Inhibition of Human Immunodeficiency Virus Type 1 Replication in Human Mononuclear Blood Cells by the Iron Chelators Deferoxamine, Deferiprone, and Bleomycin

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Replication of human immunodeficiency virus type 1 (HIV-1) can be influenced by iron. Hence, decreasing the availability of iron may inhibit HIV-1 replication. Deferoxamine and deferiprone, both forming catalytically inactive iron-chelator complexes, and bleomycin, by use of which iron catalyzes oxidative nucleic acid destruction, were investigated. Expression of p24 antigen in human monocyte-derived macrophages and peripheral blood lymphocytes (PBL) was reduced by all 3 iron chelators. In PBL, p24 reduction was mirrored by a decrease in proliferation after incubation with deferoxamine or deferiprone, suggesting that viral inhibition is closely linked to a decrease in cellular proliferation. In contrast, clinically relevant bleomycin concentrations reduced p24 levels by ~50% without affecting proliferation. When deferoxamine and the nucleoside analogue dideoxynosine were used in combination, they acted synergistically in inhibiting HIV-1 replication. These observations suggest that iron chelators with different mechanisms of action could be of additional benefit in antiretroviral combination therapy.

There are several possibilities as to how iron is involved in the replication of the human immunodeficiency virus type 1 (HIV-1). First, the HIV-1 long terminal repeat contains nuclear factor (NF)-κB response elements regulating proviral transcription. NF-κB activation can be influenced by iron through the production of reactive oxygen species [1]. It has indeed been shown that iron chelation with 5 μM deferoxamine (DF) and 60 μM deferiprone (L1) inhibited NF-κB activation and the subsequent replication of HIV-1, as measured by p24 antigen production and reverse transcriptase measurements in peripheral blood mononuclear cells (PBMC) and other cell types [2]. However, in a more recent study, DF at concentrations of up to 1 mM and another 2 unrelated iron chelators failed to inhibit NF-κB in Jurkat and NIH 3T3 cells [3]. Earlier reports on the effect of DF on HIV-1 replication were also conflicting. The reduction in p24 antigen by 30 μM DF was attributed to a decrease in absolute cell numbers by other investigators [4]. These latter authors failed to observe an anti-HIV effect by DF (0.5–60 μM), as assessed by the measurement of reverse transcriptase activity in various types of cells and with use of various HIV strains [5].

A second route by which iron chelation could influence HIV replication is inhibition of DNA synthesis by inactivation of iron-dependent ribonucleotide reductase [6]. It is known that nonheme iron is important for the function of ribonucleotide reductase, which is involved in DNA synthesis by reducing ribonucleotides to deoxyribonucleotides [7, 8]. The R2 subunit of this enzyme contains 2 binding sites for iron. By chelating iron from the low-molecular-weight iron pool [9], DF can indirectly inactivate the cellular enzyme ribonucleotide reductase [6, 10] and thus inhibit DNA synthesis, as was shown in human mononuclear cells [11].

Another strategy that can be used in targeting iron chelators against HIV-1 is direct attack on viral DNA or RNA. This could be achieved by bleomycin, a cytostatic agent, isolated from Streptomyces verticillus [12], that has DNA- and RNA-damaging properties. Currently, bleomycin is being successfully used to treat various cancers [13]. The ability of bleomycin to form a complex with DNA [14] and RNA [15, 16] and mediate their oxidative cleavage is believed to be the mechanism by which it directs its antitumor activity. Nucleic acid degradation by bleomycin has been found to be metal ion dependent [17, 18]. Bleomycin chelates Fe2+, and in oxidative environments, an electron will be donated to an oxygen atom, resulting in the formation of an Fe(III)-bleomycin complex and the generation of reactive oxygen species that can attack DNA and RNA by hydrogen abstraction [19].

In the present study, we investigated the effects of DF, L1,
and bleomycin on HIV-1 replication in primary blood cells. We used human peripheral blood lymphocytes (PBL) and monocyte-derived macrophages (MDMs), the main target cells for HIV. We were also interested in exploring the possibility of using a combination of iron chelators with established antiviral agents in antiviral combination therapy.

