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<p><b>(21) International Application Number:</b> PCT/US96/09837</p> <p><b>(22) International Filing Date:</b> 6 June 1996 (06.06.96)</p> <p><b>(30) Priority Data:</b>  08/572,427                      14 December 1995 (14.12.95)      US</p> <p><b>(71) Applicant:</b> CERUS CORPORATION [US/US]; Suite 300, 2525 Stanwell Drive, Concord, CA 94520 (US).</p> <p><b>(72) Inventors:</b> WOLLOWITZ, Susan; 764 Beale Court, Walnut Creek, CA 94598 (US). ISAACS, Stephen, T.; 79 Loma Vista Drive, Orinda, CA 94563 (US). WIESEHAHN, Gary; 1170 Ninth Street #5, Alameda, CA 94501 (US).</p> <p><b>(74) Agents:</b> CARROLL, Peter, G. et al.; Cerus Corporation, Suite 300, 2525 Stanwell Drive, Concord, CA 94520 (US).</p>		<p><b>(81) Designated States:</b> AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b>  <i>With international search report.</i></p>
<p><b>(54) Title:</b> INACTIVATION OF NON-ENVELOPED VIRUS</p>		
<p><b>(57) Abstract</b></p> <p>Methods are described for inactivation of pathogens using new and known psoralens which do not compromise the function of blood products for transfusion. Specifically, the present application describes methods using aminopsoralens and ultraviolet light to inactivate human parvovirus B19 in blood products.</p>		

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## INACTIVATION OF NON-ENVELOPED VIRUS

5 The present application is a continuation in part of U.S. Application  
No. 08/337,987, filed November 14, 1994, which is a continuation in part of  
U.S. Application No. 08/212,113, filed March 11, 1994 which is a  
continuation in part of U.S. Patent No. 5,399,719, issued March 21, 1995, the  
entire contents of which is hereby incorporated by reference.

### 10 FIELD OF THE INVENTION

The present invention provides methods of using new and known  
compounds to inactivate non-enveloped viruses in health related products  
to be used in vivo and in vitro. The present invention particularly  
provides methods of inactivating human parvoviruses in blood products  
15 and blood products.

### BACKGROUND

Pathogen contamination within the blood supply remains an  
important medical problem throughout the world. Although improved  
20 testing methods for hepatitis B (HBV), hepatitis C (HCV), and Human  
Immunodeficiency Virus (HIV) have markedly reduced the incidence of  
transfusion associated diseases, the public is losing trust in the safety of the  
blood supply due to publicity of cases of transfusion related transmission of  
these viruses.

25 For example, the recent introduction of a blood test for HCV will  
reduce transmission of this virus; however, it has a sensitivity of only 67%  
for detection of probable infectious blood units. HCV is responsible for 90%  
of transfusion associated hepatitis. Melnick, J.L., abstracts of Virological  
Safety Aspects of Plasma, Cannes, November 3-6 (1992) (page 9). It is  
30 estimated that, with the test in place, the risk of infection is 1 out of 3300  
units transfused.

Similarly, while more sensitive serological assays are in place for  
HIV-1 and HBV, these agents can nonetheless be transmitted by  
seronegative blood donors. International Forum: Vox Sang 32:346 (1977).  
35 Ward, J.W., et al., N. Engl. J. Med., 318:473 (1988). Up to 10% of total  
transfusion-related hepatitis and 25% of severe icteric cases are due to the  
HBV transmitted by hepatitis B surface antigen (HBsAg) negative donors.  
To date, fifteen cases of transfusion-associated HIV infections have been

reported by the Center for Disease Control (CDC) among recipients of blood pre tested negative for antibody to HIV-1.

Furthermore, other viral, bacterial, and agents are not routinely tested for, and remain a potential threat to transfusion safety. Schmunis, G.A., *Transfusion* 31:547-557 (1992). For example, human parvovirus B19 (B19) is a widespread pathogen which is known to be transmitted by blood products. (Heegaard, ED and Hornsleth, A, "Parvovirus: The Expanding Spectrum of Disease", *Acta Pædiatr* 84:109-117 (1995)). B19 has been shown to be present in two of three untreated batches of clotting-factor concentrates, and in one of four to five solvent/detergent-treated batches. As a result, a sero-prevalence among hemophiliacs of up to 88-97% has been observed. Heegaard, *Acta Pædiatr* 84:111. The risk of transmission from single donor blood products is varied, generally in the range of 1 in 3-50,000. Some clinical manifestations of parvovirus include: erythema infectiosum, arthropathy, hydrops fetalis, aplastic crisis, chronic anemia, neurologic disease, and rheumatologic disease. Clearly, this and other viruses which are not commonly screened for still represent a significant health risk to the population receiving transfused blood products.

In addition, testing will not insure the safety of the blood supply against future unknown pathogens that may enter the donor population resulting in transfusion associated transmission before sensitive tests can be implemented.

Even if seroconversion tests were a sufficient screen, they may not be practical in application. For example, Cytomegalovirus (CMV - a herpes virus) and parvovirus B19 in humans are common. When they occur in healthy, immunocompetent adults, they nearly always result in asymptomatic seroconversion. Because such a large part of the population is seropositive, exclusion of positive units would result in substantial limitation of the blood supply.

An alternative approach to eliminate transmission of viral diseases through blood products is to develop a means to inactivate pathogens in transfusion products. Development of an effective technology to inactivate infectious pathogens in blood products offers the potential to improve the safety of the blood supply, and perhaps to slow the introduction of new tests, such as the recently introduced HIV-2 test, for low frequency pathogens. Ultimately, decontamination technology could significantly reduce the cost of blood products and increase the availability of scarce blood products.

To be useful, such an inactivation method i) must not adversely effect the function for which the blood product is transfused, ii) must thoroughly inactivate existing pathogens in the blood product, and iii) must not adversely effect the recipients of the blood product. Several methods  
5 have been reported for the inactivation or elimination of viral agents in erythrocyte-free blood products. However, most of these techniques are completely incompatible with maintenance of the function of platelets, an important blood product. Examples of these techniques are: heat  
(Hilfenhous, J., et al., *J. Biol. Std.* 70:589 (1987)), solvent/detergent treatment  
10 (Horowitz, B., et al., *Transfusion* 25:516 (1985)), gamma-irradiation (Moroff, G., et al., *Transfusion* 26:453 (1986)), ultraviolet (UV) radiation combined with beta propriolactone, (Prince A.M., et al., *Reviews of Infect. Diseases* 5: 92-107 (1983)), visible laser light in combination with hematoporphyrins  
(Matthews J.L., et al., *Transfusion* 28: 81-83 (1988); North J., et al.,  
15 *Transfusion* 32: 121-128 (1992)), use of the photoactive dyes aluminum phthalocyananine and merocyanine 540 (Sieber F., et al., *Blood* 73: 345-350 (1989); Rywkin S., et al., *Blood* 78(Suppl 1): 352a (Abstract) (1991)) or UV alone (Proudouz, K.N., et al., *Blood* 70:589 (1987)).

Other methods inactivate viral agents by treatment with  
20 furocoumarins, such as psoralens, in the presence of ultra-violet light. Psoralens are tricyclic compounds formed by the linear fusion of a furan ring with a coumarin. Psoralens can intercalate between the base pairs of double-stranded nucleic acids, forming covalent adducts to pyrimidine bases upon absorption of long wave ultraviolet light (UVA). G. D. Cimino  
25 et al., *Ann. Rev. Biochem.* 54:1151 (1985); Hearst et al., *Quart. Rev. Biophys.* 17:1 (1984). If there is a second pyrimidine adjacent to a psoralen-pyrimidine monoadduct and on the opposite strand, absorption of a second photon can lead to formation of a diadduct which functions as an interstrand crosslink. S.T. Isaacs et al., *Biochemistry* 16:1058 (1977); S.T.  
30 Isaacs et al., *Trends in Photobiology* (Plenum) pp. 279-294 (1982); J. Tessman et al., *Biochem.* 24:1669 (1985); Hearst et al., US Patents Nos. 4,124,598, 4,169,204, and 4,196,281, hereby incorporated by reference.

The covalently bonded psoralens act as inhibitors of DNA replication and thus have the potential to stop the replication process. Due to this  
35 DNA binding capability, psoralens are of particular interest in relation to solving the problems inherent in creating and maintaining a pathogen blood supply. Some known psoralens have been shown to inactivate viruses in some blood products. H.J. Alter et al., *The Lancet* (ii:1446) (1988);

L. Lin et al., Blood 74:517 (1989) (decontaminating platelet concentrates); G.P. Wieseahn et al., US Patents Nos. 4,727,027 and 4,748,120, hereby incorporated by reference, describe the use of a combination of 8-methoxypsoralen (8-MOP) and irradiation. P. Morel et al., Blood Cells 18:27  
5 (1992) show that 300 µg/mL of 8-MOP together with ten hours of irradiation with ultraviolet light can effectively inactivate viruses in human serum. Similar studies using 8-MOP and aminomethyltrimethyl psoralen (AMT) have been reported by other investigators. Dodd RY, et al., Transfusion 31:483-490 (1991); Margolis-Nunno, H., et al., Thromb Haemostas 65:1162  
10 (Abstract)(1991). Indeed, the photoinactivation of a broad spectrum of microorganisms has been established, including HBV, HCV, and HIV, under conditions different from those used in the present invention and using previously known psoralen derivatives. [Hanson, C.V., Blood Cells: 18: 7-24 (1992); Alter, H.J., et al., The Lancet ii:1446 (1988); Margolis-Nunno, H. et al., Thromb Haemostas 65: 1162 (Abstract) (1991).]  
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Psoralen photoinactivation is only feasible if the ability of the psoralen to inactivate viruses is sufficient to ensure a safety margin in which complete inactivation will occur. On the other hand, the psoralen must not be such that it will cause damage to blood products. The methods  
20 just described, when applied using known psoralens, require the use of difficult and expensive procedures to avoid causing damage to blood products. For example, some compounds and protocols have necessitated the removal of molecular oxygen from the reaction before exposure to light, to prevent damage to blood products from oxygen radicals produced during  
25 irradiation. See L. Lin et al., Blood 74:517 (1989); US Patent No. 4,727,027, to Wieseahn. This is a costly and time consuming procedure.

Finally, some commonly known compounds used in photochemical decontamination (PCD) exhibit undesirable mutagenicity which appears to increase with increased ability to kill virus. In other words, the more  
30 effective the known compounds are at inactivating viruses, the more injurious the compounds are to the recipient, and thus, the less useful they are at any point in an inactivation system of products for *in vivo* use.

A method of inactivating non-enveloped viruses, such as human parvoviruses, is needed which displays low mutagenicity and which does  
35 not result in significant damage to blood products for which it is used.

SUMMARY OF THE INVENTION

The present invention provides methods of using new and known compounds to inactivate non-enveloped viruses, such as human parvoviruses, in health related products to be used in vivo and in vitro, and particularly, in blood products and blood products in synthetic media.

The present invention contemplates methods of inactivating non-enveloped viruses in blood products, comprising, in the following order: a) providing, in any order, i) a psoralen; ii) photoactivating means for photoactivating said psoralen; and iii) a blood product; b) adding said psoralen to said blood product; and c) photoactivating said psoralen, so as to inactivate any non-enveloped virus present. Specifically, a method is contemplated wherein said blood product comprises platelets or plasma. The present invention further contemplates photoactivating means comprises a photoactivation device capable of emitting a spectrum of electromagnetic radiation comprising wavelengths between 180 nm and 400 nm and intensity between 1 and 30 mW/cm<sup>2</sup>, and where said blood product is exposed to said intensity for between 1 second and thirty minutes.

The invention contemplates that said spectrum of electromagnetic radiation comprises wavelengths between 320 nm and 380 nm. The invention further contemplates that said blood product is in a synthetic media. The invention contemplates that the synthetic media comprises a phosphate buffered salt solution. The invention also contemplates that said psoralen is added to said blood product to a final concentration of between .1 and 250 μM. The invention specifically contemplates that said psoralen is added to said blood product to a final concentration of between 100 and 200 μM.

The invention contemplates that said aminopsoralen is a 4'-primaryamino-substituted psoralen or a 5'-primaryamino-substituted psoralen. The invention further contemplates that said aminopsoralen comprises: a) a substituent R<sub>1</sub> on the 4' carbon atom, selected from the group comprising: -(CH<sub>2</sub>)<sub>u</sub>-NH<sub>2</sub>, -(CH<sub>2</sub>)<sub>w</sub>-R<sub>2</sub>-(CH<sub>2</sub>)<sub>z</sub>-NH<sub>2</sub>, -(CH<sub>2</sub>)<sub>w</sub>-R<sub>2</sub>-(CH<sub>2</sub>)<sub>x</sub>-R<sub>3</sub>-(CH<sub>2</sub>)<sub>z</sub>-NH<sub>2</sub>, and -(CH<sub>2</sub>)<sub>w</sub>-R<sub>2</sub>-(CH<sub>2</sub>)<sub>x</sub>-R<sub>3</sub>-(CH<sub>2</sub>)<sub>y</sub>-R<sub>4</sub>-(CH<sub>2</sub>)<sub>z</sub>-NH<sub>2</sub>; wherein R<sub>2</sub>, R<sub>3</sub>, and R<sub>4</sub> are independently selected from the group comprising O and NH, in which u is a whole number from 1 to 10, w is a whole number from 1 to 5, x is a whole number from 2 to 5, y is a whole number from 2 to 5, and z is a whole number from 2 to 6; and b)

substituents R<sub>5</sub>, R<sub>6</sub>, and R<sub>7</sub> on the 4, 5', and 8 carbon atoms respectively, independently selected from the group comprising H and (CH<sub>2</sub>)<sub>v</sub>CH<sub>3</sub>, where v is a whole number from 0 to 5; or a salt thereof.

The invention contemplates that said compound comprises 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen. The invention further contemplates the administration of the blood product by intravenous infusion to a mammal.

The invention alternately contemplates that said aminopsoralen comprises: a) a substituent R<sub>1</sub> on the 5' carbon atom, selected from the group comprising: -(CH<sub>2</sub>)<sub>u</sub>-NH<sub>2</sub>, -(CH<sub>2</sub>)<sub>w</sub>-R<sub>2</sub>-(CH<sub>2</sub>)<sub>z</sub>-NH<sub>2</sub>, -(CH<sub>2</sub>)<sub>w</sub>-R<sub>2</sub>-(CH<sub>2</sub>)<sub>x</sub>-R<sub>3</sub>-(CH<sub>2</sub>)<sub>z</sub>-NH<sub>2</sub>, and -(CH<sub>2</sub>)<sub>w</sub>-R<sub>2</sub>-(CH<sub>2</sub>)<sub>x</sub>-R<sub>3</sub>-(CH<sub>2</sub>)<sub>y</sub>-R<sub>4</sub>-(CH<sub>2</sub>)<sub>z</sub>-NH<sub>2</sub>; wherein R<sub>2</sub>, R<sub>3</sub>, and R<sub>4</sub> are independently selected from the group comprising O and NH, and in which u is a whole number from 1 to 10, w is a whole number from 1 to 5, x is a whole number from 2 to 5, y is a whole number from 2 to 5, and z is a whole number from 2 to 6; and, b) substituents R<sub>5</sub>, R<sub>6</sub>, and R<sub>7</sub> on the 4, 4', and 8 carbon atoms respectively, independently selected from the group comprising H and (CH<sub>2</sub>)<sub>v</sub>CH<sub>3</sub>, where v is a whole number from 0 to 5, where when R<sub>1</sub> is selected from the group comprising -(CH<sub>2</sub>)<sub>u</sub>-NH<sub>2</sub>, R<sub>7</sub> is (CH<sub>2</sub>)<sub>v</sub>CH<sub>3</sub>, and where when R<sub>5</sub>, R<sub>6</sub>, and R<sub>7</sub> are (CH<sub>2</sub>)<sub>v</sub>CH<sub>3</sub>, u is a whole number from 3 to 10; or a salt thereof.

The invention provides methods of inactivating human parvovirus B19 in blood products, comprising, in the following order: a) providing, in any order, i) an aminopsoralen; ii) photoactivating means for photoactivating said aminopsoralen; and iii) a blood product suspected of being contaminated with human parvovirus B19; b) adding said aminopsoralen to said blood product; and c) photoactivating said aminopsoralen, so as to inactivate any human parvovirus B19 present.

The invention further contemplates that said blood product comprises platelets. The invention further contemplates that said blood product comprises plasma.

The invention further contemplates that said photoactivating means comprises a photoactivation device capable of emitting a spectrum of electromagnetic radiation comprising wavelengths between 180 nm and 400 nm and intensity between 1 and 30 mW/cm<sup>2</sup>. The invention further contemplates that said blood product is exposed to said intensity for between 1 second and thirty minutes. The invention further contemplates that said spectrum of electromagnetic radiation comprises wavelengths between 320 nm and 380 nm.



The invention further contemplates that said blood product is in a synthetic media. The invention further contemplates that said synthetic media comprises a phosphate buffered salt solution. The invention further contemplates that said aminopsoralen is added to said blood product to a final concentration of between .1 and 250  $\mu$ M. The invention further contemplates that said aminopsoralen is added to said blood product to a final concentration of between 100 and 150  $\mu$ M.

The invention further contemplates that said aminopsoralen is a 4'-primaryamino-substituted psoralen. The invention further contemplates that said aminopsoralen is a 5'-primaryamino-substituted psoralen. The invention further contemplates that said aminopsoralen comprises:

- a) a substituent R<sub>1</sub> on the 4' carbon atom, selected from the group comprising:  $-(\text{CH}_2)_u\text{-NH}_2$ ,  $-(\text{CH}_2)_w\text{-R}_2\text{-(CH}_2)_z\text{-NH}_2$ ,  $-(\text{CH}_2)_w\text{-R}_2\text{-(CH}_2)_x\text{-R}_3\text{-(CH}_2)_z\text{-NH}_2$ , and  $-(\text{CH}_2)_w\text{-R}_2\text{-(CH}_2)_x\text{-R}_3\text{-(CH}_2)_y\text{-R}_4\text{-(CH}_2)_z\text{-NH}_2$ ; wherein R<sub>2</sub>, R<sub>3</sub>, and R<sub>4</sub> are independently selected from the group comprising O and NH, in which u is a whole number from 1 to 10, w is a whole number from 1 to 5, x is a whole number from 2 to 5, y is a whole number from 2 to 5, and z is a whole number from 2 to 6; and
- b) substituents R<sub>5</sub>, R<sub>6</sub>, and R<sub>7</sub> on the 4, 5', and 8 carbon atoms respectively, independently selected from the group comprising H and  $(\text{CH}_2)_v\text{CH}_3$ , where v is a whole number from 0 to 5; or a salt thereof. The invention further contemplates that said compound comprises 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen. The invention further contemplates the administration of said platelet preparation by intravenous infusion to a mammal. The invention alternatively contemplates that said aminopsoralen comprises: a) a substituent R<sub>1</sub> on the 5' carbon atom, selected from the group comprising:  $-(\text{CH}_2)_u\text{-NH}_2$ ,  $-(\text{CH}_2)_w\text{-R}_2\text{-(CH}_2)_z\text{-NH}_2$ ,  $-(\text{CH}_2)_w\text{-R}_2\text{-(CH}_2)_x\text{-R}_3\text{-(CH}_2)_z\text{-NH}_2$ , and  $-(\text{CH}_2)_w\text{-R}_2\text{-(CH}_2)_x\text{-R}_3\text{-(CH}_2)_y\text{-R}_4\text{-(CH}_2)_z\text{-NH}_2$ ; wherein R<sub>2</sub>, R<sub>3</sub>, and R<sub>4</sub> are independently selected from the group comprising O and NH, and in which u is a whole number from 1 to 10, w is a whole number from 1 to 5, x is a whole number from 2 to 5, y is a whole number from 2 to 5, and z is a whole number from 2 to 6; and,
- b) substituents R<sub>5</sub>, R<sub>6</sub>, and R<sub>7</sub> on the 4, 4', and 8 carbon atoms respectively, independently selected from the group comprising H and  $(\text{CH}_2)_v\text{CH}_3$ , where v is a whole number from 0 to 5, where when R<sub>1</sub> is selected from the group comprising  $-(\text{CH}_2)_u\text{-NH}_2$ , R<sub>7</sub> is  $(\text{CH}_2)_v\text{CH}_3$ , and where when R<sub>5</sub>, R<sub>6</sub>,

and R7 are  $(\text{CH}_2)_v\text{CH}_3$ , u is a whole number from 3 to 10; or a salt thereof.

The invention provides methods of inactivating human parvovirus B19 in a blood product, comprising, in the following order: a) providing, in any order, i) 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen, ii) photoactivating means for photoactivating said 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen, and iii) a platelet preparation suspected of being contaminated with said virus; b) adding said 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen to said platelet preparation; and c) photoactivating said 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen, so as to inactivate said virus, without causing significant damage to said platelet preparation.

### DESCRIPTION OF THE DRAWINGS

5           FIG. 1 details the compound synthesis scheme of two isomers of bromomethyl-trialkylpsoralen.

          FIG. 2 details the compound synthesis scheme of several 4'-primary amino substituted psoralens.

10           FIG. 3 details the compound synthesis scheme of several 4'-primaryamino substituted psoralens.

          FIG. 4 details the compound synthesis scheme of several psoralens having a terminal amine linked to the psoralen at the 4'-position by an alkyl chain.

15           FIG. 5 details the compound synthesis scheme of several 5'-primaryamino-substituted psoralens.

          FIG. 6 is a perspective view of one embodiment of the device of the present invention.

          FIG. 7 shows the impact of concentration on the log kill of R17 when Compounds 1 - 3 of the present invention are photoactivated.

20           FIG. 8 shows the impact of concentration on the log kill of R17 when Compounds 3 - 6 of the present invention are photoactivated.

          FIG. 9 shows the impact of concentration on the log kill of R17 when Compounds 2 and 6 of the present invention are photoactivated.

25           FIG. 10 shows the impact of concentration on the log kill of R17 when Compounds 6 and 18 of the present invention are photoactivated.

          FIG. 11 shows the impact of concentration on the log kill of R17 when Compound 16 of the present invention is photoactivated.

          FIG. 12 shows the impact of varying Joules/cm<sup>2</sup> (Watt second/cm<sup>2</sup>) of irradiation on the log titer of R17 for Compound 6 of the present

invention.

FIG. 13 shows the impact of varying Joule/cm<sup>2</sup> of irradiation on the log titer of R17 for Compounds 7, 9 and 10 of the present invention.

5 FIG. 14 shows the impact of varying Joules/cm<sup>2</sup> of irradiation on the log titer of R17 for Compounds 7 and 12 of the present invention.

FIG. 15 shows the impact of varying Joules/cm<sup>2</sup> of irradiation on the log titer of R17 for Compound 15 of the present invention.

FIG. 16 shows the impact of varying Joules/cm<sup>2</sup> of irradiation on the log titer of R17 for Compound 17 of the present invention.

10 FIG. 17 shows the impact of varying Joules/cm<sup>2</sup> of irradiation on the log titer of R17 for Compounds 6 and 17 of the present invention.

FIG. 18 shows the impact of varying Joules/cm<sup>2</sup> of irradiation on the log titer of R17 for Compounds 6 and 15 of the present invention.

15 FIG. 19 shows the effect of varying the concentration of Compounds 2 and 6 of the present invention, in plasma.

FIG. 20 shows the effect of varying the concentration of Compounds 2 and 6 of the present invention, in synthetic medium.

FIG. 21A schematically shows the standard blood product separation approach used presently in blood banks.

20 FIG. 21B schematically shows an embodiment of the present invention whereby synthetic media is introduced to platelet concentrate prepared as in FIG. 25A.

FIG. 21C schematically shows one embodiment of the decontamination approach of the present invention applied specifically to platelet concentrate diluted with synthetic media as in FIG. 25B.

25 FIG. 22A is a graph comparing a day one control (C1), a control stored for 5 days (D5), ultraviolet light alone (UV) and treatment with ultraviolet light and Compound 2 at 100 μM (PCD) by their effects on platelet function as measured by platelet count. "n" represents the number of experiments represented by the data point.

30 FIG. 22B is a graph comparing a day one control (C1), a control stored for 5 days (D5), ultraviolet light alone (UV) and treatment with ultraviolet light and Compound 2 at 100 μM (PCD) by their effects on platelet function as measured by platelet aggregation. "n" represents the number of experiments represented by the data point.

35 FIG. 22C is a graph comparing a day one control (C1), a control stored for 5 days (D5), ultraviolet light alone (UV) and treatment with ultraviolet light and Compound 2 at 100 μM (PCD) by their effects on platelet function

as measured by GMP-140 expression. "n" represents the number of experiments represented by the data point.

FIG. 22D is a graph comparing a day one control (C1), a control stored for 5 days (D5), ultraviolet light alone (UV) and treatment with ultraviolet light and Compound 2 at 100  $\mu$ M (PCD) by their effects on platelet function as measured by pH. "n" represents the number of experiments represented by the data point.

FIG. 23A is a graph comparing a day one control (C1), a control stored for 5 days (D5), ultraviolet light alone (UV) and treatment with ultraviolet light and Compound 6 at 100  $\mu$ M (PCD) by their effects on platelet function as measured by platelet count. "n" represents the number of experiments represented by the data point.

FIG. 23B is a graph comparing a day one control (C1), a control stored for 5 days (D5), ultraviolet light alone (UV) and treatment with ultraviolet light and Compound 6 at 100  $\mu$ M (PCD) by their effects on platelet function as measured by platelet aggregation. "n" represents the number of experiments represented by the data point.

FIG. 23C is a graph comparing a day one control (C1), a control stored for 5 days (D5), ultraviolet light alone (UV) and treatment with ultraviolet light and Compound 6 at 100  $\mu$ M (PCD) by their effects on platelet function as measured by GMP-140 expression. "n" represents the number of experiments represented by the data point.

FIG. 23D is a graph comparing a day one control (C1), a control stored for 5 days (D5), ultraviolet light alone (UV) and treatment with ultraviolet light and Compound 6 at 100  $\mu$ M (PCD) by their effects on platelet function as measured by pH. "n" represents the number of experiments represented by the data point.

FIG. 24A is a graph comparing a day one control (C1), a control stored for 5 days (D5), ultraviolet light alone (UV) and treatment with ultraviolet light and Compound 17 at 100  $\mu$ M (PCD) by their effects on platelet function as measured by platelet count. "n" represents the number of experiments represented by the data point.

FIG. 24B is a graph comparing a day one control (C1), a control stored for 5 days (D5), ultraviolet light alone (UV) and treatment with ultraviolet light and Compound 17 at 100  $\mu$ M (PCD) by their effects on platelet function as measured by platelet aggregation. "n" represents the number of experiments represented by the data point.

FIG. 24C is a graph comparing a day one control (C1), a control stored

for 5 days (D5), ultraviolet light alone (UV) and treatment with ultraviolet light and Compound 17 at 100  $\mu$ M (PCD) by their effects on platelet function as measured by GMP-140 expression. "n" represents the number of experiments represented by the data point.

5           FIG. 24D is a graph comparing a day one control (C1), a control stored for 5 days (D5), ultraviolet light alone (UV) and treatment with ultraviolet light and Compound 17 at 100  $\mu$ M (PCD) by their effects on platelet function as measured by pH. "n" represents the number of experiments represented by the data point.

10           FIG. 25A is a graph comparing a day one control (C1), a control stored for 5 days (D5), ultraviolet light alone (UV) and treatment with ultraviolet light and Compound 18 at 100  $\mu$ M (PCD) by their effects on platelet function as measured by platelet count. "n" represents the number of experiments represented by the data point.

15           FIG. 25B is a graph comparing a day one control (C1), a control stored for 5 days (D5), ultraviolet light alone (UV) and treatment with ultraviolet light and Compound 18 at 100  $\mu$ M (PCD) by their effects on platelet function as measured by platelet aggregation. "n" represents the number of experiments represented by the data point.

20           FIG. 25C is a graph comparing a day one control (C1), a control stored for 5 days (D5), ultraviolet light alone (UV) and treatment with ultraviolet light and Compound 18 at 100  $\mu$ M (PCD) by their effects on platelet function as measured by GMP-140 expression. "n" represents the number of experiments represented by the data point.

25           FIG. 25D is a graph comparing a day one control (C1), a control stored for 5 days (D5), ultraviolet light alone (UV) and treatment with ultraviolet light and Compound 18 at 100  $\mu$ M (PCD) by their effects on platelet function as measured by pH. "n" represents the number of experiments represented by the data point.

30           FIG 26 is a graph showing the kinetics of HIV expression with and without TNF- $\alpha$ .

            FIG 27 is a bar graph showing the inhibition of HIV p24 expression in psoralen treated OM-10.1 cells.

            FIG 28 is a bar graph showing Compound 2 inactivation of proviral  
35 HIV-1 in OM-10.1 cells.

            FIG. 29 is a graph showing Compound 2 inactivation of parvovirus B19.

            FIG. 30 is a graph showing psoralen inactivation of parvovirus B19.

## DESCRIPTION OF THE INVENTION

The present invention provides methods of inactivating non-enveloped viruses in health related products to be used in vivo and in vitro, and in particular, blood products. Specifically, the present invention provides methods of inactivating human parvoviruses in blood products.

The inactivation methods of the present invention provide methods of inactivating pathogens, and, in particular, viruses, including several forms of cytomegalovirus (CMV), bovine viral diarrhea virus (BVDV), proviral human immunodeficiency virus (proviral HIV), and parvovirus, such as human parvovirus B19 (B19), in blood products prior to use *in vitro* or *in vivo*. In contrast with previous approaches, there is no need to limit the concentration of molecular oxygen.

The description of the invention is divided into the following sections: I) Photoactivation Devices, II) Compound Synthesis, III) Binding of Compounds to Nucleic Acid, IV) Inactivation of Contaminants, and V) Preservation of Biochemical Properties of Material Treated.

### I. PHOTOACTIVATION DEVICES

Methods of the present invention use devices for photoactivation and specifically, for photoactivation of photoreactive nucleic acid binding compounds. The specific devices used in the examples described below are either described in detail in issued U.S. Patent No. 5,399,719 (the entire contents of which is hereby incorporated by reference) or have been tested (results not shown) and prove to have approximately equivalent properties to the devices described. In general, the present invention contemplates a photoactivation device for treating photoreactive compounds, comprising:

- a) means for providing appropriate wavelengths of electromagnetic radiation to cause photoactivation of at least one photoreactive compound;
- b) means for supporting a plurality of samples in a fixed relationship with the radiation providing means during photoactivation; and
- c) means for maintaining the temperature of the samples within a desired temperature range during photoactivation.

The present invention also contemplates methods, comprising:

- a) supporting a plurality of sample containers, containing one or more photoreactive compounds, in a fixed relationship with a fluorescent source of electromagnetic radiation;
- b) irradiating the plurality of sample containers simultaneously with electromagnetic radiation to cause photoactivation of at least one photoreactive compound;

and c) maintaining the temperature of the sample within a desired temperature range during photoactivation.

The present application is not limited to any particular light sources described here or in U.S. Patent No. 5,399,719 or similar thereto. However, 5 the present invention contemplates that any light source which delivers comparable intensity, wavelength and distribution of light in a temperature controlled fashion is additionally appropriate for use in the present invention.

The major features of one embodiment of the device of the present 10 invention involve: A) an inexpensive source of ultraviolet radiation in a fixed relationship with the means for supporting the sample containers, B) rapid photoactivation, C) large sample processing, D) temperature control of the irradiated samples, and E) inherent safety.

#### 15 A. Electromagnetic Radiation Source

Many sources of ultraviolet radiation can be successfully used in decontamination protocols with psoralens. Without intending to be limited to any type of photoactivation device, the present invention contemplates several preferred arrangements for the photoactivation 20 device, as follows.

A preferred photoactivation device of the present invention has an inexpensive source of ultraviolet radiation in a fixed relationship with the means for supporting the sample vessels.

Particular types of ultraviolet radiation are herein described in terms 25 of wavelength. Wavelength is herein described in terms of nanometers ("nm";  $10^{-9}$  meters). For purposes herein, ultraviolet radiation extends from approximately 180 nm to 400 nm. When a radiation source, by virtue of filters or other means, does not allow radiation below a particular wavelength (e.g. 320 nm), it is said to have a low end "cutoff" at that 30 wavelength (e.g. "a wavelength cutoff at 300 nanometers"). Similarly, when a radiation source allows only radiation below a particular wavelength (e.g. 360 nm), it is said to have a high end "cutoff" at that wavelength (e.g. "a wavelength cutoff at 360 nanometers").

For any photochemical reaction it is desired to eliminate or least 35 minimize any deleterious side reactions. Some of these side reactions can be caused by the excitation of endogenous chromophores that may be present during the photoactivation procedure. In a system where only nucleic acid and psoralen are present, the endogenous chromophores are

the nucleic acid bases themselves. Restricting the photoactivation process to wavelengths greater than 320 nm minimizes direct nucleic acid damage since there is very little absorption by nucleic acids above 313 nm.

5 In human serum or plasma, for example, the nucleic acid is typically present together with additional biological constituents. If the biological fluid is just protein, the 320 nm cutoff will be adequate for minimizing side reactions (aromatic amino acids do not absorb above 320 nm). If the biological fluid includes other analytes, there may be constituents that are sensitive to particular wavelengths of light. In view of the presence of these  
10 endogenous constituents, it is intended that the device of the present invention be designed to allow for irradiation within a small range of specific and desirable wavelengths, and thus avoid damage blood components. The preferred range of desirable wavelengths is between 320 and 350 nm.

15 Some selectivity can be achieved by choice of commercial irradiation sources. For example, while typical fluorescent tubes emit wavelengths ranging from 300 nm to above 400 nm (with a broad peak centered around 360 nm), BLB type fluorescent lamps are designed to remove wavelengths above 400 nm. This, however, only provides an upper end cutoff.

20 In a preferred embodiment, the device of the present invention comprises an additional filtering means, such as a glass cut off filter or a liquid filter solution that transmits only a specific region of the electromagnetic spectrum, which yields a transmission window of 320-400 nm.

25 It is not intended that the present invention be limited by the particular filter employed. Several inorganic salts and glasses satisfy the necessary requirements.

When ultraviolet radiation is herein described in terms of irradiation, it is expressed in terms of intensity flux (milliwatts per square  
30 centimeter or "mW cm<sup>-2</sup>" or J/cm<sup>2</sup>sec). "Output" is herein defined to encompass both the emission of radiation (yes or no; on or off) as well as the level of irradiation. In a preferred embodiment, intensity is monitored at 4 locations: 2 for each side of the plane of irradiation.

A preferred source of ultraviolet radiation is a fluorescent source.

35 As used here, fixed relationship is defined as comprising a fixed distance and geometry between the sample and the light source during the sample irradiation. Distance relates to the distance between the source and the sample as it is supported. It is known that light intensity from a point



source is inversely related to the square of the distance from the point source. Thus, small changes in the distance from the source can have a drastic impact on intensity. Since changes in intensity can impact photoactivation results, changes in distance are avoided in the devices of the present invention. This provides reproducibility and repeatability.

Geometry relates to the positioning of the light source. For example, it can be imagined that light sources could be placed around the sample holder in many ways (on the sides, on the bottom, in a circle, etc.). The geometry used in a preferred embodiment of the present invention allows for uniform light exposure of appropriate intensity for rapid photoactivation. The geometry of a preferred device of the present invention involves multiple sources of linear lamps as opposed to single point sources. In addition, there are several reflective surfaces and several absorptive surfaces. Because of this complicated geometry, changes in the location or number of the lamps relative to the position of the samples to be irradiated are to be avoided in that such changes will result in intensity changes.

#### B. Rapid Photoactivation

The light source of the preferred embodiment of the present invention allows for rapid photoactivation. The intensity characteristics of the irradiation device have been selected to be convenient with the anticipation that many sets of multiple samples may need to be processed. With this anticipation, a fifteen minute exposure time or less is a practical goal.

In designing the devices of the present invention, relative position of the elements of the preferred device have been optimized to allow for approximately fifteen minutes of irradiation time, so that, when measured for the wavelengths between 320 and 350 nanometers, an intensity flux greater than approximately  $1 \text{ mW cm}^{-2}$  ( $.001 \text{ J/cm}^2 \text{ sec.}$ ) is provided to the sample vessels.

#### C. Processing of Large Numbers of Samples

As noted, another important feature of the photoactivation devices of the present invention is that they provide for the processing of large numbers of samples. In this regard, one element of the devices of the present invention is a means for supporting a plurality of blood bags. In the preferred embodiment of the present invention the supporting means

comprises a blood bag support placed between two banks of lights. By accepting commonly used commercially available bags, the device of the present invention allows for convenient processing of large numbers of samples.

5

#### D. Temperature Control

As noted, one of the important features of the photoactivation devices of the present invention is temperature control. Temperature control is important because the temperature of the sample at the time of exposure to light can dramatically impact the results. With single stranded 5S ribosomal RNA, irradiation at low temperatures enhances the covalent addition of HMT to 5S rRNA by two fold at 4°C compared to 20°C. Thompson et al., J. Mol. Biol. 147:417 (1981). Even further temperature induced enhancements of psoralen binding have been reported with synthetic polynucleotides. Thompson et al., Biochemistry 21:1363 (1982).

10

15

#### E. Inherent Safety

Ultraviolet radiation can cause severe burns. Depending on the nature of the exposure, it may also be carcinogenic. The light source of a preferred embodiment of the present invention is shielded from the user. This is in contrast to the commercial hand-held ultraviolet sources as well as the large, high intensity sources. In a preferred embodiment, the irradiation source is contained within a housing made of material that obstructs the transmission of radiant energy (i.e. an opaque housing). No irradiation is allowed to pass to the user. This allows for inherent safety for the user.

20

25

## II. COMPOUND SYNTHESIS

### A. Photoactivation Compounds in General

30

"Photoactivation compounds" (or "photoreactive compounds") defines a family of compounds that undergo chemical change in response to electromagnetic radiation. Table 1 is a partial list of photoactivation compounds.

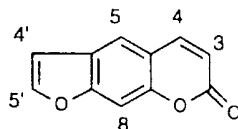
35 Table 1. Photoactivation Compounds

Actinomycins

Anthracyclines

- Anthramycin
- Benzodipyrones
- Fluorenes and fluorenones
- Furocoumarins
- 5 Mitomycin
- Monostral Fast Blue
- Norphillin A
- Many organic dyes not specifically listed
- Phenanthridines
- 10 Phenazathionium Salts
- Phenazines
- Phenothiazines
- Phenylazides
- Quinolines
- 15 Thioxanthenones

The preferred species of photoreactive compounds described herein is commonly referred to as the furocoumarins. In particular, the present invention contemplates those compounds described as psoralens: [7H-  
20 furo(3,2-g)-(1)-benzopyran-7-one, or  $\beta$ -lactone of 6-hydroxy-5-benzofuranacrylic acid], which are linear:



and in which the two oxygen residues appended to the central aromatic moiety have a 1, 3 orientation, and further in which the furan ring moiety  
25 is linked to the 6 position of the two ring coumarin system. Psoralen derivatives are derived from substitution of the linear furocoumarin at the 3, 4, 5, 8, 4', or 5' positions.

8-Methoxypsoralen (known in the literature under various named, e.g., xanthotoxin, methoxsalen, 8-MOP) is a naturally occurring psoralen  
30 with relatively low photoactivated binding to nucleic acids and low mutagenicity in the Ames assay, which is described in the following experimental section. 4'-Aminomethyl-4,5',8-trimethylpsoralen (AMT) is one of most reactive nucleic acid binding psoralen derivatives, providing up to 1 AMT adduct per 3.5 DNA base pairs. S.T. Isaacs, G. Wieseahn and

L.M. Hallick, NCI Monograph 66: 21 (1984). However, AMT also exhibits significant levels of mutagenicity. A new group of psoralens was desired which would have the best characteristics of both 8-MOP and AMT: low mutagenicity and high nucleic acid binding affinity, to ensure safe and thorough inactivation of pathogens. The compounds of the present invention were designed to be such compounds.

The term "4'-primaryamino-substituted psoralen" shall herein be defined as a psoralen compound which has an NH<sub>2</sub> group linked to the 4'-position of the psoralen by a hydrocarbon chain having a total length of 2 to 20 carbons, where 0 to 6 of those carbons are independently replaced by NH or O, and each point of replacement is separated from each other point of replacement by at least two carbons, and is separated from the psoralen by at least one carbon. 4'-primaryamino-substituted psoralens encompass additional substitutions on the 4, 5', and 8 positions of the psoralen, said substitutions include, but are not limited to, the following groups: H and (CH<sub>2</sub>)<sub>n</sub>CH<sub>3</sub>, where n = 0-6.

TABLE 2

#	COMPOUND
1	4'-(4-amino-2-aza)butyl-4,5',8-trimethylpsoralen
2	4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen
3	4'-(2-aminoethyl)-4,5',8-trimethylpsoralen
4	4'-(5-amino-2-oxa)pentyl-4,5',8-trimethylpsoralen
5	4'-(5-amino-2-aza)pentyl-4,5',8-trimethylpsoralen
6	4'-(6-amino-2-aza)hexyl-4,5',8-trimethylpsoralen
7	4'-(7-amino-2,5-oxa)heptyl-4,5',8-trimethylpsoralen
8	4'-(12-amino-8-aza-2,5-dioxa)dodecyl-4,5',8-trimethylpsoralen
9	4'-(13-amino-2-aza-6,11-dioxa)tridecyl-4,5',8-trimethylpsoralen
10	4'-(7-amino-2-aza)heptyl-4,5',8-trimethylpsoralen
11	4'-(7-amino-2-aza-5-oxa)heptyl-4,5',8-trimethylpsoralen
12	4'-(9-amino-2,6-diaza)nonyl-4,5',8-trimethylpsoralen
13	4'-(8-amino-5-aza-2-oxa)octyl-4,5',8-trimethylpsoralen
14	4'-(9-amino-5-aza-2-oxa)nonyl-4,5',8-trimethylpsoralen
15	4'-(14-amino-2,6,11-triaza)tetradecyl-4,5',8-trimethylpsoralen
16	5'-(4-amino-2-aza)butyl-4,4',8-trimethylpsoralen
17	5'-(6-amino-2-aza)hexyl-4,4',8-trimethylpsoralen
18	5'-(4-amino-2-oxa)butyl-4,4',8-trimethylpsoralen

The term "5'-primaryamino-substituted psoralen" shall herein be defined as a psoralen compound which has an NH<sub>2</sub> group linked to the 5'-position of the psoralen by a hydrocarbon chain having a total length of 1 to 20  
5 carbons, where 0 to 6 of those carbons are independently replaced by NH or O, and each point of replacement is separated from each other point of replacement by at least two carbons, and is separated from the psoralen by at least one carbon. 5'-Primaryamino-substituted psoralens encompass additional substitutions on the 4, 4', and 8 positions of the psoralen, said  
10 substitutions include, but are not limited to, the following groups: H and (CH<sub>2</sub>)<sub>n</sub>CH<sub>3</sub>, where n = 0-6.

