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Hydroxyurea as an Inhibitor of Human Immunodeficiency Virus–Type 1 Replication

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Hydroxyurea, a drug widely used in therapy of several human diseases, inhibits deoxynucleotide synthesis—and, consequently, DNA synthesis—by blocking the cellular enzyme ribonucleotide reductase. Hydroxyurea inhibits human immunodeficiency virus–type 1 (HIV-1) DNA synthesis in activated peripheral blood lymphocytes by decreasing the amount of intracellular deoxynucleotides, thus suggesting that this drug has an antiviral effect. Hydroxyurea has now been shown to block HIV-1 replication in acutely infected primary human lymphocytes (quiescent and activated) and macrophages, as well as in blood cells infected in vivo obtained from individuals with acquired immunodeficiency syndrome (AIDS). The antiviral effect was achieved at nontoxic doses of hydroxyurea, lower than those currently used in human therapy. Combination of hydroxyurea with the nucleoside analog didanosine (2′,3′-dideoxyinosine, or ddI) generated a synergistic inhibitory effect without increasing toxicity. In some instances, inhibition of HIV-1 by hydroxyurea was irreversible, even several weeks after suspension of drug treatment. The indirect inhibition of HIV-1 by hydroxyurea is not expected to generate high rates of escape mutants. Hydroxyurea therefore appears to be a possible candidate for AIDS therapy.

Further attempts to design drugs for therapy of AIDS are necessary (*1*). Despite their differences in structure, antiviral activity, and pharmacokinetic properties, ddI, zidovudine (azidothymidine, or AZT), noncompetitive HIV-1 reverse transcriptase inhibitors, and HIV-1 protease inhibitors (*2*) share a common feature: They directly target viral proteins. As an alternative approach, we have suggested targeting one or more cellular components (*3*). The rationale for this strategy is to avoid triggering the onset of viral escape mutants as a result of direct selective pressure against viral proteins. Another rationale is to achieve specific antiviral effects of the drug with low or no toxic effects on the cell.

Hydroxyurea has been widely used over the last 30 years for the treatment of human malignancies, especially chronic myelogenous leukemia and other myeloproliferative syndromes (*4*). More recently, hydroxyurea has been proposed for the treatment of sickle cell anemia (*5*). High doses of hydroxyurea are commonly used in leukemia treatment (*4*). Oral administration of the drug at a dose of 500 mg/m^2 every 4 hours generated plasma peak concentrations ranging from 0.5 to 2.5 mM and trough concentrations of 0.2 to 0.5 mM (*6*). Hydroxyurea is a free radical quencher and inhibits the cellular enzyme ribonucleotide reductase [a rate-limiting enzyme in the synthesis of deoxynucleoside triphosphates (dNTPs)]. We have shown that, by decreasing the intracellular pool of dNTPs, hydroxyurea inhibits HIV-1 DNA synthesis, resulting in the generation not only of decreased amounts of viral DNA, but also mainly incomplete chains (*3*). Goulaouic *et al.* (*7*), with Moloney murine leukemia virus, confirmed that inhibition of reverse transcription by hydroxyurea depends on the intracellular nucleotide pool (rather than on the precise arrest of the host cell cycle). High single doses of hydroxyurea delay HIV-1 spread in vitro (*8*). Furthermore, by decreasing the amount of cellular dNTPs, hydroxyurea was expected to increase the uptake and metabolism of nucleoside analogs, such as ddI or AZT, and consequently to enhance the effect of these compounds, hopefully in a synergistic manner. We now demonstrate that low, subtoxic doses of hydroxyurea, alone or in combination with AZT or ddI, block HIV-1 replication.

Because nonstimulated lymphocytes are not productively infected by HIV-1, but only allow viral entry and reverse transcription (*3*, *9*), we assessed HIV-1 infection in peripheral blood mononuclear cells (PBMCs) by monitoring HIV-1 DNA synthesis (*3*, *10*). Compared to untreated cells, HIV-1 DNA synthesis was slower and less efficient, and the final DNA was mostly incomplete, in hydroxyurea-treated quiescent lymphocytes (Fig. 1). A similar phenomenon has been described for activated lymphocytes (*3*). The effect of hydroxyurea was dose-dependent, especially for the synthesis of the full-length minus strand DNA (R-*gag*, the longer DNA synthesis product analyzed in our experiments). The block of DNA synthesis was almost complete at 1 mM. The DNA shown in Fig. 1 mainly represents DNA carried by the incoming virions (*3*, *10*); the amount of DNA did not vary during the time course and remained mostly incomplete. More elongation was observed at 0.1 mM, although at much lower levels compared to the untreated control. No cytotoxic effects were observed at the drug concentrations used in these experiments (*11*) because quiescent cells do not undergo genomic DNA synthesis.

