

Suppression Of HIV-1 Replication And Inhibition Of eIF5A Hydroxylation : Dual Effects of Two Widely Used Drugs.

D. Saxena ^{*1}, P. Palumbo ¹, H.M. Hanauske-Abel ¹, M. Hoque ¹, D.G. D'Alliessi ², G.L. McLendon ², M.H. Park ³, E.C. Wolff ³, Z. Garcia ¹, T. Pe'ery ¹, and M.B. Mathews ¹

¹ New Jersey Medical School - UMDNJ; Newark, USA ; ² Princeton University; Princeton, NJ, USA , ³ National Institute of Dental and Craniofacial Research - NIH; Bethesda, MD , USA



ABSTRACT

Background: Viral replication relies on cellular elements that may be targeted for inhibition. Cellular deoxyhypusine hydroxylase (DOHH) enzymatically activates eukaryotic initiation of translation factor 5A (eIF5A), which has been implicated as a co-factor for HIV Rev and replication. Molecules docking into the DOHH active site and blocking function are predicted to possess antiretroviral activity. Testing this hypothesis, we investigated the drugs deferiprone (DEF), an oral agent used to treat iron overload, and ciclopirox (CPX), a topical antifungal agent, as well as the CPX analog P2, predicted by the structure-activity relationship (SAR) for DOHH inhibitors to be less active or inactive.

Methods: Compounds were assayed for inhibition of DOHH. Suppression of retroviral protein synthesis (p24) and of viron formation (RNA copy number) was measured in chronically infected H9 cells and in short- and long-term infection of freshly harvested, uninfected peripheral blood mononuclear cells (PBMCs) with clinical HIV isolates.

Results: DEF and CPX suppressed DOHH *in vitro* at distinctly lower concentrations than P2 (28 vs. 170 μ M). Similarly, DEF and CPX but not P2 inhibited p24 synthesis in both infected H9 cells and in short / long-term infection of PBMCs with wild-type HIV-1. In 18-hour H9 cell experiments, 200 μ M DEF and 30 μ M CPX exhibited >40% inhibition of p24 antigen than the controls. These active compounds prevented detectable p24 antigen production in acutely infected PBMCs (drug added 48 hours post-infection) and markedly suppressed viral RNA production in a PBMC chronic infection model over 7 to 10 days of treatment. Proviral DNA became undetectable 12 to 24 days post-treatment initiation in the PBMC chronic infection model, which includes serial addition of non-infected cells.

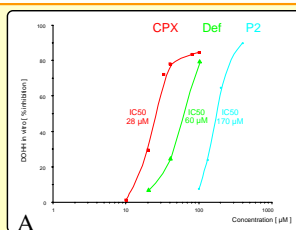
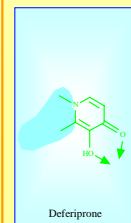
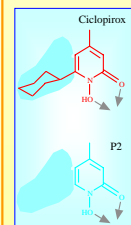
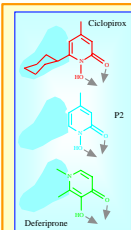
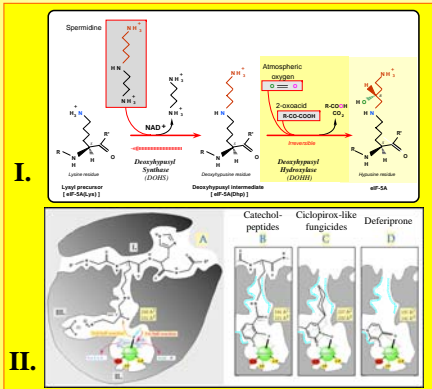
Conclusions: Our results suggest that the functional relation between the posttranslational hydroxylation of eIF5As and the replication of HIV-1 can be employed to realize an antiretroviral effect. DEF and CPX may serve as pilot agents for accelerated clinical trials and for the SAR - guided synthesis of a novel class of antiretrovirals targeting cellular components.

INTRODUCTION

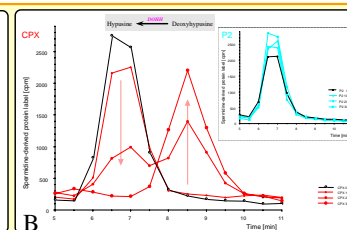
The Rev/Rex protein of lentiviruses are absolutely essential for the translational utilization of their intron-containing RNAs by infected cells, and for the formation of infectious progeny [1]. A significant body of evidence indicates that the eukaryotic translation initiation factors 5A (eIF5As) serve as strategic cellular partners for Rev/Rex function [2, 3]. Genetic or pharmacological manipulations of the structure of eIF5A abrogate the ability of HIV-1 to replicate in human lymphocytes [4, 5].

The eIF5As are, like the collagens, subject to a posttranslational hydroxylation that produces a genetically non-encoded residue which, in turn, determines their function. The biochemical pathway for eIF5A hydroxylation, summarized below (see I.), relies on several substrates and cofactors, including molecular oxygen, to form the single hypusine residue within each eIF5A molecule [6]. Hypusine occurs only in the eIF5As. The enzyme generating hypusine and thus bioactive eIF5A, is deoxyhypusine hydroxylase (DOHH), a member of the 2-oxoacid utilizing, non-heme iron dioxygenases [7]. Catalysis by these enzymes is known in subatomic detail [8]. It centers on a reaction among substrate ligands coordinated to the active site non-heme iron atom (HAG mechanism [9] , see II. A).

