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The site of antiviral action of 3-nitrosobenzamide on the infectivity process of human immunodeficiency virus in human lymphocytes

(zinc finger/proviral DNA block)

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ABSTRACT **The C-nitroso compound 3-nitrosobenzamide, which has been shown to remove zinc from the retroviral-type zinc finger of p7NC nucleocapsid proteins, inhibits acute infection of human immunodeficiency virus type 1 in cultured human lymphocytes. The attachment of the virus to lymphocytes and the activities of critical viral enzymes, such as reverse transcriptase, protease, and integrase, are not affected by 3-nitrosobenzamide. However, the process of reverse transcription to form proviral DNA is effectively abolished by the drug, identifying the mode of action of 3-nitrosobenzamide as interrupting the role of p7NC in accurate proviral DNA synthesis during the infectious phase of the virus life cycle.**

Rational drug design for the chemotherapy of AIDS requires the identification of conserved viral target structures (see ref. 1) and the development of drugs that exploit their unique features. The binding of human immunodeficiency virus (HIV) to cell surfaces, specifically the interaction of the viral envelope glycoprotein gp120 with the cellular CD4 receptor, is blocked by dextran sulfate and soluble CD4. However, application of such compounds to clinical chemotherapy failed (2). A second specific phase of the HIV infectious cycle, reverse transcription, has been an intensely explored target by reverse transcriptase oriented drugs, such as nucleoside analogs and non-nucleoside inhibitors. Rapidly developing drug resistance appears to thwart this approach (3–5). The HIV protease, required for post-integrational processing of viral precursor polypeptides, has been shown to be inhibited by synthetic polypeptides, but adverse pharmacological properties of these inhibitors interfered with their clinical application (6). Consequently, there is a sustained need for novel anti-HIV agents.

The p7NC nucleocapsid protein of HIV-1 contains two retroviral zinc finger sequences, each consisting of 14 amino acids (Cys-Xaa_2-Cys-Xaa_4-His-Xaa_4-Cys) that chelate zinc through three cysteine thiolates and one histidine imidazolate (CCHC) (7–12). Since the CCHC sequence is completely conserved and is essential for viral replication, this sequence in principle is a prime target for antiviral chemotherapy. We recently demonstrated that treatment of HIV-1 with 3-nitrosobenzamide (NOBA) results in a loss of zinc from the virion, as well as from a synthetic HIV-1 zinc finger polypeptide, coincidental with viral inactivation (13). Since the biochemical mechanisms of cellular infectivity of HIV have been reasonably well defined (1), we designed experiments to identify the site of action of NOBA. We demonstrate here that the anti-infectivity action of NOBA on HIV-1 is explained by the inhibition of proviral DNA synthesis alone, without a direct impairment of the reverse transcriptase, integrase, or protease enzymes. The binding of HIV-1 to human lymphocytes is also unaffected by NOBA. Integrase (14), another critical enzymatic component of HIV-1 that contains a zinc finger motif and is specifically required for the integration of proviral DNA into the target cell genome (1), was also found to be unresponsive to NOBA. Two other DNA-binding nuclear enzymes, topoisomerases I and II (15, 16), the former identified to contain zinc-binding cysteine residues (17), were equally insensitive to the drug. Since the structures of integrase and topoisomerases I and II are dissimilar from the zinc fingers of p7NC protein (10) or that of poly(ADP-ribose) polymerase (18), we conclude that NOBA specifically attacks the anisotropic "retroviral" zinc finger configuration, representing a uniquely specific molecular pharmacological mechanism.

MATERIALS AND METHODS

Virus Replication Inhibition Assays. Phytohemagglutinin-stimulated human peripheral blood mononuclear cells (PBMCs) were distributed into 96-well plates (10^5 per well) in the presence of the indicated concentrations of NOBA and 250 50% tissue culture infectious doses ($TCID_{50}$) of the HIV-1_{WeJo} pediatric clinical isolate (kindly provided by Robert Buckheit of the Southern Research Institute AIDS Antiviral Screen Program, Frederick, MD), which has been propagated only in human PBMCs. After 7 days, cultures were assayed for p24 antigen content with a p24 antigen-capture kit (Coulter). Cell viability was quantitated by using biscarboxyethyl-5(6)-carboxyfluorescein acetoxymethyl ester (Molecular Probes) (19).