#### B. Synthesis of the Psoralens

For ease of reference, Table 2 sets forth the nomenclature used for the psoralen derivatives discussed herein. Note that in this section  
15 (entitled "B. Synthesis of the Psoralens") the roman numerals used to identify compounds correlate with Schematics 1-6 only, and do not correlate with the compound numbers listed in Table 2.

It is most logical to first describe the synthesis of intermediates useful in synthesizing many of the compounds of the present invention.  
20 While the invention is not limited to 4,5',8-trimethyl-4'-primaryamino-substituted psoralens or 4,4',8-trimethyl-5'-primaryamino-substituted psoralens, some important intermediates include tri- and tetramethyl psoralens, 4'-halomethyl-4,5',8-trimethylpsoralens and 5'-halomethyl-4,4',8-trimethylpsoralens. The preparation of these critical intermediates  
25 presents difficult challenges.

#### Synthesis of Intermediates

Previous syntheses of 4'-chloromethyl-4,5',8-trimethylpsoralen (4'-CMT) and 4'-bromomethyl-4,5',8-trimethylpsoralen (4'-BrMT) start from  
30 4,5',8-trimethylpsoralen (5'-TMP) which is commercially available (Aldrich Chemical Co., Milwaukee, WI) or can be prepared in four steps as described below for other alkylated psoralens. 5'-TMP is converted to 4'-CMT using a large excess (20-50 equivalents) of highly carcinogenic, and volatile chloromethyl methyl ether. Halomethylation of the 4,5',8-  
35 trialkylpsoralens with chloromethyl methyl ether or bromomethyl methyl ether is described in US Patent No. 4,124,598, to Hearst. The bromo compound, 4'-BrMT, is likewise prepared using bromomethyl methyl ether which is somewhat less volatile. Yields of only 30-60% of the

desired intermediate are obtained. The 5'-chloromethyl-4,4',8-trimethylpsoralen (5'-CMT) and 5'-bromomethyl-4,4',8-trimethylpsoralen (5'-BrMT) are prepared similarly, using the isomeric starting compound, 4,4',8-trimethylpsoralen (4'-TMP) [U.S. Patent No. 4,294,822, to Kaufman; 5 McLeod, et al., "Synthesis of Benzofuranoid Systems. I. Furocoumarins, Benzofurans and Dibenzofurans," Tetrahedron Letters 237 (1972)]. Some of the figures referred to in the synthesis discussion that follows contain roman numerals used for labeling structures that embody more than one compound. This numbering system is distinct from, and not to be 10 confused with, the numbering system of Table 2, above, which was used to identify several specific compounds.

Described herein is a much improved procedure which allows for the synthesis of either isomer of the bromomethyl-trialkylpsoralens from the same psoralen precursor by careful control of reaction conditions. See 15 FIG. 1. In FIG. 1, A<sub>1</sub> and A<sub>2</sub> are independently selected from the group comprising H and an alkyl chain having 1-6 carbon atoms. Reaction of the 4,8-dialkyl-7-hydroxycoumarin with 2-chloro-3-butanone under typical basic conditions, provides 4,8-dialkyl-7-(1-methyl-2-oxo)propyloxycoumarin (I). This material is cyclized by heating in aqueous 20 NaOH to provide 4,8-dialkyl-4',5'-dimethylpsoralen (II). Treatment of the tetrasubstituted psoralen and N-bromosuccinimide (NBS) in a solvent at room temperature up to 150°C leads to bromination at the 4'- or 5'-position, depending upon the conditions used. A catalyst such as dibenzoyl peroxide may be added, but is not necessary. If the solvent used 25 is carbon tetrachloride at reflux, 4,8-dialkyl-5'-bromomethyl-4'-methylpsoralen (IV) is obtained in yields of 50% or greater. If methylene chloride is used at room temperature, only 4,8-dialkyl-4'-bromomethyl-5'-methylpsoralen (III) is obtained in ≥80% yield. Benzylic bromination in other solvents can also be done, generating one of the isomeric products 30 alone or in a mixture. These solvents include, but are not limited to 1,2-dichloroethane, chloroform, bromotrichloromethane and benzene.

#### General Scheme of Synthesis of 4'-Substituted Psoralens

Turning now to the synthesis of a subclass of the linear psoralens, 4,5',8-trialkylpsoralens can be made as follows. The 4,8-dialkylcoumarins 35 are prepared from 2-alkylresorcinols and a 3-oxoalkanoate ester by the Pechmann reaction (Organic Reactions Vol VII, Chap 1, ed. Adams et al, Wiley, NY, (1953)). The hydroxy group is treated with an allylating reagent, CH<sub>2</sub>=CHX-CH(R)-Y, where X is a halide or hydrogen, Y is a halide

or sulfonate, and R is H or  $(\text{CH}_2)_v\text{CH}_3$ , where v is a whole number from 0 to 4. Claisen rearrangement of the resultant allyl ether gives 4,8-dialkyl-6-allyl-7-hydroxycoumarin. The coumarins are converted to the 4,5',8-trialkylpsoralens using procedures similar to one of several previously described procedures (i.e., see, Bender et al, J. Org. Chem. 44:2176 (1979); Kaufman, US Patent Nos. 4,235,781 and 4,216,154, hereby incorporated by reference). 4,5',8-Trimethylpsoralen is a natural product and is commercially available (Aldrich Chemical Co., Milwaukee, WI).

#### General Scheme of Synthesis of 5'-Substituted Psoralens

10 The 4,4',8-trialkylpsoralens can be prepared in two steps also starting from the 4,8-dialkyl-7-hydroxycoumarins discussed above. The coumarin is treated with an alpha-chloro ketone under basic conditions to give the 4,8-dialkyl-7-(2-oxoalkoxy)coumarin. Cyclization of this intermediate to the 4,4',8-trialkylcoumarin occurs by heating in aqueous base.

15 Longer chain 4'-( $\omega$ -haloalkyl)trialkylpsoralens (herein referred to as longer chain 4'-HATP) where the alkyl groups are selected from the group  $(\text{CH}_2)_2$  to  $(\text{CH}_2)_{10}$  can be prepared under Friedel-Crafts conditions as discussed elsewhere (Olah and Kuhn, J. Org. Chem., 1964, 29, 2317; Friedel-Crafts and Related Reactions, Vol. II, Part 2, Olah, ed., Interscience, NY, 20 1964, p 749). While reactions of the halomethyl- intermediates with amines (e.g., Hearst et al., US patent 4,124,598), and alcohols (e.g., Kaufman, US Patent No. 4,269,852) have been described, there are only two original reports on the formation of extended chain primary amines. They describe the reaction of the 4'-chloromethyl-4,5',8-trimethyl psoralen with  $\text{H}_2\text{N}-$  25  $(\text{CH}_2)_n-\text{NH}_2$  (where n=2, 4, 6) (Lee, B., et al. "Interaction of Psoralen-Derivatized Oligodeoxyribonucleoside Methylphosphonates with Single-Stranded DNA," Biochemistry 27:3197 (1988), and with  $\text{H}_2\text{NCH}_2\text{CH}_2\text{SSCH}_2\text{CH}_2\text{NH}_2$  (Goldenberg, M., et al., "Synthesis and Properties of Novel Psoralen Derivatives," Biochemistry 27:6971 (1988)). 30 The utility of the resulting compounds for nucleic acid photoreaction has not previously been reported. The properties of these materials, such as decreased mutagenicity, are unexpected based on what is known about previously prepared compounds, such as AMT.

Several synthesis routes are shown in FIG 2. Starting from the 4'-HATP (where w is a number from 1-5; A<sub>1</sub>, A<sub>2</sub> and A<sub>3</sub> are independently selected from the group comprising H and  $(\text{CH}_2)_v\text{CH}_3$ , where v is a number from 0 to 5; and where X = Br, Cl or I), reaction with an excess of a bis-hydroxy compound, HO-(B)-OH, where B is either: an alkyl chain (e.g.,

HO-(B)-OH is 1,3-propanediol), a monoether (e.g., diethylene glycol) or a polyether (e.g., tetraethylene glycol); which is less than or equal to 18 carbon atoms long, either neat or with a solvent such as acetone at 20-80°C, and a base for the carbon chains longer than halomethyl, gives a ( $\omega$ -hydroxyalkoxy)alkyl psoralen. The terminal hydroxy group can be transformed to an amino group under a variety of conditions (for example see Larock, 'Comprehensive Organic Transformations,' VCH Publishers, NY, 1989). Particularly, the hydroxy group can be converted to the ester of methanesulfonic acid (structure VI) in the presence of methanesulphonyl chloride ( $\text{CH}_3\text{SO}_3\text{Cl}$ ). This can subsequently be converted to the azide in refluxing ethanol and the azide reduced to the final amine, structure VII (examples are Compounds 2, 4 and 7). The method described herein utilizes triphenylphosphine and water in tetrahydrofuran (THF) for the reduction but other methods are contemplated.

A preferred method of preparation of structure VII uses the reaction of 4'-HATP with a primary linear alcohol containing a protected amine (e.g., a phthalimido group) at the terminal position in a suitable solvent such as DMF at 25 - 150°C to give V. The amine is then deprotected under standard conditions (e.g., hydrazine or aqueous  $\text{MeNH}_2$  to deprotect a phthalimido group [higher alkyl hydrazines, such as benzyl hydrazines, are also contemplated]) to give VII.

Conversely, structure VI can be reacted with diamines,  $\text{H}_2\text{N}-(\text{B}')-\text{NH}_2$  where  $\text{B}'$  is an alkyl chain (e.g., 1,4,-butanediamine), a monoether (e.g., 3-oxa-1,5-pentanediamine) or a polyether (e.g., 3,6-dioxa-1,8-octanediamine) to give the final product, compound VIII (examples of compounds in this structure group are Compounds 8, 13 and 14). This reaction is carried out with an excess of diamine in acetonitrile at reflux, but other solvents and temperatures are equally possible.

Some final compounds are desired in which the carbon chain is linked to the 4'- position of the psoralen ring by an aminoalkyl group [ $\text{NH}(\text{CH}_2)_w$ ] rather than by an oxyalkyl group [ $\text{O}(\text{CH}_2)_w$ ]. Synthesis pathways for these compounds are shown in FIG. 3. When the linkage between this nitrogen and the terminating nitrogen contains only  $\text{CH}_2$  subunits and oxygen but no other nitrogens (structure X) (examples are Compounds 1, 5, 6, 9, 10 and 11), the product can conveniently be prepared from the 4'-HATP and the appropriate diamine of structure IX. This method is also applicable to final products that contain more than two nitrogens in the chain (structure XIII) (examples are Compounds 12 and 15)



starting from polyamines of structure XII (e.g., norspermidine or spermine [commercially available from Aldrich, Milwaukee, WI]), however, in this case isomeric structures are also formed in considerable amounts. The preferred method for the preparation of structure XIII is reductive amination of the psoralen-4'-alkanal (XI) with a polyamine of structure XII and a reducing agent such as sodium cyanoborohydride. This reductive amination is applicable to the synthesis of compounds X as well. The carboxaldehydes (structure XI, w = 0) have been prepared previously by hydrolysis of the 4'-halomethyl compounds and subsequent oxidation of the resultant 4'-hydroxymethyl compound. (Isaacs et al, J. Labelled Cmpds. Radiopharm., 1982, 19, 345). These compounds can also be conveniently prepared by formylation of the 4'-hydrido compounds with a formamide and POCl<sub>3</sub>, or with hexamethylene tetraamine in acid. Longer chain alkanals can be prepared from the 4'-HATP compounds by conversion of the terminal halo group to an aldehyde functionality (for example, Durst, Adv. Org. Chem. 6:285 (1969)).

Other final products have a terminal amine linked to the psoralen by an alkyl chain. As shown in FIG. 4, these compounds (structures XIV) (an example is Compound 3) are prepared either 1) by reaction of the 4'-HATP with potassium phthalimide or azide and subsequent liberation of the desired amine as before, for example, with hydrazine, or 2) conversion of the 4'-HATP to the cyanide compound, followed by reduction, for example with NaBH<sub>4</sub>-CF<sub>3</sub>CO<sub>2</sub>H.

The discussion of the conversion of 4,5',8-trialkylpsoralens to 4'-aminofunctionalized-4,5',8-trialkylpsoralens applies equally well when the 4- and/or 8-position is substituted with only a hydrogen, thus providing 4'-primaryamino-substituted-5', (4 or 8)- dialkylpsoralens and 4'-primaryamino-substituted-5'-alkylpsoralens.

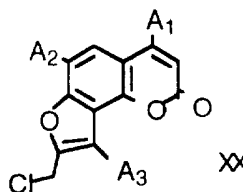
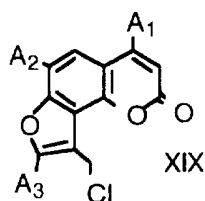
### 30 Synthesis of 5' Derivatives

Under identical conditions to those described above, the 4,4',8-trialkylpsoralens or the 4,4',8-trialkyl-5'-methylpsoralens can be converted to the 5'-( $\omega$ -haloalkyl)-4,4',8-trialkylpsoralens, (herein called 5'-HATP), as detailed in Schematic 5, below. (See Kaufman, U.S. Patent No. 4,294,822 and 4,298,614 for modified version).

The discussion of the conversion of 4,4',8-trialkylpsoralens to 5'-primaryamino-substituted-4,4',8-trialkylpsoralens applies equally well

when the 4-, 4'- and/or 8-positions are just substituted with a hydrogen, thus providing 5'-primaryamino-substituted- dialkylpsoralens and 5'-primaryamino-substituted-alkylpsoralens, with the alkyl group(s) at the 4-, 4'- and/or 8- positions.

- 5 The discussion above of the syntheses of 4'-primaryamino- and 5'-primaryamino-psoralens can be extended to the non-linear coumarins, specifically the isopsoralens or angelicins. Thus, the 4'-halomethylangelicins (XIX) and the 5'-halomethylangelicins (XX) can be prepared in a similar manner to their linear counterparts. By analogy  
10 with the synthetic pathways



presented above one can envision the synthesis of 4'-( $\omega$ -amino)alkylangelicins and 5'-( $\omega$ -amino)alkylangelicins where the alkyl linkage can contain one or more oxygen or nitrogen atoms.

15

### III. BINDING OF COMPOUNDS TO NUCLEIC ACID

The present invention contemplates binding new and known compounds to nucleic acid, including (but not limited to) viral nucleic acid and bacterial nucleic acid. One approach of the present invention to  
20 binding photoactivation compounds to nucleic acid is photobinding. Photobinding is defined as the binding of photobinding compounds in the presence of photoactivating wavelengths of light. Photobinding compounds are compounds that bind to nucleic acid in the presence of photoactivating wavelengths of light. The present invention contemplates  
25 methods of photobinding with photobinding compounds of the present invention.

One embodiment of the method of the present invention for photobinding involves the steps: a) providing a photobinding compound of the present invention; and b) mixing the photobinding compound with  
30 nucleic acid in the presence of photoactivation wavelengths of electromagnetic radiation.

The invention further contemplates a method for modifying nucleic

acid, comprising the steps: a) providing photobinding compound of the present invention and nucleic acid; and b) photobinding the photobinding compound to the nucleic acid, so that a compound:nucleic acid complex is formed. Without intending to be limited to any method by which the  
5 compounds of the present invention prevent replication, it is believed that the structure of said compound:nucleic acid complex serves to prevent replication of the nucleic acid by preventing the necessary polymerase from acting in the region where the compound has bound.

#### 10 IV. INACTIVATION OF PATHOGENS

The present invention contemplates treating a blood product with a photoactivation compound and irradiating to inactivate contaminating pathogen nucleic acid sequences before using the blood product.

15

##### A. Inactivation In General

The term "inactivation" is here defined as the altering of the nucleic acid of a unit of pathogen so as to render the unit of pathogen incapable of replication. This is distinct from "total inactivation", where all pathogen  
20 units present in a given sample are rendered incapable of replication, or "substantial inactivation," where most of the pathogen units present are rendered incapable of replication. "Inactivation efficiency" of a compound is defined as the level of inactivation the compound can achieve at a given concentration of compound or dose of irradiation. For example, if 100  $\mu\text{M}$   
25 of a hypothetical compound X inactivated 5 logs of HIV virus whereas under the same experimental conditions, the same concentration of compound Y inactivated only 1 log of virus, then compound X would have a better "inactivation efficiency" than compound Y.

To appreciate that an "inactivation" method may or may not achieve  
30 "total inactivation," it is useful to consider a specific example. A bacterial culture is said to be inactivated if an aliquot of the culture, when transferred to a fresh culture plate and permitted to grow, is undetectable after a certain time period. A minimal number of viable bacteria must be applied to the plate for a signal to be detectable. With the optimum detection method,  
35 this minimal number is 1 bacterial cell. With a sub optimal detection method, the minimal number of bacterial cells applied so that a signal is observed may be much greater than 1. The detection method determines a "threshold" below which the "inactivation method" appears to be

completely effective (and above which "inactivation" is, in fact, only partially effective).

#### B. Inactivation of Potential Pathogens

5           The same considerations of detection method and threshold exist when determining the sensitivity limit of an inactivation method for nucleic acid. Again, "inactivation" means that a unit of pathogen is rendered incapable of replication.

10           In the case of inactivation methods for material to be used by humans, whether *in vivo* or *in vitro*, the detection method can theoretically be taken to be the measurement of the level of infection with a disease as a result of exposure to the material. The threshold below which the inactivation method is complete is then taken to be the level of inactivation which is sufficient to prevent disease from occurring due to  
15           contact with the material. It is recognized that in this practical scenario, it is not essential that the methods of the present invention result in "total inactivation". That is to say, "substantial inactivation" will be adequate as long as the viable portion is insufficient to cause disease. Thus "substantially all" of a pathogen is inactivated when any viable portion of  
20           the pathogen which remaining is insufficient to cause disease. The inactivation method of the present invention renders nucleic acid in pathogens substantially inactivated. In one embodiment, the inactivation method renders pathogen nucleic acid in blood preparations substantially inactivated.

25           Without intending to be limited to any method by which the compounds of the present invention inactivate pathogens, it is believed that inactivation results from light induced binding of psoralens to pathogen nucleic acid. Further, while it is not intended that the inactivation method of the present invention be limited by the nature of  
30           the nucleic acid; it is contemplated that the inactivation method render all forms of nucleic acid (whether DNA, mRNA, etc.) substantially inactivated.

          When photoactivation compounds are used to modify nucleic acid, the interaction of the pathogen nucleic acid (whether DNA, mRNA, etc.) with the photoactivation compound preferably prevents replication of the  
35           pathogen, such that, if a human is exposed to the treated pathogen, infection will not result.

          "Synthetic media" is herein defined as an aqueous synthetic blood or blood product storage media. In one embodiment, the present invention

completely effective (and above which "inactivation" is, in fact, only partially effective).

#### B. Inactivation of Potential Pathogens

5           The same considerations of detection method and threshold exist when determining the sensitivity limit of an inactivation method for nucleic acid. Again, "inactivation" means that a unit of pathogen is rendered incapable of replication.

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15           contact with the material. It is recognized that in this practical scenario, it is not essential that the methods of the present invention result in "total inactivation". That is to say, "substantial inactivation" will be adequate as long as the viable portion is insufficient to cause disease. Thus  
20           "substantially all" of a pathogen is inactivated when any viable portion of the pathogen which remaining is insufficient to cause disease. The inactivation method of the present invention renders nucleic acid in pathogens substantially inactivated. In one embodiment, the inactivation method renders pathogen nucleic acid in blood preparations substantially inactivated.

25           Without intending to be limited to any method by which the compounds of the present invention inactivate pathogens, it is believed that inactivation results from light induced binding of psoralens to pathogen nucleic acid. Further, while it is not intended that the inactivation method of the present invention be limited by the nature of  
30           the nucleic acid; it is contemplated that the inactivation method render all forms of nucleic acid (whether DNA, mRNA, etc.) substantially inactivated.

35           When photoactivation compounds are used to modify nucleic acid, the interaction of the pathogen nucleic acid (whether DNA, mRNA, etc.) with the photoactivation compound preferably prevents replication of the pathogen, such that, if a human is exposed to the treated pathogen, infection will not result.

**TABLE 3**

## Viruses Photochemically Inactivated by Psoralens

Family	Virus
5	
Adeno	Adenovirus 2
	Canine hepatitis
Arena	Pichinde
	Lassa
10	Bunya
	Turlock
	California encephalitis
	Herpes
	Herpes simplex 1
	herpes simplex 2
	Cytomegalovirus
15	Pseudorabies
Orothomyxo	Influenza
Papova	SV-40
Paramyxo	Measles
	Mumps
20	Parainfluenza 2 and 3
Picornal <sup>1</sup>	Poliovirus 1 and 2
	Coxsackie A-9
	Echo 11
25	Pox
	Vaccinia
	Fowl Pox
	Reo
	Reovirus 3
	Blue tongue
	Colorado tick fever
30	Retro
	HIV
	Avian sarcoma
	Murine sarcoma
	Murine leukemia
	Rhabdo
	Vesticular stomatitis virus
35	Toga
	Western equine encephalitis
	Dengue 2
	Dengue 4
	St. Louis encephalitis
	Hepadna
	hepatitis B
40	Bacteriophage
	Lambda
	T2
	(Rickettsia)
	R. akari (rickettsialpox)

"Synthetic media" is herein defined as an aqueous synthetic blood or blood product storage media. In one embodiment, the present invention contemplates inactivating blood products in synthetic media comprising a

buffered saline solution. This method reduces harm to blood products and permits the use of much lower concentrations of photoactivation compounds.

5 The psoralen photoinactivation method inactivates nucleic acid based pathogens present in blood through a single procedure. Thus, it has the potential to eliminate bacteria, protozoa, and viruses as well. Had an effective decontamination method been available prior to the advent of the AIDS pandemic, no transfusion associated HIV transmission would have occurred.

10 Psoralen-based decontamination has the potential to eliminate all infectious agents from the blood supply, regardless of the pathogen involved. Additionally, psoralen-based decontamination has the ability to sterilize blood products after collection and processing, which in the case of platelet concentrates could solve the problem of low level bacterial  
15 contamination and result in extended storage life. Morrow J.F., et al., JAMA 266: 555-558 (1991); Bertolini F., et al., Transfusion 32: 152-156 (1992).

A list of viruses which have been photochemically inactivated by one or more psoralen derivatives appears in Table 3. (From Table 1 of  
20 Hanson, C.V., Blood Cells 18:7 (1992)). In the article, it was pointed out that Picornaviruses, which are non-enveloped viruses, were photoinactivated only if psoralens were present during virus growth. "Non-enveloped viruses" are defined as viruses which do not have a lipid envelope surrounding the nucleic acid.

25 This list is not exhaustive, and is merely representative of the great variety of pathogens psoralens can inactivate. The present invention contemplates the inactivation of these and other viruses by the compounds described herein, including both enveloped and non-enveloped viruses.

### 30 C. Selecting Photoinactivation Compounds for Inactivation of Pathogens

In order to evaluate a compound to decide if it would be useful in the photochemical decontamination (PCD) methods of the present invention, two important properties should be considered: 1) the compound's ability  
35 to inactivate pathogens and 2) its mutagenicity. The ability of a compound to inactivate pathogens may be determined by several methods. One technique is to perform a bacteriophage screen; an assay which determines nucleic acid binding of test compounds. A screen of this type, an R17

screen, is described in detail in EXAMPLE 12, below. If the R17 screen shows inactivation activity, it is useful to directly test the compound's ability to inactivate a virus. One method of performing a direct viral inactivation screen is described in detail in EXAMPLE 27 for human parvovirus B19.

5 However, the present invention contemplates that where the target virus cannot itself be used in an inactivation assay, a model pathogen assay may be found below, or in the published literature, which is particularly appropriate to test a compound's inactivation efficiency against the target pathogen or class of pathogens. Such pathogen models can be identified and selected by finding a model which shares with the target pathogen the following elements: as close a match as possible between model and target pathogen in nucleic acid structure, composition and size (for example, RNA, DNA, single stranded or double stranded); and similar packaging, including the presence or absence of an envelope, and the composition and structure of any protein coat. A final element that must be present in a model is relative ease of use, which encompasses the following factors: ability of the model pathogen to grow in culture and relative safety, which includes considerations of the facilities and skills available to use the model. These elements can be used to determine a predictive model of target pathogen inactivation.

10  
15  
20

The R17 bacteriophage screen is believed to be predictive of the efficiency of compounds against many viruses. R17 was chosen because it was expected to be a very difficult pathogen to inactivate. It is a small, single stranded RNA phage. Without intending to be limited to any means by which the present invention operates, it is expected that shorter pieces of nucleic acid are harder to inactivate because they require a higher frequency of formation of psoralen adducts than do longer pieces of nucleic acid. Further, single stranded RNA pathogens are more difficult to inactivate because psoralens can neither intercalate between base pairs, as with double-stranded nucleic acids, nor form diadducts which function as interstrand crosslinks. Thus it is expected that when inactivation of R17 is achieved, these same conditions will cause the inactivation of many viruses and bacteria.

25  
30

The B19 screen complements the R17 screen by affirming that a given compound which has tested positive in R17 will actually work effectively to inactivate human parvoviruses. Thus, if a compound shows activity in the R17 screen, it is next tested in the viral inactivation screen.

35

The second property that is important in testing a compound for use



in methods of the present invention is mutagenicity. The most widely used mutagen/carcinogen screening assay is the Ames test. This assay is described by D.M. Maron and B.N. Ames in *Mutation Research* 113: 173 (1983) and a specific screen is described in detail in Example 17, below. The Ames test utilizes several unique strains of *Salmonella typhimurium* that are histidine- dependent for growth and that lack the usual DNA repair enzymes. The frequency of normal mutations that render the bacteria independent of histidine (i.e., the frequency of spontaneous revertants) is low. The test allows one to evaluate the impact of a compound on this revertant frequency.

Because some substances are not mutagenic by themselves, but are converted to a mutagen by metabolic action, the compound to be tested is mixed with the bacteria on agar plates along with the liver extract. The liver extract serves to mimic metabolic action in an animal. Control plates have only the bacteria and the extract.

The mixtures are allowed to incubate. Growth of bacteria (if any) is checked by counting colonies. A positive Ames test is one where the number of colonies on the plates with mixtures containing the compound significantly exceeds the number on the corresponding control plates.

When known carcinogens are screened in this manner with the Ames test, approximately ninety percent are positive. When known non carcinogens are similarly tested, approximately ninety percent are negative.

A new compound (X) can be evaluated as a potential blood photodecontamination compound for B19, as shown in Table 4, below. X is initially evaluated in Step I. X is screened in the R17 assay at several different concentrations between 4 and 320  $\mu\text{M}$ , as explained in EXAMPLE 12. If the compound shows inactivation activity greater than 1 log inactivation of R17 (log kill) in the R17 screen at any concentration, the compound is then screened in the B19 assay, as explained in Example 27. If the compound shows suppression of infection by allowing the appearance of BFU-E, the compound and AMT are then screened in the Ames assay. Finally, if the compound shows lower mutagenicity in the Ames assay than does AMT, the new compound is identified as a useful agent for inactivation of pathogens.

TABLE 4

STEP	SCREEN	RESULT	INTERPRETATION
1	R17	>1 log kill by any concentration	potential PCD compound, go to step 2
		<1 log kill	compound is ineffective as an inactivation treatment
2	Viral Inactivation	> 1 log kill by any concentration	potential PCD compound, go to step 3
		< 1 log kill	compound is ineffective as an inactivation treatment
3	Ames	less mutagenic than AMT	useful agent for PCD

By following these instructions, a person can quickly determine which compounds would be appropriate for use in methods of the present invention.

#### D. Delivery of Compounds for Photoinactivation

The present invention contemplates several different formulations and routes by which the compounds described herein can be delivered in an inactivation method. This section is merely illustrative, and not intended to limit the invention to any form or method of introducing the compound.

The compounds of the present invention may be introduced in an inactivation method in several forms. The compounds may be introduced as an aqueous solution in water, saline, a synthetic media such as "Sterilyte™ 3.0" (contents set forth at the beginning of the Experimental section, below) or a variety of other media. The compounds can further be provided as dry formulations, with or without adjuvants.

The new compounds may also be provided by many different routes. For example, the compound may be introduced to the reaction vessel, such as a blood bag, at the point of manufacture. Alternatively, the compound may be added to the material to be sterilized after the material has been placed in the reaction vessel. Further, the compounds may be introduced alone, or in a "cocktail" or mixture of several different compounds.

## V. PRESERVATION OF BIOCHEMICAL PROPERTIES OF MATERIAL TREATED

When treating blood products to be used in vivo, two factors are of  
5 paramount importance in developing methods and compounds to be used.  
First, one must ask whether the process or the compounds used alter the in  
vivo activity of the treated material. For example, platelet transfusion is a  
well established efficacious treatment for patients with thrombocytopenic  
bleeding. However, if the decontamination treatment used greatly reduces  
10 the platelets clotting activity, then the treatment has no practical value.  
Psoralens are useful in inactivation procedures, because the reaction can be  
carried out at temperatures compatible with retaining biochemical  
properties of blood and blood products. Hanson, C.V., Blood Cells 18:7  
(1992). But not all psoralens or methods will decontaminate without  
15 significantly lowering the biological activity of the decontaminated  
material. Previous compounds and protocols have necessitated the  
removal of molecular oxygen from the reaction before and during exposure  
to light, to prevent damage to blood products from oxygen radicals  
produced during irradiation. See L. Lin et al., Blood 74:517 (1989); US Patent  
20 No. 4,727,027, to Wieseahn. The present invention may be used to  
decontaminate blood products, in the presence of oxygen, without  
destroying the activity for which the products are prepared. The present  
invention contemplates that in vivo activity of a blood product is not  
destroyed or significantly lowered if a sample of blood product which is  
25 decontaminated by methods of the present invention tests as would a  
normally functioning sample of blood product in known assays for blood  
product function. For example, where platelets are concerned, in vivo  
activity is not destroyed or significantly lowered if pH of the platelets are  
substantially the same in platelets treated by the methods of the present  
30 invention and stored 5 days as they are in untreated samples stored for 5  
days. "Substantially the same" pH means that the values fall within the  
range of error surrounding that particular data point.

The present invention contemplates a preferred way of protecting  
blood and blood products during irradiation by introducing a synthetic  
35 media comprising a buffered saline solution for the period of irradiation  
and any storage thereafter. In a preferred embodiment of the present  
invention, dibasic phosphate is used as a buffer. Dibasic phosphate contains  
both singly charged ions and a doubly charged ions.

The second factor is whether the compounds used are toxic or mutagenic to the patient treated. A "compound displaying low mutagenicity" is defined as a compound which is less mutagenic than AMT when it is tested at concentrations below 250  $\mu$ M in the Ames assay, 5 described in the Experimental section, below. The inactivation compounds and methods of the present invention are especially useful because they display the unlinking of pathogen inactivation efficiency from mutagenicity. The compounds exhibit powerful pathogenic inactivation without a concomitant rise in mutagenicity. The commonly known 10 compounds tested in photoinactivation protocols, such as AMT, appear to exhibit a link between pathogen inactivation efficiency and mutagenetic action that until now seemed indivisible.

While it is not intended that the present invention be limited to any theory by which pathogen inactivation efficiency is unlinked from 15 mutagenicity, it is postulated that unlinking occurs as a result of the length of the groups substituted on the psoralen, and the location of charges on the compounds. It is postulated that positive charges on one or both ends of mutagenic compounds have non-covalent interactions with the phosphate backbone of DNA. These interactions are presumed to occur independent 20 of the presence of light (called "dark binding"). In theory, the psoralen thereby sterically blocks polymerase from opening up the DNA, causing mutagenicity. In contrast, compounds of the present invention carry a positive or neutral charge on a long substitute group. These substituted groups form a steric barrier during dark binding that is much easier to free 25 from the DNA, permitting polymerase to pass. Thus no mutagenicity results.

### EXPERIMENTAL

30 The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: eq (equivalents); M (Molar);  $\mu$ M (micromolar); N 35 (Normal); mol (moles); mmol (millimoles);  $\mu$ mol (micromoles); nmol (nanomoles); g (grams); mg (milligrams);  $\mu$ g (micrograms); Kg (kilograms); L (liters); mL (milliliters);  $\mu$ L(microliters); cm (centimeters); mm (millimeters);  $\mu$ m (micrometers); nm (nanometers); J (Joules, also watt

second, note that in FIGS. 7, 9 - 18 Joules or J refers to Joules/cm<sup>2</sup>); °C (degrees Centigrade); TLC (Thin Layer Chromatography); EAA (ethyl-acetoacetate); EtOH (ethanol); HOAc (acetic acid); W (watts); mW (milliwatts); NMR (Nuclear Magnetic Resonance; spectra obtained at room  
5 temperature on a Varian Gemini 200 MHz Fourier Transform Spectrometer); m.p. (melting point); UV (ultraviolet light); THF (tetrahydrofuran); DMEM (Dulbecco's Modified Eagles Medium); FBS (fetal bovine serum); LB (Luria-Bertani); EDTA (ethelene diamine tetracidic acid); Phorbol Myristate Acetate (PMA); PBS (phosphate buffered saline); D-PBS  
10 (Dulbecco's phosphate buffered saline); BSA (bovine serum albumin); PCR (polymerase chain reaction); RCF (relative centrifugal force); IMDM (Iscove's Modified Dulbecco's Medium). Further, in the examples describing synthesis of compounds of the present invention, yields presented are for the preceeding step only, rather than for the entire  
15 synthesis.

For ease of reference, some compounds of the present invention have been assigned a number from 1 - 18. The reference numbers that are assigned in Table 2 are used throughout the experimental section.

When isolating compounds of the present invention in the form of  
20 an acid addition salt, the acid is preferably selected so as to contain an anion which is non-toxic and pharmacologically acceptable, at least in usual therapeutic doses. Representative salts which are included in this preferred group are the hydrochlorides, hydrobromides, sulphates, acetates, phosphates, nitrates, methanesulphonates, ethanesulphonates, lactates,  
25 citrates, tartrates or bitartrates, and maleates. Other acids are likewise suitable and may be employed as desired. For example, fumaric, benzoic, ascorbic, succinic, salicylic, bismethylenesalicylic, propionic, gluconic, malic, malonic, mandelic, cinnamic, citraconic, stearic, palmitic, itaconic, glycolic, benzenesulphonic, and sulphamic acids may also be employed as acid  
30 addition salt-forming acids.

One of the examples below refers to HEPES buffer. This buffer contains 8.0 g of 137 mM NaCl, 0.2 g of 2.7 mM KCl, 0.203 g of 1 mM MgCl<sub>2</sub>(6H<sub>2</sub>O), 1.0g of 5.6 mM glucose, 1.0 g of 1mg/ml Bovine Serum Albumin (BSA) (available from Sigma, St. Louis, MO), and 4.8 g of 20 mM  
35 HEPES (available from Sigma, St. Louis, MO).

In one of the examples below, phosphate buffered synthetic media is formulated for platelet treatment. This can be formulated in one step, resulting in a pH balanced solution (e.g. pH 7.2), by combining the following

reagents in 2 liters of distilled water:

Preparation of Sterilyte™ 3.0			
	Formula W.	mMolarity	Grams/2 Liters
NaAcetate*3H <sub>2</sub> O	136.08	20	5.443
Glucose	180.16	2	0.721
D-mannitol	182.17	20	7.287
KCl	74.56	4	0.596
NaCl	58.44	100	11.688
Na <sub>3</sub> Citrate	294.10	10	5.882
Na <sub>2</sub> HPO <sub>4</sub> *7H <sub>2</sub> O	268.07	14.46	7.752
NaH <sub>2</sub> PO <sub>4</sub> *H <sub>2</sub> O	137.99	5.54	1.529
MgCl <sub>2</sub> *6H <sub>2</sub> O	203.3	2	0.813

- 5 The solution is then mixed, sterile filtered (0.2 micron filter) and refrigerated.

Another synthetic media (hereinafter referred to as "synthetic media plus phosphate") contains the following reagents:

Preparation of synthetic media plus phosphate			
	Formula W.	mMolarity	Grams/Liters
NaAcetate*3H <sub>2</sub> O	136.08	30	4.08
NaCl	58.44	86	5.02
Citrate*2H <sub>2</sub> O	294.10	10	2.94
Na <sub>2</sub> HPO <sub>4</sub>	142.07	19.8	2.81
NaH <sub>2</sub> PO <sub>4</sub> *H <sub>2</sub> O	137.99	6.2	0.858

- 10 The Polymerase Chain Reaction (PCR) is used in one of the examples to measure whether viral inactivation by some compounds was complete. PCR is a method for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. See K.B. Mullis et al., US Patents Nos. 4,683,195 and 4,683,202, hereby incorporated by reference. This process for amplifying the target sequence consists of introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase. The two primers are complementary to their respective strands
- 15

of the double stranded target sequence. To effect amplification, the mixture is denatured and the primers then to annealed to their complementary sequences within the target molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing, and polymerase extension can be repeated many times (i.e. denaturation, annealing and extension constitute one "cycle;" there can be numerous "cycles") to obtain a high concentration of an amplified segment of the desired target sequence. The length of the amplified segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to by the inventors as the "Polymerase Chain Reaction". Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified".

With PCR, it is possible to amplify a single copy of a specific target sequence in genomic DNA to a level detectable by several different methodologies (e.g. hybridization with a labeled probe; incorporation of biotinylated primers followed by avidin-enzyme conjugate detection; incorporation of  $^{32}\text{P}$  labeled deoxynucleotide triphosphates, e.g. dCTP or dATP, into the amplified segment). In addition to genomic DNA, any oligonucleotide sequence can be amplified with the appropriate set of primer molecules.

The PCR amplification process is known to reach a plateau concentration of specific target sequences of approximately  $10^{-8}$  M. A typical reaction volume is 100  $\mu\text{l}$ , which corresponds to a yield of  $6 \times 10^{11}$  double stranded product molecules.

PCR is a polynucleotide amplification protocol. The amplification factor that is observed is related to the number (n) of cycles of PCR that have occurred and the efficiency of replication at each cycle (E), which in turn is a function of the priming and extension efficiencies during each cycle. Amplification has been observed to follow the form  $E^n$ , until high concentrations of PCR product are made. At these high concentrations (approximately  $10^{-8}$  M/l) the efficiency of replication falls off drastically. This is probably due to the displacement of the short oligonucleotide primers by the longer complementary strands of PCR product. At concentrations in excess of  $10^{-8}$  M, the rate of the two complementary PCR

amplified product strands finding each other during the priming reactions become sufficiently fast that this occurs before or concomitant with the extension step of the PCR procedure. This ultimately leads to a reduced priming efficiency, and therefore, a reduced cycle efficiency. Continued  
5 cycles of PCR lead to declining increases of PCR product molecules. PCR product eventually reaches a plateau concentration.

The sequences of the polynucleotide primers used in this experimental section are as follows:

DCD03: 5' ACT AGA AAA CCT CGT GGA CT 3'  
10 DCD05: 5' GGG AGA GGG GAG CCC GCA CG 3'  
DCD06: 5' CAA TTT CGG GAA GGG CAC TC 3'  
DCD07: 5' GCT AGT ATT CCC CCG AAG GT 3'

With DCD03 as a common forward primer, the pairs generate amplicons of  
15 length 127, 327, and 1072 bp. These oligos were selected from regions that are absolutely conserved between 5 different dHBV isolates (DHBV1, DHBV3, DHBV16, DHBV22, and DHBV26) as well as from heron HBV (HHBV4).

The following examples serve to illustrate certain preferred  
20 embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

### EXAMPLE 1

As noted above, the present invention contemplates devices and  
25 methods for the photoactivation of photoreactive nucleic acid binding compounds. In this example, a photoactivation device is described for decontaminating blood products according to the method of the present invention. This device comprises: a) means for providing appropriate wavelengths of electromagnetic radiation to cause photoactivation of at  
30 least one photoreactive compound; b) means for supporting a plurality of blood products in a fixed relationship with the radiation providing means during photoactivation; and c) means for maintaining the temperature of the blood products within a desired temperature range during photoactivation.

35 FIG. 6 is a perspective view of one embodiment of the device integrating the above-named features. The figure shows an opaque housing (100) with a portion of it removed, containing an array of bulbs (101) above and below a plurality of representative blood product



containing means (102) placed between plate assemblies (103, 104) which filter certain wavelengths of light. The plate assemblies (103, 104) are described more fully, subsequently.

5 The bulbs (101), which are connectable to a power source (not shown), serve as a source of electromagnetic radiation. While not limited to the particular bulb type, the embodiment is configured to accept an industry standard, dual bipin lamp.

10 The housing (100) can be opened via a latch (105) so that the blood product can be placed appropriately. As shown in FIG. 6, the housing (100), when closed, completely contains the irradiation from the bulbs (101). During irradiation, the user can confirm that the device is operating by looking through a safety viewport (106) which does not allow transmission of ultraviolet light to the user.

15 The housing (100) also serves as a mount for several electronic components on a control board (107), including, by way of example, a main power switch, a count down timer, and an hour meter. For convenience, the power switch can be wired to the count down timer which in turn is wired in parallel to an hour meter and to the source of the electromagnetic radiation. The count down timer permits a user to preset the irradiation  
20 time to a desired level of exposure. The hour meter maintains a record of the total number of radiation hours that are provided by the source of electromagnetic radiation. This feature permits the bulbs (101) to be monitored and changed before their output diminishes below a minimum level necessary for rapid photoactivation.

