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(54) Title: INHIBITION OF HIV USING SYNERGISTIC COMBINATIONS OF NUCLEOSIDE DERIVATIVES

(57) Abstract

The present invention relates to the use of synergistic combinations of nucleoside derivatives for inhibiting human immunodeficiency virus (HIV) replication, thereby limiting HIV infection. In a particular embodiment, the purine nucleoside analogue dideoxyinosine combined with the pyrimidine nucleoside analogue 2',3'-dideoxy-2',3'-didehydrothymidine (d4T) exhibit strong synergistic activity and diminished cytotoxic activity toward mammalian cells.

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INHIBITION OF HIV USING SYNERGISTIC COMBINATIONS OF NUCLEOSIDE DERIVATIVES

1. INTRODUCTION

The present invention relates to the use of combinations of nucleoside derivatives for inhibiting human immunodeficiency virus (HIV) replication, thereby limiting the effects of HIV infection. The crux of the invention lies in the discovery that nucleoside derivatives used in combination have synergistic effects, such that the combined effective dose of these agents is lower than the sum of the therapeutic dosages of either drug used individually; increased anti-viral activity is not associated with a commensurate increase in cytotoxicity; in fact, combinations of nucleoside derivatives are less toxic to uninfected cells when compared to identical compounds administered separately.

2. BACKGROUND OF THE INVENTION

2.1. HUMAN IMMUNODEFICIENCY VIRUS

Human immunodeficiency virus (HIV) is a human retrovirus believed to be the causative agent of acquired immune deficiency syndrome (AIDS) and AIDS related complex (ARC). The HIV virion or virus particle is a sphere that is roughly 1000 angstrom units across. The particle is covered by a lipid bilayer membrane derived from the outer membrane of the infected host cell. Studding the viral membrane is an envelope glycoprotein which is synthesized as a precursor of 160 Kd and subsequently processed into two glycoproteins: gp41 which spans the lipid bilayer, and gp120 which extends beyond the lipid bilayer. The envelope covers a core made up of proteins designated p24 and p18. The viral RNA is carried in the core, along with several copies of the enzyme, reverse transcriptase, which catalyzes the assembly of viral DNA.

The HIV genome contains three genes that encode the components of retrovirus particles:env (which codes
for the envelope proteins), gag (which codes for the core proteins), and pol (which codes for reverse transcriptase). These three genes are flanked by stretches of nucleotides called long terminal repeats (LTRs). The LTRs include sequences that have a role in controlling the expression of viral genes. However, unlike other retroviruses, the genome of HIV includes at least five additional genes, three of which have known regulatory functions, and the expression of which is thought to have an impact on the pathogenic mechanisms exerted by the virus. The tat gene encodes a protein that functions as a potent trans-activator of HIV gene expression, and, therefore, plays an important role in the amplification of virus replication. The rev, or rre/art gene can upregulate HIV synthesis by a transacting antirepression mechanism; rev enables the integrated HIV virus to selectively produce either regulatory proteins or virion components. In contrast, the nef, or 3'-orf, gene appears to down-regulate virus expression by producing a cytoplasmic protein which, presumably via a second messenger, inhibits transcription of the HIV genome. The vif, or sor gene is not essential for virion formation, but is critical to the efficient generation of infectious virions and influences virus transmission in vitro. The pr, or R gene encodes an immunogenic protein of unknown function.

The critical basis for the immunopathogenesis of HIV infection is believed to be the depletion of the helper/inducer subset of T lymphocytes, which express the CD4 antigen, resulting in profound immunosuppression. Viral killing of these immune cells is thought to be a major factor contributing to the crippling effect HIV has on the immune system. The envelope glycoprotein plays an important role in the entry of HIV into CD4 positive host cells. The gp120 portion has been shown to bind directly
to the cellular CD4 receptor molecule, thereby producing HIV's tropism for host cells that express the CD4 receptor, e.g., T helper cells (T4 cells), macrophages, etc.

After HIV binds to the CD4 molecule, the virus is internalized and uncoated. Once internalized, the genomic RNA is transcribed into DNA by the enzyme reverse transcriptase. The proviral DNA is then integrated into the host chromosomal DNA and the infection may assume a "dormant" or latent phase. However, once activation occurs, the proviral DNA is transcribed. Translation and post translational processing results in virus assembly and budding of mature virions from the cell surface.

When active replication of virus occurs, the host CD4+ cell is usually killed. However, the precise mechanism by which HIV exerts its cytopathic effect is unknown. A number of mechanisms for the immunopathogenesis and cytopathic effect of HIV infection have been proposed: the accumulation of large amounts of unintegrated viral DNA in the infected cells; massive increase in permeability of the cell membrane when large amounts of virus bud off the cell surface; speculations that HIV may induce terminal differentiation of infected T4 cells, leading to a shortened life span. There is growing evidence that both the CD4 molecule and the virus envelope play a role in cytopathic effect in HIV infected cells by somehow promoting cell fusion. A prominent feature in the cytopathology of HIV infection is the formation of multinucleated syncytia formed by the fusion of as many as 500 cells which appear to be induced by the gp120/gp41 envelope proteins. In contrast, HIV-infected macrophages may continue to produce HIV without cytopathic effects for long periods of time; it is believed that the macrophage is a major reservoir for HIV and may be responsible for transporting virus into the central

To date, there is no cure for AIDS. Vaccine trials are currently underway in an attempt to control the spread of the virus among the population. However, efforts at controlling the course of disease within an infected patient have been directed mainly towards the use of antiviral agents.