Enzyme Assays. The *in vitro* activity of reverse transcriptase was determined with the Boehringer Mannheim ELISA kit (20), and 3′-azido-3′-deoxythymidine 5′-triphosphate (AZTTP) was included as a positive control for inhibition of reverse transcriptase. For assay of endogenous reverse transcription, 10 μg of HIV-1_{IIIB} (Universal Biotechnology, Rockville, MD) was treated with NOBA at the indicated concentrations for 10 min at 25°C, followed by permeabilization of the virus with melittin (Sigma) and subsequent

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Abbreviations: AZTTP, 3′-azido-3′-deoxythymidine 5′-triphosphate; HIV, human immunodeficiency virus; NOBA, 3-nitrosobenzamide; PBMC, peripheral blood mononuclear cell.

incubation of the reaction mixture without dithiothreitol for 6 hr at 39°C as described (21). Reactions were terminated with 0.1% SDS/10 mM EDTA, electrophoresis was performed with 0.7% agarose gels, and the gels were dried and exposed for autoradiography. HIV-1 protease activity was quantitated by a reverse-phase HPLC assay (22), but without dithiothreitol, and HIV-1 integrase activity was measured as reported (14). For comparison, topoisomerases I and II were assayed as described (15, 16).

DNA Amplification Procedures. Proviral DNA synthesis was monitored with an undiluted HIV-1_{IIIB} stock that had been premixed with NOBA or the solvent (dimethyl sulfoxide). PBMCs (3×10^6) were added and cultured for 24 hr. Cells were then washed, the DNA was extracted and PCR-amplified with long terminal repeat/*gag* primer pairs (M667/M661), and the products were analyzed by electrophoresis in 2% agarose gels, which were visualized by autoradiography of the dried gels (23).

Virus Attachment Assays. Binding of HIV-1_{RF} to PBMCs was measured by a p24-based assay. In brief, 5×10^5 PBMCs were incubated with a concentrated stock of virus for 30 min, the unbound virus was washed away, and the cell-associated virus was solubilized and analyzed by the p24 antigen-capture assay. The binding of HIV-1 to PBMCs was blocked in a concentration-dependent manner by dextran sulfate (see Table 1). Cell surface binding of HIV-1_{LAV} to PBMCs was also quantitated by flow cytometry using fluorescein-conjugated anti-HIV-1_{LAV} as reported (24).

NOBA was synthesized as reported.**

RESULTS

Inhibitory Effect of NOBA on Viral Replication. The p7NC protein contains two separate zinc finger sequences that are required not only for packaging of viral genomic RNA but also for early events in viral replication, suggesting that NOBA may induce a specific inhibitory effect in early stages of viral infection. To define this antiviral effect, studies were designed to measure the concentration-dependent action of the drug on HIV-1 replication under conditions in which the target cells (PBMCs) were simultaneously mixed with the HIV-1_{WeJo} pediatric clinical isolate and various concentrations of NOBA. As shown in Fig. 1, NOBA inhibited p24 viral antigen production with an EC_{50} (level of drug that inhibits infection by 50%) of 1.56 μM, and there was a depression of lymphocytes at 50 μM NOBA. Since the *in vitro* culturing of lymphocytes requires phytohemagglutinin, necessarily introducing some degree of artificiality, *in vitro* efficacy of NOBA has to be studied in cell types that need no artificial growth stimulants. For these reasons the apparent efficacy of NOBA, estimated to be about 32, in stimulated lymphocytes may be an underestimation.