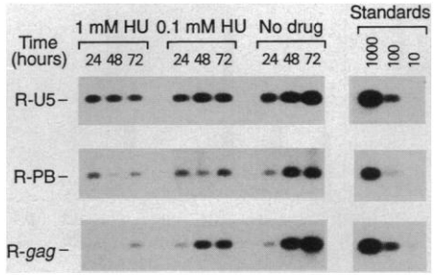

Fig. 1. Time course of inhibition of HIV-1 DNA synthesis by hydroxyurea in quiescent PBMCs. PBMCs were isolated from healthy donors and infected after 2 days with the HIV-1 strain HTLV-III$_B$ (*18*) at a multiplicity of infection of 1 in the absence of cell stimulation. After 2 hours at 37°C, the cells were washed, and fresh medium containing hydroxyurea (HU) at the indicated concentrations was added. Cells were harvested after 24, 48, and 72 hours and analyzed by quantitative polymerase chain reaction (PCR). Primers were used as described (*3*) to amplify different regions of the HIV-1 genome: R-U5 [between the R and U5 regions of the long terminal repeat (LTR)], R-PB (between the R region of the LTR and the primer binding site), and R-*gag* (between the R region of the LTR and the *gag* gene). After 30 cycles of PCR amplification and subsequent electrophoresis on 2% agarose, the samples were blotted on a nylon membrane and hybridized with a ^{32}P-labeled oligonucleotide as described (*3*). Quantitation of HIV-1 DNA during PCR amplification was achieved by comparison with a standard curve of serial dilutions of pHXB2(Rip7) plasmid DNA (*19*). The numbers above the lanes labeled "Standards" indicate the number of plasmid copies.

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Inhibition of HIV-1 replication by hydroxyurea in activated PBMCs was analyzed by measuring production of p24 antigen over time. Hydroxyurea was used either alone at low concentrations or in combination with ddI (Fig. 2A). Both hydroxyurea and ddI inhibited or delayed (or both) HIV-1 replication in a dose-dependent manner. The combination of the two drugs completely blocked HIV-1 replication (>99.9%); this effect was also dose-dependent. Cell toxicity, analyzed by combining cell counts with trypan blue exclusion, reflected the known properties of the two drugs. Hydroxyurea acts mainly as a cytostatic drug, producing arrest in early S phase of the cell cycle and, often, cell death by secondary mechanisms (*12*). However, on the basis of the total number of viable cells, toxic effects were minimal at drug concentrations of <0.1 mM (Fig. 2B). The cytotoxicity associated with the combination of the two drugs did not differ from that apparent with hydroxyurea alone, providing a further advantage for the use of hydroxyurea and ddI together because, by contrast, the antiviral effects were significantly augmented (Fig. 2, A and B).

To determine whether the different number of viable cells observed at different drug concentrations or during the course of infection affected our results (fewer cells alive yielding less virus production), we corrected the p24 values by dividing them by the number of viable cells (Fig. 2C). These results showed that the inhibitory action is mainly attributable to specific antiviral effects of hydroxyurea on viable cells and, therefore, not mediated by hydroxyurea cytotoxicity. The combination of hydroxyurea and ddI in activated PBMCs was several times more potent than the combination of hydroxyurea and AZT (*11*), consistent with previous observations (*13*) and possibly attributable to the more effective inhibition by hydroxyurea of the synthesis of dATP (cellular competitor of ddATP, the pharmacologically active metabolite generated from ddI) (*3*, *14*) than of the synthesis of other dNTPs (dGTP, dTTP, and dCTP) in activated lymphocytes.

