Biochemical Pharmacology, Vol. 55, pp. 1807–1818, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(98)00053-7

Antiretroviral Effects of Deoxyhypusyl Hydroxylase Inhibitors

A HYPUSINE-DEPENDENT HOST CELL MECHANISM FOR REPLICATION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1)

Linda Andrus,‡ Paul Szabo,†‡‖ Robert W. Grady, Axel-R. Hanauske,§¶ Tellervo Huima-Byron,‡ Bozena Slowinska,* Sylwia Zagulska* and Hartmut M. Hanauske-Abel*‖*

Departments of *Pediatrics and †Medicine, Cornell University Medical College—The New York Hospital, New York, NY; ‡The New York Blood Center, New York, NY, U.S.A.; §Department of Medicine, Technische Universitat, München, Germany

ABSTRACT. The HIV-1 protein Rev, critical for translation of incompletely spliced retroviral mRNAs encoding capsid elements, requires a host cell protein termed "eukaryotic initiation factor 5A" (eIF-5A). This is the only protein containing hypusine, a lysine-derived hydroxylated residue that determines its proposed bioactivity, the translation of a subset of cellular mRNAs controlling G_1-to-S transit of the cell cycle. We postulated that inhibiting the hypusine-forming deoxyhypusyl hydroxylase (DOHH) should, by depleting eukaryotic initiation factor 5A, compromise Rev function and thus reduce HIV-1 multiplication. We now report that the α-hydroxypyridones, specifically mimosine, a natural product, and deferiprone, an experimental drug, inhibited deoxyhypusyl hydroxylase in T-lymphocytic and promonocytic cell lines and, in a concentration-dependent manner, suppressed replication of HIV-1. However, the α-hydroxypyridones did not affect the formation of unspliced or multiply spliced HIV-1 transcripts. Rather, these agents caused Rev-dependent incompletely spliced HIV-1 mRNA such as gag, but not cellular "housekeeping" mRNAs, to disappear from polysomes. Consequently, α-hydroxypyridone-mediated depletion of eIF-5A decreased biosynthesis of structural HIV-1 protein encoded by *gag*, measured as p24, whereas the induced formation of cellular protein like tumor necrosis factor alpha remained unaffected. By interfering with the translation of incompletely spliced retroviral mRNAs, these compounds restrict HIV-1 to the early, nongenerative phase of its reproductive cycle. In the inducibly HIV-1 expressing T-cell line ACH-2, the deoxyhypusyl hydroxylase inhibitors triggered extensive apoptosis, particularly of cells that actively produce HIV-1. Selective suppression of retroviral protein biosynthesis and preferential apoptosis of retrovirally infected cells by α-hydroxypyridones point to a novel mode of antiretroviral action. BIOCHEM PHARMACOL **55**;11:1807–1818, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. HIV/AIDS; apoptosis; antiviral agents; deferiprone; mimosine; hypusine

The function of several biological systems, e.g. blood coagulation or collagen formation, rests entirely on the post-translational modification of certain amino acid residues. Among the various post-translational modifications, the stereospecific hydroxylations of aspartate, asparagine, proline, and lysine are particularly prominent. Apart from these common residues, the deoxyhypusyl moiety also becomes hydroxylated, the latter occurring exclusively in one intracellular protein, the precursor for eIF-5A**. Deoxyhypusyl itself is formed by the post-translational modification of a specific lysyl residue, mediated by deoxyhypusyl synthase (EC 1.5.1.–). Subsequent stereospecific hydroxylation of its C9 carbon atom by DOHH (EC 1.14.99.29) produces hypusine [N^{ε}-(4-amino-2(*R*)-hydroxybutyl)-L-lysine] [1–3]. The active sites of the metalloenzymes catalyzing the formation of hydroxylated residues display a remarkably similar architecture [4–7]. Hypusine formation, which does not occur in prokaryotes [2],

‖ Corresponding author: Dr. Hanauske-Abel or Dr. Szabo. Dr. Hanauske-Abel can be reached at: Hartmut M. Hanauske-Abel, M.D. Ph.D., Cornell University Medical College, Department of Pediatrics, Division of Endocrinology-MBL, Rm. LC-604, 525 East 68th St., New York, NY 10021. Tel. (212) 746-1894; FAX (212) 746-0300; E-mail: hmhanaus@mail.med.cornell.edu.

Dr. Paul Szabo can be reached at: Cornell University Medical College, Department of Medicine, Division of Geriatrics, Rm. A-453, 1300 York Ave., New York, NY 10021. Tel. (212) 746-8455; FAX (212) 746-6382; E-mail: pszabo@mail.med.cornell.edu.

¶ Current address: Department of Oncology, Universitaire Ziekenhuizen Gasthuisberg, Leuven, Belgium.

Received 9 September 1997; accepted 29 December 1997.

** *Abbreviations:* CIM, cell infection medium; DOHH, deoxyhypusyl hydroxylase; eIF-5A, eukaryotic initiation factor 5A; GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; PMA, phorbol myristate acetate; RRE, Rev-responsive element; RT-PCR, reverse transcriptase-polymerase chain reaction; TCID, tissue culture infectious doses; TNF-α, tumor necrosis factor alpha; and TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling.

determines a unique conformational energy minimum in the folding pathway of eIF-5A [8]. The hypusine residue is indispensable for the function of eIF-5A in eukaryotic cell proliferation [3, 9, 10], and the hypusine-containing domain of eIF-5A has remained unchanged from archaebacteria and fungi to plants, insects, and mammals [3].

We recently presented direct evidence that transition from the G_1 to the S phase of the eukaryotic cell cycle is a hypusine-sensitive event [3] and proposed that eIF-5A, while not required for general protein biosynthesis [11], is required for translation of specific mRNAs encoding proteins that enable and commit cells to initiate DNA replication [3]. We demonstrated the existence of hypusine-dependent messenger RNAs (*hymns*) that apparently encode a diverse family of replication-related proteins. Polysomal localization of *hymns* directly correlates with hypusine formation and precedes exit from the G_1 phase of the eukaryotic cell cycle [12]. Hypusine-depleted cells were subsequently shown to lack the proteins required for replication *in vitro* [13]. When postulating the existence of *hymns*, we also suggested that viruses could parasitize *hymn* translation by recruiting eIF-5A.

eIF-5A is one of the host cell molecules that specifically interact with the Rev protein of HIV-1 [14, 15]. Rev, essential for HIV-1 replication, enables expression of the intron-containing HIV-1 mRNAs, i.e. the unspliced 9.2 kb full-length viral transcript, which encodes the Gag and Pol polyproteins, and the singly spliced, ≈4.7-kb viral mRNAs, which encode Env and the proteins Vif, Vpr, and Vpu. Only expression of the fully spliced, ≈2-kb class of mRNAs, which encode Rev itself together with Nef and the p16 version of Tat, is Rev independent [16]. Rev binding to intron-containing viral transcripts initially occurs at the RRE located within the sequence encoding Env, and it results in their transport from the nucleus to the cytoplasm [17, 18]. Mutational disruption of Rev–RRE complex formation has established its pivotal role in the nuclear export of the 9.2- and ≈4.7-kb classes of mRNAs [19, 20] and in the polysomal loading of viral transcripts [21–25]. The ability of HIV-1 to generate infective progeny rests on functional Rev–RRE complexes [16] and, in addition, is reported to require cellular proteins involved in nucleocytoplasmic transport, such as hRip [26] and eIF-5A [14, 15]. Mutations of eIF-5A that abolish its role as cellular cofactor for Rev were proposed as a gene therapy approach to HIV-1 infection [27].

Compounds displaying a planar benzo-substructure with two vicinal oxygen atoms or a planar domain of analogous dimensions are able to enter the active site pocket of both prolyl 4-hydroxylase and lysyl hydroxylase and, by coordinating the catalytically essential metal ion, inhibit these dioxygenases [4, 5, 28, 29]. Based on this inhibitory effect, we selected the α-hydroxypyridones as pilot agents to probe the active site of DOHH [6, 7] and the functional role of hypusine in cell proliferation [3, 30–32]. In the current study, we hypothesized that HIV-1 replication itself might be sensitive to suppression of hypusine formation. Inhibition of DOHH activity by the α-hydroxypyridones mimosine and deferiprone should mimic the disruption of the Rev–RRE complex achieved by mutations of Rev, the RRE, or eIF-5A [19, 21, 33, 34], or by Rev antisense oligonucleotides [35–37] and eIF-5A antisense expression constructs [14], all of which decrease HIV-1 multiplication.

Herein we report that inhibition of DOHH by mimosine or deferiprone, and thus pharmacological depletion of functional eIF-5A, resulted in reduced polysomal loading of the intron-containing 9.2-kb mRNA of HIV-1, decreased the biosynthesis of p24 protein, and markedly reduced generation of infectious viral particles. In addition, the α-hydroxypyridones preferentially promote apoptosis of retrovirally infected human T lymphocytes, suggesting that these agents have the potential to selectively ablate HIV-1-producing cells.

MATERIALS AND METHODS

Cell Culture

The T-lymphocytic cell line ACH-2 and the promonocytic cell line U1 were obtained from the AIDS Research and Reference Reagent Program (Ogden BioServices). Each of these cell lines carries a single integrated HIV-1 provirus. Stimulation of these cells with 100 ng/mL of PMA results in the enhanced production of full-length genomic HIV-1 RNA and increased generation of infectious particles [38–41]. The T-lymphocytic cell line H9 and a seed stock of HTLV-III_B (HIV-1/Lai) were supplied by Dr. Robert Gallo, and were used to generate cultures of chronically HIV-1-infected H9 cells as described [42, 43]. CEM cells (CCL119) were obtained from the American Type Culture Collection. All cell lines were maintained in RPMI-1640 containing 20% heat-inactivated fetal bovine serum (Hyclone), L-glutamine, penicillin, and streptomycin.

