Liza M. Walsh
Eleonore Ofosu-Antwi
WALSH PIZZI O'REILLY FALANGA LLP
One Riverfront Plaza
1037 Raymond Boulevard, 6th Floor
Newark, NJ 07102
Tel: 973-757-1100
Fax: 973-757-1090

Attorneys for Plaintiffs Gilead Sciences, Inc. and Emory University

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF NEW JERSEY

GILEAD SCIENCES, INC. and EMORY UNIVERSITY, Plaintiffs, v. AUROBINDO PHARMA LTD. and AUROBINDO PHARMA USA INC., Defendants.	Civil Action No.: *Electronically Filed*

COMPLAINT FOR PATENT INFRINGEMENT

Plaintiffs Gilead Sciences, Inc. ("Gilead") and Emory University ("Emory") (collectively, "Plaintiffs"), for their complaint against Aurobindo Pharma Ltd. and Aurobindo Pharma USA Inc. (collectively, "Aurobindo"), hereby allege as follows:

Nature of Action

1. This is an action for patent infringement under the patent laws of the United States, Title 35 of the United States Code.

The Parties

2. Gilead is a corporation organized and existing under the laws of the State of Delaware, having a principal place of business at 333 Lakeside Drive, Foster City, California 94404.

3. Emory is a non-profit corporation of the State of Georgia, having an office at 201 Dowman Drive, Atlanta, Georgia 30322.

4. On information and belief, defendant Aurobindo Pharma Ltd. is a corporation organized and existing under the laws of India, having a principal place of business at Plot #2, Maitri Vihar, Ameerpet, Hyderabad—500 038, Andhra Pradesh, India. On information and belief, Aurobindo Pharma Ltd. has actual control over the activities of Aurobindo Pharma USA Inc.

5. On information and belief, defendant Aurobindo Pharma USA Inc. is a corporation organized and existing under the laws of the State of Delaware, having a principal place of business at 6 Wheeling Road, Dayton, New Jersey 08810. On information and belief, Aurobindo Pharma USA Inc. is a wholly-owned subsidiary and agent of Aurobindo Pharma Ltd.

Jurisdiction and Venue

6. This action arises under the Patent Laws of the United States and the Food and Drug Laws of the United States, Titles 35 and 21 of the United States Code. Jurisdiction is based on 28 U.S.C. §§ 1331 and 1338(a).

7. On information and belief, this Court has personal jurisdiction over both Aurobindo Pharma Ltd. and Aurobindo Pharma USA Inc.

8. On information and belief, Aurobindo Pharma Ltd., itself or through one of its wholly-owned subsidiaries, including but not limited to Aurobindo Pharma USA Inc., derives substantial revenue from selling various pharmaceutical drug products and doing business throughout the United States, including in New Jersey.

9. On information and belief, Aurobindo Pharma Ltd., itself or through one of its wholly-owned subsidiaries, including but not limited to Aurobindo Pharma USA Inc., manufactures pharmaceutical drug products that are sold and used throughout the United States, including in New Jersey.

10. On information and belief, Aurobindo Pharma Ltd., itself or through one of its wholly-owned subsidiaries, including but not limited to Aurobindo Pharma USA Inc., owns manufacturing facilities in New Jersey that are used to make pharmaceutical drug products.

11. On information and belief, residents of New Jersey purchase pharmaceutical drug products from Aurobindo Pharma Ltd. in New Jersey.

12. On information and belief, Aurobindo Pharma USA Inc. is registered with the State of New Jersey to do business as a foreign corporation in New Jersey.

13. On information and belief, Aurobindo Pharma USA Inc. derives substantial revenue from selling various pharmaceutical drug products and doing business throughout the United States, including in New Jersey.

14. On information and belief, Aurobindo Pharma USA Inc. manufactures pharmaceutical drug products that are sold and used throughout the United States, including in New Jersey.

15. On information and belief, Aurobindo Pharma USA Inc. owns manufacturing facilities in New Jersey that are used to make pharmaceutical drug products.

16. On information and belief, Aurobindo Pharma USA Inc. is registered with the State of New Jersey Department of Health as a drug wholesaler under registration number 5003120.

17. On information and belief, residents of New Jersey purchase pharmaceutical drug products from Aurobindo Pharma USA Inc. in New Jersey.

18. On information and belief, Aurobindo Pharma USA Inc.'s submission of Abbreviated New Drug Application ("ANDA") No. 90-513, discussed below, indicates Aurobindo's intention to engage in the commercial manufacture, use, sale and/or importation of products that will compete directly with Gilead's Truvada® product, which is currently being sold throughout the United States, including in New Jersey. On information and belief, Aurobindo Pharma Ltd. and Aurobindo Pharma USA Inc. will sell tablets containing 200 mg of emtricitabine and 300 mg of tenofovir disoproxil fumarate for the use for which Aurobindo seeks approval in ANDA No. 90-513, if approved, throughout the United States, including in New Jersey.

19. On information and belief, Aurobindo Pharma Ltd. and Aurobindo Pharma USA Inc. have previously consented to personal jurisdiction in this District.

20. In the alternative, this Court has jurisdiction over Aurobindo Pharma Ltd. because the requirements of Federal Rule of Civil Procedure 4(k)(2)(A) are met.

21. Venue is proper in this District under 28 U.S.C. § 1391(b), (c), (d), and 28 U.S.C. § 1400(b).

Background

22. Gilead is the holder of New Drug Application ("NDA") No. 21-752, which relates to tablets containing 200 mg of emtricitabine and 300 mg of tenofovir disoproxil fumarate. On August 2, 2004, the United States Food and Drug Administration ("FDA") approved the use of the tablets described in NDA No. 21-752 for the treatment of HIV-1 infection in adults. These tablets are prescribed and sold in the United States under the trademark Truvada®.

23. United States Patent No. 6,642,245 ("the '245 Patent," copy attached as Exhibit A), entitled "Antiviral Activity and Resolution of 2-Hydroxymethyl-5-(5-fluorocytosin-1-yl) -1,3-oxathiolane," was duly and legally issued by the United States Patent and Trademark Office on November 4, 2003. The '245 Patent claims, *inter alia*, methods for treating HIV infection in

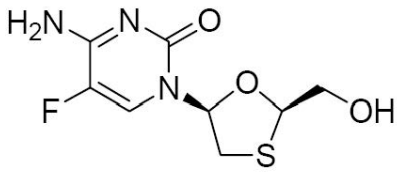
humans with emtricitabine (the active ingredient in Truvada®), and is listed in the *Approved Drug Products with Therapeutic Equivalence Evaluations* ("FDA Orange Book") for Truvada®.

24. United States Patent No. 6,703,396 ("the '396 Patent," copy attached as Exhibit B), entitled "Method of Resolution and Antiviral Activity of 1,3-Oxathiolane Nucleoside Enantiomers," was duly and legally issued by the United States Patent and Trademark Office on March 9, 2004. The '396 Patent claims, *inter alia*, emtricitabine (the active ingredient in Truvada®), and is listed in the FDA Orange Book for Truvada®.

25. United States Patent No. 8,592,397 ("the '397 Patent," copy attached as Exhibit C), entitled "Compositions and Methods for Combination Antiviral Therapy," was duly and legally issued by the United States Patent and Trademark Office on November 26, 2013. The '397 Patent claims, *inter alia*, a pharmaceutical combination tablet containing emtricitabine and tenofovir disoproxil fumarate (the two active ingredients in Truvada®) and methods for treating HIV infection in humans with the emtricitabine and tenofovir disoproxil fumarate combination. The '397 Patent is also listed in the FDA Orange Book for Truvada®.

26. United States Patent No. 8,716,264 ("the '264 Patent," copy attached as Exhibit D), entitled "Compositions and Methods for Combination Antiviral Therapy," was duly and legally issued by the United States Patent and Trademark Office on May 6, 2014. The '264 Patent claims, *inter alia*, a pharmaceutical combination tablet containing emtricitabine and tenofovir disoproxil fumarate (the two active ingredients in Truvada®) and methods for treating HIV infection in humans with the emtricitabine and tenofovir disoproxil fumarate combination. The '264 Patent is also listed in the FDA Orange Book for Truvada®.

27. Emtricitabine is a compound that has a molecular formula of $C_8H_{10}FN_3O_3S$, and which has the following chemical structure:



28. Emtricitabine can be referred to by any of several chemical names. The chemical name given to emtricitabine in the Truvada® label is "5-fluoro-1-[(2*R*,5*S*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine." Two chemical names recited for emtricitabine in the '245 Patent are "(–)-β-L-2-hydroxymethyl-5-(5-fluorocytosin-1-yl) -1,3-oxathiolane" and "β-L-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane." Two chemical names recited for emtricitabine in the '396 Patent are "(–)-cis-4-amino-5-fluoro-1-(2-hydroxymethyl-1,3-oxathiolane-5-yl)-(1H)-pyrimidin-2-one" and "(–)-enantiomer of cis-4-amino-5-fluoro-1-(2-hydroxymethyl-1,3-oxathiolane-5-yl)-(1H)-pyrimidin-2-one."

29. The named inventors on the '245 and '396 Patents are Dennis C. Liotta, Raymond F. Schinazi, and Woo-Baeg Choi.

30. Dennis C. Liotta, Raymond F. Schinazi, and Woo-Baeg Choi assigned the '245 and '396 Patents to Emory.

31. Pursuant to an agreement entered into between Gilead and Emory, Gilead has substantial rights in the '245 and '396 Patents, including, but not limited to, rights associated with being a licensee of the '245 and '396 Patents, and the right to sue for infringement of the '245 and '396 Patents.

32. The named inventors of the '397 and '264 Patents are Terrence C. Dahl, Mark M. Menning, and Reza Oliyai.

33. Terrence C. Dahl, Mark M. Menning, and Reza Oliyai assigned the '397 and '264 Patents to Gilead.

COUNT 1
Infringement of U.S. Patent No. 6,642,245

34. Plaintiffs repeat and reallege paragraphs 1-33 above as if set forth herein.

35. On information and belief, Aurobindo submitted or caused to be submitted ANDA No. 90-513 to the FDA seeking approval to engage in the commercial manufacture, use, sale and/or importation of tablets containing 200 mg of emtricitabine and 300 mg of tenofovir disoproxil fumarate for the purpose of treating HIV infection.

36. By letter dated May 27, 2016, pursuant to 21 U.S.C. § 355(j)(2)(B)(ii) (the "May 27, 2016 Notice Letter"), Aurobindo notified Plaintiffs that it had submitted ANDA No. 90-513 to the FDA seeking approval to engage in the commercial manufacture, use, sale and/or importation of tablets containing 200 mg of emtricitabine and 300 mg of tenofovir disoproxil fumarate prior to the expiration of the '245 Patent. This complaint has been filed within 45 days of Plaintiffs' receipt of the May 27, 2016 Notice Letter.

37. In its May 27, 2016 Notice Letter, Aurobindo notified Plaintiffs that, as a part of ANDA No. 90-513, it had filed a certification of the type described in 21 U.S.C. § 355(j)(2)(A)(vii)(III) and that it now intends to convert that into a certification of the type described in 21 U.S.C. § 355(j)(2)(A)(vii)(IV) ("Paragraph IV") with respect to the '245 Patent. Paragraph IV requires, *inter alia*, certification by the ANDA applicant, in its opinion and to the best of its knowledge, that the subject patent, here the '245 Patent, "is invalid or will not be infringed by the manufacture, use or sale of the new drug for which the application is submitted . . . ." The statute (21 U.S.C. § 355(j)(2)(B)(iv)(II)) also requires a Paragraph IV Notice Letter to "include a detailed statement of the factual and legal basis of the opinion of the applicant that the patent is invalid or will not be infringed." The FDA Rules and Regulations (21 C.F.R. § 314.95(c)(6)) further require that the detailed statement include "(i) [f]or each claim of a patent

alleged not to be infringed, a full and detailed explanation of why the claim is not infringed" and "(ii) [f]or each claim of a patent alleged to be invalid or unenforceable, a full and detailed explanation of the grounds supporting the allegations."

38. Aurobindo alleged in its May 27, 2016 Notice Letter that Claims 1-8 and 15 of the '245 Patent are invalid and that Claims 4, 5, and 9-22 of the '245 Patent would not be infringed by the commercial manufacture, use, sale and/or importation of its proposed product that is the subject of ANDA No. 90-513.

39. The May 27, 2016 Notice Letter does not allege non-infringement of Claims 1-3 and 6-8 of the '245 Patent.

40. The May 27, 2016 Notice Letter does not provide the full and detailed statement of Aurobindo's factual and legal basis to support its non-infringement and invalidity allegations as to the '245 Patent.

41. Accordingly, the May 27, 2016 Notice Letter fails to comply with the law, as specified in 21 U.S.C. § 355(j) and FDA rules and regulations, as specified in 21 C.F.R. § 314.95.

42. By filing ANDA No. 90-513 under 21 U.S.C. § 355(j) for the purposes of obtaining approval to engage in the commercial manufacture, use, sale and/or importation of tablets containing 200 mg of emtricitabine and 300 mg of tenofovir disoproxil fumarate before the '245 Patent's expiration, Aurobindo has committed an act of infringement of the '245 Patent under 35 U.S.C. § 271(e)(2).

43. On information and belief, Aurobindo lacked a good faith basis for alleging invalidity when ANDA No. 90-513 was filed and when the Paragraph IV certification was made. Aurobindo's ANDA and Paragraph IV certification is a wholly unjustified infringement of the '245 Patent.

44. Aurobindo's submission of ANDA No. 90-513 and service of the May 27, 2016 Notice Letter indicates a refusal to change its current course of action.

45. On information and belief, the commercial manufacture, use, sale and/or importation of tablets containing 200 mg of emtricitabine and 300 mg of tenofovir disoproxil fumarate for which Aurobindo seeks approval in ANDA No. 90-513, if approved, will infringe one or more claims of the '245 Patent.

46. On information and belief, Aurobindo will directly or indirectly infringe at least Claim 1 of the '245 Patent. Claim 1 recites a "method for treating HIV infection in humans comprising administering an effective amount of [emtricitabine], or its physiologically acceptable salt, optionally in a pharmaceutically acceptable carrier." On information and belief, Aurobindo will infringe Claim 1 of the '245 Patent because the product for which it seeks approval in ANDA No. 90-513 will be labeled for and used to treat HIV infection in humans with an effective amount of emtricitabine. In its May 27, 2016 Notice Letter, Aurobindo does not allege that Claim 1 would not be infringed by the commercial manufacture, use, sale, offer for sale and/or importation of its proposed product that is the subject of ANDA No. 90-513. For the same reasons, on information and belief, Aurobindo will likewise infringe Claims 2, 3, 6, 7, and 8 of the '245 Patent.

47. On information and belief, the tablets containing 200 mg of emtricitabine and 300 mg of tenofovir disoproxil fumarate for the use for which Aurobindo seeks approval in ANDA No. 90-513, if approved, will be administered to human patients in an effective amount for treating HIV infection. Such administration will infringe at least one claim of the '245 Patent, as described in the preceding paragraph. On information and belief, this administration will occur at Aurobindo's active behest and with its intent, knowledge and encouragement. On information and belief, Aurobindo will actively encourage, aid and abet this administration with knowledge that it

is in contravention of Plaintiffs' rights under the '245 Patent. Further, by filing ANDA No. 90-513 with a Paragraph IV certification, Aurobindo admits that it has knowledge of the '245 Patent.

48. The May 27, 2016 Notice Letter does not allege and does not address unenforceability of any claims of the '245 Patent. By not addressing unenforceability of any claims of the '245 Patent in its May 27, 2016 Notice Letter, Aurobindo admits that all of the claims of the '245 Patent are enforceable.

COUNT 2
Infringement of U.S. Patent No. 6,703,396

49. Plaintiffs repeat and reallege paragraphs 1-48 above as if set forth herein.

50. On information and belief, Aurobindo submitted or caused to be submitted ANDA No. 90-513 to the FDA seeking approval to engage in the commercial manufacture, use, sale and/or importation of tablets containing 200 mg of emtricitabine and 300 mg of tenofovir disoproxil fumarate for the purpose of treating HIV infection.

51. By letter dated May 27, 2016, pursuant to 21 U.S.C. § 355(j)(2)(B)(ii) (the "May 27, 2016 Notice Letter"), Aurobindo notified Plaintiffs that it had submitted ANDA No. 90-513 to the FDA seeking approval to engage in the commercial manufacture, use, sale and/or importation of tablets containing 200 mg of emtricitabine and 300 mg of tenofovir disoproxil fumarate prior to the expiration of the '396 Patent. This complaint has been filed within 45 days of Plaintiffs' receipt of the May 27, 2016 Notice Letter.

52. In its May 27, 2016 Notice Letter, Aurobindo notified Plaintiffs that, as a part of its ANDA No. 90-513, it had filed a certification of the type described in 21 U.S.C. § 355(j)(2)(A)(vii)(III) and that it now intends to convert that into a certification of the type described in 21 U.S.C. § 355(j)(2)(A)(vii)(IV) ("Paragraph IV") with respect to the '396 Patent. Paragraph IV requires, *inter alia*, certification by the ANDA applicant, in its opinion and to the

best of its knowledge, that the subject patent, here the '396 Patent, "is invalid or will not be infringed by the manufacture, use or sale of the new drug for which the application is submitted . . . ." The statute (21 U.S.C. § 355(j)(2)(B)(iv)(II)) also requires a Paragraph IV Notice Letter to "include a detailed statement of the factual and legal basis of the opinion of the applicant that the patent is invalid or will not be infringed." The FDA Rules and Regulations (21 C.F.R. § 314.95(c)(6)) further require that the detailed statement include "(i) [f]or each claim of a patent alleged not to be infringed, a full and detailed explanation of why the claim is not infringed" and "(ii) [f]or each claim of a patent alleged to be invalid or unenforceable, a full and detailed explanation of the grounds supporting the allegations."

53. Aurobindo alleged in its May 27, 2016 Notice Letter that Claims 1-7, 11, 13, 15, and 17 of the '396 Patent are invalid and that Claims 8-10, 12, 14, 16, and 18-28 would not be infringed by the commercial manufacture, use, sale and/or importation of its proposed product that is the subject of ANDA No. 90-513.

54. The May 27, 2016 Notice Letter does not allege non-infringement of Claims 1-7, 11, 13, 15, and 17 of the '396 Patent.

55. The May 27, 2016 Notice Letter does not provide the full and detailed statement of Aurobindo's factual and legal basis to support its non-infringement and invalidity allegations as to the '396 Patent.

56. Accordingly, the May 27, 2016 Notice Letter fails to comply with the law, as specified in 21 U.S.C. § 355(j) and FDA rules and regulations, as specified in 21 C.F.R. § 314.95.

57. By filing ANDA No. 90-513 under 21 U.S.C. § 355(j) for the purposes of obtaining approval to engage in the commercial manufacture, use, sale and/or importation of tablets containing 200 mg of emtricitabine and 300 mg of tenofovir disoproxil fumarate before the '396

Patent's expiration, Aurobindo has committed an act of infringement of the '396 Patent under 35 U.S.C. § 271(e)(2).

58. On information and belief, Aurobindo lacked a good faith basis for alleging invalidity when ANDA No. 90-513 was filed and when the Paragraph IV certification was made. Aurobindo's ANDA and Paragraph IV certification is a wholly unjustified infringement of the '396 Patent.

59. Aurobindo's submission of ANDA No. 90-513 and service of the May 27, 2016 Notice Letter indicates a refusal to change its current course of action.

60. On information and belief, the commercial manufacture, use, sale and/or importation of tablets containing 200 mg of emtricitabine and 300 mg of tenofovir disoproxil fumarate for which Aurobindo seeks approval in ANDA No. 90-513, if approved, will infringe one or more claims of the '396 Patent.

61. On information and belief, Aurobindo will directly or indirectly infringe at least Claim 2 of the '396 Patent. Claim 2 recites "[emtricitabine] or a pharmaceutically acceptable salt, ester or salt of an ester thereof." On information and belief, Aurobindo will infringe Claim 2 of the '396 Patent because the product for which it seeks approval in ANDA No. 90-513 will contain emtricitabine as the active ingredient. In its May 27, 2016 Notice Letter, Aurobindo does not allege that Claim 2 would not be infringed by the commercial manufacture, use, sale, offer for sale and/or importation of its proposed product that is the subject of ANDA No. 90-513. For the same reasons, on information and belief, Aurobindo will also infringe Claims 1, 3-7, 11, 13, 15, and 17 of the '396 Patent.

62. On information and belief, the tablets containing 200 mg of emtricitabine and 300 mg of tenofovir disoproxil fumarate for the use for which Aurobindo seeks approval in ANDA No.

90-513, if approved, will infringe at least one claim of the '396 Patent, as described in the preceding paragraph. On information and belief, the manufacture of these tablets will occur at Aurobindo's active behest and with its intent, knowledge and encouragement. On information and belief, Aurobindo will actively encourage, aid and abet the manufacture of these tablets with knowledge that it is in contravention of Plaintiffs' rights under the '396 Patent. Further, by filing ANDA No. 90-513 with a Paragraph IV certification, Aurobindo admits that it has knowledge of the '396 Patent.

63. The May 27, 2016 Notice Letter does not allege and does not address unenforceability of any claims of the '396 Patent. By not addressing unenforceability of any claims of the '396 Patent in its May 27, 2016 Notice Letter, Aurobindo admits that all of the claims of the '396 Patent are enforceable.

COUNT 3
Infringement of U.S. Patent No. 8,592,397

64. Plaintiffs repeat and reallege paragraphs 1-63 above as if set forth herein.

65. On information and belief, Aurobindo submitted or caused to be submitted ANDA No. 90-513 to the FDA seeking approval to engage in the commercial manufacture, use, sale and/or importation of tablets containing 200 mg of emtricitabine and 300 mg of tenofovir disoproxil fumarate for the purpose of treating HIV infection.

