1′*S*-1′-Acetoxychavicol acetate isolated from *Alpinia galanga* inhibits human immunodeficiency virus type 1 replication by blocking Rev transport

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AIDS remains a major global health concern. Despite a number of therapeutic advancements, there is still an urgent need to develop a new class of therapy for human immunodeficiency virus (HIV). Here, it was shown that 1′*S*-1′-acetoxychavicol acetate (ACA), a small molecular compound isolated from the rhizomes of *Alpinia galanga*, inhibited Rev transport at a low concentration by binding to chromosomal region maintenance 1 and accumulating full-length HIV-1 RNA in the nucleus, resulting in a block in HIV-1 replication in peripheral blood mononuclear cells. Additionally, ACA and didanosine acted synergistically to inhibit HIV-1 replication. Thus, ACA may represent a novel treatment for HIV-1 infection, especially in combination with other anti-HIV drugs.

INTRODUCTION

AIDS, caused by the human immunodeficiency virus (HIV), has become a serious threat to global public health in the last few decades. The current strategy for the treatment of HIV infection is called highly active antiretroviral therapy (HAART) and involves the use of agents that target the viral entry step and the reverse transcriptase and protease enzymes (Pani *et al.*, 2002; Witvrouw *et al.*, 2004). This therapy has changed the course of HIV infection dramatically. However, the rapid development of drug resistance has led to the emergence of HIV strains that are resistant to multiple anti-AIDS drugs (Biesert *et al.*, 1991; St Clair *et al.*, 1991; Najera *et al.*, 1994; Menéndez-Arias, 2002). Furthermore, these drugs have limited or transient benefits due to their adverse side effects (Tözsér, 2001). Therefore, the discovery and characterization of new anti-HIV agents is required for alternative therapeutic approaches to overcome the shortcomings of the currently available drugs.

Rev is an essential regulatory HIV-1 protein that binds unspliced and incompletely spliced viral mRNAs and mediates the transport of these mRNAs from the nucleus into the cytoplasm for translation into viral proteins. Translocation of Rev from the nucleus to the cytoplasm is crucially important for its function and for virus replication (Kalland *et al.*, 1994; Meyer & Malim, 1994). Blocking the Rev transport process would inhibit Rev function and this is a potential target for the development of antiviral therapies. In fact, several different strategies have been employed to block HIV-1 replication by inhibiting the Rev function (Matsukura *et al.*, 1989; Bevec *et al.*, 1992, 1996; Lee *et al.*, 1992; Zapp *et al.*, 1993; Duan *et al.*, 1994). However, only a few small organic compounds have been reported to block Rev export from the nucleus (Wolff *et al.*, 1997; Murakami *et al.*, 2002). In this study, we screened 600 different extracts from medicinal plants by using a Rev transport assay and identified 1′*S*-1′-acetoxychavicol acetate (ACA) isolated from *Alpinia galanga* as a novel and effective Rev transport inhibitor. We tested this activity further and found that ACA could compete with leptomycin B (LMB) for chromosomal region maintenance 1 (CRM1) binding and blocked full-length HIV-1 RNA export from the nucleus to the cytoplasm. Furthermore, we demonstrated that ACA inhibited >80 % of HIV-1 replication in peripheral blood mononuclear cells (PBMCs) at a concentration of 4 μM and its antiviral activity was found to be synergistic when delivered with other antiretroviral agents.

METHODS

Screening assay. A fission yeast, *Schizosaccharomyces pombe*, that expresses a fusion protein consisting of glutathione *S*-transferase (GST), the simian virus 40 T-antigen nuclear-localization signal (NLS), green fluorescent protein (GFP) and the Rev nuclear-export signal (RevNES) was utilized to perform a screening assay for Rev transport inhibition as described by Kudo *et al.* (1998). Briefly, *S. pombe* was grown in thiamine-free minimal medium for 24 h and cells were seeded in 96-well plates. The screening samples were added to each well at a concentration of 100 μg ml^{-1} in 1 % DMSO and incubated at 37 °C for 3 h. The distribution of the GST–NLS–GFP–RevNES fusion protein was monitored by fluorescence microscopy.

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Chemicals. ACA was isolated from the rhizomes of greater galangal (*A. galanga*) that was collected in China. Greater galangal (100 g dry weight) was extracted with methanol. The extract was purified by silica-gel column chromatography and eluted with n-hexane/ethyl acetate (at ratios of 20 : 1, 10 : 1, 5 : 1 and 1 : 1) and chloroform/methanol (1 : 1). The n-hexane/ethyl acetate 5 : 1 fraction was purified further by reverse-phase HPLC [Cosmosil 5C18-AR, methanol/water (4 : 1)] to obtain a pure compound (1·5 g). The chemical structure of this pure compound (R_F 0·4, silica gel, n-hexane/ethyl acetate 5 : 1) was identified as ACA by comparison of the spectral data of mass and nuclear magnetic resonance with that of reported compounds (De Pooter *et al.*, 1985; Morita & Itokawa, 1988). Didanosine (ddI) was purchased from Sigma.