Processing of Cells and Supernatants

Logarithmically growing cells were washed extensively to remove viral antigens and infectious particles, and resuspended in serum-supplemented, antibiotic-containing medium at a density of 2×10^5 cells/mL. After incubation with either mimosine or deferiprone, the cells were harvested and lysed, and both supernatant and lysate were analyzed to determine the intra- and extracellular amounts of p24 antigen by an antigen-specific ELISA (Coulter). Quantitation of infectious viral particles in these culture supernatants was carried out as previously described [44]. Briefly, serial half-log dilutions of the culture supernatants were prepared in 100 μL of CIM containing 20 μg/mL of polybrene, and added to the wells of 96-well plates containing 8×10^5 CEM cells in 50 μL of CIM. Four replicate cultures were prepared for each dilution. Following incubation for 2 hr at 37°, the cultures were washed three times with fresh medium and finally resuspended in 150 μL of CIM. Cultures were fed every 3–5 days, with endpoint virus titers being determined after 2 weeks of culture by both

scoring for syncytium formation and by p24 ELISA as described above. The $TCID_{50}$ values of virus were calculated by the Spearman-Karber method [44]. TNF-α was assayed in the same supernatants by a standard ELISA (Genzyme Diagnostics). For electron microscopy, cells were washed twice and fixed as pellets with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) for 1 hr, postfixed with 2% osmium tetroxide in the same buffer for 1 hr, and stained *en bloc* for 1 hr with 2% aqueous uranyl acetate. Thin sections were examined using a Philips EM 410 transmission electron microscope.

Hypusine Determination

Conversion of peptidyl deoxyhypusine to peptidyl hypusine was determined by incubating ACH-2 cells ($3–4 \times 10^5$/mL) with 3.75 μCi/mL of [*terminal methylene*-^{3}H]spermidine (DuPont) for 24 hr in serum-supplemented, antibiotic-containing RPMI 1640, with or without 200 μM of mimosine or deferiprone. Eight hours after addition of the radiolabel, the cells were induced with PMA at a final concentration of 100 ng/mL to activate viral replication [38–41]. Following incubation, the cells were split into two groups. One was harvested immediately to assess the degree of deoxyhypusyl hydroxylase inhibition; the other one was harvested after transfer into inhibitor-free medium for 2 hr, to permit measurement of resumed deoxyhypusyl hydroxylase activity [3]. Cellular proteins were hydrolyzed in 6 N of HCl at 110° for 16 hr, and the hydrolysates were analyzed for labeled amino acids using an amino acid analyzer [45]. The retention times of hypusine and deoxyhypusine were determined from authentic standards.

Preparation of Polysomal mRNA

Logarithmically growing ACH-2 cells (3×10^5/mL) were incubated with the inhibitors and then induced with PMA as detailed above. Following an additional 16 hr of incubation, cells were harvested and lysed, and post-mitochondrial supernatants were prepared as described [12]. Polysomes were fractionated by sucrose gradient centrifugation, 0.5-mL fractions being collected and extracted once with phenol:chloroform (1:1). The RNA was ethanol-precipitated in the presence of glycogen (0.5 μg/μL). An aliquot of each fraction was blotted onto nylon membranes and probed with a ^{32}P-labeled rat 18S rDNA probe [46], to determine the distribution of polysomes, monosomes, and 40S ribosomal subunits.

RT-PCR Assays

To assess the effect of α-hydroxypyridones on polysomal distribution of host cell and retroviral mRNAs, oligo dT-primed-cDNA was prepared by reverse transcription as previously reported [47]. The cDNA of each gradient fraction was analyzed by semi-quantitative PCR employing three sets of oligonucleotide primer pairs: one specific for Rev-dependent transcripts containing the *gag* open reading frame [SK38: 5′-ATAATCCACCTATCCCAGTAGGA-3′; and SK39: 5′-TTTGGTCCTTGTCTTATGTCCAG AATG-3′], and two specific for the cellular mRNAs encoding the "housekeeping" proteins GAPDH [Primer A: 5′-CAAAGTTGTCATGGATGACC-3′; and Primer B: 5′-CCATGGAGAAGGCTGGGG-3′] [47] and β-actin [Primer C: 5′-GGTCACCCACACTGTGCCCAT-3′; and Primer D: 5′-GGATGCCACAGGACTCCATGC-3′]. Primers SK38, A, and C were end-labeled by using [γ-^{32}P]ATP and polynucleotide kinase. PCR products were run on 6% denaturing polyacrylamide gels. After drying, the PCR products were visualized by autoradiography, and the band intensities were quantified by densitometric analysis, using NIH Image software. Results were expressed relative to total product per gradient.

To assess the effect of α-hydroxypyridones on unspliced and multiply spliced HIV-1 transcripts induced with PMA in ACH-2 cells, RT-PCR on total mRNA was performed as described [48] using primers for *gag* and *tat*, respectively (*gag* primers: SK38 and SK39; *tat* primers: SCO4F [5′-CCTGG AAICATCCAGGAAGTCAGCCTA-3′] and SC52R [5′-TAAGTCTCTCAAGCGGTGGTAGCTGAA-3′ [48]]. The products were separated by electrophoresis on 2.5% agarose gels, which were fixed in 7% trichoroacetic acid, dried, and visualized using a Storm 860 imager (Molecular Dynamics).

Apoptosis Assays

Logarithmically growing CEM or ACH-2 cells (3×10^5/mL) were incubated with the inhibitors for a total of 26 hr, PMA being added to some cultures after 8 hr as described, and the incubation was continued for up to 18 hr. To detect fragmentation of genomic DNA, cells were washed, and the free 3′-OH ends of DNA were tailed, using a flow cytometry-based commercial kit based on the TUNEL (Oncor) method.

RESULTS

Effect of α-Hydroxypyridones on HIV Replication in Chronically Infected Cells

Initial experiments with these agents were carried out on chronically HIV-1 producing H9 cells [42]. Cells were harvested, washed extensively to remove extracellular virions, and were replated at a density of 2×10^5/mL for 16 hr in medium containing no or increasing concentrations of either deferiprone or mimosine, covering the concentration range found to inhibit DOHH activity *in vitro* and in cells [3]. At the end of the incubation period, antiviral effects were assessed by ELISA assay for p24 in cell supernatants and lysates, and by quantitation of infectious virions in culture supernatants. Deferiprone and mimosine displayed a concentration-dependent inhibition of infectious virion production, with an IC_{50} in the range of 50–100 μM. Titers of infectious viral particles in supernatants decreased by two

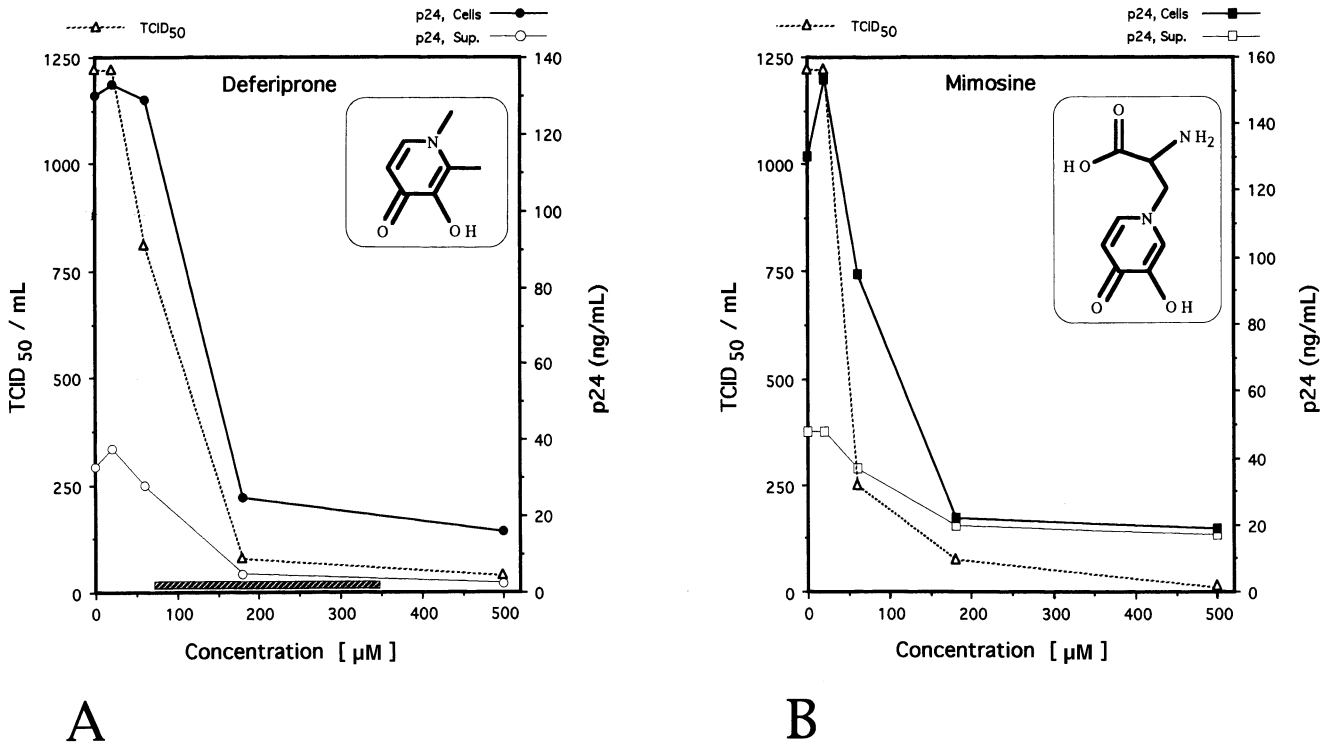

FIG. 1. Concentration-dependent suppression of infectious HIV-1 virion formation (triangles) and both intra- (closed symbols) and extracellular (open symbols) p24 levels by deferiprone (A) and mimosine (B) in the H9 human T cell line, chronically and productively infected with the HTLV-III$_B$ (HIV-1/Lai) strain [42]. Log phase proliferating cells at 2 × 10^5/mL were incubated with the indicated concentrations of compound for 24 hr prior to analysis. Identical results were obtained with the ACH-2 cells following PMA induction of HIV expression (data not shown). Viability was similar in control and α-hydroxypyridinone-exposed cells (≥70% in the chronically infected H9 cells and ≥90% in the latently infected, inducible ACH-2 cells). The hatched bar in A indicates the approximate range of serum levels measured in humans within 3 hr of ingesting a single 3-g dose of deferiprone [49]. Sup. = supernatant.

orders of magnitude (Fig. 1). Intra- and extracellular levels of the viral core protein p24 also decreased, but generally to a lesser degree. Deferiprone and mimosine showed similarly suppressive effects on intracellular p24 levels, but deferiprone was distinctly more potent than mimosine in reducing extracellular p24 (Fig. 1, compare A and B).