2.2. ANTI-HIV MEDICATIONS

Various therapeutic approaches are currently being explored in efforts to decrease the morbidity and mortality of HIV infection (Yarchoan et al., 1988, Scientific American 259:110-119; Bartlett, 1988, J.A.M.A. 260:3051-3052; De Clercq, E., 1986, J. Med. Chem. 29:1561-1569; Yarchoan, R. and Broder, S., 1987, N. Engl. J. Med. 316:557-564). Knowledge of the physiology of the HIV virus has resulted in a number of rationales designed to interfere with viral replication or dissemination of infection. Intervention could potentially prevent virus binding to target cells, fusion with cell membranes, uncoating of viral nucleic acid, reverse transcription of the HIV RNA genome into DNA, transcription or translation of viral mRNA, processing of viral proteins, assembly into mature virions, or other events related to replication or infectivity.

Several methods of preventing the binding of HIV to cell membranes are being tested; these are directed toward inhibiting interactions between the HIV envelope glycoprotein, gp120, and CD4 antigen on target cells, such as helper T lymphocytes. The binding of gp120 to CD4 antigen has been shown to be associated not only with viral penetration of cell membranes, but to syncytia formation as well (Sodroski et al., Nature 322:470-474; Lifson et al., 1986, Nature 323:725-728; Stevenson et al.,
1988, Cell 53:483-496). Smith et al. (1987, Science 238:1704-1707) has shown that soluble CD4 antigen can block HIV infectivity by binding to viral particles before they encounter CD4 molecules embedded in cell membranes. Alternatively, anti-idiotypic antibodies, directed toward anti-CD4 antibodies, have been shown to bind to HIV virus in vitro, presumably by possessing protein configurations similar to CD4 determinants (Dalgleish et al., 1989, UCLA Symposia on Molecular and Cellular Biology. J. Cell Biochem. Supp. 13B, p. 298). In addition, several large, sulfated, negatively charged molecules, including dextran sulfate, have been shown to inhibit HIV replication and syncytia formation in vitro (Yarchoan et al., supra).

Anti-sense oligonucleotides have been found to inhibit virally induced syncytium formation and expression of viral p24 protein (Agrawal et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7079-7083). These synthetic nucleic acid polymers, complementary to HIV mRNA, were designed to inhibit the translation of viral mRNA into protein; forming phosphoramide and phosphorothioate derivatives of anti-sense oligonucleotides has produced compounds substantially resistant to endonuclease degradation.

Ribavirin, comprising a ribose moiety and a triazol ring, is believed to act as an analogue of the nucleoside guanosine. It has activity against several RNA viruses in vitro, primarily, it is believed, by interfering with the guanylation step required for capping of viral mRNA. Ribavirin has been reported to suppress the replication of HIV in cultures of human T lymphocytes (McCormick et al., The Lancet, Dec. 15, 1984:1367-1369).

Alternatively, post-translational processing of viral proteins can be disrupted; castanospermine, a plant glycosidase activity, has been shown to reduce syncytium formation and HIV infectivity (Wall et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:5644-5648).
The final steps in HIV replication involve its exit from the host cell and infection of new cellular targets. Alpha interferon has been found to suppress HIV replication in vitro (Ho et al., Lancet, March 16, 1985:602-604), may reduce viral budding, and has been shown to have direct antitumor activity against Kaposi's sarcoma, a malignancy associated with AIDS. A lipid compound, AL 721, composed of neutral glycerides, phosphatidylcholine, and phosphatidylethanolamine in a 7:2:1 ratio has a demonstrated ability to extract cholesterol from cellular membranes, and appears to decrease HIV infectivity (Sarin et al., 1985, N. Engl. J. Med. 313:1289-1290).

Several methods for controlling HIV infection are being tested which relate to inhibition of viral reverse-transcriptase. Because mammalian cells lack endogenous reverse transcriptase activity, these methods offer potentially selective inhibition of virus replication with minimal host cell cytotoxicity. Agents which are believed to inhibit reverse transcriptase include phosphonoformate (Sandstrom et al., Lancet June 29, 1985:L1480), suramin (Mitsuya et al., 1984, Science 226:172-174), rifabutin (or ansamycin, Amand et al., Lancet, Jan. 11, 1986, p. 97) and nucleoside derivatives, described infra.
2.2.1. **NUCLEOSIDE DERIVATIVES**

Nucleoside derivatives are modified forms of the purine (adenosine and guanosine) and pyrimidine (thymidine, uridine and cytidine) nucleosides which are the building blocks of RNA and DNA. Reverse transcriptase and DNA polymerase will bind and incorporate nucleoside derivatives into the nascent DNA chain provided that the 5’ carbon of the derivative can bind to the 3’ hydroxyl group of the previous nucleotide (i.e. by forming a phosphodiester linkage); subsequent nucleotides can only be added if the nucleoside derivative contains a means for linking its 3’ carbon to the 5’ phosphate of another nucleotide. Many of the nucleoside derivatives under study as potential anti-HIV medications result in premature termination of viral DNA replication before the entire viral genome has been transcribed. These derivatives lack 3’ substituents that can bind to subsequent nucleosides and result in chain termination.

AZT (3’-azido-2’,3’-dideoxythymidine, azidothymidine, zidovudine) is a nucleoside derivative which has been shown to be effective in the treatment of patients with AIDS and ARC; data indicates that AZT increases the median survival of AIDS patients (Fischl et al., 1987, N. Engl. J. Med. **317**:185-191; Creagh-Kirk, et al., 1988, *J.A.M.A.* **260**:3009-3015). Preliminary evidence suggests that AZT may be efficacious in children with AIDS, HIV associated psoriasis (Bartlett et al., *supra*), AIDS associated dementia (Schmitt et al., 1988, N. Engl. J. Med. **319**:1573-1578) and HIV associated thrombocytopenia (Pottage et al., 1988, *J.A.M.A.* **260**:3045-3048). AZT is phosphorylated by mammalian cells to form AZT triphosphate (an analogue of thymidine triphosphate) which is believed to inhibit the production of viral DNA by at least two mechanisms: competitive inhibition and chain termination.
(Yarchoan et al., supra). Competitive inhibition occurs because viral reverse transcriptase binds AZT triphosphate more tightly than native nucleotides; chain termination results from AZT incorporation because AZT lacks a 3' hydroxyl group, and therefore cannot bind to subsequent nucleotides.

