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<p>(54) Title: CHEMOTHERAPEUTIC COMPOSITION FOR AIDS</p>		
<p>(57) Abstract</p> <p>The present invention describes a synergistic drug combination as an effective inhibitor of viral infection, particularly of retroviral infection. The drug combination may be an antiviral drug, such as azidothymidine, 2<sup>1</sup>, 3<sup>1</sup>-dideozycytidine or its triphosphate, and a potentiating agent, such as dipyridamole. The antiviral drug, potentiating agent or a combination of them may be encapsulated in liposomes. The drug combination is useful in treating or preventing AIDS and other HIV-related syndromes.</p>		

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CHEMOTHERAPEUTIC COMPOSITION FOR AIDS

This is a continuation-in-part of pending application Serial No. 07/194,171 filed May 16, 1988 which is incorporated herein by reference.

5 Technical field

The present invention is related to treating or preventing AIDS or other similar viral diseases. More particularly, the present invention is related to a combination of an antiviral agent and a potentiating agent, the combination being more effective against virus than either agent used alone.

Background of the Invention

Acquired immunodeficiency syndrome (AIDS) is believed to be caused by a retrovirus called human immunodeficiency virus (HIV). At present, there is no treatment which does more than delay progression of the viral infection and disease. Without effective therapy, AIDS is known to be fatal.

Certain chain-terminator compounds such as azidothymidine (AZT), other dideoxynucleosides, dideoxynucleotides, and their analogs have a degree of efficacy against the AIDS virus (Mitsuya, et al. 1985, PNAS, USA. 82:7096; Yarchoan, et al. 1988, Lancet(i) 76-80; Dahlberg et al. 1987, PNAS, USA., 84: 2469). They appear to be phosphorylated within cells and, in the triphosphate form, to terminate DNA chains being formed in the presence of viral reverse transcriptase. These drugs may act by additional mechanisms as well, for example, by competing for the enzymes active in phosphorylation of

physiological nucleosides and nucleotides.

Castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizine) is a plant alkaloid that modifies glycosylation by inhibiting  $\alpha$ -glycosidase I. Other compounds of the same group (referred to as trimming glycosidase inhibitors because they inhibit the normal removal of glucose residues during the processing of glycoproteins) which, too, have been shown to have anti-HIV activity, include dihydroxymethyldihydroxypyrrolidine (DMDP) and deoxynojirimycin (DNJ or DNM) (Gruters et al, 1987, Nature 330:74; Tymes et al, 1987, Lancet(i) 1025; Walker et al, 1987, PNAS, 84:8120).

Dipyridamole (DPM), also called Persantine, is routinely used in the context of cardiovascular disease for coronary vasodilation and inhibition of platelet aggregation (FitzGerald, 1987, New Engl. J. Med. 316:1247). Its best studied mechanism of action is inhibition of nucleoside transport. Through this mechanism it affects principally the "salvage" pathways for nucleotide production. It has also been reported to inhibit cAMP phosphodiesterase, to alter prostaglandin production, to alter adenosine levels and CD4 expression, to alter cell surface properties, and to stimulate interferon production. DPM has also been reported to have activity against some viruses, but activity either against the AIDS virus or against retroviruses has not been reported (Tonew et al, 1977, Acta Virol.21:146-150). Moreover, a synergistic combination of DPM or a similar potentiating agent with an antiviral drug has not heretofore been known or described.

#### SUMMARY OF THE INVENTION

It is, therefore, an object of the present invention to provide a drug combination with synergistic advantage as inhibitor of HIV and other retrovirus activity compared to each drug used alone.

5 It is a further object of the present invention to provide a method of arresting HIV proliferation, comprising contacting HIV infected cells by the drug-combination of the present invention in an amount sufficient to prevent HIV replication.

10 It is yet another object of the present invention to provide a method of treating or preventing AIDS and other HIV-related syndromes, comprising administering to a host infected or uninfected with the AIDS virus sufficient amount of the drug-combination of the present invention to inhibit  
15 AIDS-virus activity.

It is an additional object of the present invention to treat or prevent retroviral diseases other than AIDS in humans and animals.

20 Other objects and advantages of the present invention will become evident from the following detailed description of the invention.

#### BRIEF DESCRIPTION OF DRAWINGS

25 These and other objects, features and many of the attendant advantages of the invention will be better understood upon a reading of the following detailed description when considered in connection with the accompanying drawings wherein:

Figure 1 demonstrates the effect of dipyridamole (DPM), alone and in combination with chain-terminating

A

dideoxynucleosides, on HIV-1 replication in human monocyte/macrophage (M/M) cultures. A: Adherence-purified, cryopreserved monocyte/macrophages treated with AZT alone, DPM alone, or AZT plus DPM. The cells were treated on day 1 and re-fed on days 6, 11, and 14 with medium containing the appropriate drug concentrations. 100- $\mu$ l samples of supernatant were analyzed for HIV-1 p24. The figure shows the results on day 14. AZT 0 denotes DPM treatment alone. The data show that DPM potentiates the antiviral action of AZT. Days 6 and 11 showed qualitatively similar results. B: Experiment similar to that in A, except that elutriated monocytes were used. The cells were treated with the drugs on day 1 of culturing and were re-fed by replacing 100  $\mu$ l of medium (without drugs) at 3-4 day intervals. The data show p24 levels on day 17 and demonstrate DPM's antiviral activity and potentiation of antiviral activity of AZT. The pattern of effects was similar on day 14. C and D: From the same experiment as in B but using 2',3'-dideoxycytidine (ddC) and 2',3'-dideoxycytidine triphosphate (ddCTP), respectively. Again, DPM inhibited viral replication and potentiated the antiviral effect of ddC and ddCTP. The pattern of effects was similar on day 14. Mean  $\pm$ S.E.M. of quadruplicate wells; some bars are too small to be visible.

Figure 2 shows the results of time course study of HIV-1 infection in elutriated M/M treated with DPM alone. Experiment similar to that in Fig. 1B-D. The cells were treated on day 1, and samples of culture supernatant were taken for HIV-1 p 24 assay at the time points shown (i.e., at

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each re-feeding). Samples consisted of 100  $\mu$ l out of a total volume of 200  $\mu$ l in the well, and cumulative p24 production was calculated by correcting for antigen removed at prior time points. It is noted that DPM alone exhibited antiviral activity. Means of quadruplicate wells  $\pm$ S.E.M.

Figure 3 shows the interaction between AZT and DPM in their cytotoxic effects on human bone marrow granulocyte-monocyte precursors. The CFU<sub>GM</sub> assay, calculation of the combination index (C.I.), and other experimental details are described herein infra. Panels A and B show the C.I. values calculated on the basis of the mutually exclusive model (identical site/mechanism of action) and the mutually non-exclusive model (non-identical sites/mechanisms of action), respectively. The different symbols indicate cells from different healthy donors. The results show no synergy between AZT and DPM in bone marrow toxicity.

Figure 4 shows the effect of combination chemotherapy with DPM-AZT (panel A) and DPM-castanospermine (panel B) in T-lymphoblastoid cells (CEM-SS) infected with HIV-1. Panel A shows the results when the cells were infected by cocultivation with H9 cells carrying HIV-1(III<sub>B</sub>). Treatment was begun on day 1. In this and other similar experiments, the effect of DPM on antiviral efficacy of AZT was moderate, but there was striking protection of the cells from AZT toxicity. Hence, the in vitro therapeutic index was greatly increased. Panel B shows the result of an analogous study for DPM and castanospermine. At high concentrations, DPM potentiated the antiviral effect of castanospermine. This

result emphasizes that a wide range of agents may interact favorably with DPM against HIV and other viruses.

Figure 5 gives HPLC profiles showing the effect of DPM on intracellular accumulation of phosphorylated derivatives of <sup>3</sup>H-thymidine and <sup>3</sup>H-AZT during 6-hour incubations at 37°C with elutriated human monocyte/macrophages. DPM greatly decreased the appearance of phosphorylated thymidine (THY) but only slightly reduced that of phosphorylated AZT.

Figure 6 shows the inhibition of HIV-1 in human M/M by AZT in combination with nitrobenzylthioinosine (NBTI), like DPM an inhibitor of nucleoside transport. The experiment was performed essentially as for Figure 1A except that the adherence-purified M/M cultures were prepared from freshly drawn donor blood, not cryopreserved cells. At the concentrations tested NBTI inhibited virus replication and potentiated the anti-HIV activity of AZT. Mean  $\pm$  S.E.M. of quadruplicate wells on day 11.

