Heme inhibits human immunodeficiency virus 1 replication in cell cultures and enhances the antiviral effect of zidovudine

RICHARD D. LEVERSE,† YI-FEI GONG, ATTALLAH KAPPAS, DORIS J. BUCHER,‡ GARY P. WORMSER,§ AND NADER G. ABRAHAM∥

The Laboratory of Metabolism and Pharmacology, The Rockefeller University Hospital, New York, NY 10021; and the Departments of †Medicine and §Microbiology/Immunology, New York Medical College, Valhalla, NY 10595

Communicated by Frederick Seitz, November 19, 1990

ABSTRACT The effects of heme alone and heme administered together with 3′-azido-3′-deoxythymidine (AZT) on human immunodeficiency virus replication in human peripheral blood lymphocytes and in the H9 cell line were studied. Heme enhanced the antiviral action of AZT against both drug-resistant and drug-sensitive viral strains; the heme effect was more pronounced against the latter. Moreover, heme alone displayed a significant ability to inhibit viral replication in concentrations markedly smaller than those required to inhibit the reverse transcriptase of Rauscher murine leukemia virus. The results of this study extend the range of pharmacological actions that metalloporphyrins exert in biological systems and suggest that further study of the interactions of the natural compound heme and human immunodeficiency virus chemotherapeutic agents such as AZT may be useful.

Antiviral therapy for the treatment of the acquired immunodeficiency syndrome (AIDS) is based on the assumption that continued retroviral replication is involved in both the pathogenesis and progression of the disease. Therefore, reverse transcriptase (RT) has been a major target for antiviral treatment in AIDS, and indeed most of the agents now being investigated, including zidovudine [3′-azido-3′-deoxythymidine (AZT)], which is currently in clinical use, act on this enzyme. The phosphorylated forms of these compounds inhibit human immunodeficiency virus (HIV) replication, in part, by acting as chain terminators (1, 2). The RT of HIV is much more susceptible to the inhibitory effects of these phosphorylated dideoxynucleosides than are mammalian DNA polymerases. Administration of AZT has been shown to result in immunologic improvement and confers a survival advantage in HIV-infected patients with advanced immunodeficiency (3).

Among the major adverse effects of AZT administration in AIDS patients are anemia and neutropenia (4–7). AZT suppresses the proliferation of erythroid, granulocyte, macrophage, and primitive hematopoietic stem cells in a dose-related and time-dependent fashion (8). In addition, long-term treatment with AZT may create a selective pressure, which affords a replication advantage to viruses of the drug-resistant phenotype. Such variants may be isolated from patients suffering from advanced HIV-associated disease, sometimes as early as 6 months after initiation of treatment (9).

Tsutsumi and Mueller (10) reported that the virion-associated RT activity of Rauscher murine leukemia virus was inhibited by hemin∥ at a concentration of 100 μM. The inhibition of RT by this large concentration of hemin was reversible and appeared to be directed against the enzyme rather than the template. On the other hand, hemin did not inhibit the activity of RT purified from avian myeloblastosis virus.

Heme has long been known to play an important role in cellular differentiation and maturation processes (11–14). Conversely, many agents that either inhibit the synthesis or enhance the degradation of heme are known to have deleterious effects on cell function and viability, especially in the hemopoietic system (15, 16). In view of the toxic actions of AZT on bone marrow stem cells, we recently examined the possibility that heme could exert a protective effect against the bone marrow toxicity of this chemotherapeutic agent. The results of that investigation showed that AZT-induced inhibition of colony-forming unit-erythroid, burst-forming unit-erythroid, and colony-forming unit–granulocyte/macrophage in both murine and human marrow could be counteracted in vitro to a considerable degree by concurrently administered heme (16).

In this study, we examined the possible interactions of AZT and heme on HIV replication to determine whether heme could enhance the antiviral activity of AZT or might alone inhibit viral replication. Our results indicate that heme (10 μM) was able to augment AZT inhibition of replication of an AZT-resistant HIV isolate from one AIDS patient and, at a much smaller concentration (1 μM), augmented inhibition of replication of an AZT-sensitive isolate from another patient. Further, when the AZT-resistant virus from the former patient and a separate AZT-sensitive strain (HTLVIIIIB) were replicated in the H9 cell line, we found that heme without AZT directly inhibited virus replication. This effect of heme alone was most pronounced in the AZT-sensitive isolate.

The results of this study define an additional and potentially important biological property of heme and raise the possibility that other synthetic heme analogues now in experimental or clinical use (17–22) may exert similar or more potent actions, alone or when administered concurrently with appropriate chemotherapeutic agents, against HIV.