66. By letter dated May 27, 2016, pursuant to 21 U.S.C. § 355(j)(2)(B)(ii) (the "May 27, 2016 Notice Letter"), Aurobindo notified Plaintiffs that it had submitted ANDA No. 90-513 to the FDA seeking approval to engage in the commercial manufacture, use, sale and/or importation of tablets containing 200 mg of emtricitabine and 300 mg of tenofovir disoproxil fumarate prior to the expiration of the '397 Patent. This complaint has been filed within 45 days of Plaintiffs' receipt of the May 27, 2016 Notice Letter.

67. In its May 27, 2016 Notice Letter, Aurobindo notified Plaintiffs that, as a part of its ANDA No. 90-513, it had filed a certification of the type described in 21 U.S.C. § 355(j)(2)(A)(vii)(III) and that it now intends to convert that into a certification of the type described in 21 U.S.C. § 355(j)(2)(A)(vii)(IV) ("Paragraph IV") with respect to the '397 Patent. Paragraph IV requires, *inter alia*, certification by the ANDA applicant, in its opinion and to the best of its knowledge, that the subject patent, here the '397 Patent, "is invalid or will not be infringed by the manufacture, use or sale of the new drug for which the application is submitted . . ." The statute (21 U.S.C. § 355(j)(2)(B)(iv)(II)) also requires a Paragraph IV Notice Letter to "include a detailed statement of the factual and legal basis of the opinion of the applicant that the patent is invalid or will not be infringed." The FDA Rules and Regulations (21 C.F.R. § 314.95(c)(6)) further require that the detailed statement include "(i) [f]or each claim of a patent alleged not to be infringed, a full and detailed explanation of why the claim is not infringed" and "(ii) [f]or each claim of a patent alleged to be invalid or unenforceable, a full and detailed explanation of the grounds supporting the allegations."

68. Aurobindo alleged in its May 27, 2016 Notice Letter that Claims 1-26 of the '397 Patent are invalid.

69. Aurobindo did not allege in its May 27, 2016 Notice Letter that any claims of the '397 Patent would not be infringed by the commercial manufacture, use, sale and/or importation of its proposed product that is the subject of ANDA No. 90-513.

70. The May 27, 2016 Notice Letter does not provide the full and detailed statement of Aurobindo's factual and legal basis to support its invalidity allegations as to the '397 Patent.

71. Accordingly, the May 27, 2016 Notice Letter fails to comply with the law, as specified in 21 U.S.C. § 355(j) and FDA rules and regulations, as specified in 21 C.F.R. § 314.95.

72. By filing ANDA No. 90-513 under 21 U.S.C. § 355(j) for the purposes of obtaining approval to engage in the commercial manufacture, use, sale and/or importation of tablets containing 200 mg of emtricitabine and 300 mg of tenofovir disoproxil fumarate before the '397 Patent's expiration, Aurobindo has committed an act of infringement of the '397 Patent under 35 U.S.C. § 271(e)(2).

73. On information and belief, Aurobindo lacked a good faith basis for alleging invalidity when ANDA No. 90-513 was filed and when the Paragraph IV certification was made. Aurobindo's ANDA and Paragraph IV certification is a wholly unjustified infringement of the '397 Patent.

74. Aurobindo's submission of ANDA No. 90-513 and service of the May 27, 2016 Notice Letter indicates a refusal to change its current course of action.

75. On information and belief, the commercial manufacture, use, sale and/or importation of tablets containing 200 mg of emtricitabine and 300 mg of tenofovir disoproxil fumarate for which Aurobindo seeks approval in ANDA No. 90-513, if approved, will infringe one or more claims of the '397 Patent.

76. On information and belief, Aurobindo will directly or indirectly infringe at least Claim 1 of the '397 Patent. Claim 1 recites a "chemically stable fixed dose combination pharmaceutical dosage form comprising 300 mg tenofovir disoproxil fumarate and 200 mg emtricitabine; a binder selected from the group consisting of povidone, gelatin, hydroxypropyl methylcellulose, cellulose, microcrystalline cellulose, starch, and acacia; a disintegrant selected from sodium starch glycolate, crosslinked-povidone, cross-linked sodium carboxymethylcellulose, and alginic acid; and a lubricant selected from the group consisting of magnesium stearate, stearic acid, and talc; wherein said pharmaceutical dosage form exhibits less

than 10% degradation of the tenofovir disoproxil fumarate or emtricitabine after 6 months when packaged and stored with silica gel dessicant at 40° C./75% relative humidity." On information and belief, Aurobindo will infringe Claim 1 of the '397 Patent because the product for which it seeks approval in ANDA No. 90-513 will be a chemically stable, fixed-dose tablet containing 300 mg tenofovir disoproxil fumarate and 200 mg emtricitabine and at least one of each enumerated binder, disintegrant, and lubricant, or an equivalent thereof, and will exhibit less than 10% degradation of the tenofovir disoproxil fumarate or emtricitabine after six months when packaged and stored with silica gel dessicant at 40° C./75% relative humidity. In its May 27, 2016 Notice Letter, Aurobindo does not allege that Claim 1 would not be infringed by the commercial manufacture, use, sale, offer for sale and/or importation of its proposed product that is the subject of ANDA No. 90-513. For the same reasons, on information and belief, Aurobindo will also infringe Claims 2-6, 14-16, and 19 of the '397 Patent.

77. On information and belief, the tablets containing 200 mg of emtricitabine and 300 mg of tenofovir disoproxil fumarate for the use for which Aurobindo seeks approval in ANDA No. 90-513, if approved, will infringe at least one claim of the '397 Patent, as described in the preceding paragraph. On information and belief, the manufacture of these tablets and use of these tablets to treat HIV infection will occur at Aurobindo's active behest and with its intent, knowledge and encouragement. On information and belief, Aurobindo will actively encourage, aid and abet the manufacture of these tablets and use of these tablets to treat HIV infection with knowledge that it is in contravention of Plaintiffs' rights under the '397 Patent. Further, by filing ANDA No. 90-513 with a Paragraph IV certification, Aurobindo admits that it has knowledge of the '397 Patent.

78. The May 27, 2016 Notice Letter does not allege and does not address unenforceability of any claims of the '397 Patent. By not addressing unenforceability of any claims of the '397 Patent in its May 27, 2016 Notice Letter, Aurobindo admits that all of the claims of the '397 Patent are enforceable.

COUNT 4
Infringement of U.S. Patent No. 8,716,264

79. Plaintiffs repeat and reallege paragraphs 1-78 above as if set forth herein.

80. On information and belief, Aurobindo submitted or caused to be submitted ANDA No. 90-513 to the FDA seeking approval to engage in the commercial manufacture, use, sale and/or importation of tablets containing 200 mg of emtricitabine and 300 mg of tenofovir disoproxil fumarate for the purpose of treating HIV infection.

81. By letter dated May 27, 2016, pursuant to 21 U.S.C. § 355(j)(2)(B)(ii) (the "May 27, 2016 Notice Letter"), Aurobindo notified Plaintiffs that it had submitted ANDA No. 90-513 to the FDA seeking approval to engage in the commercial manufacture, use, sale and/or importation of tablets containing 200 mg of emtricitabine and 300 mg of tenofovir disoproxil fumarate prior to the expiration of the '264 Patent. This complaint has been filed within 45 days of Plaintiffs' receipt of the May 27, 2016 Notice Letter.

82. In its May 27, 2016 Notice Letter, Aurobindo notified Plaintiffs that, as a part of its ANDA No. 90-513, it had filed a certification of the type described in 21 U.S.C. § 355(j)(2)(A)(vii)(III) and that it now intends to convert that into a certification of the type described in 21 U.S.C. § 355(j)(2)(A)(vii)(IV) ("Paragraph IV") with respect to the '264 Patent. Paragraph IV requires, *inter alia*, certification by the ANDA applicant, in its opinion and to the best of its knowledge, that the subject patent, here the '264 Patent, "is invalid or will not be infringed by the manufacture, use or sale of the new drug for which the application is submitted . .

. .” The statute (21 U.S.C. § 355(j)(2)(B)(iv)(II)) also requires a Paragraph IV Notice Letter to “include a detailed statement of the factual and legal basis of the opinion of the applicant that the patent is invalid or will not be infringed.” The FDA Rules and Regulations (21 C.F.R. § 314.95(c)(6)) further require that the detailed statement include “(i) [f]or each claim of a patent alleged not to be infringed, a full and detailed explanation of why the claim is not infringed” and “(ii) [f]or each claim of a patent alleged to be invalid or unenforceable, a full and detailed explanation of the grounds supporting the allegations.”

83. Aurobindo alleged in its May 27, 2016 Notice Letter that Claims 1-38 of the ’264 Patent are invalid.

84. Aurobindo did not allege in its May 27, 2016 Notice Letter that any claims of the ’264 Patent would not be infringed by the commercial manufacture, use, sale and/or importation of its proposed product that is the subject of ANDA No. 90-513.

85. The May 27, 2016 Notice Letter does not provide the full and detailed statement of Aurobindo’s factual and legal basis to support its invalidity allegations as to the ’264 Patent.

86. Accordingly, the May 27, 2016 Notice Letter fails to comply with the law, as specified in 21 U.S.C. § 355(j) and FDA rules and regulations, as specified in 21 C.F.R. § 314.95.

87. By filing ANDA No. 90-513 under 21 U.S.C. § 355(j) for the purposes of obtaining approval to engage in the commercial manufacture, use, sale and/or importation of tablets containing 200 mg of emtricitabine and 300 mg of tenofovir disoproxil fumarate before the ’264 Patent’s expiration, Aurobindo has committed an act of infringement of the ’264 Patent under 35 U.S.C. § 271(e)(2).

88. On information and belief, Aurobindo lacked a good faith basis for alleging invalidity when ANDA No. 90-513 was filed and when the Paragraph IV certification was made.

Aurobindo's ANDA and Paragraph IV certification is a wholly unjustified infringement of the '264 Patent.

89. Aurobindo's submission of ANDA No. 90-513 and service of the May 27, 2016 Notice Letter indicates a refusal to change its current course of action.

90. On information and belief, the commercial manufacture, use, sale and/or importation of tablets containing 200 mg of emtricitabine and 300 mg of tenofovir disoproxil fumarate for which Aurobindo seeks approval in ANDA No. 90-513, if approved, will infringe one or more claims of the '264 Patent.

91. On information and belief, Aurobindo will directly or indirectly infringe at least Claim 1 of the '264 Patent. Claim 1 recites a "chemically stable fixed-dose combination comprising 300 mg of tenofovir disoproxil fumarate and 200 mg of emtricitabine wherein the combination exhibits less than 10% degradation of tenofovir disoproxil fumarate and emtricitabine after six months at 40° C./75% relative humidity when packaged and stored with silica gel dessicant at 40° C./70% relative humidity."[1] On information and belief, Aurobindo will infringe Claim 1 of the '264 Patent because the product for which it seeks approval in ANDA No. 90-513 will be a chemically stable, fixed-dose tablet containing 300 mg of tenofovir disoproxil fumarate and 200 mg of emtricitabine and will exhibit less than 10% degradation of tenofovir disoproxil fumarate and emtricitabine after six months at 40° C./75% relative humidity when packaged and stored with silica gel dessicant at 40° C./70% relative humidity. In its May 27, 2016 Notice Letter, Aurobindo does not allege that Claim 1 would not be infringed by the commercial manufacture, use, sale, offer for sale and/or importation of its proposed product that is the subject of ANDA No.

[1] Claim 1 contains a clear typographical error in stating "70% relative humidity" in the last clause rather than "75% relative humidity." Plaintiff Gilead will request that the Court correct this error.

90-513. For the same reasons, on information and belief, Aurobindo will also infringe at least Claims 1-3, 9, 16, 17, 33, and 34 of the '264 Patent.

92. On information and belief, the tablets containing 200 mg of emtricitabine and 300 mg of tenofovir disoproxil fumarate for the use for which Aurobindo seeks approval in ANDA No. 90-513, if approved, will infringe at least one claim of the '264 Patent, as described in the preceding paragraph. On information and belief, the manufacture of these tablets and use of these tablets to treat HIV infection will occur at Aurobindo's active behest and with its intent, knowledge and encouragement. On information and belief, Aurobindo will actively encourage, aid and abet the manufacture of these tablets and use of these tablets to treat HIV infection with knowledge that it is in contravention of Plaintiffs' rights under the '264 Patent. Further, by filing ANDA No. 90-513 with a Paragraph IV certification, Aurobindo admits that it has knowledge of the '264 Patent.

93. The May 27, 2016 Notice Letter does not allege and does not address unenforceability of any claims of the '264 Patent. By not addressing unenforceability of any claims of the '264 Patent in its May 27, 2016 Notice Letter, Aurobindo admits that all of the claims of the '264 Patent are enforceable.

PRAYER FOR RELIEF

WHEREFORE, Plaintiffs respectfully request the following relief:

(a) A judgment declaring that the effective date of any approval of Aurobindo's ANDA No. 90-513 under Section 505(j) of the Federal Food, Drug and Cosmetic Act (21 U.S.C. § 355(j)) be a date which is not earlier than the expiration of the '245 Patent or any later date of exclusivity to which Plaintiffs are or become entitled;

(b) A judgment declaring that the effective date of any approval of Aurobindo's ANDA No. 90-513 under Section 505(j) of the Federal Food, Drug and Cosmetic Act (21 U.S.C. § 355(j)) be a

date which is not earlier than the expiration of the ’396 Patent or any later date of exclusivity to which Plaintiffs are or become entitled;

(c) A judgment declaring that the effective date of any approval of Aurobindo’s ANDA No. 90-513 under Section 505(j) of the Federal Food, Drug and Cosmetic Act (21 U.S.C. § 355(j)) be a date which is not earlier than the expiration of the ’397 Patent or any later date of exclusivity to which Plaintiffs are or become entitled;

(d) A judgment declaring that the effective date of any approval of Aurobindo’s ANDA No. 90-513 under Section 505(j) of the Federal Food, Drug and Cosmetic Act (21 U.S.C. § 355(j)) be a date which is not earlier than the expiration of the ’264 Patent or any later date of exclusivity to which Plaintiffs are or become entitled;

(e) A judgment declaring that the ’245 Patent remains valid and enforceable, and that one or more claims have been infringed by Aurobindo;

(f) A judgment declaring that the ’396 Patent remains valid and enforceable, and that one or more claims have been infringed by Aurobindo;

(g) A judgment declaring that the ’397 Patent remains valid and enforceable, and that one or more claims have been infringed by Aurobindo;

(h) A judgment declaring that the ’264 Patent remains valid and enforceable, and that one or more claims have been infringed by Aurobindo;

(i) A permanent injunction against any infringement of the ’245 Patent by Aurobindo, their officers, agents, attorneys and employees, and those acting in privity or contract with them;

(j) A permanent injunction against any infringement of the ’396 Patent by Aurobindo, their officers, agents, attorneys and employees, and those acting in privity or contract with them;

(k) A permanent injunction against any infringement of the ’397 Patent by Aurobindo, their officers, agents, attorneys and employees, and those acting in privity or contract with them;

(l) A permanent injunction against any infringement of the ’264 Patent by Aurobindo, their officers, agents, attorneys and employees, and those acting in privity or contract with them;

(m) A judgment that Aurobindo’s conduct is exceptional in this case;

(n) An award of reasonable attorney fees pursuant to 35 U.S.C. § 285;

(o) To the extent that Aurobindo has committed any acts with respect to the subject matter claimed in the ’245 Patent, other than those acts expressly exempted by 35 U.S.C. § 271(e)(1), an award of damages for such acts, which should be trebled pursuant to 35 U.S.C. § 284;

(p) To the extent that Aurobindo has committed any acts with respect to the subject matter claimed in the ’396 Patent, other than those acts expressly exempted by 35 U.S.C. § 271(e)(1), an award of damages for such acts, which should be trebled pursuant to 35 U.S.C. § 284;

(q) To the extent that Aurobindo has committed any acts with respect to the subject matter claimed in the ’397 Patent, other than those acts expressly exempted by 35 U.S.C. § 271(e)(1), an award of damages for such acts, which should be trebled pursuant to 35 U.S.C. § 284;

(r) To the extent that Aurobindo has committed any acts with respect to the subject matter claimed in the ’264 Patent, other than those acts expressly exempted by 35 U.S.C. § 271(e)(1), an award of damages for such acts, which should be trebled pursuant to 35 U.S.C. § 284;

(s) Costs and expenses in this action; and

(t) Such other relief as this Court may deem just and proper.

Dated: July 8, 2016

Respectfully submitted,

s/ Liza M. Walsh
Liza M. Walsh

Eleonore Ofosu-Antwi
WALSH PIZZI O'REILLY FALANGA LLP
One Riverfront Plaza
1037 Raymond Boulevard, 6th Floor
Newark, NJ 07102
Tel: 973-757-1100
Fax: 973-757-1090

OF COUNSEL:

David B. Bassett (*pro hac vice forthcoming*)
David A. Manspeizer
Jonathan E. Barbee (*pro hac vice forthcoming*)
WILMER CUTLER PICKERING HALE AND DORR LLP
7 World Trade Center
250 Greenwich Street
New York, NY 10007

Christopher Borello (*pro hac vice forthcoming*)
FITZPATRICK, CELLA, HARPER & SCINTO
1290 Avenue of the Americas
New York, NY 10104

Attorneys for Plaintiffs Gilead Sciences, Inc. and Emory University

RULE 11.2 CERTIFICATION

I hereby certify that, to the best of my knowledge, the matter in controversy is not the subject of any other pending or anticipated litigation in any court or arbitration proceeding, nor are there any non-parties known to Plaintiffs that should be joined to this action. In addition, I recognize a continuing obligation during the course of this litigation to file and to serve on all other parties and with the Court an amended certification if there is a change in the facts stated in this original certification. However, this case is related to the subject matter of the following action:

- *Gilead Sciences, Inc. et al v. Aurobindo Pharma Ltd. et al.*, Civ. A. No. 16-3722.

The foregoing case involves the same parties and two of the same patents and has been assigned to Hon. Renee M. Bumb, U.S.D.J., and Hon. Ann Marie Donio, U.S.M.J.

Dated: July 8, 2016

WALSH PIZZI O'REILLY FALANGA LLP

s/ Liza M. Walsh
Liza M. Walsh
Eleonore Ofosu-Antwi
One Riverfront Plaza
1037 Raymond Boulevard, 6th Floor
Newark, NJ 07102
Tel: 973-757-1100
Fax: 973-757-1090

OF COUNSEL:

David B. Bassett (*pro hac vice forthcoming*)
David A. Manspeizer
Jonathan E. Barbee (*pro hac vice forthcoming*)
WILMER CUTLER PICKERING HALE AND DORR LLP
7 World Trade Center
250 Greenwich Street
New York, NY 10007

Christopher Borello (*pro hac vice forthcoming*)
FITZPATRICK, CELLA, HARPER & SCINTO

1290 Avenue of the Americas
New York, NY 10104

Attorneys for Plaintiffs Gilead Sciences, Inc. and Emory University

RULE 201.1 CERTIFICATION

I hereby certify that the above-captioned matter is not subject to compulsory arbitration in that the Plaintiffs seek, inter alia, injunctive relief.

Dated: July 8, 2016

WALSH PIZZI O'REILLY FALANGA LLP

s/ Liza M. Walsh

Liza M. Walsh
Eleonore Ofosu-Antwi
One Riverfront Plaza
1037 Raymond Boulevard, 6th Floor
Newark, NJ 07102
Tel: 973-757-1100
Fax: 973-757-1090

OF COUNSEL:

David B. Bassett (*pro hac vice forthcoming*)
David A. Manspeizer
Jonathan E. Barbee (*pro hac vice forthcoming*)
WILMER CUTLER PICKERING HALE AND DORR LLP
7 World Trade Center
250 Greenwich Street
New York, NY 10007

Christopher Borello (*pro hac vice forthcoming*)
FITZPATRICK, CELLA, HARPER & SCINTO
1290 Avenue of the Americas
New York, NY 10104

Attorneys for Plaintiffs Gilead Sciences, Inc. and Emory University

Exhibit A

US006642245B1

(12) **United States Patent**
Liotta et al.

(10) **Patent No.: US 6,642,245 B1**
(45) **Date of Patent: *Nov. 4, 2003**

(54) **ANTIVIRAL ACTIVITY AND RESOLUTION OF 2-HYDROXYMETHYL-5-(5-FLUOROCYTOSIN-1-YL)-1,3-OXATHIOLANE**

(75) Inventors: **Dennis C. Liotta**, Stone Mountain, GA (US); **Raymond F. Schinazi**, Decatur, GA (US); **Woo-Baeg Choi**, North Brunswick, NJ (US)

(73) Assignee: **Emory University**, Atlanta, GA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **08/475,339**

(22) Filed: **Jun. 7, 1995**

Related U.S. Application Data

(63) Continuation of application No. 07/831,153, filed on Feb. 12, 1992, now abandoned, and a continuation-in-part of application No. 07/736,089, filed on Jul. 26, 1991, now abandoned, which is a continuation-in-part of application No. 07/659,760, filed on Feb. 22, 1991, now Pat. No. 5,210,085, which is a continuation-in-part of application No. 07/473,318, filed on Feb. 1, 1990, now Pat. No. 5,204,466.

(51) **Int. Cl.**[7] **A61K 31/506**; C07D 411/04

(52) **U.S. Cl.** **514/274**; 514/86; 544/243; 544/317

(58) **Field of Search** 514/86, 274

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Primary Examiner—Richard L. Raymond
(74) *Attorney, Agent, or Firm*—King & Spalding, LLP; Sherry & Knowles, Esq.