Dideoxynucleosides are nucleoside derivatives which lack hydroxyl groups at both the 2' and 3' carbon residues, and therefore result in DNA chain termination. In addition, like AZT, the 5' triphosphate products of 2', 3'-dideoxyadenosine, dideoxyguanosine, dideoxycytidine, and dideoxythymidine selectively inhibit viral reverse transcriptase and cellular β and γ DNA polymerase, but do not interfere with the function of DNA polymerase α, the major DNA synthetic enzyme utilized during cell division (Edenberg et al., 1978, J. Biol. Chem. 253:3273-3280; Waqar et al., 1978, Nucl. Acids Res. 5:1933-1946; van der Vilet et al., 1981, Biochemistry 20:2628-2632; Waqar et al., 1984, J. Cell. Physiol. 121:402-408). In vitro, adenosine, guanosine, inosine, cytidine, and thymidine 2', 3' dideoxynucleosides inhibited HIV virus replication (although the thymidine derivative showed less inhibitory activity); essentially complete suppression of HIV virus was observed at doses that were lower by a factor of 10 to 20 than those needed to inhibit the proliferation or immune reactivity of T cells (Mitsuya and Broder, 1986, Proc. Natl. Acad. Sci. U.S.A. 83:1911-1915). Ahluwalia et al. performed initial studies on the cellular pharmacology of 2',3'-dideoxyinosine (1987, Biochm. Pharm. 36:3797-380). PCT publication WO 87/01284, international publication date March 12, 1987, relates to the inhibition of in vitro infectivity and cytopathic effects of HIV by purine 2', 3' dideoxynucleosides, including 2', 3' dideoxyinosine, 2', 3'-dideoxyguanosine, and 2', 3'- dideoxyadenosine. European patent application 0273277,
publication date July 6, 1988, relates to the use of 3’-deoxythymidin-2’-ene (3’-deoxy-2’,3’-didehydrothymidine d4T), which lacks 2’ and 3’hydroxyl groups and has a double bond between 2’ and 3’ carbon atoms, in treating patients infected with a retrovirus. D4T, also referred to as 2’,3’-didehydro-2’,3’-dideoxythymidine, or 1-(2,3-dideoxy-β-D-glycero-pent-2-enofuranosyl)thymine, was found to inhibit HIV reverse transcriptase, and had anti-HIV activity comparable to AZT (Mansuri et al., J. Med. Chem. in press). D4T may also have less toxic effects than AZT (Mansuri et al., supra; Ghazzouli et al., 1988, ICAAC, Abstract 1301, p. 344).

Recently, acid stable 2’,3’-dideoxy-2’-fluoronucleosides which exhibit antiviral effects have been described. See, European Patent Application, Publication No. 0287313 A2 which describes 2’,3’-dideoxy-2’-fluoroadenosine and 2’,3’-dideoxy-2’-fluoroinosine and European Patent Application Publication No. 0292023 A2 which describes a fluoro pyrimidine derivative, 2’3’-dideoxy-2’-fluoro-arabinofuranosylcytosine (F-ddC).

2.2.2. TOXIC EFFECTS OF ANTI-HIV MEDICATIONS

Because HIV depends on human cells to supply the machinery necessary for the completion of its life cycle, inhibition of viral replication is frequently associated with cytotoxic effects. AZT therapy is associated with bone marrow toxicity (Richman et al., 1987, N. Engl. J. Med. 317:192-197) and consequently, decreased production of red blood cells (resulting in anemia), platelets (resulting in thrombocytopenia), and white blood cells (resulting in leukopenia). According to Bartlett et al. (1988, J.A.M.A. 260:3051-3052), 40 percent of AZT-treated patients ultimately develop anemia, requiring dosage reduction or transfusion, and leukopenia, with a decreased
number of granulocytes, thereby increasing susceptibility to infection; only about 60 percent of patients with ARC or AIDS seem to be able to tolerate continued AZT use after one year of therapy (Pettinelli, C. B., Feinberg, J., and the AIDS Clinical Trials Group: Safety and tolerance of zidovudine in patients with AIDS and advanced ARC, abstracted; Fischl, M.A., and the AIDS Clinical Trials Group: The safety and efficacy of two doses of zidovudine in the treatment of patients with AIDS, abstracted; both read before the Fourth International Conference on AIDS, Stockholm, June 15, 1988, and cited by Bartlett).

In order to avoid these toxic effects, AZT is being tested in combination with other antiviral agents, such as acyclovir sodium, foscarinet sodium, interferon-α, β, or γ, interleukin 2, or ampligen. Other studies, alternating AZT with 2′, 3′ dideoxycytidine therapy (ddC) are also in progress (Yarchoan et al., 1988, Lancet 1:76–81). Continuous high doses of ddC (which is relatively marrow-sparing) for more than eight to twelve weeks is associated with the development of painful peripheral neuropathy; it is hoped that by alternating AZT and ddC, the toxic effects of both will be minimized. A therapeutic regimen which is both effective and clinically tolerable by AIDS patients is actively being sought by health care researchers.

3. SUMMARY OF THE INVENTION

The present invention relates to the use of synergistic combinations of nucleoside derivatives for inhibiting human immunodeficiency virus (HIV) replication and thereby limiting HIV infection. According to the invention, certain nucleoside derivatives used together exhibit greater anti-viral effects and less cytotoxicity at lower concentrations than either drug used separately,
thus maximizing treatment of HIV infection and minimizing toxic side-effects.

In a particular embodiment of the present invention, the purine nucleoside analogue dideoxyinosine (ddI) and the pyrimidine nucleoside analogue 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T) can be used to inhibit HIV replication. It is shown, by way of example, that ddI and d4T exhibit strong synergistic anti-HIV activity; the combination of ddI and d4T produced a greater anti-HIV effect but was associated with less cytotoxicity than either compound used separately.