Effect of α-Hydroxypyridones on HIV Replication in Latently Infected Cells

To further investigate the mechanism underlying the antiretroviral effect in chronically infected cells, we utilized ACH-2 cells. This subclone, derived from the A3.01 variant of the human T cell line CEM, contains a single integrated copy of HIV-1 proviral DNA and constitutively expresses fully spliced, Rev-independent viral mRNAs. Exposure to PMA shifts expression towards unspliced and singly spliced, i.e. Rev-dependent, mRNAs and results in markedly enhanced virion production [38–41]. Logarithmically growing ACH-2 cells were harvested and washed four times to remove secreted virus and p24 antigen. The cells were then resuspended at 2 × 10^5/mL in complete medium, with or without deferiprone or mimosine. To inhibit cellular deoxyhypusyl hydroxylase activity and thus prevent the formation of functional eIF-5A, the study cultures were exposed to deferiprone or mimosine for 8 hr before the addition of PMA. All cultures were then incubated for an additional 16 hr in the presence of PMA and each DOHH antagonist, a period of time sufficient to detect substantial retroviral protein synthesis and virion formation (data not shown; [38]). Under these conditions, the α-hydroxypyridones caused a 10- to 20-fold decrease in both intracellular and secreted p24 levels, a result similar to that previously observed in chronically infected H9 cells (comp. Fig. 1). In striking contrast, a concentration-dependent effect of the α-hydroxypyridones on the PMA-induced formation of unspliced and multiply spliced HIV-1 transcripts, represented by *gag* and *tat*, respectively, was not apparent (Fig. 2). Deferiprone and mimosine each markedly reduced DOHH activity, and the deoxyhypusine-to-hypusine conversion, which normally is greater than 90%, fell to less than 10%. Upon release of the PMA-induced ACH-2 cells from α-hydroxypyridone inhibition, deoxyhypusine conversion rapidly resumed and within 2 hr increased to at least 25% (Fig. 3A, filled circles). The time course of hypusine recovery in the released ACH-2 cells was thus very similar to the one previously reported for CHO cells [3]. Nevertheless, the total amount of the obligatory intracellular eIF-5A isoforms, determined as the sum of deoxyhypusyl substrate plus hypusyl product of DOHH, declined rapidly, in the case of mimosine prefer-

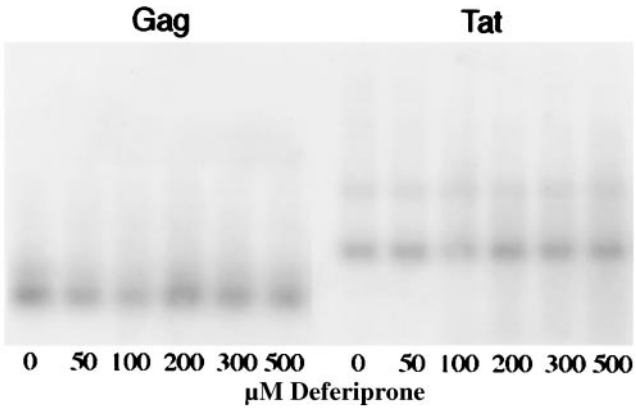

FIG. 2. Lack of concentration-dependent suppression by deferiprone of unspliced (*gag*) and multiply spliced (*tat*) HIV-1 transcripts induced with PMA in ACH-2 cells. Log phase proliferating cells at 2 × 10^5/mL were incubated with the indicated concentrations of compound and, to induce HIV-1 expression, with 100 ng/mL of PMA for 20 hr prior to analysis. The major products in each lane obtained after 35 cycles represent the RT-PCR product of the specific HIV mRNAs. No α-hydroxypyridone-dependent changes in cell viability or viral RNAs were detected.

entially during the release period and in the case of deferiprone preferentially during the incubation period (Fig. 3A, open circles). This finding suggests a dramatic loss of membrane integrity and is compatible with the observed α-hydroxypyridone-induced apoptosis of these HIV-1 producing cells (see also Fig. 6, A and B, and Table 1). At maximal inhibition of cellular DOHH activity, the level of the Gag-derived p24 viral protein, encoded by the PMA-induced, 9.2 kb mRNA class, decreased by one order of magnitude or more in both supernatant and cells. Again, deferiprone was more effective than mimosine in decreasing extracellular p24 levels. Significantly, neither agent suppressed the PMA-induced, concomitant 4- to 10-fold increase in TNF-α protein secreted by the same cells (Fig. 3B). PMA is known to induce *de novo* expression of TNF-α in human T cells [50, 51]. Thus, under the conditions used, the α-hydroxypyridones did not exert global inhibitory effects on the cellular processes required for secretion of *de novo* synthesized or stored protein, nor did they indiscriminately depress PMA-triggered cellular responses.

Both compounds also exhibited antiretroviral activity in latently infected monocytic U1 cells induced by PMA to

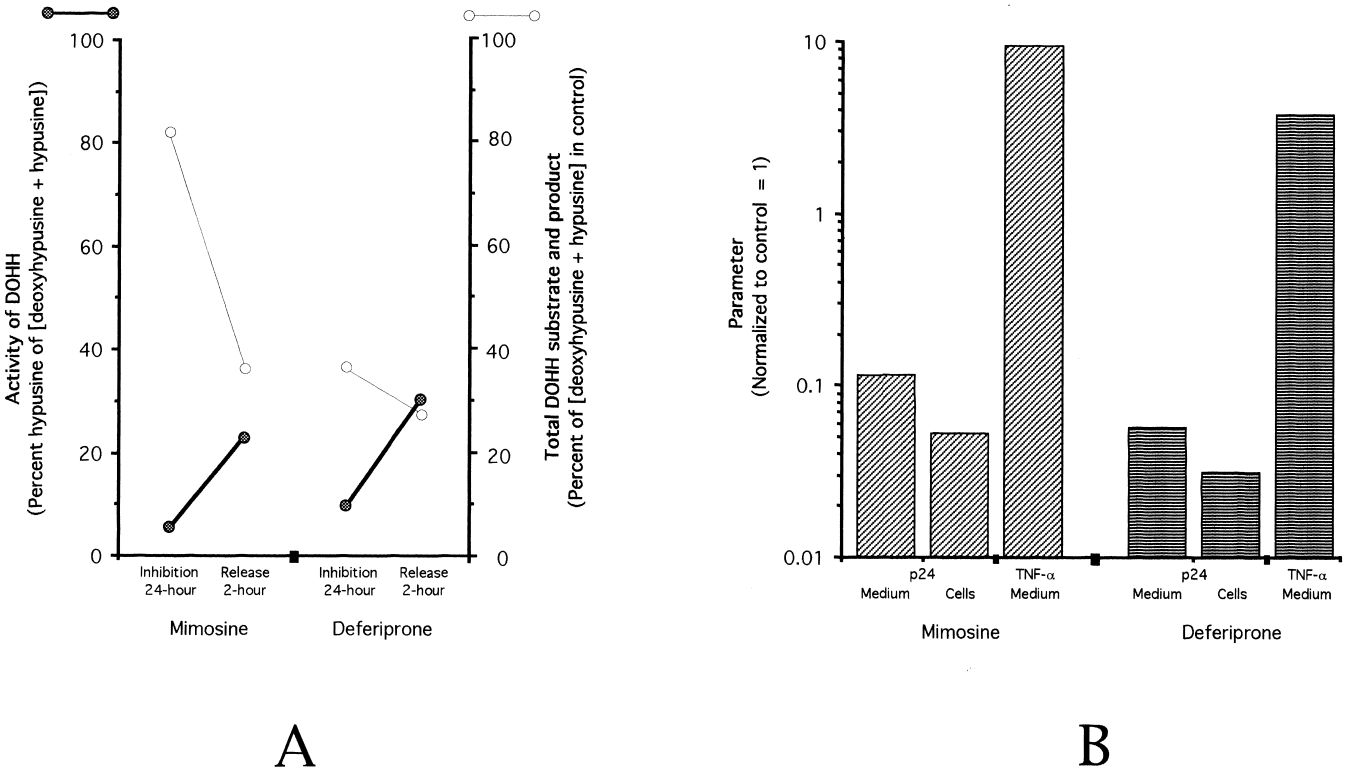

FIG. 3. (A) Effect of mimosine or deferiprone, 200 μM each, on DOHH activity (filled circles) and on total intracellular DOHH substrate and product (open circles) in PMA-induced ACH-2 cells. DOHH activity was calculated as the indicated ratio of deoxyhypusine-to-hypusine conversion [3]. In the control cells, the conversion ratio was 92.3% (hypusine, 2410 cpm; deoxyhypusine, 200 cpm). Total intracellular DOHH substrate and product, a parameter reflecting the deoxyhypusyl- plus the hypusyl-containing isoforms of eIF-5A, was measured via the specific covalent incorporation of the radiolabeled butylamine moiety of [*terminal methylene-*^{3}H]spermidine [3]. Control cells contained a total of 2610 cpm of deoxyhypusine plus hypusine (= 100%). Cell numbers in control, inhibition, and release were normalized to 6 × 10^6. (B) Effect of 200 μM mimosine (diagonally hatched bars) and 200 μM of deferiprone (horizontally hatched bars) on the levels of p24 and TNF-α in cultures of PMA-induced ACH-2 cells. Parameters were measured using commercially available ELISAs (Coulter and Genzyme Diagnostics, respectively). Control cells were not exposed to PMA or α-hydroxypyridones.