(57) **ABSTRACT**

A method and composition for the treatment of HIV and HBV infections in humans is disclosed that includes administering an effective amount of 2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane, a pharmaceutically acceptable derivative thereof, including a 5' or N^4 alkylated or acylated derivative, or a pharmaceutically acceptable salt thereof, in a pharmaceutically acceptable carrier.

A process for the resolution of a racemic mixture of nucleoside enantiomers is also disclosed that includes the step of exposing the racemic mixture to an enzyme that preferentially catalyzes a reaction in one of the enantiomers.

22 Claims, 6 Drawing Sheets

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FIGURE 1

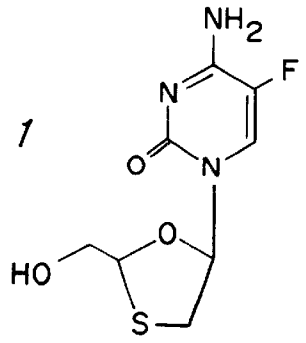
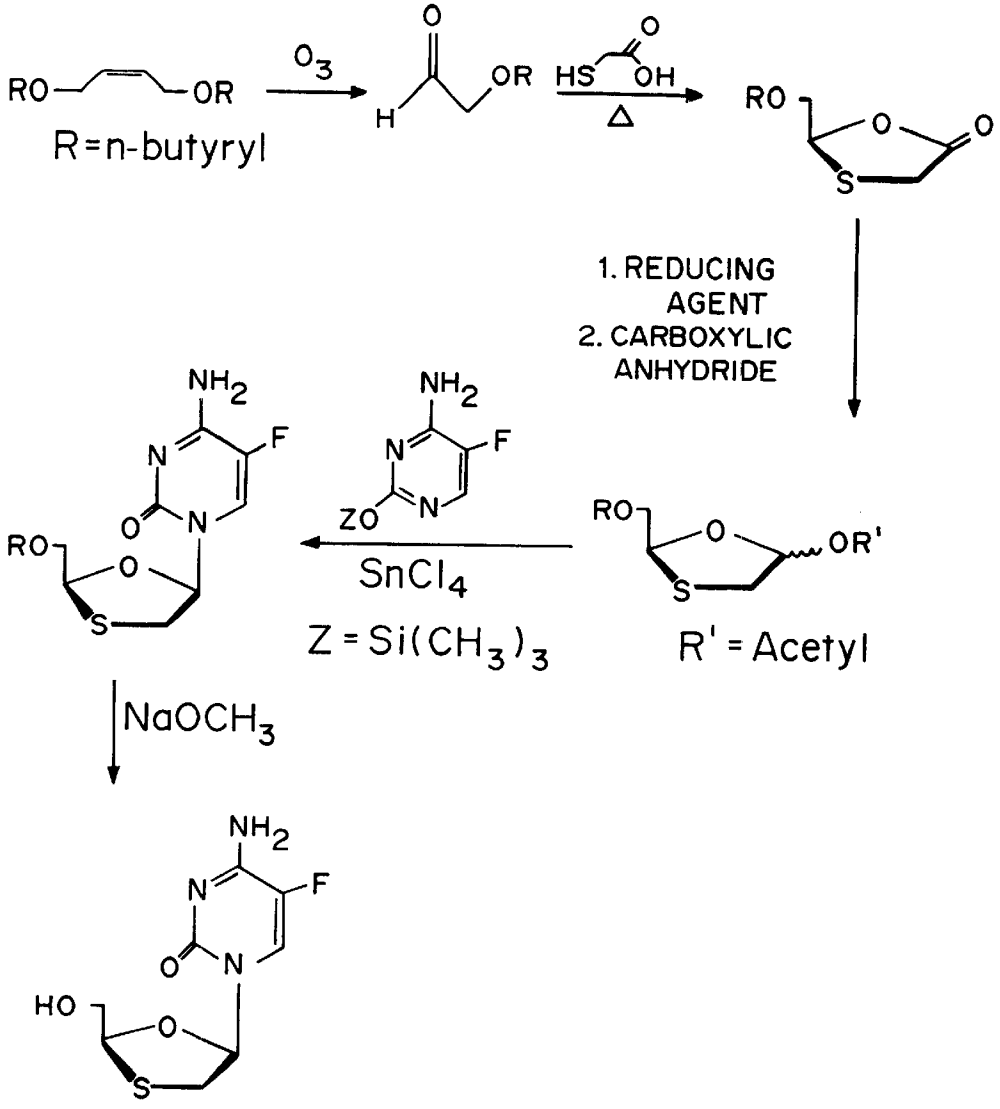


FIGURE 2



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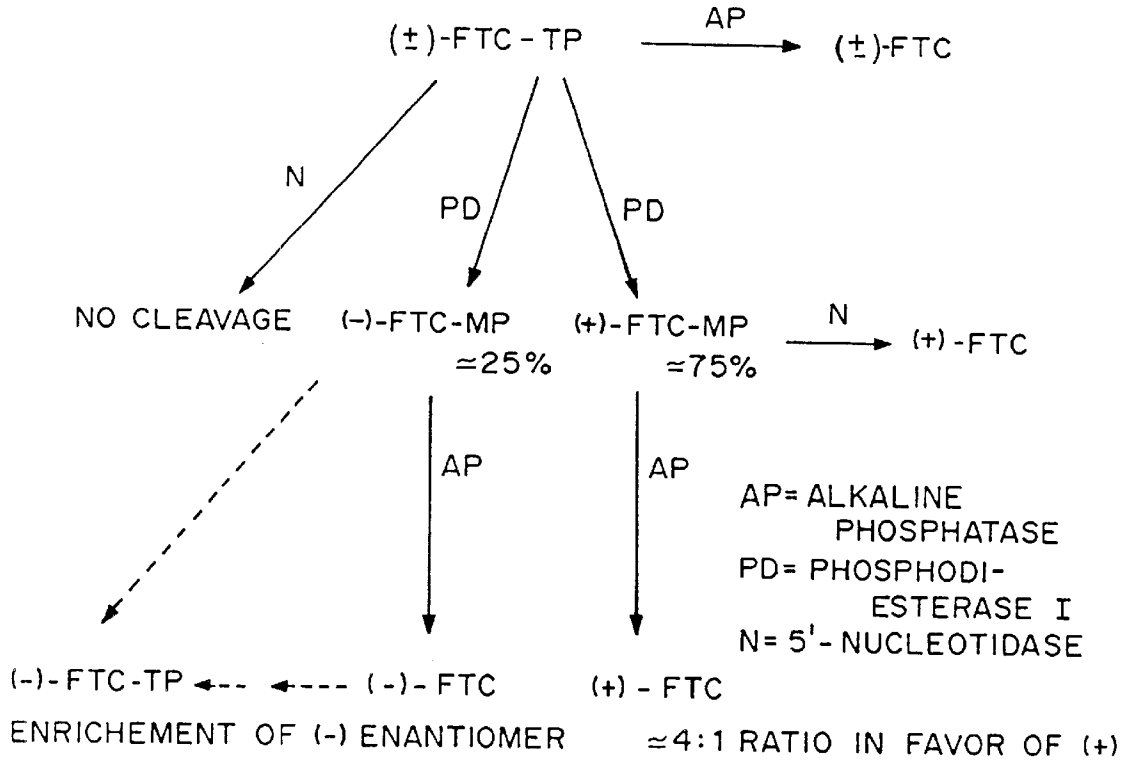

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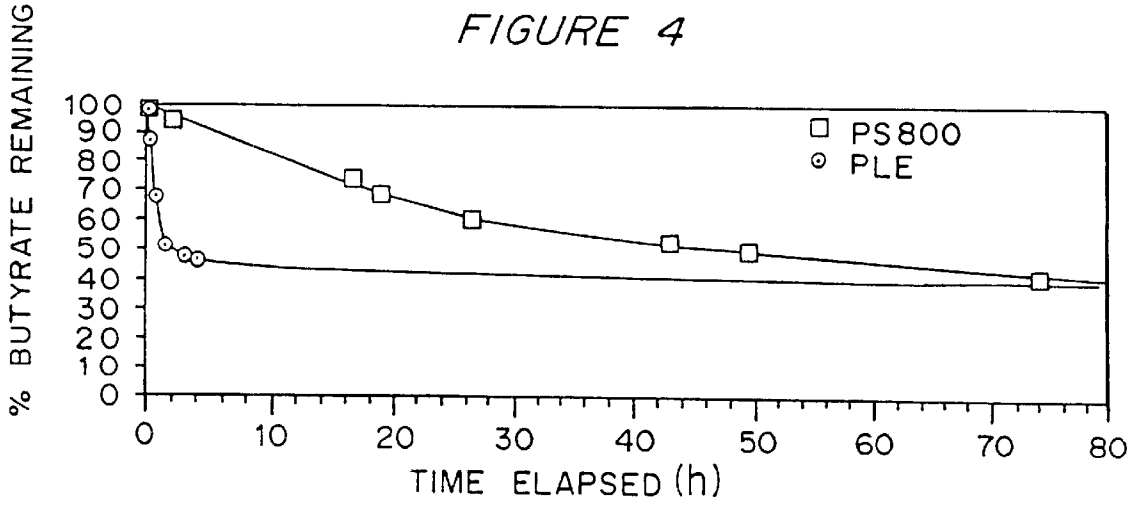

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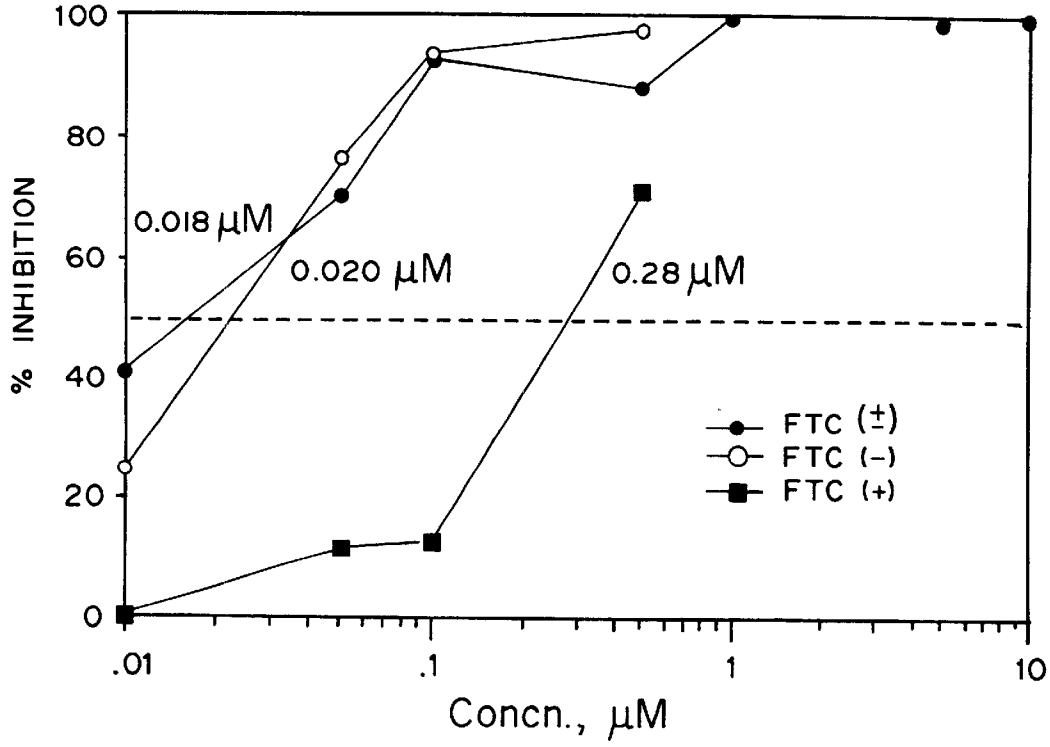

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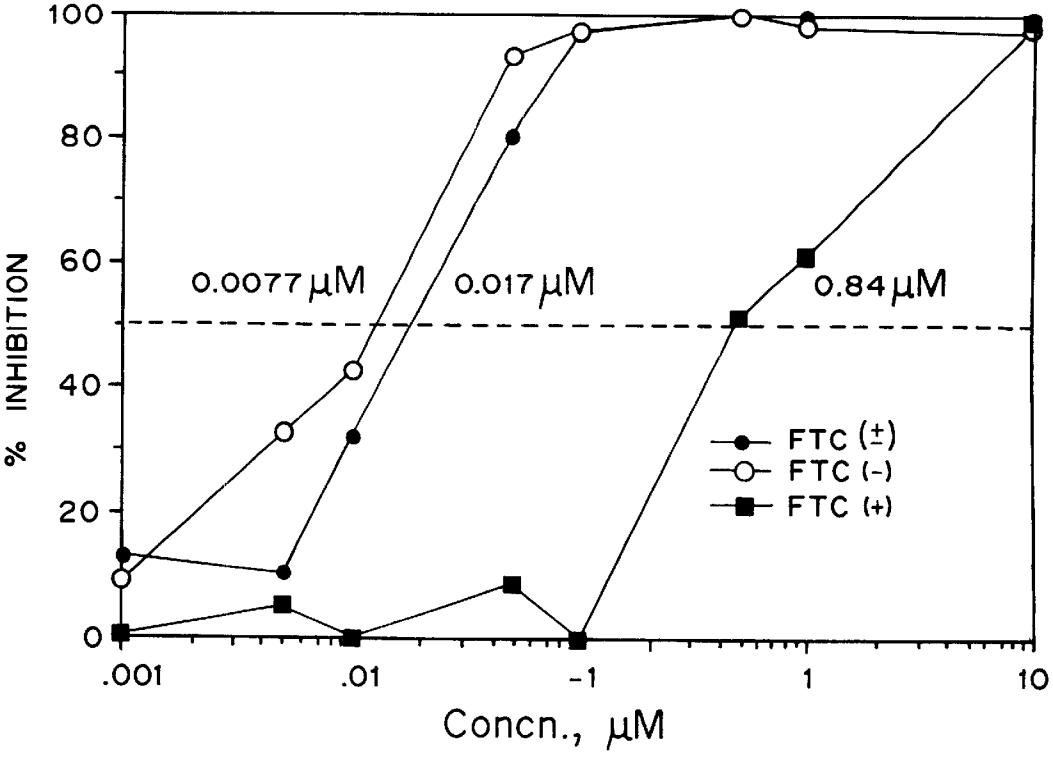

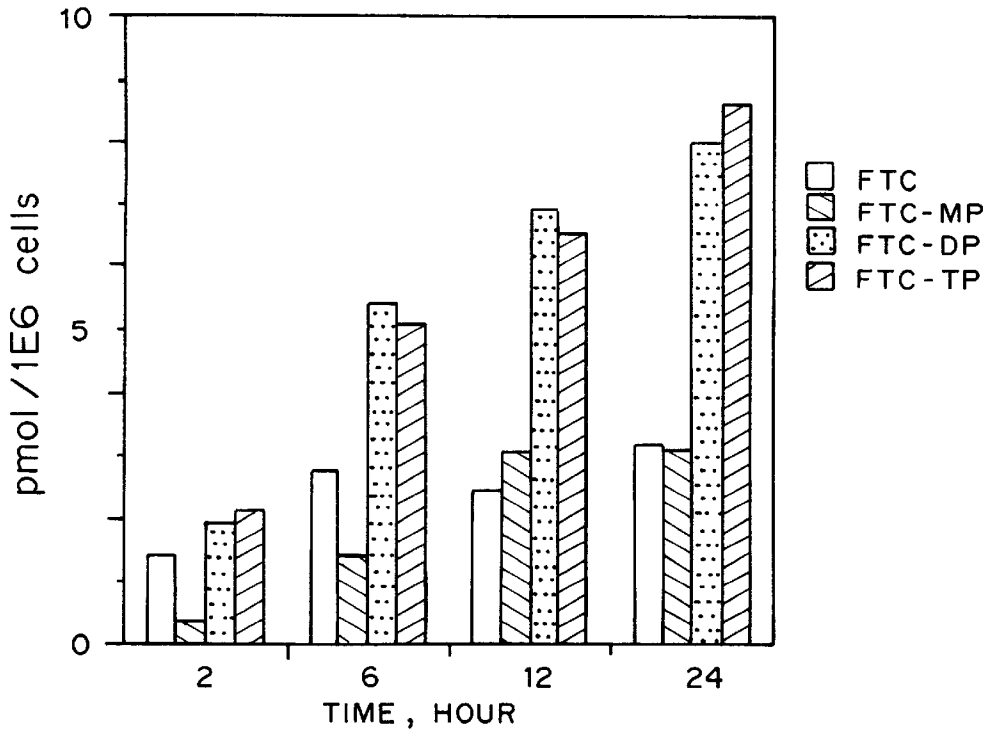

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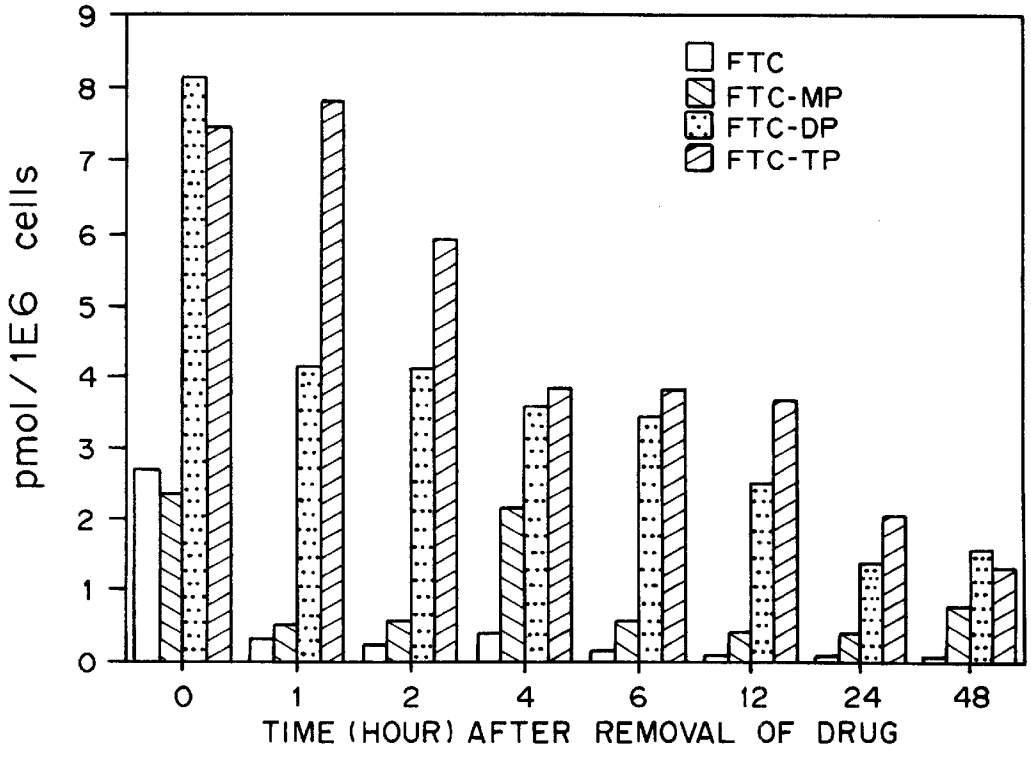

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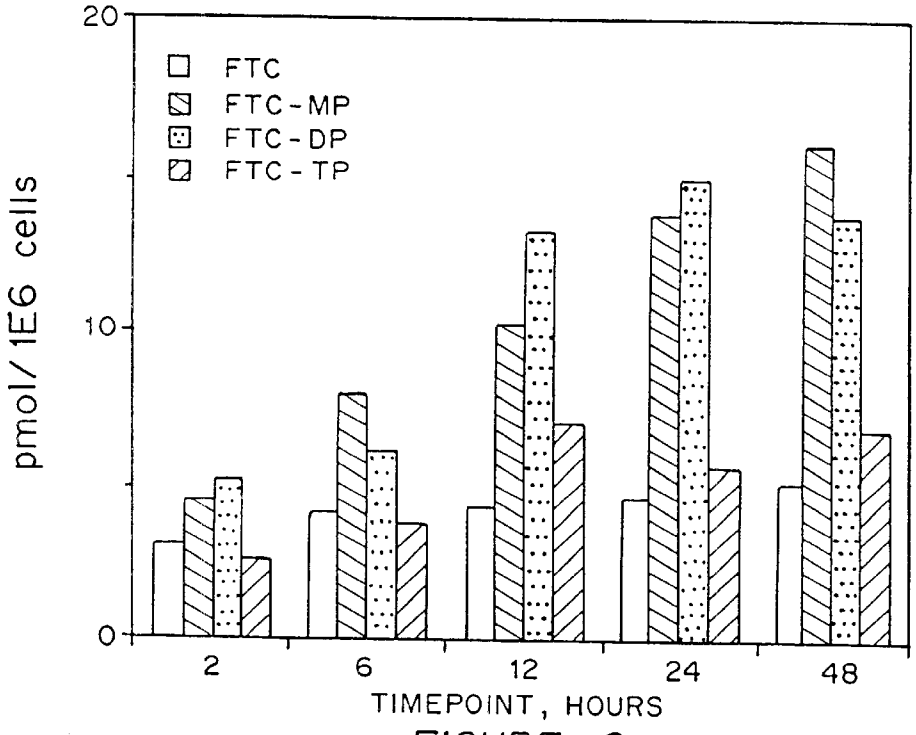