Although a higher variability among different experiments was observed with primary macrophages than with primary PBMCs (in each experiment, the same donor was used as a source of both PBMCs and macrophages), the dose-dependent inhibition of HIV-1 production in macrophages was consistently apparent at lower concentrations of hydroxyurea than in primary PBMCs (Fig. 2D). Concentrations of hydroxyurea as low as 0.05 mM blocked (>99%) HIV-1 replication in macrophages. Combination of hydroxyurea with AZT at concentrations at

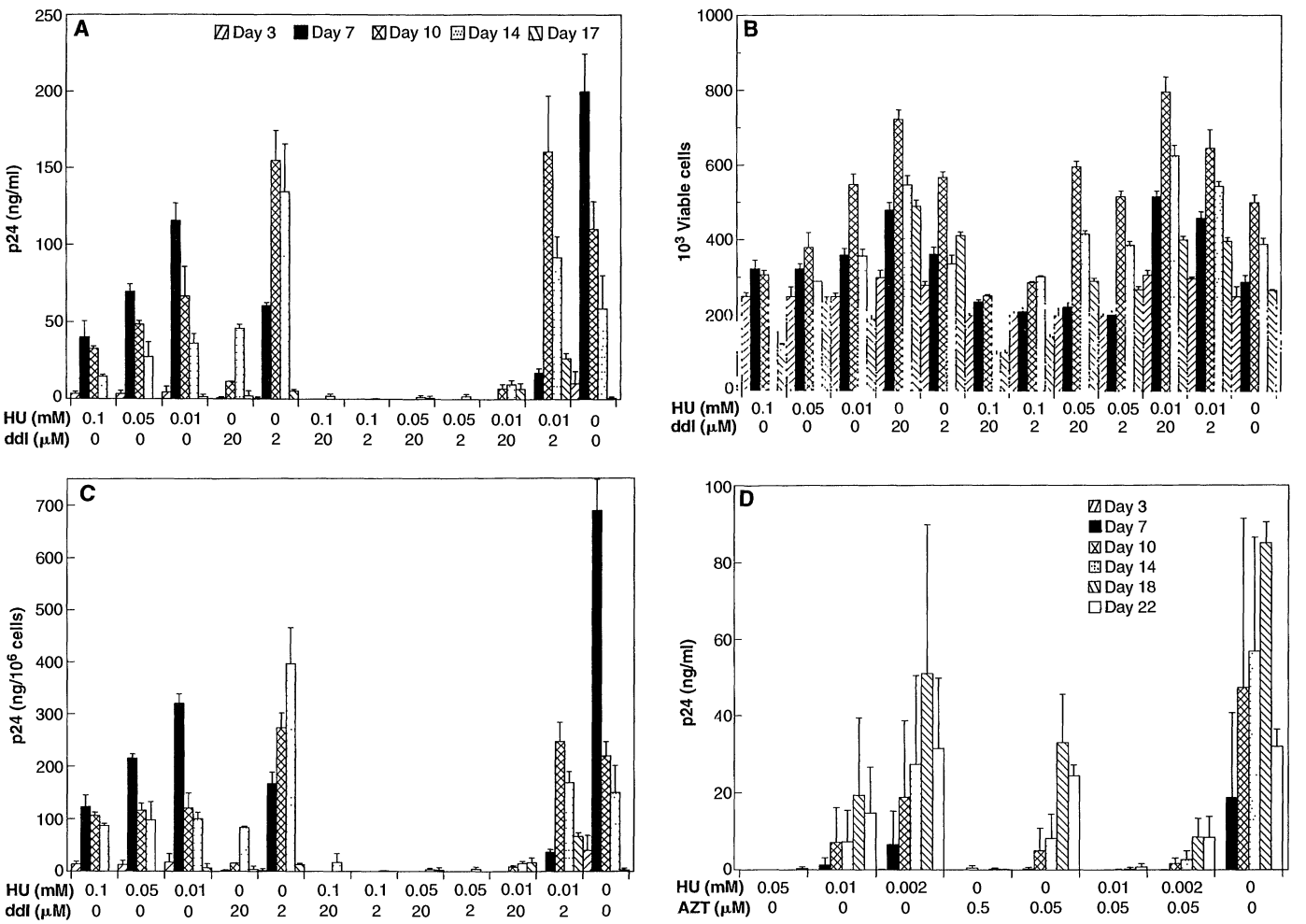


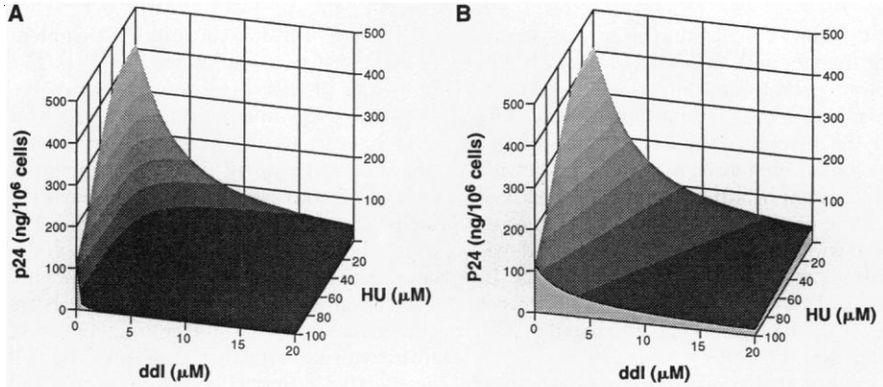
Fig. 2. Time course of inhibition of HIV-1 by hydroxyurea and nucleoside analogs in activated PBMCs and macrophages. (**A** to **C**) PBMCs from healthy donors were infected for 2 hours at 37°C with HIV-1 (HTLV-III$_B$) (*18*) (multiplicity of infection, 1) after stimulation for 2 days with phytohemagglutinin A and interleukin-2. After washing out the residual virus, cells were treated with hydroxyurea (HU), ddI, or both at the concentrations indicated. Every 3 or 4 days, supernatants were harvested for p24 analysis (A), viable cells were counted (B), and fresh medium and drugs were added. The amount of p24 in the supernatant was also expressed per 10^6 viable cells (C). (**D**) Macrophages were obtained by cell adhesion after purification of PBMCs from healthy donors (*20*). After incubation for 14 days with granulocyte-macrophage colony-stimulating factor, cells were infected overnight with the HIV-1 strain Ba-L (*20*), washed, and treated with HU and AZT at the concentrations indicated. Supernatants were harvested every 3 to 4 days for p24 analysis, and fresh medium and drugs were added. Data are means ± SD (n = 3).

which each of the two drugs showed only partial inhibition resulted in complete inhibition (>99.9%). Moreover, 1 mM hydroxyurea had no effect on cell number (Table 1). The combined effects of hydroxyurea and AZT observed in macrophages were therefore consistent with the effects of hydroxyurea and ddI in primary PBMCs.