express the virus. In this system, at equimolar concentrations deferiprone was at least three times more effective than mimosine in suppressing extracellular p24 levels and generation of infectious progeny. The PMA-induced adherence of U1 cells, a response requiring integrity of PMA signaling pathways and the expression of cellular genes, remained unaffected by either agent (data not shown). This again suggests that the inhibitory action of the α-hydroxypyridones did not universally depress PMA-inducible cellular functions but, rather, was selective for retroviral functions.

Polysomal Distribution of Unspliced HIV-1 mRNA

The precipitous decline in viral p24 biosynthesis despite continued formation of its unspliced HIV-1 transcript during exposure to α-hydroxypyridones (contrast Figs. 1 and 2) led us to examine the polysomal distribution of viral and cellular mRNAs. If the unspliced HIV-1 transcripts require functional eIF-5A to facilitate their translation, then their polysomal localization should be as hypusine dependent as that of cellular *hymns* [12].

DOHH inhibition by mimosine was associated with a marked decline on polysomes of unspliced HIV-1 mRNA encoding the Gag polyprotein. Instead, this mRNA species accumulated in the low-molecular weight, nonpolysomal fractions; compared with control, this translationally silent population of gag mRNA almost doubled (Fig. 4A). DOHH inhibition, however, did not alter the polysomal distribution of cellular mRNAs encoding the "housekeeping" proteins β-actin and GAPDH. Their mRNAs remained associated with polysomes, although the α-hydroxypyridone appeared to reduce the size of the average polysome carrying these mRNAs. In particular, the low-molecular weight, nonpolysomal fractions did not measurably differ from those in the control, and DOHH inhibition did not affect the distribution between the translationally active and inactive populations of these cellular mRNAs (Fig. 4, B and C). Similar data were obtained for deferiprone-inhibited cells (data not shown).

The discrete high-molecular weight species detected by the *gag*-specific primers in the otherwise markedly *gag*-depleted polysomal fractions of the mimosine-inhibited sample (Fig. 4A) are interpreted as representing partially assembled viral particles. Such nucleoprotein intermediates are known to sediment with polysomes under the conditions used for the velocity sucrose density centrifugation [52, 53]. It remains to be determined whether the compromised biosynthesis of structural capsid proteins, as shown in Figs. 1 and 3B, and the much larger decline in the production of infective particles, as shown in Fig. 1, could be causally related to disruption of retroviral assembly. Such a disruption is suggested by the appearance of high molecular weight gag mRNA-containing complexes in the DOHH-inhibited cells (Fig. 4A).

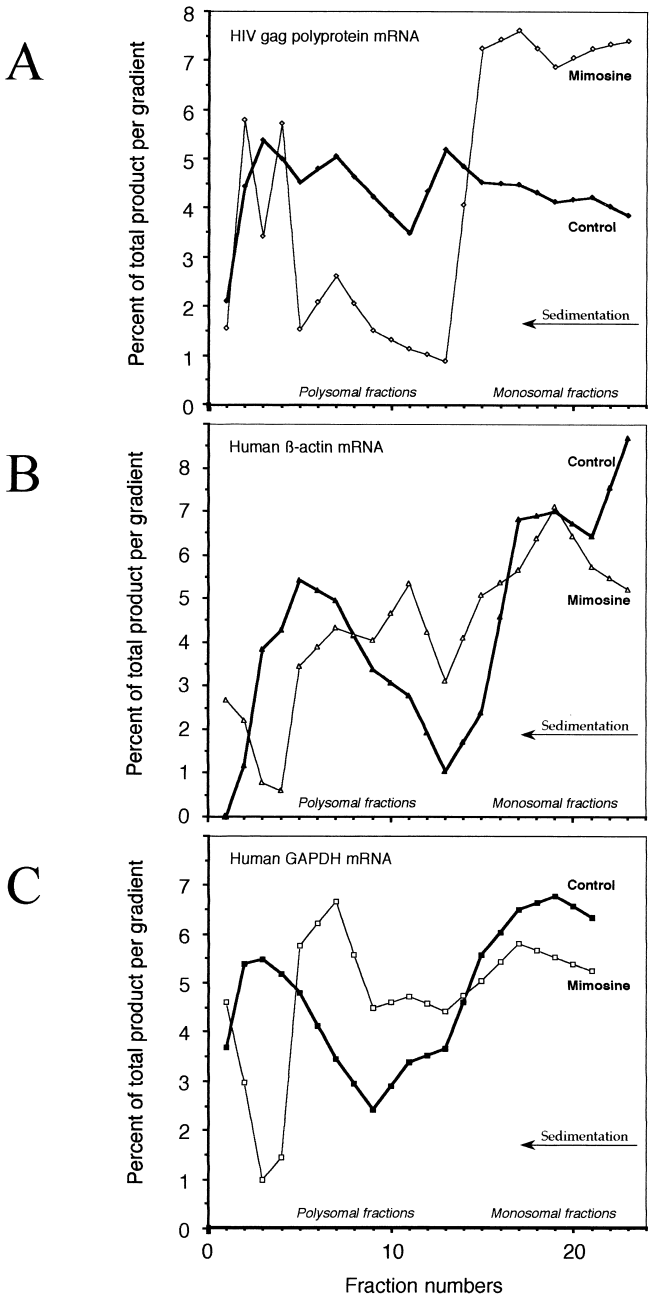

FIG. 4. Effect of 200 μM of mimosine on the polysomal loading of representative viral and cellular mRNAs obtained by sucrose gradient separation of postmitochondrial supernatants from ACH-2 cells induced with PMA to express HIV-1. The mRNAs analyzed were (A) HIV-1 *gag* mRNA, (B) β-actin mRNA and (C) GAPDH mRNA (thick lines: control cells; thin lines: mimosine-inhibited cells; arrows: direction of sedimentation). HIV-1 gag mRNA on the polysomes declines concomitant with a marked increase in its non-polysomal population. By contrast, the proportion of each cellular mRNA associated with polysomes does not change, although the sizes of the polysomes bearing the two cellular mRNAs are reduced in the mimosine-exposed cells. The three isolated high-molecular weight fractions in (A) may represent partially packaged [52, 53] rather than polysomal HIV-1 RNA.

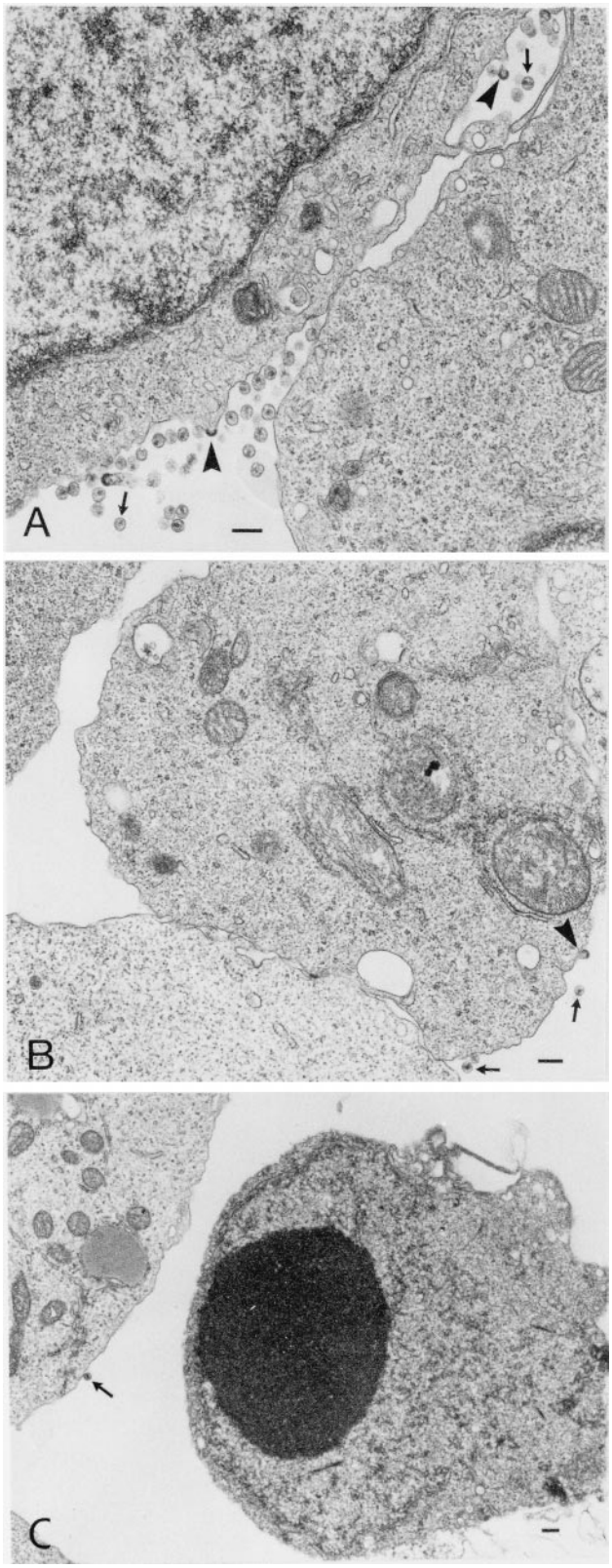

FIG. 5. Transmission electron micrographs of PMA-induced virion production by ACH-2 cells incubated in the absence (A) and presence (B and C) of 200 μM of mimosine. Two budding particles are labeled with arrow heads (A), and mature virions are labeled with arrows (A, B and C). Note unaffected ultrastructural appearance of membranes, cytoplasm, mitochondria, and rough endoplasmic reticulum in (B). Panel (C) shows a cell with an electron-dense nucleus and a homogenous cytoplasm lacking discernible organelles, characteristic of late-stage apoptosis (see Fig. 6). The bar in each micrograph represents 250 nm. Incubation with an equal concentration of deferiprone resulted in a similarly apparent reduction of virion particles (data not shown).