Figure 7 shows the inhibition of HIV-1 in human M/M by AZT in combination with papaverine. Papaverine (PA) shares with DPM the ability to inhibit activity of cellular cyclic AMP phosphodiesterase. The experiment was performed as for Figure 6. At the concentrations tested papaverine inhibited virus replication and potentiated the anti-HIV activity of AZT. The pattern of effects was similar on days 13 and 21. Mean  $\pm$  S.E.M. of quadruplicate wells on day 11.

Figure 8 shows the inhibition of HIV-1 in human M/M by AZT in combination with mioflazine. Like DPM, mioflazine is an inhibitor of nucleoside transport. The experiment was performed as was the experiment for Figure 6. At the



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concentrations tested, mioflazine inhibited virus replication and potentiated the anti-HIV activity of AZT. MF: mioflazine. The pattern of effects was similar on days 13 and 21. Mean  $\pm$  S.E.M. of quadruplicate wells.

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DETAILED DESCRIPTION OF THE INVENTION

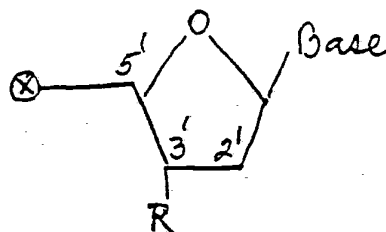
The above and various other objects and advantages of the present invention are achieved by a pharmaceutical composition, comprising an effective amount of an antiviral agent and an effective amount of a potentiating agent to  
10 inhibit viral replication in infected cells, wherein a combination of the antiviral and the potentiating agent produces greater viral inhibition than expected from the activity of each component alone, and pharmaceutically acceptable carrier. Retroviruses or diseases caused thereby,  
15 against which the composition and the methods of the present invention can be used, include HIV-1, HIV-2, HTLV-I, HTLV-II and the like. Of course, the composition may include such additives as sterilants, adjuvants, non-toxic sterile buffers and the like, as are commonly used in such preparations and  
20 which are well known to one of ordinary skill in the art.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials  
25 similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned hereunder are incorporated herein by reference. Unless mentioned otherwise, the techniques

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employed herein are standard methodologies well known to one of ordinary skill in the art.

The term "chain terminating drug" as used herein refers to a compound or a precursor thereof, which, when inserted into an elongating nucleic acid chain, terminates the growth of the chain. These drugs may act by additional mechanisms as well, for example, by competing for the enzymes active in phosphorylation of physiological nucleosides and nucleotides. These compounds usually, but not always, have the general  
10 formula:



15 wherein the "Base" is selected from the group consisting of adenine, guanine, cytosine, thymine and analogues, derivatives or salts thereof which can be incorporated into growing DNA chains, which are metabolically processed to yield molecules that can be so incorporated or which work as  
20 functionally equivalent analogues of such molecules;

R is H, azido or another group which does not allow the 3'-attachment of the next nucleotide, thereby preventing chain elongation of retroviral DNA under the influence of reverse transcriptase; and

25 X represents H, mono, di, tri or other phosphates and analogs thereof.

The ribose-based dideoxy-sugar is replaced by a different organic moiety in some of the functionally similar

chain-terminating compounds, for example in adenylline, cytallene, and PMEA.

It is preferred that the compound of the general formula (1) be AZT or another 2',3'-dideoxy nucleoside or nucleotide. Representative examples of chain-terminator nucleosides are as follows: azidothymidine, 2', 3'-dideoxycytidine, 2', 3'-dideoxyadenosine, 2', 3'-dideoxythymidine, 2', 3'-dideoxyguanosine, 2', 3'-dideoxyinosine and the like. The dideoxynucleotides include mono-, di- and triphosphates of the dideoxynucleosides. Also included are similar compounds in which one (or more) phosphate groups is (are) replaced with a phosphorothioate, methylphosphonate or other analogous group. Some such compounds (e.g., the phosphorothioates and methylphosphonates) are metabolically more stable than the parent nucleotides.

The term "potentiating agent" refers to dipyridamole (DPM) and to similar agents with overlapping mechanisms of action, including nitrobenzylthioinosine (NBTI); dilazep; lidoflazine; hexabendine; 7-bromo-1,5-dihydro-3, 6-dimethylimidazol-(2, 1, 6) quinazolin-2 (3H)-one, mioflazine and the like. These agents are inhibitors of nucleoside and nucleobase transport. Overlapping in effect on cyclic AMP phosphodiesterase are papaverine and mopidamol. Preferred among these compounds is DPM.

The term "synergistic" or "potentiated" effect, as used herein, means that a combination of an antiviral agent and a potentiating agent produces greater antiviral effect than expected from the activity of each component alone.

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Various cell types such as monocytes, T-lymphocytes, T-lymphocyte tumors, macrophages, and the like could be used as the host cell for tests of viral infection or replication. The viral activity, particularly of the HIV can be tested by p24 production, reverse transcriptase activity, trans-activator function, or other marker antigen production. Use of a particular cell type or method of measuring viral activity is not the critical part of the invention and either in vitro or in vivo systems can be used for the testing of viral activity. The significant part of the invention is the discovery that a potentiated antiviral effect is produced by chain terminators and by the  $\alpha$ -glucosidase inhibitor castanospermine when administered with a nontoxic dosage of DPM or one of the other potentiating agents mentioned above. It is also significant that DPM does not potentiate the activity of AZT against human bone marrow cells in vitro. Bone marrow suppression is known to be the dose-limiting toxicity of AZT in humans. It may further be noted that the chain terminator and/or potentiating components can be administered in combination either as such or carried by liposomes or other delivery vehicles. Methods of preparing liposomes are well known to one of ordinary skill in the art and such techniques are not a critical part of the present invention. The potentiating agent can be administered as such or in a polymeric form.

Preferred materials and methods are now described, it being understood that these materials and methods are only illustrative and not limiting.

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MATERIALS AND METHODS

Materials. Dipyridamole [2,6-bis-diethanolamino-4,8-di-piperidinopyrimido-(5,4d)-pyrimidine] was obtained from Sigma (St. Louis, MO). In most experiments it was dissolved in ethanol, and a 0.1 M stock solution was diluted to the test concentrations (ETOH <0.01%). Dilutions from 0.1 M DPM stock in 0.1 N HCl (pH readjusted to about 3) did not detectably affect the final pH of the medium and gave essentially identical results. AZT was prepared by Ash Stevens (Lot #HLR 0221) and obtained through the Developmental Therapeutics Branch, AIDS Program, NIAID. ddC and ddCTP were obtained from Moravek Biochemicals (Brea, CA). <sup>3</sup>H-ddCTP was repurified by HPLC as described below. Nitrobenzylthioinosine (NBTI; also abbreviated NBMPR) was obtained from Aldrich, Milwaukee, Wisconsin, and papaverine was obtained from Sigma, St. Louis, Missouri.

Adherence-Purified Monocyte/Macrophages. The cells were prepared and infected with HIV-1 NIH/USA/1985/HTLV-III<sub>BAL</sub>, an HIV-1 isolate recovered from and propagated in M/M, as described by Gartner et al (1986, Science 233, 215-219). In brief, peripheral blood mononuclear leukocytes were obtained from leukapheresed healthy, HIV-1 antibody-negative donors by Ficoll-Hypaque-separation. Purification by overnight adherence yielded M/M populations with >95% non-specific esterase-positive cells. The purified cells were cryopreserved according to standard procedures in RPMI 1640 containing 20% heat-inactivated fetal calf serum (FCS) and 10% (final concentration) of DMSO. Prior to infection, the

cells were thawed, washed to remove DMSO, and suspended in RPMI 1640 supplemented with 10% pooled human serum, 20% FCS and antibiotics. Infection was done by exposing  $10^7$  pelleted cells for 45-60 min at  $37^\circ\text{C}$  to 1 ml of virus inoculum containing  $0.5-1.0 \times 10^8$  cpm RT activity. After infection, the cells were washed and seeded in 96-well Costar microtiter plates ( $8 \times 10^4$  cells/well) in RPMI 1640 culture medium supplemented with 20% heat-inactivated FCS and antibiotics.