Cell culture and preparation of HIV-1 stock. PBMCs were isolated from whole blood of healthy donors by density centrifugation with Ficoll-Hypaque (Sigma). PBMCs were cultured in RPMI 1640 containing 20 % fetal bovine serum (FBS) and 4 μg phytohaemagglutinin (PHA) ml^{-1} (Invitrogen). After 3 days, PHA-stimulated PBMCs were washed three times with PBS and cultured in RPMI 1640 containing 20 % FBS and 10 U interleukin 2 ml^{-1} (Boehringer Mannheim). Human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % FBS and 50 μg gentamicin ml^{-1}. Fresh viral stocks were generated by transient transfection of 293T cells with plasmids pNL4-3 and πSVJR-CSF bearing full-length infectious molecular clones of HIV-1 NL4-3 and JR-CSF, respectively, by calcium phosphate co-precipitation. Briefly, 100 μg plasmid DNA was mixed with 2 M $CaCl_2$ and added to an equal volume of 2× HEPES-buffered saline. The DNA mixture was then incubated with 293T cells (seeded at 9×10^6 cells per T75 flask) in the presence of 25 μM chloroquine for 7 h and then washed away. Three days post-transfection, virus-containing supernatants were collected and frozen at −80 °C until needed.

HIV p24 ELISA. An HIV-1 p24 ELISA was performed by using a commercially available kit (Perkin Elmer) according to the manufacturer's instructions. For measuring HIV-1 p24 antigen production in the supernatants, 100-fold dilutions of the supernatant were used. All ELISA measurements were performed in triplicate.

Antiviral assay. Analysis of the antiviral activity of ACA on HIV-1 replication was based on inhibition of p24 viral antigen production. Briefly, PHA-activated PBMCs were grown in 24-well plates. After spinning (to further enhance attachment of the virus to the target cells; spinfection) with NL4-3 or JR-CSF virus, cells were washed twice in PBS and replaced by fresh culture medium. Different concentrations of ACA and/or the reverse transcriptase inhibitor ddI were added at various times post-infection. Five days later, supernatants were collected and HIV-1 p24 was detected by ELISA as described above.

Cell-viability assay. Cell-viability assays were conducted with the CellTiter 96 Cell Proliferation assay system (Promega) according to the manufacturer's protocol. Briefly, PBMCs were plated into a flat-bottomed 96-well plate at a density of 10^5 cells per well in 100 μl medium and various concentrations of ACA (0·5, 1, 2, 3 and 4 μM) were added. After 48 h incubation, 2 ml methyltetrazolium salt (MTS) was mixed with phenazine methosulfate (100 μl) and 20 μl of the mixture was added to each well. The plate was incubated for 3 h at 37 °C and A_{490} was read on a spectrophotometer. The percentage of viable cells treated with ACA was normalized to untreated cells.

Competition assay. 293T cells (10^7) were cultured with 100 nM LMB (Sigma), ACA (0·5, 1, 2 or 4 μM) or 0·1 % ethanol for 2 h and then treated or not with 10 nM biotinylated LMB for 3 h. After the cells had been lysed with 0·1 % NP-40 in TBS [50 mM Tris/HCl (pH 7·4), 150 mM NaCl], the supernatant was prepared by centrifugation and treated with immobilized streptavidin-conjugated agarose beads (Sigma) in TBS under rotation for 24 h at 4 °C. Bound proteins were washed thoroughly and eluted by boiling in 30 μl SDS-PAGE sample buffer. Each eluted sample was separated by SDS-PAGE (10 % gel) and proteins were transferred to Immobilon PVDF membrane (Millipore). The membrane was probed with anti-CRM1 antibody (1 : 1000 dilution; Biocompare). Horseradish peroxidase-labelled secondary antibodies were detected by the Amersham Biosciences enhanced chemiluminescence system. As an internal control, the protein concentration of the supernatants was measured and the expression of β-actin was also detected by Western blot analysis.

RNA extraction and quantitative analysis. The cytoplasmic fraction was isolated by treatment with digitonin lysis buffer [50 mM HEPES/KOH (pH 7·5), 50 mM potassium acetate, 8 mM $MgCl_2$, 2 mM EDTA, 50 μg digitonin ml^{-1}] on ice for 10 min. The lysate was centrifuged for 5 min and the supernatant was collected as the cytoplasmic fraction. Pellets were resuspended in NP-40 lysis buffer [20 mM Tris/HCl (pH 7·5), 50 mM KCl, 10 mM NaCl, 1 mM EDTA, 0·5 % NP-40) and incubated on ice for 10 min. The resultant lysate was used as the nuclear fraction. Cytoplasmic RNA and nuclear RNA were extracted and purified from the cytoplasmic fraction and the nuclear fraction, respectively, by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The amount of extracted RNA was quantified by measuring A_{260}. RNA (1 μg) was treated with 1 U DNase I (Invitrogen) in a volume of 10 μl to remove contaminating DNA (room temperature for 10 min, 70 °C for 5 min). DNase I-treated RNA (300 ng) was reverse-transcribed by using a two-step reverse transcription kit (Applied Biosystems) in a final volume of 10 μl. Reverse transcription was performed for 60 min at 37 °C. The total cDNA volume of 10 μl was frozen until real-time quantitative PCR was performed. After thawing for PCR experiments, the cDNA was diluted in 90 μl distilled water and 5 μl diluted cDNA was used for each PCR. Real-time quantitative PCR was performed by using the ABI Prism 7700 Sequence Detection system (PE Applied Biosystems) for amplification and detection. PCR conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Each PCR was carried out in triplicate and contained 15 μl SYBR Green PCR master mix (Applied Biosystems) and 0·3 μM each gene-specific primer for human DBR1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in a 30 μl reaction volume. The HIV-1-specific primers JRFL51 (5′-CTGCTAGAGATTTTCCACACTGAC-3′; nt 1204–1227) and JRFL31 (5′-GCTGCTTGATGTCCCCCCACTGTG-3′; nt 1356–1333) were used to detect full-length HIV-1 RNA. Copies of full-length HIV-1 RNA were normalized against copies of GAPDH (endogenous reference), amplified by using primers 5′-GGTGGTCTCCTCTGACTTCAA-3′ (nt 840–860) and 5′-GTTGCTGTAGCCAAATTCGTTGT-3′ (nt 966–944) specific for GAPDH.