Materials and Methods

Cell isolation. PBMC fractions were isolated from heparinized blood from HIV-1–, HIV-2–, and hepatitis B–seronegative donors (Blood Bank, Utrecht, the Netherlands) by use of ficoll-isopaque gradient separation. Cells were washed twice and then were subjected to countercurrent centrifugal elutriation, as previously described [20, 21], to obtain monocyte fractions, or monocytes in the PBMC fraction were allowed to adhere on fibronectin-coated flasks before the PBL fraction was harvested. Monocytes were >95% pure by criteria of cell morphology on May-Grünwald-Giemsa–stained cytosmears and by nonspecific esterase staining with α-naphthylacetate (Sigma, St. Louis), and PBL were >85% pure by May-Grünwald-Giemsa staining. Viability of both cell types was >95% at the point of experiment initiation, as determined by trypan blue exclusion. Isolated monocytes were cultured in suspension at a concentration of 2 × 10⁶ cells/mL in Teflon flasks (Nalgene, Rochester, NY), minimizing adherence, and were allowed to differentiate for 7 days. MDM medium consisted of Dulbecco’s modified Eagle medium supplemented with 10% heat-inactivated human AB serum, negative for anti-HIV antibodies (Blood Bank, Utrecht, the Netherlands); 10 μg/mL gentamicin (Life Technologies, Paisley, UK); 5 μg/mL ciprofloxacin (Beyer, Leverkusen, Germany); 0.6 mg/mL l-glutamine (Sigma); and 3.4 g/L bicarbonate (Merck, Darmstadt, Germany). Isolated PBL (10⁶/mL) were stimulated to proliferate for 3 days with 4 μg/mL phytohemagglutinin (Sigma) in RPMI 1640 (Life Technologies) that had been supplemented with 10% heat-inactivated fetal calf serum (Sigma) and 10 μg/mL gentamicin. After stimulation with phytohemagglutinin, PBL were cultured in medium containing 10 U/mL human recombinant interleukin-2 (Boehringer Mannheim, Mannheim, Germany). All incubations were done in flat-bottomed 96-well plates at 37°C in 5% CO₂ and 95% air.

HIV infection and p24 measurements. Cells were infected for 2 h with HIV-1₄₄₅-5. Macrophages were infected at an MOI of 0.005 and lymphocytes at an MOI of 0.001. The cells were then washed twice to remove excess virus and subsequently were incubated with either DF (Novartis Pharma, Arnhem, the Netherlands), L1 (donated by Ducheña Farma, Haarlem, the Netherlands), or bleomycin (H. Lundbeck, Copenhagen, Denmark) for 5 days. Virus in culture supernatant was inactivated in a final concentration of 0.05% emepigen (Calbiochem-Novabiochem, La Jolla, CA). The presence of HIV-1 in the inactivated supernatant was monitored by checking for the p24 core antigen by ELISA, as previously described [22, 23].

Lymphocyte proliferation measurements. Because iron chelators may influence cellular proliferation [11], we also determined the effect of the 3 iron chelators on PBL proliferation. This was done by monitoring overnight [³H]thymidine incorporation into DNA. [³H]thymidine (25 μCi [0.5 μCi]; Amersham International, Amersham, UK) was added in the presence of DF, L1, bleomycin, or the combination of DF and dideoxynosine (ddl; Sigma), in a total volume of 200 μL, to cell suspensions after 4 days of incubation with the different compound concentrations. Cells were incubated in 96-well plastic plates (Costar, Cambridge, MA). After overnight incubation with [³H]thymidine, the cells were harvested onto a filter by means of an automatic cell microharvester (Titrertek cell harvester 530; Titrertek, Huntsville, AL). The filter was subsequently dried and immersed in scintillation cocktail (Wallac beta plate scint; Wallac, Milton Keyens, Bucks, UK). Radioactivity was counted in a Wallac 1205 betaplate liquid scintillation counter (Pharmacia, Buckinghamshire, UK).