25 When the housing is open, a reflector completely surrounds each array of bulbs. Blood product containing means are placed between upper and lower plate assemblies (e.g. BK-7 glass, Shott Glass Technologies, Inc., Duryea, PA). Each plate assembly is comprised of an upper and lower plates. The plate assemblies are connected via a hinge which is designed to  
30 accommodate the space created by the blood product containing means. The upper plate assembly is brought to rest just above the top of the blood product containing means supported by the lower plate of the lower plate assembly.

35 Detectors may be conveniently placed between the plates of the plate assemblies. They can be wired to a printed circuit board which in turn is wired to the control board.

Six blood product containing means (e.g. Teflon™ platelet unit bags) may be placed in a fixed relationship above an array of bulbs. The

temperature of the blood product can be controlled via a fan alone or, more preferably, by employing a heat exchanger having cooling inlet and outlet ports connected to a cooling source.

Upper plate assembly plates and lower plate assembly plates each  
5 create a temperature control chamber. The fan can circulate air within and between the chambers. When the heat exchanger is employed, the circulating air is cooled and passed between the plates.

Further description of this device appears in U.S. Patent No.  
5,399,719.

10

## EXAMPLE 2

### Synthesis of 4'-Bromomethyl-4,5',8-trimethylpsoralen

In this example, the three step synthesis of 4'-Bromomethyl-4,5',8-  
15 trimethylpsoralen is described. This synthesis is performed without a bromomethylation step, making it safer than known methods of synthesis.

Step 1: 3-Chloro-2-butanone (29.2 mL, 0.289 mol) was added to a  
20 mechanically stirred suspension of 7-hydroxy-4,8-dimethylcoumarin (50.00 g, 0.263 mol) and powdered K<sub>2</sub>CO<sub>3</sub> (54 g, 0.391 mol) in acetone (500 mL). The slurry was refluxed for 15 hours, after which the solvent was stripped off. To remove the salt, the solid was stirred in 1.2 L of water, filtered, and rinsed with water until the pH of the mother liquor was  
25 neutral (pH 5-7). The brown filtrate was dissolved in boiling methanol (150 mL), allowed to cool to room temperature to form a thick paste and rinsed with ice cold methanol to remove most of the brown impurity, giving 4,8-dimethyl-7-(1-methyl-2-oxo)propyloxy-coumarin (67.7 g, 99.0% yield) as an off-white solid, melting point 95-96°C. NMR: d 1.57 (d, J = 6.7  
30 Hz, 3H), 2.19 (s, 3H), 2.39 (s, 6H), 4.73(q, J = 6.9 Hz, 1H), 6.17 (s, 1H), 6.63 (d, J = 8.8 Hz, 1H), 7.38 (d, J = 8.9 Hz, 1H).

Step 2: A suspension of 4,8-dimethyl-7-(1-methyl-2-oxo)propyloxy-coumarin (67.5g, 0.260 mol), 10% aqueous NaOH (114 mL, 0.286 mol) and water (900 mL) was heated for 2-4 hours at 70-85°C. The mixture was then  
35 allowed to cool to room temperature. The solid was filtered, and then rinsed with chilled water (1.5 L) until the mother liquor became colorless and pH was neutral (pH 5-7). The product was air and vacuum dried to give 4, 4',5',8-tetramethylpsoralen (56.3 g, 89.5%) as a white solid, mp 197-

199°C. NMR: d 2.19 (s, 3H), 2.42 (s, 3H), 2.51 (s, 3H), 2.56 (s, 3H), 6.23 (s, 1H), 7.40 (s, 1H).

Step 3: Dry 4,4',5',8-tetramethylpsoralen (10.00g, 41.3mmol) was dissolved  
5 in methylene chloride (180 mL) at room temperature. N-Bromosuccinimide (8.09g, 45.3 mmol) was added and the reaction mixture and stirred 4.5 hours. The solvent was completely removed and the resulting solid was stirred with water (200mL) for 0.5-1 h, filtered and cold triturated with additional water (approximately 500mL) to remove the  
10 succinimide by-product. The crude product (i.e. 4'-bromomethyl-4, 5',8-trimethylpsoralen) was dried in a vacuum dessicator with P<sub>2</sub>O<sub>5</sub> then recrystallized in a minimum amount of boiling toluene (200-300mL) to give 4'-bromomethyl-4, 5',8-trimethylpsoralen (10.2g), a pale yellow solid. The mother liquor was stripped and recrystallized again with toluene  
15 (60mL) to give a second crop of product (1.08g, combined yield = 85.1%, > 99% purity by NMR), mp 206-207°C. NMR: d 2.50 (s, 3H), 2.54 (d, J = 1.2 Hz, 3H), 2.58 (s, 3H), 4.63 (s, 2H), 6.28 (apparent q, J = 1.3 Hz, 1H), 7.59 (s,1H).

20

### EXAMPLE 3

#### Synthesis of 5'-bromomethyl-4, 4',8-trimethylpsoralen

In this example, a three step synthesis of 5'-bromomethyl-4, 4',8-trimethylpsoralen is described. Like the synthesis described in Example 2,  
25 this method is improved upon previously known synthesis schemes because it does not require bromomethylation.

4, 4',5',8-Tetramethylpsoralen (2.33 g, 9.59 mmol), (synthesis described in Example 2, Steps 1 and 2), was refluxed in carbon tetrachloride (100 mL) until it dissolved. N-Bromosuccinimide (1.88 g, 10.5 mmol) and  
30 benzoyl peroxide (80 mg) were then added and the mixture was refluxed for 15 hours. Upon cooling to room temperature methylene chloride (100mL) was added to dissolve the solid and the solution was washed with water (4 x 150 mL), then brine, and dried with anhydrous Na<sub>2</sub>SO<sub>4</sub>. The solvent was stripped off to give a mixture of 5'-bromomethyl-4, 4',8-trimethylpsoralen,  
35 4'-bromomethyl-4, 5',8-trimethylpsoralen, and 4',5'-bis(bromomethyl)-4,8-dimethylpsoralen in a ratio of 55/25/20 respectively as determined by <sup>1</sup>H NMR (3.0 g, crude product). <sup>1</sup>H NMR of 5'-bromomethyl compound: d 2.29 (s, 3H), 2.52 (d, J = 1.2 Hz, 3H), 2.60 (s, 3H), 4.64 (s, 2H), 6.27 (apparent d, J

= 1.2 Hz, 1 H), 7.51 (s,1H). <sup>1</sup>H NMR of 4',5'-bis(bromomethyl) compound: d 2.54 (d, J =1.1 Hz, 3H), 2.60 (s, 3H), 4.65 (s, 4H), 6.30 (apparent q, J =1.1 Hz, 1H), 7.67 (s, 1H).

5

**EXAMPLE 4**

Synthesis of 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen Hydrochloride (Compound 2) and Related Compounds (Compound 4)

In this example, two methods of synthesis of Compound 2 are described. The first method was performed as follows:

Step 1: 4'-Bromomethyl-4,5',8-trimethylpsoralen (3.09 g, 9.61 mmol), (synthesis described in Example 2), and N-(2-hydroxyethyl)phthalimide (4.05 g, 21.2 mmol) were stirred in dry dimethylformamide (65 mL). Dry N<sub>2</sub> gas was bubbled gently into the reaction mixture. The reaction mixture was heated to 100 °C for 4.5 hours then allowed to cool to room temperature and put in the freezer for several hours. The crystalline product was filtered and washed with MeOH followed by H<sub>2</sub>O. The solid was further triturated with MeOH (100 mL) to remove the impurities. The crude product was air dried and dissolved in CHCl<sub>3</sub> (150 mL). Activated carbon and silica gel were added to decolorize and the CHCl<sub>3</sub> was completely removed. The resulting white product, 4'-[4-(N-phthalimido)-2-oxa]butyl-4,5',8-trimethylpsoralen (1.56 g, yield 37.5 %) was ≥99% pure both by NMR and HPLC; mp 224-225 °C. NMR (CDCl<sub>3</sub>) δ 2.37 (s,3H); 2.47 (s, 3H); 2.48 (s, 3H); 3.78 (s,4H); 4.59 (s,2H); 6.22 (s, 1H);7.42 (s,1H); 7.50 (m, 4H).

Step 2: 4'-[4-(N-phthalimido)-2-oxa]butyl-4,5',8-trimethylpsoralen (1.56 g, 3.61 mmol) was suspended in tetrahydrofuran (75 mL) at room temperature. Methylamine (40 % aqueous solution, 25 mL, 290 mmol) was added to the suspension and stirred overnight. The solvent and methylamine were completely removed. The resulting solid was taken up in 0.3 N HCl aqueous solution (25 mL). The acid suspension was rinsed three times with 40 mL CHCl<sub>3</sub> then taken to pH 11 with 20 % aqueous NaOH. CHCl<sub>3</sub> (3x60 mL) was used to extract the product (i.e. 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen) from the basified layer. The combined CHCl<sub>3</sub> layers were washed with H<sub>2</sub>O (100 mL) followed by brine (100 mL) then dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated to give 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen, mp 139-141 °C. Purity was greater than

99 % by NMR. NMR (CDCl<sub>3</sub>) δ 2.50 (s, 6H); 2.58 (s,3H); 2.90 (t, J = 5.27 Hz, 2H); 3.53 (t, J = 5.17 Hz, 2H); 4.66 (s, 2H); 6.25 (s, 1H); 7.61 (s, 1H). The 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen was dissolved in absolute ethanol (150 mL), a 1.0 M solution of HCl in ether (10 mL) was added and the suspension was cooled in the freezer overnight. After filtration and washing with ether, the solid was vacuum dried to give pale yellow crystals (0.76 g, yield 62 %), mp 235-236 °C.

Alternatively. Step 2 may be performed using either hydrazine or butylamine rather than methylamine. The method which uses butylamine is preferred for larger scale syntheses because, while an excess of methylamine is needed due to volatilization, the same is not true for butylamine. The method using butylamine was carried out as follows: 28.3 g phthalimide has been deprotected with n-butylamine in propanol. The crude reaction solution is then treated with HCl to precipitate out the product. Thus the reaction mixture in 285 mL of 1-propanol was treated with HCl gas to pH 2. The mixture was stirred at 5 °C for 0.5 hours, then filtered and washed with cold solvent (3 x 15 mL) to afford 20.5 g of crude 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen (92% yield).

The method using hydrazine was carried out as follows: The phthalimide precursor (6 mol) was deprotected with hydrazine and after concentration and acid-base extractions the crude amine was obtained in 30 L of ethylene dichloride. To this was added HCl gas (0.14Kg) via dispersion tube over 40 minutes maintaining the temperature at 15-25 °C. The resultant slurry was stirred an additional 1 hour. The solids were collected on a Buchner funnel. Upon drying in an air dryer at 80 °C for 2 hours, 0.945 kg of crude 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen was obtained. 0.94 kg of product in a mixture of 7.5 kg of isopropanol and 1.88 kg of water was refluxed for 30 minutes then hot filtered. The solution was cooled to room temperature over 1 hour, then chilled to 15-20 °C for 0.5 hours. The solids were collected on a Buchner funnel, then washed with cold isopropanol (0.3 L). The wet solids were transferred to glass trays and dried under vacuum (>28 minutes) at approximately 75 °C for 11.5 hours. Moisture content was 0.5%. Yield was 0.758 kg (81% yield). The 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen was analytically pure. Residual isopropanol about 1700 ppm by NMR.

The first method is a preferred embodiment of the present invention because of its high yield and purity.

The second method starts with the preparation of 4'-chloromethyl-

4,5',8-trimethylpsoralen from commercially available 4,5',8-trimethylpsoralen, as described above. The synthesis of 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen hydrochloride is achieved in four (4) steps, as described in U.S. Patent No. 5,399,719 (incorporated by reference above) in "Example 2", starting with 4'-Chloromethyl-4,5',8-trimethylpsoralen and yielding 1.25 g of Compound 2, mp 236° C (decomp). <sup>13</sup>C NMR: 8.54, 12.39, 19.18, 38.75, 62.26, 65.80, 108.01, 112.04, 112.42, 112.97, 116.12, 125.01, 148.76, 153.97, 154.37, 155.76, 160.34.

Similarly prepared, by reacting 4'-CMT with 1,3-propanediol comparably to Step 1 and proceeding analogously through Step 4, was 4'-(5-amino-2-oxa)pentyl-4,5',8-trimethylpsoralen, (Compound 4), m.p. 212-214 °C (decomposed). NMR of the free base:  $\delta$  1.73 (pent, J=6.4 Hz, 2H), 2.45(s, 6H), 2.51 (s, 3H), 2.78 (t,J=6.8 Hz, 2H), 3.54 (t, J=6.2 Hz, 2H), 4.59 (s,2H), 6.18 (s, 1H), 7.54 (s, 1H).

15

#### EXAMPLE 5

##### Synthesis of 5'-(4-Amino-2-oxa)butyl-4,4',8-trimethylpsoralen (Compound 18)

This example describes the synthesis of Compound 18. To a stirred solution of N-methylformanilide (16.0 mL, 0.134 mol) in acetonitrile (130 mL) was added phosphorus oxychloride (12.5 mL, 0.134 mol), then 4,4',8-trimethylpsoralen (5.0 g, 21.9 mmol) (described in McLeod, et al., Tetrahedron Letters No. 3:237 (1972)). The temperature was kept between 0-10 °C during addition of the psoralen by use of an ice/water bath. The slurry was stirred at 50°C for 2 days protected from moisture with a drierite drying tube. The reaction mix was allowed to cool to room temperature, then chilled in an ice/water bath. The acetonitrile was decanted off, then ice/water (150 mL) was added to the orange slurry and stirred for 1h. The orange solid was filtered off and rinsed with chilled water, then chilled acetonitrile. The crude product was recrystallized and charcoal decolorized in dichloroethane (600 mL) to give 4,4',8-trimethyl-5'-psoralencarboxaldehyde (3.59g, 64.0%) as a pale yellow-orange solid, sublimes  $\geq$  250°C, decomp. > 300°C. <sup>1</sup>H NMR (CDCl<sub>3</sub>): 2.54 (d, J = 1 Hz, 3H), 2.64 (s, 3H), 2.68 (s, 3H), 6.32 (apparent d, J = 1 Hz, 1H), 7.75 (s, 1H), 10.07 (s, 1H).

35

4,4',8-trimethyl-5-psoralencarboxaldehyde (7.50 g, 29.3 mmol) was stirred in 200 proof EtOH (250mL). Sodium borohydride was added and the

slurry was stirred overnight. Ice water (150mL) and 10% aq NaCO<sub>3</sub> (50mL) were added to quench the reaction. After stirring for 45 min, the precipitate was filtered off and rinsed with water until the filtrate was neutral (pH 5-7). The product was dried in a vacuum desiccator with P<sub>2</sub>O<sub>5</sub> to give 5'-

5 hydroxymethyl-4,4',8-trimethylpsoralen (7.46 g, 98.5%) as a pale yellow solid, mp 244-245°C. <sup>1</sup>H NMR (CDCl<sub>3</sub>): 1.97 (t, J = 6 Hz, 1H), 2.31 (s, 3H), 2.51 (d, J = 1 Hz, 3H), 2.58 (s, 3H), 4.79 (d, J = 6 Hz, 2H), 6.25 (apparent d, J = 1 Hz, 1H), 7.49 (s, 1H).

To a stirred, ice/water chilled slurry of 5'-hydroxymethyl-4,4',8-trimethylpsoralen (15.42 g, 59.7 mmol) in dichloroethane (500mL) was added phosphorus tribromide (6.17 mL, 65.7 mmol) dropwise. The reaction was protected from moisture and allowed to stir overnight at room temperature. The mixture was then stirred with 300 mL ice/water for 1h. The solid was filtered off, dried, dissolved in hot toluene, filtered through  
15 fluted filter paper and stripped to give 5'-bromomethyl-4,4',8-trimethylpsoralen (3.43 g). The reaction solvents (dichloroethane and water) were separated and the aqueous layer was extracted three times with dichloroethane. The organic layers were combined, rinsed with brine then dried (anhyd Na<sub>2</sub>SO<sub>4</sub>) and stripped under vacuum to give the bulk of the  
20 product, 5'-bromomethyl-4,4',8-trimethylpsoralen, (13.13g, combined yield of 86.4%), as a pale yellow solid, mp 201-202 °C. <sup>1</sup>H NMR (CDCl<sub>3</sub>): 2.29 (s, 3H), 2.52 (d, J = 1 Hz, 3H), 2.60 (s, 2H), 4.64 (s, 2H), 6.27(apparent d, J = 1Hz, 1H), 7.51 (s, 1H)

N-Hydroxyethylphthalimide (3.00 g, 15.5 mmol) was dissolved in  
25 DMF (5 mL) at 60-64°C while N<sub>2</sub> was bubbled into the solution. Sodium iodide (0.01 g, 0.067 mmol) and 5'-bromomethyl-4,4',8-trimethylpsoralen (1.00 g, 3.11 mmol) were added and the slurry was stirred under these conditions overnight. The thick yellow reaction mixture was allowed to cool to room temperature, chilled in an ice/water bath, filtered and rinsed  
30 with ice cold MeOH to give crude product (1 g). The solid was recrystallized in dichloroethane (100 mL) to give 4,4',8-trimethyl-5'-[2-(N-phthalimido)-2-oxa]butylpsoralen (0.68 g, 50.8%), as an off-white solid, mp 225-228°C. <sup>1</sup>H NMR (CDCl<sub>3</sub>): 2.26 (s, 3H), 2.46 (s, 3H), 2.51 (d, J = 1 Hz, 3H), 3.87 (m, 4H), 4.64 (s, 2H), 6.26 (apparent d, J = 1 Hz, 1H), 7.42 (s, 1H), 7.64 (multiplet, 4H).

35 4,4',8-Trimethyl-5'-[4'-(N-phthalimido)-2-oxa]butylpsoralen (1.61 g, 3.73 mmol) was stirred with THF (40 mL) and 40 wt% aq methylamine (20 mL, 257 mmol) overnight. The solvent was stripped and the residue was partitioned between dilute aq HCl and dichloromethane. The aqueous layer

was rinsed several more times with dichloromethane then made basic with  $K_2CO_3$ . The base layer was extracted three times with dichloromethane.

The combined organic extracts from the base shaken with brine then dried (anhydrous  $Na_2SO_4$ ) and stripped to give 5'-(4-amino-2-oxa)butyl-4,4',8-trimethylpsoralen (0.71 g, 63.4%), mp 126-129°C.  $^1H$  NMR ( $CDCl_3$ ): 2.30 (s, 3H), 2.51 (s, 3H), 2.58 (s, 3H), 2.91 (t, J = 5 Hz, 2H), 3.59 (t, J = 5Hz, 2H), 4.64 (s, 2H), 6.25 (s, 1H), 7.50 (s, 1H).

The above amine (0.71 g, 2.36 mmol) was dissolved in hot ethanol, converted to the acid with 1M HCl in diethylether (3 mL, 3 mmol), decolorized with charcoal, cooled and collected. The solid was decolorized again with charcoal and stripped to give 5'-(4-amino-2-oxa)butyl-4,4',8-trimethylpsoralen hydrochloride (0.39 g, 49.3% yield) as a white solid, mp 235-236 °C. (Note: Other preparations of this material have given a product with a significantly lower melting point, but identical NMR spectra).  $^1H$  NMR ( $d_6$ -DMSO): 2.32 (s, 3H), 2.45 (s, 3H), 2.50 (s, 3H), 3.00 (m, 2H), 3.71 (t, J = 5 Hz, 2H), 4.71 (s, 2H), 6.33 (s, 1H), 7.79 (s, 1H), 8.15 (br).  $^{13}C$  NMR ( $d_6$ -DMSO): 7.93, 8.57, 19.01, 38.74, 62.66, 66.28, 108.22, 112.42, 113.69, 115.34, 116.06, 125.60, 149.38, 150.95, 154.26 (tentatively 2 carbons), 160.26.

20

### EXAMPLE 6

#### Synthesis of 4'-(7-amino-2,5-oxa)heptyl-4,5',8-trimethylpsoralen Hydrochloride (Compound 7)

In this example, the synthesis of Compound 7 is described. The synthesis of 4'-(7-amino-2,5-oxa)heptyl-4,5',8-trimethylpsoralen hydrochloride proceeds in four (4) steps, as described in U.S. Patent No. 5,399,719 (incorporated by reference above) in "Example 3", starting from 4'-Chloromethyl-4,5',8-trimethylpsoralen (589 mg, 2.13 mmol).and yielding 63.9 mg of product, 4'-(7-amino-2,5-oxa)heptyl-4,5',8-trimethylpsoralen, (56 %). TLC showed only one spot. NMR  $\delta$  2.50 (s, 3H); 2.50 (s, 3H); 2.57 (s, 3H); 2.86 (t, J=5.3 Hz, 2H); 3.50 (t, J=5.3 Hz, 2H); 3.63 (s, 4H); 4.70 (s, 2H); 6.24 (s, 1H); 7.62 (s, 1H). m.p. 170-173 °C.

The solid was dissolved in absolute ethanol, then 1M HCl in ethyl ether was added, the suspension was filtered and the product rinsed with ether and dried.

35



**EXAMPLE 7**

Synthesis of 4'-(12-amino-8-aza-2,5-dioxa)dodecyl-4,5',8-trimethylpsoralen  
Dihydrochloride (Compound 8)

5

The synthesis of 4'-(12-amino-8-aza-2,5-dioxa)dodecyl-4,5',8-trimethylpsoralen dihydrochloride proceeds as described in U.S. Patent No. 5,399,719, in "Example 4", starting with 4'-(7-methanesulfonyloxy-2,5-oxa)heptyl-4,5',8-trimethylpsoralen (108 mg, 0.253 mmol) and yielding 63  
10 mg of product, 4'-(12-amino-8-aza-2,5-dioxa)dodecyl-4,5',8-trimethylpsoralen dihydrochloride (60%). NMR  $\delta$  1.45 (m, 2H), 2.49 (s, 6H), 2.55 (s, 3H), 2.58 (t, 2H), 2.66 (t, J=5.6 Hz, 2H), 2.76 (m, 4H), 3.55-3.61 (m, 6H), 4.68 (s, 2H), 6.22 (s, 1H), 7.61 (s, 1H).

15

**EXAMPLE 8**

Synthesis of 4'-(2-aminoethyl)-4,5',8-trimethylpsoralen Hydrochloride  
(Compound 3)

The synthesis of 4'-(2-aminoethyl)-4,5',8-trimethylpsoralen proceeds  
20 in one (1) step, as described in "Example 5" of U.S. Patent No. 5,399,719, starting with 4'-cyanomethyl-4,5',8-trimethylpsoralen (Kaufman et al., J. Heterocyclic Chem. 19:1051 (1982)) (188 mg, 0.703 mmol) and yielding Compound 3, a light purple solid (32.7 mg, yield 15 %), m.p. > 237 °C (decomp.)

25

**EXAMPLE 9**

4'-(6-Amino-2-aza)hexyl-4,5',8-trimethylpsoralen Dihydrochloride  
(Compound 6)

30 The synthesis of 4'-(6-amino-2-aza)hexyl-4,5',8-trimethylpsoralen dihydrochloride proceeds in one (1) step, as follows: a solution of 4'-chloromethyl-4,5',8-trimethylpsoralen (188 mg, 0.68 mmol) in 30 mL of acetonitrile was added to a solution of 1,4-diaminobutane (120 mg, 1.4 mmol) in 7 mL of acetonitrile. After stirring overnight the solvent was  
35 removed under reduced pressure. Chloroform (10 mL) and 1N NaOH (10 mL) were added to the residue and the mixture was shaken and separated. The aqueous solution was extracted with a further 2x10 mL of CHCl<sub>3</sub> and the combined extracts were rinsed with water. The product was then

extracted from the  $\text{CHCl}_3$  solution with 0.3 N aqueous HCl and the acidic layer was then taken to pH 12 with concentrated NaOH solution. The base suspension was extracted with  $\text{CHCl}_3$  which was then rinsed with water, dried over  $\text{Na}_2\text{SO}_4$  and concentrated under reduced pressure to give the  
5 amine as the free base; NMR ( $\text{CDCl}_3$ );  $\delta$  1.33 (m, 3H), 1.52 (m, 4H), 2.47 (s, 3H), 2.49 (d,  $J=1.1$  Hz, 3H), 2.54 (s, 3H), 2.68 (q,  $J=6.5$  Hz, 4H), 3.86 (s, 2H), 6.21 (apparent d,  $J=1.1$  Hz, 1 H), 7.60 (s, 1H).

The free base, dissolved in about 6 mL of absolute EtOH was treated with a solution of HCl in ether (1.0M, 3 mL). The resultant HCl salt was  
10 filtered, rinsed with absolute EtOH and dried under vacuum to yield 150 mg of compound 6, (55%), m.p. 290 °C (decomposed). Analysis calculated for  $\text{C}_{19}\text{H}_{26}\text{Cl}_2\text{N}_2\text{O}_3 \cdot \text{H}_2\text{O}$ : C, 54.42; H, 6.73; N, 6.68. Found: C, 54.08; H, 6.45; N, 6.65.

The following compounds were prepared in a similar manner, with  
15 the differences in synthesis noted:

a) 4'-(4-amino-2-aza)butyl-4,5',8-trimethylpsoralen dihydrochloride (Compound 1), mp 320-322°C (decomp). In this synthesis ethylene diamine was used as the diamine.

b) 4'-(5-amino-2-aza)pentyl-4,5',8-trimethylpsoralen dihydrochloride  
20 (Compound 5), mp 288°C (decomp). NMR of free base: d 1.33 (br s, 3H), 1.66 (pent,  $J=6.8$  Hz, 2H), 2.47 (s, 3H), 2.50 (d,  $J=1$  Hz, 3H), 2.55 (s, 3H), 2.6-2.85 (m, 4H), 3.89 (s, 2H), 6.22 (apparent d,  $J=1$  Hz, 1H), 7.62 (s, 1H). For this synthesis, 1,3-diaminopropane was used as the diamine.

c) 4'-(7-amino-2-aza)heptyl-4,5',8-trimethylpsoralen dihydrochloride  
25 (Compound 10), mp 300°C (decomp). NMR of free base: d 1.22 (br s, ), 1.3 - 1.6 (m) total 9 H, 2.44 (s), 2.50 (s), total 9H, 2.63 (m, 4H), 6.17 (s, 1H), 7.56 (s, 1H). Here, 1,5-diaminopentane was used as the diamine.

#### EXAMPLE 10

30 5'-(6-Amino-2-aza)hexyl-4,4',8-trimethylpsoralen  
Dihydrochloride (Compound 17)

The synthesis of 5'-(6-amino-2-aza)hexyl-4,4',8-trimethylpsoralen dihydrochloride proceeds in one (1) step, as described in U.S. Patent No.  
35 5,399,719, in "Example 6", starting with 5'-chloromethyl-4,4',8-trimethylpsoralen (190 mg, 0.68 mmol) and yielded the free amine. NMR ( $\text{CDCl}_3$ ):  $\delta$  1.35 (m, 3H); 1.49 (m, 4H); 2.22 (s, 3H); 2.46 (d,  $J=1.1$  Hz, 3H); 2.51 (S, 3H); 2.65 (m, 4H); 3.88 (s, 2H); 6.17 (apparent d, 1Hz); 7.40 (s, 1H). The free

base, dissolved in absolute EtOH (~6 mL) was treated with a solution of HCl in ether (1.0 M, ~3 mL). The resultant HCl salt was filtered, rinsed with absolute EtOH and dried under vacuum to yield 100 mg (36.3%) of product, 5'-(6-Amino-2-aza)hexyl-4,4',8-trimethylpsoralen dihydrochloride, m.p.

5 288°C (decomposed).

5'-(4-Amino-2-aza)butyl-4,4',8-trimethylpsoralen dihydrochloride (Compound 16) was prepared in the same manner, except that ethylene diamine was used as the diamine. NMR of free base:  $\delta$  1.83 (br s, 3H), 2.27 (s, 3H), 2.51 (s, 3H), 2.58 (s, 3H), 2.74 (m, 2H), 2.87 (m, 2H), 3.95 (s, 2H), 6.24 (s, 10 1H), 7.46 (s, 1H).

### EXAMPLE 11

4'-(14-Amino-2,6,11-triaza)tetradecyl-4,5',8-trimethylpsoralen  
Tetrahydrochloride (Compound 15)

15

The synthesis of 4'-(14-amino-2,6,11-triaza)tetradecyl-4,5',8-trimethylpsoralen tetrahydrochloride proceeds in one (1) step, as described in U.S. Patent No. 5,399,719, in "Example 7", starting with 0.128 g (0.5 mmol) of 4,5',8-trimethylpsoralen-4'carboxaldehyde and yielding the free 20 base of the amine,  $\geq$  95% pure by NMR. NMR (CDCl<sub>3</sub>): d 1.31 (m, 5H), 1.45 (pent, J=3.41 Hz, 4H), 1.65 (m, 4 H), 2.46 (s, 3H), 2.49 (d, J=1.14 Hz, 3H), 2.66 (m, 15 H), 3.85 (s, 2H), 6.21 (s, 1H)m 7.60 (s, 1H). The free amine was dissolved in absolute ethanol and HCl (anhydrous, 1N in ethyl ether) was added. The hydrochloride salt was filtered and washed with absolute 25 ethanol and dried under vacuum at room temperature giving 80.2 mg of product, 4'-(14-amino-2,6,11-triaza)tetradecyl-4,5',8-trimethylpsoralen tetrahydrochloride, as a light yellow solid.

### EXAMPLE 12

30 An R17 bacteriophage assay was used in this example to predict pathogen inactivation efficiency and to determine nucleic acid binding of the photoreactive binding compounds of the present invention. In the R17 assay, the bacteriophage was placed in a solution with each compound tested and was then irradiated. The ability of the phage to subsequently 35 infect bacteria and inhibit their growth was measured. The bacteriophage was selected for its relatively accessible nucleic acid such that the culture growth inhibition would accurately reflect nucleic acid damage by the test compounds. The bacteriophage assay for nucleic acid binding to test

compounds offers a safe and inexpensive procedure to identify compounds likely to display efficient pathogen inactivation. Previous experiments support that the R17 assay accurately measures HIV-1 sensitivity to similar compounds.

5           The R17 was grown up in Hfr 3000 bacteria, approximate titer  $5 \times 10^{11}$ . (R17 and Hfr 3000 were obtained from American Tissue Culture Collection (ATCC), Washington, D.C.) The R17 phage stock was added to a solution of 15% fetal bovine serum in Dulbecco's Modified Eagles Medium (DMEM) to a final phage concentration of  $10^9$ /mL. An aliquot (0.5 mL) was  
10 transferred to a 1.5 mL snap-top polyethylene tube. An aliquot (0.004-0.040 mL) of the test compound stock solution prepared in water, ethanol or dimethylsulfoxide at 0.80-8.0 mM was added to the tube. Compounds were tested at concentrations between 4  $\mu$ M and 320  $\mu$ M. (AMT is commercially available from HRI, Inc., Concord, CA; 8-MOP is commercially available  
15 from Sigma, St. Louis, MO). The tubes were placed in a light device as described in EXAMPLE 1 and irradiated for between 1 and 10 minutes. Sterile 13 mL dilution tubes were prepared; each test compound required one tube with 0.4 mL of Luria broth (LB) and five tubes containing 0.5 mL of LB broth. To make the dilutions, a 0.100 mL aliquot of the irradiated  
20 solution of phage and test compound was added to the first dilution tube of 0.4 mL of media then 0.020 mL of this solution was added to the second tube of 0.5 mL medium (1:25). The second solution was then diluted serially (1:25) into the remaining tubes. To each diluted sample was added 0.050 mL of Hfr 3000 bacteria cultured overnight and 3 mL of molten LB top agar and  
25 the mixed materials were poured onto LB broth plates. After the top agar hardened, the plates were incubated at 37 °C overnight. The plaque forming units were then counted the following morning and the titer of the phage remaining after phototreatment was calculated based on the dilution factors.

30           The following controls were run: the "phage only" in which phage was not treated with test compound and not irradiated (listed as "starting titer" in the tables below); the "UV only" in which the phage was irradiated in the absence of test compound; and the "dark" control in which the phage/test compound solution was not irradiated before it was diluted and  
35 plated.

Table 5, below, shows three different experiments which tested Compound 1 according to the R17 protocol just described. A comparison of values for the control samples in runs 1 - 3 (values in bold) shows that

neither the "UV only" nor the "dark" controls result in significant bacterial kill (at most, .3 logs killed in the "UV only" control and .1 logs killed in the "dark" control).

**TABLE 5**

5

EXPERIMENT #	TREATMENT	LOG TITER	LOGS KILLED
1	phage only	7.7	--
	uva only (10')	7.4	<b>0.3</b>
	compound only (32 $\mu$ M)	7.6	<b>0.1</b>
	32 $\mu$ M cmpd 1' uva	<1	>6.7
	32 $\mu$ M cmpd 10' uva	<1	>6.7
2	phage only	7.8	--
	uva only (10')	7.6	<b>0.2</b>
	compound only (3.2 $\mu$ M)	7.7	<b>0.1</b>
	3.2 $\mu$ M cmpd 1' uva	6.9	0.9
	3.2 $\mu$ M cmpd 10' uva	6.1	1.7
3	phage only	7.3	--
	uva only (1')	7.3	<b>0</b>
	compound only (16 $\mu$ M)	7.3	<b>0</b>
	4 $\mu$ M cmpd 1' uva	6.3	1.0
	8 $\mu$ M cmpd 1' uva	5.6	1.7
	16 $\mu$ M cmpd 1' uva	3.9	3.4

The "UV only" control was repeated in many similar experiments with other compounds of the present invention and consistently showed no significant kill. (Data not shown). Thus, the "UV

only" control is not shown in the tables and figures that follow, although it was performed in every experiment in this example. As for the "dark" control, after many trials with various compounds of the present invention, it became apparent that regardless of the type of substitution on the 4' position of the psoralen, no experimentally significant bacterial inactivation was observed in the dark. (Data not shown). For example, in Table 5, experiment 1 shows .1 logs kill with compound 1 in the dark. In contrast, when Compound 1 is irradiated for just 1 minute, the resulting drop in titer is >6.7 logs. Therefore, "dark" controls were not run for the later tested compounds and where run, are not shown in the tables and figures that follow

Tables 6 - 9, below, and FIGS. 7-9 show the results of the R17 assay for several of the 4'-primaryamino-substituted psoralen compounds of the present invention. The data in Tables 7 and 8 appears in FIGS. 7 and 8, respectively. 5'-Primaryamino-substituted psoralen compounds of the present invention, which have substitutions on the 5' position similar to the 4'-primaryamino-substituted psoralen compounds, were also tested at varying concentration, as described above in this example, and are shown to exhibit comparable inactivation efficiency. The results for these compounds are shown in FIGS. 10 and 11, below.

The compounds of the present invention having substitutions on the 4' position of the psoralen ring proved to be active in killing R17, as shown in

**TABLE 6**

Starting titer of R17: approx. 7.5 logs  
1 Minute Irradiation

Cmpd.	R17 log kill (32 $\mu$ M)
AMT	>6.7
8-MOP	0
1	>6.6

**TABLE 7**  
Starting titer approx. 7.2 logs R17  
1 minute irradiation

Compound	R17	log	kill
	8 $\mu$ M	16 $\mu$ M	32 $\mu$ M
AMT	2.7	4.6	>6.2
1	1.7	2.8	5.3
2	3.8	>6.2	>6.2
3	>6.2	>6.2	>6.2

5

**TABLE 8**  
Starting titer approx. 7.1 logs  
1 minute irradiation = 1.2 J/cm<sup>2</sup>

10

Cmpd.	R17	log	kill	
	8 $\mu$ M	16 $\mu$ M	32 $\mu$ M	64 $\mu$ M
AMT	--	4.5	4.8	--
3	5.6	>6.1	--	--
4	--	2.3	4.3	>6.1
5	--	5.6	>6.1	>6.1
6	--	>6.1	>6.1	>6.1

**TABLE 9**  
Starting titer approx. 7.1 logs R17.  
1 Minute Irradiation.

5

Cmpd.	R17 log kill			
	8 $\mu$ M	16 $\mu$ M	32 $\mu$ M	64 $\mu$ M
AMT	--	>6	>6	--
6	>6	>6	--	--
7	--	>6	>6	>6

the tables above. In Table 7, it is apparent that compound 1 of the present invention exhibits much higher R17 inactivation efficiency than does 8-MOP. As shown in Table 7 and FIG. 7, Compound 1 is one of the less active compounds of the present invention. Both Compounds 2 and 3 show higher log inactivation than Compound 1 at each concentration point. These results support that the compounds of the present invention are generally much more active than 8-MOP.

15 The compounds of the present invention also have similar or better R17 inactivation efficiency than AMT. In Tables 7 and 8, and FIGS. 7 - 11, all compounds of the present invention achieve R17 log inactivation at levels comparable to AMT. Compounds 2 and 3 (Table 6, FIG. 7), Compounds 5 and 6 (Table 8, FIG. 8), and Compound 16 (FIG. 11) exhibit significantly higher inactivation efficiency than does AMT.

20 Compounds of the present invention were also tested at a constant concentration for varying doses of UV light. Three sets of 1.5 mL tubes were prepared containing .6 ml aliquots of R17 in DMEM (prepared as described above). The compound tested was added at the desired concentration and the samples were vortexed. The samples were then irradiated at intervals of 1.0 J/cm<sup>2</sup>, until 3.0 J/cm<sup>2</sup> was reached. Between each 1.0 J/cm<sup>2</sup> interval, 100 $\mu$ L was removed from each sample and placed in the first corresponding dilution tube, then five sequential dilutions were performed for each compound tested, at all 3 irradiation doses, as described above in this example.

30



Then 50 $\mu$ L of Hfr 3000 bacteria was added to each tube, 3 mL of top agar was added and the tube contents were vortexed. The contents of each tube was poured into its own LB plate and the plates were incubated overnight at 37 °C. Plaques were counted by visual inspection the following morning.

The results of the assay for several 4' and 5'-primaryamino-substituted psoralen compounds are shown in FIGS. 12 - 18. This data further supports that the compounds of the present invention are comparable to AMT in their ability to inactivate R17. Further, Compounds 6 (FIG. 12), 10 (FIG. 13), 12 (FIG. 14), 15 (FIG. 15 and 18), and Compound 17 (FIG. 16), all were more efficient at inactivating R17 than was AMT.

### EXAMPLE 13

Pathogen inactivation efficiency of several compounds of the present invention was evaluated by examining the ability of the compounds to inactivate cell-free virus (HIV). Inactivation of cell-free HIV was performed as follows.

As in the R17 assay, small aliquots of the compounds listed in Tables 10 and 11, below, at the concentrations listed in the table, were added to stock HIV-1 to a total of 0.5 mL. The stock HIV ( $10^5$  -  $10^7$  plaque forming units/mL) was in DMEM/15% FBS. The 0.5 mL test aliquots were placed in 24 well polystyrene tissue culture plates and irradiated with 320 - 400 nm (20 mW/cm<sup>2</sup>) for 1 min on a device similar to the device of Example 1. The photoactivation device used here was previously tested and found to result in light exposure comparable to the Device of Example 1. (Data not shown). Controls included HIV-1 stock only, HIV-1 plus UVA only, and HIV-1 plus the highest concentration of each psoralen tested, with no UVA. Post irradiation, all samples were stores frozen at -70° C until assayed for infectivity by a microtiter plaque assay. Aliquots for measurement of residual HIV infectivity in the samples treated with a compound of the present invention were withdrawn and cultured.

Residual HIV infectivity was assayed using an MT-2 infectivity assay. (Previously described in Hanson, C.V., Crawford-Miksza, L. and Sheppard, H.W., J. Clin. Micro 28:2030 (1990)). The assay medium was 85% DMEM (with a high glucose concentration) containing 100  $\mu$ g of streptomycin, 100 U of penicillin, 50  $\mu$ g of gentamicin, and 1  $\mu$ g of amphotericin B per mL, 15% FBS and 2  $\mu$ g of Polybrene (Sigma Chemical Co., St. Louis, Mo.) per mL. Test and control samples from the inactivation procedure were diluted in

50% assay medium and 50% normal human pooled plasma. The samples were serially diluted directly in 96-well plates (Corning Glass Works, Corning, N.Y.). The plates were mixed on an oscillatory shaker for 30 seconds and incubated at 37°C in a 5% CO<sub>2</sub> atmosphere for 1 to 18 hours.

5 MT-2 cells (0.025 mL) [clone alpha-4, available (catalog number 237) from the National Institutes of Health AIDS Research and Reference Reagent Program, Rockville, Md.] were added to each well to give a concentration of 80,000 cells per well. After an additional 1 hour of incubation at 37°C in 5% CO<sub>2</sub>, 0.075 mL of assay medium containing 1.6% SeaPlaque agarose (FMC

10 Bioproducts, Rockland, Maine) and prewarmed to 38.5°C was added to each well. The plates were kept at 37°C for a few minutes until several plates had accumulated and then centrifuged in plate carriers at 600 × g for 20 minutes in a centrifuge precooled to 10°C. In the centrifuge, cell

15 monolayers formed prior to gelling of the agarose layer. The plates were incubated for 5 days at 37°C in 5% CO<sub>2</sub> and stained by the addition of 0.05 mL of 50 µg/mL propidium iodide (Sigma Chemical Co.) in phosphate-buffered saline (pH 7.4) to each well. After 24 to 48 hours, the red

20 fluorescence-stained microplaques were visualized by placing the plates on an 8,000 µW/cm<sup>2</sup> 304 nm UV light box (Fotodyne, Inc., New Berlin, Wis.). The plaques were counted at a magnification of x20 to x25 through a stereomicroscope. The results are shown in Tables 10 and 11, below. "n" represents the number of runs for which the data point is an average.

