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Inhibition of the replication of native and 3′-azido-2′,3′-dideoxythymidine (AZT)-resistant simian immunodeficiency virus (SIV) by 3-nitrosobenzamide

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The 3-nitrosobenzamide (NOBA) drug abolishes SIV replication sharply at 20 μM concentration when CEM x174 cells are preincubated for 1 h with the drug prior to viral infection. Treatment of CEM x174 cells with 20 μM NOBA resulted in the inhibition of the synthesis of the DNA sequence coding for the *gag* gene, as determined by the PCR technique. Cell viability was directly proportional to the antiviral action of NOBA. Replication of AZT-resistant SIV 23740 in MMU 23740 cells in vitro was suppressed by NOBA in a concentration-dependent manner without significant effects on cell viability. Reverse transcriptase activity of $SIV_{mac}239$ was unaffected by NOBA up to 800 μM concentration. Preincubation of two SIV strains with NOBA completely abolished their infectivity in human PHA-PBL cells. Replication of two strains of SIV in PHA-PBL cells was also inhibited by NOBA.

AZT-resistant SIV; 6-Nitrosobenzamide; Antiviral; Zinc finger; Anti-AIDS

1. INTRODUCTION

Animal models are essential in evaluating antiviral agents for chemotherapy [1]. In the case of the acquired immunodeficiency syndrome (AIDS), the first drug established to be active but not curative was AZT [1–3]. A decrease in HIV core antigen levels and virus titer in the plasma of patients treated with AZT has been reported [4]. Since prolonged treatment with AZT leads to the emergence of drug-resistant strains of HIV [5], novel agents for AIDS chemotherapy appear to be necessary.

We have reported that two aromatic C-nitroso compounds, 6-nitroso-1,2-benzopyrone (NOBP) and 3-nitrosobenzamide (NOBA), induce zinc ejection from isolated zinc fingers of p7 or from HIV-1 virions and inhibit HIV-1 replication in human lymphocytes [6]. Since NOBA was more effective in suppressing HIV-1 propagation than NOBP and was well tolerated by uninfected lymphocytes [6], this drug appeared to be the agent of choice for further studies.

SIV in terms of genetics and CD4 cell tropism is by far the closest animal retrovirus to HIV [7]. Furthermore, SIV and HIV contain similar Mg^{2+}-dependent reverse transcriptases [8] and their major core proteins reveal common antigenic regions [9]. Thus SIV infection of rhesus macaques, which leads to an immunodeficiency syndrome similar to that found in HIV-infected humans, remains the best animal model available for the study of the pathogenesis of AIDS [10]. Since preclinical testing of new antiviral agents in animal models is generally preceded by cell culture studies [11], we determined the effect of NOBA on SIV replication in CEM × 174 cells in vitro and compared these results with the effect of NOBA on AZT-resistant SIV 23740. We also tested the hypothesis that each of the two drugs (AZT, NOBA) should exert specific independent modes of action on SIV replication and therefore AZT-resistant viral strains should still respond to the antiviral action of NOBA. For comparison with earlier studies

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Abbreviations: AZT, 3′-azido-2′,3′-dideoxythymidine; HIV, human immunodeficiency virus; MMU, *Macaca mulatta*; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NOBA, 3-nitrosobenzamide; NOBP, 6-nitroso-1,2-benzopyrone; PBMCs, peripheral blood mononuclear cells; PCR, polymerase chain reaction; PHA-PBL, phytohaemagglutinin-stimulated human peripheral blood lymphocytes; RT, reverse transcriptase; SIV, simian immunodeficiency virus; SIV_{smm} and SIV_{mmbi} are SIV strains; $TCID_{50}$, 50% tissue culture infectious dosage.