Cytotoxicity. Possible cytotoxicity of DF, L1, and bleomycin was monitored by the MTT assay (Sigma) [24].

Studies of DF-ddI combination. Three separate experiments were done, and their setup was as described above. Multiply diluted fixed-ratio combinations of the drugs or single drugs were added to each incubation point. The IC₅₀ of each drug was calculated by

Figure 1. Effect of deferoxamine (DF) on human immunodeficiency virus type 1 (HIV-1) replication (A), cytotoxicity (B), and cellular proliferation (C) in peripheral blood lymphocytes (PBL) after 5 days of incubation. Cells were infected for 2 h with HIV-1₄₄₅-5 at an MOI of 0.001, washed twice, and incubated with different concentrations of DF. After 5 days, supernatant was harvested for p24 ELISA. Viral replication was expressed as percentage of reduction in p24 after incubation with DF compared with p24 in control HIV-infected cells incubated in absence of DF (100% p24 [19 ng/mL]). Cytotoxicity was measured by MTT assay (Sigma, St. Louis) and is expressed as percentage of viable cells after 5 days of incubation with DF compared with viable cells from control incubations in absence of DF (100% viable cells). PBL proliferation was measured by overnight [³H]thymidine incorporation after 4 days of incubation with DF and is expressed as cpm after incubation with DF compared with cpm from control cells incubated in absence of DF. Results are average of 3 experiments in duplicate. *P < .05 (repeated-measures analysis of variance and Student-Newman-Keuls test).
Figure 2. Effect of deferiprone (L1) on human immunodeficiency virus type 1 (HIV-1) replication (A), cytotoxicity (B), and cellular proliferation (C) in peripheral blood lymphocytes (PBL) after 5 days of incubation. Cells were infected for 2 h with HIV-1 Ba-L at an MOI of 0.001, washed twice, and incubated with different concentrations of L1. After 5 days, supernatant was harvested for p24 ELISA. Viral replication was expressed as percentage of reduction in p24 after incubation with L1 compared with p24 in control HIV-infected cells incubated in absence of L1 (100% p24 [19 ng/mL]). Cytotoxicity was measured by MTT assay (Sigma, St. Louis) and is expressed as percentage of viable cells after 5 days of incubation with L1 compared with viable cells from control incubations in absence of L1 (100% viable cells). PBL proliferation was measured by overnight [3H] thymidine incorporation after 4 days of incubation with L1 and is expressed as cpm after incubation with L1 compared with cpm from control cells incubated in absence of L1. Results are average of 3 experiments in duplicate. * (repeated-measures analysis of variance and Student-Newman-Keuls test).

Figure 3. Effect of bleomycin on human immunodeficiency virus type 1 (HIV-1) replication (A), cytotoxicity (B), and cellular proliferation (C) in peripheral blood lymphocytes (PBL) after 5 days of incubation. Cells were infected for 2 h with HIV-1 Ba-L at an MOI of 0.001, washed twice, and incubated with different concentrations of bleomycin. After 5 days, supernatant was harvested for p24 ELISA. Viral replication was expressed as percentage of reduction in p24 after incubation with bleomycin compared with p24 in control HIV-infected cells incubated in absence of bleomycin (100% p24 [19 ng/mL]). Cytotoxicity was measured by MTT assay (Sigma, St. Louis) and is expressed as percentage of viable cells after 5 days of incubation with bleomycin compared with viable cells from control incubations in absence of bleomycin (100% viable cells). PBL proliferation was measured by overnight [3H] thymidine incorporation after 4 days of incubation with bleomycin and is expressed as cpm after incubation with bleomycin compared with cpm from control cells incubated in absence of bleomycin. Results are average of 3 experiments in duplicate. *P < .05 (repeated-measures analysis of variance and Student-Newman-Keuls test).