The synergistic effects of the combination of hydroxyurea and ddI were derived mathematically (*15*). The quantitative analysis shown in Fig. 3 reveals well-defined antiviral synergism between hydroxyurea and ddI in PBMCs. PI_2 is the potentiation index for hydroxyurea (drug 2) acting on ddI (drug 1). A PI_2 of 0 would indicate additivity; a PI_2 of >0 would indicate synergy (potentiation); and a PI_2 of 1 would mean that hydroxyurea was as effective through its potentiating activity as through its intrinsic activity. The PI_2 value obtained for p24 in this experiment was 19.4 ± 4.1 ($P \ll 0.01$; two-tail test). When the results were expressed as p24 per million cells, PI_2 was 24.3 ± 5.3 ($P \ll 0.01$). A useful derived parameter is PC_{50_2}, which is defined as the ratio of the 50% inhibitory concentration (IC_{50}) of drug 2 to PI_2. PC_{50_2} represents the concentration of drug 2 required to increase the apparent potency of drug 1 (that is, to decrease its apparent IC_{50}) by a factor of 2 beyond what would be expected on the basis of the intrinsic activity of drug 2. The lower the value of PC_{50_2}, the stronger the potentiation; additivity corresponds to a PC_{50_2} approaching infinity. The values for p24 and p24 per million cells in this experiment were 3.7 and 3.2 μM, respectively. The experiments shown in Fig. 2 showed trends toward synergism when analyzed in the same way, but the data sets were too small to achieve statistical significance.

The previous experiments were all performed with primary human cells and with the distantly related HIV-1 isolates $HTLV\text{-}III_B$ and Ba-L, suggesting that hydroxyurea alone or in combination could be effective on the variety of viral strains detected in vivo. We next studied an in vitro system that may be the most relevant to the clinical situation: primary cells from HIV-1–infected individuals. This model combines the use of primary cells with primary isolates, in the situation of an infection naturally established in vivo.