Elutriated Monocyte/Macrophages. Peripheral blood monocytes were obtained by standard counterflow centrifugal elutriation (Wahl et al, 1984, Cell Immunol. 85, 373-383; Wahl et al, 1984, Cell Immunol. 85, 384-395), with the exception that pyrogen-free phosphate buffered saline was used in the elutriation procedure. The freshly prepared M/M were infected as described above. They were then washed and seeded in 96-well Costar plates at  $2.5 \times 10^5$  cells/well in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% FCS, 2 mM glutamine, and antibiotics. After plating and washing, elutriated monocyte cultures are found to be >99% positive for Leu M3 and non-specific esterase.

Treatment of Cells and Measurement of HIV Replication. On day 1 following infection, the cultured cells were washed one time with complete medium, and the drugs under study were added to the microtiter wells. The cultures were re-fed at 3- to 4-day intervals by substituting fresh medium (with or without drugs, as indicated) for half of the cell supernatant (100  $\mu\text{l}$ ). Supernatant samples were analyzed for HIV-1 p24 antigen using an enzyme-linked immunosorbent assay (ELISA) kit

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(Dupont Co., Wilmington, DE; Cat. #NEK 041). Experiments with NBTI, papaverine and mioflazine were performed similarly.

ddCTP Stability and Cellular Uptake. ddCTP was incubated with uninfected, elutriated M/M ( $5 \times 10^6$  cells/well) under conditions mimicking those of the viral studies. Samples of the supernatants were centrifuged through Centrifree filters (Amicon Co., Danvers, MA) and analyzed for ddCTP metabolites by HPLC, using a VYDAC 303NT405 nucleotide column (Separations Group, Hesperia, CA). The column was eluted with a 0-50% linear gradient of 0.035 mM ammonium formate pH 4.65 and 0.5 M sodium phosphate pH 2.8 (formed over 10 min). Cellular uptake of ddCTP was assessed under similar conditions by incubating 1  $\mu\text{M}$   $^3\text{H}$ -labeled ddCTP with uninfected, elutriated M/M cultures ( $5 \times 10^6$  cells/well), followed by thorough washing of the cells (4 times with ice-cold saline), detachment from the plates (with 0.5% Triton X-100), and measurement of cell-associated radioactivity.

Accumulation and phosphorylation of  $^3\text{H}$ -thymidine and  $^3\text{H}$ -AZT in M/M. Elutriated monocytes were seeded in 6-well microtiter plates (Costar) at  $5 \times 10^6$  cells/well. Uptake experiments were started on day 2 after preparation of the cells. The medium was replaced with 1 ml DMEM-10% FCS containing either  $^3\text{H}$ -thymidine (Dupont) or  $^3\text{H}$ -AZT (Moravek) plus 0.5  $\mu\text{M}$  unlabeled AZT. Incubation was then continued at 37°C in a  $\text{CO}_2$  incubator. At various time points, the supernatants were removed and the wells quickly washed 3 times with iced DMEM containing 20  $\mu\text{M}$  DPM (to block further transport). 0.9 ml of 0.5% Triton X-100 was then added to

each well, and the plates were shaken for 30 minutes at room temperature. The contents were transferred to 1.5 ml polypropylene centrifuge tube (Eppendorf), and 0.1 ml of 50% trichloroacetic acid was added. The tubes were then spun in  
5 a Microfuge at maximum speed for 10 minutes. The supernatants were collected, neutralized with 1.2 ml 20% tri-n-octylamine (in trichlorotrifluoroethane), and vortex-mixed. After this extraction procedure, the aqueous phase was transferred to another tube and lyophilized. The sample was  
10 then redissolved in 120  $\mu$ l of distilled water and vortex-mixed. Samples (100  $\mu$ l) were assayed by HPLC using the VYDAC column and elution scheme described above. Fifty fractions from each run were counted in scintillation vials. Generally, duplicate wells were harvested and processed  
15 separately for each time point.

Superoxide Production.  $O_2^-$  production in uninfected, elutriated monocyte/macrophage cultures was assessed by measuring  $O_2^-$ -dependent reduction of cytochrome c, as described by Wahl et al (1987, J. Immunol. 139, 1342-1347).  
20 On day 14, the medium in each of quadruplicate wells was replaced by 100  $\mu$ l of Earle's balanced salt solution containing 160  $\mu$ M ferricytochrome c. The cells were then incubated for 3 h at 37°C, and  $O_2^-$  production was determined by measuring  $A_{520}$ . Values were expressed as nmole  $O_2^-$ /well  
25 calculated from the molar extinction coefficient of reduced cytochrome c, corrected for the presence of oxidized protein and the 3 mm vertical light path through the well volume. Baseline  $O_2^-$  production was assessed by adding superoxide dismutase (final concentration 600 ug/ml) to the wells to



destroy any  $O_2^-$  present; phorbol ester-stimulated production was assessed by adding phorbol myristate acetate (final concentration 10 ng/ml); spontaneous production was determined with no stimulus added.

5 Bone Marrow Cell Toxicity. Standard procedures were used for the collection of bone marrow specimens from healthy volunteers and for the granulocyte/monocyte colony formation (CFU<sub>GM</sub>) assay as described by Fine et al (1987, Clin. Oncol. 5, 489-495). Briefly, the cells were separated on Ficoll-10 Hypaque and suspended in McCoy's 5A medium supplemented with 20% heat-inactivated FCS, 2 mM glutamine, and 15% (v/v) HIV-negative colony stimulating factor (CSF) derived from supernatants of a human myelogenous leukemia line (P-38). The cells were plated by the soft-agar method (Pike et al, 15 1970, J. Cell Physiol. 76, 77-84) at a final concentration of 0.3% agar with 20% FCS and 15% CSF. After 9-12 days of incubation, colonies of >40 cells were counted. Approximately 100 colonies formed from the  $2 \times 10^5$  mononuclear cells plated. The cells were stained by the method of Kubota 20 et al (1980, Exp. Hematol. 8, 339-344) for morphological examination. For each donor the effects of AZT and DPM alone, as well as 2 to 10 different combinations with AZT/DPM ratios ranging from 0.16 to 20, were tested.

Anti-HIV efficacy and cell cytotoxicity in T-lymphoblastoid  
25 cell lines. Cells of the CD4+ CEM-SS and MT-2 T-lymphoblastoid lines were grown in RPMI-1640 containing 10% FCS. The cells were then infected either with free HIV-1(III<sub>b</sub>) stock or with HIV-1(RF)-infected H9 cells and seeded in 96-well microtiter plates. After 7 days in culture, 50 ml of

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solution containing the tetrazolium salt "XTT" (1 mg/ml) and the electron acceptor phenazine methosulfate (0.01-0.02 mM) was added to each well. Uninfected cells or cells which are protected by drugs and have continued to proliferate produce a soluble orange formazan whose O.D. can be read at 450 nm. The data, correlated with cell health, are presented as % of untreated control formazan = (Test O.D.x100)/(Cell control O.D.). It should be noted that there is a correlation between this tetrazolium dye test and other assays such as p24 antigen capture, syncytium formation and trypan blue exclusion.

PHA-stimulated human T-lymphocytes. Mononuclear cells from a healthy volunteer were grown in the presence of phytohemagglutinin (PHA) (5µg/ml) for 2 days and then stimulated with IL2 in RPMI 1640 with 15% FCS, 1% L-glutamine, and 0.1% gentamycin. After 3 days the cells were infected with various titers of HIV-1(III<sub>2</sub>) for 90 minutes at 37°C. After washing, cells were reseeded in microtiter wells at  $3 \times 10^5$  cells/0.5ml. Drugs were added immediately after plating, and their concentrations were thereafter kept constant at each re-feeding. The cells were re-fed every 2-3 days by replacement of half of the supernatant. Ten days after infection, p24 was measured by standard ELISA, and the cells were examined for trypan blue exclusion.

Data Analysis. The 50% inhibitory doses (ID<sub>50</sub>) for the tested drugs were calculated by plotting  $\log[f_{50}/(1-f_{50})]$  versus  $\log D$  (median-effect plot), where  $f_{50}$  is the fraction affected (i.e., percent inhibition /100), and  $D$  is the drug dose [Chou et al, 1984, Adv. Enzyme Regul. 22, 27-55; Chou et

al., 1987) in New Avenues in Developmental Cancer  
Chemotherapy, eds. Harrap, K. R. & Connors, T.A. (Bristol  
Myers Symposium Series, Academic Press, N.Y.), pp. 37-64].  
The plots, which linearize the dose-effect relationship and  
5 give  $ID_{50}$  as the intercept at the x-axis, were constructed  
with the use of a microcomputer program [Chou et al., 1986)  
Dose-Effect Analysis with Microcomputers: A Computer  
Software for Apple II or IBM PC (Elsevier Biosoft,  
Cambridge, England)]. Drug interactions in the  $CFU_{GM}$  assay  
10 were analyzed by calculating the "combination index (C.I.)",  
a parameter that derives from comparison of isoeffective  
doses of the drugs when applied alone and when applied in  
combination (Chou et al., supra; Chou et al., supra). The  
isoeffective doses of AZT alone and DPM alone at different  
15 fractional effect levels between 0.1 and 0.9 were obtained  
from the median effect plots.