Electron Microscopy

Ultrastructural studies confirmed that the α-hydroxypyridones suppressed production of extracellular HIV-1 particles. Quantification of more than 90 profiles from PMA-induced, control and mimosine-inhibited ACH-2 cells indicated a 4-fold reduction in the total number of both mature and immature virions, and an 18-fold reduction in the number of budding virions. This effect was not consistently associated with other ultrastructural changes (compare A and B of Fig. 5), suggesting a lack of structurally disruptive compound toxicity. The only consistent ultrastructural change, occurring in a subpopulation of mimosine-treated cells, was the appearance of morphological features typically noted in apoptosis, such as nuclear condensation [54] (Fig. 5C).

Induction of Apoptosis by α-Hydroxypyridones

We investigated the effect of mimosine and deferiprone on HIV-1-expressing ACH-2 cells and on CEM cells, the uninfected parental T cell line from which ACH-2 was derived. Following exposure to either agent for 18 hr after the addition of PMA, cytoplasmic membrane integrity was intact in approximately 90% of CEM and 80% of ACH-2 cells (Fig. 6A, release point 1). At this time, however, only ACH-2 cells exhibited a marked propriocidal response (Fig. 6B, release point 1). Upon inhibitor removal, membrane integrity remained virtually unchanged in the CEM cells, but dramatically deteriorated in the ACH-2 cells (Fig. 6A, release point 2). DNA fragmentation did not occur in the CEM cells within the time scale of these experiments, whereas it further accelerated in the HIV-1-infected cells (Fig. 6B, compare release points 1 and 2), causing nuclear and nucleolar changes indicative of apoptosis (see Fig. 5C). These results suggested that HIV-1 expression might render infected cells particularly susceptible to α-hydroxypyridone-induced apoptosis.

To specifically address this question, an additional set of experiments was performed to differentiate the effects of deferiprone and PMA, individually and in combination, on the parental, uninfected CEM line and on their inducibly HIV-1-expressing ACH-2 progeny. Results are summarized in Table 1. Irrespective of whether PMA (column A) and deferiprone (column B) were given alone or in combination (column C), the ACH-2 cells displayed an enhanced susceptibility to programmed cell death. Although deferiprone by itself induced some degree of apoptosis in both T cell lines (column B), addition of PMA caused a marked further increase of apoptosis only in association with HIV-1 expression induced in the ACH-2 cells (column C). There

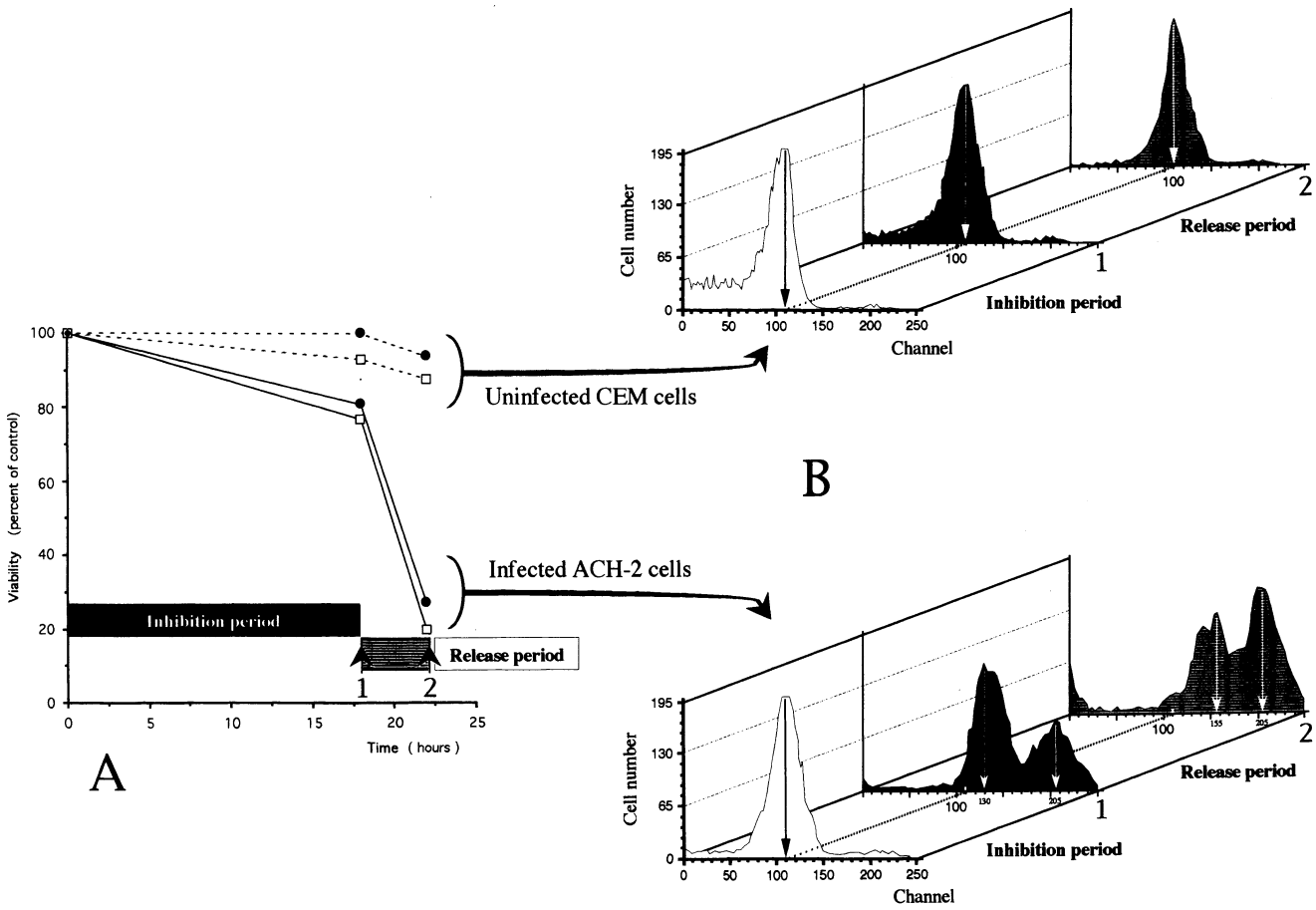

FIG. 6. Time course and differential action of α-hydroxypyridones on uninfected CEM and HIV-1-expressing ACH-2 cells. Logarithmically growing cells (3×10^5/mL) were incubated with 200 μM of deferiprone or mimosine for 8 hr. PMA (100 ng/mL final concentration) was then added and the incubation continued for an additional 18 hr ("Inhibition period"). Measurements were made at the time point indicated by "1," after which the cells were transferred into inhibitor-free medium and incubated for an additional 4 hr ("Release period"). At the time point indicated by "2," measurements were repeated. Determinations described in panels A and B were performed in parallel with the same set of cells. (A) Effect of deferiprone (closed circles) and mimosine (open squares) on the integrity of the cytoplasmic membranes of CEM (dashed lines) and ACH-2 (solid lines) cells. Trypan blue exclusion was determined at time points 1 and 2. All results are expressed relative to uninhibited PMA-induced CEM or ACH-2 cells. (B) Detection of the internucleosomal degradation of genomic DNA by flow cytometry in uninhibited PMA-induced CEM or ACH-2 cells (front) and in mimosine-treated cells after inhibition (time point 1) and release (time point 2). DNA degradation, characteristic of apoptosis, generates 3′-OH groups that are specifically end-labeled in a template-independent manner using a terminal deoxynucleotidyl transferase-based technique (TUNEL, see Materials and Methods). The signal shift to higher channels directly correlated with *de novo* formation of 3′-OH ends and, thus, propriocidal DNA fragmentation. Incubation with an equal concentration of deferiprone resulted in similar findings (data not shown).

was an average 2.6-fold increase in apoptosis for productive versus an average 1.4-fold increase for nonproductive ACH-2 cells (Table 1, columns C/B vs B/A). Consequently, on-going production of HIV-1, rather than exposure to PMA and/or deferiprone, is the critical determinant for α-hydroxypyridone-induced apoptosis in the cells studied.