The results support that the compounds of the present invention are effective in inactivating HIV. In fact, the data for concentrations of 64µM of

25 compound or higher suggests that compounds 2 and 3 are significantly more active than AMT, which was previously thought to be one of the most active anti-viral psoralens. At lower concentrations, Compound 6 is able to kill a higher log of HIV (3.1 logs at 32 µM) than is AMT (2.5 logs at 32µM). The other compounds listed in Table 9 display inactivation

30 efficiency in the same range as AMT.

**TABLE 10**

1 minute irradiation

HIV starting titer: approximately 5 logs

COMPOUND	HIV log kill			
	16 $\mu$ M	32 $\mu$ M	64 $\mu$ M	128 $\mu$ M
AMT	1.4	1.9 - >3.6	3.9 - >3.6	>4.1
1	-	-	2.1	>2.8
2	1.4	3.8	>4.5	>4.5
3	-	2.7	>3.8	>3.8
4	-	2.2	>3.6	>3.6
5	0.9	1.3	>2.6	-
6	2.0	3.1	>3.8	-
7	0.8	2.1	3.5	-
8	1.1	1.9	3.7	>3.7

5

**TABLE 11**

HIV starting titer: approximately 5.4 logs

1 minute irradiation

COMPOUND	HIV log kill		
	16 $\mu$ M	32 $\mu$ M	64 $\mu$ M
6	2.1	3.2	>2.8
9	0.8	1.4	2.7
10	2.0	>3.5	>3.5
12	0.4	0.8	1.3
17	1.2	2.9	3.4
18	1.0	1.0	3.1

**EXAMPLE 14**

10 This example describes the protocol for inactivation of Duck  
Hepatitis B Virus (DHBV), a model for Hepatitis B Virus, using compounds  
of the present invention.

15 DHBV in duck yolk was added to platelet concentrate (PC) to a final  
concentration of  $2 \times 10^7$  particles per mL and mixed by gentle rocking for  
 $\geq 15$  min. Psoralens Compounds 2 and 6 and AMT were added to 3 mL  
aliquots of PC in a Teflon™ mini-bag at concentrations of 35, 70, and 100  
mM. Samples, including controls without added psoralen, were irradiated

with 5J/cm<sup>2</sup> UVA, with mixing at 1 J/cm<sup>2</sup> increments. After irradiation, leukocytes and platelets were separated from virus by centrifugation. The supernatant containing DHBV was digested overnight with 50 µg/mL proteinase K in a buffer containing 0.5% sodium dodecyl sulphate, 20 mM Tris buffer, pH 8.0, and 5 mM EDTA at 55°C. Samples were extracted with phenol-chloroform and chloroform, followed by ethanol precipitation. Purified DNA was then used in PCR amplification reactions with a starting input of 10<sup>6</sup> DHBV genomes from each sample. PCR amplicons were generated using primers pairs DCD03/DCD05 (127 bp), DCD03/DCD06 (327 bp) and DCD03/DCD07 (1072 bp). PCR was performed in a standard PCR buffer containing 0.2 mM each deoxyribonucleoside 5'-triphosphates (dATP, dGTP, dCTP, and dTTP), 0.5 mM each primer, and 0.5 units Taq polymerase per 100 ml reaction. 30 cycles of amplification were performed with the following thermal profile: 95°C 30 sec, 60° C 30 sec, 72° C 1 min. The amplification was followed by a 7 min incubation at 72° C to yield full length products. [ $\lambda$ -<sup>32</sup>P] dCTP was added at an amount of 10 mCi per 100 ml in order to detect and quantify the resulting products. Products were separated by electrophoresis on denaturing polyacrylamide slab gels and counted. The absence of signal in a given reaction was taken to indicate effective inactivation of DHBV.

The results showed that the smaller amplicons displayed increasing inactivation as a function of psoralen concentration for all psoralens tested. At the same concentrations, Compounds 2 and 6 inhibited PCR of the smaller amplicons better than did AMT. For the 1072 bp amplicon, complete inhibition of PCR was observed at all concentrations of Compounds 2 and 6, whereas the sample without psoralen gave a strong signal. AMT inhibited PCR amplification of the 1072 bp amplicon at the 70 and 100 mM levels, but a signal could be detected when AMT was used at 35 mM final concentration.

30

#### EXAMPLE 15

In Example 13, the compounds of the present invention were tested for their ability to inactivate virus in DMEM/15% FBS. In this example, the compounds are tested in both 100% plasma and predominantly synthetic media, to show that the methods of the present invention are not restricted to any particular type of medium.

For the samples in synthetic media: standard human platelet

concentrates were centrifuged to separate plasma. Eighty-five percent of the plasma was then expressed off and replaced with a synthetic medium (referred to as "Sterilyte™ 3.0") containing 20 mM Na acetate, 2 mM glucose, 4 mM KCl, 100 mM NaCl, 10 mM Na<sub>3</sub> Citrate, 20 mM NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub>, and 2 mM MgCl<sub>2</sub>. H9 cells infected with HIV were added to either the 85% Sterilyte™ 3.0 platelet concentrates or standard human platelet concentrates (2.5 x 10<sup>7</sup> cells per concentrate), final concentration 5x10<sup>5</sup> cells/mL. The platelet concentrates were placed in Teflon™ modified FL20 or Teflon™ Minibags (American Fluoroseal Co., Silver Springs, MD), treated with one of the compounds shown in FIGS. 19 and 20, at the concentrations shown, and then irradiated with 320-400 nm (20mW/cm<sup>2</sup>) for 5 J/cm<sup>2</sup> (for plasma samples) or 2 J/cm<sup>2</sup> (for 85% Sterilyte™ 3.0 samples) on a device similar to the Device of Example 1. The photoactivation device used here was previously tested and found to result in light exposure comparable to the Device of Example 1. (Data not shown). Aliquots for measurement of residual HIV infectivity in the samples treated with a compound of the present invention were withdrawn and cultured.

For samples run in plasma: H9 cells infected with HIV were added to standard human platelet concentrates (2.5 x 10<sup>7</sup> cells per concentrate), final concentration 5x10<sup>5</sup> cells/mL. Aliquots of HIV contaminated platelet concentrate (5 mL) were placed in water jacketed Pyrex chambers. The chambers had previously been coated on the inside with silicon. The platelet concentrates were treated with one of the compounds listed in Tables 10 and 11 at the concentrations listed in the table, and then irradiated with 320-400 nm (20mW/cm<sup>2</sup>) for 1 minute on a device similar to the Device of Example 1. The photoactivation device used here was previously tested and found to result in light exposure comparable to the Device of Example 1. (Data not shown). Aliquots for measurement of residual HIV infectivity in the samples treated with a compound of the present invention were withdrawn and cultured. Residual HIV infectivity was assayed for both the plasma and the 85% Sterilyte™ samples using an MT-2 infectivity assay. (Detailed in Example 13, above, and previously described in Hanson, C.V., *et al.*, J. Clin. Micro 28:2030 (1990)). The results are shown in FIGS. 19 and 20.

The results support that the compounds of the present invention are effective in inactivating HIV in both plasma and synthetic medium. Comparing FIGS. 14 and 15, the inactivation curves appear to be the same,

both achieving approximately 5 logs of inactivation at 64  $\mu\text{M}$  concentrations of compound. However, the inactivation in synthetic media was performed with only 2  $\text{J}/\text{cm}^2$  irradiation, 3  $\text{J}/\text{cm}^2$  less than that required to achieve the same inactivation in plasma. Thus, it appears from the data that synthetic media facilitates the inactivation methods of the present invention.

#### EXAMPLE 16

10 In this example bacterial inactivation by the photoreactive nucleic acid binding compounds of the present invention was measured as a function of the ability of the bacteria to subsequently replicate. A gram negative bacteria was chosen as representative of the more difficult bacterial strains to inactivate.

15 The bacteria, a strain of *Pseudomonus*, was inoculated into LB with a sterile loop and grown overnight in a shaker at 37°C. Based on the approximation that one OD at 610 nm is equivalent to  $5 \times 10^8$  colony forming units (cfu)/mL, a 1:10 dilution of the culture was measured on a spectrophotometer, (manufactured by Shimatsu). The bacterial culture was added to a solution of 15% fetal bovine serum in DMEM to a final bacteria concentration of approximately  $10^6$ /mL. An aliquot (0.8 mL) was transferred to a 1.5 mL snap-top polyethylene tube. An aliquot (0.004-0.040 mL) of the test compound stock solution prepared in water, ethanol or dimethylsulfoxide at 0.80-8.0 mM was added to the tube. Compounds were tested at a concentration of 16  $\mu\text{M}$ . The tubes were placed in a light device as described in EXAMPLE 1 and irradiated with 1.3  $\text{J}/\text{cm}^2$ , 1.2  $\text{J}/\text{cm}^2$ , and finally 2.5  $\text{J}/\text{cm}^2$ , for a total of 5  $\text{J}/\text{cm}^2$ . 150  $\mu\text{L}$  were removed for testing after each pulse period. Sterile 13 mL dilution tubes were prepared; each test compound required one tube with 0.4 mL of LB broth and four tubes containing 0.5 mL of LB broth. To make the dilutions, a 0.050 mL aliquot of the irradiated solution of phage and test compound was added to the first dilution tube of 0.5 mL of media then 0.050 mL of this solution was added to the second tube of 0.5 mL medium (1:10). The second solution was then diluted serially (1:10) into the remaining tubes. 100  $\mu\text{L}$  of the original sample and each dilution are plated separately onto LB agar plates and incubated at 37 °C overnight. The colony forming units were then counted the following morning and the titer of the phage remaining after phototreatment was calculated based on the dilution factors.

The following controls were run: the "bacteria only" in which bacteria was not treated with test compound and not irradiated (listed as "starting titer" in the tables); the "UV only" in which the bacteria was irradiated in the absence of test compound. Dark controls were not performed here for reasons set forth in Example 12 above.

The results were as follows. The starting titer of bacteria was 6.5 logs. After 5 J/cm<sup>2</sup> irradiation, the log kill for the various compounds tested were as follows: 8-MOP - 1.9 logs, AMT - 5.2 logs, Compound 2 - >5.5, Compound 6 - >5.5. From these results, it is clear that the compounds of the present invention are more efficient than both AMT and 8-MOP at inactivating a gram: negative bacteria.

### EXAMPLE 17

In the above examples, psoralens of the present invention have been demonstrated to be effective for inactivating pathogens, such as bacteria (pseudomonas), bacteriophage (R17) and viruses (HIV and DHBV). Without intending to be limited to any method by which the compounds of the present invention inactivate pathogens, it is believed that inactivation results from light induced binding of the psoralens to the nucleic acid of the pathogens. As discussed above, AMT is known both for its pathogen inactivation efficiency and its accompanying mutagenic action in the dark at low concentrations. In contrast, the less active psoralens, such as 8-MOP, that have been examined previously, show significantly less mutagenicity. This example establishes that photobinding and mutagenicity are not linked phenomena in the compounds of the present invention. The psoralens of the present invention have exceptional pathogen inactivation efficiency while displaying only minimal mutagenicity.

In this example the compounds of the present invention are tested for their dark mutagenicity using an Ames assay. The procedures used for the Salmonella mutagenicity test as described in detail by Maron and Ames were followed exactly. Maron, D.M. and B.N. Ames, Mutation Research 113: 173 (1983). A brief description for each procedure is given here. The tester strains TA97a, TA98, TA100, TA102, TA1537 and TA1538 were obtained from Dr. Ames. TA97a, TA98, TA1537 and TA1538 are frameshift tester strains. TA100 and TA102 are base-substitution tester strains. Upon receipt each strain was cultured under a variety of conditions to confirm the genotypes specific to the strains.

The standard Salmonella tester strains used in this study require histidine for growth since each tester strain contains a different type of mutation in the histidine operon. In addition to the histidine mutation, these tester strains contain other mutations, described below, that greatly increase their ability to detect mutagen.

*Histidine Dependence:* The requirement for histidine was tested by streaking each strain first on a minimal glucose plate supplemented only with biotin and then on a minimal glucose plate supplemented with biotin and histidine. All strains grew the lack of growth of the strains in the absence of histidine.

*rfa Mutation:* A mutation which causes partial loss of the lipopolysaccharide barrier that coats the surface of the bacteria thus increasing permeability to large molecules was confirmed by exposing a streaked nutrient agar plate coated with the tester strain to crystal violet. First 100  $\mu$ L of each culture was added to 2 mL of molten minimal top agar and poured onto a nutrient agar plate. Then a sterile filter paper disc saturated with crystal violet was placed at the center of each plate. After 16 hours of incubation at 37°C the plates were scored and a clear zone of no bacterial growth was found around the disc, confirming the *rfa* mutation.

*uvrB Mutation:* Three strains used in this study contain a deficient UV repair system (TA97a, TA98, TA100, TA1537 and TA1538). This trait was tested for by streaking the strains on a nutrient agar plate, covering half of the plate, and irradiating the exposed side of the plate with germicidal lamps. After incubation growth was only seen on the side of the plate shielded from UV irradiation.

*R-factor:* The tester strains (TA97a, TA98, TA100, and TA102) contain the pKM101 plasmid that increases their sensitivity to mutagens. The plasmid also confers resistance to ampicillin to the bacteria. This was confirmed by growing the strains in the presence of ampicillin.

*pAQ1:* Strain TA102 also contains the pAQ1 plasmid that further enhances its sensitivity to mutagens. This plasmid also codes for tetracycline resistance. To test for the presence of this plasmid TA102 was streaked on a minimal glucose plate containing histidine, biotin, and tetracycline. The plate was incubated for 16 hours at 37°C. The strain showed normal growth indicating the presence of the pAQ1 plasmid.

The same cultures used for the genotype testing were again cultured and aliquots were frozen under controlled conditions. The cultures were again tested for genotype to confirm the fidelity of the genotype upon



manipulation in preparing the frozen permanents.

The first tests done with the strains were to determine the range of spontaneous reversion for each of the strains. With each mutagenicity experiment the spontaneous reversion of the tester strains to histidine independence was measured and expressed as the number of spontaneous revertants per plate. This served as the background controls. A positive mutagenesis control was included for each tester strain by using a diagnostic mutagen suitable for that strain (2-aminofluorene at 5mg/plate for TA98 and sodium azide at 1.5 mg/plate for TA100).

For all experiments, the pre-incubation procedure was used. In this procedure one vial of each tester strain was thawed and 20  $\mu$ L of this culture was added to 6 mL of Oxoid Nutrient Broth #2. This solution was allowed to shake for 10 hours at 37°C. In the pre-incubation procedure, 0.1 mL of this overnight culture was added to each of the required number of sterile test tubes. To half of the tubes 0.5 mL of a 10% S-9 solution containing Aroclor 1254 induced rat liver extract (Molecular Toxicology Inc., Annapolis, MD), and MgCl<sub>2</sub>, KCl, glucose-6-phosphate, NADP, and sodium phosphate buffer (Sigma, St. Louis, Missouri) were added. To the other half of the tubes 0.5 mL of 0.2M sodium phosphate buffer, pH 7.4, was used in place of the S-9 mixture (the -S9 samples). Finally 0.1 mL of the test solution containing either 0, 0.1, 0.5, 1, 5, 10, 50, 100, 250, or 500  $\mu$ g/mL of the test compound was added. The 0.7 mL mixture was vortexed and then pre-incubated while shaking for 20 minutes at 37°C. After shaking, 2 mL of molten top agar supplemented with histidine and biotin were added to the 0.7 mL mixture and immediately poured onto a minimal glucose agar plate (volume of base agar was 20 mL). The top agar was allowed 30 minutes to solidify and then the plates were inverted and incubated for 44 hours at 37°C. After incubation the number of revertant colonies on each plate were counted. The results are set forth in table form in U.S. Patent No. 5,399,719, from "Table 10(A)" through "Table 16(B)".

Maron and Ames (1983) describe the conflicting views with regard to the statistical treatment of data generated from the test. In light of this, this example adopts the simple model of mutagenicity being characterized by a two-fold or greater increase in the number of revertants above background, as well as dose dependent mutagenic response to drug.

With regard to 8-MOP, the only mutagenic response detected was a weak base-substitution mutagen in TA102 at 500 $\mu$ g/plate.

In sharp contrast, AMT showed frameshift mutagenicity at between 5

and 10 µg/plate in TA97a and TA98, at 5µg/plate in TA1537 and at 1 µg/plate in TA1538. AMT showed no significant base-substitution mutations.

Looking at Compound 1, the only mutagenic response detected was a  
5 weak frameshift mutagen in TA1538 at 5 µg/plate in the presence of S9.  
Compound 1 also displayed mutation in the TA100 strain, but only in the  
absence of S9. Compound 2 also showed weak frameshift mutagenicity in  
the presence of S9 in TA98 and TA1537. Compounds 3 and 4 showed no  
mutagenicity. Compound 6 had no base substitution mutagenicity, but  
10 showed a frameshift response in TA98 in the presence of S9 at  
concentrations of 250µg/plate and above. It also showed a response at  
50µg/plate in TA1537 in the presence of S9. Compound 18 showed only a  
weak response at high concentrations in the presence of S9 in strains TA 90  
and TA 1537. The response was higher in the absence of S9, but still was  
15 significantly below that of AMT, which displayed mutagenicity at much  
lower concentrations (5 µg/plate).

From this data it is clear that the compounds of the present  
invention are less mutagenic than AMT, as defined by the Ames test. At  
the same time, these compounds show much higher inactivation efficiency  
20 than 8-MOP, as shown in Examples 12 and 16. These two factors support  
that the compounds of the present invention combine the best features of  
both AMT and 8-MOP, high inactivation efficiency and low mutagenicity.

### EXAMPLE 18

25 In Example 15, the compounds of the present invention exhibited the  
ability to inactivate pathogens in synthetic media. This example describes  
methods by which synthetic media and compounds of the present  
invention may be introduced and used for inactivating pathogens in blood.  
30 FIG. 21A schematically shows the standard blood product separation  
approach used presently in blood banks. Three bags are integrated by  
flexible tubing to create a blood transfer set (200) (*e.g.*, commercially  
available from Baxter, Deerfield, Ill.). After blood is drawn into the first bag  
(201), the entire set is processed by centrifugation (*e.g.*, Sorvall™ swing  
35 bucket centrifuge, Dupont), resulting in packed red cells and platelet rich  
plasma in the first bag (201). The plasma is expressed off of the first bag (201)  
(*e.g.*, using a Fenwall™ device for plasma expression), through the tubing  
and into the second bag (202). The first bag (201) is then detached and the

two bag set is centrifuged to create platelet concentrate and platelet-poor plasma; the latter is expressed off of the second bag (202) into the third bag (203).

FIG. 21B schematically shows an embodiment of the present invention by which synthetic media and photoactivation compound are introduced to platelet concentrate prepared as in FIG. 21A. A two bag set (300) is sterile docked with the platelet concentrate bag (202) (indicated as "P.C."). Sterile docking is well-known to the art. See *e.g.*, US Patent No. 4,412,835 to D.W.C. Spencer, hereby incorporated by reference. See also US Patents Nos. 4,157,723 and 4,265,280, hereby incorporated by reference. Sterile docking devices are commercially available (*e.g.*, Terumo, Japan).

One of the bags (301) of the two bag set (300) contains a synthetic media formulation of the present invention (indicated as "STERILYTE"). In the second step shown in FIG. 21B, the platelet concentrate is mixed with the synthetic media by transferring the platelet concentrate to the synthetic media bag (301) by expressing the platelet concentrate from the first blood bag into the second blood bag via a sterile connection means. The photoactivation compound can be in the bag containing synthetic media (301), added at the point of manufacture. Alternatively, the compound can be mixed with the blood at the point of collection, if the compound is added to the blood collection bag (FIG. 21A, 201) at the point of manufacture. The compound may be either in dry form or in a solution compatible with the maintenance of blood.

FIG. 21C schematically shows one embodiment of the decontamination approach of the present invention applied specifically to platelet concentrate diluted with synthetic media as in FIG. 21B. In this embodiment, platelets have been transferred to a synthetic media bag (301). The photoactivation compound either has already been introduced in the blood collection bag (201) or is present in the synthetic media bag (301). Either the platelets are then expressed into the synthetic media bag via a sterile connection means (as shown) or the synthetic media is expressed into the platelet bag. The bag containing the mixture of platelet concentrate and synthetic media (301), which has UV light transmission properties and other characteristics suited for the present invention, is then placed in a device (such as that described in Example 1, above) and illuminated.

Following phototreatment, the decontaminated platelets are transferred from the synthetic media bag (301) into the storage bag (302) of the two bag set (300). The storage bag can be a commercially available

storage bag (e.g., CLX bag from Cutter).

FIG. 21D schematically shows an embodiment of the decontamination approach of the present invention, which includes a capture device to remove photoinactivation compound from the treated material after phototreatment. The present invention contemplates several adsorptive materials which may be used in a capture device to remove photoinactivation compounds, of which the following is a non-exclusive list: Amberlite XAD-4 (available from Rohm and Haas Ltd., Croydon, Surrey, UK) ("Resin hemoperfusion for Acute Drug Intoxication," Arch Intern Med 136:263 (1976)); Amberlite XAD-7 ("Albumin-Coated Amberlite XAD-7 Resin for Hemoperfusion in Acute Liver Failure," Artificial Organs, 3:20 (1979); Amberlite 200, Amberlite DP-1, Amberlite XAD-2, Amberlite XAD-16; activated charcoals, ("Charcoal haemoperfusion in Drug Intoxication," British J. Hospital Med. 49:493 (1993); silica ("In vitro Studies of the Efficacy of Reversed Phase Silica Gel as a Sorbent for Hemo- and Plasmaperfusion," Clinical Toxicology 30:69 (1992)). In one embodiment, the present invention contemplates an absorptive material operating in conjunction with a filtering means to remove compounds.

20

### EXAMPLE 19

This example involves an assessment of the impact of the compounds and methods of the present invention on platelet function. Four indicators of platelet viability and function were employed: 1) GMP-140 expression; 2) maintenance of pH; 3) platelet aggregation, and 4) platelet count.

To measure the effects of the present compounds and methods of decontamination on platelet function using these four indicators, four samples were prepared for each compound tested, two control samples and two containing compound. Three units of human platelets were obtained from the Sacramento Blood Center, Sacramento, CA. These were each transferred under sterile conditions to 50 ml centrifuge tubes, then aliquots of each unit were transferred into a second set of 50 ml sterile centrifuge tubes. To each centrifuge tube containing platelet concentrate (PC), an aliquot of compound stock was added to reach a final concentration of 100  $\mu$ M of compound. The compounds tested in this experiment were Compound 2 (36  $\mu$ L of 10 mM stock added to 4 ml PC), Compound 6 (173.5  $\mu$ l of 9.8 mM stock added to 16.8 ml PC), Compound 17 (2.0 ml of 1mM

stock added to 18 ml PC) and Compound 18 (.842 ml of 2.0 mM stock to 16 ml PC). The samples were pipetted gently up and down to mix. Then aliquots (either 3 ml or 8 ml) of each sample was transferred to two sterile Teflon™ Medi-bags™ (American Fluoroseal Co., Silver Springs, MD) (presently owned by The West Company, Lionville, PA). Samples were treated in one of two different sized bags, having either 3 ml or 8 ml capacity. The bags both have approximately the same surface area to volume ratios, and previous experiments have shown that the two bags exhibit approximately equivalent properties during irradiation of samples. (Data not shown). For each compound tested, two control samples without compound were prepared by again removing aliquots of platelet concentrate (17 ml if using an 8 ml bag, 4 ml if using a 3 ml bag) from the same one of the first set of 50 ml centrifuge tubes from which the compound sample was drawn, and dividing into Medibags, as before. One of each pair of Medibags containing a compound, and one of each pair of control Medibags, were illuminated for 5 Joules/cm<sup>2</sup> on the device described in Example 1, above. Then all experimental and control Medibags were placed on a platelet shaker for storage for 5 days. The same experiments were repeated several times to obtain more statistically meaningful data, as represented by "n", the number of data points represented, in the graphs of FIGS 22-25, discussed below. Also in FIGS 22-25, "C1" represents an untreated control at day 1, "D5" represents an untreated control after a five day storage, "UV" represents a sample which was treated with ultraviolet light only, and "PCD" represents the test sample, treated with ultraviolet light and a compound of the present invention.

To obtain data for control samples at day one, approximately 3 ml were removed from the remaining volume of each of the three units and divided into two 1.5 ml tubes. These samples were tested for pH as described below. A platelet count was also taken, as described below, at a 1:3 dilution. The residual platelet concentrate from each unit was spun for 10 minutes at 3800 rpm (3000 g) in Sorval RC3B (DuPont Company, Wilmington, Delaware) to pellet platelets. Plasma was then decanted into 2 sterile 50 ml tubes (one for Day one, and the other stored at 4° C for Day 5) for use in the aggregation assay.

- 1) GMP-140 Expression

When platelets become activated, an alpha granule membrane

glycoprotein called p-selectin (GMP140) becomes exposed on the platelet surface. Less than (5%) of fresh, normal unstimulated platelets express detectable GMP140 levels by flow cytometry. See generally M.J. Metzelaar, *Studies on the Expression of Activation-Markers on Human Platelets*

5 (Thesis 1991).

To measure GMP140, a small aliquot of platelet rich plasma is placed in HEPES buffer containing a GMP140-binding antibody or an isotype control mouse IgG. CD62 is a commercially available monoclonal antibody which binds to GMP140 (available from Sanbio, Uden, the Netherlands;

10 Caltag Labs, So. San Francisco, CA, and Becton Dickinson, Mountain View, CA). After a fifteen minute incubation at room temperature, Goat F(ab')<sub>2</sub> Anti-Mouse IgG conjugated to FITC (Caltag Laboratories, So. San Francisco, CA) is added to the tube in saturating amounts and allowed to incubate at room temperature (RT) for 15 minutes. Finally, the cells are diluted in 1%  
15 paraformaldehyde in phosphate buffered saline and analyzed on a FACSCAN™ (Becton Dickinson, Mountain View, CA). The positive control is made by adding Phorbol Myristate Acetate (PMA) to the test system at a final concentration of  $2 \times 10^{-7}$  M.

In this example, CD62 was employed to measure the impact, if any, of  
20 irradiation in the presence of several compounds of the present invention on platelet activation. The antibody was mixed with HEPES buffer (10  $\mu$ L antibody [0.1 mg/ml] : 2.49mL buffer) and stored in 50  $\mu$ L aliquots at -40°C prior to use. A positive control consisted of: 10  $\mu$ L CD62, 8  $\mu$ L PMA and 2.482 mL Hepes buffer. A mouse IgG1 control (0.05 mg/ml) (Becton  
25 Dickinson, Mountain View, CA #9040) 5X concentrated was also employed. The antibody was diluted in HEPES buffer (20 $\mu$ L antibody : 2.48 ml buffer) and stored at -40°C. Phorbol Myristate Acetate (PMA) (Sigma, St. Louis, MO) was stored at -40°C. At time of use, this was dissolved in DMSO (working concentration was 10  $\mu$ g/mL).

30 1% Paraformaldehyde (PFA) (Sigma, St. Louis, MO) was prepared by adding 10 grams paraformaldehyde to 1 liter PBS. This was heated to 70°C, whereupon 3 M NaOH was added dropwise until the solution was clear. The solution was cooled and the pH was adjusted to 7.4 with 1 N HCl. This was filtered and stored.

35 Processing each of the samples of platelet concentrate after treatment involved adding 5  $\mu$ l of platelet concentrate, diluted 1:3 in Hepes buffer, to each microcentrifuge tube containing the antibody CD62, and appropriate reagents and mixing very gently by vortex. The samples were then

incubated for 15 minutes at room temperature.

The Goat anti-Mouse IgG-FITC (diluted 1:10 in HEPES buffer) was added (5 microliters) to each tube and the solution was mixed by gentle vortex. The samples were incubated for an additional 15 minutes at room temperature. Next, 1 ml of 1% PFA in PBS was added to each tube and mixed gently. The platelets were analyzed on the FACSCAN™. The results are shown in FIGS 22C, 23C, 24C, and 25C. (FIGS 22 correspond to Compound 2, FIGS 23 correspond to Compound 6, FIGS 24 correspond to Compound 17 and FIGS 25 correspond to Compound 18). Clearly, three of the four compounds tested, 2, 6, and 17, exhibited little or no difference between the day 5 untreated control (D5) and the sample treated with both light and psoralen compound (PCD). Only Compound 18 exhibited a notable increase above the control. But the value was still well below the positive control value.

## 2) Maintenance of pH:

Changes in pH of platelets in concentrate can alter their morphological characteristics and their survival post transfusion. Moroff, G., *et al.*, "Factors Influencing Changes in pH during Storage of Platelet Concentrates at 20-24° C," *Vox Sang.* 42:33 (1982). The range of pH at which platelets function normally is from approximately 6.0 - 6.5 to 7.6. Stack, G. and E.L. Snyder, "Storage of Platelet Concentrate," *Blood Separation and Platelet Fractionation* 99, at 107 (1991). To measure pH of the samples, a CIBA-CORNING 238 pH/Blood Gas analyzer was used (CIBA-CORNING, Norwood, MA). A small amount of platelet concentrate from each sample was introduced into the pH/Blood Gas analyzer.

Measurements of pH were taken at time zero and after 5 days of storage for all samples. FIGS 22D, 23D, 24D and 25D are bar graphs showing pH results for a dark control, a light control and an experimental light plus compound. These graphs indicate that the pH of platelet concentrate samples after illumination in the presence of any one of the compounds remains above a pH of 6.5. Thus platelets remain at a pH acceptable for stored platelets following photoinactivating treatment using compounds of the present invention.

### 3) Aggregation

Platelet aggregation is measured by the change in optical  
5 transmission that a platelet sample exhibits upon stimulation of  
aggregation. Platelet aggregation was measured using a Whole Blood  
Aggregometer (Chrono-Log Corp., Havertown, PA, model 560VS). The  
number of platelets in each sample was controlled to be constant for every  
measurement. A Model F800 Sysmex cell counter (Toa Medical Electronics,  
10 Kobe, Japan) was used to measure platelet count in the platelet samples and  
autologous plasma was used to adjust platelet counts to 300,000/mL of  
platelet concentrat

For the procedure, all the samples were incubated in a capped plastic  
tube for 30 minutes at 37°C for activation. The aggregometer was warmed  
15 up to 37°C. The optical channel was used for platelet aggregation  
measurement. The magnetic speed of the aggregometer was set at 600  
/min. Remaining platelet concentrate, from the units obtained which was  
not drawn as a sample for treatment, was centrifuged at high speed (14,000  
g) with a micro-centrifuge for 5 minutes to obtain containers of platelet  
20 poor plasma autologous to the experimental samples.

To begin, 0.45 ml of the autologous platelet poor plasma was added  
along with 0.5 ml of saline into a glass cuvette and placed in the PPP  
channel. Then 0.45 ml of the sample platelet concentrate and 0.50 ml of  
saline were added to a glass cuvette (containing a small magnet) into the  
25 sample channel. After one minute, ADP and collagen reagents (10  $\mu$ l) each  
were added to the sample cuvette. The final concentration of ADP was  
10  $\mu$ M and the final concentration of collagen was 5  $\mu$ g/ml. Platelet  
aggregation was recorded for about 8-10 minutes or until the maximum  
reading was reached.

30 The results appear in FIGS. 22B, 23B, 24B, and 25B. The 100 %  
aggregation line is the level at which the recorder was set to zero. The 0%  
aggregation line is where the platelets transmitted before the ADP and  
collagen were added. The aggregation value for the sample is determined  
by taking the maximum aggregation value as a percent of the total range.  
35 Three of the four compounds tested showed very little or no difference in  
aggregation levels between the samples treated with compound and the  
untreated samples which were stored for 5 days. Compound 2 exhibited a  
small reduction in aggregation, of approximately 8% from the day 1 control.



The aggregation for the sample treated with compound and UV was the same as that for the UV only sample. This is supporting evidence that the decontamination compounds tested do not have a significant effect on platelet aggregation when used in the methods of the present invention.

5

#### 4) Count

A Sysmex cell counter was used to measure platelet count in the platelet samples. Samples were diluted 1 : 3 in blood bank saline.

The results of the platelet count measurements appear in FIGS. 22A, 23A, 24A, and 25A. For each of the compounds, the samples show little or no drop in platelet count between the Day 5 control and the Day 5 treated sample. Interestingly, samples treated with Compounds 6, 17 and 18 all display a higher platelet count than samples treated with light alone. For example, samples treated with Compound 6 had counts equivalent to the 5 day control, but samples treated with only ultraviolet light showed approximately a 33% reduction in platelet count. Thus, not only is treatment with compounds of the present invention compatible with the maintenance of platelet count, but it actually appears to prevent the drop in count caused by ultraviolet light exposure.

20

### EXAMPLE 20

A preferred compound for decontaminating blood subsequently used *in vivo* should not be mutagenic to the recipient of the blood. In the first part of this experiment, some compounds were screened to determine their genotoxicity level in comparison to aminomethyltrimethylpsoralen. In the second part, the *in vivo* clastogenicity of some compounds of the present invention was measured by looking for micronucleus formation in mouse reticulocytes.

30

#### 1) Genotoxicity

Mammalian cell cultures are valuable tools for assessing the clastogenic potential of chemicals. In such studies, cells are exposed to chemicals with and/or without rat S-9 metabolic activation system (S-9) and are later examined for either cell survival (for a genotoxicity screen) or for changes in chromosome structure (for a chromosome aberration assay).

35

Chinese hamster ovary (CHO; ATCC CCL 61 CHO-K1, proline-requiring) cells were used for the *in vitro* genotoxicity and chromosomal

aberration tests. CHO cells are used extensively for cytogenic testing because they have a relatively low number of chromosomes ( $2n=20$ ) and a rapid rate of multiplication (~12 to 14 hours, depending on culture conditions). The cells were grown in an atmosphere of 5% CO<sub>2</sub> at approximately 37° C  
5 in McCoy's 5a medium with 15% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin-streptomycin solution to maintain exponential growth. This medium was also used during exposure of the cells to the test compound when no S-9 was used. Cell cultures were maintained and cell exposures were performed in T-75 or T-25 flasks.

10 Each of the sample compounds were tested at seven dilutions, 1, 3, 10, 33, 100, 333, and 1000 µg/ml. The compound was added in complete McCoy's 5a medium. After the compound was added, cells were grown in the dark at approximately 37° C for approximately 3 hours. The medium containing the test compound was then aspirated, the cells were washed  
15 three times with phosphate-buffered saline (PBS) at approximately 37° C, and fresh complete McCoy's 5a medium was added. The positive control was methylmethane sulfonate. The solvent control was dimethylsulfoxide (DMSO) diluted in culture medium. For assays using metabolic activation (see below) the activation mixture was also added to the solvent control.  
20 The cultures were then incubated for an additional time of approximately 12 hours before they were harvested. Colchicine (final concentration, 0.4 µg/ml) was added approximately 2.5 hours prior to the harvest.

After approximately 2.5 hours in colchicine, the cells were harvested. Cells were removed from the surface of the flasks using a cell scraper. The  
25 resulting cell suspension was centrifuged, the supernatant, aspirated, and 4 ml of a hypotonic solution of 0.075 M KCl added to the cells for 15 minutes at approximately 37°C. The cells were then centrifuged, the supernatant aspirated, and the cells suspended in a fixative of methanol: acetic acid (3:1). After three changes of fixative, air-dried slides were prepared using  
30 cells from all flasks. The cell density and metaphase quality on the initial slide from each flask was monitored using a phase-contrast microscope; at least two slides of appropriate cell density were prepared from each flask. The slides were stained in 3% Giemsa for 20 min, rinsed in deionized water, and passed through xylene. Coverslips were mounted with  
35 Permount. Slides are then examined to determine what concentration of each test compound represented a toxic dose.

An analysis of the results showed that AMT was genotoxic at 30 µg/ml. In contrast, Compounds 2 and 6 were only genotoxic at 100 µg/ml,

more than three times the toxic dose of AMT.

A psoralen compound with a structure distinct from compounds of the present invention, 8-aminomethyl-4,4',5'-trimethylpsoralen, was also tested in this experiment and proved to be toxic at 10 µg/ml. While the 8-  
5 substituted aminomethyl compound and similar structures may not be suited for methods of the present invention, they may be useful for alternative purposes. In light of the ability of the compounds to prevent nucleic acid replication, in combination with their extreme toxicity, the compounds could be used, for example, to treat diseases characterized by  
10 uncontrolled cell growth, such as cancer.

## 2) Micronucleus Assay Protocol

Saline solutions were prepared for Compounds 2, 6, 17 and 18 at various concentrations. Male Balb/c mice were then injected with 0.1 ml of  
15 a compound solution via the tail vein. At least 3 mice were injected per dose level. Saline only was used as a negative control. For a positive control, cyclophosphamide (cycloPP) was administered at a dose of 30 mg/kg. In the experimental group, the injections were repeated once per day for four days. In the positive control group, the sample was  
20 administered only once, on day three. On day 5, several microliters of blood were withdrawn from each subject and smeared on a glass slide. Cells were fixed in absolute methanol and stored in a slide rack.

For analysis, cells were stained with acridine orange and visualized under a fluorescence microscope by counting: (i) the number of  
25 reticulocytes per 5000 erythrocytes; and (ii) the number of micronucleated reticulocytes per 1000 reticulocytes. Reticulocytes were distinguished by their red fluorescence due to the presence of RNA. Micronuclei were distinguished by their green fluorescence due to the presence of DNA. The percentage of reticulocytes (%PCE) was then calculated. A decrease in the  
30 frequency of erythrocytes, represented by an increase in the percentage of reticulocytes, is an indication of bone marrow toxicity. The percentage of reticulocytes with micronuclei (%PCE with MN) was also calculated. An increase in %PCE with MN is a measure of clastogenicity.

After initial results were determined, the experiment was  
35 repeated using increased dose levels, until: (i) Micronucleus formation was seen; or (ii) Bone marrow toxicity was observed; or (ii) The lethal dose was reached; or (iv) A dose of 5 g/kg was administered. For the assays with each of the compounds 2, 6, 17 and 18, the acutely lethal dose was reached

before there were any signs of bone marrow toxicity or micronucleus formation. The results of the experiment appear in Table 12, below. As is clear from the table, no bone marrow toxicity was observed for any of the compounds at the doses tested. The percent reticulocyte value for treatment with each compound remained close to the negative control value. This is in contrast with a drop of approximately 2-2.5% PCE/RBC seen in the positive control, representing erythrocyte depletion due to bone marrow toxicity. Nor did any of the compounds display clastogenic action.

TABLE 12

10

COMPOUND	DOSE (mg/kg)	PCE/RBC (%)	PCE + MN (%)	# duplicates
2	40	3.08 ± 0.82	0.20 ± 0.14	4
2	25	3.46 ± 0.32	0.25 ± 0.11	6
CycloPP	30	1.65 ± 0.64	1.98 ± 0.40	6
saline		3.49 ± 0.55	0.18 ± 0.13	6
6	45	3.79 ± 0.41	0.36 ± 0.14	3
6	30	3.61 ± 0.12	0.27 ± 0.38	3
17	45	5.7 ± 2.14	0.31 ± 0.07	3
17	30	3.47 ± 0.83	0.30 ± 0.17	3
CycloPP	30	0.99 ± 0.33	1.76 ± 0.64	3
saline		3.47 ± 0.44	0.23 ± 0.15	3
18	20	3.48 ± 0.79	0.17 ± 0.06	3
18	7.5	3.59 ± 0.33	0.43 ± 0.12	3
18	3.75	3.61 ± 1.14	0.17 ± 0.12	3
CycloPP	30	1.39 ± 0.41	2.09 ± 0.17	3
saline		3.31 ± 0.63	0.36 ± 0.11	3

EXAMPLE 21

In EXAMPLE 13, the inactivation of cell-free HIV virus, using compounds and methods of the present invention, is shown. This example shows inactivation of cell-associated HIV also using compounds of the present invention.

H9 cells chronically infected with HIV<sub>III</sub>B were used. (H9/HTLV-III-B NIH 1983 Cat.#400). Cultures of these cells were maintained in high glucose Dulbecco Modified Eagle Medium supplemented with 2 mM L-

glutamine, 200 u/mL penicillin, 200 µg/ml streptomycin, and 9% fetal bovine serum (Intergen Company, Purchase, N.Y.) For maintenance, the culture was split once a week, to a density of  $3 \times 10^5$  to  $4 \times 10^5$  cells/ml and about four days after splitting, 3.3% sodium bicarbonate was added as  
5 needed. For the inactivation procedure, the cells were used three days after they were split. They were pelleted from their culture medium at  $400 \text{ g} \times 10$  minutes, the supernatant was discarded, and the cells were resuspended in one to five day old human platelet concentrate (PC) (pH 7.5-6.5), to a concentration of  $2 \times 10^6$  cells/ml. Aliquots of the PC-infected cell  
10 suspension were made for psoralen free dark controls, for psoralen free UVA only controls, for psoralen dark controls, and for the psoralen plus UVA experimental sample. Concentrated filter-sterilized stock solutions of each psoralen in water were diluted into the appropriate aliquots to yield a final concentration of  $150 \text{ } \mu\text{M}$ . (A 10 mM stock of Compound 18 was  
15 diluted about 67-fold and a 2 mM stock of Compound 2 was diluted about 13-fold). After an equilibration period of thirty minutes at room temperature, 0.5 ml of each of the dark controls was placed in a cryovial and stored in the dark at  $-80^\circ \text{ C}$ . For UVA illumination, 8 ml of the psoralen free aliquot and 8 ml of each psoralen containing aliquot were introduced  
20 into a modified Fl 20 Teflon™ bag (modified to be  $92 \text{ cm}^2$  total surface area, The West Co., Phoenixvill, PA) via a plastic disposable 10 ml syringe attached to one of the polypropylene ports on the bag. This gave an average path length of 0.17 cm. The bags were then illuminated for a total exposure of  $3 \text{ Joules/cm}^2$  in the device described in Example 1, above, attached to a  
25 circulating refrigerating waterbath set at  $4^\circ \text{ C}$ , which maintains the temperature in the bag at approximately  $22\text{-}25^\circ \text{ C}$ . During exposure, the device was shaken on a platelet shaker (Helmer Labs, Noblesville, IN). After exposure, the contents of the bags were withdrawn by a fresh syringe through the remaining unused port on the bag, and placed in cryovials for  
30 storage in the dark at  $-80^\circ \text{ C}$  until analysis.