RESULTS

Screening for Rev transport inhibitors

More than 600 medical plants were extracted with methanol and a screening assay was performed to select Rev transport inhibitors. The extract obtained from greater galangal inhibited transport of the fusion protein of *S. pombe* from the nucleus to the cytoplasm at a concentration of 5 μg ml^{-1}. To determine the substance accounting for this inhibition of Rev transport, the methanol extract of greater galangal was fractionated further and the different fractions were tested. A dose-dependent inhibitory effect was

observed when incubated with the n-hexane/ethyl acetate 5 : 1 fraction, whilst other fractions did not show obvious effects (Fig. 1a). Therefore, the n-hexane/ethyl acetate 5 : 1 fraction was purified further to obtain the bioactive compound ACA (Fig. 1b). This compound completely inhibited the export of the RevNES fusion protein from the nucleus at 5 μM in comparison with the DMSO control (Fig. 1c, d).

ACA inhibits HIV-1 replication in PBMCs

To test whether ACA could be used to inhibit HIV-1 replication, PBMCs were infected with NL4-3 or JR-CSF virus in the presence of increasing concentrations of ACA. The antiviral activity of ACA on HIV-1 replication was monitored by determining the amount of p24 in supernatants at 5 days post-infection. Cells treated with ACA showed a dose-dependent inhibition of virus replication in comparison with control cells. The maximal inhibition of virus production by ACA at 4 μM was >80 % in the HIV-1 X4-tropic reporter NL4-3-infected or R5-tropic reporter JR-CSF-infected cells (Fig. 2a). This result demonstrated that ACA blocked the replication of both X4 and R5 HIV-1 significantly. The inhibition activity of ACA was also observed in both NL4-3- and JR-CSF-infected MT-2 cells (data not shown).

To investigate the time course of the inhibitory effect of ACA on HIV-1 replication, ACA was added to cells at different time points after infection with NL4-3 or JR-CSF virus. Supernatants were collected at 5 days post-infection and the amount of viral p24 was measured. The results showed that p24 levels were remarkably reduced at all time points in comparison with the control cells that were not treated with ACA, particularly at 0–6 h (Fig. 2b).

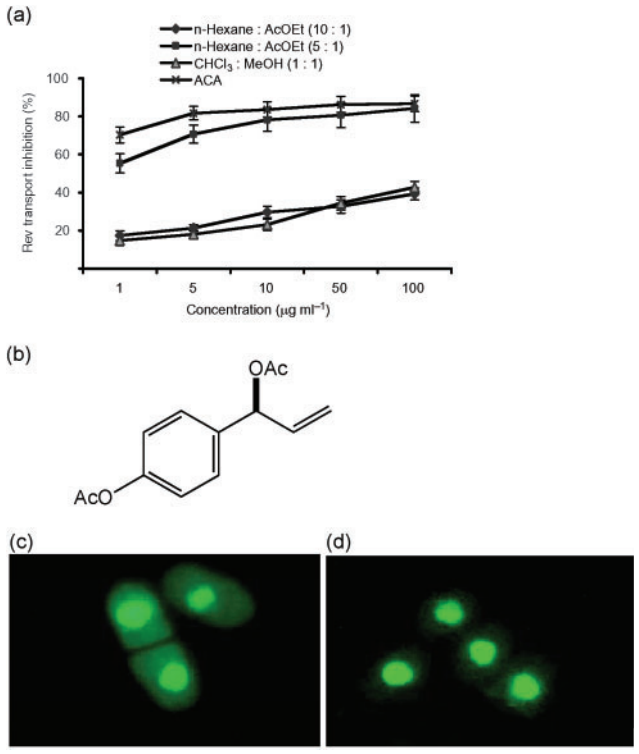

Fig. 1. (a) Dose-dependent inhibitory effect on Rev transport found in the n-hexane/ethyl acetate 5 : 1 fraction of greater galangal extract is similar to that of ACA. The n-hexane/ethyl acetate 10 : 1 fraction and all other similar fractions (data not shown) had no inhibitory effect, similar to the chloroform/methanol 1 : 1 fraction from the rest of the extract. Data represent the mean ± SD of three independent experiments. (b) The n-hexane/ethyl acetate 5 : 1 fraction was purified further by HPLC and identified as ACA. The chemical structure of ACA is shown. (c, d) A fission yeast, *S. pombe*, expressing a GST–NLS–GFP–RevNES fusion protein was treated with 1 % DMSO as a control (c) or with 5 μM ACA (d) and observed by fluorescence microscopy.