Results

Effect of DF on HIV-1 replication, cellular proliferation, and cytotoxicity in PBL. DF was capable of reducing p24 levels in culture supernatants. At a concentration of 3 μM DF, we observed a 40% reduction in viral replication in PBL (19 vs. 11 ng of p24/mL; n = 3; P < .05). A compound concentration of 30 μM reduced p24 levels by >90% (figure 1A), from 19 to 1.9 ng of p24/mL (n = 3). To rule out the possibility that these inhibitory effects were due to cytotoxicity, cell viability was monitored. A DF concentration of 100 μM was found to be cytotoxic (figure 1B). In addition, because of the antiproliferative properties of DF, cellular proliferation was also monitored. The observed reduction in p24 was mirrored by a decrease in proliferation (figure 1C). Thus, noncytotoxic DF

Use of the computer software program CalcuSyn (Biosoft, Cambridge, UK) [25]. The combined-drug effect was evaluated by the median-effect principle and the isobologram method [26]. This method involved plotting the dose-effect curve for each drug alone and in combination. The slope of the median-effect plot and the x intercept of the plot were then used for the computerized calculation of a combination index. The combination index values were based on the classic isobologram equation, and the values <1, 1, and >1 indicate synergism (greater than expected additive effect), additive effects, and antagonism (less than the expected additive effect), respectively. Fraction affected was calculated for each drug combination and for each drug alone after p24 measurements in infected PBL incubated for 5 days with compounds.

Statistical analysis. Repeated-measures analysis of variance and the Student-Newman-Keuls test were used to analyze the data. P < .05 was considered significant.
concentrations were capable of reducing HIV replication, and this reduction was accompanied by inhibition of cellular proliferation.

**Effect of L1 on HIV-1 replication, cellular proliferation, and cytotoxicity in PBL.** HIV-1 replication in lymphocytes was reduced to minimal levels by 100 μM L1 (figure 2A). This concentration was not cytotoxic in PBL (figure 2B). There seemed to be a threshold point in L1 concentration at which the inhibition slope became very steep, and this phenomenon was also mirrored by a decrease in proliferation (figure 2C). Thus, the HIV inhibition observed with L1 could also be attributed to inhibition of cellular proliferation. At lower compound concentrations, a subtle increase in p24 antigen production was noted to inhibition of cellular proliferation. At lower compound concentrations were capable of reducing HIV replication, and this reduction was accompanied by inhibition of cellular proliferation.

**Effect of bleomycin on HIV-1 replication, cellular proliferation, and cytotoxicity in PBL.** Bleomycin dose-dependently decreased HIV-1 replication in lymphocytes (figure 3A). This compound was not cytotoxic in PBL (figure 3B). At concentrations in the range of 3–300 ng of bleomycin/mL, a 20%–50% reduction was noted, reflecting a decrease from 19 to 15 (20% decrease) or to 9.5 (50% decrease) ng of p24/mL (n = 3; figure 3A). Cellular proliferation remained intact (figure 3C). Thus, HIV-1 inhibition by bleomycin, in contrast to DF and L1, seems to proceed via a pathway other than inhibition of cellular proliferation.

**Effects of DF, L1, and bleomycin on HIV-1 replication and cytotoxicity in MDM.** In MDM, DF concentrations of ~100 μM reduced viral replication by up to 50% (1.2 vs. 0.6 ng of p24/mL) in a dose-dependent manner (figure 4A, left). However, this decrease in p24 was associated with the onset of cytotoxicity (figure 4A, right). L1 in the concentrations studied strikingly up-regulated p24 antigen production by up to 2-fold at 30 μM (figure 4B, left). These concentrations were not cytotoxic (figure 4B, right).

In MDM, a dose-dependent decrease in p24 levels was also observed, with a reduction of 50% at 300 ng of bleomycin/mL, from 1.2 to 0.6 ng of p24/mL (figure 4C, left). As was the case in PBL, bleomycin concentrations were found not to affect cell viability in MDM (figure 4C, right).