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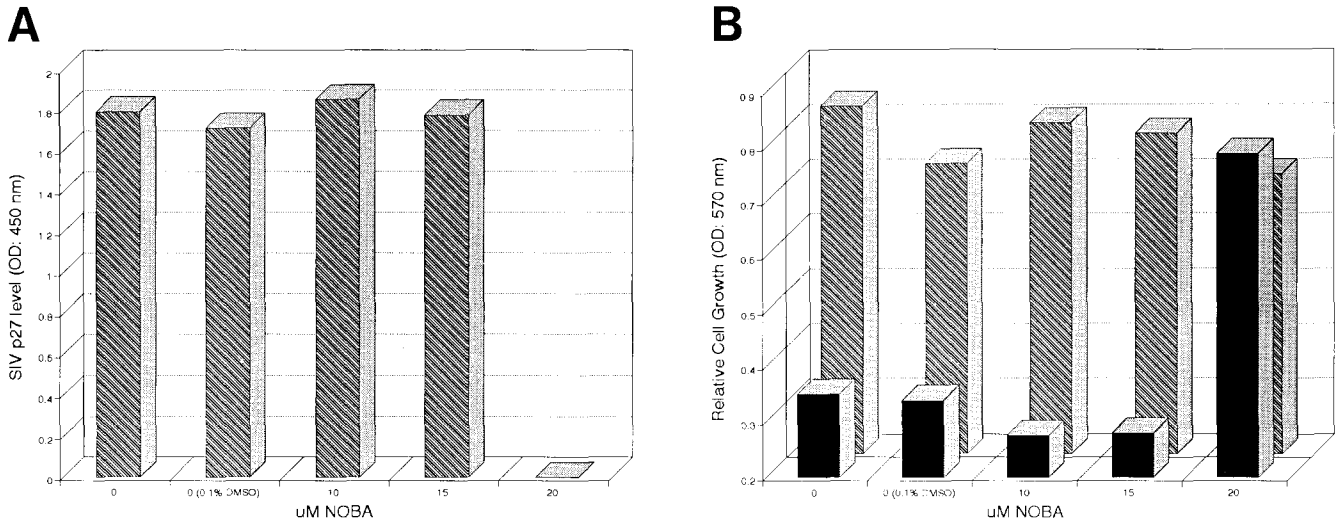

Fig. 1. The effect of NOBA on $SIV_{mac}239$ replication (A) and CEM x174 cell viability (B). Details of experimental protocol are described in the text (section 3). Each bar expresses the mean of 3 independent tests, which do not differ ± 10% (not shown). In A, ordinate = p27 antigen assay (ELISA) performed on day 10; abscissa = concentration of NOBA or DMSO. In B, cell viability test determined on day 10 by the tetrazolium assay (see section 2); first line bars = virus infected cells (SIV) in the presence of NOBA; second line bars (controls) = uninfected cells treated with NOBA.

[6] we also determined the effect of NOBA on SIV replication in human lymphocytes.

2. MATERIALS AND METHODS

CEM x174 cells are the fusion product of human B cell line 721.174 and human T cell line CEM [12]. A molecular clone of SIV_{mac} ($SIV_{mac}239$) was kindly provided by Dr. R. Desrosiers of the New England Primate Research Center. AZT (3′-azido-2′,3′-dideoxythymidine) was manufactured by the Burroughs Wellcome Co. The compound 3-nitrosobenzamide (NOBA) was prepared by Octamer Inc., Tiburon, CA*. RPMI 1640 supplemented with L-glutamine was purchased from Gibco Labs. Inc.

2.1. *Preincubation with the drug*

CEM x174 cells were suspended at 4×10^5 cells/ml and distributed into 24-well tissue culture plates. Cultures were treated with various concentrations of the test compound (along with DMSO as controls) and incubated at 37°C for 1 h in a CO_2 incubator. The cells were infected with 5 μl of a stock solution of $SIV_{mac}239$ at 300 $TCID_{50}$/ml (50% tissue culture infectious dosage per ml cell suspension). Cell viability was determined by the tetrazolium salt (MTT) assay and the cultures were divided 1:4 every 3–4 days in medium containing the drug. The cultures were examined periodically by light microscopy for the presence of syncytia. The virus titers were determined by analysis of supernatant SIV p27 core antigen protein or reverse transcriptase (RT) levels.

2.2. *Preincubation with the virus*

CEM x174 cells were distributed into 24-well tissue culture plates as above. Cells were incubated with virus for 2 days (until syncytia appeared) before treatment with NOBA. Cultures were examined periodically for the presence of syncytia. Virus titers were determined by SIV p27 or RT assays.

*Kun, E., Mendeleyev, J. Rice, W.G., US Patent, Ser. No. 07/780,809, pending.