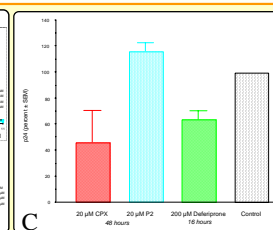
In the past, this knowledge provided a rational basis for the *de novo* design of DOHH inhibitors [10]; see II. B). Here we used the active site dimensions of DOHH [10] to identify potential inhibitors among existing drugs. We report on the DOHH inhibitory and antiretroviral properties of two clinically introduced drugs, the fungicide ciclopirox (see II. C) and the decorporation agent deferiprone (see II. D). To control for the compounds' metal binding properties, we designed and tested P2, a chelation homologue of ciclopirox.



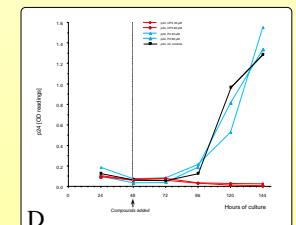
Inhibition of DOHH activity *in vitro*.



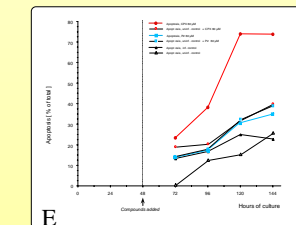
Effect of CPX and its chelation homologue P2 on DOHH activity in HIV-1 infected H9 cells



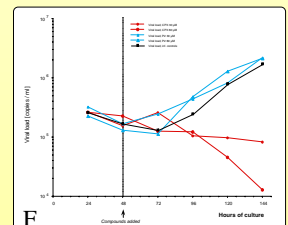
Inhibition of p24 synthesis by CPX (red), P2 (blue), and deferiprone (green) in HIV-1 infected H9 cells.



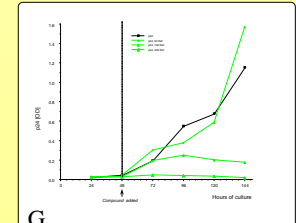
Inhibition of p24 synthesis by CPX (red), but not P2 (blue) in patient-isolate infected naive PBMCs.



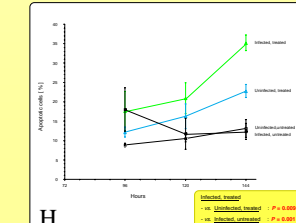
Activation of apoptosis by CPX (red), but not P2 (blue) in patient-isolate infected naive PBMCs.



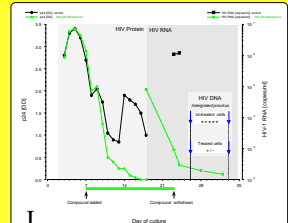
Suppression of viral RNA by CPX (red), but not P2 (blue) in patient-isolate infected naive PBMCs.



Inhibition of p24 synthesis by deferiprone in patient-isolate infected naive PBMCs.



Activation of apoptosis by deferiprone in patient-isolate infected naive PBMCs.



Suppression of p24, viral RNA, and proviral DNA by deferiprone in patient-isolate infected naive PBMCs.

RESULTS

Summary of results

The active site pocket of DOHH contains distinct hydrophobic regions (blue shaded lines in I. B). Deferiprone and ciclopirox, but not its chelation homologue P2, display domains that can serve as hydrophobic anchors at these regions. Consequently, the former compounds inhibit DOHH *in vitro* and in cells at distinctly lower concentrations than the latter agent (A. B).

Only deferiprone and ciclopirox, but not P2, suppress p24 synthesis by HIV-1 infected H9 cells and in short-term cultures of patient-isolate infected PBMCs (E, F). In the same system, deferiprone likewise activates apoptosis (H). This activation of apoptosis occurs with significant preference especially in those PBMC cultures that had been infected with patient-isolate HIV-1 (E, H).

Only ciclopirox, but not its chelation homologue P2, activate apoptosis and suppress retroviral RNA formation in short-term cultures of patient-isolate infected PBMCs (E, F). In the same system, deferiprone likewise activates apoptosis (H). This activation of apoptosis occurs with significant preference especially in those PBMC cultures that had been infected with patient-isolate HIV-1 (E, H).

In long-term cultures of patient-isolate infected PBMCs displaying active viral propagation, a 2-week course of deferiprone at a clinically achievable concentration -

- > reduces p24 levels below the detection limit;
- > suppresses retroviral RNA to extremely low copy numbers, which further decline even after discontinuation of the drug;
- > renders proviral DNA doubtfully detectable; and
- > inhibits resurgence of HIV-1 production until ten days after discontinuation of the drug, when the experiment was finally stopped (I).