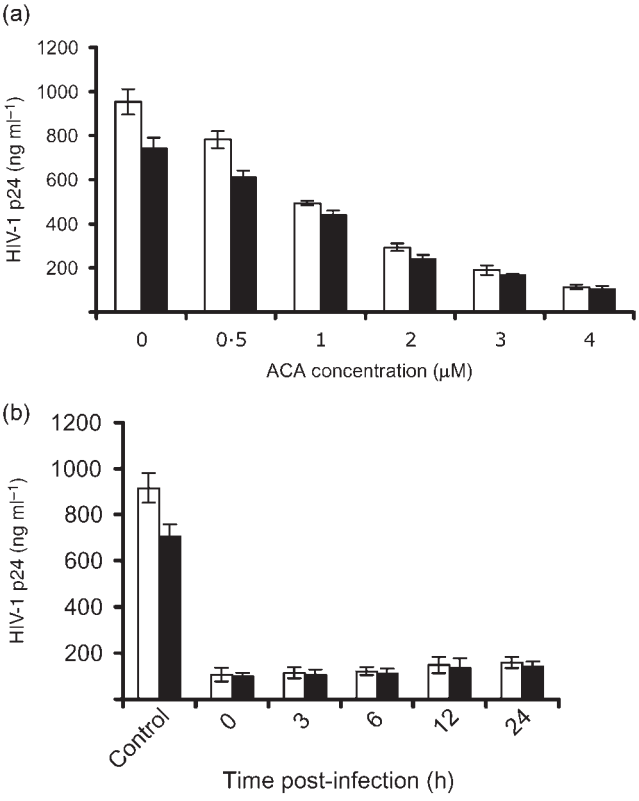

Fig. 2. ACA inhibits HIV-1 replication in PBMCs. (a) HIV-1 p24 detected by ELISA in infected cells treated with different concentrations of ACA. PHA-stimulated PBMCs were infected with HIV-1 NL4-3 (open bars) or JR-CSF (closed bars). Cells treated with ACA displayed dose-dependent inhibition. (b) HIV-1 p24 detected by ELISA in 4 μM ACA-treated infected cells at different time points. PHA-stimulated PBMCs were infected with HIV-1 NL4-3 (open bars) or JR-CSF (closed bars). Production of HIV-1 p24 was decreased significantly at all time points when ACA was added to the cells, in particular from 0 to 6 h post-infection. Data represent the mean ± SD of two experiments carried out in triplicate.

Effect of ACA in combination with ddI on HIV-1 replication

If ACA is able to target HIV-1 Rev export from the nucleus to the cytoplasm, it was expected that combination of ACA with another antiretroviral inhibitor, such as the reverse transcriptase inhibitor ddI or 3′-azido-3′-deoxythymidine (AZT), would be synergistic. To test this hypothesis, we evaluated the inhibition of NL4-3 (Fig. 3a) and JR-CSF (Fig. 3b) virus replication in PBMCs in the presence of various concentrations of ACA and ddI alone or in combination. The data showed that ACA plus ddI strengthened the inhibition of HIV-1 production significantly, compared with ACA or ddI alone. The same effect was also found by using AZT instead of ddI (data not shown). These results indicated that ACA acts synergistically with different target anti-HIV agents to inhibit HIV-1 replication.

ACA has no significant toxic effect on cells at the effective anti-HIV concentration

The cytotoxicity of ACA was determined in parallel with the antiviral activity assay. We used a commercial assay for cellular mitochondrial metabolism to determine whether ACA had a cytotoxic effect. PBMCs were incubated with 0·5, 1, 2, 3 or 4 μM ACA for 48 h and mitochondrial electron transport was analysed by its ability to reduce the tetrazolium salt MTS to produce formazan using a commercial kit. The result showed that ACA had no significant cytotoxic effect on the host cells within the concentration range at which the antiviral activity was effective (Fig. 4). Although previous studies have identified LMB as a strong inhibitor of Rev transport, it is not an acceptable candidate for anti-HIV drug development due to its severe cytotoxicity (Wolff *et al.*, 1997). In contrast, our result suggests that ACA is a safer HIV-1 replication inhibitor.

ACA inhibits CRM1-dependent nuclear export

It is known that LMB is an inhibitor of the nuclear export of HIV-1 Rev. This inhibition is reported to be mediated by LMB binding directly to CRM1, a receptor for the nuclear export signal (Kudo *et al.*, 1999). Our studies determined that ACA inhibited the nuclear export of the HIV-1 RevNES protein and showed high-level inhibition of HIV-1 replication in PBMCs. We hypothesized that the interaction between ACA and CRM1 was probably the same as that with LMB.

To confirm this hypothesis, ACA was used in an *in vitro* competition assay with biotinylated LMB for CRM1 binding. 293T cells were cultured with 10 nM biotinylated LMB for 2 h, biotin-containing complexes were isolated by using streptavidin-conjugated agarose beads and bound proteins were analysed by SDS-PAGE followed by Western blotting. Western blot analysis using an anti-CRM1 antibody showed that CRM1 was detected when the cell culture contained biotinylated LMB or a lower concentration of ACA. However, when 4 μM ACA (as a competitor) or 0·1 μM LMB (as a control competitor) was added to the cell culture 2 h before the biotinylated LMB was added, CRM1 was not detected (Fig. 5). In the competition experiment, ACA was

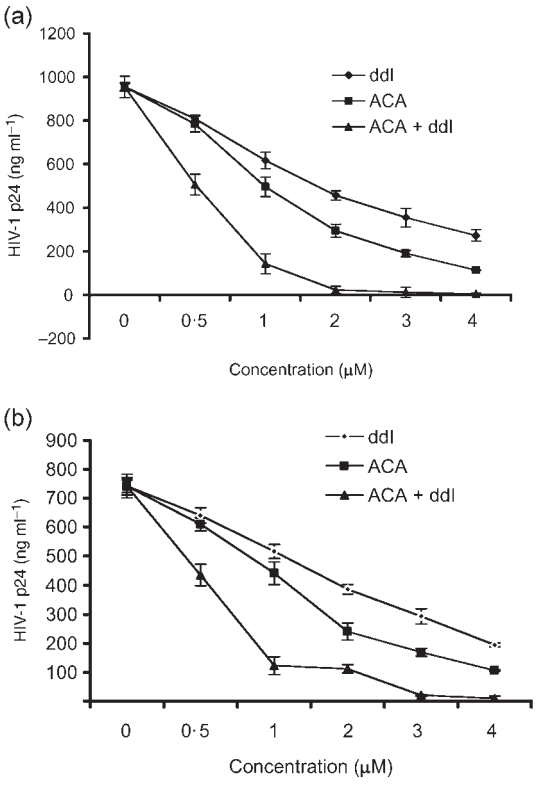

Fig. 3. PHA-stimulated PBMCs were infected with HIV-1 NL4-3 (a) or JR-CSF (b). Cells were washed three times in PBS and fresh medium was added. Various concentrations of ACA and/or ddI were added. Virus replication was quantified by HIV-1 p24 ELISA from the culture supernatant at 5 days post-infection. Data represent the mean ± SD of two experiments carried out in triplicate.