In PBMCs from AIDS patients (mainly at late stages of the disease), hydroxyurea in-



ddI (μM)	HU 0	HU 10	HU 50	HU 75	HU 100
0	475 ±2	390 ±25	330 ±24	222 ±33	137 ±5
0.2	383 ±35	295 ±13	123 ±26	53 ±7	5 ±1
1	338 ±70	222 ±15	21 ±2	8 ±1	0 ±0
5	113 ±35	54 ±18	0 ±0	2 ±0	0 ±0
20	83 ±10	0 ±.0	0 ±0	2 ±2	0 ±0

Fig. 3. Synergistic activity of ddI and hydroxyurea against HIV-1 in PBMCs. (**A**) The best fit of the robust potentiation model in the COMBO computer program package to the data (*15*). The bowl-shaped, inward curvature of the fitted surface indicates synergism, which was confirmed by statistical analysis (see text). (**B**) The null hypothesis of additivity (no synergism or antagonism), as indicated by the fact that contour lines run linearly from axis to axis. Culture conditions were as described in the legend to Fig. 2 but with a larger number of drug concentrations. (**C**) Values represent p24 production (means ± SD) for three replicates at day 7 after infection. Equivalent analysis of the data in Fig. 2 gave the following results: $PI_2 = 1.1 \pm 0.6$, $PC_{50_2} = 7.9$ μM, $P = 0.04$ (one-tail test), $P = 0.08$ (two-tail test). The results were significant in the one-tail sense. The marginal statistical significance in the two-tail sense related in part to the smaller number of data points in the experiment.

Table 1. HIV-1 inhibition and suspension of drugs. Experiments were performed as described in Fig. 2. The percentage inhibition of HIV-1 is given in parentheses after the values for viable cells in experiment 1 and amount of p24 produced in experiment 2. The percentage was calculated on the basis of p24 production compared to the untreated control (no drug). Drug treatment of macrophages was suspended either after 14 days (experiment 1) or after 20 days (experiment 2). Viability of macrophages was derived from the number of adherent cells.

Experiment 1

Hu (mM)	Viable cells (thousands/cm²) at day after infection (% inhibition of HIV-1)						
	4	7	10	14*	21	28	35
1	300 (100)	280 (100)	270 (100)	270 (100)	260 (100)	240 (100)	190 (100)
0	300	310	310	290	270	230	200

Experiment 2

Hu (mM)	Amount of p24 produced (ng/ml) at day after infection (% inhibition of HIV-1)							
	3	9	15	20*	23	27	36	44
1	0 (100)	0.14 (99)	0.01 (99.9)	0.09 (99.4)	0 (100)	0.01 (99.8)	0.01 (99.8)	0 (100)
0.1	0.002 (99)	0.23 (98.4)	0.24 (99.2)	0.21 (98.6)	0.11 (97.8)	0.15 (96.6)	0.69 (85)	0.53 (83.9)
0.01	0.06 (70)	3.92 (72.6)	4.89 (84)	5.88 (60.3)	1.73 (65.4)	1.67 (62)	2.07 (55)	2.16 (34.6)
0	0.2	14.3	30.7	14.8	5	4.4	4.6	3.3

*Time of drug suspension.