2.3. *RT assays*

To test for RT activity, 10 μl of infected cell supernatant was added to a reaction mixture containing 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 20 mM KCl, and 1% Triton X-100 in a total volume of 50 μl. Poly(rA).oligo$(dT)_{12\ 18}$ was present at 100 μg/ml and [^{3}H]TTP at 2.4 μM. The reaction mixtures were incubated at 37°C for 1 h and the TCA-precipitable radioactivity was filtered onto nitrocellulose filters which were then washed, dried, and counted.

2.4. *Tetrazolium salt (MTT) assays*

Cell viability was measured by a published procedure [13]. Briefly, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was dissolved at a concentration of 5 mg/ml in sterile phosphate buffered saline (PBS). 20 μl of MTT solution were added into each microtiter well containing 100 μl of cell culture. Following 2 h incubation at 37°C, 100 μl of solubilizing medium (cf. [13]) were added. After overnight incubation at 37°C, optical densities were measured at 570 nm using a microtiter plate reader.

2.5. *SIV_{mac} p27 core antigen levels*

These were determined by an enzyme immunoassay provided by Coulter Corp. (Hialeah, FL). The assay was performed according to the manufacturer's specifications.

2.6. *Polymerase chain reaction (PCR) analysis of infected cell genomes*

DNA extracted from drug-treated, SIV-infected CEM x174 cells or from control cells were screened for the presence of the SIV sequence by a published method [14] using individually designed primers corresponding to the SIV *gag* gene. Northern hybridization has shown the primers to be complementary to the region encoding the major core antigen protein, p27 [14].

2.7. *Assays on human lymphocytes*

These were performed as described previously [6].

3. RESULTS

The effectiveness of NOBA in preventing $SIV_{mac}239$ replication in CEM x174 cells was determined by prein-

cubating cells with varying concentrations of NOBA at 37°C for 1 h before infection with the virus. As illustrated in Fig. 1A, pretreatment of CEM x174 cells with 0 to 20 μM NOBA and maintenance of these drug concentrations during the entire experimental period strikingly abolished $SIV_{mac}239$ replication *only* at 20 μM NOBA; no effect of NOBA was detected at lower drug concentrations. The exact reasons for the sharp transition between ineffective (below 15 μM) and fully effective (20 μM) NOBA concentrations are not known, but we presume that the quantity of intracellular NOBA-inactivating systems may explain this phenomenon, which is overcome by higher than 15 μM NOBA. Coincidental with the antiviral action of 20 μM NOBA cell viability was maintained at the level of virus-free controls (Fig. 1B), however, when SIV p27 levels were high the cytocidal action of $SIV_{mac}239$ was reflected in a significantly depressed cell viability, as would be predicted.

Since virus replication was expected to coincide with the appearance of integrated viral DNA in the genome of CEM x174 cells, we assayed cellular DNA by the PCR method (see section 2) with the aid of specifically designed *gag*-selective SIV primers [14]. Results of the PCR assay are shown in Fig. 2. Lane 1 in Fig. 2A is a molecular marker (*Hin*dIII-digested ϕX174 DNA), and lane 2 is the plasmid DNA encoding the SIV p27 core antigen protein amplified by *gag*-specific primers. Lane 3 illustrates the absence of the specific amplified DNA from non-infected control cells, and lanes 4–7 show the result of PCR assay in SIV-infected cells in the absence of NOBA (lane 4), with 0.1% DMSO (lane 5) and after preincubation and treatment with 10 μM NOBA (lane 6) and finally with 20 μM NOBA (lane 7), which completely abolished the signal for the infectious DNA (compare with Fig. 1A). To rule out the possible artifact that the absence of SIV *gag* DNA may be due to incomplete DNA extraction technique, we also tested for the ubiquitous β-actin gene as shown in Fig. 2B, where lane 1 shows a molecular marker (*Hin*dIII-digested ϕX174 DNA), lane 2 is the β-actin segment amplified by β-actin specific primers, and lanes 3–7 are β-actin primer amplification of the DNA extracted from a non-infected cell culture (lane 3) and infected cell cultures treated with 0 μM NOBA (lane 4), 0 μM NOBA with 0.1% DMSO (lane 5), 10 μM NOBA (lane 6), and 20 μM NOBA (lane 7). These results confirm that the absence of the SIV genome in infected cells treated with 20 μM NOBA was not due to the lack of extractable DNA.