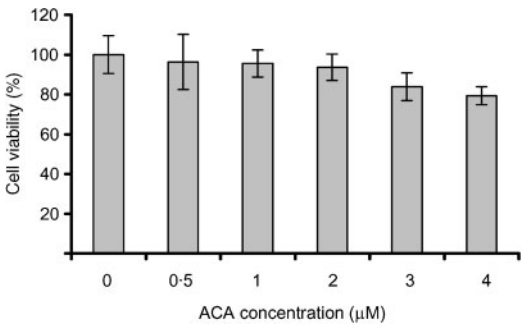

Fig. 4. Determination of cell viability. No significant cytotoxic effect was observed on the host cells within an ACA concentration ranging from 0·5 to 4 μM, the concentration at which antiviral activity was effective. Data are shown as the mean ± SD from two experiments carried out in triplicate and normalized to control cells.

required at a higher concentration than LMB, but 0·1 μM LMB showed strong cytotoxicity. These results indicated that ACA competes with LMB for CRM1 binding in the cells.

ACA causes the accumulation of full-length HIV-1 RNA in the nucleus

To demonstrate directly that ACA inhibits Rev transport, we investigated Rev activity in the nucleus and cytoplasm. Previous studies have reported that Rev facilitates the nuclear export of unspliced and partially spliced viral mRNAs (Felber *et al.*, 1989; Hadzopoulou-Cladaras *et al.*, 1989; Malim *et al.*, 1989). If ACA inhibits Rev function, the unspliced and partially spliced viral mRNAs would accumulate in the nucleus. Therefore, we determined the quantity of cytoplasmic and nuclear full-length viral RNA in host cells to confirm whether ACA contributed to Rev-specific inhibition.

In this experiment, PBMCs were infected with JR-CSF virus in the absence or presence of increasing concentrations of ACA. Five days after infection, HIV-1 RNA in the cytoplasm and nucleus was extracted and the amount of full-length RNA was determined by using the specific primer pair JRFL51 (nt 1204–1227) and JRFL31 (nt 1356–1333) by real-time quantitative RT-PCR. An internal control was performed in which an equal amount of RNA from each cytoplasmic and nuclear fraction was analysed by real-time quantitative RT-PCR using primer pairs specific for cellular GAPDH mRNA. We observed that unspliced HIV-1 RNA showed a dose-dependent accumulation in the nucleus as expected. When cells were treated with 0·5 μM ACA, the accumulation of viral nuclear unspliced RNA showed a slight increase, whilst 4 μM ACA led to a significant increase (1·9-fold) in comparison with the untreated control (Fig. 6a). On the other hand, ACA resulted in a dose-dependent reduction of full-length RNA in the cytoplasm (Fig. 6b). This result was consistent with the data obtained from the LMB competition assay and indicated that ACA inhibits HIV function by blocking Rev transport.

DISCUSSION

The Rev protein is an essential factor for HIV-1 replication and promotes the export of unspliced or partially spliced mRNA responsible for the production of the viral structural proteins (Malim *et al.*, 1989). This protein is a potential target for the development of antiviral therapies. Currently reported Rev inhibitors target mainly the interaction

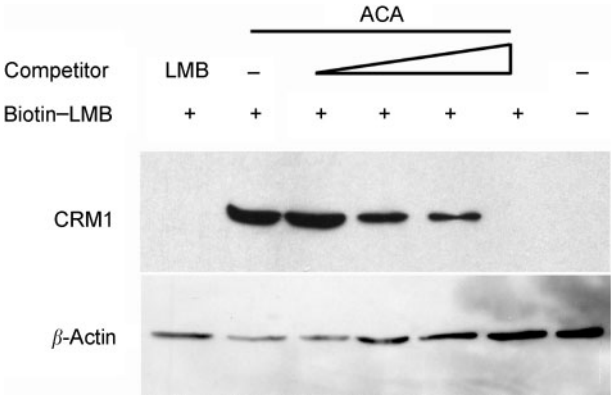

Fig. 5. Interaction of ACA with CRM1. 293T cells were incubated with 100 nM LMB (lane 1), 0, 0·5, 1, 2 or 4 μM ACA (lanes 2–6, respectively) or 0·1 % ethanol (lane 7) for 2 h and then treated with (lanes 1–6) or without (lane 7) 10 nM biotinylated LMB for 3 h. After cell extracts had been prepared, biotin-containing complexes were isolated by using immobilized streptavidin-conjugated agarose beads. Western blot analysis of the proteins probed with anti-CRM1 antibody (upper panel) or β-actin antibody (lower panel, prior to being subjected to the immobilized streptavidin) was performed.