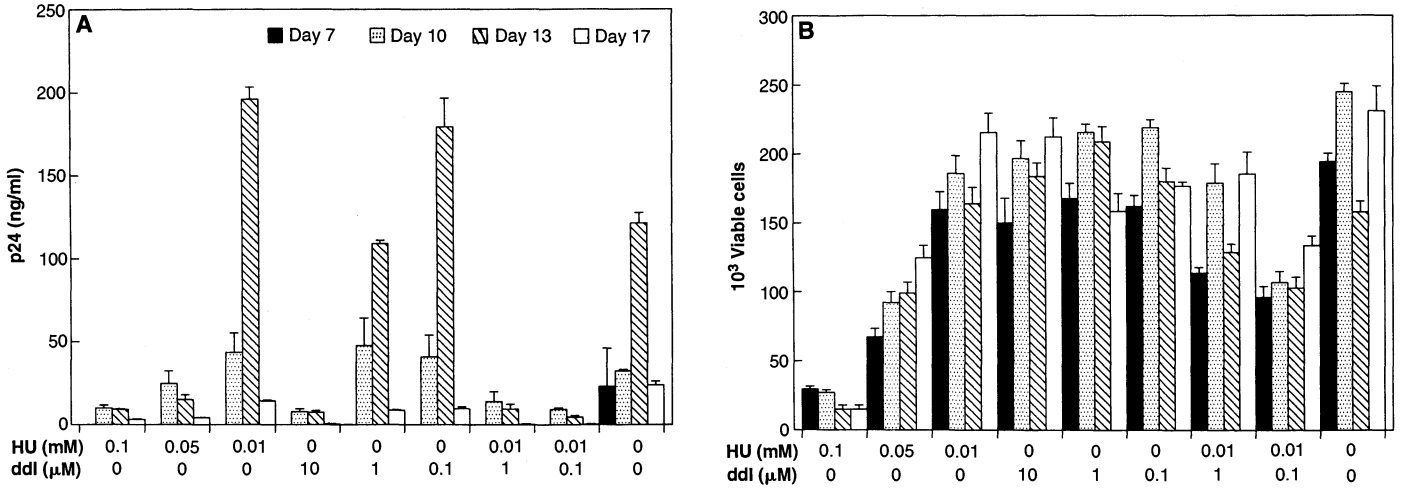


Fig. 4. Time course of inhibition of HIV-1 by hydroxyurea and ddI in activated PBMCs from an individual infected with HIV-1. PBMCs were isolated and stimulated for 2 days with phytohemagglutinin A and interleukin-2. Subsequently, HU and ddI were added at the indicated concentrations and p24 production in the supernatant (**A**) and the number of viable cells (**B**) were determined as described in the legend to Fig. 2. Data are means ± SD (n = 3).

hibited HIV-1 replication in a dose-dependent manner and, in combination with ddI, showed potentiating effects (Fig. 4A). In general, both the pharmacological (Fig. 4A) and the cytotoxic (Fig. 4B) effects of hydroxyurea were more pronounced than with PBMCs from healthy donors, and lower doses of the drug were subsequently used, especially with cells from HIV-1–infected patients in the advanced stages of AIDS. In some instances, the lowest concentrations of both hydroxyurea and ddI stimulated HIV-1 replication (Fig. 4A).

Inhibition of HIV-1 in macrophages from healthy donors was also measured after suspension of drug treatment (Table 1). At high concentrations of hydroxyurea (1 mM), HIV-1 was either undetectable or traces of HIV-1 were inconsistently detected in the culture supernatant. The absence of HIV-1 replication in infected macrophages was documented even several weeks after discontinuing the drug treatment. For experiment 1 shown in Table 1, quantitative polymerase chain reaction (PCR) analysis was performed as in Fig. 1 by amplifying the R-*gag* region of the HIV-1 genome with DNA extracted from the infected macrophages 31 days after suspension of the drug treatment (45 days after infection). Less than one copy of viral DNA per 1000 cells was detected, in contrast to the large amount of DNA detected in the positive control, in which all cells theoretically contained at least one copy of viral DNA. Such a small amount of DNA did not yield any detectable production of p24. At low doses of hydroxyurea (insufficient to block HIV-1 replication completely), no marked rebound of HIV-1 replication was observed after drug suspension, although the relative percentage of inhibition decreased with time (mainly because the p24 values in the absence of drugs were lower in the last part of the time course) (Table 1).