In order to identify an AZT-resistant SIV strain, viruses from SIV-infected rhesus macaques were isolated and tested for their sensitivity toward AZT. The molecular clone $SIV_{mac}239$ was AZT-sensitive but virus isolates from an $SIV_{mac}239$-infected rhesus macaque (MMU 23740) 14 months post-infection were AZT-resistant; AZT only partially inhibited the growth of SIV 23740 compared to $SIV_{mac}239$, suggesting that the macaque virus contained a mixture of the original infecting virus ($SIV_{mac}239$) and other, mutated viruses. A comparison of the number of syncytia formed in AZT-treated wells revealed the complete absence of the cytopathic effect of $SIV_{mac}239$ in contrast to SIV 23740 (Table I).

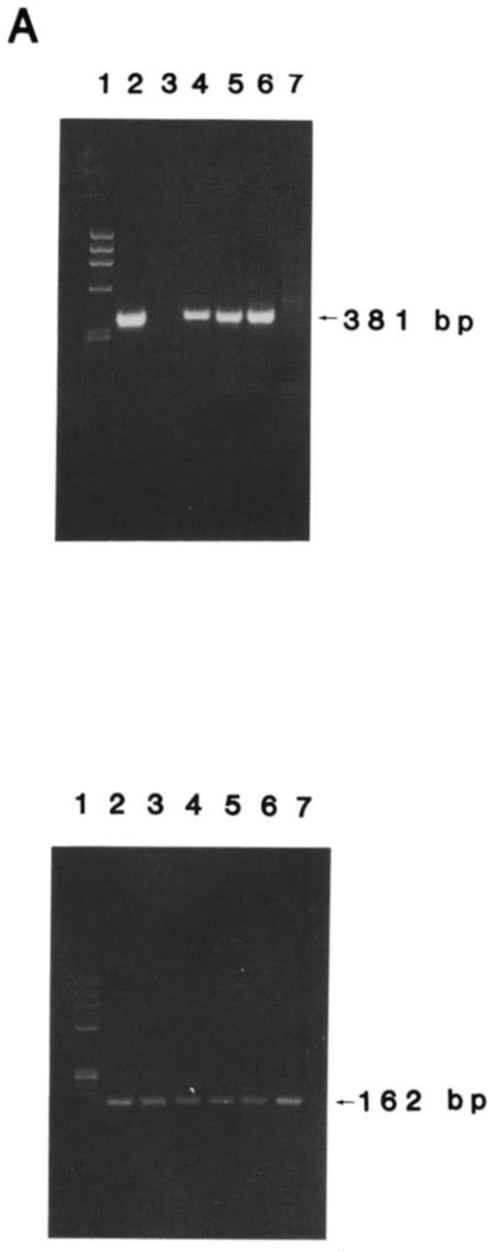

Fig. 2. Analysis of the cellular genome of SIV-infected and -uninfected cultures by PCR. CEM x174 cells from 6-day cultures of the experiment described in Fig. 1 were used for DNA extraction. (Six-day cultures were used instead of 10-day cultures to ensure the presence of enough extractable DNA). PCR analysis was performed as described in section 2. For details see section 3.

The inhibitory action of NOBA on the replication of AZT-resistant SIV strains was assayed by incubating supernatants of 6-day-old co-cultivation systems, consisting of MMU 23740 PBMCs and CEM x174, with