#### RESULTS

Antiviral Effects in human monocyte/macrophages. As shown in  
Fig. 1A, AZT effectively inhibited viral replication in  
20 cryopreserved, adherence-purified cells. Dipyridamole in the  
0.08-10  $\mu$ M range had little effect by itself but unexpectedly  
it greatly potentiated the antiviral efficacy of AZT. In the  
presence of 2  $\mu$ M DPM, for example, the  $ID_{50}$  and  $ID_{90}$  levels  
of AZT (Table 1) were decreased by more than 5-fold and 10-  
25 fold, respectively (i.e., to 18 and 8% of the values for AZT  
alone). Fig. 1B shows that the potentiating influence of  
DPM, in relative terms, was even more pronounced in  
elutriated M/M cultures. In the presence of 2 or 10  $\mu$ M DPM,  
p24 expression was near baseline levels at each AZT

concentration including the lowest, 1.6 nM. This degree of inhibition was achieved only at 1  $\mu$ M level when AZT was used alone. Dipyridamole also unexpectedly potentiated the anti-HIV effects of ddC and 2',3'-dideoxycytidine triphosphate (ddCTP). The  $ID_{50}$  values for these drugs decreased at least 5-fold in the presence of  $\geq 2$   $\mu$ M DPM (Fig. 1C-D). In the elutriated M/M system, DPM by itself appeared to be inhibitory, causing a significant and unanticipated decrease in p24 expression (Fig. 2). This effect of DPM alone showed no dependence on concentration in the range from 2 to 20  $\mu$ M.

Stability and Cellular Uptake of ddCTP. Since nucleotides do not readily enter cells, ddCTP itself was expected to be relatively ineffective against intracellular viral RT. However, it was found by HPLC analysis that ddCTP decomposes through ddC monophosphate to ddC with a  $t_{1/2}$  of about 12-14 h in the culture medium. This time course corresponded to that of  $^3H$  accumulation in cells incubated with  $^3H$ -ddCTP. Taken together, these observations indicate that the conversion of ddCTP to ddC may be rate-limiting to uptake and that the predominant species entering the cells was probably ddC.

Toxicity for monocyte/macrophages. Cell counting in the culture wells with an inverted microscope showed no consistent differences between control and treatment groups for uninfected cells two weeks after treatment with AZT and/or DPM. Cell viability of infected M/M cells, as evaluated by trypan blue exclusion, was >95% in each of the groups.

Superoxide production. Table 2 shows the  $O_2^-$  production by uninfected M/M treated with AZT and/or DPM. The principal

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significance of these data is that they showed no discernible DPM toxicity with or without AZT, at the concentrations applied. However, some numerically small but statistically significant trends were seen. Spontaneous superoxide levels decreased with increasing AZT ( $p < 0.001$ ; two tail), whereas both the spontaneous ( $p = 0.013$ ) and PMA-stimulated superoxide values increased ( $p < 0.001$ ) with increasing DPM. The relationship of these trends to the observed antiviral activity remains to be determined.

10 Bone marrow toxicity. To assess the bone marrow toxicity of various combinations of AZT and DPM, CFU<sub>GM</sub> assays were performed on bone marrow samples from 7 healthy donors. The mean  $ID_{50}$  of AZT, calculated from pooled values in the effective dynamic range of the assay (5-95% inhibition), was 15  $0.6 \mu M \pm 0.1 \mu M$  (S.E.M.;  $n = 21$ ). DPM at high concentrations also inhibited colony formation ( $ID_{50} = 10.0 \pm 4.5 \mu M$ ;  $n = 19$ ). As shown in Fig. 3A, the C.I. values obtained from 6 donors over a wide range of inhibition levels clustered near 1 in the "mutually exclusive" model, suggesting essentially 20 additive toxicities. In the "mutually non-exclusive" model the C.I. values are predominantly  $> 1$  in Fig. 3B suggesting an unexpected trend toward antagonism of the toxicities, rather than toward synergy. The relative frequencies of pure granulocyte colonies, pure monocyte colonies, and mixed 25 granulocyte-monocyte colonies were not affected by the treatments. These results together with the anti-viral studies suggest that if AZT is combined with  $\leq 2 \mu M$  DPM, identical antiviral effect with less marrow cell toxicity than with AZT alone can be obtained and that the in vitro

therapeutic index is thus unexpectedly increased.

In summary, the data presented herein clearly demonstrate that DPM has an intrinsic effect against HIV and that it significantly potentiates the antiviral action of AZT, ddC and ddCTP in human monocyte/macrophages. Of particular relevance from a therapeutic standpoint was the finding that in the AZT/DPM combination, DPM did not potentiate the marrow toxicity of AZT, and the in vitro therapeutic index was therefore unexpectedly increased. It was also found, surprisingly, that DPM potentiated the anti-HIV activity of ddC, which enters cells primarily via DPM-sensitive nucleoside transport (Zimmerman et al. 1987, J. Biol. Chem. 262, 5748-5754). However, ddC is more lipophilic than dC, the physiological nucleoside, because it has one less hydroxyl group. Therefore, a greater inhibitory effect of DPM on the transport of dC might explain the potentiation of ddC activity.

Antiviral effects in human T-lymphoblastoid cell lines.

Results shown in Figure 4A indicate a beneficial effect of DPM on the AZT treatment of T-lymphoblastoid cell line (CEM-SS); DPM diminished the toxicity of AZT in these cells while increasing or not affecting its activity against HIV-1 (HTLV-III<sub>RF</sub>). Consequently, the in vitro therapeutic index increased in an unanticipated manner. MT-2 cells, another T-lymphoblastoid cell line, were more sensitive to toxic effects of both AZT and DPM. Results presented in Figure 4B also show a surprising potentiation of castanospermine's anti-HIV activity in CEM-SS cells at high concentrations of

DPM.

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Combination chemotherapy of HIV-1 infection in PHA-stimulated human T-cells. Table 3 shows the results of an experiment on PHA-stimulated T-lymphocytes. The data indicate striking 5 potentiation of AZT activity by DPM concentrations as unexpectedly low as 0.074  $\mu\text{M}$ . Cell counts and trypan blue exclusion showed major drug toxicity to the cells only at the highest dose (10  $\mu\text{M}$ ) of AZT. These results are important because they indicate that DPM can potentiate the antiviral 10 effect of a chain-terminator drug in T-lymphoid cells as well as in M/M. These are the two most important cell lineages in HIV infections.

HPLC studies to test "differential transport inhibition".

Figure 5 shows results obtained in experiments for 15  $^3\text{H}$ -thymidine and for  $^3\text{H}$ -AZT. DPM greatly decreased the appearance of phosphorylated thymidine species, whereas it had only a minor effect on the appearance of phosphorylated AZT. Two independent experiments showed qualitatively similar inhibitory effects of DPM on the intracellular 20 appearance of phosphorylated  $^3\text{H}$ -thymidine. In three separate experiments, DPM increased or did not change the amount of phosphorylated  $^3\text{H}$ -AZT in the cells.

#### DPM Analogues

Certain analogues of DPM were also evaluated for their 25 intrinsic activity against HIV and for their ability to potentiate the activity of the chain-terminating antiviral agent AZT. As shown in Figure 6, the nucleoside transport inhibitor NBTI had an intrinsic inhibitory effect on HIV-1 replication in human M/M and also potentiated the inhibitory

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effect of AZT. As indicated in Figure 7, papaverine gave similar results. As shown in Figure 8, mioflazine also had an intrinsic inhibitory effect and potentiated the inhibitory effect of AZT. Mioflazine, like DPM, inhibits nucleoside transport, but it has the possible advantage of crossing the blood-brain barrier (Deckert et al, 1988, Life Sciences 42, 1331-1345; Wauquier et al, 1987, Psychopharm. 91, 434-439) more readily than does DPM. Thus, unanticipated and surprising antiviral effects were obtained with these DPM analogues alone and in combination with AZT.

Without being bound to any theory, it is postulated that the mechanism by which DPM or its analogues potentiate or act synergistically is most likely due to the well-established activities of DPM in blocking nucleoside transport and/or inhibiting cyclic AMP phosphodiesterase activity.