In another embodiment of the present invention, combinations of fluoronucleoside analogs may be used with d4T to inhibit HIV replication. For example, the purine nucleoside analog, 2′,3′-dideoxy-2′-beta-fluorooinosine (F-ddI) may be used with d4T.

3.1. DEFINITIONS AND ABBREVIATIONS

The following terms as used herein whether in the singular or plural, shall have the meanings designated.

A combination index may be determined with the equation:

\[ CI = \frac{(D_1^*)}{(D_1)} + \frac{(D_2^*)}{(D_2)} + \frac{\alpha(D_1)(D_2^*)}{(D_1)(D_2)} \]

where \((D_1^*)\) is the dose of agent 1 required to produce \(x\)% percent effect alone, and \((D_1^*)\) is the dose of agent 1 required to produce the same \(x\)% percent effect in combination \((D_2^*)\). Similarly, \((D_2^*)\) is the dose of agent 2 required to produce \(x\)% percent effect alone, and \((D_2^*)\) is the dose required to produce the same effect in combination with \((D_1^*)\). As described in Hartshorn et al. (supra), the slope of dose-effect curves indicate whether the agents have mutually exclusive effects (e.g. similar mode of action) or mutually nonexclusive effects (e.g. independent mode of action).

If the agents are mutually exclusive, then \(\alpha = 0\) (i.e., CI is the sum of two terms); if the agents are mutually nonexclusive, \(\alpha = 1\) (i.e., CI is the sum of three terms).

Synergy: the action of two or more substances to achieve an effect greater than that of either substance used individually. Synergism in antiviral activity should be construed to mean greater antiviral activity; synergism in cytotoxicity should be construed to mean greater cytotoxicity.

Tissue Culture Inhibitory Dose (i.e. TCID\(_{50}\)) the amount of virus necessary to reduce viral expression by a given percentage, i.e., 50 percent.

Tissue Culture Toxic Dose (i.e., TCTD\(_{50}\)) the amount i.e. of drug required to reduce the number of tissue culture cells by a given percentage (i.e., 50 percent).
4. DESCRIPTION OF THE FIGURES

Figure 1. Antiviral activity of equal total concentrations of d4T (broken line), ddI (dotted line) or d4T + ddI (solid line) as measured by p25gag binding, expressed as percent inhibition.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the use of synergistic combinations of nucleoside derivatives for inhibiting the replication of HIV, thereby limiting the effects of HIV infection. According to the invention, combinations of certain nucleoside derivatives exert stronger anti-HIV effects but are associated with less cytotoxicity than individual nucleoside derivatives at comparable total drug concentrations.

Although the applicants are under no duty or obligation to explain the mechanism by which the invention works it may be that by supplying two or more nucleoside derivatives, any one of which may substitute for naturally occurring nucleosides in viral reverse transcriptase activity, the likelihood that these derivatives will be incorporated into the viral DNA sequence (and consequently terminate reverse transcription by preventing elongation of nascent DNA) is substantially increased.

For the purpose of clarity of disclosure, and not by way of limitation, the description of the present invention will be divided into three sections: (1) nucleoside derivative combinations; (2) in vitro assay for demonstrating the HIV-inhibitory effect of nucleoside derivative combinations, and (3) therapeutic uses of HIV-inhibitory nucleoside derivative combinations.
5.1. IDENTIFICATION OF SYNERGISTIC NUCLEOSIDE DERIVATIVE COMBINATIONS

According to the present invention, nucleoside derivatives which may be used in combination include, but are not limited to, 2',3'-dideoxyadenosine (ddA); 2',3'-dideoxyguanosine (ddG); 2',3'-dideoxyinosine (ddI); 5 2',3'-dideoxycytidine (ddC); 2',3'-dideoxythymidine (ddT); 2',3'-dideoxy-2',3'-didehydrothymidine (d4T) and 3'-azido-2',3'-dideoxythymidine (AZT). Alternatively, halogenated nucleoside derivatives may be used, preferably 2',3'-dideoxy-2'-fluoronucleosides including, but not limited to 2',3'-dideoxy-2'-fluoroadenosine; 2',3'-dideoxy-2'-fluorocytidine; and 2',3'-dideoxy-2',3'-didehydro-2'-fluoronucleosides including, but not limited to 2',3'-dideoxy-2',3'-didehydro-2'-fluorothymidine (Fd4T). Preferably, the 2',3'-dideoxy-2'-fluoronucleosides of the invention are those in which the fluorine linkage is in the beta configuration, including, but not limited to, 2',3'-dideoxy-2'-beta-fluoroadenosine (F-ddA), 2',3'-dideoxy-2'-beta-fluorocytidine (F-ddC), and 2',3'-dideoxy-2'-beta-fluorothymidine (F-ddT).

The combination of 2',3'-dideoxyinosine (ddI) and 2',3'dideoxy-2',3'-didehydrothymidine (d4T) represents a preferred embodiment of the invention. The combination of 2',3'-dideoxy-2'-beta-fluorocytidine (F-ddC) and 2',3'-dideoxy-2',3'-didehydrothymidine (d4T) also represents a preferred embodiment. For a more detailed description of the 2',3'-dideoxy-2'-fluoronucleosides and the 2',3'-dideoxy-2',3'-didehydro-2'-fluoronucleosides, see copending application Serial No. 120051, filed November 12, 1987 by Sterzycki, Mansuri and Martin which is incorporated by reference herein in its entirety. In order to evaluate potential therapeutic efficacy of combinations of nucleoside derivatives, these
combinations may be tested for anti-viral activity according to methods known in the art. For example, the ability of a nucleoside combination to inhibit HIV cytotoxicity, syncytia formation, reverse transcriptase activity, or generation of viral RNA or proteins may be tested in vitro.