The stored samples were thawed at  $37^\circ \text{ C}$ , then titrated in an HIV microplaque assay, as described in Hanson, C.V., Crawford-Miksza, L. and Sheppard, H.W., J. Clin. Micro 28:2030 (1990), and as described in EXAMPLE 13, above, with the following modifications. Clot removal from each  
35 sample was performed before plating. Because plating of a target volume of 4 ml after clot removal was desired, an excess of sample (6 ml) was transferred to a polypropylene tube and diluted to a final volume of 60 ml with Test and control samples from the inactivation procedure were

diluted in 50% assay medium and 50% normal human pooled plasma. The samples were serially diluted directly in 96-well plates (Corning Glass Works, Corning, N.Y.). The plates were mixed on an oscillatory shaker for 30 seconds and incubated at 37°C in a 5% CO<sub>2</sub> atmosphere for 1 to 18 hours.

5 MT-2 cells (0.025 mL) [clone alpha-4, available (catalog number 237) from the National Institutes of Health AIDS Research and Reference Reagent Program, Rockville, Md.] were added to each well to give a concentration of 80,000 cells per well. After an additional 1 hour of incubation at 37°C in 5% CO<sub>2</sub>, 0.075 mL of assay medium containing 1.6% SeaPlaque agarose (FMC

10 Bioproducts, Rockland, Maine) and prewarmed to 38.5°C was added to each well. The plates were kept at 37°C for a few minutes until several plates had accumulated and then centrifuged in plate carriers at 600 x g for 20 minutes in a centrifuge precooled to 10°C. In the centrifuge, cell monolayers formed prior to gelling of the agarose layer. The plates were

15 incubated for 5 days at 37°C in 5% CO<sub>2</sub> and stained by the addition of 0.05 mL of 50 µg/mL propidium iodide (Sigma Chemical Co.) in phosphate-buffered saline (pH 7.4) to each well. After 24 to 48 hours, the red fluorescence-stained microplaques were visualized by placing the plates on an 8,000 µW/cm<sup>2</sup> 304 nm UV light box (Fotodyne, Inc., New Berlin, Wis.).

20 The plaques were counted at a magnification of x20 to x25 through a stereomicroscope.

The results were as follows: Compound 2 (150 µM) inactivated >6.7 logs of HIV after 3 Joules/cm<sup>2</sup> irradiation (compared to dark and light controls of 0 log inactivation, starting log titer 6.1 plaque forming

25 units/ml). At the same concentration and irradiation time, Compound 18 inactivated >7.2 logs of HIV (compared to a dark control of 0 logs and a light control of .1 logs, starting titer 6.6). This example supports that the compounds of the present invention are effective in inactivating cell associated virus.

30

#### EXAMPLE 22

This example involves an assessment of new synthetic media formulations as measured by the following *in vitro* platelet function assays: 1) maintenance of pH; 2) platelet aggregation ("Agg") and 3) GMP140 expression.

35 The assays for each of these tests have been described above.

TABLE 13\*

	S 2.19	S 2.22	S 3.0	S 4.0
Na gluconate	23	0	0	0
Na acetate	27	20	20	20
glucose	0	2	2	2
mannitol	30	20	0	20
KCl	5	4	4	4
NaCl	45	80	100	90
Na <sub>3</sub> citrate	15	15	10	10
Na phosphate	20	20	20	20
MgCl <sub>2</sub>	0	3	2	2

\*Amounts in mM

Four formulations were prepared: S 2.19, S 2.22, S 3.0 and S.4.0. The composition of these synthetic media formulations are shown in Table 13.

One unit of human platelet rich plasma (PRP) was obtained from the Sacramento Blood Bank. The unit was centrifuged at room temperature for 6 minutes at 4000 rpm and then transferred to a unit press. Using an attached transfer line, plasma was expressed from the unit, leaving approximately 9.4 mls of residual plasma.

The unit was allowed to rest for 1 hour, after which it was gently kneaded to resuspend the platelets. To 0.6 ml of the suspension, 2.4 ml of plasma was added back and the entire contents transferred to a Teflon™ minibag. The reconstituted unit was assayed for pH and other tests the next day, with the following results:

pH	7.19
GMP140	62%
Agg	58%

The remaining unit was then used to evaluate synthetic media for platelet storage with and without photodecontamination. Aliquots (0.8 ml) from the unit were added to each formulation (3.2 mls) in tubes. 3 mls of each mixture was transferred to a Teflon™ minibag (final plasma concentration of 20%).

Five days later, platelet function was assessed using the battery of tests described above. The results for each of the synthetic media formulations are shown in Table 14 below.

**TABLE 14**

	no light		light	
	S 2.19	S 2.22	S 2.19	S 2.22
pH	6.86	6.82	6.83	6.60
GMP140	87%	74%	90%	80%
Agg	30	48	16	31

It appeared that the synthetic media containing 2 mM glucose (*i.e.*, S 2.22) maintained platelet function, as measured by GMP140 and Aggregation, better than the synthetic media that did not contain glucose (*i.e.*, S 2.19).

5 To confirm the above finding, experiments were repeated ("n" being the number of replicate experiments) with these formulations as well as additional glucose-free formations (3.0 and 4.0). Platelet function was evaluated both before and after storage, and in conjunction with photodecontamination. A summary of the results is provided in Tables 15, 16 and 17, below.

10

**TABLE 15\***

	Plasma	S 2.22	S 3.0	S 4.0	S 2.19
	n=17	n=22	n=4	n=4	n=23
pH	7.31	7.14	7.12	7.13	7.04
Agg	82	83	76	78	81
GMP-140	52	49	46	45	68

\*No UVA; Day 1 of Storage

**TABLE 16\***

	Plasma	S 2.22	S 3.0	S 4.0	S 2.19
	n=18	n=20	n=4	n=4	n=23
pH	7.03	6.92	6.93	6.93	6.96
Agg	75	70	67	70	64
GMP-140	61	63	63	64	74

\*No UVA; Day 5 of Storage

15

**TABLE 17\***

	S 2.22	S 3.0	S 4.0	S 2.19
	n=20	n=4	n=4	n=22
pH	6.80	6.78	6.79	6.95
Agg	59	54	54	58
GMP-140	73	76	76	83

\*3 Joules UVA; Day 5 of Storage



**EXAMPLE 23**

In this example, bacterial inactivation efficiency of several  
 5 compounds of the present invention was evaluated by examining the  
 ability of the compounds to inactivate a variety of bacteria. For this  
 experiment, twelve phylogenetically distinct and clinically pathogenic  
 strains of bacteria were studied. Table 18 contains a list of these bacteria. All  
 bacteria were obtained from the Department of Public Health Services in  
 10 Berkeley, CA. Inactivation assays were performed as follows.

**TABLE 18**

<u>Corynebacterium minutissimum</u>	gram positive	Public Health # 88A-1157
<u>Enterobacter cloacae</u>	gram negative	Public Health # 6710-A-76
<u>Escherichia coli</u>	gram positive	Public Health # 7186-75
<u>Klebsiella pneumoniae</u>	gram negative	Public Health # 92A-2214
<u>Listeria monocytogenes</u>	gram positive	Public Health # 86A-6620
<u>Pseudomonas aeruginosa</u>	gram negative	Public Health # 91A-5818
<u>Salmonella choleraesuis</u>	gram negative	Public Health # 92A-2467
<u>Serratia marcescens</u>	gram negative	Public Health # 2574-3-79
<u>Staphylococcus aureus</u>	gram positive	Public Health # 89A-3667
<u>Staphylococcus epidermidis</u>	gram positive	Public Health # 85A-2460
<u>Streptococcus Group A</u>	gram positive	Public Health # 90A-2540
<u>Yersinia enterocolitica</u>	gram positive	Public Health # 91A-7632

15 For E. cloacae, E. coli, K pneumoniae, L. monocytogenes, P. aeruginosa,  
S. choleraesuis, S. marcescens, S. aureus, S. epidermidis and Y. enterocolitica, a  
 single colony of bacteria was taken from Luria-Bertani (LB) agar plates [LB  
 broth-Bactotryptone 10 g/L (Difco, Detroit MI), Yeast extract 5 g/L, NaCl 10  
 g/L; LB Agar plates-LB and Bacto-agar 15g/L (Difco) in petri dish] and  
 20 inoculated in to 3 ml LB broth in a Falcon 2051 capped tube (Becton Dickinson,  
 Rutherford, NJ). Bacteria were grown overnight in a Lab Line Orbit Environ-  
 shaker (Labline, Melrose Park, IL) at 37°C.

For C. minutissimum, L. monocytogenes and S. Group A, bacteria were  
 streaked on TSA II Soy Agar with 5% Sheep blood plates ( BBL stacker plate;  
 25 Becton Dickinson, Cockeysville, MD). Plates were incubated overnight at 37°C

C. Then 2.5 ml of LB broth were added to the plate. Colonies were scraped into the broth using a sterile loop. LB broth containing the bacteria was then removed from the plate into a sterile Falcon 2051 culture tube.

For all bacteria strains, 100 ml of bacteria suspension were diluted with  
5 900 ml of LB broth. Using LB broth as a reference, a spectrum was taken from 400-800 nm using a Shimadzu UV160U spectrophotometer (Shimadzu, Kyoto, Japan). Bacterial density was calculated assuming that an optical density (OD) of 1.0 at 600 nm was equivalent to  $5 \times 10^8$  cfu/ml (colony forming units/ml). OD readings ranged from  $1.1 \times 10^9$  cfu/ml to  $2.75 \times 10^9$  cfu/ml.

10 For S. choleraesuis, E. cloacae, K. pneumoniae and P. aeruginosa, recently outdated platelet concentrate units from a local Blood Bank were stored in Helmer platelet incubator and shaker at 22°C until use. Two units (each comprising 4 pooled platelet concentrates) of approximately 50ml each were pooled using a Haemonetics SCD 312 sterile docker (Terumo; Braintree,  
15 MA) in order to have sufficient volume for each experiment. The platelet concentrate was divided evenly in 50 ml conical centrifuge tubes (Starstedt). Tubes were spun at 3800 rpm for 6 minutes with brake 6 on a Sorvall RC3B Centrifuge (DuPont Company; Wilmington, DE). Plasma was removed and replaced with a synthetic media (comprising: 116 mM NaCl, 10 mM Na<sub>3</sub>  
20 citrate, 30 mM Na acetate, having pH adjusted to 7.2 with HCl) such that the final concentration was 35% plasma 65% synthetic media. Platelets were resuspended via serial pipette (Falcon, Becton Dickinson; Rutherford, NJ). For the rest of the bacteria, apheresis units were used. These also contained the same synthetic media, however, S. aureus had additional phosphate (26  
25 mM) and S. Group A, L. monocytogenes had additional phosphate (26 mM) and mannitol (20 mM).

Blood gas analysis was done on a Ciba-Corning 238 pH/Blood Gas Analyzer (Ciba-Corning; Medfield, MA) and ranged from 6.57 to 7.08 pH. Platelet count was performed by diluting platelet concentrate 1:3 in S/P  
30 Certified Blood Bank Saline (Baxter), diluting that sample using a Sysmex DD-100 with Quicklyser-II™ (TOA Medical Electronics Co., LTD; Kobe, Japan) and using a Sysmex Microcell counter F-800. Numbers were multiplied by 3 to get actual concentration of platelets which ranged from  $8.52 \times 10^5$ /ml to  $1.1 \times 10^7$ /ml.

35 An aliquot (30 ml) of this platelet suspension was put into three 50ml tubes. Bacteria was added to all three such that the final concentration was  $10^6$  plaque forming units (pfu)/ml based on the count taken above, except P. aeruginosa, which was added at  $10^5$  pfu/ml. Two of the three tubes had

Compound 2 added at a final concentration of 150 mM. From each tube, 27ml was put into mini PL732 blood bags (Baxter Fenwall, Deerfield, IL) using a serological pipette and a 20cc syringe. As much air as possible was removed from the bag before capping. Bag thickness was about 1cm at the highest point when completely filled. For S. marcescens, K. pneumoniae, S. choleraesuis, and E. cloacae, 2 additional tubes were set up with  $10^5$  cfu/ml bacteria and 150 mM Compound 2. For P. aeruginosa, duplicates were set up for  $10^5$  cfu/ml,  $10^4$  cfu/ml, and  $10^3$  cfu/ml with 150 mM Compound 2.

Bags were laid on their side in the device described in Example 1, above. Irradiation was for one Joule intervals with constant shaking. Between irradiations, bags were picked up and mixed by hand. All bacteria were irradiated for 2 joules total, except S. marcescens, K. pneumoniae, and P. aeruginosa, which were irradiated for 3 joules.

After the two joule irradiation, the exterior of the bag was wiped off using 70% ethanol. A 1 cc insulin syringe with a 28 gauge 1/2 in. needle was used to remove 2 aliquots from the bag. Approximately 300 ml was removed into a sterile Falcon 2017 culture tube for plating on LB agar. The culture tube was gently vortexed and 2 aliquots of 100 ml was removed and sterile plated on 2 LB plates. C. minutissimum, L. monocytogenes and S. Group A were plated on Soy Agar with 5% Sheep blood plates. Plates were incubated overnight at 37°C.

Treated platelet concentrate (1ml) was incubated in 9 ml of sterile LB in a 50 ml Starstedt tube. The lid was loosely attached with tape. Culture was shaken overnight at 37°C and was then streaked on sterile LB plates using a sterile loop. C. minutissimum, L. monocytogenes and S. Group A were plated on Soy Agar with 5% Sheep blood plates. These plates were incubated overnight at 37°C. Complete kill was determined by clean plates after the overnight incubation. The sampling procedure was repeated at the 3 joule point for gram negative bacteria samples.

Serial 1:10 dilutions using non-irradiated platelet concentrate/ bacteria/ Compound 2 as well as for the irradiated UVA control were done in sterile Falcon 2017 culture tubes containing LB broth down to  $10^{-6}$  dilution. The  $10^{-1}$  to  $10^{-3}$  dilutions of these were discarded as colonies would have been too numerous to count. Culture tubes were gently vortexed and 100 ml was sterile plated on LB plates. C. minutissimum, L. monocytogenes and S. Group A were plated on Soy Agar with 5% Sheep blood plates. Plates were incubated overnight in 37°C incubator.

Colonies were counted and the numbers obtained were multiplied by

the appropriate dilution factor to get cfu/ml. Titers for each treatment were averaged and the log<sub>10</sub> bacteria was calculated. Log<sub>10</sub> kill was calculated by subtracting the treated bacteria log<sub>10</sub> from the untreated bacteria log<sub>10</sub>. For samples that showed no colonies, 1 ml samples were then plated, incubated and counted. If these plates showed no colonies, the kill was considered to be complete.

The results appear in Table 19, below. Complete kill, to the detection limit of the assay used, was achieved against all bacteria tested.

10

TABLE 19

Organism	Starting Log Titer	Log Kill
<u>Corynebacterium minutissimum</u>	5.79	complete kill
<u>Enterobacter cloacae</u>	6.1	complete kill
<u>Escherichia coli</u>	6.18	complete kill
<u>Klebsiella pneumoniae</u>	6.58	complete kill
<u>Listeria monocytogenes</u>	6.82	complete kill
<u>Pseudomonas aeruginosa</u>	4.52	complete kill
<u>Salmonella choleraesuis</u>	6.22	complete kill
<u>Serratia marcescens</u>	5.32	complete kill
<u>Staphylococcus aureus</u>	6.44	complete kill
<u>Staphylococcus epidermidis</u>	5.8	complete kill
<u>Streptococcus Group A</u>	6.07	complete kill
<u>Yersinia enterocolitica</u>	6.13	complete kill

## EXAMPLE 24

The life cycle of HIV includes entry into a host cell, reverse transcription of the viral RNA into DNA and integration of the viral DNA into the host genome. This integrated viral DNA is called proviral DNA. The proviral DNA can be replicated, transcribed and translated into viral protein. The viral RNA is packaged into particles that bud out of the infected cell. Viable virus budding from infected cells is termed cell-associated virus. Intact viable virus particles after budding from the infected cell are called cell-free virus. Both cell-free and cell-associated virus have been demonstrated to be sensitive to Compound 2 treatment in platelet concentrates.

Proviral DNA can also be maintained in the absence of transcription and translation resulting in a latent infection. Since it is possible for latently

infected lymphocytes to contaminate platelet concentrates, experiments were designed to measure inactivation of provirus by Compound 2 plus UVA light.

The OM-10.1 cell line is a clone of HL-60, a promyelocyte cell line, that harbors a single copy per cell of proviral DNA of the IIIB strain of HIV-1. Butera ST, et al., (1991) "Oscillation of the HIV surface receptor is regulated by the state of viral activation in a CD4+ cell model of chronic infection", *Journal of Virology* **65**: 4645-4653. The provirus is an obligate intermediate in the replication cycle of HIV. The provirus present in the OM-10.1 is in a state of "absolute" latency as defined by the absence of proviral transcription. Butera ST, et al. (1994) "Human Immunodeficiency virus type 1 RNA expression by four chronically infected cell lines indicated multiple mechanisms of latency" *Journal of Virology* **68**: 2726-30. There is, however, a subpopulation of individual cells within a culture that do produce HIV at a low constitutive level. Upon stimulation by the cytokine TNF- $\alpha$ , OM-10.1 cells begin to produce infectious HIV. Viral expression, once initiated, produces large amounts of infectious progeny and viral p24 protein. At the same time expression of CD4, the cellular receptor for HIV, is down regulated. Butera, *Journal of Virology* 65:4645 (1991). OM10.1 is used here as a sensitive model system with a broad dynamic range for the study of the sensitivity of proviral HIV to Compound 2 treatment.

20

#### **Cultivation of the OM-10.1 cell line**

OM-10.1 cells (obtained from American Type Culture Collection, #CRL10850) were propagated in RPMI 1640 culture medium (RPMI) (available from Gibco, Inc., Gathersburg Maryland) with 10% heat-inactivated FBS and 2 mM L-glutamine, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin. Cultures were maintained in a 37°C, 5% CO<sub>2</sub>, humidified incubator. The subculturing schedule required a 1:10 split of each culture every 48-72 hours. It was important not to allow the cell culture to overgrow to greater than  $1 \times 10^6$  cells per mL. Overgrowth stimulated the expression of infectious HIV. Low passage stocks of cell suspension in FBS with 10% DMSO were archived in a vapor phase liquid nitrogen freezer.

30

#### **Photochemical treatment conditions**

A 48 hour old culture of OM-10.1 cells was counted (Neubauer type hemocytometer) to determine the concentration of cells in suspension. The concentration was then adjusted by pelleting the cells at 1600 RPM for 10 minutes in a Beckman Instruments J-6B centrifuge with a JS-4.2 rotor (Palo Alto, California). The supernatant was removed and discarded. The cells were then resuspended in tissue culture medium (RPMI/10% FBS) at a final density of  $2 \times 10^5$

35

cells per mL. Five mL of this OM-10.1 cell suspension was then added to each well of a 6-well tissue culture plate ( $1 \times 10^6$  cells in 5 mL per well). Serial ten-fold dilutions were made of a concentrated stock of Compound 2 in deionized water. This was done so that Compound 2 could be added as a 100x concentrate to separate wells to achieve final concentrations of 0.01, 0.1, 1, 10, or 100  $\mu\text{M}$  in the cell suspension. After addition of Compound 2 the contents of each well were thoroughly mixed.

The 6-well tissue culture plate was placed in the UVA light device described in Example 1 and illuminated with  $1 \text{ J}/\text{cm}^2$  total illumination without reciprocal shaking. Four control samples were also prepared, none of which received any Compound 2. Two of these controls were also illuminated with  $1 \text{ J}/\text{cm}^2$  UVA, while the other two were not illuminated.

After illumination all samples, including no-UVA controls, were transferred into labeled 15 mL conical polypropylene tubes. Cells were pelleted in a Beckman J-6 centrifuge at 1600 rpm for 10 minutes with a JS-4.2 rotor. The supernatants were removed and discarded. The OM-10.1 cell pellets were resuspended in 5 mL RPMI/10% FBS. Recombinant human TNF- $\alpha$  at 20 U/mL (#TNF-H, Genzyme Corp., Boston MA) was included in the medium of each of the samples scheduled to be stimulated for proviral expression.

Cultures were harvested after 48 hours in culture at  $37^\circ\text{C}$  to allow for the expression of any surviving proviral HIV. Production of HIV p24 antigen was measured using a commercially available Vironostika HIV-1 p24 antigen detection microelisa kit available from Organon Teknika Corp (Durham, NC, catalog. number 59464) according to the manufacturer's instructions. Production of infectious HIV was also measured using an MT-2 microplaque assay as described above in Example 13. Assays were carried out following photochemical treatment and a minimum of 48 hours of incubation after addition of TNF- $\alpha$  to allow for HIV expression. Samples were then kept in storage at  $-80^\circ\text{C}$  for a minimum of 18 hours before assay. These assays were chosen for sensitivity and capability to quantify infectious HIV production.

For the MT-2 microplaque assay, titration of the production of infectious HIV was performed as described in Example 13. Samples of each culture were serially 10-fold diluted prior to assay.

#### **HIV p24 ELISA**

Serial dilutions of each sample were made to bring the p24 signal into the linear range of the assay. HIV p24 levels were determined from standard curves run simultaneously with each p24 experiment.

### **Determination of cell culture density and viability**

Determination of cell density in a cell culture was performed as follows: cells were stained with ethidium bromide which is non-permeant to viable cells, and acridine orange which is permeant to viable cells. A Neubauer type hemacytometer was used to count individual cells for both number and viability using an inverse microscope equipped with a UV light source.

### **Characterization of the OM-10.1 system**

#### **Kinetics of cell growth with and without stimulation**

The kinetics of cell growth and viral expression were studied with and without TNF- $\alpha$  stimulation. Two cultures of OM-10.1 were set up in parallel, one stimulated by TNF- $\alpha$  and the other an unstimulated control. The cultures were sampled at day 0, and daily thereafter until day 4. Samples were taken for titration of infectious virus in the HIV microplaque assay, for quantitation of p24 concentration, and for determination of cell culture density. Sampling of the stimulated culture on the fourth day was not done because of a technical error. The growth kinetics experiment was carried out twice with very similar results (FIG. 26).

TNF- $\alpha$  stimulation clearly initiated the productive infection of the latently infected cell causing an overall decrease in cell number relative to control. This can occur by two mechanisms; an inhibition of cell growth because of metabolic demands of viral assembly, or cell death among some of the virus producing cells.

#### **Kinetics of viral expression with and without TNF- $\alpha$ ,**

The same stimulated and unstimulated cultures described above were sampled for the presence of infectious HIV virions and viral p24 protein. In an unstimulated culture it was observed that there is a linear relationship between the total cell number and the amount of p24 and infectious virus. To compensate for this all values were normalized for total cell number and the baseline values were standardized to zero. The increase in total p24 log<sub>10</sub> ng/ml paralleled the increase in infectious HIV titer over the 4 day time course of the experiment.

Stimulation of the OM-10.1 cell culture with TNF- $\alpha$  resulted in a dramatic increase in production of both infectious virus and p24 protein (FIG. 26). In both cases this represented an increase of over 100-fold from the starting level. Expression of p24 and HIV virions was roughly equivalent in both timing and magnitude. Thus, p24 could serve as a useful predictor of

infectious titer.

Based on these kinetics observations it was decided to harvest the cultures in subsequent inactivation experiments 48 to 72 hours after photochemical treatment and TNF- $\alpha$  stimulation.

**Inactivation data:**

Two experiments were conducted to test the efficacy of psoralen derivatives against the HIV provirus within the OM-10.1 cell line. The first experiment tested a range of concentrations between 0.1 and 100  $\mu\text{M}$  of four novel compounds; Compounds 2, 6, 17 and 18. The second experiment focused exclusively on Compound 2, which was evaluated at a range of between 0.01 and 100  $\mu\text{M}$ .

**Inactivation of Proviral HIV**

OM-10.1 cells were photochemically treated with 1 Joule/ $\text{cm}^2$  UVA light and Compounds 2, 6, 17 or 18, each at concentrations of 0.1, 1.0, 10 and 100  $\mu\text{M}$ . The cells were then isolated from the psoralen-containing medium and cultured in RPMI 1640 medium with 20 U/ml TNF- $\alpha$ . Samples were taken after 72 hours of stimulation and examined for total p24 (FIG. 27) and for infectious HIV titer (Table 20).



TABLE 20

	Sample	p24 ng/ml	HIV titer log pfu/ml
OM-10.1 cell controls	OM-10.1 on day 0	3	1.6-2.5
	No UV-A No TNF, 72 hrs.	204.5	4.1
	No UV-A + TNF, 72 hrs.	576.1	4.5
	#NAME?	268.3	4.3
	#NAME?	522.8	4.5
Compound 2	0.1 $\mu$ M	3.2	<0.7
	1.0 $\mu$ M	2.3	<0.7
	10 $\mu$ M	1.8	<0.7
	100 $\mu$ M	1.4	<0.7
	100 $\mu$ M NO UV-A, -TNF	78.2	2.2-3.1
	100 $\mu$ M NO UV-A, +TNF	64.5	1.7-2.3
Compound 6	0.1 $\mu$ M	38.5	1.6
	1.0 $\mu$ M	2.6	<0.7
	10 $\mu$ M	1.9	<0.7
	100 $\mu$ M	1.5	<0.7
	100 $\mu$ M NO UV-A, -TNF	1.7	<0.7
	100 $\mu$ M NO UV-A, +TNF	1.6	<0.7
Compound 17	0.1 $\mu$ M	14.3	1.5
	1.0 $\mu$ M	2.5	<0.7
	10 $\mu$ M	1.5	<0.7
	100 $\mu$ M	1.5	<0.7
	100 $\mu$ M NO UV-A, -TNF	8.2	<0.7
	100 $\mu$ M NO UV-A, +TNF	28.9	<0.7
Compound 18	0.1 $\mu$ M	2.6	<0.7
	1.0 $\mu$ M	1.6	<0.7
	10 $\mu$ M	1.5	<0.7
	100 $\mu$ M	1.4	<0.7
	100 $\mu$ M NO UV-A, -TNF	173.6	4.1
	100 $\mu$ M NO UV-A, +TNF	163.7	3.4

Controls were prepared with each psoralen derivative at a concentration of 100  $\mu$ M to test for cytotoxicity in the absence of UVA illumination. All four compounds were cytotoxic at 100  $\mu$ M to varying degrees. Compounds 6 and 17 in the absence of light completely inhibited the expression of any detectable infectious HIV. The samples treated with Compound 2 were still able to produce 2 to 3 logs (pfu/ml) of infectious virus compared to 4.5 logs in the analogous control sample.

The p24 concentration of the inactivated samples was measured following TNF- $\alpha$  stimulation for 72 hours (FIG. 27). The 100  $\mu$ M, UVA

illuminated samples were not included in FIG. 27, as they were essentially identical to the 10  $\mu\text{M}$  samples.

At 0.1  $\mu\text{M}$  psoralen there was some breakthrough expression of p24 in the cultures treated with Compounds 6 and 17. All other values were at or below the background p24 level. The background was due to the presence of p24 in the day 0 sample at the outset of the experiment. The viral titer of the 0.1  $\mu\text{M}$  Compound 6 and Compound 17 treated samples was 1.6  $\log_{10}$  pfu/ml. Compounds 2 and 18 completely inhibited expression of infectious HIV at this concentration.

The control samples in this experiment were unusual in that the unstimulated controls (both with and without UVA exposure) produced virus and p24 at almost the same level as stimulated controls. TNF- $\alpha$  stimulated controls had 550 ng/ml p24 and a titer of 4.5  $\log_{10}$  pfu/ml, whereas the unstimulated controls had 235 ng/ml p24 and a titer of 4.2  $\log_{10}$  pfu/ml. This is probably the result of sub optimal cell culture conditions which initiated proviral expression. Infectious titer was measured to correlate well with the p24 data (Table 20).

#### **Inactivation of Proviral HIV by Compound 2 from 0.01 $\mu\text{M}$ to 100 $\mu\text{M}$ :**

In the second experiment, Compound 2 was studied in greater detail. A more complete dose-response curve was generated by continuing to decrease Compound 2 dose until some breakthrough expression was seen. Cultures from this experiment were harvested after 48 hours of culture as opposed to 72 hours in the previous experiment. This resulted in lower overall p24 values for both inactivated samples and controls. In this experiment a UVA exposure of 1 Joule/cm<sup>2</sup> did not stimulate the expression of latent HIV. Additionally, this same light dose without Compound 2 did not inhibit the expression of HIV after stimulation by TNF- $\alpha$ .

The inactivation of latent HIV by Compound 2 in this experiment was comparable to the previous experiment (FIG. 28). No viral expression was detected by measurement of p24 in the 0.1, 1, 10, or 100  $\mu\text{M}$  treated samples. There was a single infectious unit detected in the 1.0  $\mu\text{M}$  Compound 2 treated sample. This was observed at the limit of detection for the microplaque assay. This sample also had a lower p24 value than all of the other treated samples, all of which had undetectable levels of viable virus.

In this experiment, Compound 2 at a concentration of 0.01  $\mu\text{M}$  allowed some breakthrough expression of p24 (9.7 ng/ml) and infectious virus (2.5  $\log_{10}$  pfu/ml). The comparable control culture had a p24 level of 185 ng/ml

and an infectious titer of  $4.8 \log_{10}$  pfu/ml. This represents close to a 95% reduction in p24 expression and more than a 200-fold reduction in infectious titer compared to the control sample.

The experiments described here supported that photochemical treatment with Compound 2 and 1 Joule/cm<sup>2</sup> UVA totally inactivated latent proviral expression of OM-10.1 with concentrations of Compound 2 as low as 0.1  $\mu$ M.

In the first experiment the unstimulated controls showed considerable expression of HIV. This led to the concern that the OM-10.1 culture had been stimulated somehow prior to treatment. If the HIV provirus were being actively transcribed, it may have been a "hotspot" for psoralen inactivation. The negative controls in the second experiment showed that the OM-10.1 cells were not expressing HIV to the same extent and yet were still readily inactivated by Compound 2 photo-treatment. These observations demonstrate that regardless of the state of HIV expression the proviral genome was quite sensitive to Compound 2 photoinactivation.

The inactivation experiments described here were not performed in platelet concentrate. Photoinactivation was instead performed in tissue culture medium. To compensate, the UVA light dose was decreased from the photochemical treatment dose of 3 Joules/cm<sup>2</sup> to 1 Joule/cm<sup>2</sup>. Despite these differences these data strongly suggest that treatment with 150  $\mu$ M of either Compound 2, 6, 17 or 18 together with 3 Joules/cm<sup>2</sup> UVA light in a platelet concentrate solution would be more than sufficient to inactivate all latent proviral HIV genomes present in infected cells contaminating a platelet concentrate.

#### EXAMPLE 25

Cytomegalovirus (CMV) is a member of the Herpes family of viruses. CMV is prevalent in the United States and, as a result, within the blood supply. While the infection in a healthy individual is seldom symptomatic, patients who are immunosuppressed CMV-seronegative and become infected via transfusion of a CMV infected blood product can suffer serious complications or death. Quinley, E.D., "Immunohematology Principles and Practice," J.B. Lippincott Co., Philadelphia, PA (1993). In this example, methods of the present invention are tested for the ability to inactivate cytomegalovirus (CMV).

A  $10^7$  pfu/ml stock of cell associated CMV was made by harvesting flasks of MRC-5 cells (obtained from American Type Culture Collection) on the eighth

day of infection with CMV strain AD 169 (obtained from American Type Culture Collection.) The infected cells were trypsinized and counted using a Neubauer type hemocytometer. The infected cells were then centrifuged and resuspended in phosphate buffered saline (PBS) so that the final concentration was  $10^7$  cells/ml. Each infected cell yields one plaque forming unit, so the final concentration of plaque forming units was also  $10^7$  per ml.

To 63.35 ml of platelets in 65% synthetic media plus phosphate and 35% plasma, 3.35 mls of cell associated CMV at  $2 \times 10^7$  cells per ml to make a final titer of  $10^6$  pfu/ml of cell associated CMV. (This represents a 1:10 dilution of cells into platelet concentrate.) This was mixed thoroughly and split into 4 aliquots: 3 aliquots of 20 mls each, plus one aliquot of 7 mls. To the 3 aliquots of 20 mls, Compound 2 at 12.09 mM was added to final concentrations of  $1.5\mu\text{m}$ ,  $15\mu\text{m}$ , and  $150\mu\text{m}$ . These aliquots were sampled (1 ml ) and the remaining 19 ml were loaded into a mini-bag (Teflon™ Minibags (American Fluoroseal Co., Silver Springs, MD)) and illuminated for 0.5, 1, and 3 Joules/cm<sup>2</sup> in the light device described in Example 1, with 1 ml of each sample removed after each time point. The remaining 7 ml was used as a no drug, no light control, as well as a no drug plus light only control: a 1 ml sample was taken and the remaining 6 mls was placed in a mini-bag and illuminated for 1 Joule/cm<sup>2</sup>. The bag was sampled again after illumination to determine kill due to UV only.

Samples were frozen at  $-80^\circ\text{C}$  overnight and assayed. The CMV was assayed using MRC-5 cells in a plaque assay format. MRC-5 cells were grown to confluence in a 24 well plate. Samples were quickly thawed and diluted 1:10 in Modified Eagles Medium with Earle's salts with Yeastolate (Gibco) and Bovine Serum Albumin (BSA) and allowed to clot at  $37^\circ\text{C}$  for 30 minutes before a second 1:10 diluting in the same medium. Each well was washed with diluent medium (to remove any inhibitory proteins) and then infected with  $125\mu\text{l}$  of diluted sample, and four wells are infected for each dilution. Eight wells (one mL total) are infected for the 3 Joule/cm<sup>2</sup> samples to be sure that there is no remaining live virus. After an hour of infection, the wells are washed and overlaid with 3% Seaplaque agarose mixed 1:1 with Modified Eagle's Medium with Earle's salts with Yeastolate and BSA. After 7 days of incubation the wells are overlaid with an overlay that is identical to the original overlay except with 7% neutral red solution (Gibco) These are incubated for one night and evaluated the following day for plaques.

Plaques have a markedly increased uptake of the neutral red stain as compared to the background lawn of cells. Plaques are visualized on a light box using a stereomicroscope. Cells that are questionable are confirmed using a light

microscope, as are wells where no plaques are found.

The results of this assay are shown in Table 21, below.

TABLE 21

Sample	Starting titer (0J)	Ending titer	Log Kill
1.5 $\mu$ m 0.5J	6.2	2.4**	3.8
1J	6.2	.99*	>5.2
3J	6.2	.99*	>5.2
15 $\mu$ m 0.5J	6.1	.99*	>5.1
1J	6.1	.99*	>5.1
3J	6.1	.99*	>5.1
150 $\mu$ m 0.5J	6.2	.99*	>5.2
1J	6.2	.99*	>5.2
3J	6.2	.99*	>5.2

\* .99 logs is the limit of detection for this assay = no plaque found in 8 wells

These results were further confirmed by PCR using MIE4 and MIE5 (Midland Reagent Co., Midland, Michigan) primers that amplifies a 423 base pair region of the CMV genome. The controls: 0J + no Compound 2, 0J + Compound 2, no Compound 2 + 1 Joule were shown to have no inhibition. The test samples were 1.5 $\mu$ m + 3J, 15 $\mu$ m + 3J and 150  $\mu$ m + 3 J. These samples were identical to the samples taken for the plaque assay. DNA was extracted using SiO<sub>2</sub> and diluted 1:10 serially prior to PCR in TE buffer. Twenty four cycles were performed. Then DNA was visualized in an agarose gel containing Ethidium Bromide on a UV box. The test samples showed increasing inhibition of PCR with increasing concentration, supporting the results of the plaque assay in Table 21.

#### EXAMPLE 26

Hepatitis C virus (HCV) is one of many viruses that is transmissible by blood transfusion. Bovine viral diarrhea virus (BVDV) has inactivation characteristics similar to HCV. This example was designed to examine the inactivation of cell-free BVDV in the presence of single-donor platelet apheresis (SDP) units using a combination of Compound 2 and UVA illumination. The study was structured as four replicate inactivation experiments. In each of the four replicates the "Test" unit contained approximately  $4 \times 10^{11}$  platelets suspended in 35% autologous plasma and 65% synthetic medium plus phosphate, with a total volume of

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approximately 300 mL. In addition, each "Test" unit contained approximately 15 mg of Compound 2 and approximately  $10^5$  pfu/mL of cell-free BVDV stock, which was prepared as follows.

One-day-old roller bottles of MDBK cells (approx. 70-80% confluent) were inoculated with approximately 5 mL of previously prepared BVDV stock. The roller bottles were then incubated for 1-2 hours at 37° C. After incubation, the inoculum was removed and fresh medium was added, and the roller bottles returned to the 37° C incubator. After approximately 72 hours, the new viral stock was harvested and centrifuged at 3000 rpm (approximately 2000 g) for 10' to remove cellular debris. The supernatant was either used immediately as BVDV inoculum, or stored frozen at or below -70° C until use.

In each replicate the "Control" units were prepared from the same SDP or SDP pool as the "Test" unit. The "Control" units differed only in that no Compound 2 was added to the "Control" units, the "Control" units were not illuminated and in two of the four replicates, and the "Control" units were prepared in Teflon™ "mini" bags containing approximately 30 mL instead of the approximately 300 mL in the "Test" unit. Mini bags were used in the Control units to conserve BVDV stock.

Samples were withdrawn from the "Test" units after cumulative UVA exposures of 0 J/cm<sup>2</sup>, 0.5 J/cm<sup>2</sup>, 1 J/cm<sup>2</sup>, 2 J/cm<sup>2</sup> and 3 J/cm<sup>2</sup> and evaluated for virus infectivity. Matched samples were withdrawn from the "Control" units at the same times as the "Test" samples. The "Control" units were never illuminated, however. The samples from the "Control" units were also evaluated for virus infectivity. The virus infectivity was assessed by a plaque assay using bovine turbinate (BT) cells as the host and using a 6-well tray format similar to the plaque assay in Example 25. The plaques were visualized after staining with neutral red and counted with the aid of a light box.

The "Control" samples had an average titer of  $10^{4.9} \pm 0.6$  pfu/mL at the first sampling time. The average titers at the subsequent sampling times were  $10^{4.9} \pm 0.6$  pfu/mL,  $10^{4.9} \pm 0.6$  pfu/mL,  $10^{5.0} \pm 0.6$  pfu/mL and  $10^{5.0} \pm 0.6$  pfu/mL, respectively. Therefore there was no significant decrease in BVDV titer as a result of dilution into SDP during the course of the Compound 2 and UVA treatment.

The "Test" samples had an average initial titer of  $10^{4.8} \pm 0.7$  pfu/mL at the first sampling time (prior to illumination). The average titers at the subsequent sampling times were  $<10^{-1.5}$  pfu/mL,  $10^{-0.2} \pm 0.2$  pfu/mL,  $<10^{-0.3}$  pfu/mL and  $<10^{-1.7}$  pfu/mL, respectively. This demonstrated a rapid  
 5 inactivation of cell-free BVDV in the presence of Compound 2 with increasing amounts of UVA illumination. The average decrease in BVDV titer was at least  $10^{4.8}$  pfu/mL/ $10^{-1.7}$  pfu/mL which represents a  $10^{6.5}$  - fold reduction in BVDV infectivity.

Application of the Poisson distribution to the result of each of  
 10 the four replicate "Test" units led to the conclusion that the level of residual viable BVDV was less than  $10^{-1.2}$  pfu/mL with 95% confidence.

TABLE 22

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Description	Average Titer (Log <sub>10</sub> pfu/mL)	Standard Deviation (Log <sub>10</sub> pfu/mL)
BVDV Stock	6.0	0.5
Control Bag (0 J)	4.9	0.6
Control Bag (0.5 J)	4.9	0.6
Control Bag (1 J)	4.9	0.6
Control Bag (2 J)	5.0	0.6
Control Bag (3 J)	5.0	0.6
Test Bag (0 J)	4.8	0.7
Test Bag (0.5 J)	<-1.5	**
Test Bag (1 J)	-0.2*	0.2
Test Bag (2 J)	<-0.3	**
Test Bag (3 J)	<-1.7	**

In each of 4 replicate experiments, no viable BVDV was recovered after the Compound 2 plus UVA treatment when 48.75 mL was analyzed by plaque assay on BT cells. The average titer of all of the control samples was  
 20  $10^{4.9} \pm 0.5$  pfu/mL (range  $10^{4.2}$  to  $10^{5.5}$  pfu/mL).

These data are consistent with inactivation of as much as  $10^{5.4}$  pfu/mL of BVDV using 150 mM Compound 2 and  $0.5 \text{ J/cm}^2$  of UVA (in 2 of the 4 replicates). These data also show inactivation of  $10^{6.6}$  pfu/mL of BVDV using 150 mM

Compound 2 and 3 J/cm<sup>2</sup> UVA (in two of the four replicates). The average of four replicate experiments showed inactivation of  $10^{6 \pm 0.7}$  pfu/mL of BVDV using 150  $\mu$ M Compound 2 and 3 J/cm<sup>2</sup> of UVA.

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EXAMPLE 27

In this example, we determine the inactivation kinetics by Compound 2 and psoralen of a human non-enveloped virus, parvovirus B19 ("B19"), by looking at infectivity of B19.

10 The following materials were used in the described experiments: 15.1 mM Compound 2; 10 mg/ml Psoralen; normal plasma - control; 1 M sodium ascorbate - prepared fresh; Iscove's Modified Dulbecco's Medium (IMDM)/2% fetal bovine serum (FBS); Dulbecco's phosphate buffered saline (D-PBS) without Ca<sup>++</sup> & Mg<sup>++</sup>; 1 ampule of peripheral blood stem cells ("PBSC"); 96-well and 6-  
15 well plates; and plasma containing infectious B19 (plasma obtained from Bernard J. Cohen, Ph.D., Virus Reference Division, 61 Colindale Avenue, London NW9 5HT, United Kingdom) (hereinafter B19(+) plasma). To confirm the infectivity of the B19(+) plasma samples, the following analysis was performed.