The use of hydroxyurea in the treatment of AIDS offers several advantages: (i) The properties of this drug are well known because it has been used in human therapy for over 30 years. (ii) Hydroxyurea is extremely diffusible, entering all tissues, including the central nervous system, with a maximal velocity that appears infinite (*16*). (iii) Because the brain damage induced by HIV-1 appears to be mediated by the effects of viral replication in macrophages (*17*), which are extremely sensitive to the antiviral effects of hydroxyurea, we predict that this drug will be effective against HIV-1 in these cells and a promising candidate for the treatment of neurological manifestations of AIDS. (iv) Hydroxyurea is a mildly toxic drug and does not cause immunodepression. Myelotoxicity is the dose-limiting toxicity for hydroxyurea. However, such toxicity can be readily monitored and is constantly and rapidly reversible after decreasing the dose or suspending the treatment (*4*). By monitoring simple parameters such as peripheral cell counts, hydroxyurea can be administered for years, and sometimes decades. Furthermore, bone marrow toxicity is severe only when hydroxyurea is used at high doses, comparable to those used in leukemia treatment (~0.5 to 2.5 mM) (6), whereas in most of our experiments concentrations of hydroxyurea 10 to 100 times lower, in combination with ddI, completely inhibited HIV-1 replication. However, in the case of bone marrow toxic effects, the drug could be temporarily suspended without an immediate significant rebound (Table 1) and started again when the toxicity was resolved (usually within a few days). And (v) hydroxyurea does not inhibit HIV-1 directly, but as a result of inhibiting the cellular enzyme ribonucleotide reductase. Genes encoding cellular enzymes do not mutate under physiological conditions, and one could expect that HIV-1 resistance to hydroxyurea would be far less likely to occur than would resistance to conventional antiviral drugs. Moreover, the generation of nucleoside analog escape mutants should also be reduced when these drugs are used in combination with hydroxyurea, because of the synergistic inhibition of viral replication and the fact that viral replication is essential for virus mutation and generation of escape mutants.

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15. Response surface fits were obtained with the COMBO program package, which operates in the Mathematical Modeling Laboratory (MLAB) environment (Civilized Software, Bethesda, MD). The methods are described in detail elsewhere [B. Bunow and J. N. Weinstein, *Ann. N.Y. Acad. Sci.* **616**, 490 (1990); J. N. Weinstein *et al.*, *ibid.*, p. 367; P. Ashorn *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7472 (1990); S. Kageyama *et al.*, *Antimicrob. Agents Chemother.* **36**, 926 (1992)]. Briefly, the data were fitted by iteratively reweighted nonlinear least squares regression to a "robust potentiation" model defined by the implicit equation:

$$1 = (1/z - 1)^{-1/B1}\frac{C_1}{IC_{50_1}}[1 + (PI_2C_2)^{BP2}]^{-1} + (1/z - 1)^{-1/B2}\frac{C_2}{IC_{50_2}}[1 + (PI_1C_1)^{BP1}]^{-1}$$

where $z = (a - y)/(a - d)$ is the normalized effect. In the equation for z, y is the measured p24 level in natural units, a is the level in the absence of drug, and d is the level at indefinitely high drug concentrations. The variable y defines a surface over C_1 and C_2, which represent the concentrations of the two drugs. IC_{50_1} and IC_{50_2} are the 50% inhibitory concentrations of the two drugs used separately; B_1 and B_2 are the corresponding 50%-effect slopes; BP_1 and BP_2 are slope parameters for the potentiating effects; PI_1 and PI_2 are potentiation indices for drug 1 acting on drug 2, and drug 2 acting on drug 1, respectively. For the present data, it sufficed to set $BP_1 = BP_2 = 1$ and $PI_1 = 0$. Weights for the fitting procedure were determined from the error structure of the data set itself with a Gaussian kernel windowing technique based on estimated responses.

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Isolation of Virus Capable of Lysing the Brown Tide Microalga, *Aureococcus anophagefferens*

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Viruses have been hypothesized to control blooms of *Aureococcus anophagefferens* gen. et sp. nov. (Chrysophyceae), a marine phytoplankton that since 1985 has caused devastating summer blooms called "brown tide." By means of ultrafiltration methods, viruses specific to this alga were isolated from both the Great South Bay and Peconic Bay systems of Long Island, New York, during the summer bloom period of 1992. Cell lysis of healthy algal cultures was demonstrated, as well as continuing reinfection with serial transfers of cultures. Electron microscope surveys yielded images of phage-like virus particles with tails that could attach to *A. anophagefferens* cells within minutes of exposure. The isolation and cultivation of this virus highlights the need for further study of viral infection of eukaryotic algae and the potential for a better understanding of algal bloom control by viral infection.