Combinations of nucleoside derivatives over wide concentration ranges for each may be tested for antiviral and/or cytotoxic activity, and combination indices may be derived, according to methods outlined in Section 6.1. infra. A preferred method for demonstrating the HIV inhibitory effect of nucleoside derivative combinations is set forth in section 5.2 and example section 6, infra. In addition, combinations of nucleoside derivatives may be tested for anti-viral activity in vivo using an animal model system for AIDS, such as the simian immunodeficiency virus (SIV) system (Kanki et al., 1985, Science 220:951-954); however, because different species of animals (or even different cell types within one species) differ in the efficiency with which they phosphorylate these drugs, extreme caution should be used in extrapolating the experimental results obtained in one species (or in one cell type within a species) to another (Yarchoan and Broden, 1987, N. Engl. J. Med. 316:557-564; Waqar, 1984, J. Cell. Physiol. 121:402-408; Furman et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:833-7; Ono et al., 1979, Biochem. and Biophys. Res. Commun. 88:1255-1262).

Therapeutic as well as cytotoxic doses for these combinations may be established, and those combinations which show greatest synergy, i.e., which are associated with highest anti-viral activity and/or lowest cytotoxicity, may be considered for use in humans.
5.2. IN VITRO ASSAY FOR DEMONSTRATING THE HIV-INHIBITORY EFFECT OF NUCLEOSIDE DERIVATIVE COMBINATIONS

The inhibitory activity of the nucleoside derivative combinations may be tested using an in vitro assay system such as any of those described in Section 6 et seq. herein. For example, the efficacy of any nucleoside derivative combination selected may be assessed by its relative ability to inhibit (a) the formation of syncytia, and (b) the production of HIV particles in HIV-infected cells in vitro using a target cell line that can be infected with HIV. In general such cell lines would be of a T-cell or myelocytic/monocytic lineage or another cell type transfected with the gene for CD4. Alternatively, if the nucleoside derivatives were linked to a "targeting" molecule e.g., a monoclonal antibody, hormone, growth factor etc. the target cell line should express the appropriate cell surface antigen or receptor for the molecule conjugated to the nucleoside derivative.

In order to assess the efficacy of the nucleoside derivative combination chosen, the target cell should be grown in vitro, infected with HIV and treated with an array of doses of the nucleoside derivatives before, during or after infection (e.g., limited dilution techniques can be used). The inhibitory effect on virion production may be assessed as described, e.g., by measuring (a) production of HIV by assaying for HIV proteins, such as the 25 gag viral core protein or reverse transcriptase, in the culture media; (b) the induction of HIV antigens in cells by immunofluorescence; or (c) the reduction in syncytia formation assessed visually. The ability of a nucleoside derivative combination to inhibit HIV is indicative of the inhibitory activity and
inhibitory dose of the nucleoside derivative combination tested.

5.3. THERAPEUTIC USES OF HIV-INHIBITORY SYNERGISTIC DERIVATIVE COMBINATIONS

The synergistic nucleoside derivative combination may be used in accordance with the invention in vivo to prevent the formation of syncytia and the production of HIV virions and, thus, inhibit the progression of HIV infection within an exposed patient. Effective doses of nucleoside derivatives formulated in suitable pharmacological carriers may be administered by any appropriate route including but not limited to injection (e.g., intravenous, intraperitoneal, intramuscular, subcutaneous, etc.), by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and vaginal epithelial linings, nasopharyngeal mucosa, intestinal mucosa, etc.); etc.

In addition, nucleoside derivatives may be mixed in any suitable pharmacological carrier, linked to a carrier or targeting molecule (e.g., antibody, hormone, growth factor, etc.) and/or incorporated into liposomes, microcapsules, and controlled release preparations prior to administration in vivo.

In another embodiment, synergistic nucleoside derivative combinations may be used in conjunction with other treatments for HIV infection. For example, and not by way of limitation, synergistic nucleoside derivatives may be used in conjunction with other antiviral compounds that inhibit reverse transcriptase activity (e.g., AZT); with soluble CD4 or monoclonal antibodies that inhibit HIV absorption to cells; or with other cytokines and growth factors (e.g., interferon α, β or γ; tumor necrosis factor, interleukin-2, granulocytic/monocytic colony stimulating factor; colony stimulating factor-1, etc.);
and with agents that are used to treat other microorganisms or viruses that opportunistically infect AIDS patients.

In another embodiment of the invention, synergistic nucleoside derivatives can be used in vitro to test the efficacy of different drugs. In this regard, it would be advantageous to obtain bone marrow samples from an AIDS patient and expose the marrow in vitro to the drug, compound or regimen being tested in order to identify those that, for example, kill the virus yet spare normal cells, or those that will stimulate marrow repopulation. However, bone marrow derived from AIDS patients exhibits very poor marrow colony formation in vitro. This is probably due to infection of the precursor CD 34 cells with HIV. As a result, the efficacy of various drugs tested in vitro is difficult or impossible to assess. The use of synergistic nucleoside derivatives to inhibit HIV in such an assay system should increase marrow colony formation in vitro and therefore, permit the screening of various other protocols and drugs on the marrow activity in vitro.

6. EXAMPLE: INHIBITION OF HIV INFECTION USING DIDEOXYDIDEHYDROTHYMIDINE AND DIDEOXYINOSINE

The experiments described below demonstrate the inhibitory effect of dideoxydidehydrothymidine (d4T) and dideoxyinosine (ddI) on HIV infection of target cells of T-cell origin in culture in vitro.

6.1. MATERIALS AND METHODS

6.1.1. CELLS AND VIRUS

CEM-F cells were originally derived from the acute human lymphoblastic leukemia and represent an established T lymphoblastoid line. They are available at
the American Type Culture Collection as CCRF-CEM cells (ATCC No. CCL 119).

The LAV\textsubscript{BRU} strain of human immune deficiency virus (HIV) was obtained from Dr. Luc Montagnier, Institut Pasteur, Paris, France. The virus was adapted to CEM-F cells, and stored in small aliquots in liquid nitrogen. The titer of the virus was determined every two to three weeks and is expressed as TCID\textsubscript{50} (tissue culture inhibitory doses; i.e., the amount of virus necessary to reduce the viral expression by 50%). In all experiments described in the subsections below, a viral dose of 50 TCID\textsubscript{50} was used.