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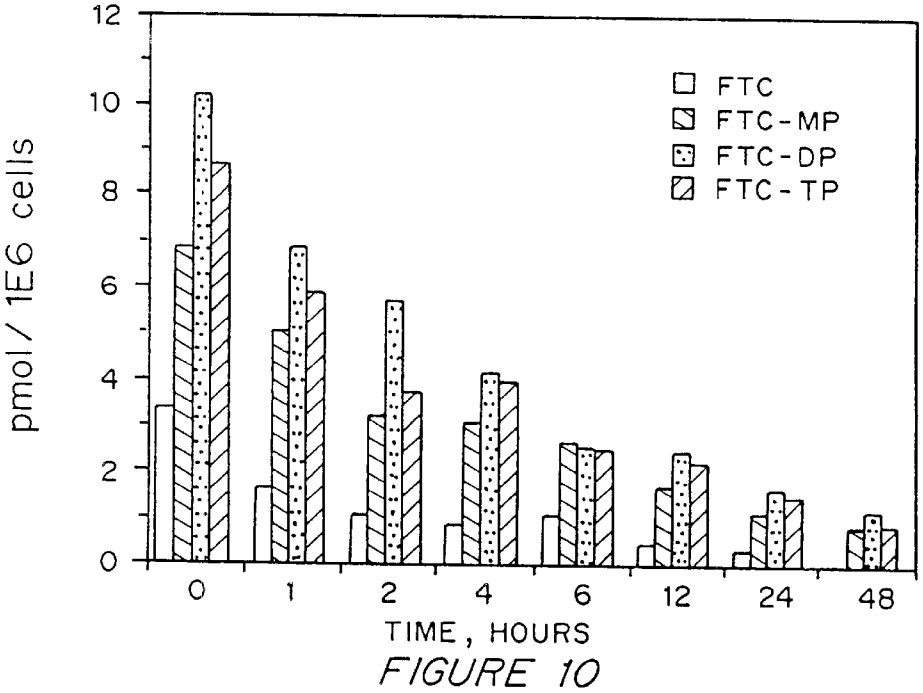


FIGURE 10

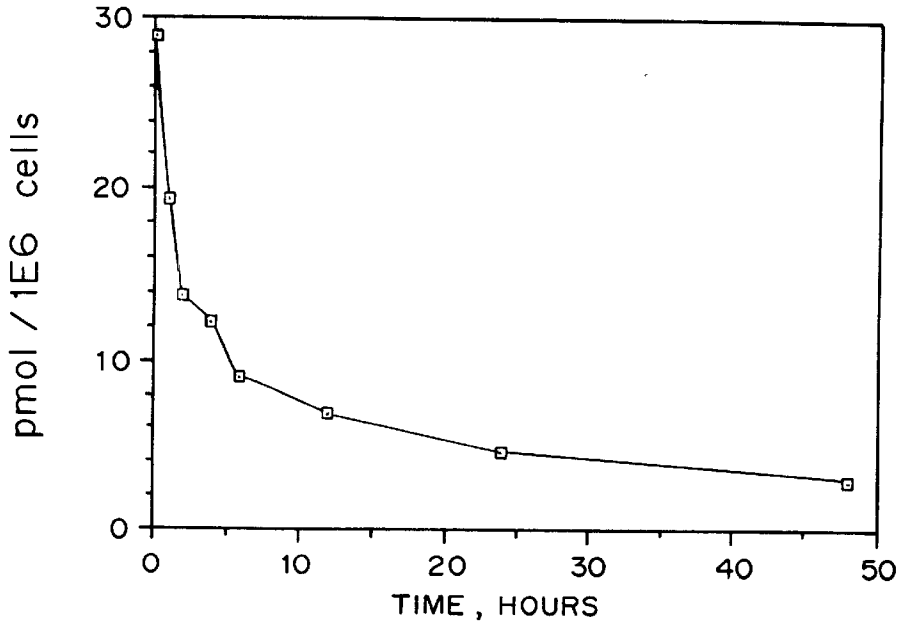


FIGURE 11

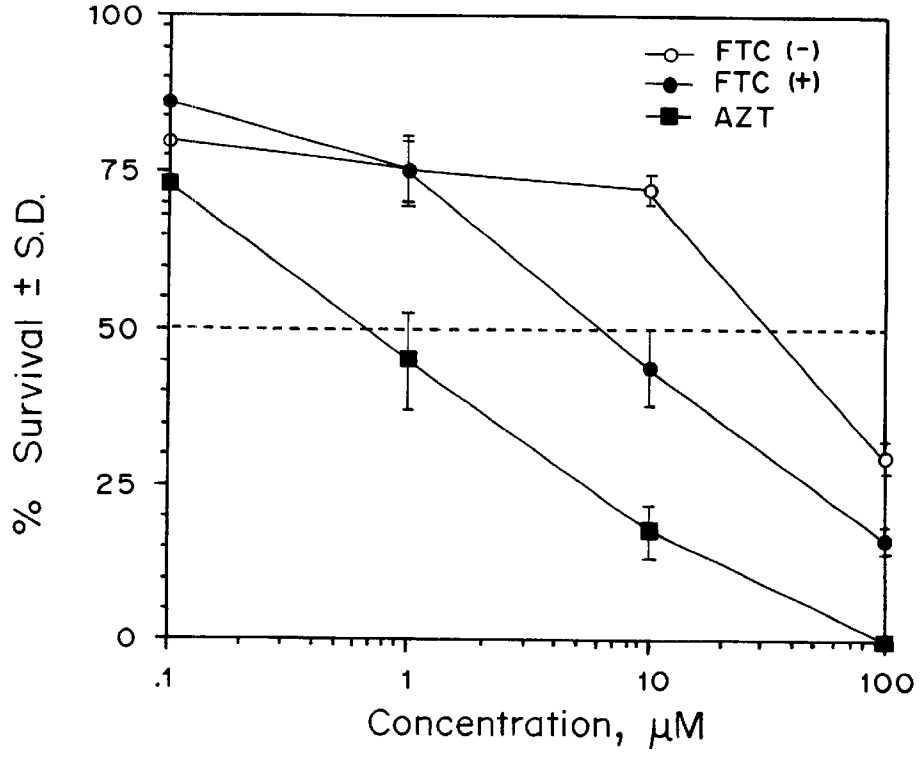


FIGURE 12

ANTIVIRAL ACTIVITY AND RESOLUTION OF 2-HYDROXYMETHYL-5-(5-FLUOROCYTOSIN-1-YL)-1,3-OXATHIOLANE

This application is a Continuation application of U.S. Ser. No. 07/831,153, filed on Feb. 12, 1992 now abandoned, by Dennis C. Liotta, Raymond F. Schinazi, and Woo-Baeg Choi for "Antiviral Activity and Resolution of 2-Hydroxymethyl-5(5-Fluorocytosin-1-yl)-1,3-Oxathiolane" which is a continuation-in-part application of (1) U.S. Ser. No. 07/659,760, now U.S. Pat. No. 5,210,085, entitled "Method for the Synthesis, Compositions and Use of 2'-Deoxy-5-Fluoro-3'-Thiacytidine and Related Compounds", filed on Feb. 22, 1991, by Dennis C. Liotta, Raymond F. Schinazi, and Woo-Baeg Choi, which is a continuation in part application of U.S. Ser. No. 07/473,318, now U.S. Pat. No. 5,204,466, entitled "Method and Compositions for the Synthesis of BCH-189 and Related Compounds", filed on Feb. 1, 1990, by Dennis C. Liotta and Woo-Baeg Choi and, (2) a continuation-in-part of U.S. Ser. No. 07/736,089, now abandoned, entitled "Method of Resolution and Antiviral Activity of 1,3-Oxathiolane Nucleoside Enantiomers" filed on Jul. 26, 1991, by Dennis C. Liotta, Raymond F. Schinazi, and Woo-Baeg Choi, which is a continuation-in-part of U.S. Ser. No. 07/659,760, now U.S. Pat. No. 5,210,085, referenced above.

The U.S. Government has rights in this invention arising out of the partial funding of work leading to this invention through the National Institutes of Health Grant Nos. AI-26055, AI-28731, NIH 5-21935, as well as a Veteran's Administration Merit Review Award.

BACKGROUND OF THE INVENTION

This invention is in the area of biologically active nucleosides, and specifically includes antiviral compositions that include 2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane ("FTC"), its physiologically acceptable derivative, or physiologically acceptable salt, and a method for the resolution and use of the (−)-β-L and (+)-β-D enantiomers of FTC.

In 1981, acquired immune deficiency syndrome (AIDS) was identified as a disease that severely compromises the human immune system, and that almost without exception leads to death. In 1983, the etiological cause of AIDS was determined to be the human immunodeficiency virus (HIV). By December of 1990, the World Health Organization estimated that between 8 and 10 million people worldwide were infected with HIV, and of that number, between 1,000,000 and 1,400,000 were in the U.S.

In 1985, it was reported that the synthetic nucleoside 3'-azido-3'-deoxythymidine (AZT) inhibits the replication of human immunodeficiency virus. Since then, a number of other synthetic nucleosides, including 2',3'-dideoxyinosine (DDI), 2',3'-dideoxycytidine (DDC), 3'-fluoro-3'-deoxythymidine (FLT), and 2',3'-dideoxy-2',3'-didehydrothymidine (D4T), have been proven to be effective against HIV. A number of other 2',3'-dideoxynucleosides have been demonstrated to inhibit the growth of a variety of viruses in vitro. It appears that, after cellular phosphorylation to the 5'-triphosphate by cellular kinases, these synthetic nucleosides are incorporated into a growing strand of viral DNA, causing chain termination due to the absence of the 3'-hydroxyl group.

The success of various 2',3'-dideoxynucleosides in inhibiting the replication of HIV in vivo or in vitro has led a number of researchers to design and test nucleosides that

substitute a heteroatom for the carbon atom at the 3'-position of the nucleoside. Norbeck, et al., disclose that (±)-1-[(2β, 4β)-2-(hydroxymethyl)-4-dioxolanyl]thymine (referred to as (±)-dioxolane-T) exhibits a modest activity against HIV (EC_{50} of 20 μm in ATH8 cells), and is not toxic to uninfected control cells at a concentration of 200 μM. *Tetrahedron Letters* 30 (46), 6246, (1989). European Patent Application Publication No. 0 337 713 and U.S. Pat. No. 5,041,449, assigned to IAF BioChem International, Inc., disclose 2-substituted-4-substituted-1,3-dioxolanes that exhibit antiviral activity.

U.S. Pat. No. 5,047,407 and European Patent Application Publication No. 0 382 526, also assigned to IAF Biochem International, Inc. disclose a number of 2-substituted-5-substituted-1,3-oxathiolane nucleosides with antiviral activity, and specifically report that the racemic mixture (about the C4'-position) of the C1'-β isomer of 2-hydroxymethyl-5-(cytosin-1-yl)-1,3-oxathiolane (referred to below as (±)-BCH-189) has approximately the same activity against HIV as AZT, and no cellular toxicity at the tested levels. (±)-BCH-189 has also been found to inhibit the replication of AZT-resistant HIV isolates in vitro from patients who have been treated with AZT for longer than 36 weeks.

Another virus that causes a serious human health problem is the hepatitis B virus (referred to below as "HBV"). HBV is second only to tobacco as a cause of human cancer. The mechanism by which HBV induces cancer is unknown, although it is postulated that it may directly trigger tumor development, or indirectly trigger tumor development through chronic inflammation, cirrhosis, and cell regeneration associated with the infection.

After a two to six month incubation period in which the host is unaware of the infection, HBV infection can lead to acute hepatitis and liver damage, that causes abdominal pain, jaundice, and elevated blood levels of certain enzymes. HBV can cause fulminant hepatitis, a rapidly progressive, often fatal form of the disease in which massive sections of the liver are destroyed.

Patients typically recover from acute hepatitis. In some patients, however, high levels of viral antigen persist in the blood for an extended, or indefinite, period, causing a chronic infection. Chronic infections can lead to chronic persistent hepatitis. Patients infected with chronic persistent HBV are most common in developing countries. By mid-1991, there were approximately 225 million chronic carriers of HBV in Asia alone, and worldwide, almost 300 million carriers. Chronic persistent hepatitis can cause fatigue, cirrhosis of the liver, and hepatocellular carcinoma, a primary liver cancer.

In western industrialized countries, high risk groups for HBV infection include those in contact with HBV carriers or their blood samples. The epidemiology of HBV is very similar to that of acquired immune deficiency syndrome, which accounts for why HBV infection is common among patients with AIDS or AIDS-related complex. However, HBV is more contagious than HIV.

A human serum-derived vaccine has been developed to immunize patients against HBV. While it has been found effective, production of the vaccine is troublesome because the supply of human serum from chronic carriers is limited, and the purification procedure is long and expensive. Further, each batch of vaccine prepared from different serum must be tested in chimpanzees to ensure safety. Vaccines have also been produced through genetic engineering. Daily treatments with a-interferon, a genetically engineered

protein, has also shown promise. However, to date there is no known pharmaceutical agent that effectively inhibits the replication of the virus.

To market a nucleoside for pharmaceutical purposes, it must not only be efficacious with low toxicity, it must also be cost effective to manufacture. An extensive amount of research and development has been directed toward new, low cost processes for large scale nucleoside production. 2',3'-Dideoxynucleosides are currently prepared by either of two routes: derivatization of an intact nucleoside or condensation of a derivatized sugar moiety with a heterocyclic base. Although there are numerous disadvantages associated with obtaining new nucleoside analogues by modifying intact nucleosides, a major advantage of this approach is that the appropriate absolute stereochemistry has already been set by nature. However, this approach cannot be used in the production of nucleosides that contain either nonnaturally occurring bases or nonnaturally occurring carbohydrate moieties (and which therefore are not prepared from intact nucleosides), such as 1,3-oxathiolane nucleosides and 1,3-dioxolane nucleosides.

When condensing a carbohydrate or carbohydrate-like moiety with a heterocyclic base-to form a synthetic nucleoside, a nucleoside is produced that has two chiral centers (at the C1'and C4'-positions), and thus exists as a diastereomeric pair. Each diastereomer exists as a set of enantiomers. Therefore, the product is a mixture of four enantiomers.

It is often found that nucleosides with nonnaturally-occurring stereochemistry in either the C1'or the C4'-positions are less active than the same nucleoside with the stereochemistry as set by nature. For example, Carter, et al., have reported that the concentration of the (−)-enantiomer of carbovir (2',3'-didehydro-2',3'-dideoxyguanosine) in cell culture required to reduce the reverse transcriptase activity by 50% (EC_{50}) is 0.8 μM, whereas the EC_{50} for the (+)-enantiomer of carbovir is greater than 60 μM. *Antimicrobial Agents and Chemotherapy*, 34:6, 1297–1300 (June 1990).

PCT International Publication No. WO 91/11186 discloses that 1,3-oxathiolane nucleosides can be prepared with high diastereoselectivity (high percentage of nucleoside with a β configuration of the bond from the C1'-carbon to the heterocyclic base) by careful selection of the Lewis acid used in the condensation process. It was discovered that condensation of a 1,3-oxathiolane nucleoside with a base occurs with almost complete β-stereospecificity when stannic chloride is used as the condensation catalyst. Other Lewis acids provide low (or no) C1'-β selectivity or simply fail to catalyze the reactions.

In light of the fact that acquired immune deficiency syndrome, AIDS-related complex, and hepatitis B virus have reached epidemic levels worldwide, and have tragic effects on the infected patient, there remains a strong need to provide new effective pharmaceutical agents to treat these diseases that have low toxicity to the host.

There is also a need to provide a cost effective, commercially viable method to produce pharmaceutically important nucleosides, and specifically attain β-stereospecificity in the C4'-position of synthetic nucleosides prepared by condensing a carbohydrate-like moiety with a base.

Therefore, it is an object of the present invention to provide a method and composition for the treatment of human patients infected with HIV.

It is another object of the present invention to provide a method and composition for the treatment of human patients or other host animals infected with HBV.

It is still another object of the present invention to provide enantiomerically enriched 1,3-oxathiolane nucleosides.

It is still another object of the present invention to provide a method for the resolution of C4'-enantiomers of 1,3-oxathiolane nucleosides.

SUMMARY OF THE INVENTION

A method and composition for the treatment of HIV and HBV infections in humans and other host animals is disclosed that includes administering an effective amount of 2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane, a pharmaceutically acceptable derivative thereof, including a 5'or N^4 alkylated or acylated derivative, or a pharmaceutically acceptable salt thereof, in a pharmaceutically acceptable carrier.

It has been discovered that 2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane ("FTC"), exhibits surprisingly high activity against human immunodeficiency virus with very low host cell toxicity. It has also been discovered that FTC exhibits very significant activity against HBV, and therefore can be used to treat patients who have a variety of illnesses associated with HBV infection.

Toxicity and pharmacokinetic studies confirm the usefulness of FTC as an antiviral agent for pharmaceutical administration. FTC and its enantiomers are nontoxic to peripheral human bone marrow cells at concentrations up to 50 μM and other cell lines at concentrations up to 200 μM. FTC-TP is a major intracellular metabolite in PBMC and HepG2 cells. FTC-TP competitively inhibits HIV-1 reverse transcriptase (RT) with a K_1, of 0.2 μM using a poly(I)oligo(dC) template-primer. Using sequencing analysis, FTC-TP can be shown to be a potent DNA chain terminator when HIV-RT is used (C-stops).

Chronic treatment with FTC is not toxic to rodents, even at oral doses of 85 mg/kg per day for at least two months. The pharmacokinetics of FTC in rhesus monkeys indicates high oral bioavailability (approximately 73±6%) and a plasma terminal half life of approximately 1.34±0.18 (mean of oral and I.V. administration).

A process for the resolution of a racemic mixture of nucleoside enantiomers, including the racemic mixture of FTC, is also disclosed that includes the step of exposing the racemic mixture to an enzyme that preferentially catalyzes a reaction in one of the enantiomers. The process can be used to resolve a wide variety of nucleosides, including pyrimidine and purine nucleosides that are optionally substituted in the carbohydrate moiety or base moiety. The process can also be used to resolve nucleoside derivatives that contain additional heteroatoms in the carbohydrate moiety, for example, (±)-FTC and (±)-BCH-189. The resolution of nucleosides can be performed on large scale at moderate cost.

Using methods described herein, FTC was resolved into its (+)-β-D and (−)-β-L enantiomers. The (−)-β-L-enantiomer appears to be more potent that the (+)-β-D-enantiomer against HIV, HBV, and SIV. The (+)-enantiomer of FTC is also active against HIV, HBV, and SIV.

BRIEF DESCRIPTION OF THE FIGURES

FIG. **1** is an illustration of the chemical structure of 2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane ("FTC").

FIG. **2** is an illustration of a method for the preparation of 2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane.

FIG. **3** is a flow chart of the specificity of alkaline phosphatase and snake venom phosphodiesterase for the (+) and (−) enantiomers of FTC.

FIG. **4** is a graph indicating the progress of lipase-catalyzed hydrolysis of the 5'-butyryl ester of FTC over time using the enzymes Amano PS-800® (-open square-) and PLE (-open circle with dot-).

FIG. **5** is a graph of the effect of concentration (μM) of racemic and enantiomerically enriched FTC (prepared by the method of Example 4) versus the percent inhibition of human PBM cells infected with HIV-1. ((-darkened circle-, (±)-FTC), (-open circle-,(−)-FTC), (-darkened square-, (+)-FTC).

FIG. **6** is a graph of the effect of concentration (μM) of racemic and enantiomerically enriched FTC (prepared by method of Example 3) on the percent inhibition of human PBM cells infected with HIV-1. ((-darkened circle-, (±)-FTC), (-open circle-,(−)-FTC), (-darkened square-, (+)-FTC).

FIG. **7** is a graph of the uptake of tritiated (±)-FTC in human PBM cells (average of two determinations) in time (hours) versus pmol/10^6 cells.

FIG. **8** is a graph of the egress of radiolabeled (±)-FTC from human PBM cells, measured in hours versus pmol/10^6 cells.

FIG. **9** illustrates the presence of [^{3}H]-(±)-FTC and its phosphorylated derivatives in human HepG-2 cells (average of two determinations) incubated in media containing 10 μM [^{3}H]-(±)-FTC, measured in pmol/10^6 cells over time.

FIG. **10** illustrates the egress of [^{3}H]-(±)-FTC and its phosphorylated derivatives in human HepG2 in pmol/10^6 cells over time cells after pulsing cells with 10 μM [^{3}H]-(±)-FTC (700 DPM/pmole) for 24 hours, and evaluating the concentration of compound 24 hours after removal.

FIG. **11** illustrates the decrease in the combined concentration of [^{3}H]-(±)-FTC and its phosphorylated derivatives from human HepG2 cells after incubation with 10 μM [^{3}H]-(±)-FTC (700 DPM/pmole) for 24 hours, in pmol/10^6 cells over time.

FIG. **12** is a graph of the effect of the enantiomers of FTC on colony formation of granulocyte-macrophage precursor cells, as measured in percent survival versus concentration in μM ((−)-FTC, open circle; (+)-FTC, darkened circle; AZT, darkened square.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the term "enantiomerically enriched nucleoside" refers to a nucleoside composition that includes at least 95% of a single enantiomer of that nucleoside.

As used herein, the term FTC refers to 2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane (the racemic form or enantiomers), also referred to as 2'-deoxy-5-fluoro-3'-thiacytidine.

As used herein, the term (±)-FTC refers to (±)-β-D,L-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1, 3-oxathiolane.

As used herein, the term (−)-FTC refers to (−)-β-L-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1, 3-oxathiolane.

As used herein, the term (+)-FTC refers to (+)-β-D-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1, 3-oxathiolane.

As used herein, the terms FTC-MP, FTC-DP, and FTC-TP refer to the monophosphate, diphosphate, and triphosphate of FTC, respectively.

As used herein, the term BCH-189 refers to 2-hydroxymethyl-5-(cytosin-1-yl)-1,3-oxathiolane.