Plasma concentrations of DPM in excess of 10  $\mu\text{M}$  can be sustained in humans. It is important to note, therefore, that the studies described in Example 1 demonstrated potentiation of dideoxynucleoside-mediated HIV inhibition at DPM concentrations of 2  $\mu\text{M}$  and in some experiments much less (in 10%-20% fetal calf serum). DPM is largely protein-bound in blood. Hence, the free drug level at a given overall concentration is expected to be higher in tissue culture experiments than in vivo. The other potentiating agents are employed similarly to DPM.

The present invention now provides a chemotherapeutic method of treating AIDS or other viral diseases. The method comprises administering to a host afflicted with viral



infection, including AIDS-virus infection, an effective amount of the drug-combination of the present invention to inhibit replication of the disease-causing virus. The drug-combination can be administered by any suitable route such as oral, parenteral, intrathecal or the like and administered as often per day as tolerated. Uninfected individuals or those exposed to the virus can be treated in like manner to prevent infection. Antiviral agents, such as AZT, ddC and the like, can be administered in combination or in alternating schedule along with DPM and/or its analogues.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

**Table 1** Levels of AZT eliciting 50% and 95% inhibition of HIV-1 p24 antigen expression in adherence-purified human M/M cultures, in the presence and absence of DPM. ID<sub>50</sub> and ID<sub>95</sub> values were calculated from the equations of the median-effect plots (see Methods for data analysis). *r*, linear regression coefficients for the plots. The percentages in brackets relate the ID<sub>50</sub> and ID<sub>95</sub> values to those of AZT alone.

Treatment	AZT ( $\mu$ M)		<i>r</i>
	ID <sub>50</sub>	ID <sub>95</sub>	
AZT alone	0.63 (100%)	5.99 (100%)	0.99
AZT + 0.08 $\mu$ M DPM	0.30 (47%)	2.55 (43%)	0.99
AZT + 0.4 $\mu$ M DPM	0.23 (37%)	0.74 (12%)	0.94
AZT + 2 $\mu$ M DPM	0.11 (18%)	0.50 (8%)	0.96
AZT + 10 $\mu$ M DPM	0.11 (18%)	0.72 (12%)	0.97

**Table 2.** *The effect of AZT and DPM on superoxide ( $O_2^-$ ) production in elutriated human MM cultures.* Superoxide production (on day 14 of culturing) is taken as a functional index of cell health. Experimental details were the same as in Fig. 1B-D, except that the cells were not infected with HIV-1. Values are expressed as nmole  $O_2^-$ /well. Means of quadruplicate wells  $\pm$ S.E.M. The data were analyzed by the method of Jonckheere (30) for non-parametric testing of trends of ordered alternatives.

AZT ( $\mu$ M)		Superoxide production (nmole/well)		
		0 $\mu$ M DPM	2 $\mu$ M DPM	10 $\mu$ M DPM
0	Spontaneous	c (100%) <sup>a</sup>	0.53 $\pm$ 0.11 (100%)	0.73 $\pm$ 0.03 (138%)
	PMA	c (100%) <sup>b</sup>	3.04 $\pm$ 0.16 (109%)	3.15 $\pm$ 0.16 (113%)
0.063	Spontaneous	0.39 $\pm$ 0.08 (89%)	0.32 $\pm$ 0.01 (97%)	0.29 $\pm$ 0.02 (100%)
	PMA	1.58 $\pm$ 0.36 (79%)	1.73 $\pm$ 0.16 (127%)	2.00 $\pm$ 0.30 (157%)
0.25	Spontaneous	0.32 $\pm$ 0.05 (73%)	0.31 $\pm$ 0.02 (94%)	0.28 $\pm$ 0.01 (97%)
	PMA	1.95 $\pm$ 0.27 (96%)	1.72 $\pm$ 0.33 (126%)	1.80 $\pm$ 0.26 (141%)
1.0	Spontaneous	0.36 $\pm$ 0.05 (82%)	0.35 $\pm$ 0.04 (66%)	0.23 $\pm$ 0.02 (79%)
	PMA	1.69 $\pm$ 0.14 (85%)	4.04 $\pm$ 0.30 (145%)	1.64 $\pm$ 0.20 (129%)

a. Percentage relative to spontaneous, untreated controls (see note c) on the same microtiter plate.

b. Percentage relative to PMA-stimulated, untreated controls (see note c) on the same microtiter plate.

c. Mean and S.E.M. for untreated control wells (spontaneous; PMA-stimulated) on the different plates were: (0.44 $\pm$ 0.07; 2.00 $\pm$ 0.32), (0.53 $\pm$ 0.12; 2.78 $\pm$ 0.20), (0.33 $\pm$ 0.01; 1.36 $\pm$ 0.23), and (0.29 $\pm$ 0.02; 1.27 $\pm$ 0.25).

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**Table 3. Potentiation by DPM of AZT activity against HIV-1 replication in PHA-stimulated human T-cells. Values in table are mean p24 levels (pg/ml) in the supernatants of duplicate wells.**

AZT ( $\mu$ M)	DPM ( $\mu$ M)					
	0	0.074	0.222	0.667	2.0	6.0
0.0	213	344	341	341	219	162
0.156	313	93	8	8	0	0
0.625	125	3	0	0	0	0
2.5	17	0	0	0	0	0
10.0	7	0	0	0	0	0

\*0\* means below threshold for reliable detection (less than twice background)

WHAT IS CLAIMED IS:

1. A pharmaceutical composition, comprising an effective amount of an antiviral drug and a potentiating agent to inhibit viral replication in infected cells, wherein a combination of the antiviral drug and the potentiating agent produces greater antiviral effect than expected from the activity of each component alone, and pharmaceutically acceptable carrier.
2. The composition of claim 1 wherein said antiviral drug is a chain terminator.
3. The composition of claim 1 wherein the antiviral drug, the potentiating agent or a combination thereof is encapsulated in liposomes.
4. The composition of claim 2 wherein said chain terminating drug is a nucleoside or a mono-, di- or tri-phosphorylated nucleoside.
5. The composition of claim 4 wherein said chain-terminating drug is a phosphorylated or unphosphorylated nucleoside analogue without ribose-based dideoxy-sugar.
6. The composition of claim 4 wherein the mono-, di-, or tri-phosphate moiety is replaced by phosphonate or phosphorothioate.
7. The composition of claim 4 wherein the chain-terminator is selected from the group consisting of azidothymidine, dideoxycytidine, dideoxyadenosine, dideoxythymidine, dideoxyguanosine, dideoxyinosine or phosphorylated derivatives and mixtures thereof.
8. The composition of claim 7 wherein said chain terminator is dideoxycytidine or dideoxycytidine triphosphate.

9. The composition of claim 7 wherein said chain terminator is azidothymidine.
10. The composition of claim 1 wherein said antiviral drug is a glucosidase inhibitor plant alkaloid or a derivative or analogue thereof selected from the group consisting of castanospermine, dihydroxymethylhydroxypyrrolidone, deoxynojirimycin and mixture thereof.
11. The composition of claim 10 wherein said alkaloid is castanospermine.
12. The composition of claim 1 wherein said potentiating agent is selected from the group consisting of nitrobenzylthioinosine; dilazep; lidoflazine; hexobendine; mioflazine; 7-bromo-1,5-dihydro-3,6-dimethylimidazol-(2,1,6)quinazolin-2(3H)-one; or derivatives and analogues thereof.
13. The composition of claim 1 wherein said potentiating agent is selected from the group consisting of dipyridamole, mopidamol, papaverine and mixture, analogue or derivative thereof.
14. The composition of claim 13 wherein said potentiating agent is dipyridamole.
15. The composition of claim 1 wherein said chain terminating drug is AZT, ddC or ddCTP and said potentiating agent is DPM.
16. The composition of claim 1 wherein said chain-terminating drug is AZT and said potentiating agent is NBTI, papaverine or mioflazine.
17. A method of treating or preventing viral disease, comprising administering to normal host or a host

afflicted with viral infection, an effective amount of the composition of claim 1 to inhibit or prevent growth of disease-causing virus.

18. The method of claim 17 wherein said viral symptoms are associated with a retroviral agent selected from the group consisting of HTLV1, HTLV2, HIV-1 and HIV-2.
19. A pharmaceutical composition, comprising as a first agent an antiviral drug, and a second agent which decreases toxicity of the antiviral drug to the host without decreasing the effectiveness of the antiviral agent to the same extent.
20. A method of treating or preventing viral disease comprising administering to normal host or a host afflicted with viral infection, an effective amount of the composition of claim 19 to inhibit or prevent growth of disease-causing virus.