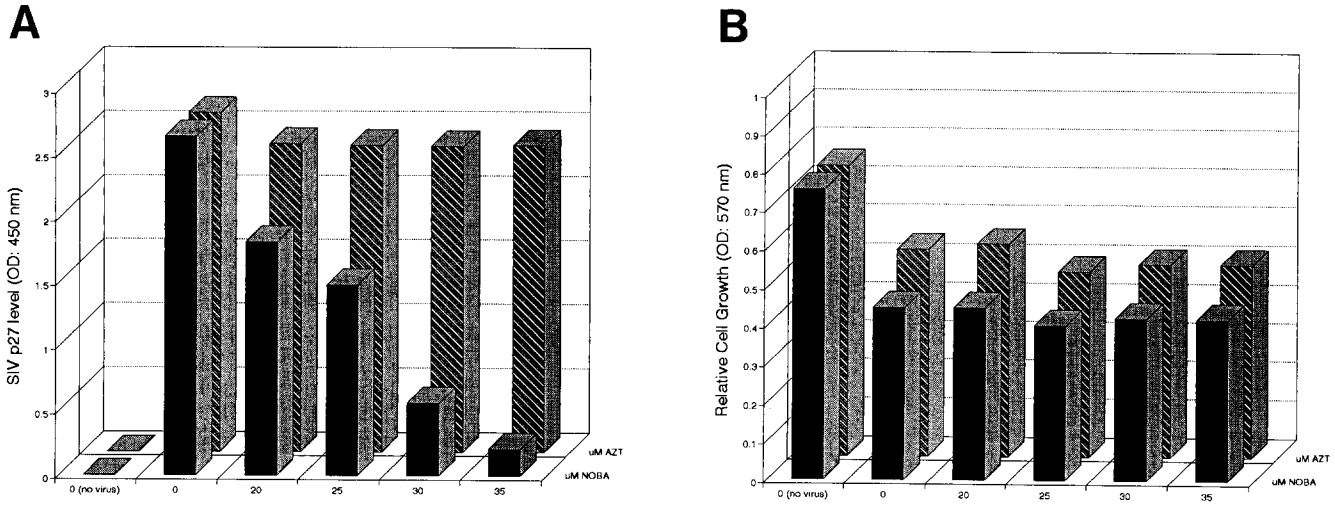

Fig. 3. Effect of NOBA on AZT-resistant strains of SIV. Peripheral blood mononuclear cells (1.2×10^6) from an $SIV_{mac}239$-infected rhesus macaque (MMU 23740) were co-cultivated in a 24-well tissue culture plate with 3×10^5 CEM x174 cells/ml for 6 days. Aliquots (500 μl) of the co-cultivation supernatant were added to 3×10^5 fresh CEM x174 cells/ml. The cells were incubated at 37°C for another 2 days (until syncytia appeared) before adding NOBA or AZT. Cultures were replenished with new medium containing drug on day 9. Fresh CEM x174 cells (1.75×10^5/well) and drug were added on day 13. (A) Virus titers in the supernatant of cultures 16 days from initial co-cultivation as determined by the SIV p27 core antigen capture ELISA. (B) Viability of cell cultures 16 days from initial co-cultivation as determined by the MTT assay. Data presented are the average of duplicate wells.

fresh CEM x174 cells. This system simulates conditions that may exist in vivo. Assays for the p27 core antigen with ELISA 16 days after the initial co-cultivation showed a NOBA dose-dependent depression of SIV 23740 production, whereas no antiviral action of AZT occurred (Fig. 3A). There was no significant drug-dependent decrease of cell viability due to either NOBA or AZT (Fig. 3B).

In contrast to the powerful anti-SIV action of NOBA, no direct effect on RT activity could be ascertained (Table II).

The direct anti-SIV action of NOBA was also assayed with human peripheral lymphocytes that were stimulated by phytohaemagglutinin (PHA-PBL) as described earlier for HIV [6]. This experiment represents a direct comparison between SIV and HIV in the same test system. As seen in Fig. 4, preincubation of SIV_{smm} with 50

Table I

The effect of AZT on nonresistant and AZT-resistant SIV_{mac} as assayed by syncytia formation[a]

Virus	AZT concentration (μM)	Syncytia[b]
$SIV_{mac}239$	0	++++
	10	-
	15	-
	20	-
	25	-
	30	-
	35	-
	40	-
SIV 23740	0	++++
(AZT-resistant)	10	++++
	15	++++
	20	++++
	25	++++
	30	++++
	35	++++
	40	++++

[a]CEM x174 cells (1.5×10^5/100 μl) were infected with equal doses of $SIV_{mac}239$ or virus isolates from an $SIV_{mac}239$-infected rhesus macaque (MMU 23740). Three days post-infection, AZT concentrations ranging from 0 to 40 μM were added to the cells and the cultures were incubated for 4 days. The wells were replenished with fresh CEM x174 cells and AZT and incubated for an additional 3 days. Cell cultures were then examined for syncytia formation.

[b]The number of syncytia in cell cultures was counted in arbitrary fields under 60 × magnification and scored as follows: over 30 (++++), 20–30 (+++), 10–20 (++), 1–9 (+), and 0 (−).