Insensitivity of the Binding of HIV-1 to Cells and of Reverse Transcriptase, HIV-1 Protease, and Integrase to NOBA. We determined the influence of NOBA on the binding of HIV-1 to PBMCs and on the *in vitro* activities of HIV-1 enzymes namely, on reverse transcriptase, protease, and integrase. Pretreatment of virus with 100 μM NOBA had no effect on the attachment of virus, as quantitated by the association of p24 with the PBMCs (Table 1), whereas dextran sulfate (10 μg/ml) produced nearly complete inhibition. The lack of an effect on viral attachment by C-nitroso drugs was also confirmed by a flow cytometry method which is based on the fluorescein isothiocyanate-anti-HIV-1 assay (data not shown). With an artificial homopolymer template–primer, poly(rA)·oligo(dT), there was no inhibitory effect of NOBA on the activity of reverse transcriptase (Table 1), whereas AZTTP effectively inhibited the enzyme activity. Likewise, although the A-74704 synthetic protease inhibitor (25) at 1 μM was found to depress the protease activity, NOBA (100 μM) demonstrated no inhibition of protease activity (Table 1). It is of particular interest that NOBA had no effect on integrase activity (Fig. 2) even after preincubation. The integrase protein contains a "classical" type of zinc finger sequence (CCHH rather than the retroviral CCHC type) (26). As a positive control, the inhibitory action of caffeic acid phenethyl ester on integrase is also shown. Since the major DNA-binding nuclear enzymes topoisomerases I and II contain zinc, the action of NOBA was also tested on these enzymes. At concentrations of NOBA which completely block HIV infectivity or the formation of proviral DNA, no effects on topoisomerases I and II could be ascertained even after preincubation for 1 hr (data not shown). Thus, NOBA was without effect on four major targets of HIV-1 (attachment, reverse transcriptase, protease, and integrase) and exhibited specificity toward the retroviral zinc finger structure.

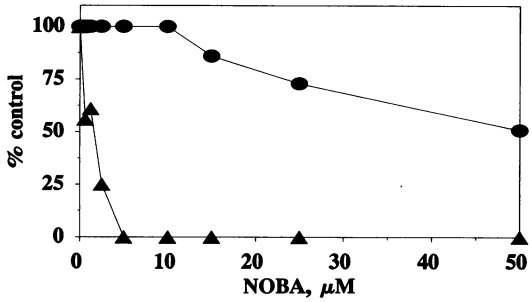

FIG. 1. Inhibition of HIV-1_{WeJo} replication by NOBA. Human PBMCs were placed in 96-well plates (10^5 per well), various amounts of NOBA immediately followed by 250 $TCID_{50}$ of HIV-1_{WeJo} were added, and the plates were incubated for 7 days. Virus production was measured by p24 antigen capture (▲) and is expressed as the percent of antigen in the infected cells in the absence of drug (10 replicates per point). Cell viability (●) was determined by use of biscarboxyethyl-5(6)-carboxyfluorescein (19), and activities are expressed as a percentage of the signal in the drug-free and virus-free control (10 replicates per point).

NOBA Blocks the Synthesis of Proviral DNA. The formation of proviral DNA within PBMCs was determined by mixing a concentrated stock suspension of HIV-1_{LAV} with the drug and adding the mixture to PBMC cultures. After 24 hr in culture the cells were analyzed by PCR with long terminal repeat/*gag* (M667/M661) primer pairs to probe for the presence of full-length or nearly full-length proviral DNA (23). The products of reverse transcription, as assayed by PCR, were completely blocked by 10 μM NOBA (Fig. 3). Virus replication was also blocked under the same conditions (data

Table 1. Effect of NOBA on various HIV-1 functions

Condition	Attachment*	RT activity†	Protease activity‡
No drug	1.01 ± 0.09	1.002 ± 0.108	0.335 ± 0.129
NOBA (100 μM)	1.17 ± 0.22	1.109 ± 0.037	0.375 ± 0.147
Dextran sulfate (10 μg/ml)	0.06 ± 0.05		
AZTTP (1 μM)		0.087 ± 0.058	
A-74704 (1 μM)			0.005 ± 0.01

*Values for virus attachment (mean ± SD, $n = 3$ of the absorbance at 450–650 nm) represent p24 levels as measured by an antigen-capture assay.
†Mean ± SD ($n = 3$) of the 405/490 nm absorbance ratio.
‡Values represent the mean ± SD ($n = 3$) of the change in absorbance at 206 nm for the cleavage of the synthetic substrate A-74704.