DISCUSSION

While access of incompletely spliced cellular mRNAs to the translational machinery of the cell is precluded by specific mechanisms, incompletely spliced mRNAs of HIV-1 are able to gain access to this machinery. This result, critically important to HIV-1 replication, relies on the formation of Rev–RRE complexes [16]. Incapacitation of Rev–RRE complex formation has therefore been considered a possible antiretroviral strategy [36, 37]. Mutational changes of eIF-5A, a cellular cofactor of Rev, are able to decrease HIV-1 replication even if not impairing the interaction of eIF-5A with Rev–RRE complexes, and therapeutic expression of such mutants is pursued as an antiretroviral modality [27, 34]. However, these antisense- or gene therapy-based efforts face formidable obstacles, beginning with the sequence variability of Rev and the RRE in different HIV-1 strains. On the other hand, the requirement of hypusine formation for the function of eIF-5A [3, 9], and especially the decisive role of hypusine formation for polysomal localization of cellular *hymns* [12], opens the possibility of pharmacologically inhibiting hypusine forma-

TABLE 1. Deferiprone-induced apoptosis in productive and non-productive ACH-2 versus uninfected CEM cells

	Fold increase in apoptosis over untreated cultures		
Cells	(A) Plus PMA minus deferiprone	(B) Minus PMA plus deferiprone	(C) Plus PMA plus deferiprone
ACH-2	1.39 ± 0.08	2.00 ± 0.94	5.29 ± 1.21
CEM	0.85 ± 0.01	1.31*	1.62 ± 0.65

CEM and ACH-2 cells were cultured at 3×10^5 cells/mL with or without deferiprone (250 μM) for 8 hr before PMA (100 ng/mL) was added to the indicated cultures. After 12 hr, cells were collected by centrifugation, extensively washed, and fixed in 1% paraformaldehyde. The extent of apoptosis was measured by the TUNEL method. For flow cytometric analysis, cells were gated for lymphocyte size and a minimum of 10,000 counted. Values are expressed as fold increases in apoptosis over untreated cultures of either cell line and represent the mean ± SD of three experiments. Deferiprone-enhanced apoptosis in ACH-2 cells followed the PMA-induced expression of HIV-1 (compare A/B vs C for ACH-2).

*Average of two experiments.

tion to deplete functional eIF-5A and thus disrupt the eIF-5A/Rev–RRE interaction. This approach does not target a retroviral enzyme or nucleic acid, but rather a protein of the host cell, and bypasses escape mutations of the virus.

Our study suggests that deferiprone and mimosine are representative of this novel class of antiretroviral compounds. These α-hydroxypyridones decreased HIV-1 replication and HIV-1-directed protein biosynthesis in a concentration-dependent manner (Fig. 1). They inhibited DOHH activity (Fig. 3A) and caused a decline of full-length HIV-1 mRNA in the polysomal fraction, whereas "housekeeping" cellular mRNAs remained polysomal (see Fig. 4). The consequences of this pharmacologically induced eIF-5A deficiency for HIV-1 replication are identical to those previously observed after expression of an eIF-5A antisense construct [14], application of antisense oligonucleotides against *rev* [37], or mutational disruption of Rev–RRE complex formation [16, 19, 33, 55, 56] and of eIF-5A [34]. Significantly, the pharmacological depletion of functional eIF-5A not only mirrored the antiretroviral pattern obtained with mutations of eIF-5A, but also resulted in p24 levels reduced to a comparable extent [27, 34].

DOHH inhibitors affect the translation of viral mRNAs and of "housekeeping" host cell mRNAs in strikingly different ways. We present evidence that α-hydroxypyridone-induced eIF-5A deficiency did not inhibit the dramatic PMA-induced increase in TNF-α secretion by the HIV-1-infected cells studied, whereas their PMA-induced formation of viral p24 and its levels in the supernatant were decreased markedly (Fig. 3B). *In vivo*, TNF-α production by cytotoxic T cells is usually a critical element of host defense against viral infections. Paradoxically, TNF-α activates viral replication in HIV-1 infection and promotes persistence of the virus [57, 58]. TNF-α concentrations as low as 50 pg/mL enhance HIV-1 expression in ACH-2 cells [39]. In our ACH-2 cells, the PMA-induced TNF-α levels were 4–10 times higher than those measured in these earlier studies, but still failed to overcome the antiretroviral effect of either DOHH inhibitor (Fig. 3B). Thus, these agents obviate not only the HIV-1-inducing effect of PMA, but also that of TNF-α, indicating that they must act at a point in the HIV-1 replication pathway that is common to both modes of induction. The interaction of eIF-5A with Rev–RRE complexes fulfills this requirement.

While α-hydroxypyridones can trigger apoptosis in some cultured cells [59], other cells merely respond by rapidly reversible proliferative arrest [3, 7, 31, 32, 60]. Furthermore, the divergent effect of deferiprone on PMA-induced versus uninduced ACH-2 cells suggests that induction of apoptosis was related to the expression of HIV-1 (Table 1, compare columns B and C). It has been proposed that viral proteins encoded by Rev-dependent mRNAs, e.g. Vpr, delay or prevent apoptosis of infected cells [61], whereas uninfected bystander cells, for instance those exposed to Tat [62], appear to be susceptible to apoptosis [63]. It is possible that DOHH inhibition results in a reduction of anti-apoptotic viral products via compromised translation of their Rev-dependent mRNAs. Consequently, agents that inhibit DOHH would be expected to deblock the normal apoptotic response to viral invasion, a basic protective mechanism of cells [64]. Computer modeling of such a treatment strategy, selective ablation of HIV-1-producing cells by a cyclical regimen that enhances their apoptosis, indicates that eventual eradication of the virus is possible [65]. In general, the Rev–RRE system serves to prevent premature death of infected cells not yet capable of supporting levels of viral mRNA and protein synthesis sufficient for optimal HIV-1 replication [66]. DOHH inhibitors apparently counter the biological function of that system.

The pattern of antiretroviral activity mediated by mimosine and deferiprone is strikingly similar to the one reported for baicalin (5,6-dihydroxyflavone-7-β-D-glucuronidate). Baicalin, which decreases the biosynthesis of viral p24 in infected H9 cells [67, 68], also causes apoptosis in CEM cells only if they are productively infected with HIV-1 [69]. Like the structurally analogous α-hydroxypyridones, baicalin displays a planar benzo-substructure carrying two vicinal oxygen atoms and thus could well inhibit DOHH activity [6].

Our study does not directly address whether the suppressive effects of the α-hydroxypyridones on viral protein translation result from failure to initiate translation of viral transcripts present in the cytoplasm, or from disruption of their nucleocytoplasmic transport. Because both eIF-5A and Rev shuttle between the nucleus and the cytoplasm [14, 70], the latter alternative is more attractive. Mimosine and deferiprone may also have other, mechanistically unrelated effects contributing to their antiretroviral activity. Mimosine, for instance, has been shown to inhibit ribonucleotide reductase (EC 1.17.4) and causes a decrease in intracellular deoxynucleotide pools [71, 72], a situation which itself is known to adversely affect replication of HIV-1 [73, 74]. Whatever the actual mechanism(s), systemic administration of deferiprone, the more effective of

the antiretroviral α-hydroxypyridones reported here, is well tolerated by humans. The oral bioavailability of this agent has made it an attractive alternative to deferoxamine in the decorporation treatment of transfusional iron overload, e.g. in thalassemia major [75]. Deferiprone achieved its most marked suppressive effect on p24 biosynthesis and viral replication at concentrations that occur in human sera after a single oral dose of 3 g and are sustained for up to 3 hr (75–150 μM, with peaks of 350 μM [49]; see hatched bar in Fig. 1A). Interestingly, a case report is available on a thalassemic patient with transfusion-contracted HIV-1 infection, who received deferiprone as an iron decorporation agent. This patient "showed no progression of HIV" during 12 months on deferiprone, then stopped medication "because of depression and succumbed to AIDS with *Pneumocystis* pneumonia and encephalitis several months later" [76].

The antiretroviral activity of deferiprone in HIV-1-infected human T cell lines (Figs. 1–6) and the known pharmacokinetic and toxicological profile of this agent in humans invite rapid and specific clinical trials. It should also be noted that its potentially low price sets deferiprone apart from all currently marketed antiretroviral agents, whose cost-per-dose is of major concern in industrialized nations [77, 78] and becomes so prohibitive as to limit treatment options for HIV-1-afflicted patients in developing countries [79].

H. M. H-A. is deeply grateful to Drs. J. E. Folk, M-H. Park, and E. C. Wolff, National Institutes of Health, Bethesda, MD, for numerous stimulating suggestions and for the repeated use of their facilities, and is indebted to Dr. M. Lalande, Harvard Medical School, Boston, MA, for critically reading the manuscript. Discussions with Dr. A. Gosiewska, Johnson & Johnson, Skillman, NJ, are acknowledged. L. A. was supported by NIH Grant AI34773 and by funds from the M. K. Simpson Charitable Trust and the Achellis Foundation. P. S. was supported by NIA Grants PO1AG00541 and R37AG08707, and R. W. G. by NIH Grant HL-43027 and FDA Grant FD-R-001050. A-R. H. was supported by a grant from the Wilhelm-Sander Stiftung. H. M. H-A. was supported by the Evariste Galois Fund, and, in part, by grants from Hoechst Marion Roussel and the Council for Tobacco Research. Additional funds for P. S., R. W. G., and H. M. H-A. were provided by Helix Therapeutics Inc.