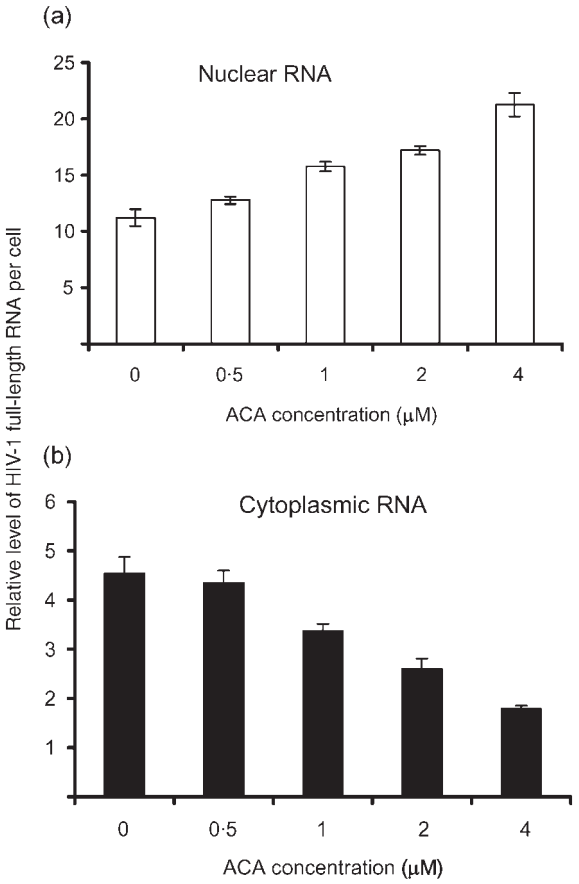

Fig. 6. Nuclear accumulation of unspliced HIV-1 RNA by ACA. PHA-stimulated PBMCs were infected with HIV-1 JR-CSF and left untreated or treated with various concentrations of ACA (0·5, 1, 2 or 4 μM). At 5 days post-infection, nuclear RNA (a) and cytoplasmic RNA (b) were extracted, treated with DNase I and used as template in real-time quantitative RT-PCR. Each reaction included 300 ng RNA and 0·5 μM each primer for full-length HIV-1 RNA or GAPDH amplification. GAPDH was used as an internal control. Results are shown as the mean ± SD from triplicate samples in three experiments.

between Rev and the Rev-responsive element (Ratmeyer *et al.*, 1996; Zapp *et al.*, 1997). In this paper, we reported that the low-molecular-mass compound ACA, isolated from the rhizomes of *A. galanga*, inhibited Rev transport completely in an *in vitro* yeast model and reduced HIV-1 replication by $>80\,\%$ in PBMCs. To investigate the mechanism of inhibition, we performed a competition binding assay with biotinylated LMB to test whether ACA had the same target as LMB. Our results showed clearly that ACA competed with biotinylated LMB in binding with CRM1. This result suggested that, similar to LMB, ACA may inhibit HIV-1 replication by blocking Rev transport via the CRM1 pathway. It has been reported that the Rev transport inhibitor LMB was identified as blocking HIV-1 replication. The inhibitory mechanism suggested that LMB bound covalently and selectively to the thiol group of Cys-529 in CRM1 via its α,β-unsaturated δ-lactone (Kudo *et al.*, 1999). Whether ACA binds to the same thiol group of Cys-529 or to another site on CRM1, and the type of functional group of ACA that is involved in its binding to CRM1, are currently under investigation.

ACA was first isolated from the rhizomes of *A. galanga* and found to prevent the growth of various fungi (Janssen & Scheffer, 1985). It was also reported that ACA showed anti-tuberculosis and anti-allergy activity (Palittapongarnpim *et al.*, 2002; Matsuda *et al.*, 2003; Yoshikawa *et al.*, 2004). Furthermore, numerous studies have demonstrated that ACA suppresses the development of many tumours, such as skin cancer, oral cancer, colon cancer, liver cancer, bile-duct cancer and oesophageal cancer *in vivo* (Murakami *et al.*, 1996; Ohnishi *et al.*, 1996; Tanaka *et al.*, 1997a, b; Kobayashi *et al.*, 1998; Kawabata *et al.*, 2000; Miyauchi *et al.*, 2000), but the mechanism is less well understood. Recently, ACA was found to inhibit beta interferon mRNA expression and nuclear factor κB activation in lipopolysaccharide-activated mouse peritoneal macrophages, resulting in inhibition of the production of nitric oxide (Ando *et al.*, 2005). In this study, we have discovered a new function for ACA. ACA was able to compete with LMB, an inhibitor of Rev transport, in binding to CRM1, suggesting that ACA, similar to LMB, probably blocks Rev transport via CRM1-mediated export pathways. We examined and compared quantitatively the amount of unspliced HIV-1 RNA in the nucleus and cytoplasm in the presence and absence of ACA. The results showed that ACA was able to increase full-length RNA accumulation in the nucleus and reduce the amount of full-length RNA in the cytoplasm, which suggests that ACA is involved in the inhibition of Rev–RNA transport from the nucleus to the cytoplasm.

The currently available HAART uses two non-nucleoside agents and one nucleoside reverse transcriptase inhibitor or two non-nucleoside reverse transcriptase inhibitors and one protease inhibitor. The significant disadvantages associated with these therapies are severe drug side effects and viral escape mutants. As a Rev transport inhibitor that targets a different stage of the HIV-1 replication cycle, ACA may produce favourable interactions with other agents to overcome these issues. We observed the synergistic effect of ACA with the reverse transcriptase inhibitor ddI and with AZT. Despite the fact that the two drugs act at different stages of the HIV-1 life cycle, the reasons for the synergistic interaction are not clear. However, these results indicate that ACA may significantly enhance the antiviral activity with other anti-HIV-1 drugs and suggests that ACA possesses advantages when combined with some of the currently used nucleoside analogues.