20 1) Sample dilution - Diluted 10  $\mu$ l of each sample in 90  $\mu$ l IMDM/2% FBS ( $10^{-1.0}$ ). Serial dilutions of each sample to  $10^{-4.0}$  were made in the same way.

2) Cells - One ampule of PBSCs cryopreserved in liquid nitrogen was thawed rapidly at 37°C. After transfer to a 50 ml centrifuge tube, the cells were vortexed while cold D-PBS was added up to 50 ml. The cells were centrifuged at  
25 300 relative centrifugal force (RCF) (1190 rpm) for 5 min. The supernatant was discarded and the pellet resuspended with vigorous trituration in 2 ml of D-PBS. This step was followed by bringing the volume up to 50 ml and centrifugation. The wash was then repeated. After the last centrifugation the supernatant was discarded and the pellet was resuspended in 2 ml IMDM/2%  
30 FBS. 0.1 ml was diluted 1:10 in IMDM. These cells were counted using Trypan blue as a vital stain. The concentration was then adjusted with IMDM to yield  $\sim 1.0 \times 10^6$  cells/ml. Then 41 aliquots of 270  $\mu$ l of this suspension was dispensed.

3) Thirty (30)  $\mu$ l of each virus dilution was inoculated into a corresponding aliquot of PBSCs, and the samples were incubated for 2 hours at  
35 4°C.

4) The virus/cell suspension was then transferred to a tube of Methocult™ GF (H4434) medium, vortexed vigorously and allowed to sit until the bubbles all rose to the top. Three (3) wells were plated per sample at  $\sim 1$  ml



per well in a 6-well plate. Sterile water was added to the spaces between the wells to provide adequate humidity. The samples were incubated at 37°C in a humidified 5% CO<sub>2</sub> incubator. Samples were read at 10 - 18 days for burst forming unit-erythroid (BFU-E) formation. Results appear in Table 23. Sample 5 10 showed the most infectivity, and therefore was chosen for the experiments described below.

TABLE 23

SAMPLE	10 <sup>-1.0</sup>	10 <sup>-2.0</sup>	10 <sup>-3.0</sup>	10 <sup>-4.0</sup>
cell control	439/3 ave = 146.3			
1	484/3 ave = 161.3	176	175	161
2	458/3 ave = 152.7	133	166	161
3	475/3 ave = 158.3	165	162	151
4	142/3 ave = 47.3	161	155	180
5	475/3 ave = 158.3	172	139	205
6	498/3 ave = 166	166	129	123
7	438/3 ave = 146	159	143	167
8	143/3 ave = 47.7	146	132	138
9	0/3 ave = 0	364/3 ave = 121.3	162	144
10	0/3 ave = 0	235/3 ave = 78.3	168	166

10

Reagents were prepared for the experiment as follows. First, 3 mM Compound 2 /10 mM sodium ascorbate was prepared by combining 100 µl 15.1 mM Compound 2; 5 µl 1 M sodium ascorbate; and 395 µl dH<sub>2</sub>O. Then 2.5 mg/ml Psoralen/ 25 mM sodium ascorbate was prepared by combining 250 µl 10 mg/ml Psoralen; 25 µl 1 M sodium ascorbate; and 725 µl DMSO. (The present invention contemplates that the use of ascorbate during inactivation aids in the preservation of blood product function during photochemical inactivation. While ascorbate is not believed to be necessary for the methods of the present

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invention, previous experiments to evaluate the use of ascorbate for the preservation of blood product function during photochemical inactivation have suggested improvements attributable to the presence of ascorbate (data not shown). Without restricting the present invention to any particular mode of operation, it is hypothesized that ascorbate acts as a quencher, binding molecular oxygen from the treated sample so that oxygen radicals which may cause damage to the blood product do not have an opportunity to react with the blood product, thereby protecting the function of the blood product).

Next the samples were prepared. To 1.5 ml eppendorf tubes the following was added: 1 X 285  $\mu$ l normal plasma, 1 X 294  $\mu$ l normal plasma, 1 X 95  $\mu$ l B19(+) plasma, 1 X 98  $\mu$ l B19(+) plasma. To 285  $\mu$ l of normal plasma and 95  $\mu$ l of B19(+) plasma, 15  $\mu$ l and 5 $\mu$ l respectively of 3 mM Compound 2 /10 mM sodium ascorbate was added (to give a final concentration of 150  $\mu$ M Compound 2 /0.5 mM sodium ascorbate). These were mixed well and transferred to a 96-well flat bottom tissue culture plate. To 294  $\mu$ l of normal plasma and 98  $\mu$ l of B19(+) plasma was added 6  $\mu$ l and 2  $\mu$ l respectively of 2.5 mg psoralen/ 25 mM sodium ascorbate (to give a final concentration of 50  $\mu$ M Psoralen/0.5 mM sodium ascorbate). Samples were mixed well.

Samples were then illuminated according to the following schedule (all illuminations were performed within 30 minutes of final mixing using a device providing approximately the same intensity, wavelength and distribution of light and temperature as the device described in Example 1) :

**"0" J/cm<sup>2</sup> sampling:**

Compound 2 /B19(+) sample - 10  $\mu$ l was removed for assay into 90  $\mu$ l IMDM/2% FBS (10<sup>-1.0</sup>). Continue dilution series (10  $\mu$ l + 90  $\mu$ l) out to 10<sup>-3.0</sup>. A second 10  $\mu$ l was added to 40  $\mu$ l IMDM/2% FBS for PCR analysis. Freeze PCR sample.

Psoralen/B19(+) sample - 10  $\mu$ l was removed for assay into 90  $\mu$ l IMDM/2% FBS (10<sup>-1.0</sup>). Dilutions were continued out to 10<sup>-3.0</sup>. Another 10  $\mu$ l was removed into 40  $\mu$ l IMDM/2% FBS for PCR analysis and the sample was frozen.

Compound 2 /normal plasma and Psoralen/normal plasma samples - 20  $\mu$ l was removed from each well into an eppendorf tube for HPLC analysis (60  $\mu$ l). Ten (10)  $\mu$ l was removed from the pool into 90  $\mu$ l of PBSC cells for "Drug only" control.

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**"0.7 J/cm<sup>2</sup>" treatment and sampling:** The 96-well plate was illuminated for 0.7 J/cm<sup>2</sup>.

Compound 2 /B19(+) sample - 10  $\mu$ l was removed for assay into 90  $\mu$ l IMDM/2%

FBS ( $10^{-1.0}$ ). The dilution series was repeated ( $10\ \mu\text{l} + 90\ \mu\text{l}$ ) out to  $10^{-3.0}$ . A second  $10\ \mu\text{l}$  was removed into  $90\ \mu\text{l}$  of PBSC cells for undiluted sample.

Psoralen/B19(+) sample -  $10\ \mu\text{l}$  was removed for assay into  $90\ \mu\text{l}$  IMDM/2% FBS ( $10^{-1.0}$ ). The dilution series was repeated ( $10\ \mu\text{l} + 90\ \mu\text{l}$ ) out to  $10^{-3.0}$ . A second  $10$

5  $\mu\text{l}$  was removed into  $90\ \mu\text{l}$  of PBSCs for undiluted sample.

Compound 2 /normal plasma and Psoralen/normal plasma samples -  $20\ \mu\text{l}$  was removed from each well into an eppendorf tube for HPLC analysis ( $60\ \mu\text{l}$  total).

**"2.5 J/cm<sup>2</sup>" treatment and sampling:** The 96-well plate was illuminated for 1.8  
10 J/cm<sup>2</sup> (total UVA =  $2.5\ \text{J}/\text{cm}^2$ ).

Compound 2 /B19(+) sample -  $10\ \mu\text{l}$  was removed for assay into  $90\ \mu\text{l}$  IMDM/2% FBS ( $10^{-1.0}$ ). The dilution series was continued ( $10\ \mu\text{l} + 90\ \mu\text{l}$ ) out to  $10^{-3.0}$ . A second  $10\ \mu\text{l}$  was removed into  $90\ \mu\text{l}$  of PBSCs for undiluted sample.

15 Psoralen/B19(+) sample -  $10\ \mu\text{l}$  was removed for assay into  $90\ \mu\text{l}$  IMDM/2% FBS ( $10^{-1.0}$ ). The dilution series was continued ( $10\ \mu\text{l} + 90\ \mu\text{l}$ ) out to  $10^{-3.0}$ . A second  $10\ \mu\text{l}$  was removed into  $90\ \mu\text{l}$  of PBSC cells for undiluted sample.

Compound 2 /normal plasma and Psoralen/normal plasma samples -  $20\ \mu\text{l}$  was removed from each well into an eppendorf tube for HPLC analysis ( $60\ \mu\text{l}$  total).

20 **"5.0 J/cm<sup>2</sup>" treatment and sampling:** The 96-well plate was illuminated for 2.5 J/cm<sup>2</sup> (total UVA =  $5.0\ \text{J}/\text{cm}^2$ ).

Compound 2 /B19(+) sample -  $10\ \mu\text{l}$  was removed for assay into  $90\ \mu\text{l}$  IMDM/2% FBS ( $10^{-1.0}$ ). The dilution series was repeated ( $10\ \mu\text{l} + 90\ \mu\text{l}$ ) out to  $10^{-3.0}$ . A second  $10\ \mu\text{l}$  was removed into  $90\ \mu\text{l}$  of PBSCs for undiluted sample. The last  $10$   
25  $\mu\text{l}$  was removed into  $40\ \mu\text{l}$  IMDM/2% FBS for PCR analysis and samples were frozen.

Psoralen/B19(+) sample -  $10\ \mu\text{l}$  was removed for assay into  $90\ \mu\text{l}$  IMDM/2% FBS ( $10^{-1.0}$ ). The dilution series was repeated ( $10\ \mu\text{l} + 90\ \mu\text{l}$ ) out to  $10^{-3.0}$ . A second  $10\ \mu\text{l}$  was removed into  $90\ \mu\text{l}$  of PBSC cells for undiluted sample. The last  $10\ \mu\text{l}$  was  
30 removed into  $40\ \mu\text{l}$  IMDM/2% FBS for PCR analysis and samples were frozen.

Compound 2 /normal plasma and Psoralen/normal plasma samples -  $20\ \mu\text{l}$  was removed from each well and placed in an eppendorf tube for HPLC analysis ( $60\ \mu\text{l}$  total).

Next, the infectious assay was performed in peripheral blood stem cells.  
35 One ampule of cryopreserved PBSCs was thawed rapidly in  $37^\circ\text{C}$  water bath, and the contents were transferred into a  $50\ \text{ml}$  conical centrifuge tube. While vortexing the cell suspension gently  $\sim 48\ \text{ml}$  ice cold  $1\times$  D-PBS was added slowly. The mixture was centrifuged at  $300\ \text{RCF}$  for  $5\ \text{min}$  and the supernatant was

discarded. The pellet was resuspended with 2 ml cold D-PBS and triturated vigorously. The volume was brought to 50 ml with more D-PBS, the mixture was centrifuged at 300 RCF for 5 min, and the supernatant was discarded. This was repeated once. The pellet was then resuspended with 2 ml ice cold IMDM/  
5 2% FBS by triturating vigorously. A count of 100  $\mu$ l was taken using trypan blue as a vital stain. The volume of the cell suspension was adjusted to give  $\sim 1 \times 10^7$  cells/ml. A 1:10 dilution of this cell suspension was prepared for each sample to be tested (infected) to yield  $\sim 1 \times 10^6$  cells/ml. Aliquots of 270  $\mu$ l of this suspension were prepared for each dilution to be tested and 90  $\mu$ l for the  
10 undiluted samples. The infected cultures were then incubated for 2 hours at 4°C.

After incubation, the 300  $\mu$ l samples were added to a 3 ml tube of Methocult™GF (H4434) medium and the 100  $\mu$ l samples to a 1 ml tube of Methocult™GF (H4434) medium, followed by vigorous vortexing. Bubbles were allowed to rise to the top of the mixture. Then the mixtures were transferred into  
15 wells (1 ml per well) of a 6-well plate. The plates were incubated at 37°C in a CO<sub>2</sub> incubator. The plates were then observed daily and scored on days 10-18.

The results appear in FIGS. 29 and 30. Note that the assay measures the number of cells that manage to divide and give rise to colony forming units-erythroid (CFU-E). Positive controls show that no BFU-E are present in the B19  
20 infected samples that remain untreated. In the highest dilution samples ( $10^{-3}$ ) even the controls show BFU-E at around the average number. It is presumed that at this concentration either no virus was present in the samples or the virus present was not sufficient to cause infection. The results show that both psoralen and Compound 2 have inactivation activity, which is displayed by the contrast  
25 between surviving BFU-E in the photochemically treated infective samples and the untreated infective samples. This is particularly clear at the first dilution ( $10^{-1}$ ), where photochemical treatment with either psoralen or Compound 2 resulted in greatly lowered infectivity which increased with increasing light doses, whereas the positive control displayed infection so widespread that no BFU-E  
30 appeared. It is also apparent from the results that Compound 2 is significantly more effective in reducing or eliminating the infectivity of the B19 pathogen, as evidenced by a comparison of FIG. 29 with FIG. 30. Again, at the level of the first dilution ( $10^{-1}$ ) photochemical treatment with Compound 2 appears to result in complete or almost complete prevention of infection, whereas psoralen  
35 treatment results in only a 50% reduction in infectivity. Thus aminopsoralens present a particularly effective tool for inactivation.

It is to be understood that the invention is not to be limited to the exact

details of operation or exact compounds, composition, methods, or procedures shown and described, as modifications and equivalents will be apparent to one skilled in the art. All patents described are hereby incorporated by reference.

## CLAIMS

We claim:

1. A method of inactivating non-enveloped viruses in a blood product, comprising, in the following order:
  - a) providing, in any order, i) a psoralen; ii) photoactivating means for photoactivating said psoralen; and iii) a blood product;
  - b) adding said psoralen to said blood product; and
  - c) photoactivating said psoralen, at conditions which inactivate any non-enveloped virus present.
2. The method of Claim 1, wherein said blood product comprises platelets.
3. The method of Claim 1, wherein said blood product comprises plasma.
4. The method of Claim 1, wherein said photoactivating means comprises a photoactivation device capable of emitting a spectrum of electromagnetic radiation comprising wavelengths between 180 nm and 400 nm and intensity between 1 and 30 mW/cm<sup>2</sup>.
5. The method of Claim 1, wherein said blood product is exposed to said intensity for between 1 second and thirty minutes.
6. The method of Claim 4, wherein said spectrum of electromagnetic radiation comprises wavelengths between 320 nm and 380 nm.
7. The method of Claim 1, wherein said blood product is in a synthetic media.
8. The method of Claim 7, wherein said synthetic media comprises a phosphate buffered salt solution.
9. The method of Claim 1, wherein said psoralen is added to said blood product to a final concentration of between .1 and 250 µM.
10. The method of Claim 9, wherein said psoralen is added to said blood

product to a final concentration of between 100 and 200  $\mu\text{M}$ .

11. The method of Claim 1, wherein said psoralen is an aminopsoralen.

12. The method of Claim 1, wherein said psoralen is selected from the group comprising 4'-primaryamino-substituted psoralens and 5'-primaryamino-substituted psoralens.

13. The method of Claim 1, wherein said psoralen comprises 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen.

14. The method of Claim 13, wherein said non-enveloped virus is human parvovirus B19.

15. The method of Claim 14, further comprising:

d) administering said blood product by intravenous infusion to a mammal.

16. A method of inactivating human parvovirus B19 in blood products, comprising, in the following order:

a) providing, in any order, i) an aminopsoralen; ii) photoactivating means for photoactivating said aminopsoralen; and iii) a blood product suspected of being contaminated with human parvovirus B19;

b) adding said aminopsoralen to said blood product; and

c) photoactivating said aminopsoralen, at conditions which inactivate any human parvovirus B19 present.

17. The method of Claim 16, wherein said blood product comprises platelets.

18. The method of Claim 16, wherein said blood product comprises plasma.

19. The method of Claim 16, wherein said photoactivating means comprises a photoactivation device capable of emitting a spectrum of electromagnetic radiation comprising wavelengths between 180 nm and 400 nm and intensity between 1 and 30  $\text{mW}/\text{cm}^2$ .

20. The method of Claim 19, wherein said blood product is exposed to said intensity for between 1 second and thirty minutes.
21. The method of Claim 19, wherein said spectrum of electromagnetic radiation comprises wavelengths between 320 nm and 380 nm.
22. The method of Claim 16, wherein said blood product is in a synthetic media.
23. The method of Claim 22, wherein said synthetic media comprises a phosphate buffered salt solution.
24. The method of Claim 16, wherein said aminopsoralen is added to said blood product to a final concentration of between .1 and 250  $\mu$ M.
25. The method of Claim 24, wherein said aminopsoralen is added to said blood product to a final concentration of between 100 and 200  $\mu$ M.
26. The method of Claim 16, wherein said aminopsoralen is a 4'-primaryamino-substituted psoralen.
27. The method of Claim 16, wherein said aminopsoralen is a 5'-primaryamino-substituted psoralen.
28. The method of Claim 27, wherein said aminopsoralen comprises 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen.
29. The method of Claim 28, further comprising:
  - d) administering said blood product by intravenous infusion to a mammal.
30. A method of inactivating human parvovirus B19 in a blood preparation, comprising, in the following order:
  - a) providing, in any order, i) 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen, ii) photoactivating means for photoactivating said 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen, and iii) a blood preparation, selected from the group consisting of platelet preparation and plasma preparation, suspected of being contaminated with said virus;



b) adding said 4'-(4-amino-2-oxa)butyl-4,5,8-trimethylpsoralen to said platelet preparation; and

c) photoactivating said 4'-(4-amino-2-oxa)butyl-4,5,8-trimethylpsoralen, at conditions which inactivate said human parvovirus B19, without causing significant damage to said blood preparation.

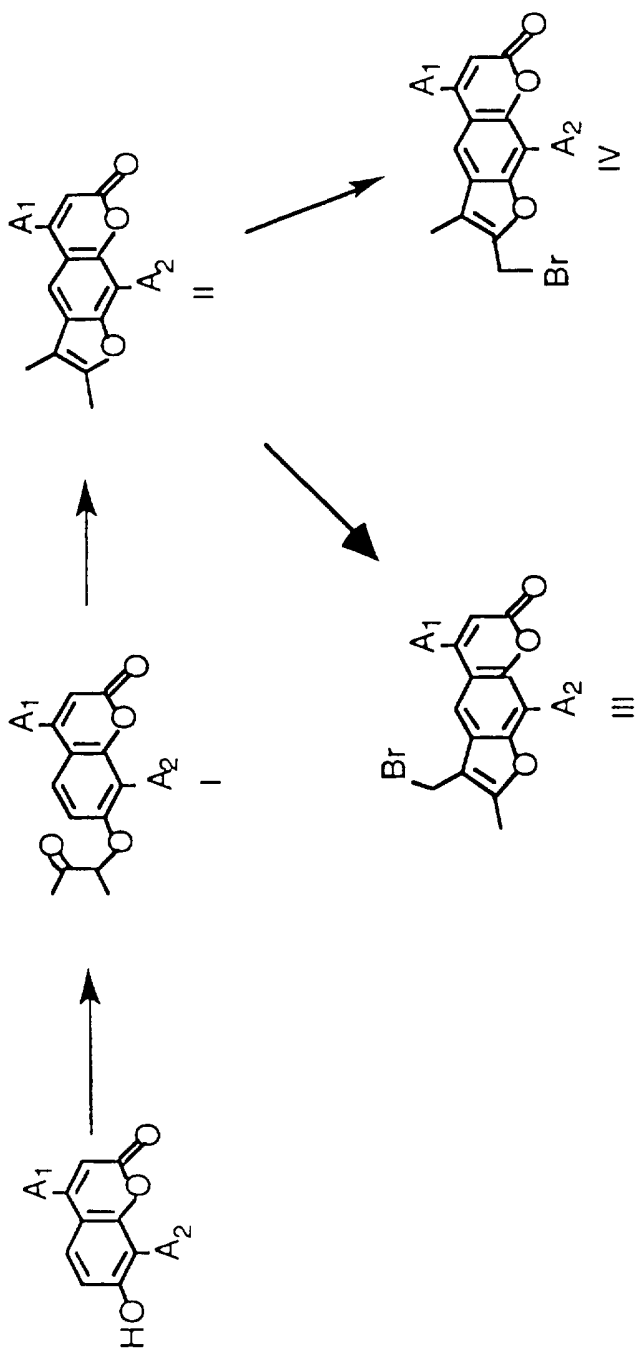


FIG. 1

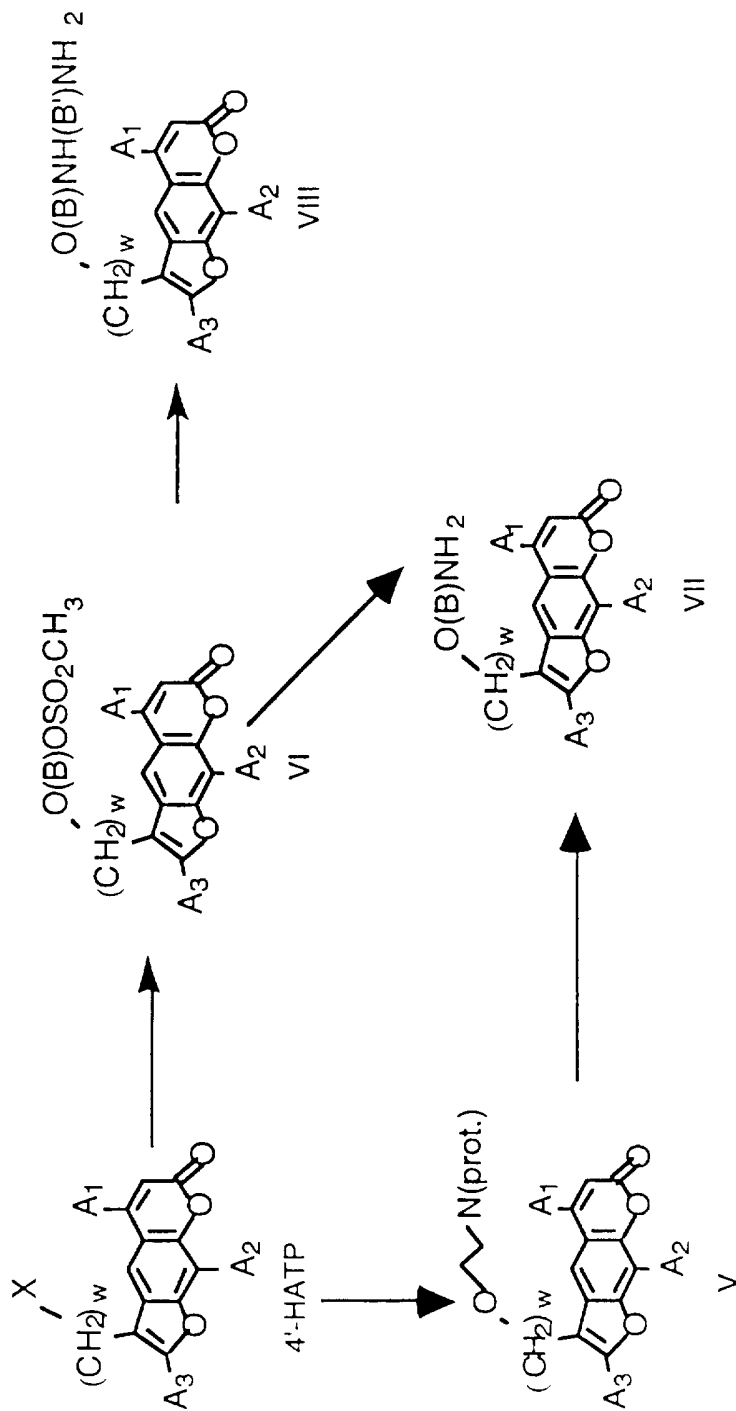


FIG. 2

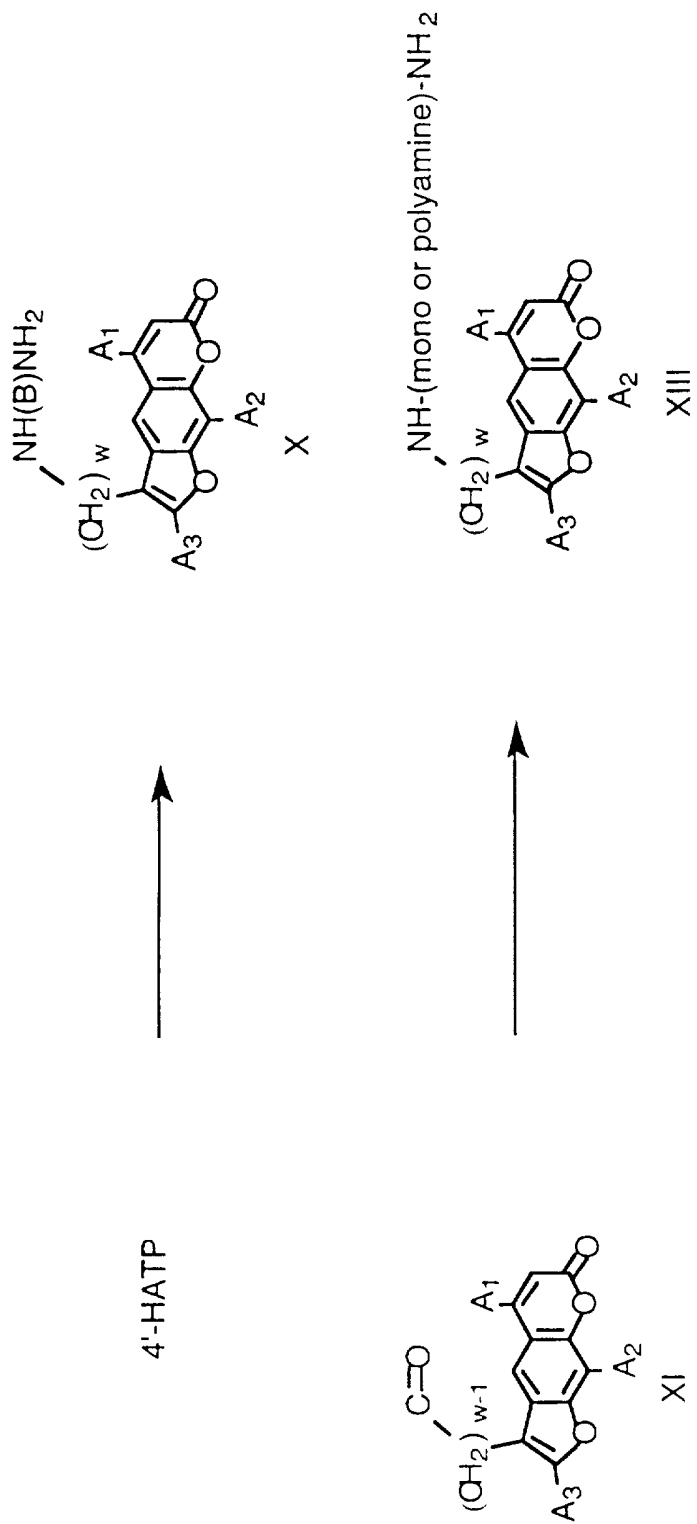
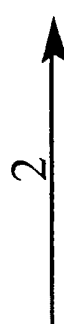
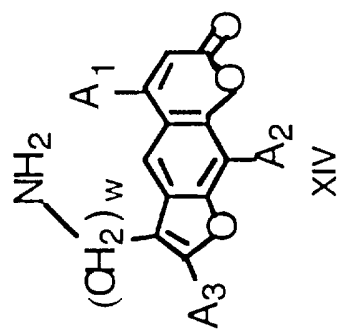