References

1. Park MH, Wolff EC and Folk JE, Is hypusine essential for eukaryotic cell proliferation? *Trends Biochem Sci* **18:** 475–479, 1993.
2. Park MH, Wolff EC and Folk JE, Hypusine: Its post-translational formation in eukaryotic initiation factor 5A and its potential role in cellular regulation. *Biofactors* **4:** 95–104, 1993.
3. Hanauske-Abel HM, Park M-H, Hanauske A-R, Popowicz AM, Lalande M and Folk JE, Inhibition of the G1-S transition of the cell cycle by inhibitors of deoxyhypusine hydroxylation. *Biochim Biophys Acta* **1221:** 115–124, 1994.
4. Hanauske-Abel HM and Günzler V, A stereochemical concept for the catalytic mechanism of prolyl hydroxylase. Applicability to classification and design of inhibitors. *J Theor Biol* **94:** 421–455, 1982.
5. Majamaa K, Turpeenniemi-Hujanen TM, Latipää P, Günzler V, Hanauske-Abel HM, Hassinen IE and Kivirikko KI, Differences between collagen hydroxylases and 2-oxoglutarate dehydrogenase in their inhibition by structural analogues of 2-oxoglutarate. *Biochem J* **229:** 127–133, 1985.
6. Abbruzzese A, Hanauske-Abel HM, Park MH, Henke S and Folk JE, The active site of deoxyhypusyl hydroxylase: Use of catecholpeptides and their component chelator and peptide moieties as molecular probes. *Biochim Biophys Acta* **1077:** 159–166, 1991.
7. McCaffrey TA, Pomerantz KB, Sanborn TA, Spokojny AM, Du B, Park MH, Folk JE, Lamberg A, Kivirikko KI, Falcone DJ, Mehta SB and Hanauske-Abel HM, Specific inhibition of eIF-5A and collagen hydroxylation by a single agent: Antiproliferative and fibrosuppressive effects on smooth muscle cells from human coronary arteries. *J Clin Invest* **95:** 446–455, 1995.
8. João HC, Csonga R, Klier H, Koettnitz K, Auer M and Eder J, The polypeptide chain of eukaryotic initiation factor 5A occurs in two distinct conformations in the absence of the hypusine formation. *Biochemistry* **34:** 14703–14711, 1995.
9. Schnier J, Schwelberger HG, Smit-McBride Z, Kang HA and Hershey JWB, Translation initiation factor 5A and its hypusine modification are essential for cell viability in the yeast *Saccharomyces cerevisiae*. *Mol Cell Biol* **11:** 3105–3114, 1991.
10. Magdolen V, Klier H, Wohl T, Klink F, Hirt H, Hauber J and Lottspeich F, The function of the hypusine-containing proteins of yeast and other eukaryotes is well conserved. *Mol Gen Genet* **244:** 646–652, 1994.
11. Kang HA and Hershey JWB, Effect of initiation factor eIF-5A depletion on protein synthesis and proliferation of *Saccharomyces cerevisiae*. *J Biol Chem* **269:** 3934–3940, 1994.
12. Hanauske-Abel HM, Slowinska B, Zagulska S, Wilson RC, Staiano-Coico L, Hanauske A-R, McCaffrey T and Szabo P, Detection of a sub-set of polysomal mRNAs associated with modulation of hypusine formation at the G1-S boundary. Proposal of a role for eIF-5A in onset of DNA replication. *FEBS Lett* **366:** 92–98, 1995.
13. Kalejta RF and Hamlin JL, The dual effect of mimosine on DNA replication. *Exp Cell Res* **231:** 173–183, 1997.
14. Ruhl M, Himmelspach M, Bahr GM, Hammerschmid F, Jaksche H, Wolff B, Aschauer H, Farrington GK, Probst H, Bevec D and Hauber J, Eukaryotic initiation factor 5A is a cellular target of the human immunodeficiency virus type 1 Rev activation domain mediating *trans*-activation. *J Cell Biol* **123:** 1309–1320, 1993.
15. Bevec D, Klier H, Holter W, Tschachler E, Valent P, Lottspeich F, Baumruker T and Hauber J, Induced gene expression of the hypusine-containing protein eukaryotic initiation factor 5A in activated human T lymphocytes. *Proc Natl Acad Sci USA* **91:** 10829–10833, 1994.
16. Cullen BR and Malim MH, The HIV-1 Rev protein: Prototype of a novel class of eukaryotic post-transcriptional regulators. *Trends Biochem Sci* **16:** 346–350, 1991.
17. Mann DA, Mikaélian I, Zemmel RW, Green SM, Lowe AD, Kimura T, Singh M, Butler PJG, Gait MJ and Karn J, A molecular rheostat. Co-operative Rev binding to stem I of the Rev-response element modulates human immunodeficiency virus type-1 late gene expression. *J Mol Biol* **241:** 193–207, 1994.
18. Peterson RD, Bartel DP, Szostak JW, Horvath SJ and Feigon J, ^{1}H NMR studies of the high-affinity Rev binding site of the Rev-responsive element of HIV-1 mRNA: Base pairing in the core binding element. *Biochemistry* **33:** 5357–5366, 1994.
19. Malim MH and Cullen BR, Rev and the fate of pre-mRNA in the nucleus: Implications for the regulation of RNA processing in eukaryotes. *Mol Cell Biol* **13:** 6180–6189, 1993.
20. Fischer U, Meyer S, Teufel M, Heckel C, Lührmann R and

Rautmann G, Evidence that HIV-1 Rev directly promotes the nuclear export of unspliced RNA. *EMBO J* **13:** 4105–4112, 1994.
21. Arrigo SJ and Chen ISY, Rev is necessary for translation but not cytoplasmic accumulation of HIV-1 *vif*, *vpr*, and *env/vpu-2* RNAs. *Genes Dev* **5:** 808–819, 1991.
22. D'Agustino DM, Felber BK, Harrison JE and Pavlakis GN, The Rev protein of human immunodeficiency virus type 1 promotes polysomal association and translation of *gag/pol* and *vpu/env* mRNAs. *Mol Cell Biol* **12:** 1375–1386, 1992.
23. Lawrence JB, Cochrane AW, Johnson CV, Perkins A and Rosen CA, The HIV-1 Rev protein: A model system for coupled RNA transport and translation. *New Biol* **3:** 1220–1232, 1991.
24. Stutz F and Rosbash M, A functional interaction between Rev and yeast pre-mRNA is related to splicing complex formation. *EMBO J* **13:** 4096–4104, 1994.
25. Felber BK, Hadzopoulou-Cladaras M, Cladaras C, Copeland T and Pavlakis GN, Rev protein of human immunodeficiency virus type 1 affects the stability and transport of the viral mRNA. *Proc Natl Acad Sci USA* **86:** 1495–1499, 1989.
26. Görlich D and Mattaj IW, Nucleocytoplasmic transport. *Science* **271:** 1513–1518, 1996.
27. Junker U, Bevec D, Barske C, Kalfoglou C, Escaich S, Dobrovnik M, Hauber J and Böhnlein E, Intracellular expression of cellular eIF-5A mutants inhibits HIV-1 replication in human T cells: A feasibility study. *Hum Gene Ther* **15:** 1861–1869, 1996.
28. Hanauske-Abel HM, Prolyl 4-hydroxylase, a target enzyme for drug development. Design of suppressive agents and the *in vitro* effects of inhibitors and proinhibitors. *J Hepatol* **13** (Suppl 3): S8–S16, 1991.
29. Majamaa K, Günzler V, Hanauske-Abel HM, Myllylä R and Kivirikko KI, Partial identity of the 2-oxoglutarate and ascorbate binding sites of prolyl 4-hydroxylase. *J Biol Chem* **261:** 7819–7823, 1986.
30. Lalande M and Hanauske-Abel HM, A new compound which reversibly arrests T lymphocyte cell cycle near the G_1/S boundary. *Exp Cell Res* **188:** 117–121, 1990.
31. Watson PA, Hanauske-Abel HM, Flint A and Lalande M, Mimosine reversibly arrests cell cycle progression at the G1-S phase border. *Cytometry* **12:** 242–246, 1991.
32. Hoffman BD, Hanauske-Abel HM, Flint A and Lalande M, A new class of reversible cell cycle inhibitors. *Cytometry* **12:** 26–32, 1991.
33. Sakai H, Furuta RA, Tokunaga K, Kawamura M, Hatanaka M and Adachi A, Rev-dependency of expression of human immunodeficiency virus type 1 *gag* and *env* genes. *FEBS Lett* **365:** 141–145, 1995.
34. Bevec D, Jaksche H, Oft M, Wöhl T, Himmelspach M, Pacher A, Schebesta M, Koettnitz K, Dobrovnik M, Csonga R, Lottspeich F and Hauber J, Inhibition of HIV-1 replication in lymphocytes by mutants of the Rev cofactor eIF-5A. *Science* **271:** 1858–1860, 1996.
35. Fenster SD, Wagner RW, Froehler BC and Chin DJ, Inhibition of human immunodeficiency virus type-1 *env* expression by C-5 propyne oligonucleotides specific for Rev-response element stem-loop V. *Biochemistry* **33:** 8391–8398, 1994.
36. Chin DJ, Inhibition of human immunodeficiency virus type 1 Rev-Rev-response element complex formation by complementary oligonucleotides. *J Virol* **66:** 600–607, 1992.
37. Matsukura M, Zon G, Shinozuka K, Robert-Guroff M, Shimada T, Stein CA, Mitsuya H, Wong-Staal F, Cohen JS and Broder S, 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 USA* **86:** 4244–4248, 1989.
38. Michael NL, Morrow P, Mosca J, Vahey M, Burke DS and Redfield RR, Induction of human immunodeficiency virus type 1 expression in chronically infected cells is associated primarily with a shift in RNA splicing pattern. *J Virol* **65:** 1291–1303, 1991.
39. Folks TM, Clouse KA, Justement J, Rabson A, Duh E, Kehrl JH and Fauci AS, Tumor necrosis factor α induces expression of human immunodeficiency virus in a chronically infected T-cell clone. *Proc Natl Acad Sci USA* **86:** 2365–2368, 1989.
40. Pomerantz RJ, Trono D, Feinberg MB and Baltimore D, Cells non-productively infected with HIV-1 exhibit an aberrant pattern of viral RNA expression: A molecular model for latency. *Cell* **61:** 1271–1276, 1990.
41. Clouse KA, Powell D, Washington I, Poli G, Strebel K, Farrar W, Barstad P, Kovacs J, Fauci AS and Folks TM, Monokine regulation of human immunodeficiency virus-1 expression in a chronically infected human T cell clone. *J Immunol* **142:** 431–438, 1989.
42. Prince AM, Horowitz B, Dichtelmueller H, Stephan W and Gallo RC, Quantitative assays for evaluation of HTLV-III inactivation procedures: Tri(*N*-butyl)phosphate:sodium cholate and β-propiolactone. *Cancer Res* **45:** 4592s–4594s, 1985.
43. Mann DL, O'Brien SJ, Gilbert DA, Reid Y, Popovic M, Read-Connole E, Gallo RC and Gazdar AF, Origin of the HIV-susceptible human CD4+ cell line H9. *AIDS Res Hum Retroviruses* **5:** 253–255, 1989.
44. Prince AM, Horowitz B, Baker L, Shulman RW, Ralph H, Valinsky J, Cundell A, Brotman B, Boehle W, Rey F, Piet M, Reesink H, Lelie N, Tersmette M, Miedema F, Barbosa L, Nemo G, Nastala CL, Allan JS, Lee DR and Eichberg JW, Failure of a human immunodeficiency virus (HIV) immune globulin to protect chimpanzees against experimental challenge with HIV. *Proc Natl Acad Sci USA* **85:** 6944–6948, 1988.
45. Park MH, Cooper HL and Folk JE, The biosynthesis of protein-bound hypusine (N^{ε}-(4-amino-2-hydroxybutyl)lysine). *J Biol Chem* **257:** 7217–7222, 1982.
46. Katz RA, Erlanger BF and Guntaka RV, Evidence for extensive methylation of ribosomal RNA genes in a rat XC cell line. *Biochim Biophys Acta* **739:** 258–264, 1983.
47. Ben-Yehuda A, Szabo P, Dyall R and Weksler ME, Bone marrow declines as a site of B-cell precursor differentiation with age: Relationship to thymus involution. *Proc Natl Acad Sci USA* **91:** 11988–11992, 1994.
48. Michael NL, Mo T, Merzouki A, O'Shaughnessy M, Oster C, Burke DS, Redfield RR, Birx DL and Cassol SA, Human immunodeficiency virus type 1 cellular RNA load and splicing patterns predict disease progression in a longitudinally studied cohort. *J Virol* **69:** 1868–1877, 1995.
49. Kontoghiorghes GJ, Goddard JG, Bartlett AN and Sheppard L, Pharmacokinetic studies in humans with the oral iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one. *Clin Pharmacol Ther* **48:** 255–261, 1990.
50. Goldfeld AE, Strominger JL and Doyle C, Human tumor necrosis factor α gene regulation in phorbol ester stimulated T and B cell lines. *J Exp Med* **174:** 73–81, 1991.
51. Rhoades KL, Golub SH and Economou JS, The regulation of the human tumor necrosis factor α promoter region in macrophage, T cell, and B cell lines. *J Biol Chem* **267:** 22102–22107, 1992.
52. Mergener K, Fäcke M, Welker R, Brinkmann V, Gelderblom HR and Kräusslich H-G, Analysis of HIV particle formation using transient expression of subviral constructs in mammalian cells. *Virology* **186:** 25–39, 1992.
53. Babe LM and Craik CS, Constitutive production of nonenveloped human immunodeficiency virus type I particles by a mammalian cell line and effects of a protease inhibitor on