Both the CEM-F cells and the HIV virus were grown in LAV/CEM medium. The medium consists of RPMI 1640, supplemented with 1% glutamine, 100 U/ml of Penicillin, 100 \(\mu\)g/ml Streptomycin, 2 \(\mu\)g/ml Polybrene, and 10% fetal bovine serum.

6.1.2. NUCLEOSIDE DERIVATIVES D4T AND DDI

Dideoxydidehydrothymidine (d4T, BMY27857-3/8, lot \#26630-23A) and dideoxyinosine (ddI, BMY40900, lot \#25879-46-1) were obtained from Pharmacetical Research and Development Division of Bristol-Myers Company. Both compounds were resuspended in 2-3 drops of dimethyl sulfoxide (DMSO, D-8779), Sigma Chem. Co.) and LAV/CEM media. The suspension was then sonicated (Microultrasoundic disruptor, Kontes) and brought to 1 mM final concentration. All dilutions were made from that 1 mM stock. In order to test the combined anti-HIV effect of the drugs, two different sets of experiments were done. In the first set, the drugs were mixed so that one was kept at constant concentration, while the other drug concentration was varied. For example, d4T was kept at either 0.01, 0.1, 1 or 10 \(\mu\)M, while ddI range was 0.1, 1, 10 to 100 \(\mu\)M. In this setup the ratio of the drugs in
each mixture was different. In order to more precisely determine the degree of possible synergy between d4T and ddI, in the second set of assays the drugs were mixed at 1:5 ratio starting at 10 and 50 µM, and diluted twofold. This way the ratio of the drugs was kept constant through all dilutions. The data was evaluated using the "Dose-Effect Analysis with Microcomputers" software (Chou, J., and T-C. Chow, Elsevier-Biosoft Publishers, 1986). The same two protocols were used to evaluate toxicity of d4T and ddI on uninfected cells, except that in the equal drug ratio experiment the ratio of the drugs was 1:1, with a starting concentration of 100 µM for both drugs. Azidothymidine (AZT, BMY27755/7, lot #2300-38) was used as a positive control in all assays.

6.1.3. DETERMINATION OF THE PROLIFERATIVE EFFECT OF D4T AND DDI

For each assay 2 x 10^4 CEM-F cells/well were plated in 96 well plates. The cells were mixed with each different combination, each set up in quadruplicate. The total volume/well was 250 µl. Plates were incubated for six days at 37°C and 5% CO₂. On day six, cells were labeled for four hours with 1 µCi/well of ³²PHTdR (New England Nuclear Corp. specific activity 6.7 Ci/mmol) harvested onto glass fiber filters and counted in the scintillation counter (Hartman, R.J., Segall, M., Bach, M.L., and Bach, F.H., 1971, Histocompatibility matching. VI. Miniaturization of the mixed leukocyte culture test: A preliminary report. Transplantation, 11:268-273). The results were expressed as percent ³²PHTdR uptake of the control which consisted of cells incubated without drugs. In addition, the results can be expressed as a tissue culture toxic dose 50 (TCTD_{50}), which represents the amount in µg of the drug or drugs necessary to reduce the number of cells by 50% as compared to the control.
6.1.4. INHIBITION OF HIV REPLICATION

The CEM-F cells were plated in 96 well plates at $2 \times 10^4$ cells/well and mixed with 50 TCID$_{50}$ of virus for 45 minutes. After 45 minutes nucleoside derivatives were added to each well and incubated as described for the proliferation assay. At the end of day six the supernatants were tested for the presence of viral antigen using an antigen capture ELISA assay described below. The assay uses two monoclonal antibodies against the viral core protein p24gag (Hu, S.H. et al., 1987, Nature 328:721-723; and Kinney Thomas, E. et al., 1988, AIDS 2:25-29).

6.1.5. ANTIGEN-CAPTURE ASSAY

For this assay Microtiter plates (96 well plates) were coated with two monoclonal antibodies: 25-2 (ATCC #9407) and 25-3 (ATCC #9408), each diluted at 1:2500. These antibodies (capture reagents) are specific for p24, p40, and p55 HIV gag proteins. Horseradish peroxidase (HRP)-conjugated human IgG purified from a serum of a seropositive individual was used as a signal. The absorbance (450/630 nm) is determined after the addition of substrate - tetramethyl benzidine (TMB). The OD readings fall into three categories: Experimentals = values from wells containing cells, viral inoculum and nucleoside derivative(s); and Controls = values from wells containing cells and virus (100%); and Background = values from wells containing viral inoculum alone. The background value was subtracted from all the OD values.

The antiviral effect of the nucleoside derivative(s) is expressed as the percent p24gag binding of the control. For example, if that value is 20%, it means that the viral replication, measured indirectly through p25gag binding, is inhibited by 80%.
6.1.6. **SYNCYTIA FORMATION**

Prior to collecting supernatants for the antigen-capture assay, all the wells were visually examined. This was done to assure that the infection took place and also to check the state of the cells. The syncytia were easy to observe, and although they were not counted, the differences in their numbers between wells were very apparent.

6.1.7. **ANALYSIS OF CYTOTOXICITY**

The toxicities of D4T and DDI were tested against the host cell CEM-F (ATCC CCL119). CEM-F is a T-cell line that constitutively expresses the CD4 receptor, and therefore, is an appropriate host cell target for HIV infection. The effect of D4T and DDI on proliferation of uninfected cells was assessed by measuring thymidine incorporation in uninfected CEM-F cells treated with D4T and DDI.

6.2. **RESULTS**

6.2.1. **HIV INHIBITION BY A NUCLEOSIDE DERIVATIVE COMBINATION**

In vitro assays for p25gag protein revealed a strong synergistic effect of d4T in combination with ddI for inhibiting p25gag expression, representing antiviral activity greater than either compound used individually. The results of binding assays for p25gag are shown in Table I; a comparison of a combination of d4T and ddI to either drug used at the same total concentrations is shown in Figure 1.