FIG. 3



4'-HATP

**FIG. 4**

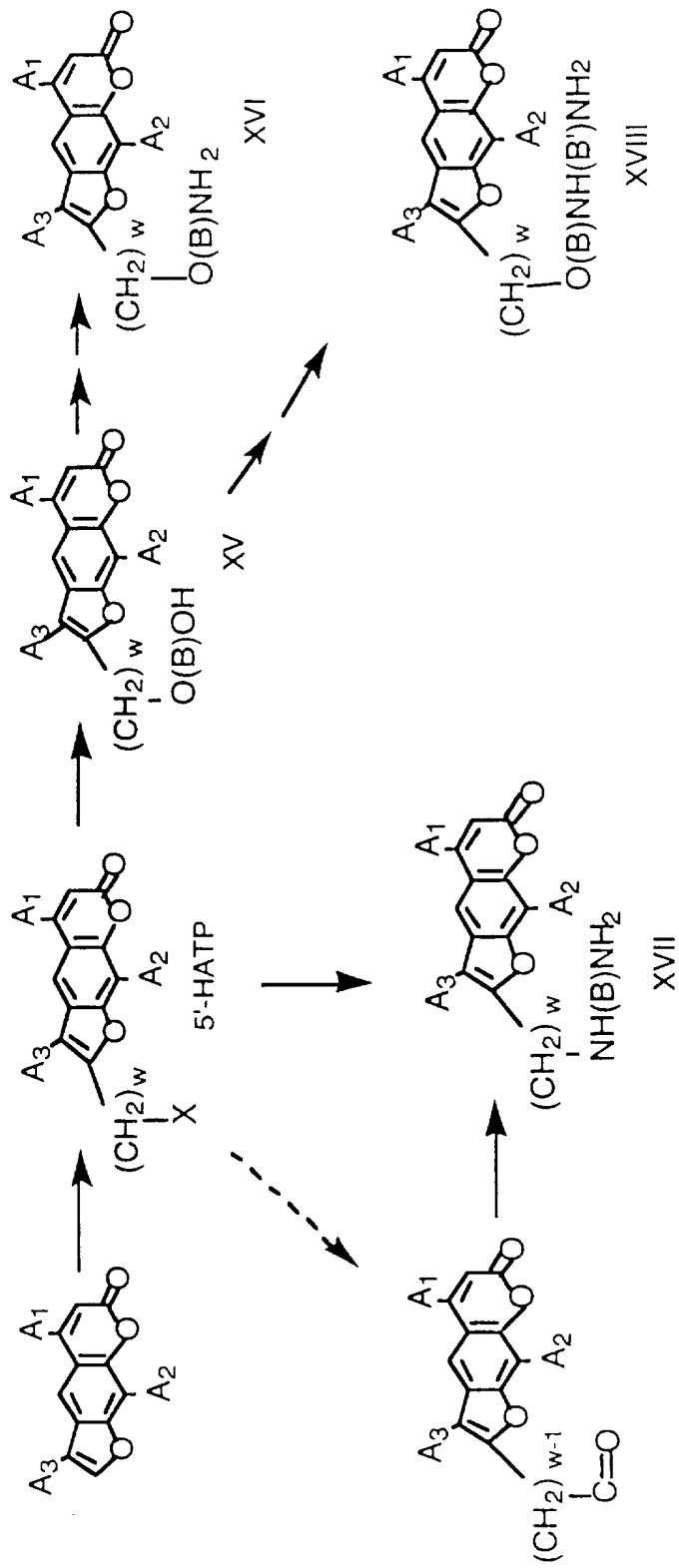


FIG. 5

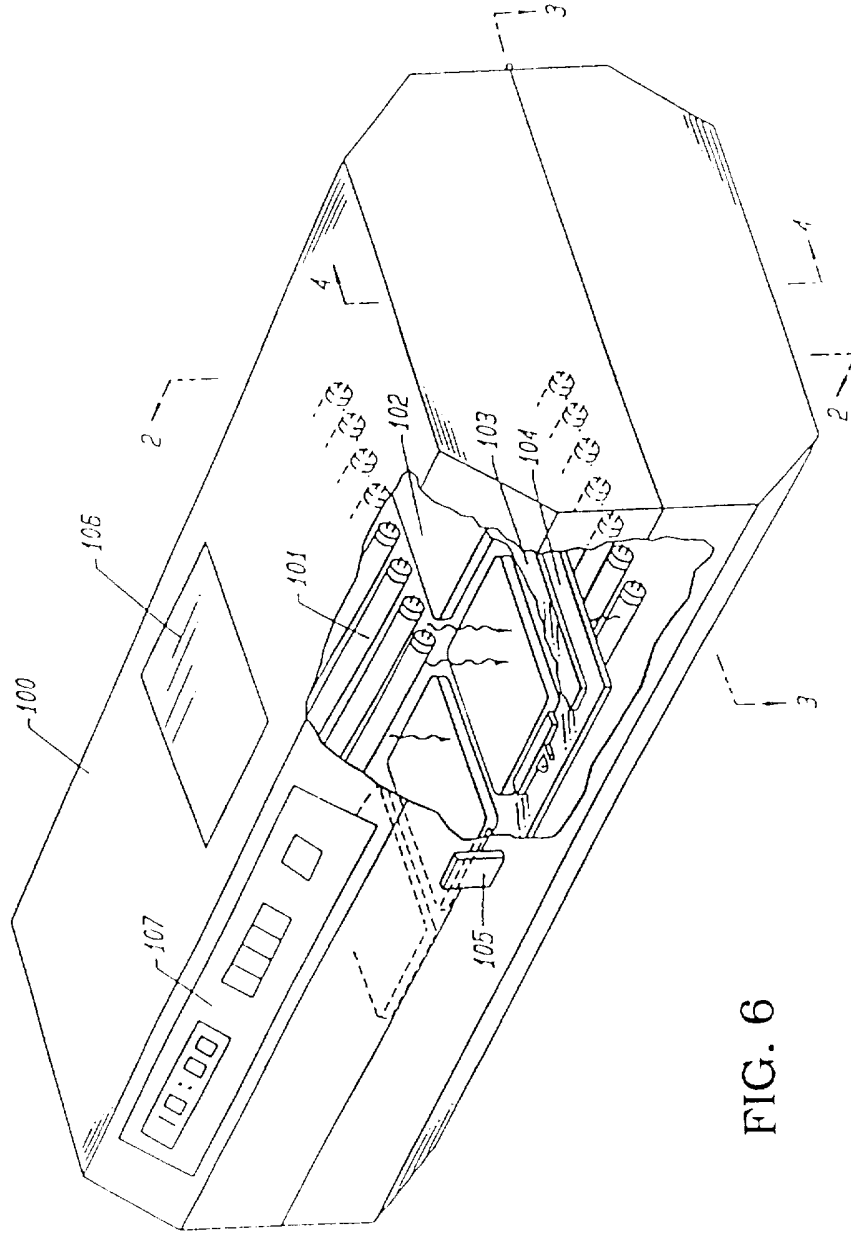


FIG. 6

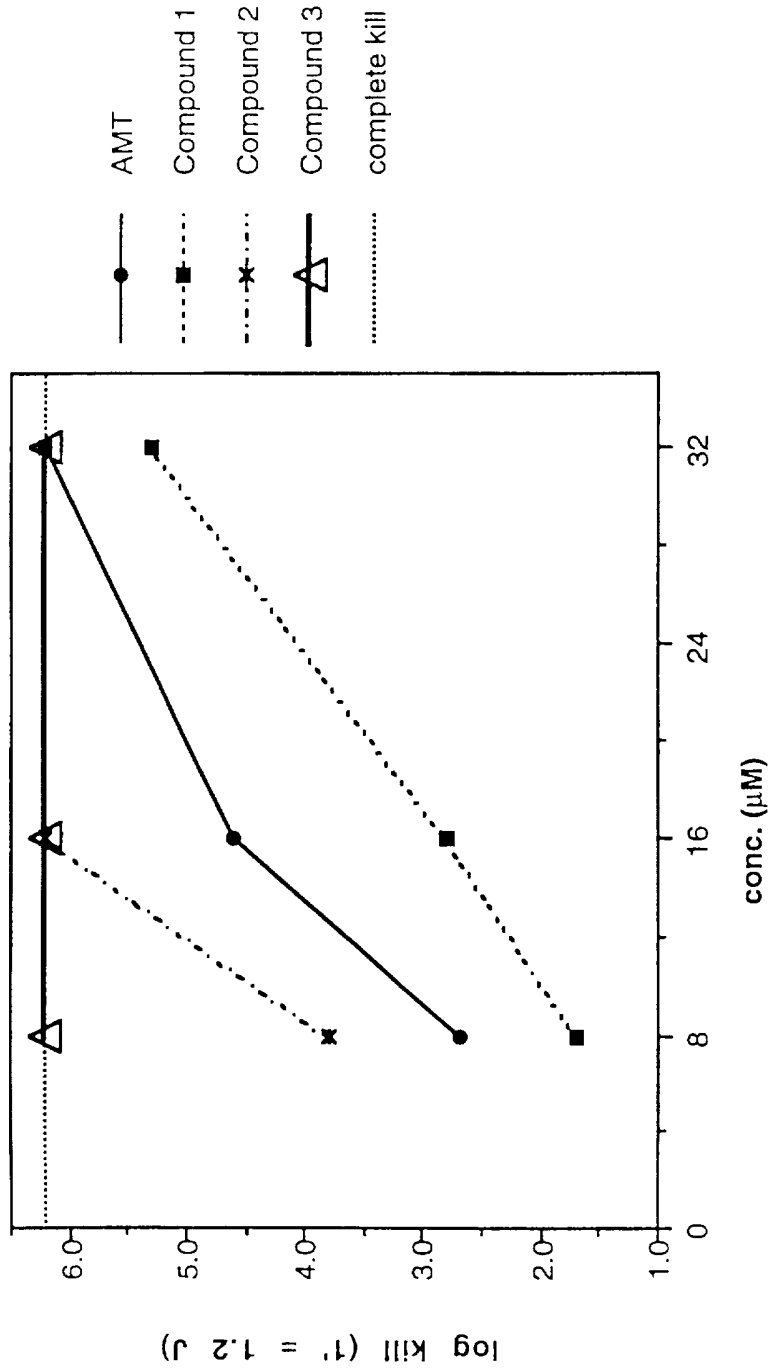


FIG. 7



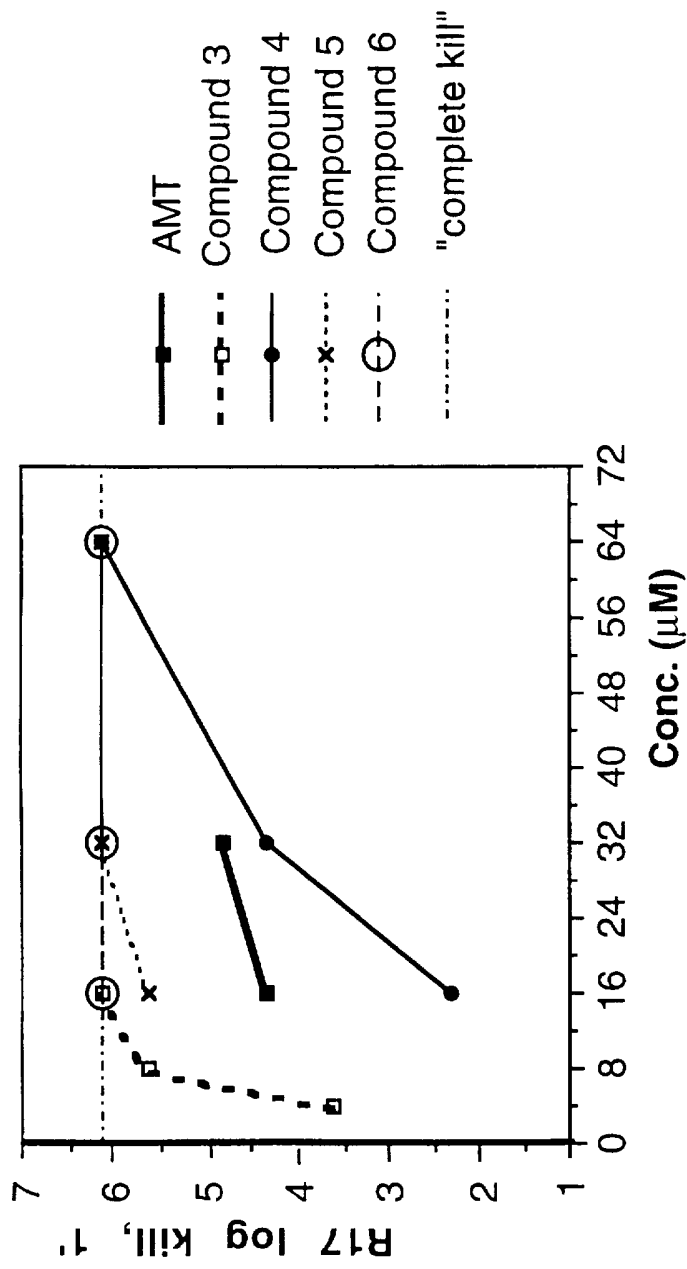


FIG. 8

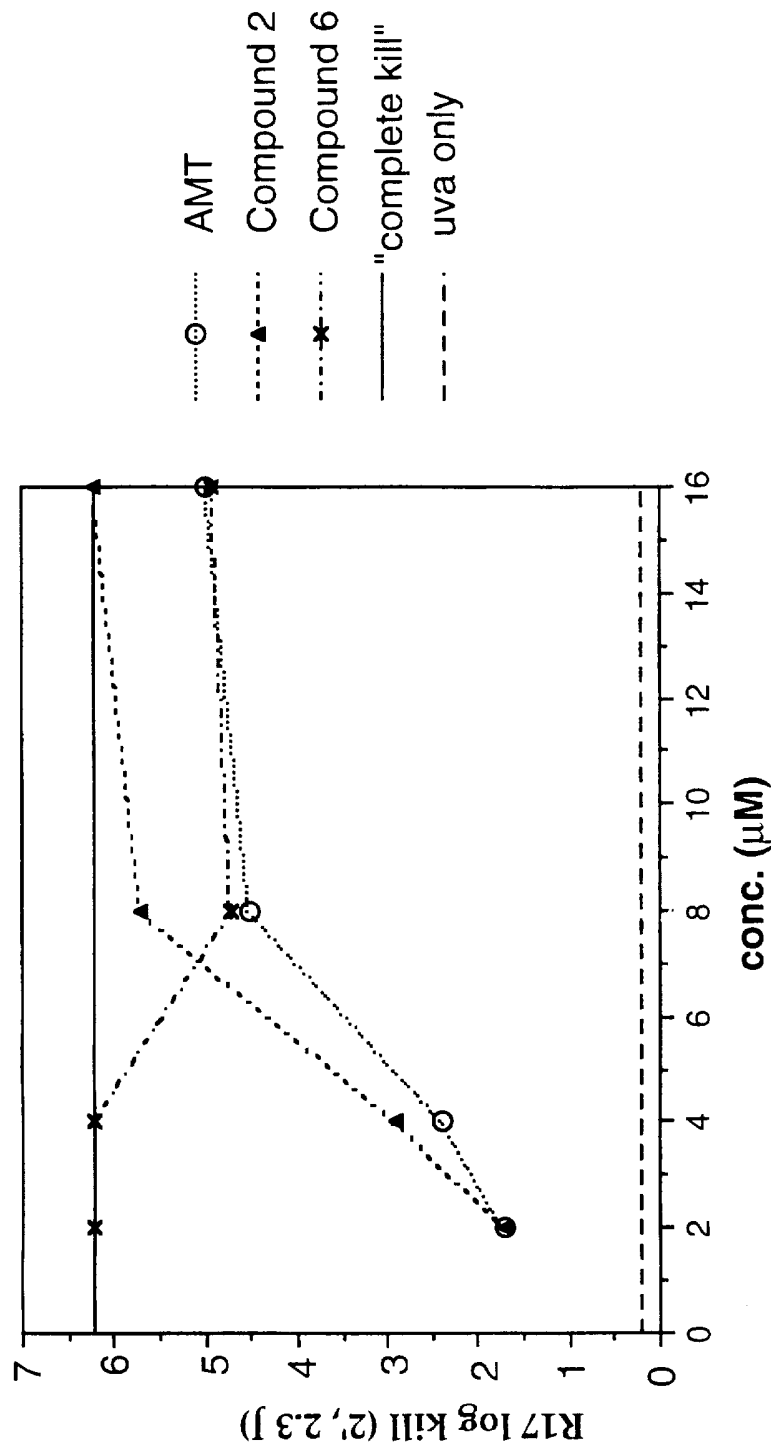


FIG. 9

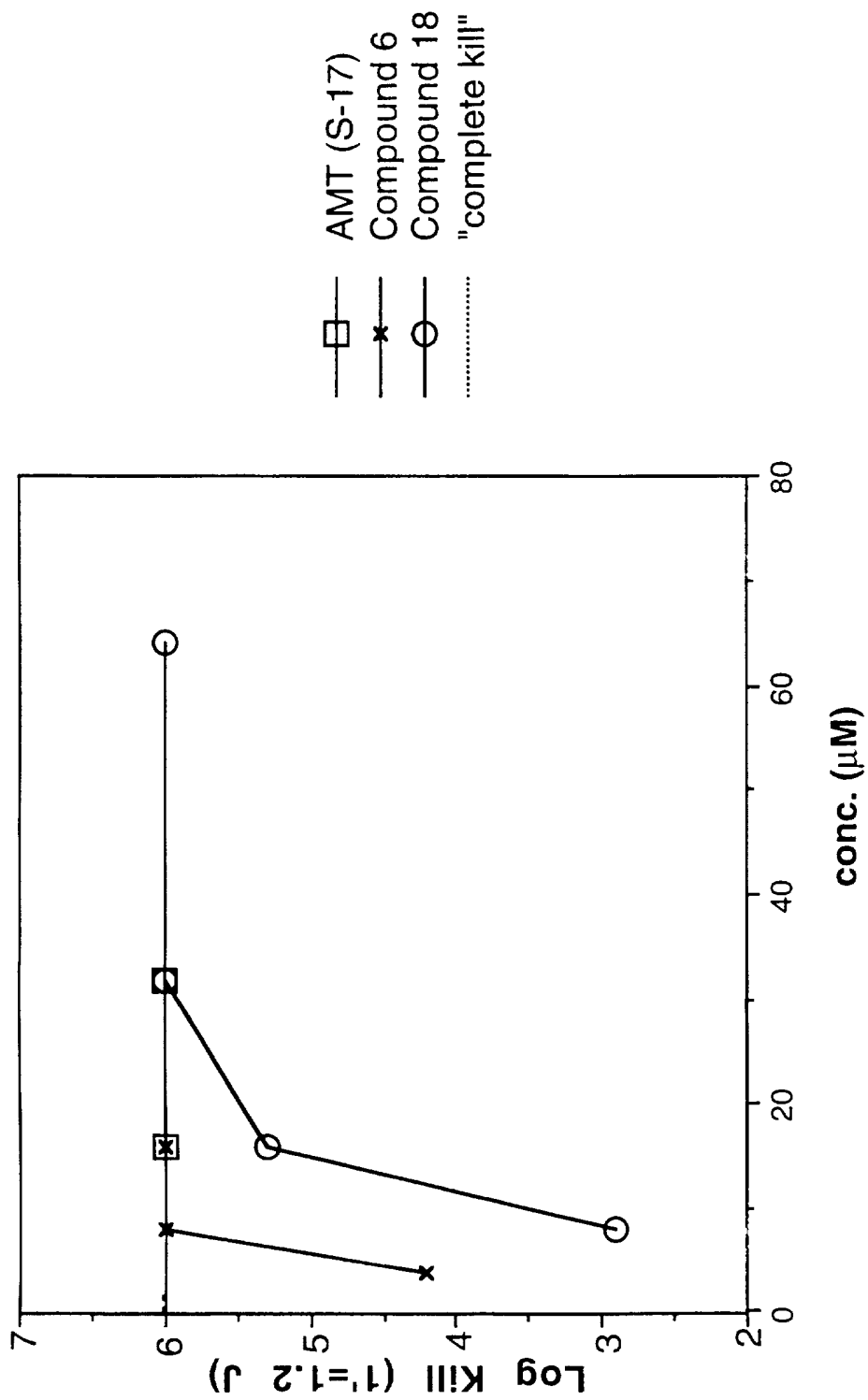


FIG. 10

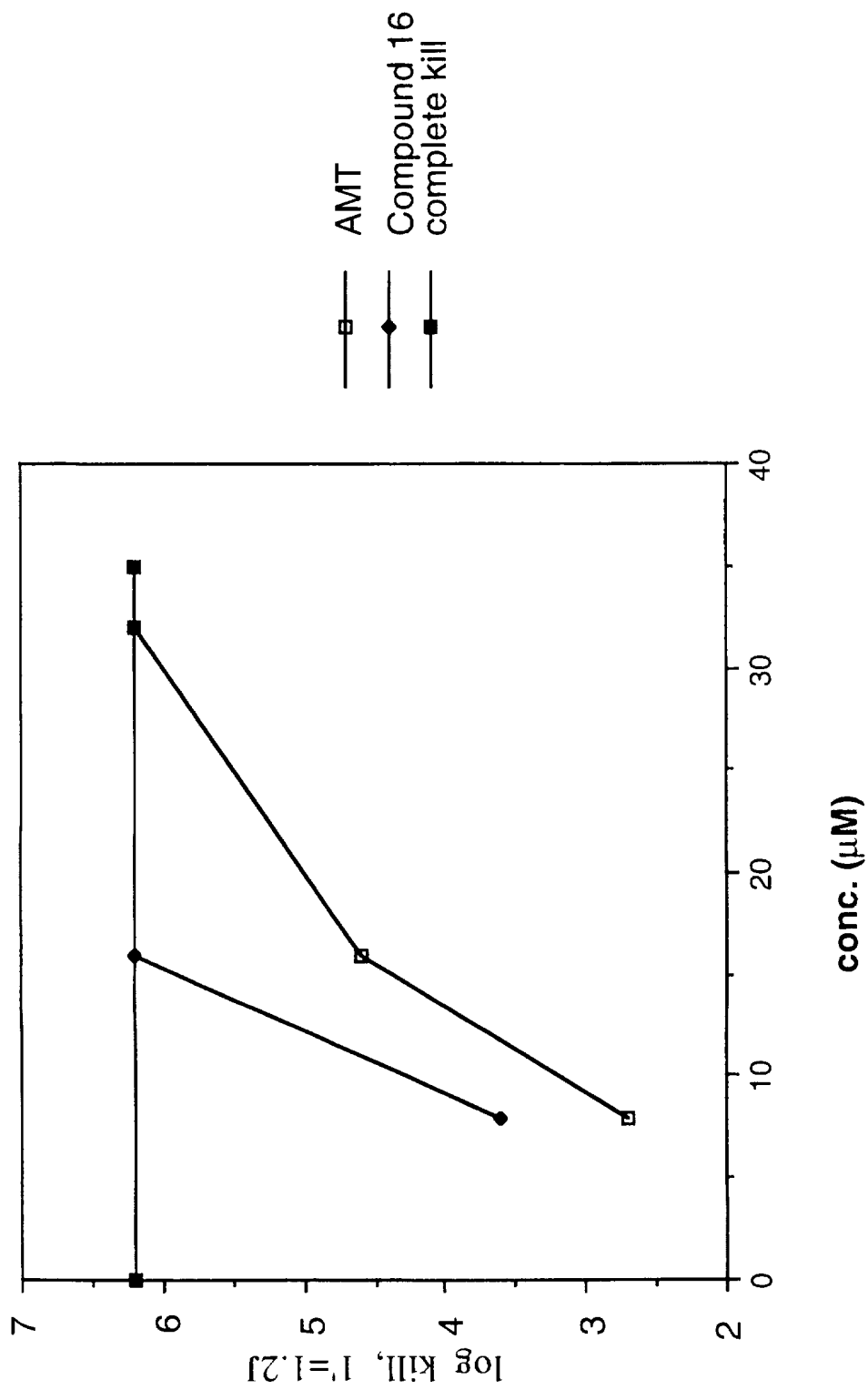


FIG. 11

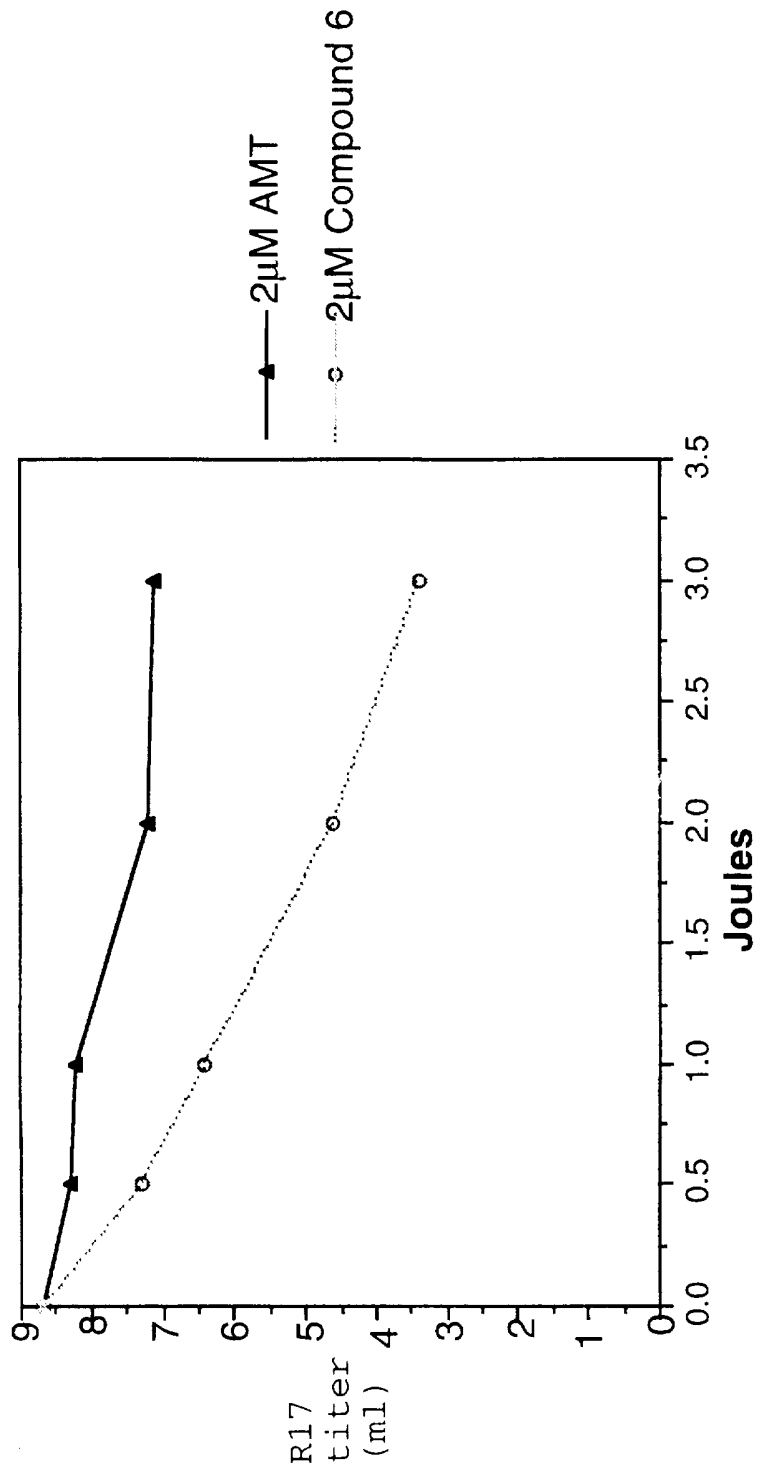


FIG. 12

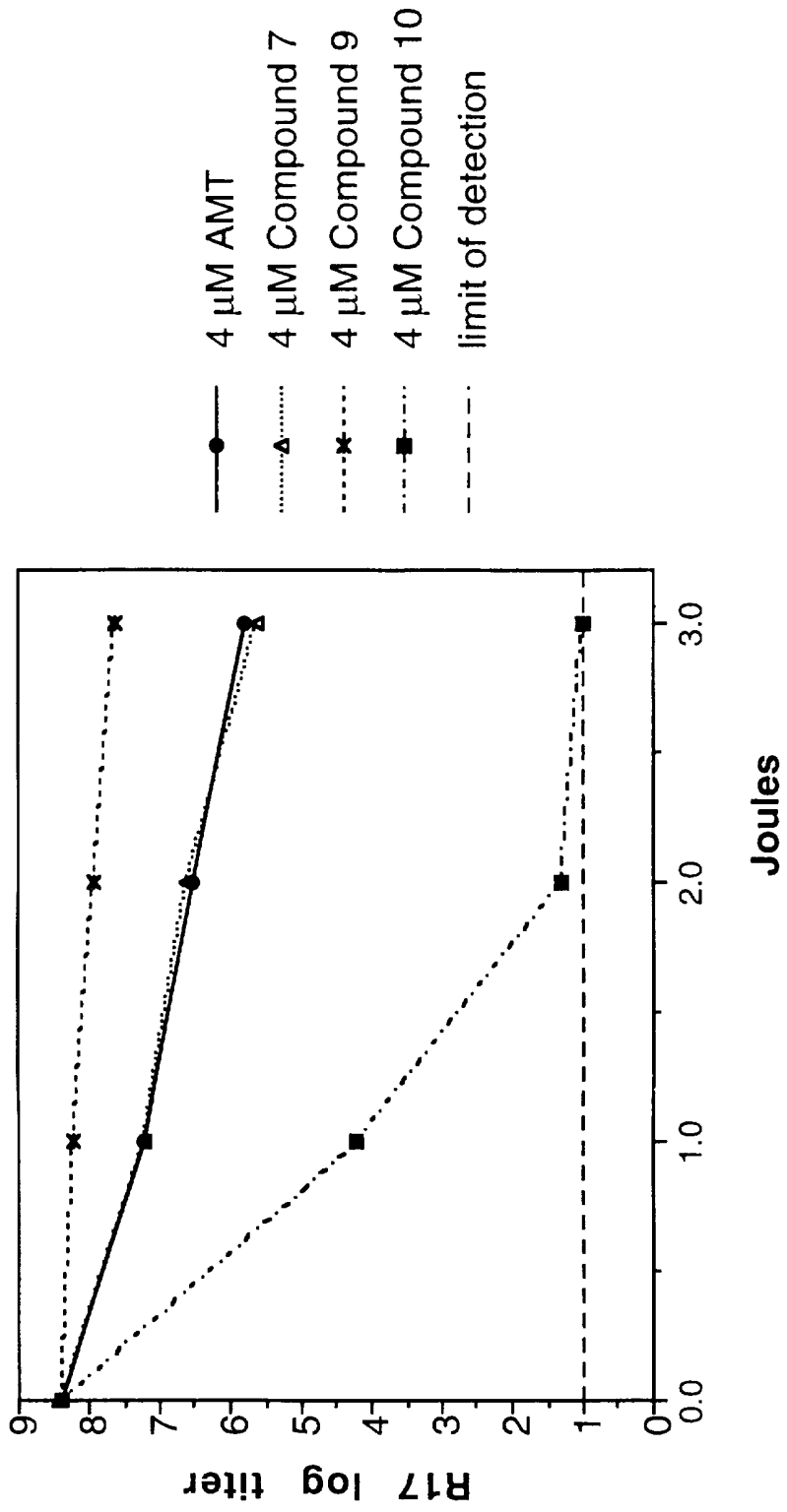


FIG. 13

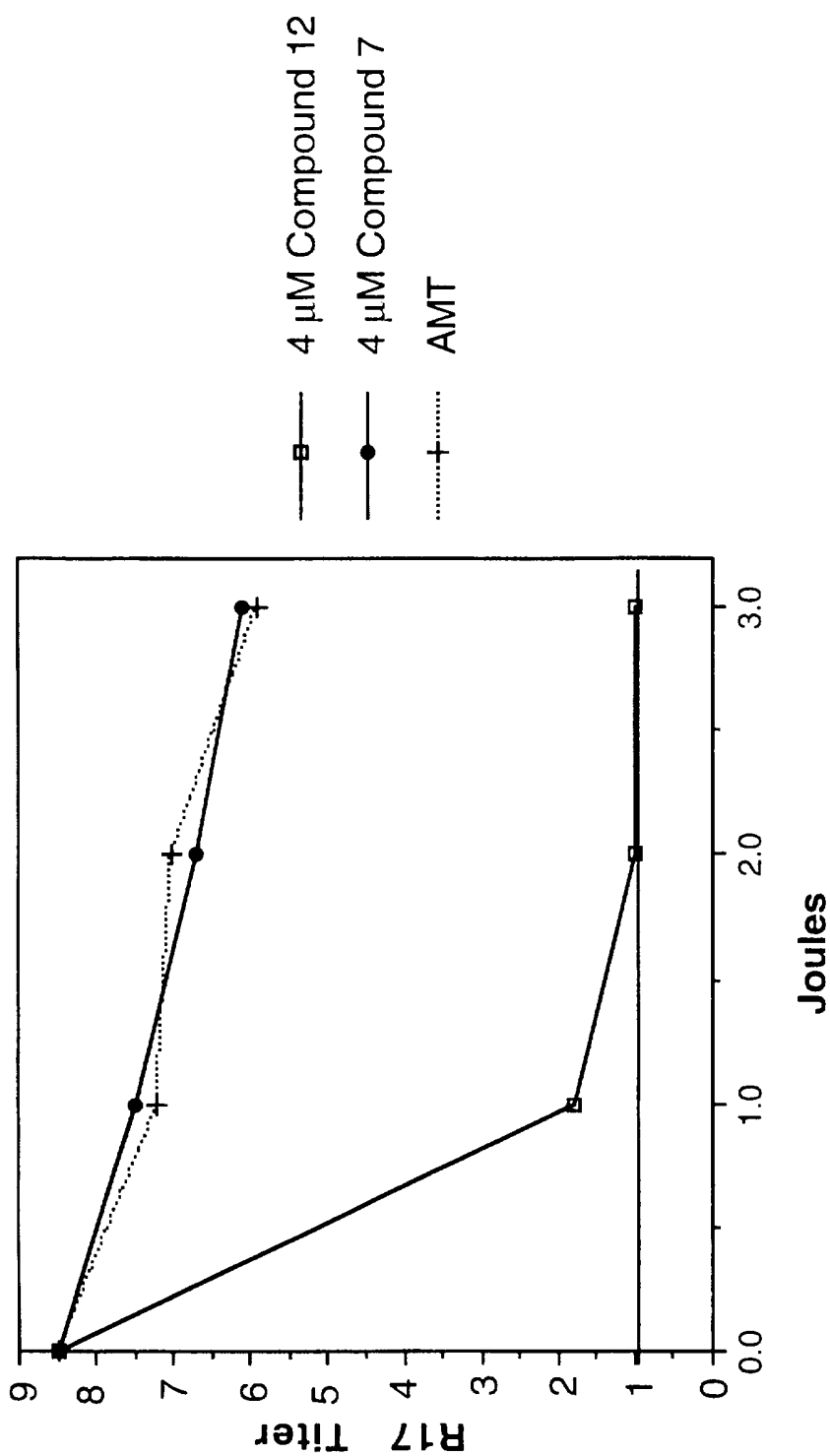


FIG. 14

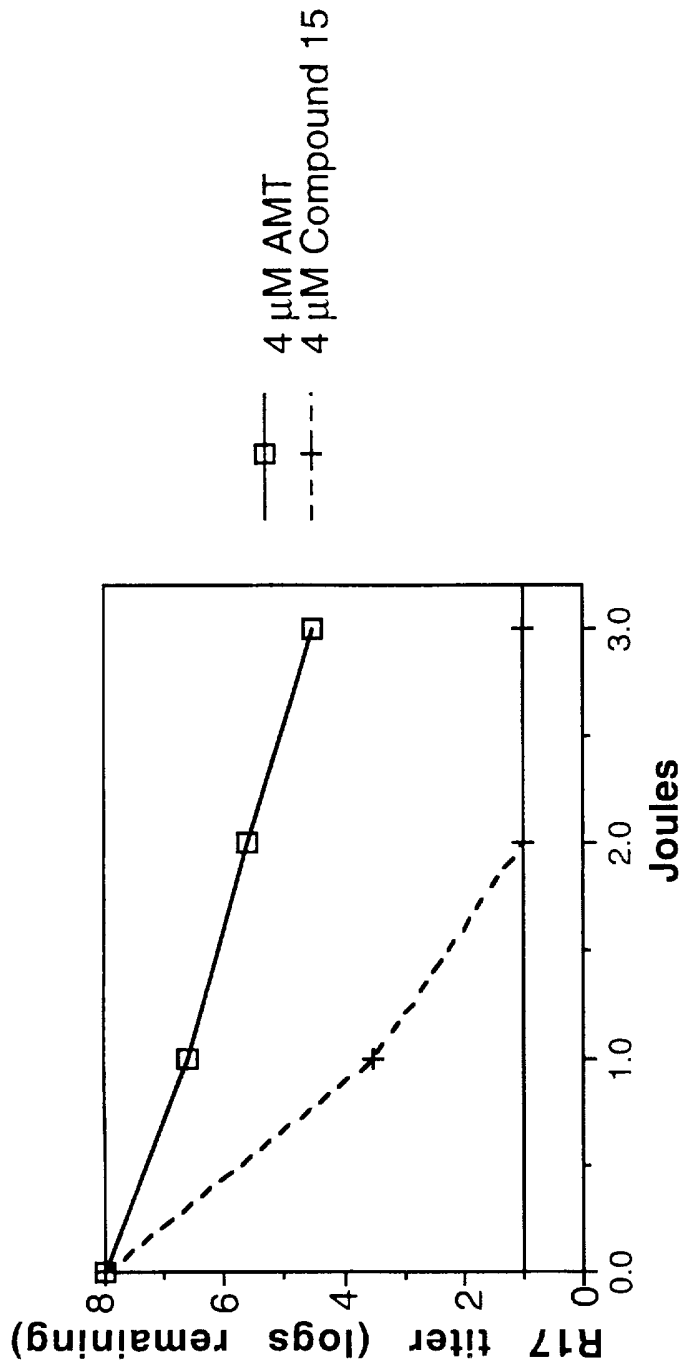


FIG. 15



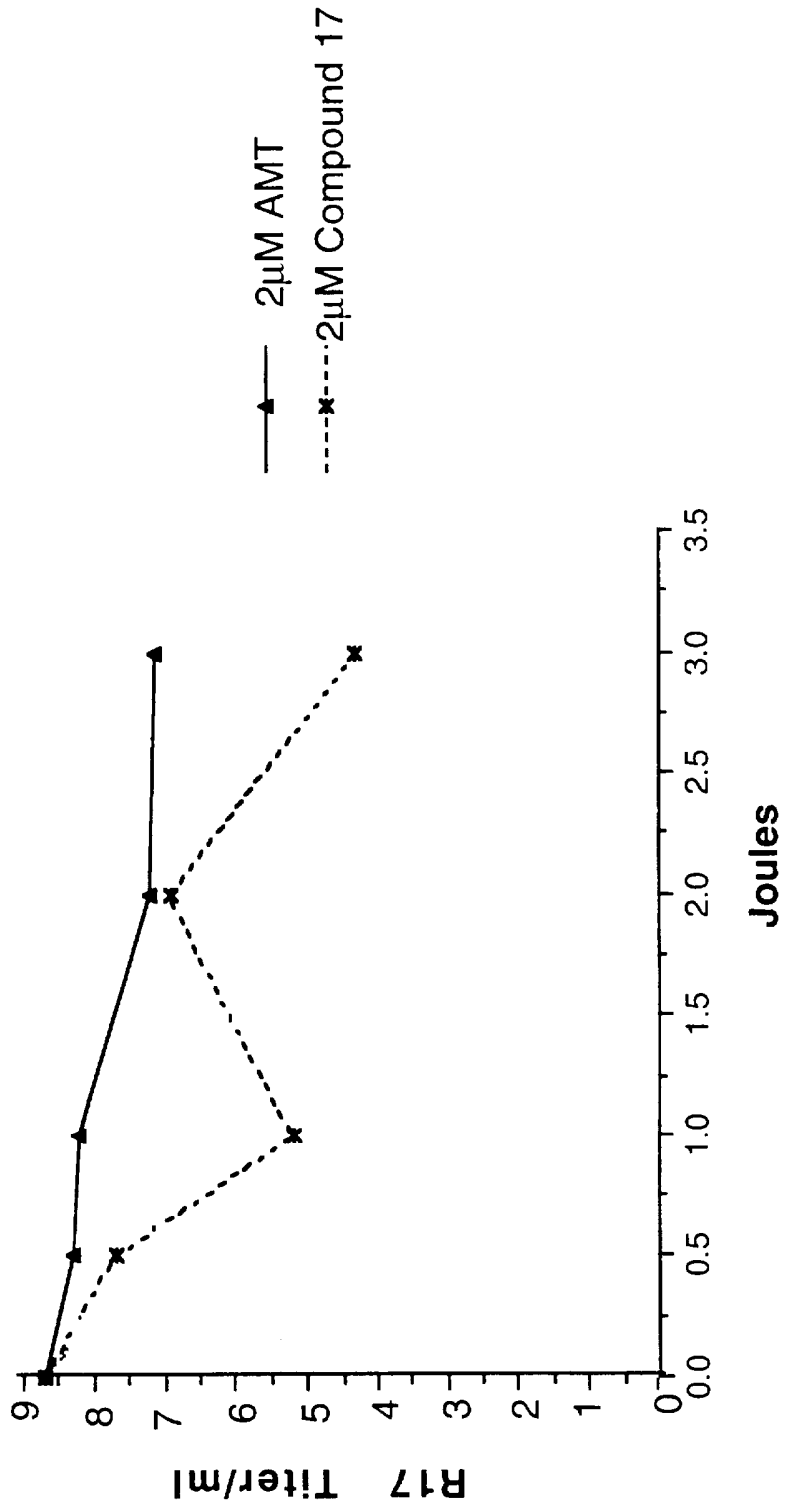


FIG. 16

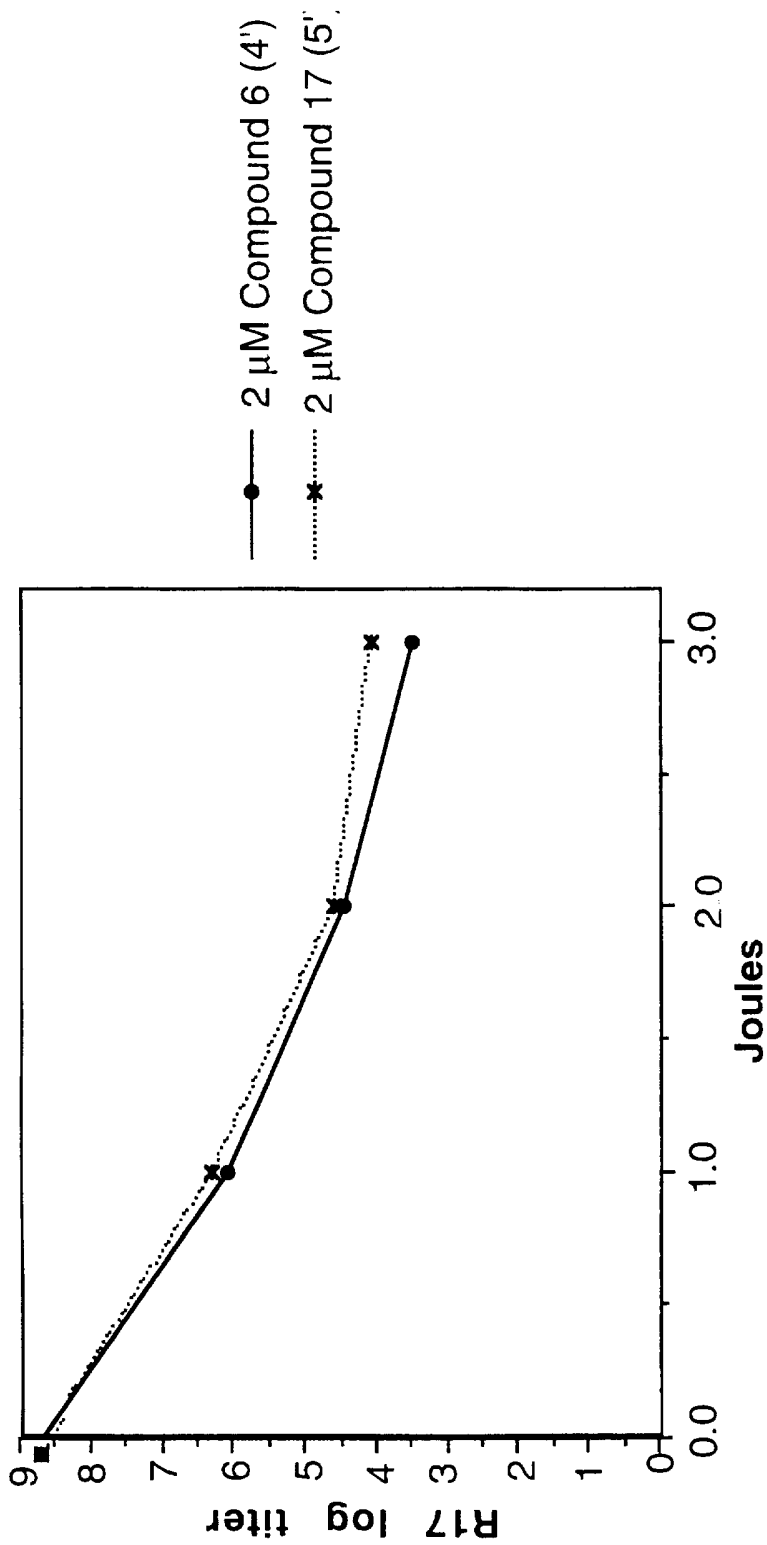
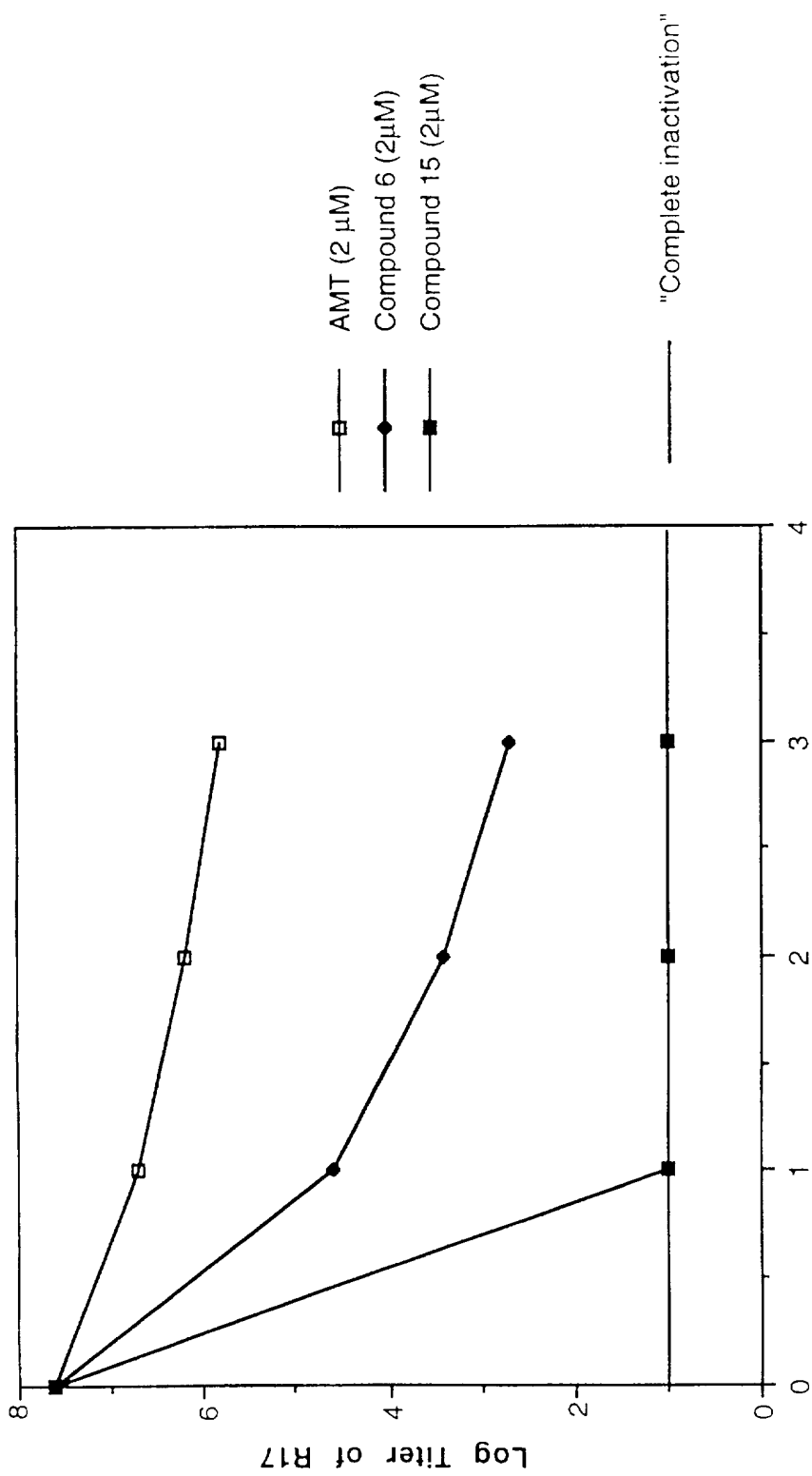


