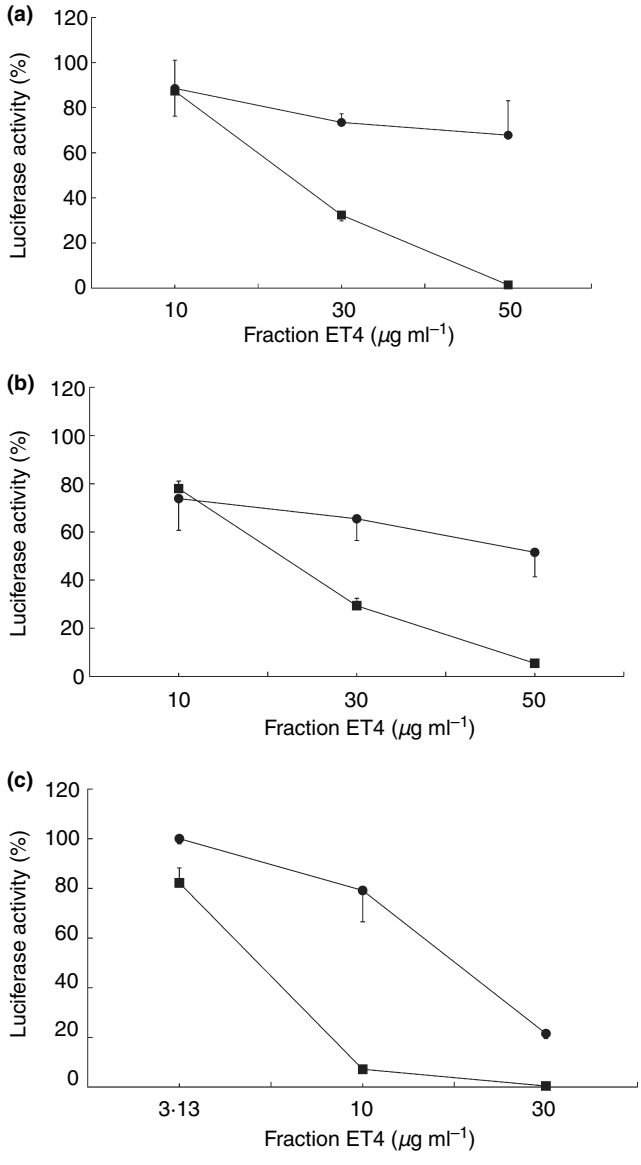

Fig. 4 Comparative analysis of the inhibition of lentiviral, adenoviral and retroviral vector infectivity by ET4. Vector infectivity (■) in the presence of the indicated amounts of fraction ET4 with a lentiviral vector (a), adenoviral vector (b) or retroviral vector (c) on HeLa (lentiviral and adenoviral vectors) or Hep2 target cells (retroviral vector). Cytotoxicity (●) of the extracts was evaluated in parallel on HeLa or Hep2 cells, respectively as described in Fig. 2

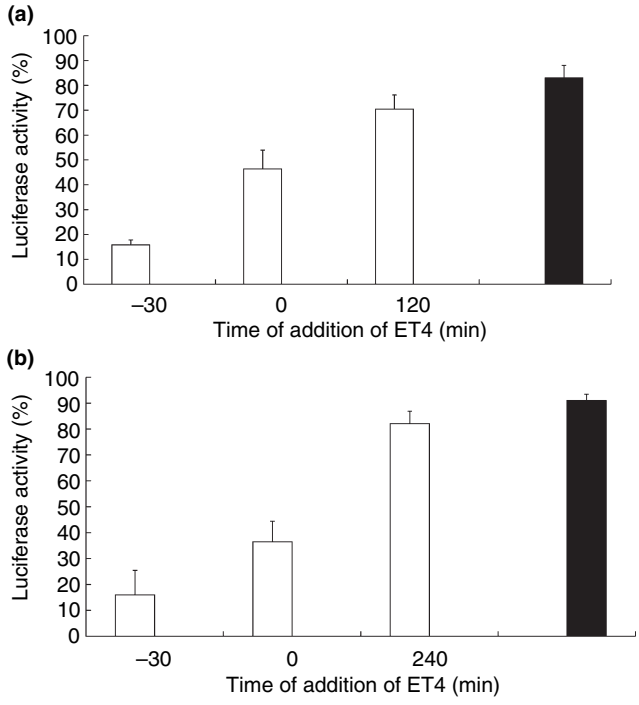

Fig. 5 Inhibitory kinetics of ET4 against lentiviral and adenoviral vectors. Lentiviral (a) or adenoviral (b) vectors were either preincubated with 20 μg ml^{-1} (a) or 30 μg ml^{-1} (b) of fraction ET4 for 30 min (−30) or added simultaneously (0) with same amount of ET4 to HeLa target cells. Target cells were also preincubated for 120 min with the lentiviral vector or for 240 min with the adenoviral vector prior to addition of ET4. Vector infectivity (white bars) is given as percentage luciferase activity of target cells 2 days (a) or 1 day (b) after transduction with the vectors in the absence of ET4. Cytotoxicity (black bars) was evaluated in parallel as described in Fig. 2