**Combined antiviral effects of DF-ddI.** Moderate to strong synergism was detected when DF was used in combination with ddI. Figure 5 shows a representative graph of the combination index with respect to fraction affected for the inhibition of HIV by a mixture of DF and ddI (molar ratio, 4:1). The IC$_{50}$ of DF was 8.5 μM, and that of ddI was 3.7 μM. From figure 5, it can be seen that there is marked synergism at all fraction-affected values obtained with the different combinations, with the synergism getting stronger as the fraction affected increases. At around the IC$_{50}$ concentrations of both DF and ddI (combination points on figure 5, 10 μM DF, 2.5 μM ddI), the highest fraction-affected value was obtained, and the combination was strongly synergistic. Cellular proliferation was also monitored with these combinations and with each drug alone. It was found that ddI in combination with DF did not have an additional inhibitory effect on cellular proliferation, compared with that of DF alone (data not shown).

**Discussion**

A reduction in HIV-1 replication was noted on incubation of infected human PBL with all iron chelators used in this study. However, there was a distinct difference between either DF or L1 and bleomycin. In PBL, the reduction in HIV-1 replication by DF and L1 was mirrored by a decrease in cell proliferation (figures 1 and 2). DF and L1 have been reported to inhibit lymphocyte proliferation [27]. Because HIV replicates only in proliferating T cells [28], we believe that inhibition of cellular proliferation could be the pathway of p24 inhibition in lymphocytes. This leads to the conclusion that neither DF nor L1 directly attacks the virus; rather, they target the host where the virus replicates. On the other hand, bleomycin inhibited viral replication without affecting cellular proliferation (figure 3), suggesting that bleomycin attacks the virus directly.

The effect of DF and L1 was also found to vary between the 2 different cell types studied. In general, PBL were more sensitive to iron chelation by these compounds than were MDMs. Lymphocytes are proliferating cells, and macrophages are fully differentiated cells. Proliferating cells, in general, have higher needs for iron, because they synthesize high amounts of DNA, requiring the ribonucleotide reductase enzyme. The specific ribonucleotide reductase inhibitor hydroxyurea has been found to reduce HIV-1 levels in human cells in vitro by lowering the dATP pool in the cell [29] and inhibiting [3H]thymidine incorporation into DNA [6]. Interestingly, in combination with nucleoside reverse transcriptase inhibitors such as ddI, hydroxyurea was found to synergistically [30] and potently effect long-term inhibition of HIV-1 replication in PBMC and macrophages, even after a 1-year suspension of treatment (see also [30–33]). In the case of hydroxyurea when used with nucleoside analogues, viral replication is attacked by incorporation of nucleoside analogues, which will terminate viral DNA synthesis, in competition with cellular deoxynucleoside triphosphates.

Iron chelators such as DF could use the same mechanism of action in inhibiting HIV-1 replication, because DF has also been found to decrease the dATP pool in human lymphocytes [6]. DF, with a similar mechanism of action to hydroxyurea (nucleotide depletion), could have the same beneficial effects in combination with ddI. This was the rationale for the choice of the DF-ddI combination investigated herein. DF and ddI were also found to synergistically work toward inhibiting p24 production in infected PBL.

The proliferation inhibition noted to be induced by both hydroxyurea and DF does not necessarily imply undesirable effects. The effect of hydroxyurea has been found to be re-
Figure 4. Effect of deferoxamine (DF) (A), deferiprone (L1) (B), and bleomycin (C) on human immunodeficiency virus type 1 (HIV-1) replication (left) and compound cytotoxicity (right) in monocyte-derived macrophages after 5 days of incubation with compounds. Cells were infected for 2 h with HIV-1<sub>in</sub> at an MOI of 0.005, washed twice, and incubated with different compounds. After 5 days, supernatant was harvested for p24 ELISA. Viral replication was expressed as percentage of reduction in p24 after incubation with bleomycin compared with p24 in control HIV-infected cells incubated in absence of bleomycin (100% p24 [1.2 ng/mL]). Cytotoxicity was measured by MTT assay (Sigma, St. Louis) and is expressed as percentage of viable cells after 5 days of incubation with bleomycin compared with viable cells from control incubations in absence of bleomycin (100% viable cells). Results are average of 3 experiments in duplicate. *P < .05 (repeated-measures analysis of variance and Student-Newman-Keuls test).