MATERIALS AND METHODS

Cells and Viruses. In this study a total of three strains of HIV were used. Two strains were isolated (23) from blood obtained from patients in the Infectious Diseases Clinic at the Westchester County Medical Center (Valhalla, NY). One isolate was obtained from a patient who had been on AZT therapy for 4 months; this isolate is defined as AZT-resistant (at 1 μM AZT) in Table 1. The other isolate was derived from a patient who had never received AZT and is referred to as AZT-sensitive (at 1 μM AZT) in Table 1. The third HIV strain (HTLVIIIIB) was obtained from ERC Bioservices (Rockville, MD) through the National Institute of Allergy and Infectious Diseases AIDS Research and Reference Reagent Program.

Abbreviations: RT, reverse transcriptase; HIV, human immunodeficiency virus; FBS, fetal bovine serum; AZT, 3′-azido-3′- deoxythymidine; PBL, peripheral blood lymphocyte.

∥Hemin is the chloride of ferriprotoporphyrin IX (heme); the terms are used interchangeably in the text.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
were examined: 100 U/ml or when AZT, in HIV AZT-resistant a with the combination the cultures were maintained in heme buffer to -70°C of 1640 concentration of 100 U/ml were infected with the two strains of HIV (HTLV III and the AZT-resistant patient isolate). The initial concentration of HIV p24 was between 800 pg/ml and 3500 pg/ml, assayed by the Abbott HIV antigen detection kit (see below). Heme, supplied as hemin (ferriprotoporphyrin IX chloride) (Sigma) was dissolved in 0.01 M NaOH and diluted in phosphate buffer to a stock concentration of 0.1 mol/liter (pH 7.4). Heme was then added to the two groups of cultures to a final concentration of 0-10 μM. The cultures were maintained in RPMI 1640 medium supplemented with 10% FBS at 37°C with replacement of 50% of the medium with medium containing heme twice during 7 days. An aliquot of 0.5 ml was collected with an equal volume of FBS at day 7 and frozen at −70°C until assayed.

p24 Assay. Samples were assayed in duplicate for the HIV antigen p24 with the Abbott HIV antigen detection system according to the Abbott protocol (Publ. 83-4895/R3).

Cultivation of HIV in Peripheral Blood Lymphocytes (PBLs). Human PBLs (0.5 × 10⁶ per ml) from healthy donors were infected in vitro with HIV isolated from two AIDS patients (Table 1). AZT (Sigma; 99% purity) was dissolved in RPMI 1640 medium and then added to the cultures at a final concentration of 1 μM, whereas heme was added at a final concentration of 1 μM or 10 μM in a total volume of 2 ml. The cultures were maintained at 37°C with replacement of 50% of the medium with medium containing AZT, heme, or a combination of AZT and heme twice within 7 days. Aliquots were collected with an equal volume of FBS on day 7 and frozen at −70°C until assayed.

RESULTS AND DISCUSSION

In the experiments shown in Table 1, AZT (1 μM) completely inhibited HIV replication in the drug-sensitive strain (Table 1, patient 1) on day 7 in in vitro cultures of PBLs. Heme did not alter the antiviral action of AZT when administered concurrently with the drug. Further, heme alone (10 μM) had a substantial inhibitory effect on viral replication. For the AZT-resistant HIV isolate (Table 1, patient 2), heme alone or AZT, in the concentrations studied, had no antiviral activity. However, when a combination of AZT (1 μM) and heme (1 μM or 10 μM) was used, HIV replication was inhibited completely. The effects of hemin and AZT on cell viability were examined after culture termination and measurement of p24. Cell viability after inclusion of hemin at 1 μM and 10 μM was 85% ± 3.5% and 85% ± 5.9%, respectively, which is not different from the untreated cells (78.3% ± 3.5%).