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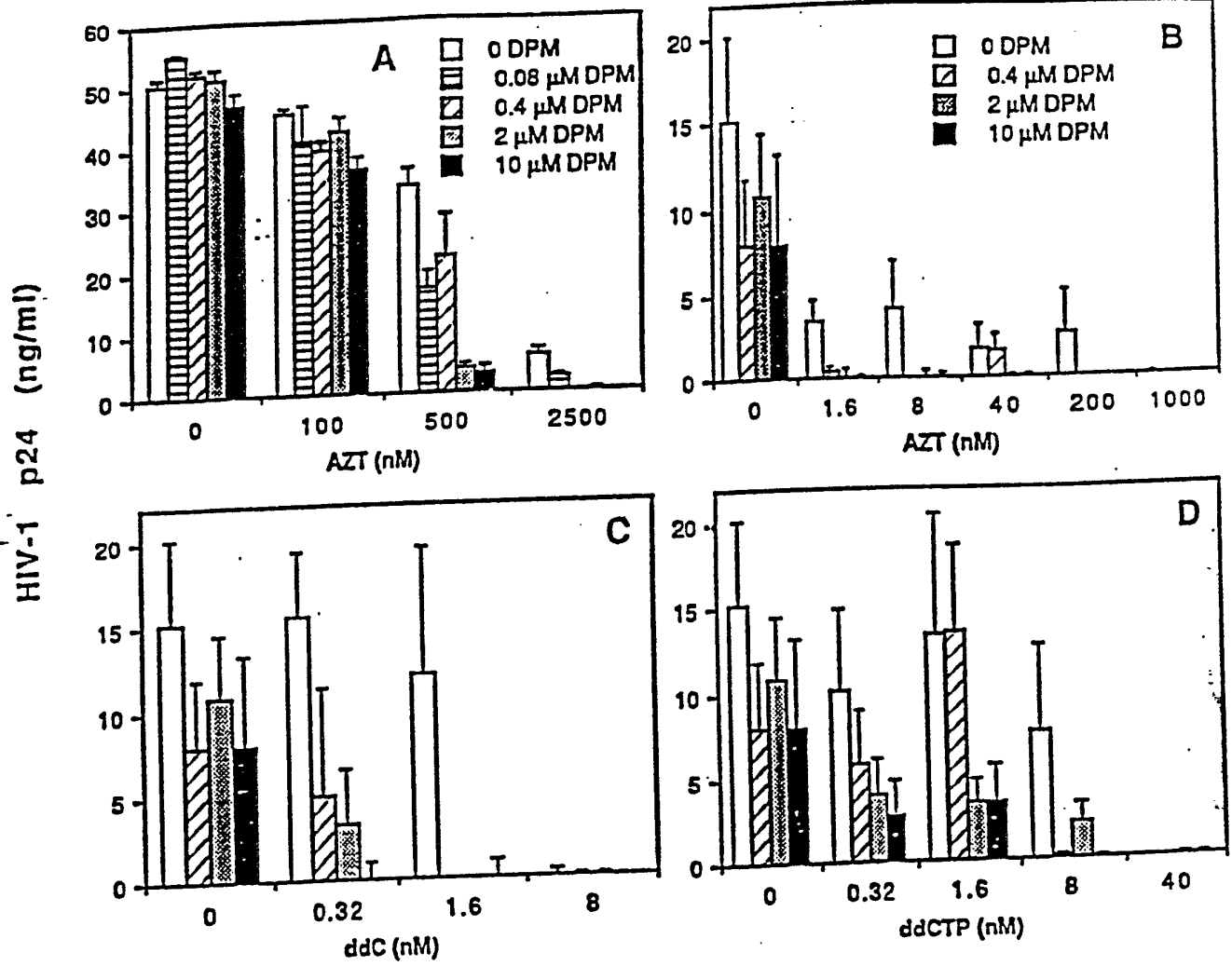


Figure 1.



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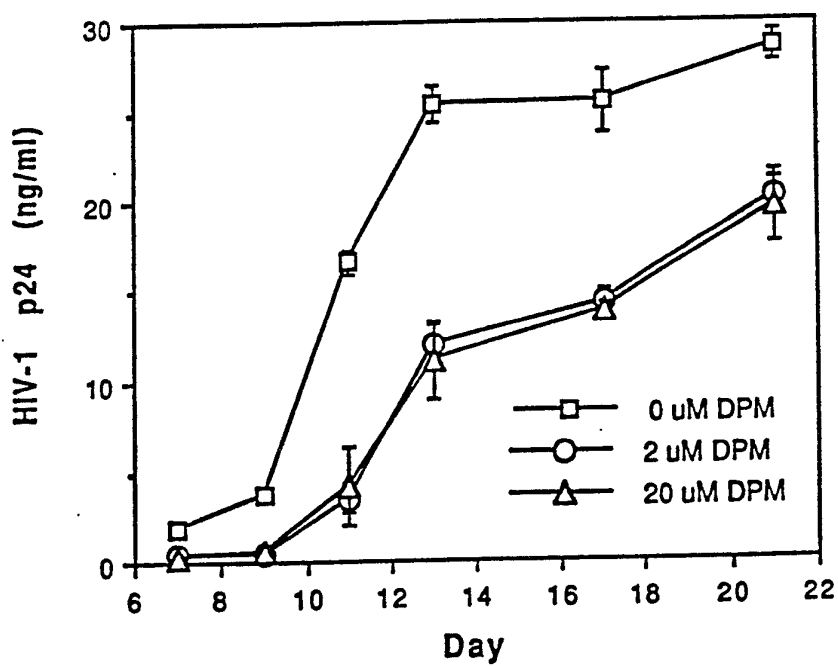


Figure 2

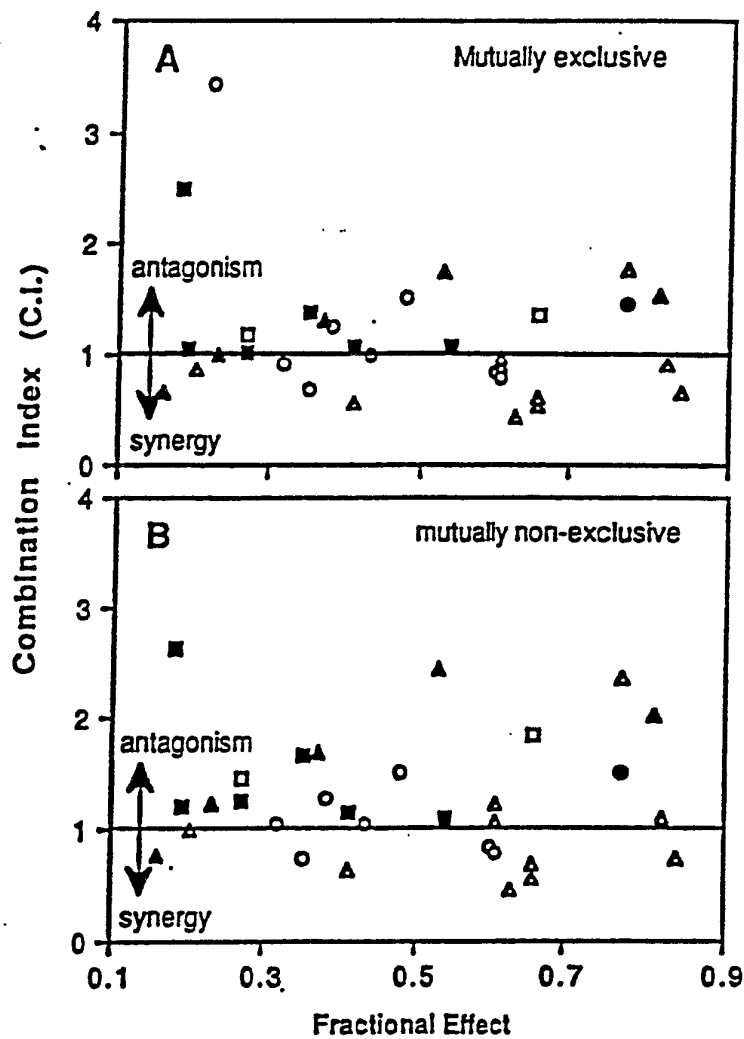


Fig. 3.