versible [30], and the S-phase inhibition of human lymphocytes by DF has also been shown to be reversible [11] and thus can be monitored closely in patients. De Boer and Boucher [34], in 1996, used a mathematical model for the anti-CD4 treatment of AIDS, and they suggest that reducing the CD4 target cell count by ~25% will strongly reduce the HIV load, and a decreased amount of escape mutants will be formed (see also [35]). This could explain the encouraging long-term effects of the ddI-hydroxyurea combination. By killing dividing CD4 T cells and by depleting intracellular dATP concentrations, DF, just like hydroxyurea, could be reducing the availability of suitable target cells for HIV. In a similar manner, DF and ddI, as a potential antiviral combination, could have the added benefit of long-term reduction of HIV replication.

The 2-fold increase in HIV-1 replication induced by L1 at low concentrations and not by DF in MDMs could be ex-
synergistic, additive, and antagonistic effects, respectively. Subdivision
and of ddI was 3.7 μM. Data are representative of 3 independent
experiments.

explained by the fact that the latter chelator is a hexadentate and
the former a bidentate ligand [36]. The iron-inactivating ca-
cacity of L1 is, as a consequence of this structural difference,
concentration dependent. At lower concentrations, it will in-
sufficiently encompass all of the 6 coordination sites of iron,
leaving the iron still catalytically active [37] and able to stim-
ulate HIV replication. This characteristic should be kept in
mind when bidentate iron chelators are used in anti-HIV treat-
ment. Another major difference between these 2 chelators is
their different membrane permeability properties. The partition
coefficient of free ligand for DF is 0.01 and for L1 is 0.21 [38];
thus, L1 penetrates the cell membrane faster.

The antiviral potential of bleomycin is of importance, be-
cause the drug did not affect proliferation or viability of human
lymphocytes and macrophages in clinically relevant concen-
trations. We have investigated the iron dependence of bleomycin
by coincubations of DF and bleomycin. In the presence of DF,
bleomycin was unable to exert its p24-inhibiting action (data
not shown). Possible limitations to the long-term use of bleo-
mycin would be the onset of lung damage as a consequence of
the reduced levels of bleomycin hydrolase in lung tissue, which
is capable of breaking down the drug [39], and increased
amounts of cytokines in alveolar macrophages in the presence
of bleomycin [40]. In addition, the cumulative dose should not
exceed 450 mg, to avoid bleomycin toxicity [41]. However, al-
though bleomycin, as a single drug, did not completely inhibit
HIV replication in the cell systems described herein, it may give
a valuable contribution to combination therapy, because its
mode of action is completely different from those of the already
used protease and reverse transcriptase inhibitors.

The concentration ranges of the iron chelators investigated
in this study were of clinical relevance. During a 24-h subcu-
taneous infusion of DF (100 mg/kg), the plasma concentrations
reached a maximum of 20 μM in normal subjects (8 μM in
iron-loaded patients); a single intravenous injection (10 mg/kg)
results in plasma concentrations between 80 and 130 μM [42].
L1 serum concentrations after oral ingestion of 50 mg/kg have
been reported to reach between 50 and 350 μM [43–45]. Intra-
muscular administration of 2–10 mg/m² bleomycin results in
peak plasma levels of 130–600 ng/mL, or about one-tenth the
peak levels achieved by the intravenous bolus doses [46].

We conclude that these observations suggest that iron che-
lators with different effects on iron biochemistry—rendering
iron catalytically inactive (DF and L1) or, on the other hand,
favoring its catalytic function (bleomycin)—could be of addi-
tional benefit in antiretroviral combination therapy. The com-
bination DF with ddI proved to work synergistically in reducing
HIV in human PBL in vitro. Further study of other combi-
nations of iron chelators with established antiviral agents is
worthwhile.

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