FIG. 17



Joules  
FIG. 18

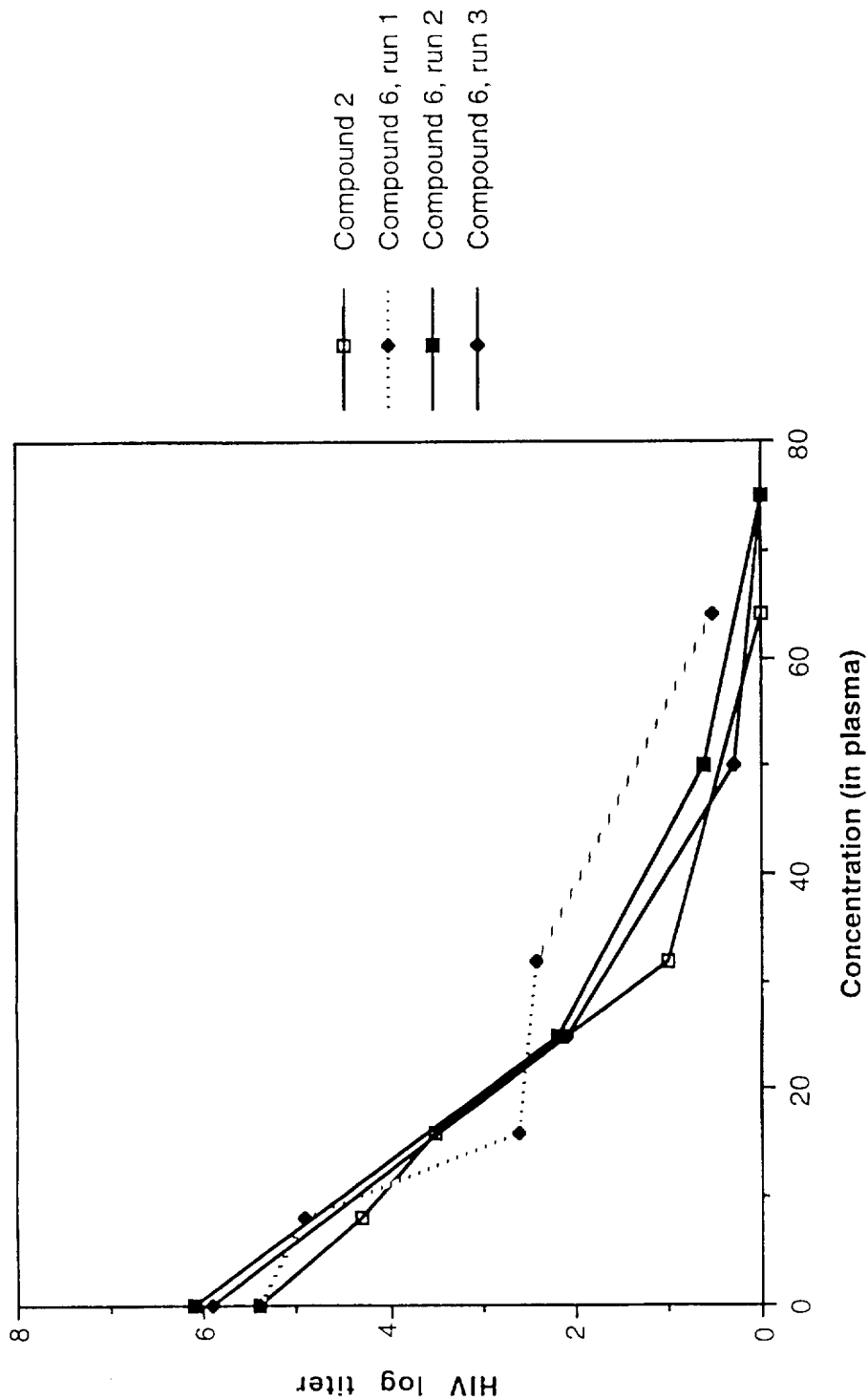


FIG. 19

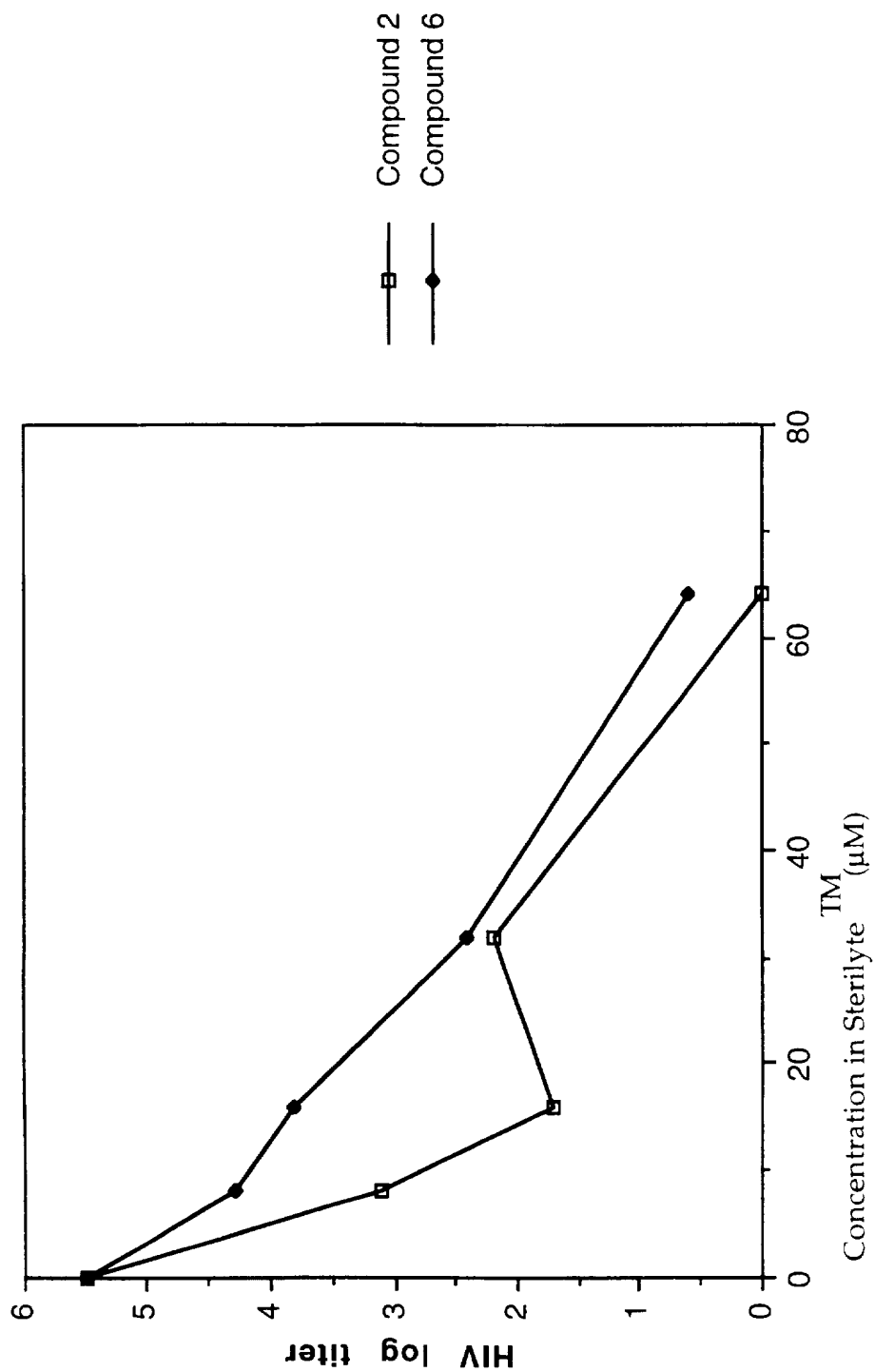


FIG. 20

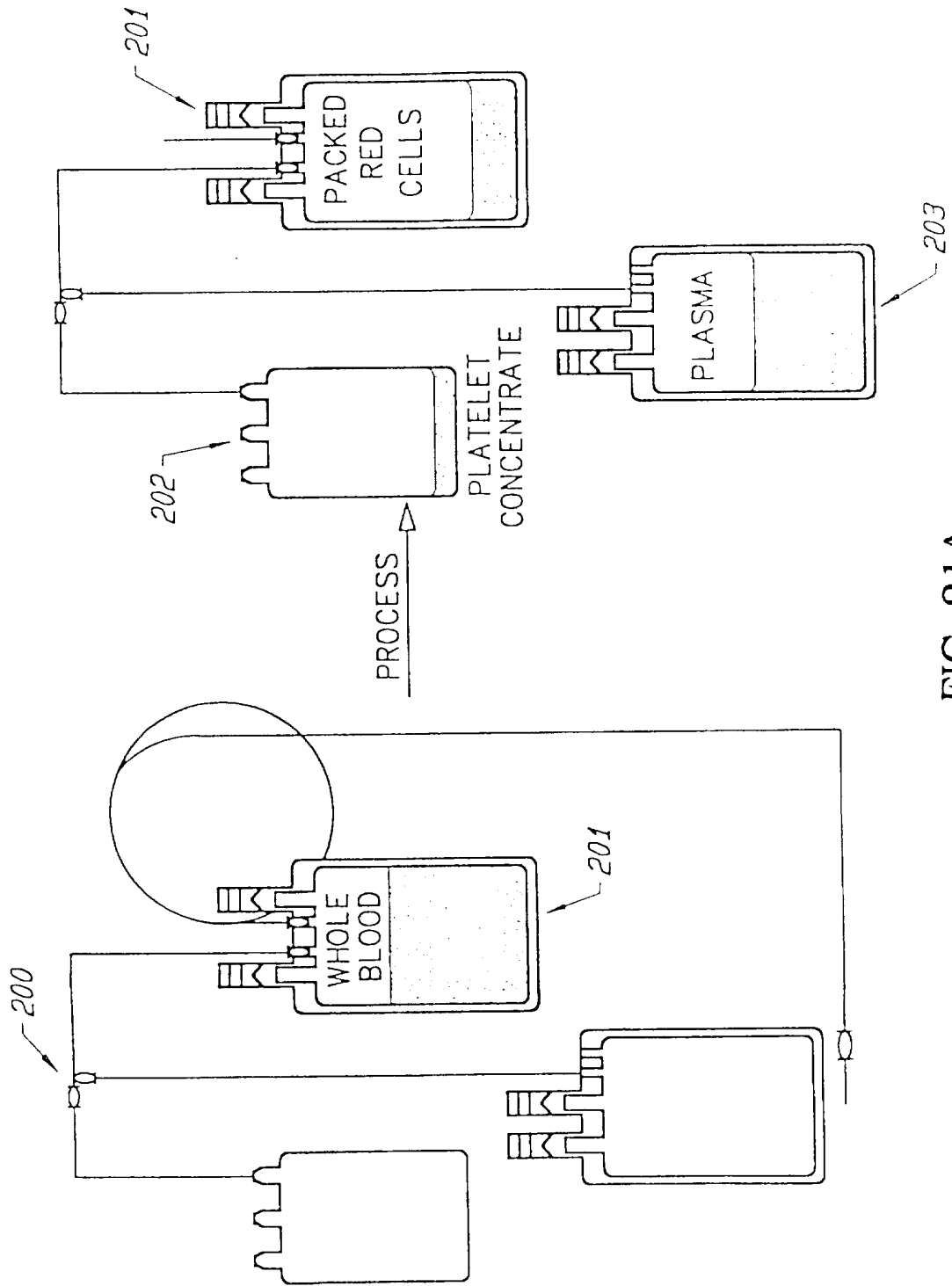


FIG. 21A

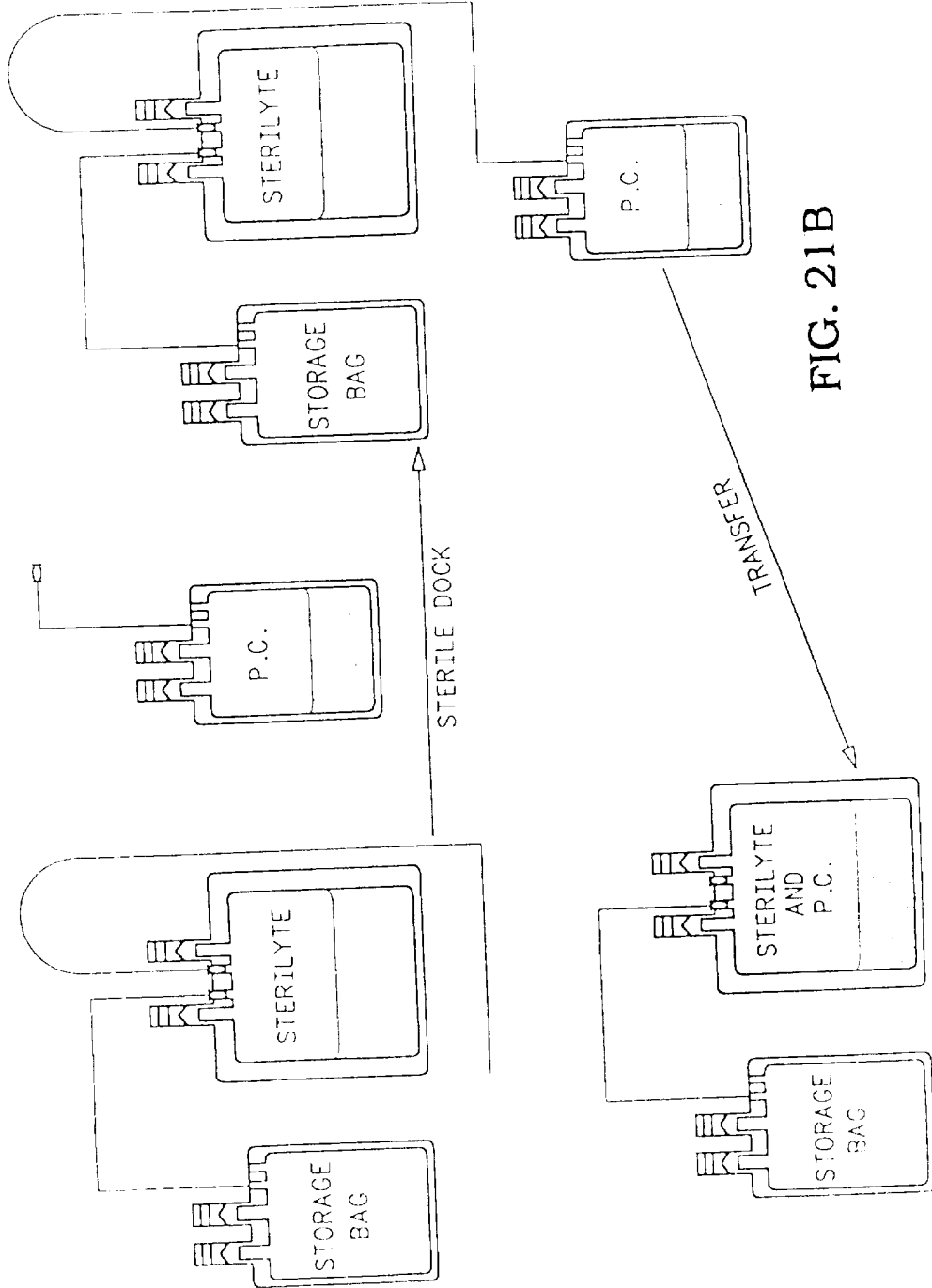


FIG. 21B

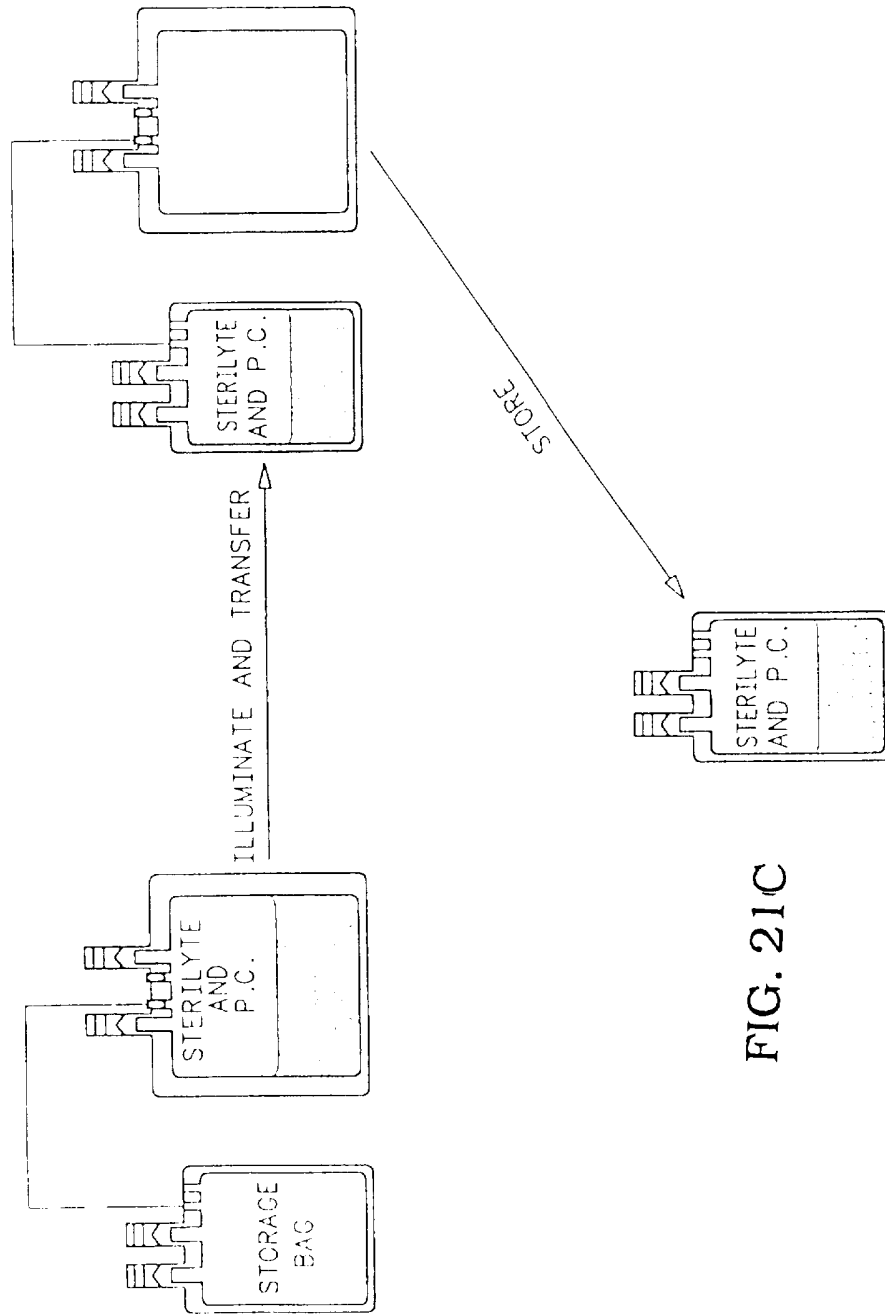


FIG. 21C



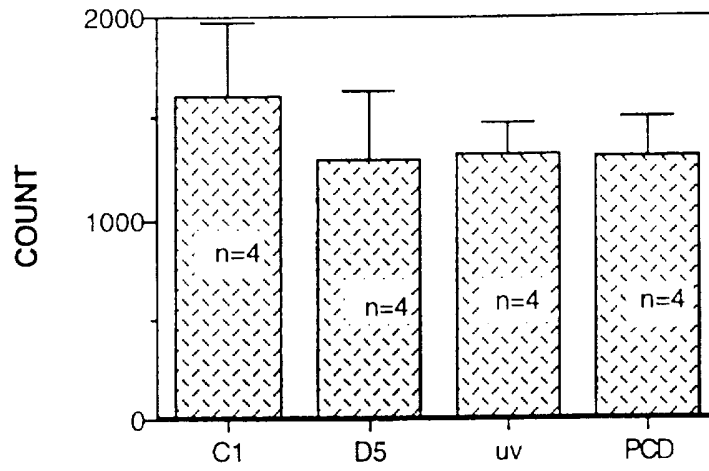


FIG. 22A

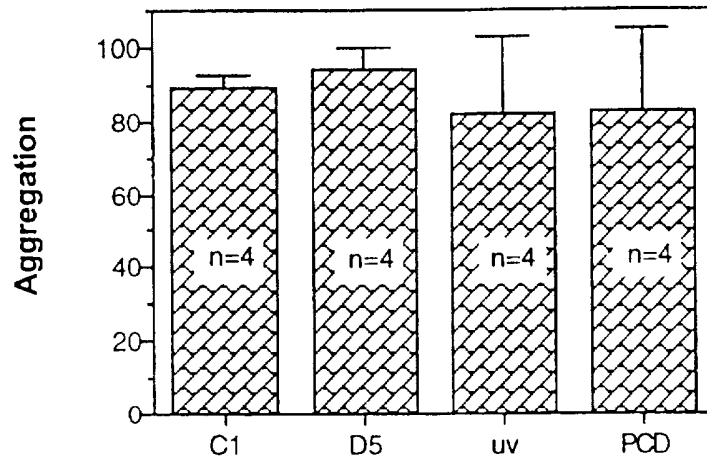


FIG. 22B

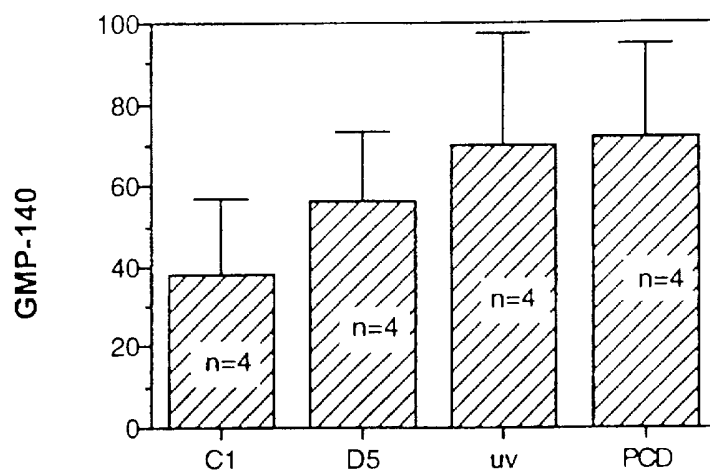


FIG. 22C

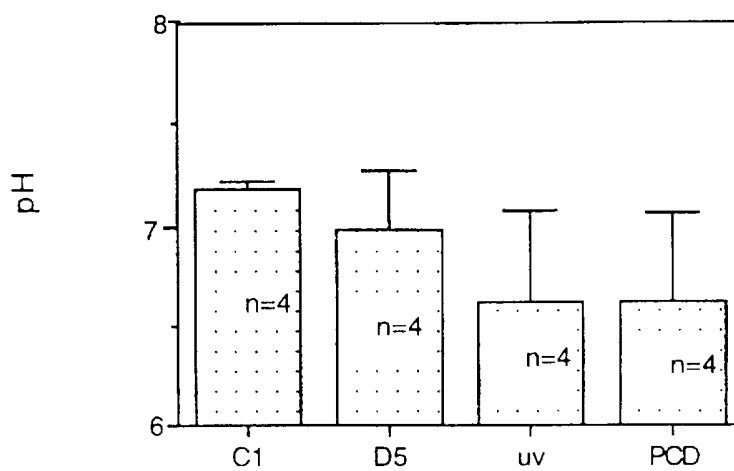


FIG. 22D

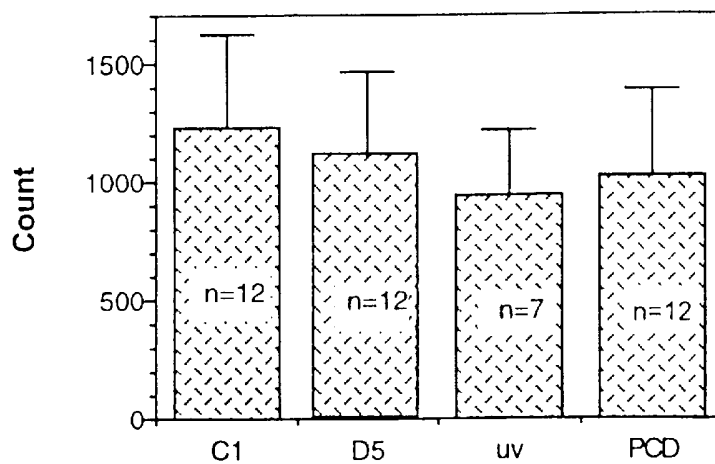


FIG. 23A

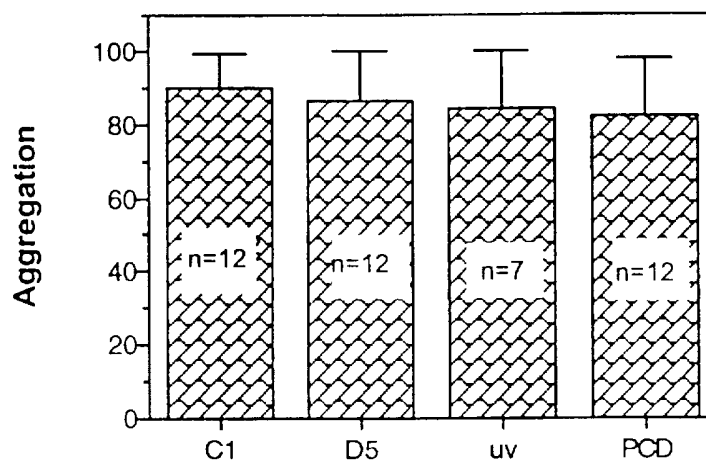


FIG. 23B

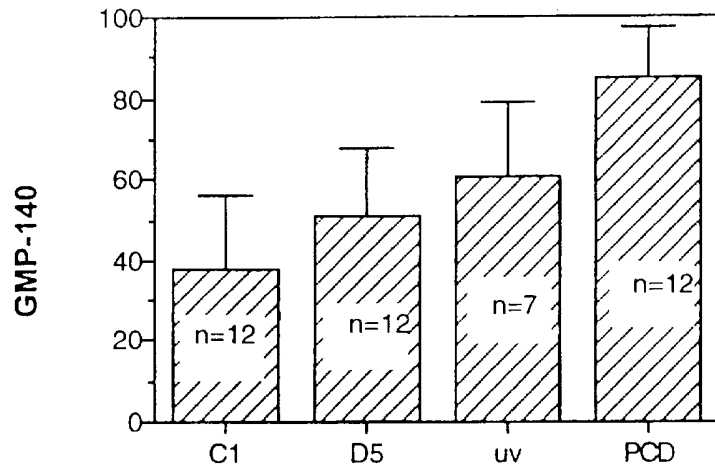


FIG. 23C

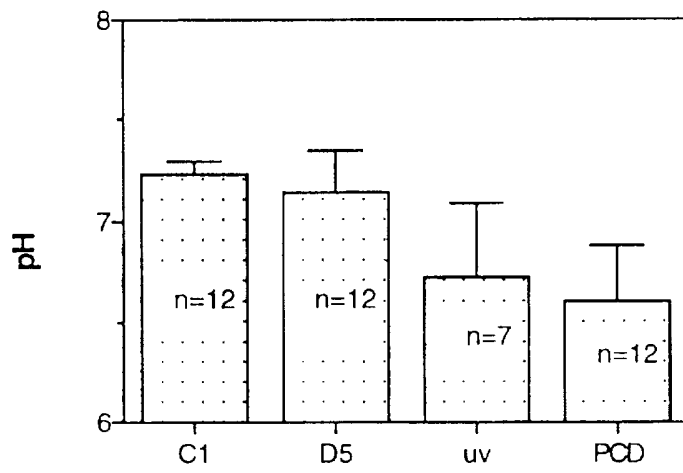


FIG. 23D

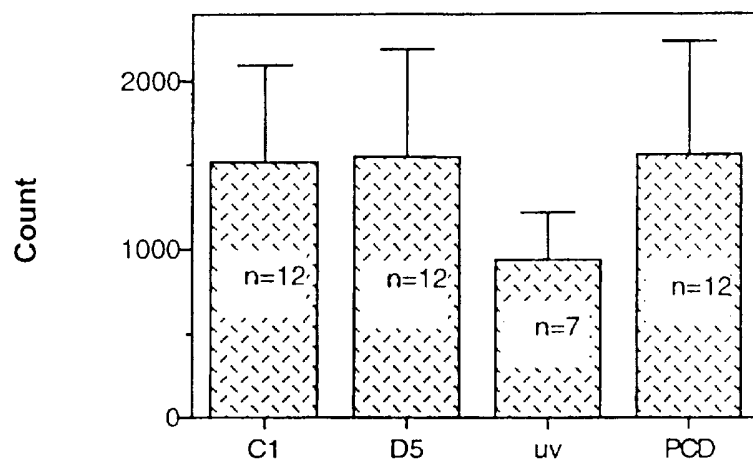


FIG. 24A

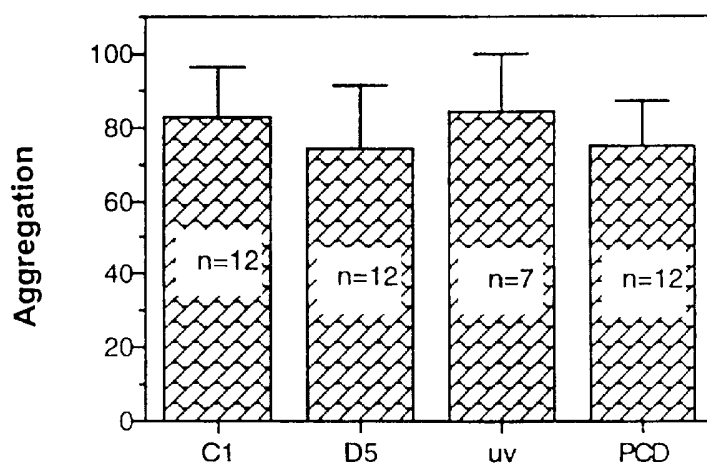


FIG. 24B

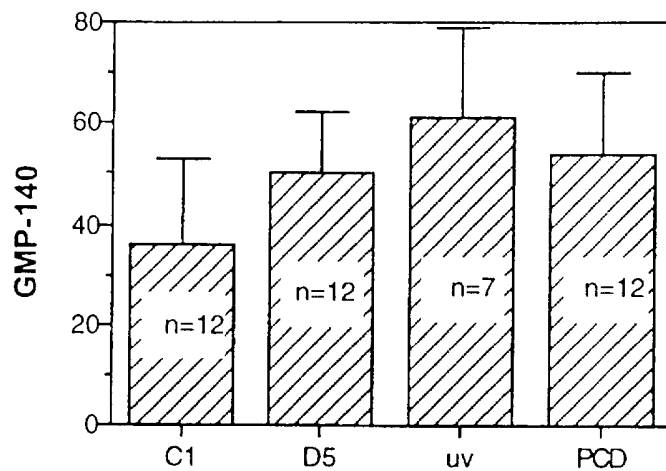


FIG. 24C

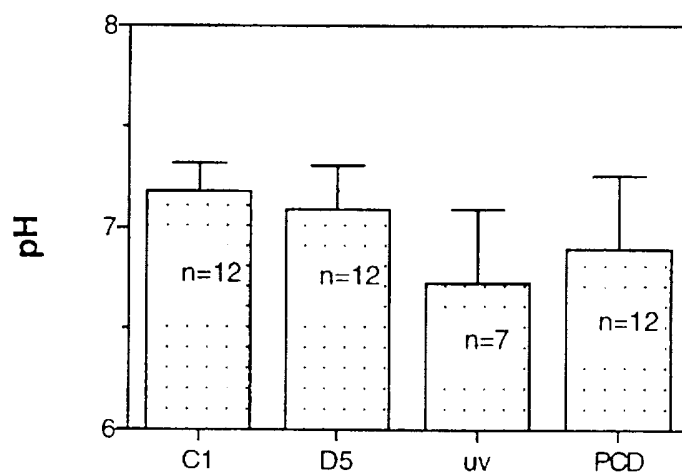


FIG. 24D

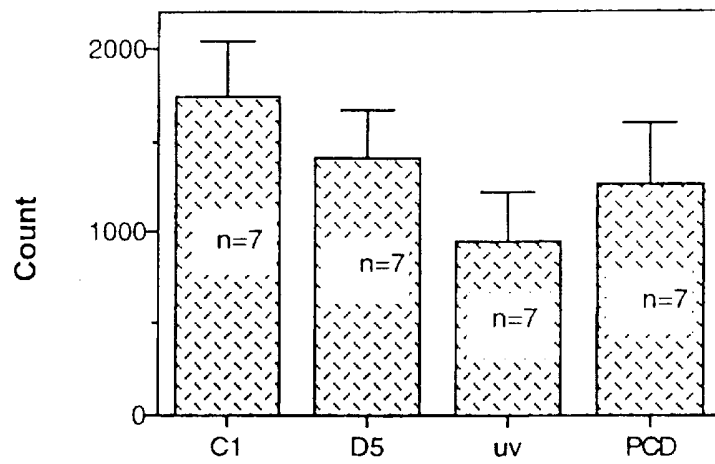


FIG. 25A

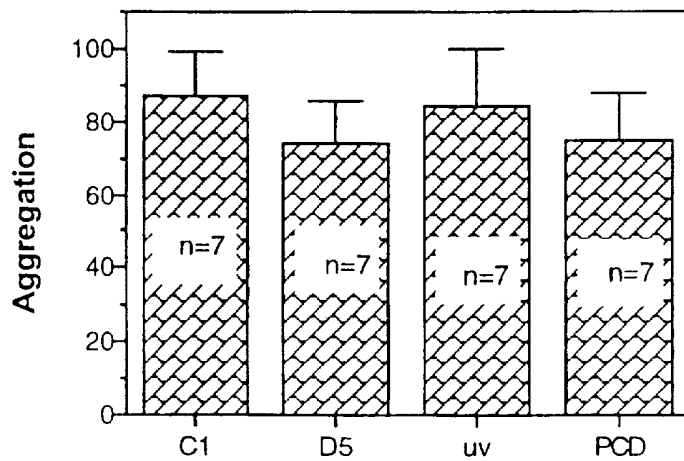


FIG. 25B

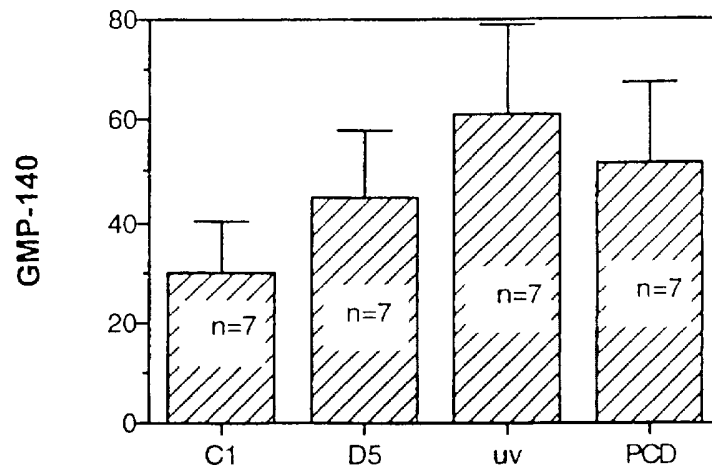


FIG. 25C

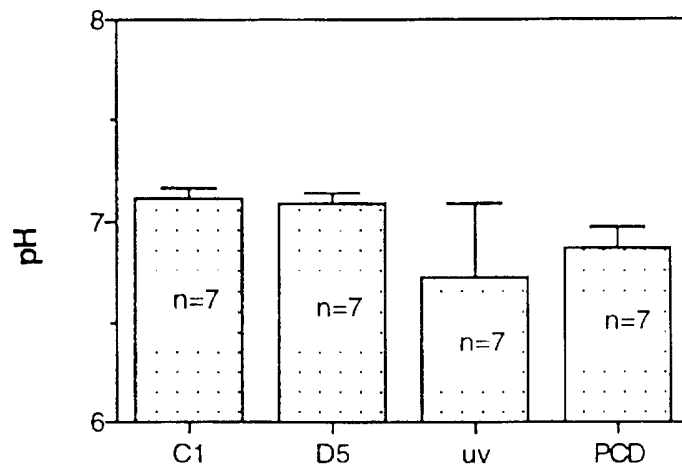


FIG. 25D



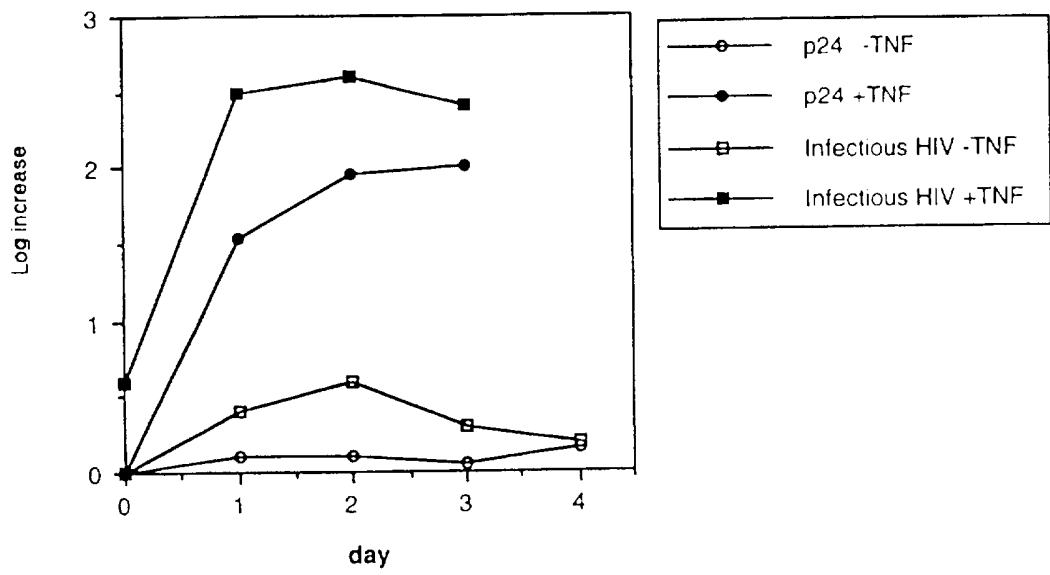


FIG. 26

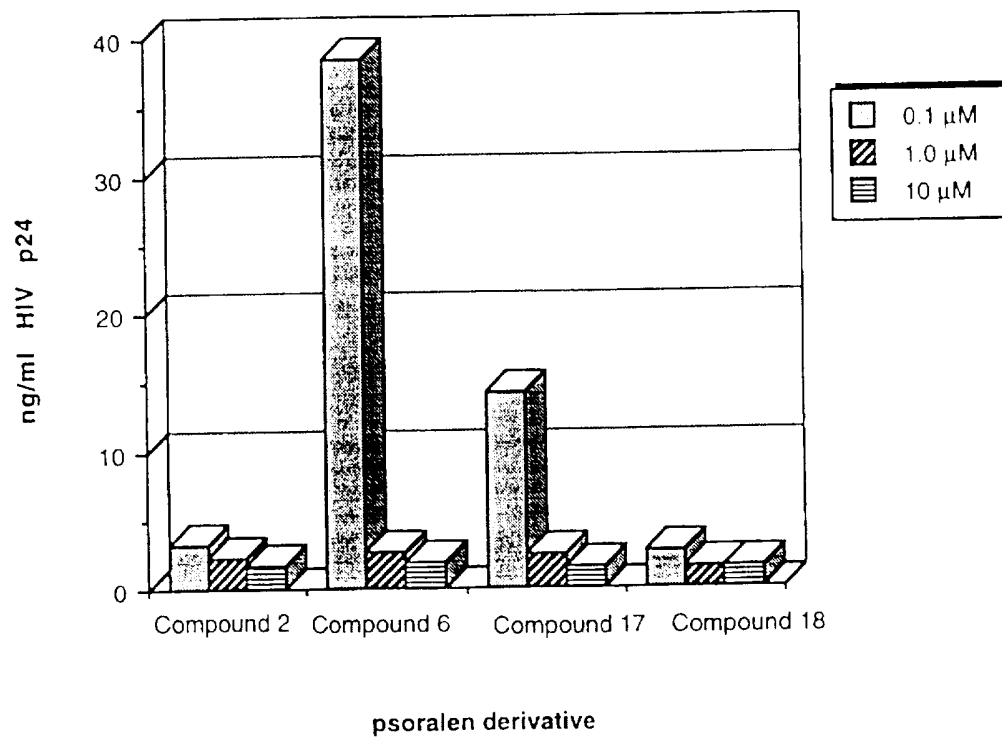


FIG. 27

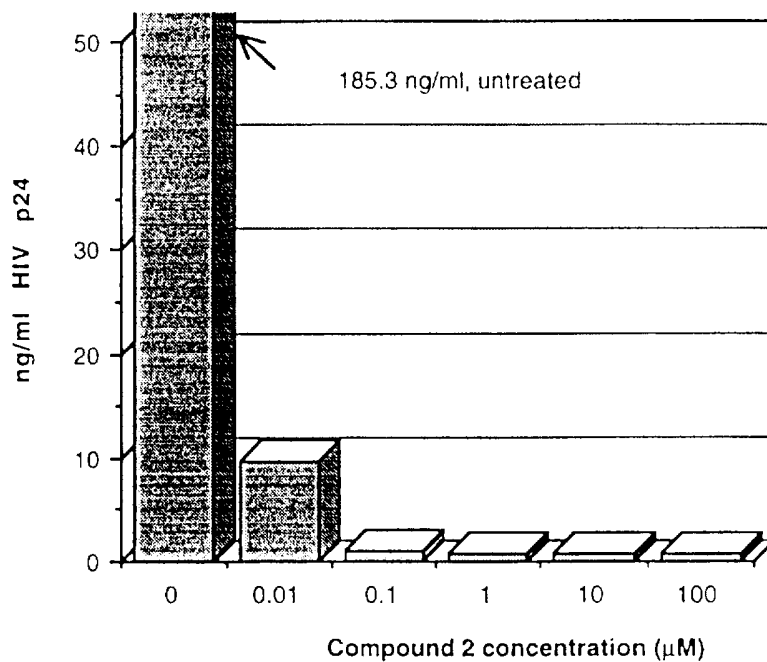


FIG. 28

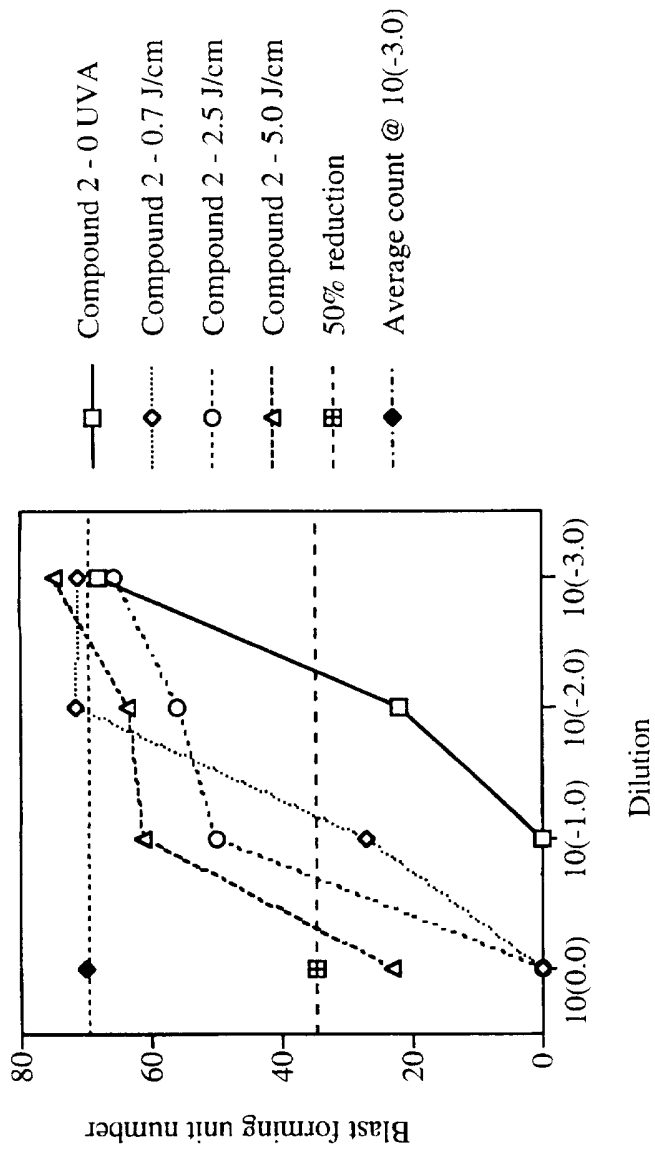


FIGURE 29

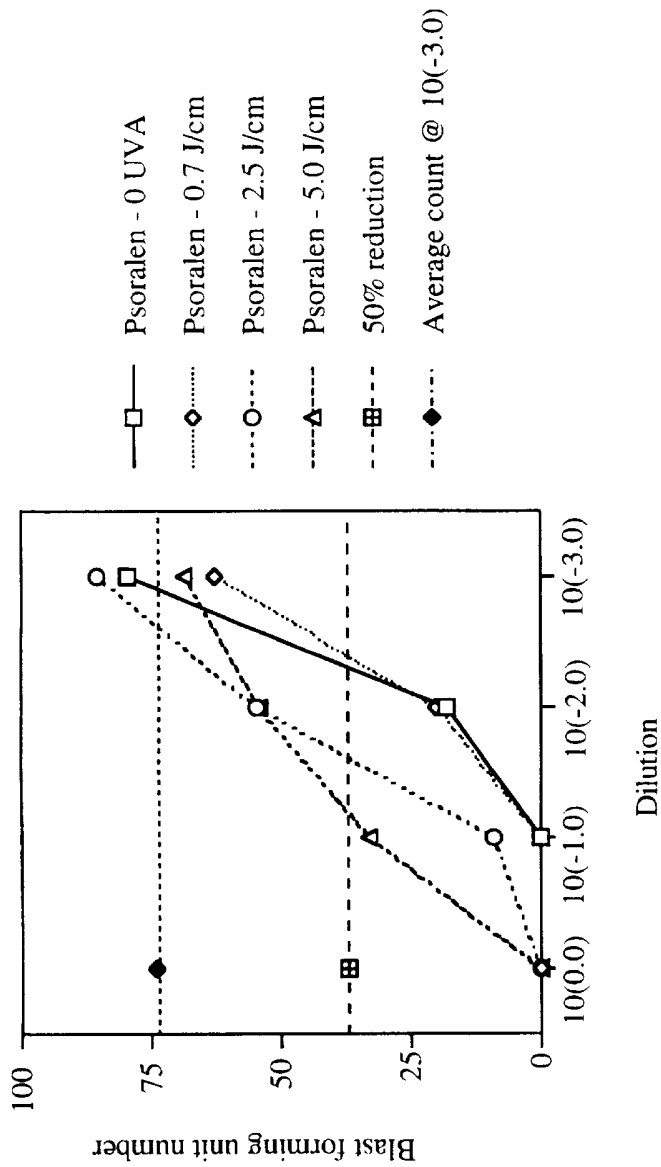


Fig. 30

INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/09837

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : A01N 1/02; C12N 7/06

US CL : 435/2, 238

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/2, 238

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, CA-ONLINE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,545,987 A (GILES ET AL.) 08 October 1985, column 1, lines 13-17, column 2, lines 8-15.	1-12, 16-27
Y	COTTON M. et al. Psoralen treatment of adenovirus particles eliminates virus replication and transcription while maintaining the endosomolytic activity of the virus capsid. Virology. 1994 Vol. 205, No. 1, pages 254-261, especially abstract.	1-12, 16-27
Y	NILSEN T. W. et al., Cross Linking of Viral RNA by 4'-Aminomethyl-4,5',8-trimethyl psoralen in HELA Cells Infected with Encephalomyocarditis Virus and the tsG-114 Mutant of Vesticular Stomatitis Virus. Virology 1981. Volume 109, No. 1, pages 82-93, especially abstract.	1-12, 16-27

Further documents are listed in the continuation of Box C.  See patent family annex.

* Special categories of cited documents:	"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
"A" document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"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
"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)	"&"	document member of the same patent family
"O" 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		

Date of the actual completion of the international search 08 AUGUST 1996	Date of mailing of the international search report 10 SEP 1996
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Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SANDRA SAUCIER Telephone No. (703) 308-0196
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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/09837

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NAIR C.N. et al., Photochemical Inhibition of Poliovirus Replication by 4, 5, 8-Trimethyl psoralen Plus Light. Intervirology. 1978 Volume 9, No. 2, pages 65-75, especially abstract.	1-12, 16-27
Y	US 5,399,719 A (WOLLOWITZ ET AL.) 21 March 1995. Columns 5 and 6, example 12 and abstract.	1-30
Y	HANSON C.V., Photochemical Inactivation of Viruses with Psoralens: An Overview. Blood Cells. 1992. Volume 18, pages 7-25, especially abstract.	1-30