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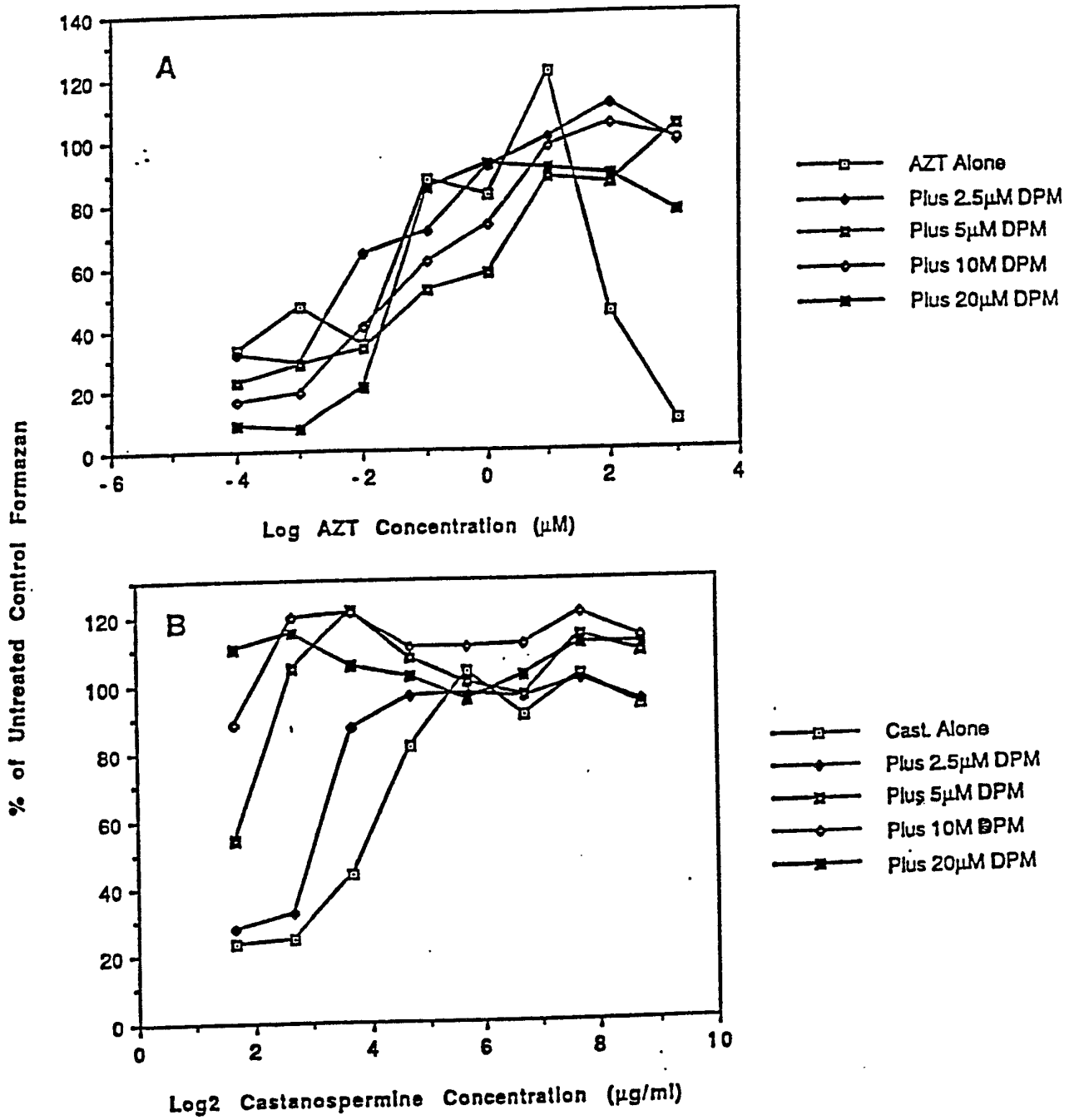


Figure 4

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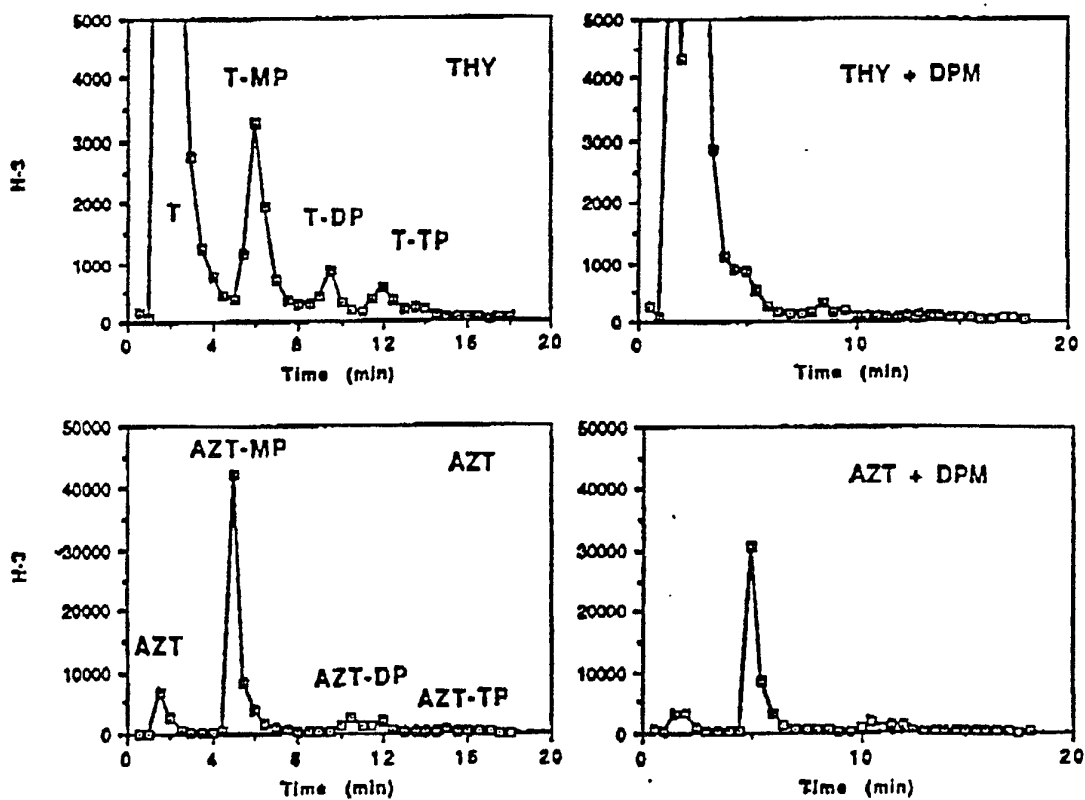


Figure 5

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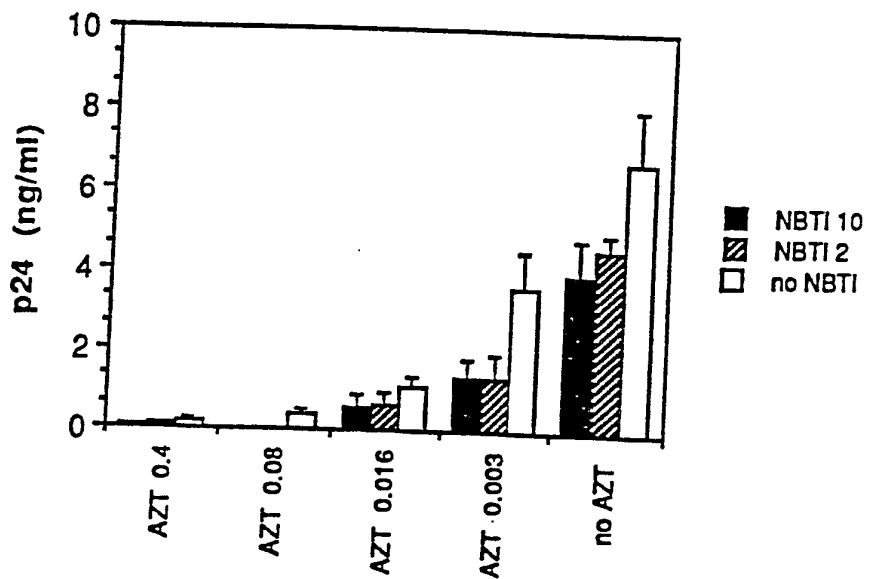