Table II

[^{3}H]TTP incorporation by SIV 239-RT in the presence of NOBA[a]

Concentration of NOBA (μM)	cpm ($\times 10^3$)
0 (no enzyme)	0.4
0	761.4
0 (0.1% DMSO)	743.8
0.8	897.0
8.0	836.8
20.0	763.8
40.0	748.8
80.0	764.9
400.0	690.7
800.0	706.6

[a]Reverse transcriptase assays were performed with DMSO controls in the presence or absence of NOBA as described in section 2.

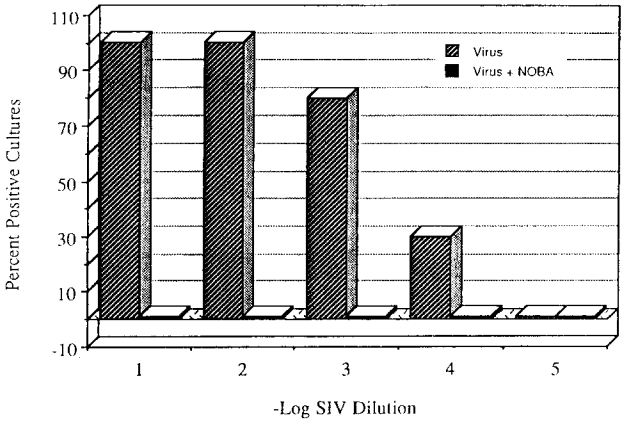

Fig. 4. The effect of NOBA on SIV assayed in human lymphocytes. A concentrated stock of SIV_{smm} ($TCID_{50}$ = 3,300) was incubated for 30 min at 37°C with 50 μM NOBA. Afterwards, the mixture was serial 10-fold diluted to yield the 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions. Each dilution was used to infect 10^6 PHA-PBL for 18 h at 37°C, after which the free virus and drug were removed. Cells were then aliquoted into 96 well plates (10^5 cells/well with 10 replicates per dilution). Cultures were scored positive for infection if their absorbance at 490 nm in the antigen capture ELISA was >3 S.D. above the mean absorbance of 10 uninfected cultures. The untreated virus cultures (▨) scored positive with dilutions as low as 10^{-4}, whereas NOBA-treated virus (■) did not score positive with any cultures.

μM NOBA for 30 min at 37°C completely suppressed SIV replication in PHA-PBL. As determined in separate studies, designed to quantitate the dose–responsive effect of NOBA on SIV replication in PBMCs, the EC_{50} value (concentration of drug that suppresses 50% virus replication) varied between 17 and 8 μM NOBA for SIV_{smm} and SIV_{mmbi} strains, respectively.

4. DISCUSSION

It is apparent (Fig. 4) that a direct action of NOBA on SIV is indistinguishable from its effect on HIV [6]. The morphologic, antigenic and molecular properties of SIV_{mac} are nearly identical to those of HIV [9,15,18]. It is important that the retroviral zinc finger destabilizing action of NOBA is not confined to the zinc fingers of *gag* proteins only, but includes one of the retroviral-type zinc fingers of eukaryotic poly(ADP-ribose) polymerase [16]. Inactivation of poly(ADP-ribose) polymerase by zinc ejection [16] derepresses an ADP-ribosylation inhibited Ca^{2+}/Mg^{2+}-dependent endonuclease in tumor cells and produces selective apoptosis [17]. The participation of poly(ADP-ribose) polymerase in the regulation of DNA, including retroviral DNA, is being analyzed.

Infection of CEM x174 cells with AZT-resistant strains of SIV isolated from a persistently infected rhesus macaque has shown NOBA to be effective against AZT-resistant viruses. Since NOBA has a different molecular site of action than AZT, a synergism between the two drugs is predictable, as well as preventive action against the emergence of AZT-resistant viral mutants. The development of novel anti-AIDS agents such as NOBA, which targets retroviral zinc fingers and does not act on reverse transcriptase, thus represents a new approach to AIDS chemotherapy. Combination strategies of the mechanistically distinct AZT and NOBA compounds may also provide for enhanced chemotherapeutic efficacy, with decreased drug doses, lessened toxicity and a decreased likelihood for the development of drug-resistant variant strains.

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