**Kun, E., Mendeleyev, J. & Rice, W. G., U. S. Patent Appl., No. 07/780,809, pending.

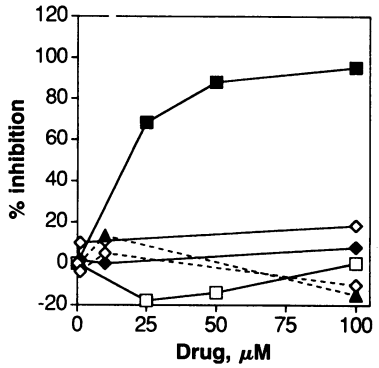

FIG. 2. NOBA does not inhibit integrase activity. HIV-1 integrase protein (2 pmol per reaction mixture) produced by an *Escherichia coli* expression vector (14) was obtained through the generosity of R. Craigi (National Institute of Diabetes and Digestive and Kidney Diseases) and stored at −70°C in 1 M NaCl/20 mM Hepes, pH 7.6/1 mM EDTA/1 mM dithiothreitol/20% (wt/vol) glycerol. Caffeic acid phenethyl ester was kindly supplied by Dezider Grunberger (Columbia University, New York). The assay was carried out as reported (14). ◇, NOBA, preincubation, DNA cleavage assay; ▲, NOBA, preincubation, integration assay; ◇, NOBA, no preincubation, cleavage assay; ◆, NOBA, no preincubation, integration assay; □, caffeic acid phenethyl ester, no preincubation, cleavage assay; ■, caffeic acid phenethyl ester, no preincubation, integration assay.

not shown). NOBA inhibited the reverse transcription process assayed in permeabilized HIV-1 virions (Fig. 4) composed of the native RNA template, $tRNA_3^{Lys}$ primer, and reverse transcriptase and nucleocapsid proteins. This "endogenous" assay used a 100-fold higher concentrated stock of HIV-1_{IIIB} than the tests illustrated in Fig. 3; therefore higher concentrations of NOBA were required, since there is a stoichiometry between the concentration of NOBA and that of retroviral zinc fingers (13). Even though NOBA does not directly affect reverse transcriptase, it prevents the formation of mature proviral DNA that is required for integration into the cellular genomic DNA.

DISCUSSION

The retroviral zinc finger is one of the most highly invariant structures of retroviruses, and mutations of the zinc-chelating amino acid residues (CCHC) result in noninfectious virions (7–12). Since HIV-1 p7NC nucleocapsid proteins

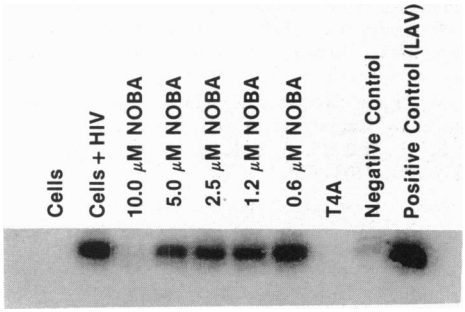

FIG. 3. PCR analysis of the effect of NOBA on HIV-1 proviral DNA formation in PBMCs. Various concentrations of NOBA were added to a concentrated stock of HIV-1 (30 min at 37°C), which was then mixed with a pellet of 3×10^6 PBMCs and incubated for 24 hr. The concentration of drug in the final mixture is indicated. After incubation the samples were analyzed by PCR as described in *Materials and Methods*. As a control, cells were exposed to virus in the presence of anti-CD4 monoclonal antibodies (T4A) to block infection. The negative control represents the PCR performed in the absence of primers. The positive control is the 8E5 bone marrow isolate from a patient infected with HIV-1_{LAV}.