Fig. 6

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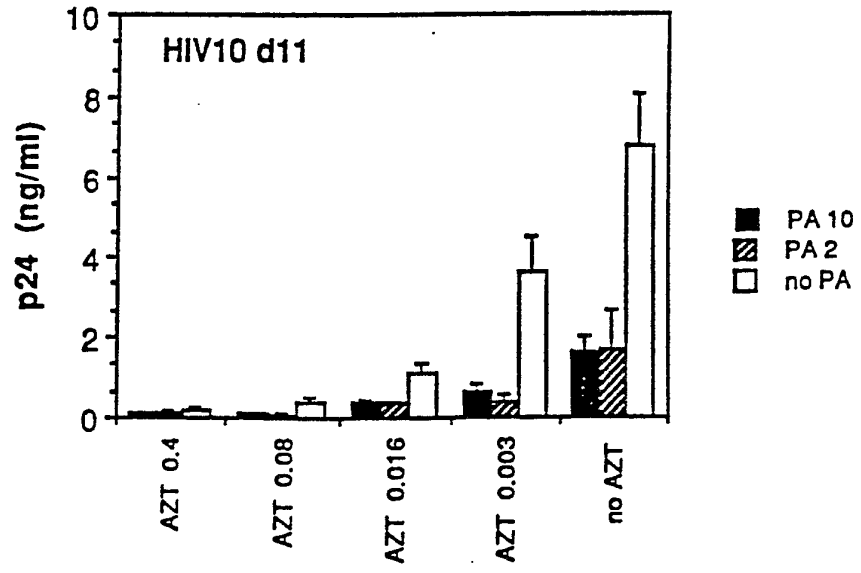


Fig. 7

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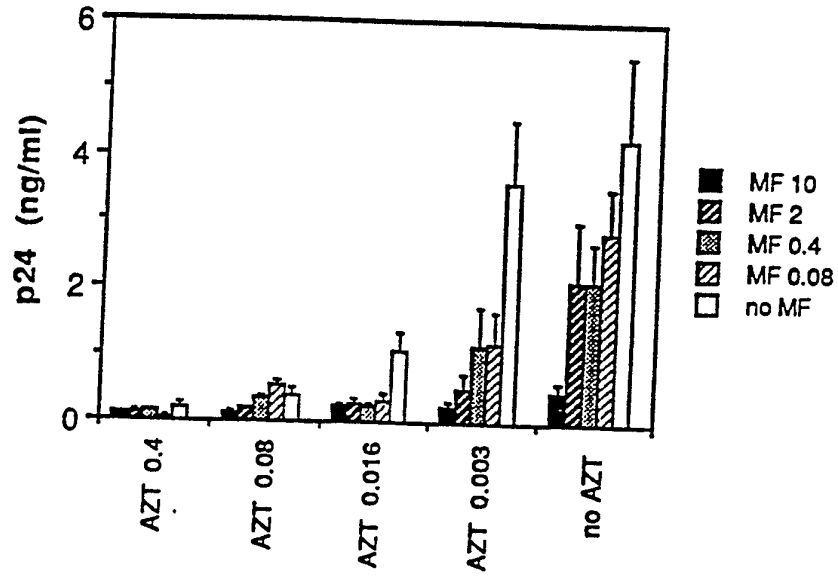


Fig. 8

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/02034

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> <small>(International Classification of Substances (IPC) and the 1969 6)</small>				
According to International Patent Classification (IPC) or to both National Classification and IPC Int. Cl. 4 A61K 9/66; 37/22; 45/05; B01J 13/02. U.S. Cl. 424/450; 428/402.2; 436/829; 514/885.				
<b>II. FIELDS SEARCHED</b>				
<small>Minimum Documentation Searched 7</small>				
<small>Classification System</small>	<small>Classification Symbols</small>			
U.S. Cl.	424/450; 428/402.2; 436/829; 514/885.			
<small>Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8</small>				
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT 9</b>				
<small>Category *</small>	<small>Citation of Document, 11 with indication, where appropriate, of the relevant passages 12</small>	<small>Relevant to Claim No. 13</small>		
Y	Biochemical Pharmacology, Vol. 35, NO. 13, pages 2065-2068, 1986, Cooney et al., "Initial Studies on the Cellular Pharmacology of 2 <sup>1</sup> , 3 <sup>1</sup> -Dideoxycytidine, an Inhibitor of HTL V-III Infectivity" (Note pages 2065 and sentence bridging pages 2067 and 2068.)	1-8,12-15, 17-20		
Y	Proc. Natl. Acad. Sci. USA, Vol. 83, pages 8333-8337, Nov. 1986, Furman et al., "Phosphorylation of 3'-azido-3'-deoxythymidine and Selective Interaction of the 5'-triphosphate with Human Immunodeficiency Virus Reverse Transcriptase" (Note abstract and page 8337.)	1-7,9, 12-20		
Y,P	US, A, 4,792,558 SUNKARA ET AL. 20 DECEMBER 1988 SEE COL. 1, LINES 30-55.	1-6,10-14, 17-20		
Y	US, A, 4,515,736 DEAMER 07 MAY 1985 SEE COL. 4, LINES 31 AND 32.	1-11,13-15, 17-20		
Y	US, A, 3,669,969 LUNN 13 JUNE 1972 SEE ABSTRACT; COL. 1, LINES 20-30; AND TABLES I-V.	1-12, 17-20		
Y,P	US, A, 4,751,224 AGARWAL ET AL. 14 JUNE 1988 SEE COL. 3, LINE 65 - COL. 4, LINE 12.	1-20		
Y,P	US, A, 4,824,675 WONG ET AL. 25 APRIL 1989 SEE COL. 17, LINES 26-56.	1-12, 16-20		
<table style="width: 100%; border: none;"> <tr> <td style="width: 50%; vertical-align: top; padding: 5px;"> <ul style="list-style-type: none"> <li>* Special categories of cited documents: 10</li> <li>"A" document defining the general state of the art which is not considered to be of particular relevance</li> <li>"E" earlier document but published on or after the international filing date</li> <li>"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)</li> <li>"O" document referring to an oral disclosure, use, exhibition or other means</li> <li>"P" document published prior to the international filing date but later than the priority date claimed</li> </ul> </td> <td style="width: 50%; vertical-align: top; padding: 5px;"> <ul style="list-style-type: none"> <li>"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</li> <li>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</li> <li>"Y" document of particular relevance: the claimed invention 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.</li> <li>"&amp;" document member of the same patent family</li> </ul> </td> </tr> </table>			<ul style="list-style-type: none"> <li>* Special categories of cited documents: 10</li> <li>"A" document defining the general state of the art which is not considered to be of particular relevance</li> <li>"E" earlier document but published on or after the international filing date</li> <li>"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)</li> <li>"O" document referring to an oral disclosure, use, exhibition or other means</li> <li>"P" document published prior to the international filing date but later than the priority date claimed</li> </ul>	<ul style="list-style-type: none"> <li>"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</li> <li>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</li> <li>"Y" document of particular relevance: the claimed invention 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.</li> <li>"&amp;" document member of the same patent family</li> </ul>
<ul style="list-style-type: none"> <li>* Special categories of cited documents: 10</li> <li>"A" document defining the general state of the art which is not considered to be of particular relevance</li> <li>"E" earlier document but published on or after the international filing date</li> <li>"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)</li> <li>"O" document referring to an oral disclosure, use, exhibition or other means</li> <li>"P" document published prior to the international filing date but later than the priority date claimed</li> </ul>	<ul style="list-style-type: none"> <li>"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</li> <li>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</li> <li>"Y" document of particular relevance: the claimed invention 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.</li> <li>"&amp;" document member of the same patent family</li> </ul>			
<b>IV. CERTIFICATION</b>				
<small>Date of the Actual Completion of the International Search</small>	<small>Date of Mailing of this International Search Report</small>			
19 JULY 1989	16 AUG 1989			
<small>International Searching Authority</small>	<small>Signature of Authorized Officer</small>			
ISA/US	Richard D. Lovering			



## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	US, A, 4,235,871 PAPAHADJOPOULOS ET AL. 25 NOVEMBER 1980 SEE COL. 3, LINES 28-40 AND COL. 6, LINES 31-35.	3
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V.  OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.  Claim numbers \_\_\_\_\_, because they relate to subject matter <sup>12</sup> not required to be searched by this Authority, namely:

2.  Claim numbers \_\_\_\_\_, 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 <sup>13</sup>, 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).

VI.  OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>2</sup>

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 claims:

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 invite payment of any additional fee.

## Remark on Protest

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