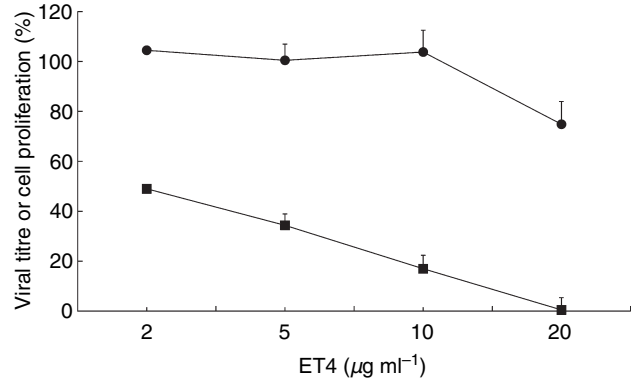

Fig. 6 Inhibitory effect of ET4 on wild-type HIV-1. Wild-type HIV-1 derived from the molecular clone NL4-3 was incubated with increasing concentrations of ET4. The virus titre (■) was subsequently determined on P4CCR5 indicator cells and is expressed as percentage of the titre of the untreated HIV-1 virus stock. Cytotoxicity (●) of ET4 was determined in parallel using the 5-bromo-2′-deoxyuridine (BrdU) incorporation assay

ted antilentiviral activity. Antiviral activity is measured as reduction in the luciferase expression of target cells transduced with the viral vectors in the presence of various concentrations of the extracts while cytotoxicity is equally measured as reduction in luciferase activity of same target cells stably expressing luciferase treated with same concentrations of the extracts. The reduction in luciferase activities in vector transduced cells could either be due to inhibition of

the vector or due to cytotoxic effects or modulatory influences on cell metabolism. To discriminate between these possibilities target cell lines were first infected with the lentiviral vector stably transferring the luciferase gene. The stably transduced cells were than exposed to the extracts in the absence of vectors. Cytotoxic extracts (such as NH, Fig. 3a,b) demonstrated no differences in the reduction of luciferase activities in cultures infected with the lentiviral/adenoviral vectors and in cultures stably expressing luciferase. On the contrary, extracts with specific antiviral activities (such as ME and ET4, Figs 2b,c and 3a–c) reduced the luciferase activity of cultures infected with the lentiviral/adenoviral vectors much more than cultures stably expressing luciferase.

All the plants screened were indigenous to Nigeria and were selected either based on evidence of their use in Nigerian traditional medical practice for the treatment of various viral diseases (*Combretum* spp., *Ritchiea capparoides* and *Cajanus cajan*) or on evidence of reported antiviral activity of similar types of plants in other parts of the world (the lichens: *R. farinacea* and *P. perlata*). *Combretum micranthum* is one of the main constituents of an indigenous Nigerian antiviral remedy called 'Seven Keys to Power', i.e. used for the treatment of small pox, chicken pox and measles. Also, a herbalist in the eastern part of Nigeria uses extracts of *Combretum micranthum* in the treatment of his AIDS patients. *R. capparoides* is equally used by herbalists in the eastern part of Nigeria for the treatment of chicken pox, small pox and hepatitis, while *C. cajan* is used by cattle rearers in the northern parts to treat diverse viral diseases of cattles. Various workers have shown that lichens posses potent antiviral properties (Pengsuparp *et al.* 1995; Cohen *et al.* 1996; Neamati *et al.* 1997) and in a preliminary study, we showed, using a cytopathic effect reduction assay that a crude polysaccharide fraction from *P. perlata* inhibited the infectivity of yellow fever virus but not that of polio or infectious bursal disease virus (unpublished data).