In conclusion, the development of the low-cost, low-cytotoxicity Rev transport inhibitor ACA is a promising approach towards providing novel antiretroviral therapies. When combined with other anti-HIV agents, ACA is extremely effective in inhibiting HIV-1 replication.

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REFERENCES

Ando, S., Matsuda, H., Morikawa, T. & Yoshikawa, M. (2005). 1′*S*-1′-Acetoxychavicol acetate as a new type inhibitor of interferon-β production in lipopolysaccharide-activated mouse peritoneal macrophages. *Bioorg Med Chem* **13**, 3289–3294.

Bevec, D., Dobrovnik, M., Hauber, J. & Böhnlein, E. (1992). Inhibition of human immunodeficiency virus type 1 replication in human T cells by retroviral-mediated gene transfer of a dominant-negative Rev trans-activator. *Proc Natl Acad Sci U S A* **89**, 9870–9874.

Bevec, D., Jaksche, H., Oft, M. & 9 other authors (1996). Inhibition of HIV-1 replication in lymphocytes by mutants of the Rev cofactor eIF-5A. *Science* **271**, 1858–1860.

Biesert, L., Zimmermann, F., Schroder, B., Matthes, E., Suhartono, H., Dietrich, U. & Rubsamen-Waigmann, H. (1991). Biochemical and genetical analysis of AZT-resistant HIV-mutants. *Behring Inst Mitt* **89**, 74–80.

De Pooter, H. L., Omar, M. N., Coolsaet, B. A. & Schamp, N. M. (1985). The essential oil of greater galanga (*Alpinia galanga*) from Malaysia. *Phytochemistry* **24**, 93–96.

Duan, L., Bagasra, O., Laughlin, M. A., Oakes, J. W. & Pomerantz, R. J. (1994). Potent inhibition of human immunodeficiency virus type 1 replication by an intracellular anti-Rev single-chain antibody. *Proc Natl Acad Sci U S A* **91**, 5075–5079.

Felber, B. K., Hadzopoulou-Cladaras, M., Cladaras, C., Copeland, T. & Pavlakis, G. N. (1989). rev protein of human immunodeficiency virus type 1 affects the stability and transport of the viral mRNA. *Proc Natl Acad Sci U S A* **86**, 1495–1499.

Hadzopoulou-Cladaras, M., Felber, B. K., Cladaras, C., Athanassopoulos, A., Tse, A. & Pavlakis, G. N. (1989). The *rev* (*trs*/*art*) protein of human immunodeficiency virus type 1 affects viral mRNA and protein expression via a *cis*-acting sequence in the *env* region. *J Virol* **63**, 1265–1274.

Janssen, A. M. & Scheffer, J. J. C. (1985). Acetoxyhydroxychavicol acetate, an antifungal component of *Alpinia galanga*. *Planta Med* **6**, 507–511.

Kalland, K.-H., Szilvay, A. M., Brokstad, K. A., Sætrevik, W. & Haukenes, G. (1994). The human immunodeficiency virus type 1 Rev protein shuttles between the cytoplasm and nuclear compartments. *Mol Cell Biol* **14**, 7436–7444.

Kawabata, K., Tanaka, T., Yamamoto, T. & 7 other authors (2000). Suppression of *N*-nitrosomethylbenzylamine-induced rat esophageal tumorigenesis by dietary feeding of 1′-acetoxychavicol acetate. *Jpn J Cancer Res* **91**, 148–155.

Kobayashi, Y., Nakae, D., Akai, H. & 9 other authors (1998). Prevention by 1′-acetoxychavicol acetate of the induction but not growth of putative preneoplastic, glutathione *S*-transferase placental form-positive, focal lesions in the livers of rats fed a choline-deficient, L-amino acid-defined diet. *Carcinogenesis* **19**, 1809–1814.

Kudo, N., Wolff, B., Sekimoto, T., Schreiner, E. P., Yoneda, Y., Yanagida, M., Horinouchi, S. & Yoshida, M. (1998). Leptomycin B inhibition of signal-mediated nuclear export by direct binding to CRM1. *Exp Cell Res* **242**, 540–547.

Kudo, N., Matsumori, N., Taoka, H., Fujiwara, D., Schreiner, E. P., Wolff, B., Yoshida, M. & Horinouchi, S. (1999). Leptomycin B inactivates CRM1/exportin 1 by covalent modification at a cysteine residue in the central conserved region. *Proc Natl Acad Sci U S A* **96**, 9112–9117.

Lee, T. C., Sullenger, B. A., Gallardo, H. F., Ungers, G. E. & Gilboa, E. (1992). Overexpression of RRE-derived sequences inhibits HIV-1 replication in CEM cells. *New Biol* **4**, 66–74.

Malim, M. H., Hauber, J., Le, S.-Y., Maizel, J. V. & Cullen, B. R. (1989). The HIV-1 rev *trans*-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. *Nature* **338**, 254–257.

Matsuda, H., Morikawa, T., Managi, H. & Yoshikawa, M. (2003). Antiallergic principles from *Alpinia galanga*: structural requirements of phenylpropanoids for inhibition of degranulation and release of TNF-α and IL-4 in RBL-2H3 cells. *Bioorg Med Chem Lett* **13**, 3197–3202.

Matsukura, M., Zon, G., Shinozuka, K. & 7 other authors (1989). Regulation of viral expression of human immunodeficiency virus *in vitro* by an antisense phosphorothioate oligodeoxynucleotide against *rev* (*art*/*trs*) in chronically infected cells. *Proc Natl Acad Sci U S A* **86**, 4244–4248.