CONCLUSIONS

- The current concept on the catalysis by and the active site architecture of DOHH (HAG mechanism [8,9]) facilitates the rapid identification of inhibitors among existing drugs, exemplified by ciclopirox and deferiprone. Both drugs interfere with the hydroxylation and thus bioactivity of the eIF5As [6], and deny HIV-1 the utilization of functional eIF5A for its replication [1, 3].
- Ciclopirox and its chelation homologue P2 display distinct and consistent differences in activity, from inhibition of DOHH to antiretroviral effect. Plain coordination of transition metal ions is therefore not sufficient for the observed effects, which show a structure-activity relation to the presence of a properly positioned hydrophobic anchor.
- The significant augmentation of apoptosis in HIV-1 infected PBMCs, previously also noted for H9 cells [5], suggests that these drugs interfere with the expression of retroviral antiapoptotic proteins, and thus re-activate the original apoptotic response triggered by the infection. The preferential ablation suffices to explain the unprecedented disappearance of retroviral RNA and DNA from the infected PBMC cultures, and the failure to re-establish infection even 10 days after drug discontinuation.
- Hypusine formation within eIF5As is a promising target for the knowledge-guided *de novo* development of a new class of antiretrovirals.
- Clinical trials with deferiprone are pending.

MATERIAL AND METHODS

DOHH activity was measured via enzymatic conversion of radioactive deoxyhypusine, metabolically generated by using tritiated spermidine. P2 was synthesized by standard chemical procedures. H9 cells were cultured in RPMI medium containing 20% FCS v/v. PBMCs were isolated from the blood of healthy donors by Ficoll-plaque density centrifugation. Cells were activated overnight in Media A (RPMI, 10% FCS v/v, 1% penicillin / streptomycin, glutamate, IL-2 and phytohemagglutinin) at 37 C and 5% CO₂. Activated cells were spun down and resuspended in media B (Media A without PHA) at a final concentration of 0.5 x 10⁶ cells/ml. 2ml of the cell suspension (1 x 10⁶) were plated in 24-well plates and infected with PBMCs isolated from HIV-1 patients. The ratio of uninfected to infected PBMCs was 10:1. The cultures were maintained at 37 C and 5% CO₂ for the indicated number of days. Compounds were added to the cultures at the indicated concentrations and times. Cultures were fed everyday by harvesting half of the supernatant and replenishing it with Media B and the respective compound. Cell-free supernatant was used for p24 and viral load measurements. Cells were also processed for apoptosis, determined by TUNEL-based flow cytometry. The p24 protein, viral RNA and proviral DNA were measured by commercial assays. Viability was quantified by computerized trypan blue exclusion.

REFERENCES

- Hamber J. Nuclear export mediated by the Rev/Rex class of retroviral Trans-activator proteins. *Curr Top Microbiol Immunol*. 2001; 259: 15-76.
- Kashner J, Libratnik D, Sakai H, Adachi A, Yonemura K, Shink H. Effects of translation initiation factor eIF5A on the functioning of human T-cell leukemia virus type I Rev and human immunodeficiency virus Rev inhibited trans dominantly by a Rev mutant deficient in RNA binding. *J Virol*. 1995; 69: 3123-31.
- Bever D, Hamber J. Eukaryotic initiation factor 5A activity and HIV-1 Rev function. *Biol Signals*. 1997; 6: 124-53.
- Bever D, Libratnik H, Gu M, Wall T, Hamamoto M, Packer A, Schmitt M, Kunitani K, Dubrovnik M, Cheng R, Lempicki F, Hamber J. Inhibition of HIV-1 replication in lymphocytes by mutants of the Rev cofactor eIF-5A. *Science*. 1996; 271: 1853-60.
- Adachi A, Zlotnik P, Guady RW, Hamamoto M, Hamer-Spencer T, Shirokawa H, Zuparski J, Hanauske-Abel HM. Antiretroviral effects of deoxyhypusine hydroxylase inhibitors: a hypusine-dependent host cell mechanism for replication of human immunodeficiency virus type 1 (HIV-1). *Biochem Pharmacol*. 1998; 57: 1007-16.
- Park MH, Wall EC, Park H. Hypusine is post-translational formation in eukaryotic initiation factor 5A and its potential role in cellular regulation. *Biochem Biophys Res Commun*. 1998; 249: 4-10.
- Hanauske-Abel HM, Lee YR, Wall EC, Clement PM, Park MH. Deoxyhypusine hydroxylase, required for cell proliferation, is a 2-oxoacid utilizing dioxygenase. *FASEB J*. 1991; 5: 4779-4800.
- Hanauske-Abel HM, Ghisler V. A noncovalent mechanism for the catalytic mechanism of prolyl hydroxylase. Applicability to classification and design of inhibitors. *J Biol Chem*. 1992; 267: 421-425.
- Hanauske-Abel HM, Popescu AM. The HAG mechanism: a molecular rationale for the discovery, application of iron chelators in human diseases involving the iron-catalyzed dioxygenase. *Curr Med Chem*. 2003; 10: 1017-1050.
- Altshuler A, Hanauske-Abel HM, Park MH, Haska S, Folk JE. The active site of deoxyhypusine hydroxylase. Use of cathecolpeptides and their copper complex and peptide-mimetic as substrate probes. *Biochemistry*. 1993; 32: 11077-11081.