The vector-based assay clearly identified plants with anti-HIV vector activity and those without. Amongst all the extracts of *C. micranthum* tested, no activity against the lentiviral vector was detected, although this plant is used traditionally by an herbalist to treat AIDS patients. Moreover, another species of *Combretum* (*Combretum paniculatum*) has also been shown to inhibit HIV-1 infectivity *in vitro* (Asres *et al.* 2001), although this activity was confined to the acetone extract (which was not used in the present study). Ferrea *et al.* (1993) demonstrated that the *in vitro* antiviral activity of a methanolic extract of *C. micranthum* leaves against HSV-1 and HSV-2 was present only in the extract dissolved 7 days before the assay but not in the freshly prepared extract. They attributed this to the presence of inactive precursors in the fresh extracts (identified as condensed catechinic tannins), which undergo alkaline auto-oxidation (when dissolved for 7 days) to form the active antiviral catechinic acid. We similarly dissolved the aqueous extract of *C. micranthum* stem bark and root for 7 days before vector-based assay against the HIV-1-based vector and still found them inactive (data not shown). Based on the present study therefore, the WA/methylene chloride extracts of *C. micranthum* and the methanolic extract of *Combretum dolchipeles* root are inactive against the lentiviral vector. However, as our assay only screens for envelope-independent inhibitors of early steps in the HIV replication cycle, potential benefits as antivirals cannot be ruled out.

Although some studies have shown that lichen metabolites posses anti-HIV activity (Pengsuparp *et al.* 1995; Cohen *et al.* 1996; Neamati *et al.* 1997), this is the first report of the antiviral activity of a tropical lichen in general, and of *R. farinacea* in particular. It has been proposed that plant derivatives that are effective against HIV are promising alternatives, especially for use in developing nations with little access to expensive western medicines (De Clercq 1995; Cowan 1999). In the light of this, this tropical antiviral lichen could be of great economic importance because of its easy accessibility. Moreover, the lichen also displays specific antiadenoviral activity. Given that disseminated adenovirus disease has been reported in patients with AIDS (Krilov *et al.* 1990; Hierholzer 1992) and that there are currently very limited and nonspecific therapeutic options for the treatment of adenovirus infections (Cook 1993; Wildner *et al.* 2003), the dual inhibition of HIV-1 and adenovirus by these lichen extracts is desirable. What is unclear at the moment is whether the antilentiviral and antiadenoviral activities are mediated by the same or different compounds in the bioactive ET4. Further bioassay-guided fractionation will reveal this. However, if medicinal plants are to be effectively utilized in developing countries for the treatment of life-threatening ailments such as viral diseases, then whole plant extracts (and not pure compounds derived from them) would have to be employed, because of their ease of production, possible intracomponent synergism and broad-spectrum of action.

Based on the time-of-addition studies, the active lichen fraction (ET4) was shown to principally inhibit early steps in the lentiviral replication cycle. This suggests that ET4 either directly interacted with the vector particles, inhibiting the envelop protein or that it interacts with producer cell-derived components of the viral particle. Because lentiviral vectors and retroviral vectors were inhibited to a similar degree although they do not share any viral protein, the target for ET4 on the vector particles is probably cell-derived. This could either be the lipid membrane derived from the cell, which surrounds the vector particles or cellular membrane proteins that are frequently incorporated in lentiviral and retroviral particles during budding (Gould *et al.* 2003). For the adenoviral vector, the results are

consistent with inhibition of the viral absorption step. Charge-based interactions of one or more components of the extract with viral surface proteins leading to inhibition of absorption might also explain the broad inhibitory effects observed, but virucidal effects cannot be excluded either.

We further demonstrated that the activity of ET4 against the wild-type HIV-1 was more than 10-fold stronger than against the lentiviral vector. Given that the recombinant infectious lentiviral vector contained less viral sequences than the wild-type HIV-1, we had expected the activity of the extract against the wild-type HIV-1 to at best, be equal to the activity against the lentiviral vector, but not considerably higher (10-fold) as observed. However, a similar correlation between anti-HIV-1 vector activity and anti-HIV activity of the porcine defensin, protegrin-1, has been recently demonstrated by Steinstraesser *et al.* (2005). In their study, the activity of protegrin-1 against the wild-type HIV-1 was shown to be more than threefold higher than the corresponding activity against lentiviral vector. These preliminary results at least suggest a positive correlation between the vector-based assay and anti-HIV activity of compounds that target early steps in the HIV-1 replication cycle.

Taken together, the vector-based assay is a suitable (rapid, safe, selective and reproducible) pharmacodynamic evaluation technique for pilot and mechanistic evaluation of antiviral medicinal plants. Using this technique, the ET fraction of the lichen, *R. farinacea*, indigenous to Nigeria, has been shown to contain antilentiviral, antiretroviral and antiadenoviral principles. The observed antiviral activities involve inhibition of early steps in the viral replication cycle.

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