Table I presents the results of a series of experiments used to discern the optimal concentrations and ratios of concentrations of d4T and DDI for achieving maximal anti-viral activity. A wide range of
concentrations were tested by evaluating logarithmic increments in the concentration of each derivative in multiple combinations, using a "checkerboard" pattern of testing.

<table>
<thead>
<tr>
<th>Concentration of D4T (μM)</th>
<th>0</th>
<th>.1</th>
<th>1</th>
<th>10</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>86</td>
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<tr>
<td>10</td>
<td>87</td>
<td>92</td>
<td>88</td>
<td>92</td>
<td></td>
</tr>
</tbody>
</table>

* as measured by p25gag binding

When either d4T or ddI were used in conjunction with AZT, at a concentration of 1:10:50, a synergistic effect was achieved which was less than the synergism observed for the combination of d4T and ddI (see Table III and Section 6.3).

6.2.2. DIMINISHED CYTOTOXICITY OF A COMBINATION OF NUCLEOSIDE DERIVATIVES

Cytotoxicity assays measuring $^3$H-thymidine incorporation show that d4T and ddI used in combination are not more cytotoxic to CEM-F cells than the same total concentration of either compound used individually, despite increased toxicity to virus. The results of these assays over a wide range of concentrations of d4T, ddI, or
d4T in combination with ddI in 1:1 ratio are shown in Table II.

<table>
<thead>
<tr>
<th>Concentration of D4T (μM)</th>
<th>0</th>
<th>.1</th>
<th>1</th>
<th>10</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<tr>
<td>100</td>
<td>19</td>
<td>26</td>
<td>31</td>
<td>28</td>
<td>30</td>
</tr>
</tbody>
</table>

* as measured by $^3$H-thymidine uptake.

6.3. DISCUSSION

The data presented above indicates that the nucleoside derivatives d4T and ddI exhibit a strong synergistic antiviral effect when used in combination. For example, referring to Table I, a concentration of 1μM of d4T resulted in 24 percent inhibition of viral activity, and a concentration of 10μM of ddI was associated with 2 percent inhibition of viral activity; however, 1μM of d4T combined with 10μM of ddI achieved 75 percent HIV inhibition. Figure 1 contains a graph which shows the antiviral activities of d4T, ddI, and (d4T + ddI) at the same total concentration of nucleoside, and clearly shows the synergistic effects of d4T used in conjunction with ddI.
The combination index (CI) is a numerical representation of the synergistic or antagonistic effects of drug combinations. The CI value is obtained using an isobologram equation and computer simulation according to Chou (see Section 3.1). A CI value of less than one represents synergy (i.e., the whole is greater than the sum of its parts) CI greater than one represents antagonism (i.e., the whole is less than the sum of its parts) and CI equal to one represents additivism, (i.e., the whole is equal to the sum of its parts). Table III shows the CI values of (d4T + ddI), (d4T + AZT), and (ddI + AZT) for antiviral effect, when AZT, d4T, and ddI concentrations were in the ratio of 1:10:50.
Table III

<table>
<thead>
<tr>
<th>Compound</th>
<th>50%</th>
<th>70%</th>
<th>90%</th>
</tr>
</thead>
<tbody>
<tr>
<td>D4T + DDI</td>
<td>0.024</td>
<td>0.049</td>
<td>0.16</td>
</tr>
<tr>
<td>D4T + AZT</td>
<td>0.53</td>
<td>0.43</td>
<td>0.30</td>
</tr>
<tr>
<td>AZT + DDI</td>
<td>0.77</td>
<td>0.74</td>
<td>0.75</td>
</tr>
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</table>

Because of the synergistic relationship between d4T and ddI, lower concentrations of nucleoside can be used to achieve effective viral inhibition. As shown in Table II, increased antiviral activity of d4T and ddI combinations is not associated with increased cytotoxicity, and therefore, selective antiviral activity has been achieved. In addition, studies to establish the TD\textsubscript{50} (toxic dose) for d4T and ddI singly, or in combination, revealed that whereas the TD\textsubscript{50} for both d4T and ddI individually were greater than 100\,\mu M, the TD\textsubscript{50} for the combination of D4T and DDI was 730 \,\mu M (data not shown). The CI for cytotoxic effects of combinations of AZT, d4T, and ddI, at concentration ratios of 1:10:10, respectively, show that at concentrations that inhibit 90\% of HIV activity, the combination of d4T and ddI is markedly less than equally virocidal concentrations of either (d4T + AZT), or (ddI + AZT) (see Table IV). Therefore, not only is the combination of d4T and ddI more virocidal than either d4T or ddI considered individually, (d4T + ddI) is less cytotoxic. The therapeutic window for treating HIV infection is therefore broadened by using these nucleoside derivatives in combination; therapeutic
anti-HIV effects may be achieved in patients without risking dangerous or painful complications.

Table IV

<table>
<thead>
<tr>
<th>Compound</th>
<th>50%</th>
<th>90%</th>
</tr>
</thead>
<tbody>
<tr>
<td>D4T + DDI</td>
<td>2.17</td>
<td>6.40</td>
</tr>
<tr>
<td>D4T + AZT</td>
<td>2.45</td>
<td>1.66</td>
</tr>
<tr>
<td>AZT + DDI</td>
<td>2.21</td>
<td>2.37</td>
</tr>
</tbody>
</table>
WHAT IS CLAIMED IS:

1. A method for inhibiting HIV comprising bringing HIV-infected cells in contact with a synergistic combination of nucleoside derivatives.

2. The method according to claim 1 which is performed in vitro.

3. The method according to claim 1 which is performed in vivo.

4. The method according to claim 3 which is performed in a mammal.

5. The method according to claim 3 which is performed in a human.

6. The method according to claim 1, 2, 3, 4, or 5 in which at least one of the nucleoside derivatives is a 2',3'-dideoxynucleoside.