As used herein, the term "preferential enzyme catalysis" refers to catalysis by an enzyme that favors one substrate over another.

As used herein, a leaving group means a functional group that forms an incipient carbonation when it separates from the molecule that it is attached to.

The invention as disclosed herein is a method and composition for the treatment of HIV and HBV infections, and other viruses replicating in like manner, in humans or other host animals, that includes administering an effective amount of the (±)-β-D,L, the (−)-β-Lor (+)-β-Denantiomer of 2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane, a pharmaceutically acceptable derivative, including a 5' or N^4 alkylated or acylated derivative, or a pharmaceutically acceptable salt thereof, in a pharmaceutically acceptable carrier. As shown below, the compounds of this invention either possess antiretroviral activity, such as anti-HIV-1, anti-HIV-2 and anti-simian immunodeficiency virus (anti-SIV) activity, themselves or are metabolized to a compound that exhibits antiretroviral activity.

FTC and its pharmaceutically acceptable derivatives or salts or pharmaceutically acceptable formulations containing these compounds are useful in the prevention and treatment of HIV infections and other related conditions such as AIDS-related complex (ARC), persistent generalized lymphadenopathy (PGL), AIDS-related neurological conditions, anti-HIV antibody positive and HIV-positive conditions, Kaposi's sarcoma, thrombocytopenia purpurea and opportunistic infections. In addition, these compounds or formulations can be used prophylactically to prevent or retard the progression of clinical illness in individuals who are anti-HIV antibody or HIV-antigen positive or who have been exposed to HIV.

FTC and its pharmaceutically acceptable derivatives or pharmaceutically acceptable formulations containing these compounds are also useful in the prevention and treatment of HBV infections and other related conditions such as anti-HBV antibody positive and HBV-positive conditions, chronic liver inflammation caused by HBV, cirrhosis, acute hepatitis, fulminant hepatitis, chronic persistant hepatitis, and fatigue. These compounds or formulations can also be used prophylactically to prevent or retard the progression of clinical illness in individuals who are anti-HBV antibody or HBV-antigen positive or who have been exposed to HBV.

FTC can be converted into a pharmaceutically acceptable ester by reaction with an appropriate esterifying agent, for example, an acid halide or anhydride. FTC or its pharmaceutically acceptable derivative can be converted into a pharmaceutically acceptable salt thereof in a conventional manner, for example, by treatment with an appropriate base. The ester or salt of FTC can be converted into FTC, for example, by hydrolysis.

In summary, the present invention includes the following features:

(a) (±)-β-D,L-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1, 3-oxathioiane and pharmaceutically acceptable derivatives and salts thereof;

(b) (−)-β-L-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1, 3-oxathiolane and pharmaceutically acceptable derivatives and salts thereof;

(c) (+)-βD-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1, 3-oxathiolane and pharmaceutically acceptable derivatives and salts thereof;

(d) (±)-β-D,L-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1, 3-oxathiolanei its (−) and (+) enantiomers, and pharmaceutically acceptable derivatives and salts thereof for use in medical therapy, for example for the treatment or prophylaxis of a HIV or HBV infection;

(e) use of (±)-β-D,L-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1, 3-oxathiolane, its (−) and (+)

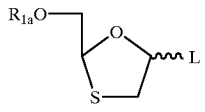
enantiomers, and pharmaceutically acceptable derivatives and salts thereof in the manufacture of a medicament for treatment of a HIV or HBV infection;

(f) pharmaceutical formulations comprising (±)-β-D,L-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1, 3-oxathiolane, its (−) or (+) enantiomer, or a pharmaceutically acceptable derivative or salt thereof together with a pharmaceutically acceptable carrier or diluent;

(g) a process for the preparation of 2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane which comprises:

(i) reacting optionally protected 5-fluorocytosine with a 1,3-oxathiolane of formula A

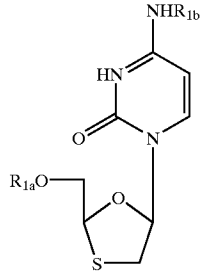
A



wherein R_{1a} is hydrogen or a hydroxyl protecting group, including an acyl group, and L is a leaving group; and optionally removing any hydroxyl protecting group.

(ii) reacting a compound of formula B

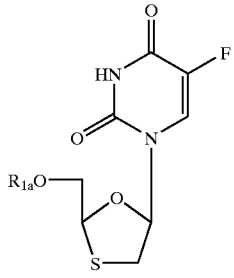
B



(wherein R_{1a} is as defined above and R_{1b} is an amino protecting group) with a fluorinating agent serving to introduce a fluorine atom in the 5-position of the cytosine ring; or

(iii) reacting a compound of formula C

C



(wherein R_{1a} is as defined above) with an agent serving to convert the oxo group in the 4-position of the uracil ring to an amino group; any remaining protecting groups being removed to produce the desired product.

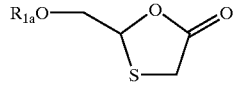
f) a process for the preparation of a (−) or (+) enantiomer of 2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1, 3-oxathiolane which comprises subjecting the compound or derivative (e.g. 5'-ester) thereof in the form of a mixture of (−) and (+) enantiomers to conditions or reacting with reagents serving to separate the enantiomers and if necessary converting the resulting derivative to the parent compound.

With regard to process e) (i), the hydroxy protecting group includes protecting groups described in detail below, including acyl (e.g. acetyl), arylacyl (e.g. benzoyl or substituted benzoyl), trityl or monomethoxytrityl, benzyl or substituted benzyl, trisubstituted silyl, including trialkylsilyl (e.g. dimethyl-t-butylsilyl) or diphenylmethylsilyl. The 5-fluorocytosine compound can be optionally protected with silyl, e.g., trisubstituted silyl groups. The protecting groups can be removed in a conventional manner. The leaving group L is a leaving group typical of those known in the art of nucleoside chemistry, e.g. halogen such as chlorine or bromine, alkoxy such as methoxy or ethoxy, or acyl such as acetyl or benzoyl.

The reaction in process e) (i) can be carried out in an organic solvent (e.g., 1,2-dichloroethane or acetonitrile) in the presence of a Lewis acid, preferably stannic chloride, or trimethylsilyl triflate.

Compounds of formula A (wherein L represents an acyl group, e.g., an acetyl group) can be obtained by reaction of a compound of formula D

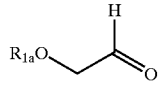
D



(wherein R_1 is defined above) with a reducing agent, e.g., a lithium aluminum hydride compound, following by treatment with the appropriate conventional reagent for the desired intermediate, for example, a carboxylic acid anhydride, e.g. acetic anhydride, for acylation, chlorinating or brominating reagents for halogenation, or alkylating reagents.

The compound of formula D can be prepared by reaction of a compound of formula E

E



with $HSCH_2CO_2H$ at an elevated temperature.

The compound of formula E can be prepared by ozonolysis of an allyl ether or ester having the formula $CH_2{=}CH{-}CH_2{-}OR$ or a diether or diester of 2-butene-1,3-diol having the formula $ROCH_2{-}CH{=}CH{-}CH_2OR$, in which R is a protecting group, such as an alkyl, silyl, or acyl group.

With regard to process e) (ii), the 5-fluoro substituent can be introduced by methods known in the art (M. J. Robins, et al., in Nucleic Acid Chemistry, Part 2, L. B. Townsend and R. S. Tipson, editors,. J. Wiley and Sons, New York, 895–900 (19/8) and references therein; R. Duschinsky in Nucleic Acid Chemistry, Part 1, L. B. Townsend and R. S. Tipson, editors, J. Wiley and Sons, New York 43–46 (1978) and references therein). The fluorinating agent may be, for example, trimethylhypofluorite in fluorotrichloromethane.

With regard to process e) iii), the compound of formula C can be treated with 1,2,4-triazole, together with 4-chlorophenyl dichlorophosphate, to form the corresponding 4-(1,2,4-triazoylyl) compound which is then converted to the desired 4-amino (cytidine) compound by reaction with for example methanol.

The starting materials of formulas B and C can be prepared for example by reaction of an appropriate

(optionally protected) base with a compound of formula A in an analogous manner to that described in process e) i). 5-Fluorouracil and 5-fluorocytosine are commercially available from Aldrich Chemical Co., Milwaukee, Wis. 53233, USA.

Resolution of the (±)-enantiomers can be accomplished as specified in detail in Section III. below.

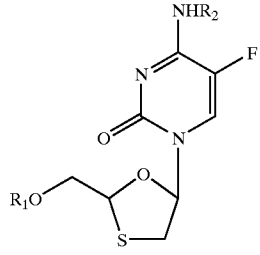
FTC can be converted into a pharmaceutically acceptable ester by reaction with an appropriate esterifying agent, for example, an acid halide or anhydride. FTC or its pharmaceutically acceptable derivative can be converted into a pharmaceutically acceptable salt thereof in a conventional manner, for example, by treatment with an appropriate base. The ester or salt of FTC can be converted into FTC, for example, by hydrolysis.

I. Active Compound, and Physiologically Acceptable Derivatives and Salts Thereof

The antivirally active compound disclosed herein is 2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane (see FIG. **1**), in the racemic form or as an isolated enantiomer.

The active compound can be administered as any derivative that upon administration to the recipient, is capable of providing directly or indirectly, the parent FTC compound, or that exhibits activity itself. Nonlimiting examples are the pharmaceutically acceptable salts (alternatively referred to as "physiologically acceptable salts"), and the 5' and N^4 acylated or alkylated derivatives of the active compound (alternatively referred to as "physiologically active derivatives"). In one embodiment, the acyl group is a carboxylic acid ester in which the non-carbonyl moiety of the ester group is selected from straight, branched, or cyclic alkyl, alkoxyalkyl including methoxymethyl, aralkyl including benzyl, aryloxyalkyl such as phenoxymethyl, aryl including phenyl optionally substituted with halogen, C_1 to C_4 alkyl or C_1 to C_4 alkoxy, sulfonate esters such as alkyl or aralkyl sulphonyl including methanesulfonyl, the mono, di or triphosphate ester, trityl or monomethoxytrityl, substituted benzyl, trialkylsilyl (e.g. dimethyl-t-butylsilyl) or diphenylmethylsilyl. Aryl groups in the esters optimally comprise a phenyl group. The alkyl group can be straight, branched, or cyclic, and is optimally a C_1 to C_{18} group.

Specific examples of pharmaceutically acceptable derivatives of FTC include, but are not limited to:



wherein R_1 and R_2 are independently selected from the group consisting of alkyl and acyl, specifically including but not limited to methyl, ethyl, propyl, butyl, pentyl, hexyl, isopropyl, isobutyl, sec-butyl, t-butyl, isopentyl, amyl, t-pentyl, 3-methylbutyryl, hydrogen succinate, 3-chlorobenzoate, cyclopentyl, cyclohexyl, benzoyl, acetyl, pivaloyl, mesylate, propionyl, butyryl, valeryl, caproic, caprylic, capric, lauric, myristic, palmitic, stearic, oleic, amino acids including but not limited to alanyl, valinyl, leucinyl, isoleucinyl, prolinyl, phenylalaninyl, tryptophanyl, methioninyl, glycinyl, serinyl, threoninyl, cysteinyl, tyrosinyl, asparaginyl, glutaminyl, aspartoyl, glutaoyl, lysinyl, argininyl, and histidinyl, and wherein one of R_1 and R_2 can be H.

FTC or its derivatives can be provided in the form of pharmaceutically acceptable salts. As used herein, the term pharmaceutically acceptable salts or complexes refers to salts or complexes of FTC that retain the desired biological activity of the parent compound and exhibit minimal, if any, undesired toxicological effects. Nonlimiting examples of such salts are (a) acid addition salts formed with inorganic acids (for example, hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid, and the like), and salts formed with organic acids such as acetic acid, oxalic acid, tartaric acid, succinic acid, malic acid, ascorbic acid, benzoic acid, tannic acid, pamoic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acids, naphthalenedisulfonic acids, and polygalacturonic acid; (b) base addition salts formed with polyvalent metal cations such as zinc, calcium, bismuth, barium, magnesium, aluminum, copper, cobalt, nickel, cadmium, sodium, potassium, and the like, or with an organic cation formed from N,N-dibenzylethylenediamine, ammonium, or ethylenediamine; or (c) combinations of (a) and (b); e.g., a zinc tannate salt or the like.

Modifications of the active compound, specifically at the N^4 and 5'-O positions, can affect the bioavailability and rate of metabolism of the active species, thus providing control over the delivery of the active species. Further, the modifications can affect the antiviral activity of the compound, in some cases increasing the activity over the parent compound. This can easily be assessed by preparing the derivative and testing its antiviral activity according to the methods described herein, or other method known to those skilled in the art.

II. Preparation of the Active Compounds

The racemic mixture of FTC can be prepared according to the method disclosed in detail in PCT International Publication No. WO 91/11186, published on Aug. 8, 1991, and filed by Emory University, or by the method disclosed in Example 1. In general, the method includes ozonizing either an allyl ether or ester having the formula CH_2═CH—CH_2—OR or a diether or diester of 2-butene-1,3-diol having the formula ROCH$_2$—CH═CH—CH$_2$OR, in which R is a protecting group, such as an alkyl, silyl, or acyl group, to form a glycoaldehyde having the formula OHC—CH_2—OR; adding thioglycolic acid to the glycoaldehyde to form a lactone of the formula 2-(R-oxy)-methyl-5-oxo-1,3-oxathiolane; reducing the lactone to various compounds containing a leaving group at the 5 position of the oxathiolane ring; coupling these compounds with silyated 5-fluorocytosine in the presence of $SnCl_4$ to form the β-isomer of FTC; and optionally removing the protecting groups.

EXAMPLE 1

Preparation of (±)-β-D,L-2-Hydroxymethyl-5-(5-fluorocytosin-1-yl)-1, 3-oxathiolane

A method for the preparation of the racemic mixture of FTC is illustrated in FIG. **2**, and described in detail below.

Protection of 2-Butene-1,4-diol

In a dry, 2L, 3-neck flask under inert atmosphere, 100 grams (93.5 ml=1.135 mol=1.00 eq.) of 2-butene-1,4-diol and 15 grams (approx. 0.1 eq.) of DMAP (4-dimethylaminopyridine) were dissolved in 800 ml of dry pyridine and stirred while cooling to 0° C. Butyryl chloride

(260 ml=2.2 eq) was then added slowly to prevent overheating and allowed to stir for one hour. The reaction was quenched with a small amount of ice water. The liquid was decanted off from the salt and evaporated in vacuo. The remaining salt was dissolved in water and the aqueous solution was extracted twice with ethyl ether. The combined other layers were washed once with saturated $CuSO_4$, twice with saturated $NaHCO_3$ containing Norit®, and then vacuum filtered through a celite® plug.

The concentrated reaction mixture was dissolved in ether and washed following the same procedure as above for the salt solution. The combined organic layers were concentrated by rotary evaporation, then placed under vacuum. This reaction is typically very close to quantitative. The scale can be easily increased as necessary. The product, 1,4-dibutyryl-2-butene-1,4-diol is a colorless to slightly yellow, clear liquid.

Ozonolysis of the Protected Diol

1,4-Dibutyryl-2-butene-1,4-diol (1.365 mol) was dissolved in 4 L of dry CH_2Cl_2 in a dry, 5 L 3-neck flask equipped with a large drying tube and an open tube for the introduction of gas. The tube is optimally not a fritted, gas bubbling tube that will clog on exposure to the concentrated solution. The solution was stirred and cooled to −78° C. while inert gas was bubbled through the solution. The gas inlet was sealed once the solution had cooled sufficiently, and the flask and stirring apparatus were moved to the ozone generator. Oxygen was bubbled through the stirring solution for at least 20 minutes while maintaining the ice bath. A Cryocool is ideal to maintain the low temperature for this lengthy reaction. The ozone was then introduced at 8 to 8.5 psi. Upon completion, the ozone flow was stopped, and oxygen was bubbled through the solution for about a half an hour before 3 equivalents of Me_2S were added. The flask was removed from the cooling bath and transported to a hood where it was stirred for about 2 days to affect complete reduction. The solution was evaporated and put under vacuum for several hours.

This reaction typically yields approximately 95% of protected aldehyde (2-butyryloxyacetaldehyde), a colorless to yellow, clear liquid.

Cyclization of the Aldehyde With Mercaptoacetic Acid

The aldehyde (1.0 equivalent) was dissolved in toluene to provide a 0.80 to 0.85M solution in a flask equipped with a Dean Stark-type trap. Thioglycolic acid (1.1 equiv.) was added and the mixture was heated to reflux. Water was azeotropically removed via the trap. The reaction was completed in 3 hours and was allowed to cool to room temperature. The organic solution was washed twice with equal volumes of sat. $NaHCO_3$ water and once with water, dried over $MgSO_4$ and Norit, and vacuum filtered through celite before being evaporated in vacuo. The first $NaHCO_3$ wash was back extracted once with ether; the ether was washed once with water, dried over $MgSO_4$ and Norit®, vacuum filtered through celite®, and evaporated along with the other organic material from the toluene solution. The combined material was placed under vacuum overnight.

The reaction typically provides a 90% yield of 2-(butyryloxy)-methyl-5-oxo-1,3-oxathiolane.

Reduction of Lactone and Conversion to the Acetate

2-Butyryloxy-methyl-5-oxo-1,3-oxathiolane (1.00 equivalent) was dissolved in dry THF to give a 0.23M solution in a dry, 3-neck flask equipped with a mechanical stirrer and maintained under an inert atmosphere. The solution was stirred and cooled to 0° C. before 1.1 equivalent of 1.0M $Li(t\text{-}BuO)_3AlH$ in THF was added via canula. The reduction was complete in approximately three hours, as indicated by TLC using 2:1 ether/hexane solvent system and an anisaldehyde stain.

Approximately 10 equivalents of freshly distilled Ac_2O were then added and allowed to stir for 2 days to provide the acetylated product. The reaction was quenched by addition of saturated $NaHCO_3$, which was stirred overnight. The solution was then evaporated and stirred with more $NaHCO_3$ solution overnight. This was extracted with ether which was washed (carefully) twice with sat. $NaHCO_3$ and once with water, dried over $MgSO_4$ and Norit®, vacuum filtered through celite®, and evaporated. The product is a dark yellow, clear liquid. Gas chromatography (Init. T - 80°; Init. time=5 min.; Prog. rate - 10°/min; Final T=240° C.) typically indicates a purity of approximately 70%.

Silylation of 5-Fluorocytosine

5-Fluorocytosine (1.05 equivalents based on amount of acetylated lactol obtained in the previous step using GC indication of purity) was silylated by reflux in at least 10 equivalents of hexamethyldisilazane containing a catalytic amount of pure ammonium sulfate (0.05 to 0.10 eq.) for two hours after the solution turned clear. The flask was then sealed tightly and the solvent removed using a vacuum pump with an auxiliary trap. The product, a white solid, was left under vacuum over night until ready for use in the following coupling reaction.

Coupling of Silylated 5-Fluorocytosine With Acetylated Lactol

To silylated 5-fluorocytosine (33.86 gm. 0.124 mol) in dry dichloromethane (350 ml) was added $SnCl_4$ solution (135.6 ml, a 1 molar solution in CH_2Cl_2) under nitrogen atmosphere. The solution was stirred for 15 minutes at room temperature. This solution was cannulated to the solution of the lactol acetate (38 gm, 0.113 mol) in dichloromethane (400 ml) under nitrogen atmosphere over a period of 30 minutes.

The reaction solution was stirred for 2 hours, at which point the completion of reaction was indicated by TLC. The reaction solution was then diluted with dichloromethane (500 ml) and quenched with ammonium hydroxide solution. The ammonium hydroxide solution (100 ml) was added slowly maintaining the temperature of reaction below 30° C., resulting in the formation of a white precipitate.

The mixture was allowed to stir for another 30 minutes, and then passed through silica gel plug column (7 inch diameter 5 inch height). It was eluted sequentially with dichloromethane (2 L), ethyl acetate (2 L) and ethyl acetate:ethanol (9:1) (4 L). The ethyl acetate and ethyl acetate:ethanol eluents contained the desired product. These solutions were combined and evaporated at reduced pressure. The residual sticky solid was then washed with dry ether (200 ml) to give a white solid (25.35 gm; 71%), FTC-5'-butyrate.

FTC-5'-butyrate (8.74 gm; 0.026 mol) was dissolved in 250 ml methanol. Sodium methoxide (2.85 gm; 0.052 gm) was added at room temperature. The reaction was stirred for 1 hour, at which point the completion of reaction was confirmed by TLC. NH_4Cl solution (10 ml) was added to quench the reaction, and then the solvent was removed under reduced pressure. The residue was absorbed on silica gel (5 gm) and passed through a small column using ethyl acetate:ethanol as an eluent (9:1). The product-containing fractions were combined and evaporated to give a sticky solid which was washed with dry ether to give white solid FTC (6.00 gm, 88%). (^1H NMR: (DMSO-d^6) 8.18 (1H, d, H_6, J=8.4 Hz), 7.81 & 7.57 (2H, broad, NH_2), 6.12 (1H, dd, $H_{1'}$, J=5.7 & 4.2 Hz), 5.40 (1H, t, OH, J=5.7 Hz), 5.17 (1H, t, $1H_{4'}$, J=3–6 Hz), 3.74 (2H, m, $2H_{5'}$), 3.41 (1H, dd, $1H_{2'}$,

J=5.7 & 11.7 Hz), 3.11 (1H, dd, $1H_{2'}$, J=4.2 & 11.7 Hz); ^{13}C NMR: (DMSO-d^6) 157.85 (d, J=13.4 Hz), 153.28, 136.12 (d, J=241 HZ), 126.01 (d, J=32.6 Hz), 86.90, 86.84, 62.48, 37.07; mp 195–196° C.