particle maturation. *Antimicrob Agents Chemother* **38:** 2430–2439, 1994.
54. Kimura K, Sasano H, Shimosegawa T, Kato K, Noguchi T, Mochizuki S, Sawai T, Koizumi M, Toyota T and Nagura H, Ultrastructural and confocal laser scanning microscopic examination of TUNEL-positive cells. *J Pathol* **181:** 235–242, 1997.
55. Malim MH, Böhnlein S, Hauber J and Cullen BR, Functional dissection of the HIV-1 Rev *trans*-activator—Derivation of a *trans*-dominant repressor of Rev function. *Cell* **58:** 205–214, 1989.
56. Malim MH, Hauber J, Le S-Y, Maizel JV and Cullen BR, The HIV-1 *rev trans*-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. *Nature* **338:** 254–257, 1989.
57. Poli G, Kinter A, Justement JS, Kehrl JH, Bressler P, Stanley S and Fauci AS, Tumor necrosis factor α functions in an autocrine manner in the induction of human immunodeficiency virus expression. *Proc Natl Acad Sci USA* **87:** 782–785, 1990.
58. Harrer T, Jassoy C, Harrer E, Johnson RP and Walker BD, Induction of HIV-1 replication in a chronically infected T-cell line by cytotoxic T lymphocytes. *J Acquir Immune Defic Syndr* **6:** 865–871, 1993.
59. Hileti D, Panayiotidis P and Hoffbrand AV, Iron chelators induce apoptosis in proliferating cells. *Br J Haematol* **89:** 181–187, 1995.
60. Lalande M, A reversible arrest point in the late G1 phase of the mammalian cell cycle. *Exp Cell Res* **186:** 332–339, 1990.
61. He J, Choe S, Walker R, Di Marzio P, Morgan DO and Landau NR, Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G_2 phase of the cell cycle by inhibiting $p34^{cdc2}$ activity. *J Virol* **69:** 6705–6711, 1995.
62. McCloskey TW, Ott M, Tribble E, Khan SA, Teichberg S, Paul MO, Pahwa S, Verdin E and Chirmule N, Dual role of HIV Tat in regulation of apoptosis in T cells. *J Immunol* **158:** 1014–1019, 1997.
63. Finkel TH, Tudor-Williams G, Banda NK, Cotton MF, Curiel T, Monks C, Baba TW, Ruprecht RM and Kupfer A, Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes. *Nat Med* **1:** 129–134, 1995.
64. Williams GT, Programmed cell death: A fundamental protective response to pathogens. *Trends Microbiol* **2:** 463–464, 1994.
65. Huss R, Hoy CA, Ottinger H, Grosse-Wilde H and Deeg HJ, Cyclosporine-induced apoptosis in CD4+ T lymphocytes and computer-simulated analysis: Modeling a treatment scenario for HIV infection. *Res Immunol* **146:** 101–108, 1995.
66. Cullen BR, Regulation of HIV-1 gene expression. *FASEB J* **5:** 2361–2368, 1991.
67. Zhang X, Tang X and Chen H, Inhibition of HIV replication by baicalin and *S. baicalensis* extracts in H9 cell cultures. *Chin Med Sci J* **6:** 230–232, 1991.
68. Li BQ, Fu T, Yan YD, Baylor NW, Ruscetti FW and Kung HF, Inhibition of HIV infection by baicalin, a flavonoid compound purified from Chinese herbal medicine. *Cell Mol Biol Res* **39:** 119–124, 1993.
69. Wu X, Akatsu H and Okada H, Apoptosis of HIV-infected cells following treatment with Sho-Saiko-to and its components. *Jpn J Med Sci Biol* **48:** 79–87, 1995.
70. Meyer BE and Malim MH, The HIV-1 Rev *trans*-activator shuttles between the nucleus and the cytoplasm. *Genes Dev* **8:** 1538–1547, 1994.
71. Dai Y, Gold B, Vishwanatha JK and Rhode SL, Mimosine inhibits viral DNA synthesis through ribonucleotide reductase. *Virology* **205:** 210–216, 1994.
72. Gilbert DM, Neilson A, Miyazawa H, DePamphilis M and Burhans WC, Mimosine arrests DNA synthesis at replication forks by inhibiting deoxyribonucleotide metabolism. *J Biol Chem* **270:** 9597–9606, 1995.
73. Gao WY, Cara A, Gallo RC and Lori F, Low levels of deoxynucleotides in peripheral blood lymphocytes: A strategy to inhibit human immunodeficiency virus type 1 replication. *Proc Natl Acad Sci USA* **90:** 8925–8928, 1993.
74. Lori F, Malykh A, Cara A, Sun D, Weinstein JN, Lisziewicz J and Gallo RC, Hydroxyurea as an inhibitor of human immunodeficiency virus-type 1 replication. *Science* **266:** 801–805, 1994.
75. Templeton DM, Therapeutic use of chelating agents in iron overload. In: *Handbook of Experimental Pharmacology* (Eds. Goyer RA and Cherian MG), Vol. 115, pp. 321–323. Springer, Berlin, 1995.
76. al-Refaie FN, Hershko C, Hoffbrand AV, Kosaryan M, Olivieri NF, Tondury P and Wonke B, Results of long-term deferiprone (L1) therapy: A report by the International Study Group on Oral Iron Chelators. *Br J Haematol* **91:** 224–229, 1995.
77. Weiss PJ, Kennedy CA, Wallace MR, Nguyen MT and Oldfield EC 3rd, Medication costs associated with the care of HIV-infected patients. *Clin Ther* **15:** 912–916, 1993.
78. Griffin MT, AIDS drugs and the pharmaceutical industry: A need for reform. *Am J Law Med* **17:** 363–410, 1991.
79. Schopper D and Walley J, Care for AIDS patients in developing countries: A review. *AIDS Care* **4:** 89–102, 1992.