7. The method according to claim 1, 2, 3, 4, or 5 in which one of the nucleoside derivatives is 2',3'-dideoxynosine.

8. The method according to claim 1, 2, 3, 4, or 5 in which one of the nucleoside derivatives is 2,3'-dideoxy-2',3'-didehydrothymidine.

9. The method according to claim 1, 2, 3, 4, or 5 in which one of the nucleoside derivatives is a 2',3'-dideoxy-2'-fluoronucleoside derivative.
10. The method according to claim 9 in which the fluoronucleoside derivative is 2',3'-dideoxy-2'-fluorooinosine.

11. The method according to claim 10 in which the fluoronucleoside derivative is 2',3'-dideoxy-2'-beta-fluorooinosine (F-ddI).

12. The method according to claim 9 in which the fluoronucleoside derivative is 2',3'-dideoxy-2'-fluoro adenosine.

13. The method according to claim 12 in which the fluoronucleoside derivative is 2',3'-dideoxy-2'-beta-fluoro adenosine (F-ddA).

14. The method according to claim 9 in which the fluoronucleoside derivative is 2',3'-dideoxy-2'-fluorocytosine.

15. The method according to claim 14 in which the fluoronucleoside derivative is 2',3'-dideoxy-2'-beta- fluorocytosine (F-ddC).

16. The method according to claim 3 which is used in conjunction with other anti-HIV treatment.

17. The method according to claim 3 which is used in conjunction with one or more therapies for HIV-associated disorders, including opportunistic infections.

18. The method according to claim 1, 2, 3, 4, or 5 in which at least one nucleoside derivative is chemically linked to a second molecule.
19. A method for inhibiting HIV comprising bringing HIV-infected cells in contact with a combination of 2',3'-dideoxyinosine (ddI) and 2',3'-dideoxy-2',3'-didehydrothymidine (d4T).

20. The method according to claim 19 in which the ratio of d4T to ddI is between about 1:1 to 1:10.

21. The method according to claim 19 in which the ratio of d4T to ddI is about 1:5.

22. A method for inhibiting HIV comprising bringing HIV-infected cells in contact with a combination of 2',3'-dideoxy-2'-beta-fluororinosine (F-ddI) and 2',3'-dideoxy-2',3'-didehydrothymidine (d4T).

23. A method for inhibiting HIV comprising bringing HIV-infected cells in contact with a combination of 2',3'-dideoxy-2'-beta-fluoroadenosine (F-ddA) and 2',3'-dideoxy-2',3'-didehydrothymidine (d4T).

24. A method for inhibiting HIV comprising bringing HIV-infected cells in contact with a combination of 2',3'-dideoxy-2'-beta-fluorocytosine (F-ddC) and 2',3'-dideoxy-2',3'-didehydrothymidine (d4T).
**INTERNATIONAL SEARCH REPORT**

**I. CLASSIFICATION OF SUBJECT MATTER**

According to International Patent Classification (IPC) or in both National Classification and IPC

IPC(5th Ed.): A61K 31/70

US Cl.: 514/45, 514/46, 514/49, 514/50

**II. FIELDS SEARCHED**

<table>
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<th>Classification System</th>
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</table>

**III. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of Document</th>
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<tbody>
<tr>
<td>Y</td>
<td>US, A, 4,837,311, TAM ET AL., Published 06 June 1989, see columns 1-36.</td>
<td>1-24</td>
</tr>
<tr>
<td>Y</td>
<td>EP, A, 0,316,017 BRISTOL-MYERS COMPANY, Published 17 May 1989, see pp. 1-17.</td>
<td>1-24</td>
</tr>
<tr>
<td>Y</td>
<td>WO, A, 87/01284, UNITED STATES OF AMERICA, Published 12 March 1987, see pp. 1-12.</td>
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<td>A</td>
<td>EP, A, 0,292,023, F. HOFFMANN-LA ROCHE &amp; CO., Published 23 Nov 1988, see pp. 1-9.</td>
<td>1-24</td>
</tr>
</tbody>
</table>

* Special cautions of cited documents:
  - "A" document defining the general state of the art which is not considered to be an particular relevance
  - "E" earlier document but published on or after the international filing date
  - "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  - "Q" document referring to an oral disclosure, use, exhibition or other means
  - "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Z" document member of the same patent family

**IV. CERTIFICATION**

Date of the Actual Completion of the International Search: 05 June 1990

Date of Mailing of this International Search Report: 19 JUL 1990

International Searching Authority: ISA/US

Signature of Authorized Officer: L. Eric Crane
<table>
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<th>Relevant to Claim No</th>
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<td>Y</td>
<td>Chemical Abstracts, Vol. 107, Issued 31 August 1987 (The American Chemical Society), Columbus, Ohio, Hamamoto et al., &quot;Inhibitory effect of 2',3'-didehydro-2',3'-dideoxynucleosides on infectivity, cytopathic effects, and replication of human immunodeficiency virus,&quot; see abstract 70319n (p. 24).</td>
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<td>Y, P</td>
<td>Chemical Abstracts, Vol. 111, Issued 28 August 1989 (The American Chemical Society), Columbus, Ohio, Ho et al., &quot;Cellular pharmacology of 2',3'-dideoxy-2',3'-didehydrothymidine, a nucleoside analogue active against human immunodeficiency virus,&quot; see abstract 70252t (p. 11).</td>
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</table>
FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A. P. EP, A. 0,314,011, F. HOFFMANN-LA ROCHE & CO., Published 03 May 1989, see pp. 1-10.


1. Claim numbers 1-24, because they relate to subject matter not required to be searched by this Authority, namely:

2. Claim numbers 1-8, 16-21, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claim numbers, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This International Searching Authority found multiple inventions in this international application as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically:

3. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not issue payment of any additional fee.

Remarks on Protest:

☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.