III. Resolution of Nucleoside Enantiomers

A method is provided herein for the resolution of racemic mixtures of nucleoside enantiomers, including but not limited to the (+) and (−) enantiomers of FTC. The method can also be used to resolve racemic mixtures of carbohydrates or carbohydrate-like moieties, such as derivatives of 1,3-oxathiolane and 1,3-dioxolane. The method involves the use of an enzyme that preferentially catalyzes a reaction of one enantiomer in a racemic mixture. The reacted enantiomer is separated from the unreacted enantiomer on the basis of the new difference in physical structure. Given the disclosure herein, one of skill in the art will be able to choose an enzyme that is selective for the nucleoside enantiomer of choice (or selective for the undesired enantiomer, as a method of eliminating it), by selecting one of the enzymes discussed below or by systematic evaluation of other known enzymes. Given this disclosure, one of skill in the art will also know how to modify the substrate as necessary to attain the desired resolution. Through the use of either chiral NMR shift reagents, polarimetry, or chiral HPLC, the optical enrichment of the recovered ester can be determined.

The following examples further illustrate the use of enzymes to resolve racemic mixtures of enantiomers. Other known methods of resolution of racemic mixtures can be used in combination with the method of resolution disclosed herein. All of these modifications are considered within the scope of the invention.

Resolution Based on Hydrolysis of C5'-Nucleoside Esters

In one embodiment, the method includes reacting the C5'-hydroxyl group of a mixture of nucleoside racemates with an acyl compound to form C5'-esters in which the nucleoside is in the "carbinol" end of the ester. The racemic mixture of nucleoside C5'-esters is then treated with an enzyme that preferentially cleaves, or hydrolyses, one of the enantiomers and not the other, in a given time period.

An advantage of this method is that it can be used to resolve a wide variety of nucleosides, including pyrimidine and purine nucleosides that are optionally substituted in the carbohydrate moiety or base moiety. The method can also be used to resolve nucleoside derivatives that contain additional heteroatoms in the carbohydrate moiety, for example, FTC and BCH-189. The broad applicability of this method resides in part on the fact that although the carbinol portion of the ester plays a role in the ability of an enzyme to differentiate enantiomers, the major recognition site for these enzymes is in the carboxylic acid portion of the ester. Further, one may be able to successfully extrapolate the results of one enzyme/substrate study to another, seemingly-different system, provided that the carboxylic acid portions of the two substrates are the same or substantially similar.

Another advantage of this method is that it is regioselective. Enzymes that hydrolyse esters typically do not catalyze extraneous reactions in other portions of the molecule. For example, the enzyme lipase catalyses the hydrolysis of the ester of 2-hydroxymethyl-5-oxo-1,3-oxathiolane without hydrolysing the internal lactone. This contrasts markedly with "chemical" approaches to ester hydrolysis.

Still another advantage of this method is that the separation of the unhydrolysed enantiomer and the hydrolysed enantiomer from the reaction mixture is quite simple. The unhydrolysed enantiomer is more lipophilic than the hydrolysed enantiomer and can be efficiently recovered by simple extraction with one of a wide variety of nonpolar organic solvents or solvent mixtures, including hexane and hexane/ether. The less lipophilic, more polar hydrolysed enantiomer can then be obtained by extraction with a more polar organic solvent, for example, ethyl acetate, or by lyophilization, followed by extraction with ethanol or methanol. Alcohol should be avoided during the hydrolysis because it can denature enzymes under certain conditions.

Enzymes and Substrates

With the proper matching of enzyme and substrate, conditions can be established for the isolation of either nucleoside enantiomer. The desired enantiomer can be isolated by treatment of the racemic mixture with an enzyme that hydrolyses the desired enantiomer (followed by extraction of the polar hydrolysate with a polar solvent) or by treatment with an enzyme that hydrolyses the undesired enantiomer (followed by removal of the undesired enantiomer with a nonpolar solvent).

Enzymes that catalyze the hydrolysis of esters include esterases, for example pig liver esterase, lipases, including porcine pancreatic lipase and Amano PS-800 lipase, substillisin, and α-chymotrypsin.

FIG. **3** is a flow chart of the specificity of alkaline phosphatase and snake venom phosphodiesterase for the (+) and (−) enantiomers of FTC. As indicated, alkaline phosphatase hydrolyses the triphosphate of both of the enantiomers to FTC, and therefore is not effective as a separation means. Phosphodiesterase I preferentially hydrolyses the (+)-isomer of FTC to its monoester, which can then be exposed to 5'-nucleotidase to provide (+)-FTC.

The most effective acyl group to be used to esterify the C5'-position of the nucleoside can be determined without undue experimentation by evaluation of a number of homologs using the selected enzyme system. For example, when 1, 3-oxathiolane nucleosides are esterified with butyric acid, resolutions with both pig liver esterase and Amano PS-800 proceed with high enantioselectivity (94–100% enantiomeric excess) and opposite selectivity. Pig liver esterase preferentially hydrolyses the (+)-enantiomer of FTC, and Amano PS-800® preferentially hydrolyses the (−)-enantiomer of FTC. The percent enantiomeric excess reported in Table 1 is the amount of purified butyrate ester remaining in the enzyme treated mixture (i.e., the butyrate ester of (−)-FTC in the case of PLE and the butyrate ester of (+)-FTC in the case of Amano PS-800®).

Non-limiting examples of acyl groups that can be evaluated for use with a particular nucleoside enantiomeric mixture and particular enzyme include alkyl carboxylic acids and substituted alkyl carboxylic acids, including acetic acid, propionic acid, butyric acid, and pentanoic acid. With certain enzymes, it may be preferred to use an acyl compound that is significantly electron-withdrawing to facilitate hydrolysis by weakening the ester bond. Examples of electron-withdrawing acyl groups include α-haloesters such as 2-chloropropionic acid, 2-chlorobutyric acid, and 2-chloropentanoic acid. α-Haloesters are excellent substrates for lipases.

Resolution Conditions

The enzymatic hydrolyses are typically carried out with a catalytic amount of the enzyme in an aqueous buffer that has a pH that is close to the optimum pH for the enzyme in question. As the reaction proceeds, the pH drops as a result of liberated carboxylic acid. Aqueous base should be added to maintain the pH near the optimum value for the enzyme.

The progress of the reaction can be easily determined by monitoring the change in pH and the amount of base needed to maintain pH. The hydrophobic ester (the unhydrolysed enantiomer) and the more polar alcohol (the hydrolysed enantiomer) can be sequentially and selectively extracted from the solution by the judicious choice of organic solvents. Alternatively, the material to be resolved can be passed through a column that contains the enzyme immobilized on a solid support.

Enzymatic hydrolyses performed under heterogeneous conditions can suffer from poor reproducibility. Therefore, it is preferred that the hydrolysis be performed under homogeneous conditions. Alcohol solvents are not preferred because they can denature the enzymes. Homogeneity can be achieved through the use of non-ionic surfactants, such as Triton X-100. However, addition of these surfactants not only assists in dissolving the starting material, they also enhance the aqueous solubility of the product. Therefore, although the enzymatic reaction can proceed more effectively with the addition of a non-ionic surfactant than under heterogeneous conditions, the isolation of both the recovered starting material and the product can be made more difficult. The product can be isolated with appropriate chromatographic and chemical (e.g., selective salt formation) techniques. Diacylated nucleosides can be used but are often quite lipophilic and hard to dissolve in the medium used.

EXAMPLE 2

Enantioselective Lipase-Catalyzed Hydrolysis of FTC Esters

A number of 5'-O-acyl derivatives of FTC were prepared by selective O-acylation of the N-hydrochloride salt (see Table 1 and FIG. **4**) of (±)-FTC. The efficiency of the hydrolysis of the derivatives by lipases was investigated. As shown in Table 1, pig liver esterase (PLE) exhibits a high level of selectivity for the hydrolysis of the ester of the (+)-enantiomer of FTC, leaving predominately the butyrate of (−)-FTC in the HPLC-analyzed mixture. In contrast, PS-800 hydrolyses the ester of the (−)-enantiomer of FTC preferentially, leaving predominately the butyrate of the (+)-FTC in the HPLC-analyzed mixture. The rate of the hydrolysis was also found to be dependent on the nature of the acyl group; the acetyl derivative was significantly slower than the butyryl derivative. It has now been discovered that the rate of the hydrolysis of the propionic acid ester of FTC is even faster than that observed for the butyrate derivative. Percent recovery and percent of enantiomeric excess were both determined using HPLC. Although the enantioselectivity is excellent when employing PLE (typically 97% e.e. or higher), additional enrichment can be accomplished.by sequential enzymatic hydrolysis reactions in which the enantiomerically-enriched butyrate from a PLE-catalyzed hydrolysis is subjected to enzymatic hydrolysis by PS-800.

TABLE 1

Comparison of Effect of Ester on Enzyme Hydrolysis

Substrate	% Recovery	% E.E. (s.m.)
FTC Esters with PLE:		(−) −FTC
		(butyrate)
acetate	32.68	N.D.
propionate	39.87	N.D.
butyrate	48.00	98
butyrate	45.71	98.6
FTC Esters with PS800:		(+) −FTC
		butyrate
acetate	73.17	N.D.
propionate	52.67	N.D.
butyrate	58.34	N.D.
valerate	41.50	94

EXAMPLE 3

Procedure for the Preparation of (+)- and (−)-FTC via Enantioselective, Lipase-Catalyzed Hydrolysis of FTC Butyrate

The 5'-O-butyrate of (±)-FTC (0.47 mmol, 149 mg) was dissolved in 16 mL of a solution of 4:1 pH 8 buffer:CH_3CN. The clear solution was stirred and treated with 26 mg of pig liver esterase (PLE-A). The progress of the reaction was monitored by HPLC (FIG. **4**). After 20 hours (52% conversion), the reaction mixture was extracted with 2×80 mL of $CHCl_3$ and 80 mL of ethyl acetate. The organic layer extracts were combined, dried over anhydrous $MgSO_4$, filtered, and concentrated by rotary evaporation. The resulting residue was eluted on 2×1000 m pTLC plates using ethyl acetate as eluant (double elution) to give, after isolation, 53 mg (36% based on starting material) of FTC butyrate which was determined to have 98% enantiomeric excess (e.e.) by HPLC analysis. The enantiomerically-enriched butyrate was then treated with 1.6 mL of methanol followed by 0.38 mmol (20 mg) of sodium methoxide. The resulting mixture was stirred at room temperature, and the progress of the reaction was monitored by HPLC. The reaction was completed within 30 minutes. The solvent was removed by rotary evaporation to give a crude white solid (76 mg) that was eluted on a 1000 m PTLC using 5:1 ethyl acetate:ethanol. (−)-FTC was isolated as a white solid (33 mg; 82% yield). HPLC analysis of the FTC as its 5'-O-acetate derivative showed 97% e.e.; $[\alpha]$ (^{20},$_D$) −120.5° (c=0.88; abs. ethanol).

Emulsions in the work-up step can be avoided by adding $HCCl_3$ to the reaction mixture on completion (which also serves to denature the enzyme), stripping the solvents under vacuum, and then extracting with $HCCl_3$.

Similarly, 1.2 mmol (375 mg) of the 5'-O-butyrate of (±)-FTC was dissolved in 40 mL of 4:1 pH 8 buffer-CH_3CN. The clear solution was stirred and treated with 58 mg of pig liver esterase (PLE-A). The progress of the reaction was monitored by HPLC. After 90 minutes (38% conversion), the reaction mixture was added to 150 mL of $CHCl_3$. The layers were separated and the aqueous layer lyophilized to remove solvent. The white residue from the lyophilization was extracted with 3×10 mL of absolute ethanol. The extracts were filtered, combined, and concentrated in vacuo to yield 179 mg of crude oil. The crude material was eluted on a 45×30 mm column of silica gel using 3×75 mL of ethyl acetate followed by 5:1 ethyl acetate-ethanol. (+)-FTC was isolated as a white solid (109 mg; 37% based on starting butyrate). HPLC analysis of the (+)-FTC as its 5'-O-acetate derivative showed 97.4% e.e.; [a]O(^{20},$_D$) +113.4° (c=2.53; absolute ethanol)

A similar reaction was performed using 0.12 mmol (37 mg) of the 5'-O-butyrate of FTC and 7 mg of PS-800 in 4.0 mL of 4:1 pH 8 buffer:CH_3CN. The reaction was considerably slower than that with PLE-A and required 74 hours for 59% conversion. The recovered butyrate (11.4 mg; 31% of the initial amount) was found to exhibit 94% e.e. by HPLC.

Resolution of Nucleoside Enantiomers With Cytidine-Deoxycytidine Deaminase

In an alternative embodiment, cytidine-deoxycytidine deaminase is used to resolve racemic mixtures of 2-hydroxymethyl-5-(cytosin-1-yl)-1,3-oxathiolane and its derivatives, including 2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1, 3-oxathiolane. The enzyme catalyses the deamination of the cytosine moiety to a uracil. It has been discovered that one of the enantiomers of 1,3-oxathiolane nucleosides is a preferred substrate for cytidine-deoxycytidine deaminase. The enantiomer that is not converted to a uracil derivative (and therefore is still basic) is extracted from the solution with an acidic solution. Care should be taken to avoid strong acidic solutions (pH below 3.0), that may cleave the oxathiolane ring.

Cytidine-deoxycytidine deaminase can be isolated from rat liver or human liver, or expressed from recombinant sequences in a procaryotic system such as in *E. coli.*

The method of resolution of cytidine nucleoside enantiomers using cytidine-deoxycytidine deaminase can be used as the sole method of resolution or can be used in combination with other methods of resolution, including resolution by enzymatic hydrolysis of 5'-O-nucleoside esters as described above.

Combination of Enzymatic Resolution With Classical Resolution Methods

The process described above for resolving racemic mixtures of nucleoside enantiomers can be combined with other classical methods of enantiomeric resolution to increase the optical purity of the final product.

Classical methods of resolution include a variety of physical and chemical techniques. Often the simplest and most efficient technique is recrystallization, based on the principle that racemates are often more soluble than the corresponding individual enantiomers. Recrystallization can be performed at any stage, including on the acylated compounds or the final enantiomeric product. If successful, this simple approach represents a method of choice.

When recrystallization fails to provide material of acceptable optical purity, other methods can be evaluated. If the nucleoside is basic (for example, a cytidine) one can use chiral acids that form diastereomeric mixtures that may possess significantly different solubility properties. Nonlimiting examples of chiral acids include malic acid, mandelic acid, dibenzoyl tartaric acid, 3-bromocamphor-8-sulfonic acid, 10-camphorsulfonic acid, and di-p-toluoyltartaric acid. Similarly, acylation of the free hydroxyl group with a chiral acid derivative also results in the formation of diastereomeric mixtures whose physical properties may differ sufficiently to permit separation.

Small amounts of enantiomerically enriched nucleosides can be obtained or purified by passing the racemic mixture through an HPLC column that has been designed for chiral separations, including cyclodextrin bonded columns marketed by Rainin Corporation.

EXAMPLE 4

Separation of Racemic Mixtures of Nucleosides by HPLC

The resolutions of the C4'-enantiomers of (±)-FTC were performed using a chiral cyclodextrin bonded (cyclobond AC-I) column obtained from Rainin Corporation (Woburn, Mass.). The conditions were as follows: Isocratic 0.5% methanol in water; flow rate 1 ml/min., UV detection at 262 nm. HPLC grade methanol was obtained from J. T. Baker (Phillipsburg, N.J.). The racemic mixtures were injected and fractions were collected. Fractions containing each of the enantiomers were pooled, frozen, and then lyophilized. The compounds were characterized by UV spectroscopy and by their retention times on HPLC. In general, the (−)-enantiomers have lower retention times than the (+)-enantiomers (see *J. Liouid Chromatography* 7:353–376, 1984). The concentrations of the compounds were determined by UV spectroscopy, using a stock solution of known concentration (15 μM) prepared in water for biological evaluation. The retention times for the separated enantiomers are provided in Table 2.

TABLE 2

Retention Times of Enantiomers of FTC

Compound	R_f (min)
(−) −FTC	8.3
(+) −FTC	8.7

EXAMPLE 5

Alternative Methods for Separating FTC Enantiomers Using a Chiral Column

Using a Cyclobond I-Ac column (5 μm, 25 cm×4.6 mm, Rainin Corporation, Woburn, Mass., catalog no. AST-41049), with a flow rate of 0.6 ml/min of 0.5% isocratic methanol (Fisher Scientific, Inc. HPLC grade, cat no. A-452-4 in water), and UV detection at 262 nm, the FTC enantiomers exhibited retention times of 12.68 minutes ((−)-FTC) and 13.20 minutes ((+)-FTC).

Using a Chiralpak AS column (10 μm, 25 cm×4.6 mm, J. T. Baker Inc., Phillisburg, N.J., catalog no. 7406-00, serial no. 09-29-10320) with a flow rate of 0.8 ml/min of isopropyl alcohol (HPLC grade, Fisher Scientific, Inc., cat no. A-451-4) and UV detection of 262 nm, the FTC enantiomers exhibited retention times of 5.9 minutes ((−)-FTC), and 9.8 minutes ((+)-FTC)

IV. Ability of 2-Hydroxymethyl-5-(5-fluorocytosin-1-yl)-1, 3-oxathiolane ("FTC") to Inhibit the Replication of HIV

It is often desirable to screen a number of racemic mixtures of nucleosides as a preliminary step to determine which warrant further resolution into enantiomerically enriched components and further evaluation of antiviral activity. The ability of nucleosides to inhibit HIV can be measured by various experimental techniques. The technique used herein, and described in detail below, measures the inhibition of viral replication in phytohemagglutinin (PHA) stimulated human peripheral blood mononuclear (PBM) cells infected with HIV-1 (strain LAV). The amount of virus produced is determined by measuring the virus-coded reverse transcriptase enzyme. The amount of enzyme produced is proportional to the amount of virus produced. Table 3 provides the EC_{50} values (concentration of nucleo

side that inhibits the replication of the virus by 50% in PBM cells, estimated 10% error factor) and IC_{50} values (concentration of nucleoside that inhibits 50% of the growth of mitogen-stimulated uninfected human PBM cells) of a number of (±)-1,3-oxathiolane and nucleosides.

EXAMPLE 6

Anti-HIV Activity of (±)-1,3-Oxathiolans Nucleosides

A. Three-day-old phytohemagglutinin-stimulated PBM cells (10^6 cells/ml) from hepatitis B and HIV-1 seronegative healthy donors were infected with HIV-1 (strain LAV) at a concentration of about 100 times the 50% tissue culture infectious dose (TICD 50) per ml and cultured in the presence and absence of various concentrations of antiviral compounds.

B. Approximately one hour after infection, the medium, with the compound to be tested (2 times the final concentration in medium) or without compound, was added to the flasks (5 ml; final volume 10 ml). AZT was used as a positive control.

C. The cells were exposed to the virus (about 2×10^5 dpm/ml, as determined by reverse transcriptase assay) and then placed in a CO_2 incubator. HIV-1 (strain LAV) was obtained from the Center for Disease Control, Atlanta, Ga. The methods used for culturing the PBM cells, harvesting the virus and determining the reverse transcriptase activity were those described by McDougal et al. (*J. Immun. Meth.* 76, 171–183, 1985) and Spira et al. (*J. Clin. Meth.* 25, 97–99, 1987), except that fungizone was not included in the medium (see Schinazi, et al., *Antimicrob. Agents Chemother.* 32, 1784–1787 (1988); Id., 34:1061–1067 (1990)).

D. On day 6, the cells and supernatant were transferred to a 15 ml tube and centrifuged at about 900 g for 10 minutes. Five ml of supernatant were removed and the virus was concentrated by centrifugation at 40,000 rpm for 30 minutes (Beckman 70.1 Ti rotor). The solubilized virus pellet was processed for determination of the levels of reverse transcriptase. Results are expressed in dpm/ml of sampled supernatant. Virus from smaller volumes of supernatant (1 ml) can also be concentrated by centrifugation prior to solubilization and determination of reverse transcriptase levels.

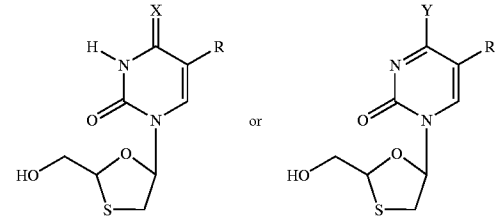
The median effective (EC_{50}) concentration was determined by the median effect method (*Antimicrob. Agents Chemother.* 30, 491–498 (1986). Briefly, the percent inhibition of virus, as determined from measurements of reverse transcriptase, is plotted versus the micromolar concentration of compound. The EC_{50} is the concentration of compound at which there is a 50% inhibition of viral growth.

E. Mitogen stimulated uninfected human PBM cells (3.8×10^5 cells/ml) were cultured in the presence and absence of drug under similar conditions as those used for the antiviral assay described above. The cells were counted after 6 days using a hemacytometer and the trypan blue exclusion method, as described by Schinazi et al., *Antimicrobial Agents and Chemotherapy*, 22(3), 499 (1982). The IC_{50} is the concentration of compound which inhibits 50% of normal cell growth.