Brown tide blooms were first documented in 1985, in Narragansett Bay, Rhode Island, in Barnegat Bay, New Jersey, and in the Peconic Bay and Great South Bay systems of Long Island, New York. These bays experienced practically simultaneous algal blooms of the eukaryotic microalga, *Aureococcus anophagefferens* gen. et sp. nov. (Chrysophyceae) (*1*). The blooms colored the water a deep, golden-brown (*2*), drastically reduced light through the water column, and caused widespread death of eelgrass, *Zostera marina* (*3*). The bloom also devastated populations of *Argopecten irradians irradians* (bay scallop) and *Mytilus edulis* (blue mussel) by apparently causing starvation and total loss of larval recruitment (*4*, *5*). This resulted in severe monetary losses to local shellfishermen, especially the bay scallop industry in Peconic Bay, New York (*4*).

Massive "brown tide" blooms reappeared in Long Island bays in 1986 and, as in 1985, remained throughout the summer. Since then these blooms have recurred sporadically in isolated Long Island embayments, but have never returned to Narragansett Bay (*6*, *7*). Major environmental factors that have been found to contribute to these blooms include elevated salinities from drought conditions (*2*), elevation of organic compounds or micronutrients in bay waters from runoff (*7*, *8*), reduced grazing (*7*), and restricted circulation of bay waters (*9*).

Some cells observed during the original 1985 transmission electron microscope (TEM) survey from Rhode Island bloom water contained virus-like particles (VLPs) 130 to 150 nm in diameter, and the existence of a virus specific for *A. anophagefferens* was hypothesized to explain sudden decreases in algal populations (*1*). We therefore decided to isolate and cultivate these viruses.

At two Long Island bloom sites [West Neck Bay (WNB) in the Peconic Bay system and Blue Point (BP) in Great South Bay], a seawater sample of 20 liters was collected at the beginning of the bloom, on 6 July 1992 at WNB and on 7 July 1992 at BP. Sampling was repeated on 14 July 1992 at WNB. Each sample was filtered through a 0.2-μm Gelman Sciences capsule filter. The sample was then concentrated according to the methods of Suttle *et al.* (*10*), except that the lower filtration cutoff was 10,000 molecular weight (MW) instead of 30,000 MW to obtain a 200-ml concentrate that could contain potentially infective viruses. The ultrafiltrate (<10,000 MW) was also collected. Concentrate and ultrafiltrate were filter-sterilized through a 0.2-μm Nuclepore filter, transferred into sterile flasks, and stored at 5°C in the dark.

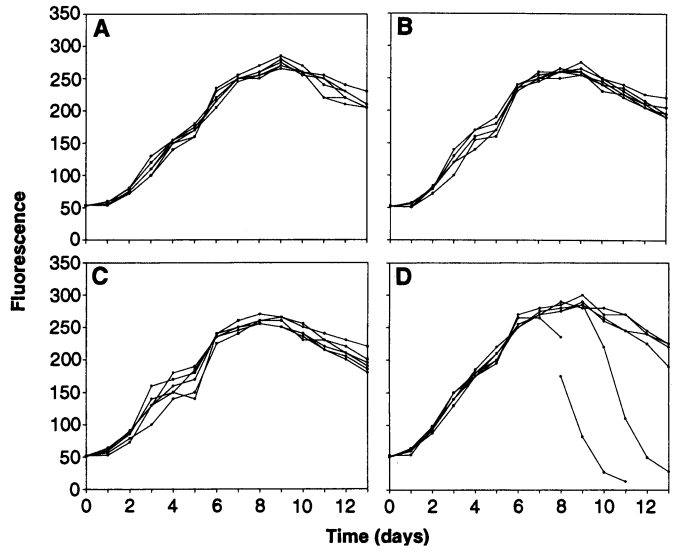

Fig. 1. Example of growth curves of *A. anophagefferens* in culture, testing for potential infectivity by viral concentrate from WNB 7/14/92 seawater. In each treatment, symbols (●) represent six replicate tubes, each containing 6 ml [some symbols overlap in (A), (B), (C), and (D)]. Treatment and control inocula were added on day 0 to healthy cultures. (**A**) Control, no virus added. (**B**) Control, 240 μl (4% of 6 ml) of microwaved (with inactivated viruses) concentrate added. (**C**) Control, 240 μl (4%) of ultrafiltrate (without viral particles), size fraction <10,000 MW added. (**D**) Example of experimental treatment, 360 μl (6%) of viral fraction concentrate added, with two infected replicates. Broken line represents inoculum extracted for serial transfer to healthy culture. Other experimental concentrations not shown were 120 μl (2%) and 240 μl (4%).

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