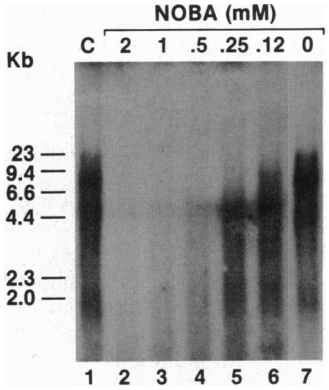

FIG. 4. Inhibition of endogenous reverse transcription in HIV-1 virions by NOBA. Permeabilized HIV-1 virions were allowed to reverse transcribe their native RNA to viral DNA in the absence or presence of various concentrations of NOBA. The controls were virus alone (C) or virus in the presence of 1% dimethyl sulfoxide (0 drug). The [^{32}P]dCTP-labeled transcripts were viewed by autoradiography after electrophoresis in 1% agarose gel.

contain two CCHC zinc fingers that are required for encapsidation of viral genomic RNA and also for early processes during virus infection (27), inactivation by C-nitroso effector drugs inhibit two consecutive events of viral replication on the same protein. The attack by several drugs at multiple enzyme sites on a single target protein has recently been suggested to circumvent the emergence of drug-resistant mutant strains (28). The above-described dual action of a single drug on two consecutive sites on the same protein appears to be a molecular mechanism that has not been previously recognized.

We have reported recently that treatment of HIV-1 with C-nitroso compounds, in particular with NOBA, results in viral inactivation and the loss of zinc from intact virions (13) and in the ejection of zinc from a synthetic HIV-1 CCHC zinc finger peptide, by an oxidative attack on the cysteine thiolate of the CCHC sequence but not through a zinc chelation, since NOBA-type molecules are weak zinc-complexing agents (unpublished results). It is evident that NOBA does not affect other discrete steps of the HIV infectivity path, namely the attachment of HIV to lymphocytes and the activities of HIV-1 reverse transcriptase, protease, and integrase (Table 1). The unresponsiveness of these target sites to NOBA can hardly be overemphasized and clearly indicates the specificity with which the drug inhibits the synthesis of proviral DNA (Fig. 4), pinpointing the site of action of NOBA as a blockage in the transcription of the viral RNA to infectious proviral DNA. These findings are consistent with a large body of experimental evidence that p7NC plays a pivotal role in early steps of HIV-1 infection (27, 29–33).

Oxidative ejection of zinc from CCHC-type zinc fingers by C-nitroso drugs, as first demonstrated for zinc finger I of poly(ADP-ribose) polymerase (34, 35), represents a specific and selective inactivation mechanism with profound biological consequences (13), as shown also in the present experiments. However, the unique sensitivity of the retroviral-type zinc fingers toward oxidation by C-nitroso compounds requires further analysis. The CCHC structure, for convenience defined as an "anisotropic" zinc finger with respect to the zinc-coordinating amino acids, is unique for the retroviral zinc fingers of nucleocapsid proteins (12), and only rare eukaryotic DNA-binding proteins, such as poly(ADP-ribose) polymerase (18) and a protein that binds to the steroid regulatory DNA element, share this zinc finger structure (36). A recently identified cytoplasmic protein, restin, of Reed–Stenberg cells of Hodgkin disease and dynein-associated proteins which regulate embryonic development in *Drosoph-*

ila also contain the CCHC motif (37) and thus could be putative targets of C-nitroso molecules. On the other hand, proteins containing the more common zinc finger structures where two cysteines and two histidines or four cysteines are complexed with the zinc ion, such as HIV-1 integrase, were found to be unresponsive to oxidative zinc removal by C-nitroso molecules.

Although the mode of antiviral action of NOBA on the process of the HIV-1 infectivity pathway is readily explained by the inactivation of p7NC zinc fingers by NOBA, a more complex additional mechanism that implicates the integrated viral DNA cannot be strictly ruled out at this time. It is possible that the potential chemotherapeutic application of C-nitroso drugs or their metabolic precursors[††] and their congeners may involve poly(ADP-ribose) polymerase in a manner similar to their apoptosis-inducing action in cancer cells (38), a subject for further experimental exploration.

[††]Kun, E., Mendeleyev, J. & Kirsten, E. (1993) U. S. Patent Appl., No. 08/060,409, pending.

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