TABLE 3

EC_{50} and IC_{50} of Various Analogues of 1,3-Oxathiolane Nucleosides in Human PBM Cells

			Antiviral Cytotoxicity	
Code	X or Y	R	EC_{50}, μM	IC_{50}, μM
DLS-009	X = O	H	>100	>100
DLS-010	X = O	Me	64.4	>100
DLS-027	X = O	F	>100	>100
DLS-028	X = O	Cl	60.8	>100
DLS-044	X = O	Br	>100	>100
DLS-029	X = O	I	>100	>100
DLS-020	Y = NH_2	H	0.02	>100
DLS-011	Y = NH_2	Me	>10	>100
DLS-022	Y = NH_2	F	0.01	>100
DLS-023	Y = NH_2	Cl	38.7	>100
DLS-021	Y = NH_2	Br	77.4	>100
DLS-026	Y = NH_2	I	0.72	>100
DLS-058(−)	Y = NH_2	F	0.008	>100
DLS-059(+)	Y = NH_2	F	0.84	>100
DLS-053	Y = NH_2	CF_3	60.7	>100



As indicated in Table 3, in general, the substituted cytosine 1,3-oxathiolane nucleosides are more active than the corresponding uracil nucleosides. The error in EC_{50} and IC_{50} measurements are estimated at ±10%.

One of the compounds, (±)-FTC, (referred to as "DLS-022", compound 8) not only exhibits exceptional activity (approximately 10 nM in PBM cells), but also quite low toxicity (>100 μM in PBM, Vero and CEM cells). Further, the (−)-enantiomer of FTC (DLS-058), exhibits significantly greater activity than the racemic mixture.

The IC_{50} of (±)-FTC was over 100 μM, indicating that the compound was not toxic in uninfected PBM cells evaluated up to 100 μM.

EXAMPLE 7

Antiviral Activity of the Enantiomers of FTC Resolved by UPLC

The enantiomers of FTC were isolated by the method of Example 4, and the antiviral activity evaluated by the method of Example 6. The results are provided in Table 4, and illustrated in FIG. **5**.

TABLE 4

Antiviral Activity of the (+) and (−) Enantiomers of FTC

Treatment	Concn., μM	DPM/ml	% Inhibition (Corrected)	EC_{50}: μM
FTC (±)	0.0001	73,755	26.6	0.018
	0.005	83,005	16.3	
	0.01	60,465	41.3	
	0.05	34,120	70.4	
	0.1	14,160	92.4	
	0.5	18,095	88.1	
	1	7,555	99.7	
	5	7,940	99.3	
	10	5,810	101.7	
FTC (−)	0.001	76,275	23.8	0.02

TABLE 4-continued

Antiviral Activity of the (+) and (−) Enantiomers of FTC

Treatment Concn., μM	DPM/ml	% Inhibition (Corrected)	EC_{50}: μM
0.005	58,590	43.3	
0.01	75,350	24.8	
0.05	28,890	76.2	
0.1	13,175	93.5	
0.5	9,485	97.6	
FTC (+) 0.001	94,340	3.8	0.28
0.005	107,430	−10.6	
0.01	99,465	−1.8	
0.05	87,120	11.8	
0.1	86,340	12.7	
0.5	33,225	71.4	

As indicated in Table 4, in this experiment the (−)-enantiomer of FTC appears to be approximately one order of magnitude more potent than the (+)-FTC enantiomer, and has approximately the same anti-HIV activity as the racemic mixture. Neither the enantiomers nor the racemic mixture is toxic up to 100 μM as measured by the Trypan Blue exclusion method in human PBM cells.

EXAMPLE 8

Antiviral Activity of FTC Enantiomers Resolved by Method of Example 3

The enantiomers of (±)-FTC were also resolved by the method of Example 3, and the antiviral activity evaluated by the method of Example 6. The results are illustrated in FIG. **6**. As indicated in FIG. **6**, the EC_{50} of the racemic mixture of FTC was 0.017 μM, the EC_{50} of (−)-FTC at 0.0077 4μM, and the EC_{50} of (+)-FTC at 0.84 μM.

EXAMPLE 9

Uptake of (±)-FTC Into Human PBX Cells

Studies were undertaken using radiolabeled FTC to follow the intracellular profiles of the parent drug and metabolites detected within the cell. All studies were conducted in duplicate. Human peripheral blood mononuclear cells (PBM cells) were suspended in RPMI 1640 medium containing 10% fetal calf serum and antibiotics (2×10^6 cells/ml), 10 ml per timepoint) and incubated with addition of 10 μM FTC (specific activity about 700 dpm/pmol). Cells were exposed to the drug for 2, 6, 12, and 24 hours. At these timepoints, the medium was removed and the cells were washed two times with cold Hank's balanced salt solution. Extraction was performed with addition of 0.2 ml of 60% cold methanol/water and stored overnight at −70° C. The following morning, the suspensions were centrifuged and extractions were repeated two times for 0.5 hours at −700° C. The total supernatants (0.6 ml) were lyophilized to dryness. The residues were resuspended in 250 μl of water and aliquots of between 50 and 100 μl were analyzed by HPLC. Quantitation of intracellular parent drug and metabolic derivatives were conducted by HPLC. Because of the potential acid lability of some compounds, a buffer system close to physiological pH was used for the separation of the metabolites.

FIG. **7** is a graph of the presence (uptake) of tritiated (±)-FTC in human PBM cells (average of two determinations) in time (hours) versus pmol/10^6 cells. The uptake studies indicate that radiolabeled FTC is readily taken up in human lymphocytes, that produce very large amounts of the 5'-triphosphate derivative of FTC.

EXAMPLE 10

Antiretroviral Activity of PTC in Various Cell Lines

The antiretroviral activity of FTC was measured in a number of cell lines using procedures similar, but not identical, to that set out in Example 6. Cell lines were obtained from either human donors, AIDS Research and Reference Reagent Program, NIH, Rockville, Md., ATCC, or the Red Cross. The CEM thymidine kinase deficient cells were prepared by sequential passage of CEM cells in the presence of 5-bromo-2'-deoxyuridine. The results are provided in Table 5.

TABLE 5

Antiretroviral Activity cf FTC In Different Cell Systems

Cell system (Virus strain)	EC_{50} (μM) (+) −FTC
HIV-1	
PBMC (LAV-1)	0.027
MT2 ($HTLV_{IIIB}$)	0.89
CEM (LAV-1)	0.08
CEM-$TK^{(-)}$ (LAV-1)	0.026
CEM ($HTLV_{IIIB}$) NIH	0.09
HIV-2	
PBMC (ROD2)	0.0038 (±) −FTC
	0.0007 (−) −FTC
	0.026 (+) −FTC
SIV	
AA-2 (SIV251)	4.6
C-8166 (SIV251)	<8.0
FIV	
CrFK (61E)	≦1

EXAMPLE 11

Egress of (±)-FTC, from Human PBM Cells

Studies were performed using radiolabeled FTC to follow the intracellular profiles of the parent drug and metabolites detected within the cell after incubation in media with drug for 24 hours, and then removal of drug. This study measures the time needed for intracellular levels of triphosphates to decline. Studies were conducted in duplicate. Uninfected cells (2×10^6 ml) were suspended in the appropriate medium supplemented with serum (10 ml per timepoint) and incubated at 37° C. in a 5% CO_2 incubator. The radiolabeled FTC concentration was 10 μM. After pulsing the cells with the labeled compound for 24 hours, the cells were thoroughly washed and then replenished with fresh medium without the antiviral drugs (0 hr). At 0, 2, 4, 6, 12, 24, and 48 hours (second incubation time), the cells were removed, and immediately extracted with 60% cold methanol/water. The extract was obtained by centrifugation and removal of the cell pellet. The extracts were lyophilized and then stored at −70° C. Prior to analysis, the material was resuspended in 250 microliters of HPLC buffer and immediately analyzed. Quantitation of intracellular parent drug and metabolic derivatives was conducted by HPLC, using either a Micromeritics or Hewlett-Packard model 1090 PHLC system with an anion exchange Partisil 10 SAX column (Whatman, Inc.), at a flow rate of 1 ml/min, 1 kpsi pressure, with UV detection at 262 nm. The mobile phase consisted of

deionized water (A), 2 mM NaH_2PO_4/16 mM NaOAc (pH=6.6) (B), 15 mM NaH_2PO_4/120.2 mM NaOAc (pH=6.6) (C), and 100 mM NaH_2PO_4/800 mM NaOAc (pH=6.6) (D).

Separation method: isocratic for 5 minutes with A, followed by a 15 minute linear gradient to 100% B, followed by a 20 minute linear gradient to 100% C, followed by 10 minute linear gradient to 100% D, followed by 30 minutes isocratic with 100% D.

Retention times (minutes) in Human Cells:

Compound	Unchanged	Mono-phosphate	Diphosphate	Triphosphate
(±) –FTC	5.0	39.0	55.0	68.0

FIG. **8** is a graph of the egress of radiolabeled (±)-FTC from human PBM cells, measured in hours after drug removal versus concentration (pmol/10^6 cells). As indicated in the FIG., FTC-triphosphate has an intracellular half-life of approximately 12 hours and can be easily detected intracellularly at concentrations of 1–5 μM 48 hours after the removal of the extracellular drug, which is well above the EC_{50} for the compound. Further, the affinity (K^I) for (±)-FTC triphosphate using HIV RT is 0.2 μM, which is below the 48 hour concentration level.

EXAMPLE 12

Anti-HIV Activity of Pharmaceutically Acceptable Derivatives of (±)-PTC

a. A number of pharmaceutically acceptable derivatives of (±)-FTC prepared by derivatizing the 5'and N^4 positions were evaluated for anti-HIV activity in PBM cells using a procedure similar to that described in Example 6. The results are as follows. The 5'-O-butyrate ester of (±)-FTC exhibited an EC_{50} of 0.0017. The N^4-acetyl derivative of (±)-FTC exhibited an EC_{50} of 0.0028. The 5'-O-butyrate, N^4-ester of (±)-FTC exhibited an EC_{50} =0.0058.

b. The anti-HIV activity of the 5'-O-butyrate ester of (±)-FTC in the MT4 system (EC_{50}) was 0.04 μM. In the same assay, the unacylated (±)-FTC exhibited an IC50 of 0.52 μM. The IC50 for AZT in this system was 0.09 μM.

V. Ability of FTC to Inhibit the Replication of HBV

EXAMPLE 13

Evaluation of Activity of (+) and (−)-Enantiomers of FTC in 2.2.15 Cell Cultures

The ability of the enantiomers of FTC to inhibit the growth of virus in 2.2.15 cell cultures (HepG2 cells transformed with hepatitis virion) is described in detail below.

A summary and description of the assay for antiviral effects in this culture system and the analysis of HBV DNA has been described (Korba and Milman, 1991, *Antiviral Res.*, 15:217). The antiviral evaluations were performed on two separate passages of cells. All wells, in all plates, were seeded at the same density and at the same time.

Assay Parameters

Due to the inherent variations in the levels of both intracellular and extracellular HBV DNA, only depressions greater than 3.5-fold (for HBV virion DNA) or 3.0-fold (for HBV DNA replication intermediates) from the average levels for these HBV DNA forms in untreated cells are considered to be statistically significant [P<0.05]. The levels of integrated HBV DNA in each cellular DNA preparation (which remain constant on a per cell basis in these experiments) were used to calculate the levels of intracellular HBV DNA forms, thereby ensuring that equal amounts of cellular DNA were compared between separate samples.

Typical values for extracellular HBV virion DNA in untreated cells ranged from 50 to 150 pg/ml culture medium (average of approximately 76 pg/ml). Intracellular HBV DNA replication intermediates in untreated cells ranged from 50 to 100 pg/μg cell DNA (average approximately 74 pg/μg cell DNA). In general, depressions in the levels of intracellular HBV DNA due to treatment with antiviral compounds are less pronounced, and occur more slowly, than depressions in the levels of HBV virion DNA (Korba and Milman, 1991, *Antiviral Res.*, 15:217).

The manner in which the hybridization analyses were performed for these experiments resulted in an equivalence of approximately 1.0 pg of intracellular HBV DNA to 2–3 genomic copies per cell and 1.0 pg/ml of extracellular HBV DNA to 3×10^5 viral particles/ml.

Toxicity Analysis

Toxicity analyses were performed to assess whether any observed antiviral effects were due to a general effect on cell viability. The method used herein was the measurement of the uptake of neutral red dye, a standard and widely used assay for cell viability in a variety of virus-host systems, including HSV and HIV. Toxicity analyses were performed in 96-well flat bottomed tissue culture plates. Cells for the toxicity analyses were cultured and treated with test compounds with the same schedule as described for the antiviral evaluations below. Each compound was tested at 4 concentrations, each in triplicate cultures (wells "A", "B", and "C"). Uptake of neutral red dye was used to determine the relative level of toxicity. The absorbance of internalized dye at 510 nm (A_{sin}) was used for the quantitative analysis. Values are presented as a percentage of the average A_{sin} values in 9 separate cultures of untreated cells maintained on the same 96-well plate as the test compounds. Dye uptake in the 9 control cultures on plate 5 ranged from 91.6% to 110.4%, and on plate 6 from 96.6% to 109%. The results are provided in Table 6.

TABLE 6

Toxicity Analysis of Test Compounds in 2.2.15 Cells

		CONC.	DYE UPTAKE (% OF CONTROL)		
PLATE	COMPOUND	(μM)	WELL A	WELL B	WELL C
5	DMSO	10.0*	0.7	1.6	0.9
		3.3	55.9	68.7	61.7
		1.0	91.2	96.4	106.8
		0.3	98.7	102.9	93.5
6	(−) –FTC	300	53.0	51.1	51.5
		100	64.1	66.6	77.6
		30	98.7	94.3	96.4
		10	94.3	94.9	92.2
6	(+) –FTC	300	43.4	56.7	58.5
		100	77.7	66.3	72.1
		30	81.1	88.3	88.1
		10	90.9	99.4	90.5

*For DMSO, concentrations are presented as percent of original stock solution.

Toxicity Evaluation

As indicated in Table 6, no significant toxicity (greater than 50% depression of the dye uptake levels observed in untreated cells) was observed for the test compounds at the concentrations used for the antiviral evaluations. Both test compounds, (−)-FTC and (+)-FTC, appeared to be toxic at the highest concentration used for the toxicity tests (330 μM).

Antiviral Evaluations

Controls

Within normal variations, levels of HBV virion DNA and intracellular HBV replication intermediates [HBV RI] remained constant in the untreated cells over the challenge period. DMSO, at a concentration of 1%, did not affect the levels of HBV replication in 2.2.15 cell cultures.

Test Compounds

As indicated in Table 7, both (−)-FTC and (+)-FTC significantly inhibited the replication of HBV at the tested levels. As indicated in Table 8, (−)-FTC still significantly inhibits the synthesis of HBV virion DNA and intracellular HBV DNA at concentrations of 4, 1, and 0.25 μM.

TABLE 7

Effect of Test Compounds on HBV Production In 2.2.15 Cell Cultures

		HBV Virion DNA* (pg/ml Culture Medium)			Intracellular HBV DNA (pg/ug Cell DNA)	
WELL	TREATMENT	DAY 0	DAY 4	DAY 9	MONO.	RI
7A	Untreated Cells	59	75	94	2.7	93
7B	Untreated Cells	47	64	88	2.5	93
8A	Untreated Cells	65	100	71	2.2	97
8B	Untreated Cells	77	65	110	2.4	62
7K	DMSO @ 1.00%	100	50	48	1.9	95
7L	DMSO @ 1.00%	48	96	54	2.8	98
8K	DMSO @ 1.00%	93	63	68	2.2	86
8L	DMSO @ 1.00%	66	57	59	1.6	97
9U	(−) −FTC @ 10 μM	120	36	1	1.1	14
9V	(−) −FTC 10 μM	89	48	1	1.5	19
10U	(−) −FTC 10 μM	58	41	0.1	1.9	13
10V	(−) −FTC 10 μM	110	32	0.1	1.2	16
9W	(+) −FTC @ 10 μM	88	42	0.1	0.8	14
9X	(+) −FTC 10 μM	58	57	0.2	0.4	19
10W	(+) −FTC 10 μM	69	55	0.1	0.7	17
10X	(+) −FTC 10 μM	45	39	0.1	0.4	15

*Sensitivity cutoff for HBV virion DNA was 0.1 pg/ml.

@ Intracellular HBV DNA was analyzed 24 hours following the 9th day of treatment. The levels of integrated HBV DNA in each cell DNA preparation were used to calculate the levels of episomal 3.2 Kb HBV genomes (MONO.) and HBV DNA replication intermediates (RI).

TABLE 8

Effect of Test Compounds on KBV Production in 2.2.15 Cell Cultures

		HBV VIRION DNA* (pg/ml CULTURE MEDIUM)			INTRACELLULAR HBV DNA* (pg/μg CELL DNA)	
WELL	TREATMENT	DAY 0	DAY 4	DAY 9	MONO.	RI
31A	untreated cells	64	54	65	2.8	65
31B	"	51	54	77	2.0	53
32A	"	100	76	56	3.5	81
32B	"	53	97	83	3.1	68
35A	(−) −FTC @ 4 μM	74	27	>0.1	1.4	1
35B	"	87	28	>0.1	0.5	1
36A	"	120	20	1	0.9	1
36B	"	59	16	0.2	0.2	2
35C	(−) −FTC @ 1 μM	70	13	>0.1	1.7	2
35D	"	62	15	>0.1	1.2	3
36C	"	60	22	1	1.4	2
36D	"	89	28	0.3	1.5	4
35E	(−) −FTC @ 0.25 μM	84	15	>0.1	1.5	4
35F	"	89	16	4	2.2	4
36E	"	66	13	1	1.8	8
36F	"	49	19	0.1	0.3	9

*Sensitivity cutoff for HBV virion DNA was 0.1 pg/ml.

+ Analysis of intracellular HBV DNA was 24 hours following the 9th day of treatment. The levels of integrated HBV DNA in each cell DNA preparation were used to calculate the levels of episomal 3.2 kb HBV genomes (MONO.) and HBV DNA replication intermediates (RI).

EXAMPLE 14

Uptake of (±)-FTC into Human Liver Cells; HVB Activity of FTC

The procedure of Example 9 was repeated with human liver cells (HepG2 cells, available from the ATCC) to determine the uptake and metabolism of FTC in these cells. As shown in FIG. **9**, (±)-FTC is taken up by HepG2 cells in large amounts. These human liver cells metabolize a large percentage of the (±)-FTC to (±)-FTC triphosphate.

This data, in conjunction with other data provided herein, indicate that (±)-FTC, as well as its (−) and (+) enantiomers, are phosphorylated in liver cells. These cells can be transformed with hepatitis B virus.

EXAMPLE 15

Egress of FTC in Human HepG2 Cells

FIG. **10** illustrates the egress of [^{3}H]-(±)-FTC and its phosphorylated derivatives in human HepG2 in pmol/10^6 cells over time cells after pulsing cells with 10 μM [^{3}H]-(±)-FTC (700 DPM/pmole) for 24 hours, and evaluating the concentration of compound 24 hours after removal.

FIG. **11** illustrates the decrease in the combined concentration of [^{3}H]-(±)-FTC and its phosphorylated derivatives from human HepG2 cells after incubation with 10 μM [^{3}H]-(±)-FTC (700 DPM/pmole) for 24 hours, in pmol/10^6 cells over time.

As illustrated, even at 48 hours, over 1 μM of active compound (which is significantly higher than the EC_{50} for the compound) is still present in the cells.

V. Toxicity in Granulocyte-Macrophage Precursor Cells

EXAMPLE 16

Effect of FTC on Colony Formation of Granulocyte-Macrophage Precursor Cells

FIG. **12** is a graph of the effect of the (−) and (+) enantiomers of FTC on colony formation of granulocytes-macrophage precursor cells, as measured in percent survival versus concentration in μM ((−)-FTC, open circle; (+)-FTC, darkened circle; AZT, darkened square. As indicated, the (−)-enantiomer of FTC appears to be less toxic i.e., have a higher IC_{50}, than either the (+)-enantiomer or AZT in this cell line.

VI. Pharmacokinetics of FTC

EXAMPLE 17

Metabolism of FTC on Administration to Rats

(±)-FTC was administered intravenously at dosages of 10, 50 and 100 mg/kg to rats, and the area under the plasma drug concentration versus time (AUC), total clearance (CL_T), steady-state volume of distribution (V_{SS}), mean residence time (MRT) and half-life ($t_{1/2}$), evaluated. The results are provided in Table 9.

TABLE 9

Pharmacokinetic Parameters of FTC After Intravenous Administration of 10, 50, 100 mg/kg to Rats*

Dose mg/kg	AUC mg h/L	CL_T L/h/kg	V_{SS} L/kg	MRT h	$t_{1/2}$ h
10	9.65	0.988	0.758	0.768	0.757
50	57.11	0.874	0.699	0.800	0.815
100	120.72	0.830	0.663	0.798	0.969

*AUC = area under the plasma drug concentration versus time curve;
CL = total clearance;
V_{SS} = steady-state volume of distribution;
MRT = mean residence time;
and $t_{1/2}$ = half-life.

EXAMPLE 18

Pharmacokinetic Parameters for FTC After Intravenous and Oral Administration of FTC

Model-independent pharmacokinetic parameters were derived for (±)-FTC by administration (intravenous (I.V.) and oral (P.O.)) of 33.3 mg/kg to Rhesus Monkeys. The results are provided in Table 10. Importantly, the mean bioavailability of the compound in monkeys was 73% (±6).

TABLE 10

Model-Independent Pharmacokinetic Parameters Derived for FTC After Intravenous (I.V.) or Oral (P.O.) Administration of 33.3 mg/kg to Rhesus Monkeys*

Monkey	AUC mg h/L	CL_T L/h/kg	V_{SS} L/kg	MRT h	$t_{1/2}$ h	Ka_1 h^-	F %
I.V.							
RUh	19.14	1.74	2.71	1.56	1.28		
RMi	26.31	1.26	1.97	1.56	1.22		
RJd	22.51	1.48	2.00	1.36	1.47		
Mean ±	22.65	1.49	2.23	1.49	1.32		
S.D.	3.59	0.24	0.42	0.12	0.13		
P.O.							
RUh	13.21			2.07	1.58	0.48	71
RMi	21.11			2.32	1.08	0.43	80
RJd	15.29			3.23	1.47	0.31	68
Mean ±	16.54			2.54	1.38	0.41	73.00 (±6)
S.D.	4.09			0.61	0.26	0.09	6.24

*AUC = area under the plasma drug concentration versus time curve;
CL = total clearance;
V_{SS} = steady-state volume of distribution;
MRT = mean residence time;
and $t_{1/2}$ = half-life;
F = bioavailability;
and K_a = first order absorption rate constant.