To extend these observations, additional experiments with heme and AZT were carried out to study HIV replication in the H9 cell line. Since we were unable to adapt the AZT-sensitive HIV isolate from patient 1 to the H9 cells, we obtained H9 cells infected with the AZT-sensitive strain HTLVIII. For each subculture of H9 cells, the number of cells at initiation of the culture was ~2 × 10⁶ cells per ml. In these experiments the AZT-resistant HIV from patient 2 was also adapted to H9 cells. To cultures of H9 cells infected with the two strains of HIV, graded amounts (0-1 μM) of AZT were added. The cultures were maintained at 37°C in RPMI 1640 supplemented with 10% FBS with replacement of 50% of the medium with medium containing hemin (1 or 10 μM) twice within 7 days. An aliquot of 0.5 ml was collected, an equal volume of FBS was added at day 7, and the sample was frozen at −70°C until assayed. For the HTLVIII AZT-sensitive HIV, the 50% inhibition concentration (IC50) of AZT was 0.008 μM (Fig. 1). Heme alone at a concentration of 10 μM almost completely blocked replication (Fig. 1). The addition of heme with AZT reduced the IC50 of the drug markedly (Fig. 1); because of the efficacy of heme alone at the 10 μM dose, augmentation of the inhibitory effect of AZT could not be determined.

In the AZT-resistant HIV strain from patient 2, the IC50 of AZT alone in the H9 cells exceeded 1 μM. Heme alone at 1 μM had no effect on virus replication; at a concentration of 10 μM, substantial inhibition (~40%) of virus growth was

![Figure 1: Effect of heme on AZT-sensitive HIVIII (American Type Culture Collection) as a function of increasing concentrations of AZT. This AZT-sensitive strain was replicated in H9 cells, and aliquots were collected at day 7 and assayed for HIV antigen. The SD for each determination is illustrated.](image-url)
Medical Sciences: Levere et al.  

---

**Fig. 2.** Effect of heme on AZT-resistant HIV (patient 2) as a function of AZT concentration. The AZT-resistant strain from patient 2 was replicated in the H9 cell line, and aliquots were collected on day 7 and assayed for the HIV p24 antigen. The SD for each determination is illustrated.

The differing effect of heme alone on the AZT-resistant strain in H9 cells as compared with PBLs (Table 1) may reflect inherent differences in these cell types. The dose–response effect of heme alone on the replication of HIV in H9 cells of both the AZT-resistant and the AZT-sensitive strains of HIV was also examined. Viral replication of the drug-sensitive strain was reduced by 50% at a heme concentration <0.05 μM (Fig. 3); for the drug-resistant strain the comparable inhibitory concentration of heme was ~15 μM (Fig. 4).

These data indicate that heme (10 μM) alone was able to inhibit replication of an AZT-sensitive isolate in cultured PBLs and that in combination with AZT heme in concentrations of 1 μM or 10 μM greatly enhanced AZT efficacy against a drug-resistant HIV strain in such cultures. Against both AZT-sensitive and AZT-resistant viral strains grown in H9 cells, heme alone displayed inhibitory properties and also augmented the antiviral actions of AZT against the AZT-sensitive viral isolates. The doses of heme required for these actions were markedly smaller than the dose of heme required to inhibit virus-associated RT activity of Rauscher murine leukemia virus (10) and are significantly lower than the concentrations of heme used experimentally in mamma-

---

**Fig. 3.** Dose–response curve of inhibition of AZT-sensitive HTLV-III replication in H9 cells by heme. The SD for each determination is illustrated.

The mechanisms by which heme exerts an inhibitory effect on HIV replication in vitro or enhances the antiviral properties of AZT are not presently known. However, as noted, heme, in high concentrations, has been shown to inhibit the RT of Rauscher murine leukemia virus (10), and the compound may act similarly on HIV in addition to enhancing the interaction of AZT with this enzyme. Heme also has been shown to exert significant actions on other cellular processes concerned with gene expression and protein synthesis. These include, among others, inhibition of RNA–protein interactions, enzyme transcription, and DNA and RNA polymerase and restriction endonuclease activities (29, 30). It is of interest in this respect that while neither heme nor AZT alone exerted an inhibitory effect on the AZT-resistant HIV (patient 2, Table 1) the compounds, when administered together, profoundly inhibited virus replication. This finding raises the possibility that the viral mutation leading to AZT resistance affects the site or the manner in which heme may interact with the virus.

It should be noted that aberrations of heme metabolism, manifest as a porphyric clinical syndrome, have been identified by Wissel et al. (31) and other groups (32, 33) in some AIDS patients. Further, erythropoietin has been shown to have a beneficial effect on the anemia associated with this disorder (34, 35). Erythropoietin, in vitro, increases the activity of the rate-limiting enzyme in heme synthesis, δ-aminolevulinate synthase (36, 37), and elevates cellular heme content. Thus, it may be of potential clinical importance to examine further the integrity of heme synthetic and degradative processes in AIDS patients and to determine if the actions of heme described in this report extend to other heme-like molecules and other HIV isolates.

---


4. Walker, R. E., Parker, R. I., Kovacs, J. A., Masur, H., Love,