Menéndez-Arias, L. (2002). Targeting HIV: antiretroviral therapy and development of drug resistance. *Trends Pharmacol Sci* **23**, 381–388.

Meyer, B. E. & Malim, M. H. (1994). The HIV-1 Rev trans-activator shuttles between the nucleus and the cytoplasm. *Genes Dev* **8**, 1538–1547.

Miyauchi, M., Nishikawa, A., Furukawa, F., Nakamura, H., Son, H.-Y., Murakami, A., Koshimizu, K., Ohigashi, H. & Hirose, M. (2000). Inhibitory effects of 1′-acetoxychavicol acetate on *N*-nitrosobis(2-oxopropyl)-amine-induced initiation of cholangiocarcinogenesis in Syrian hamsters. *Jpn J Cancer Res* **91**, 477–481.

Morita, H. & Itokawa, H. (1988). Cytotoxic and antifungal diterpenes from the seeds of *Alpinia galanga*. *Planta Med* **54**, 117–120.

Murakami, A., Ohura, S., Nakamura, Y., Koshimizu, K. & Ohigashi, H. (1996). 1′-Acetoxychavicol acetate, a superoxide anion generator inhibitor, potently inhibits tumor promotion by 12-*O*-tetradecanoylphorbol-13-acetate in ICR mouse skin. *Oncology* **53**, 386–391.

Murakami, N., Ye, Y., Kawanishi, M., Aoki, S., Kudo, N., Yoshida, M., Nakayama, E. E., Shioda, T. & Kobayashi, M. (2002). New Rev-transport inhibitor with anti-HIV activity from *Valerianae radix*. *Bioorg Med Chem Lett* **12**, 2807–2810.

Najera, I., Richman, D. D., Olivares, I., Rojas, J. M., Peinado, M. A., Perucho, M., Najera, R. & López-Galíndez, C. (1994). Natural occurrence of drug resistance mutations in the reverse transcriptase of human immunodeficiency virus type 1 isolates. *AIDS Res Hum Retroviruses* **10**, 1479–1488.

Ohnishi, M., Tanaka, T., Makita, H. & 7 other authors (1996). Chemopreventive effect of a xanthine oxidase inhibitor, 1′-acetoxychavicol acetate, on rat oral carcinogenesis. *Jpn J Cancer Res* **87**, 349–356.

Palittapongarnpim, P., Kirdmanee, C., Kittakoop, P. & Rukseree, K. (2002). *1′-Acetoxychavicol Acetate for Tuberculosis Treatment*. US Patent Application no. 2002192262.

Pani, A., Loi, A. G., Mura, M., Marceddu, T., La Colla, P. & Marongiu, M. E. (2002). Targeting HIV: old and new players. *Curr Drug Targets Infect Disord* **2**, 17–32.

Ratmeyer, L., Zapp, M. L., Green, M. R., Vinayak, R., Kumar, A., Boykin, D. W. & Wilson, W. D. (1996). Inhibition of HIV-1 Rev-RRE interaction by diphenylfuran derivatives. *Biochemistry* **35**, 13689–13696.

St Clair, M. H., Martin, J. L., Tudor-Williams, G., Bach, M. C., Vavro, C. L., King, D. M., Kellam, P., Kemp, S. D. & Larder, B. A. (1991). Resistance to ddI and sensitivity to AZT induced by a mutation in HIV-1 reverse transcriptase. *Science* **253**, 1557–1559.

Tanaka, T., Kawabata, K., Kakumoto, M. & 8 other authors (1997a). Chemoprevention of azoxymethane-induced rat colon carcinogenesis by a xanthine oxidase inhibitor, 1′-acetoxychavicol acetate. *Jpn J Cancer Res* **88**, 821–830.

Tanaka, T., Makita, H., Kawamori, T. & 7 other authors (1997b). A xanthine oxidase inhibitor 1′-acetoxychavicol acetate inhibits azoxymethane-induced colonic aberrant crypt foci in rats. *Carcinogenesis* **18**, 1113–1118.

Tözsér, J. (2001). HIV inhibitors: problems and reality. *Ann N Y Acad Sci* **946**, 145–159.

Witvrouw, M., van Maele, B., Vercammen, J., Hantson, A., Engelborghs, Y., de Clercq, E., Pannecouque, C. & Debyser, Z. (2004). Novel inhibitors of HIV-1 integration. *Curr Drug Metab* **5**, 291–304.

Wolff, B., Sanglier, J. J. & Wang, Y. (1997). Leptomycin B is an inhibitor of nuclear export: inhibition of nucleo-cytoplasmic translocation of the human immunodeficiency virus type 1 (HIV-1) Rev protein and Rev-dependent mRNA. *Chem Biol* **4**, 139–147.

Yoshikawa, M., Matsuda, H. & Morikawa, T. (2004). *Antiallergy agent obtained from Alpinia galanga and method for production thereof*. Jpn Kokai Tokkyo Koho patent no. 2004189669 (in Japanese).

Zapp, M. L., Stern, S. & Green, M. R. (1993). Small molecules that selectively block RNA binding of HIV-1 Rev protein inhibit Rev function and viral production. *Cell* **74**, 969–978.

Zapp, M. L., Young, D. W., Kumar, A., Singh, R., Boykin, D. W., Wilson, W. D. & Green, M. R. (1997). Modulation of the rev-RRE interaction by aromatic heterocyclic compounds. *Bioorg Med Chem* **5**, 1149–1155.