TABLE 11

CSF/Serum Ratio of FTC and Its Deaminated Metabolite 1 Hour After Treatment

Monkey	Route	FTC	Metabolite (FTU)
RUh	I.V.	0.076	0.024
RMi	I.V.	0.062	0.032
RJd	I.V.	0.162	0.052
Mean ±		0.100	0.036
S.D.		0.054	0.014
RUh	P.O.	0.048	0.026
RMi	P.O.	0.039	0.037
RJd	P.O.	0.117	0.055

TABLE 11-continued

CSF/Serum Ratio of FTC and Its Deaminated Metabolite 1 Hour After Treatment

Monkey	Route	FTC	Metabolite (FTU)
Mean ± S.D.		0.068 0.043	0.039 0.015

EXAMPLE 19

CSF/Serum Ratio of FTC and its Metabolites in Rhesus Monkeys

The ability of (±)-FTC to cross the blood-brain barrier was evaluated by administering 33.3 mg/kg of the active compound to rhesus monkeys, and measuring the amount of (±)-FTC in the cerebral spinal fluid (CSF) and blood serum one hour after administration. The results are provided in Table 11. The data indicates that a significant amount of active compound passes through the blood-brain barrier in this mammal.

III. Preparation of Pharmaceutical Compositions

Humans suffering from diseases caused by HIV or HBV infection can be treated by administering to the patient an effective amount of (±)-FTC, or its (−) or (+) enantiomer or a pharmaceutically acceptable derivative or salt thereof in the presence of a pharmaceutically acceptable carrier or diluent. The active materials can be administered by any appropriate route, for example, orally, parenterally, intravenously, intradermally, subcutaneously, or topically, in liquid or solid form.

The active compound is included in the pharmaceutically acceptable carrier or diluent in an amount sufficient to deliver to a patient a therapeutically effective amount of compound to inhibit viral replication in vivo, especially HIV and HBV replication, without causing serious toxic effects in the patient treated. By "inhibitory amount" is meant an amount of active ingredient sufficient to exert an inhibitory effect as measured by, for example, an assay such as the ones described herein.

A preferred dose of (−), (+), or (±)-FTC for all of the above-mentioned conditions will be in the range from about 1 to 50 mg/kg, preferably 1 to 20 mg/kg, of body weight per day, more generally 0.1 to about 100 mg per kilogram body weight of the recipient per day. The effective dosage range of the pharmaceutically acceptable derivatives can be calculated based on the weight of the parent nucleoside to be delivered. If the derivative exhibits activity in itself, the effective dosage can be estimated as above using the weight of the derivative, or by other means known to those skilled in the art.

The compound is conveniently administered in unit any suitable dosage form, including but not limited to one containing 7 to 3000 mg, preferably 70 to 1400 mg of active ingredient per unit dosage form. A oral dosage of 50–1000 mg is usually convenient.

Ideally the active ingredient should be administered to achieve peak plasma concentrations of the active compound of from about 0.2 to 70 μM, preferably about 1.0 to 10 μM. This may be achieved, for example, by the intravenous injection of a 0.1 to 5% solution of the active ingredient, optionally in saline, or administered as a bolus of the active ingredient.

The concentration of active compound in the drug composition will depend on absorption, inactivation, and excretion rates of the drug as well as other factors known to those of skill in the art. It is to be noted that dosage values will also vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition. The active ingredient may be administered at once, or may be divided into a number of smaller doses to be administered at varying intervals of time.

A preferred mode of administration of the active compound is oral. Oral compositions will generally include an inert diluent or an edible carrier. They may be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition.

The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring. When the dosage unit form is a capsule, it can contain, in addition to material of the above type, a liquid carrier such as a fatty oil. In addition, dosage unit forms can contain various other materials which modify the physical form of the dosage unit, for example, coatings of sugar, shellac, or other enteric agents.

(±)-FTC, or its (−) or (+)-enantiomer or pharmaceutically acceptable salts thereof can be administered as a component of an elixir, suspension, syrup, wafer, chewing gum or the like. A syrup may contain, in addition to the active compounds, sucrose as a sweetening agent and certain preservatives, dyes and colorings and flavors.

(±)-FTC, or its (−) or (+)-enantiomers, or pharmaceutically acceptable derivatives or salts thereof can also be mixed with other active materials that do not impair the desired action, or with materials that supplement the desired action, such as antibiotics, antifungals, antiinflammatories, or other antivirals, including other nucleoside anti-HIV compounds.

Solutions or suspensions used for parenteral, intradermal, subcutaneous, or topical application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The parental preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

If administered intravenously, preferred carriers are physiological saline or phosphate buffered saline (PBS).

In a preferred embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc.

Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) are also preferred as pharmaceutically acceptable carriers. These may be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811 (which is incorporated herein by reference in its entirety). For example, liposome formulations may be prepared by dissolving appropriate lipid(s) (such as stearoyl phosphatidyl ethanolamine, stearoyl phosphatidyl choline, arachadoyl phosphatidyl choline, and cholesterol) in an inorganic solvent that is then evaporated, leaving behind a thin film of dried lipid on the surface of the container. An aqueous solution of the active compound or its monophosphate, diphosphate, and/or triphosphate derivatives are then introduced into the container. The container is then swirled by hand to free lipid material from the sides of the container and to disperse lipid aggregates, thereby forming the liposomal suspension.

IV. Preparation of Phosphate Derivatives of FTC

Mono, di, and triphosphate derivative of FTC can be prepared as described below.

The monophosphate can be prepared according to the procedure of Imai et al., *J. Org. Chem.*, 34(6), 1547–1550 (June 1969). For example, about 100 mg of FTC and about 280 μl of phosphoryl chloride are reacted with stirring in about 8 ml of dry ethyl acetate at about 0° C. for about four hours. The reaction is quenched with ice. The aqueous phase is purified on an activated charcoal column, eluting with 5% ammonium hydroxide in a 1:1 mixture of ethanol and water. Evaporation of the eluant gives ammonium FTC-5'-monophosphate.

The diphosphate can be prepared according to the procedure of Davisson et al., *J. Org. Chem.*, 52(9), 1794–1801 (1987). FTC diphosphate can be prepared from the corresponding tosylate, that can be prepared, for example, by reacting the nucleoside with tosyl chloride in pyridine at room temperature for about 24 hours, working up the product in the usual manner (e.g., by washing, drying, and crystallizing it).

The triphosphate can be prepared according to the procedure of Hoard et al., *J. Am. Chem. Soc.*, 87(8), 1785–1788 (1965). For FTC is activated (by making a imidazolide, according to methods known to those skilled in the art) and treating with tributyl ammonium pyrophosphate in DMF. The reaction gives primarily the triphosphate of the nucleoside, with some unreacted monophosphate and some diphosphate. Purification by anion exchange chromatography of a DEAE column is followed by isolation of the triphosphate, e.g., as the tetrasodium salt.

This invention has been described with reference to its preferred embodiments. Variations and modifications of the invention, will be obvious to those skilled in the art from the foregoing detailed description of the invention. It is intended that all of these variations and modifications be included within the scope of the appended claims.

We claim:

1. A method for treating HIV infection in humans comprising administering an effective amount of (−)-β-L-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane, or its physiologically acceptable salt, optionally in a pharmaceutically acceptable carrier.

2. The method of claim **1**, wherein the carrier is suitable for oral delivery.

3. The method of claim **1**, wherein the carrier comprises a capsule.

4. The method of claim **1**, wherein the carrier is in the form of a tablet.

5. The method of claim **1**, wherein the administration is parenteral.

6. The method of claim **1**, wherein β-L-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane is administered in a form that is at least 95% free of its corresponding β-D-enantiomer.

7. The method of claim **1**, wherein β-L-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane is administered in a form that is at least 95% free of its corresponding β-D-enantiomer.

8. The method of claim **1**, wherein β-L-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane is administered as an isolated enantiomer.

9. A method for treating HIV infection in humans comprising administering an effective amount of (+)-β-D-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane, or its physiologically acceptable salt, optionally in a pharmaceutically acceptable carrier.

10. The method of claim **9**, wherein the carrier is suitable for oral delivery.

11. The method of claim **9**, wherein the carrier comprises a capsule.

12. The method of claim **9**, wherein the carrier is in the form of a tablet.

13. The method of claim **9**, wherein the administration is parenteral.

14. The method of claim **9**, wherein β-D-2-hydroxymethyl-5-(5-fluorocytosin- 1-yl)-1,3-oxathiolane is administered in a form that is at least 95% free of its corresponding β-L enantiomer.

15. The method of claim **9**, wherein β-D-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane is administered in a form that is at least 95% free of its corresponding β-L-enantiomer.

16. The method of claim **9**, wherein β-D-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane is administered as an isolated enantiomer.

17. A method for treating HIV infection in humans comprising administering an effective amount of the monphosphate, diphosphate or triphosphate of β-L-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane, or its physiologically acceptable salt, optionally in a pharmaceutically acceptable carrier.

18. The method of claim **17**, wherein the phosphate of β-L-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane is administered in a form that is at least 95% free of its corresponding β-D-enantiomer.

19. The method of claim **17**, wherein the phosphate of β-L-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane is administered as an isolated enantiomer.

20. A method for treating HIV infection in humans comprising administering an effective amount of the monophosphate, diphosphate, or triphosphate of β-D-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane, or its physiologically acceptable salt, optionally in a pharmaceutically acceptable carrier.

21. The method of claim **20**, wherein the phosphate of β-D-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane is administered in a form that is at least 95% free of its corresponding β-L-enantiomer.

22. The method of claim **20**, wherein the phosphate of β-D-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane is administered as an isolated enantiomer.

* * * * *

Exhibit B

US006703396B1

(12) **United States Patent**
Liotta et al.

(10) **Patent No.: US 6,703,396 B1**
(45) **Date of Patent: Mar. 9, 2004**

(54) **METHOD OF RESOLUTION AND ANTIVIRAL ACTIVITY OF 1,3-OXATHIOLANE NUCLESOSIDE ENANTIOMERS**

(75) Inventors: **Dennis C. Liotta**, Stone Mountain, GA (US); **Raymond F. Schinazi**, Decatur, GA (US); **Woo-Baeg Choi**, North Brunswick, NJ (US)

(73) Assignee: **Emory University**, Atlanta, GA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 641 days.

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(63) Continuation of application No. 08/092,248, filed on Jul. 15, 1993, now abandoned, which is a continuation of application No. 07/736,089, filed on Jul. 26, 1991, now abandoned, which is a continuation-in-part of application No. 07/659,760, filed on Feb. 22, 1991, now Pat. No. 5,210,085, which is a continuation-in-part of application No. 07/473,318, filed on Feb. 1, 1990, now Pat. No. 5,204,466.

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Primary Examiner—James O. Wilson
(74) *Attorney, Agent, or Firm*—Sherry M. Klowles, Esq.; King & Spalding

(57) **ABSTRACT**

A process for the resolution of a racemic mixture of nucleoside enantiomers that includes the step of exposing the racemic mixture to an enzyme that preferentially catalyzes a reaction in one of the enantiomers. The nucleoside enantiomer (−)-2-hydroxymethyl-5-(5-flurocytosin-1-yl)-1,3-oxathiolane is an effective antiviral agent against HIV, HBV, and other viruses replicating in a similar manner.

28 Claims, 5 Drawing Sheets

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Declaration of Dr. Irving Wainer in relation to the Opposition by Emory University against European Patent No. 0382526 B.
Declaration of Ian M. Mutton in relation to the Opposition by Emory University against European Patent No. 0382526 B.
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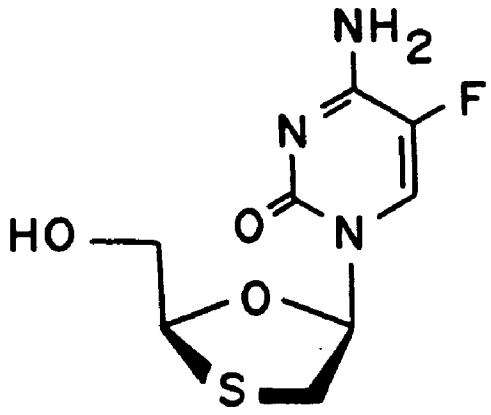


FIG. 1

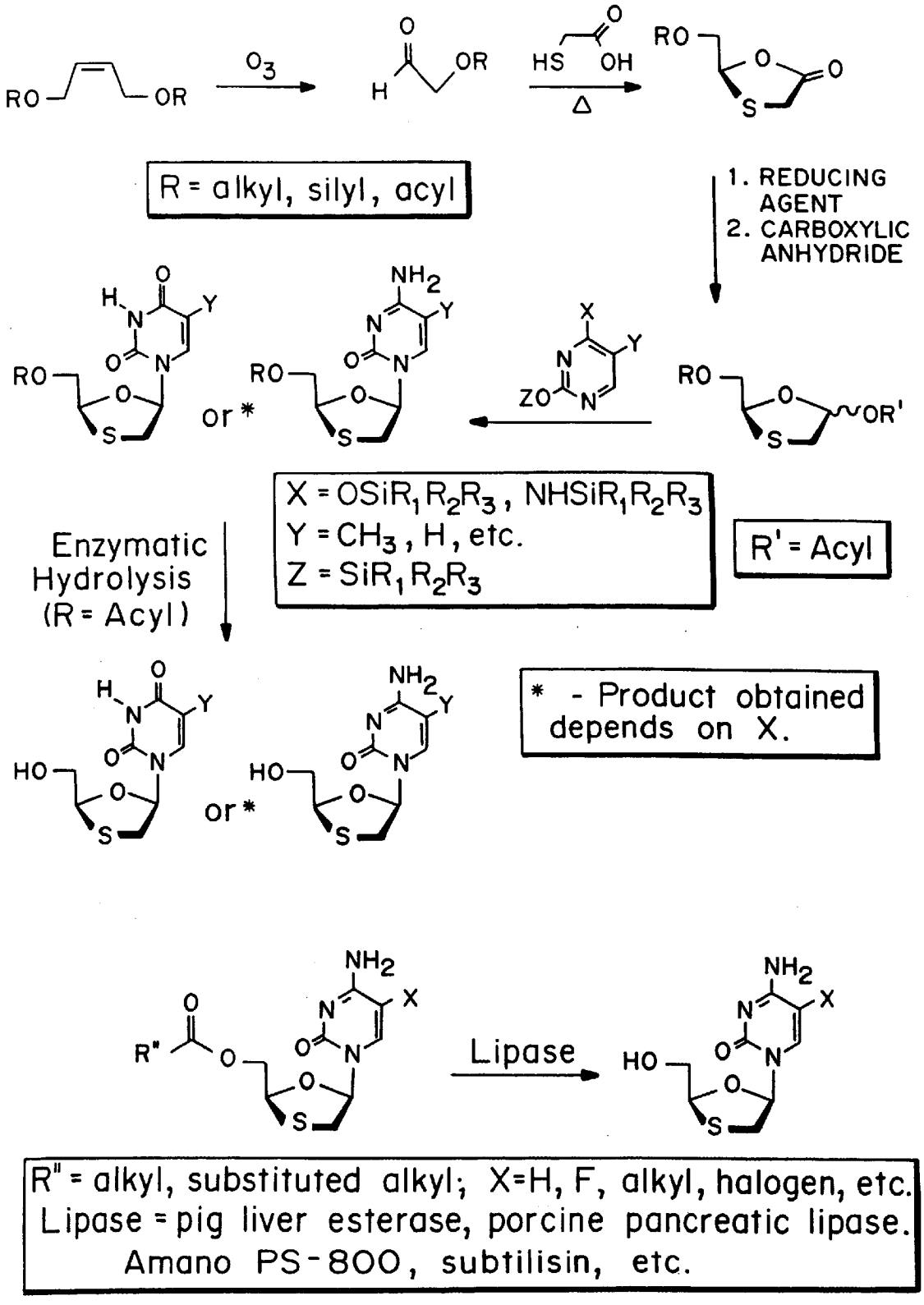

RO
OR
O_3
H
HS
OH
Δ
S
R = alkyl, silyl, acyl
1. REDUCING AGENT
2. CARBOXYLIC ANHYDRIDE
or *
ZO
OR'
X = $OSiR_1R_2R_3$, $NHSiR_1R_2R_3$
Y = CH_3, H, etc.
Z = $SiR_1R_2R_3$
R' = Acyl
Enzymatic Hydrolysis (R = Acyl)
HO
* - Product obtained depends on X.
R"
Lipase
R" = alkyl, substituted alkyl; X=H, F, alkyl, halogen, etc.
Lipase = pig liver esterase, porcine pancreatic lipase. Amano PS-800, subtilisin, etc.

FIG. 2

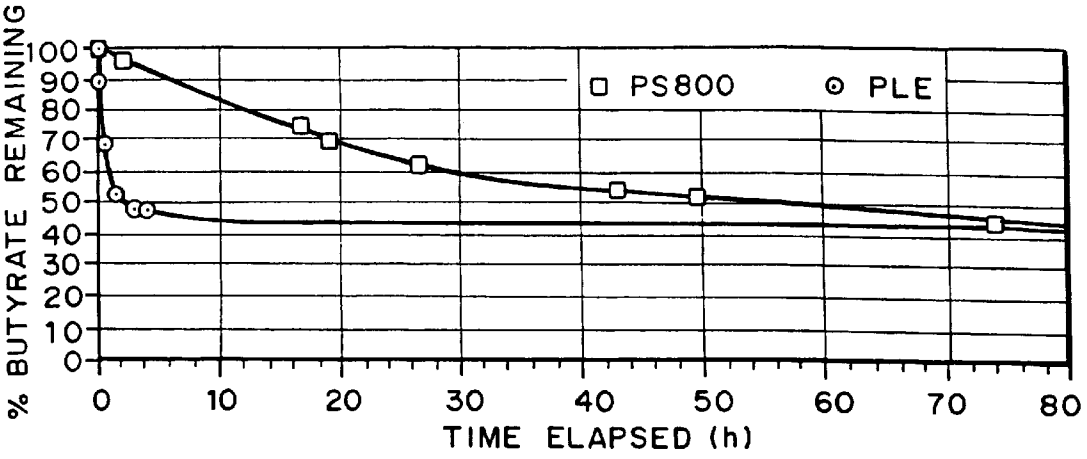

% BUTYRATE REMAINING
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
TIME ELAPSED (h)
PS800
PLE

FIG. 3

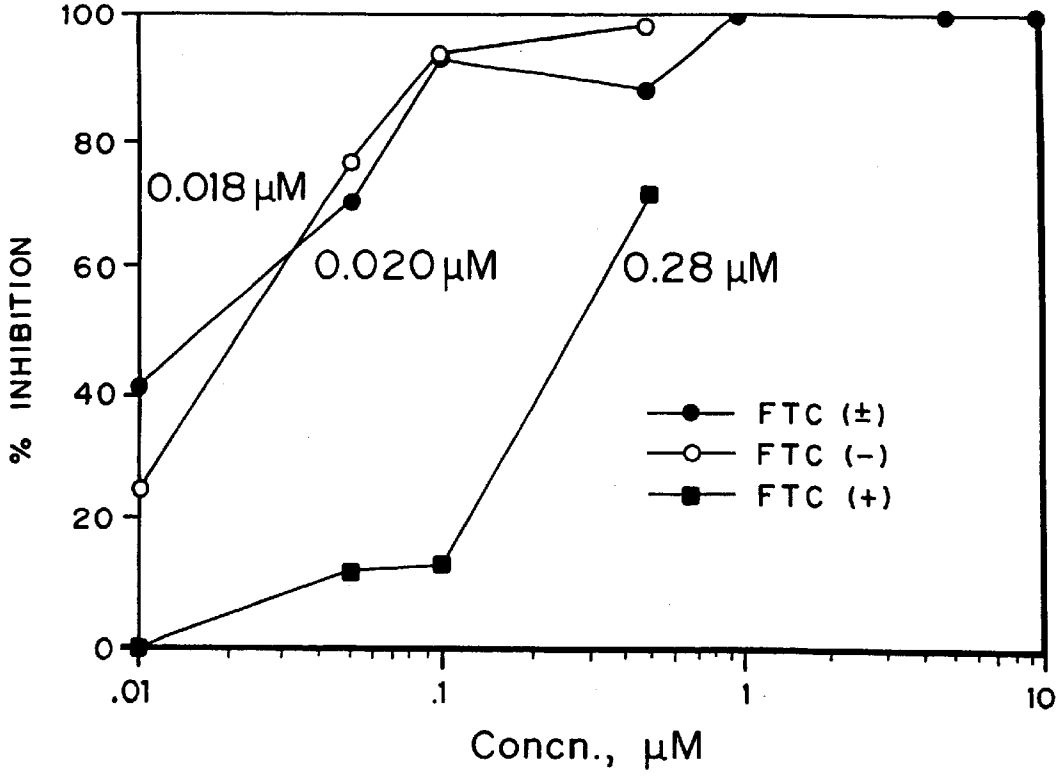

% INHIBITION
100
80
60
40
20
0
.01
.1
1
10
Concn., µM
0.018 µM
0.020 µM
0.28 µM
FTC (±)
FTC (-)
FTC (+)

FIG. 4

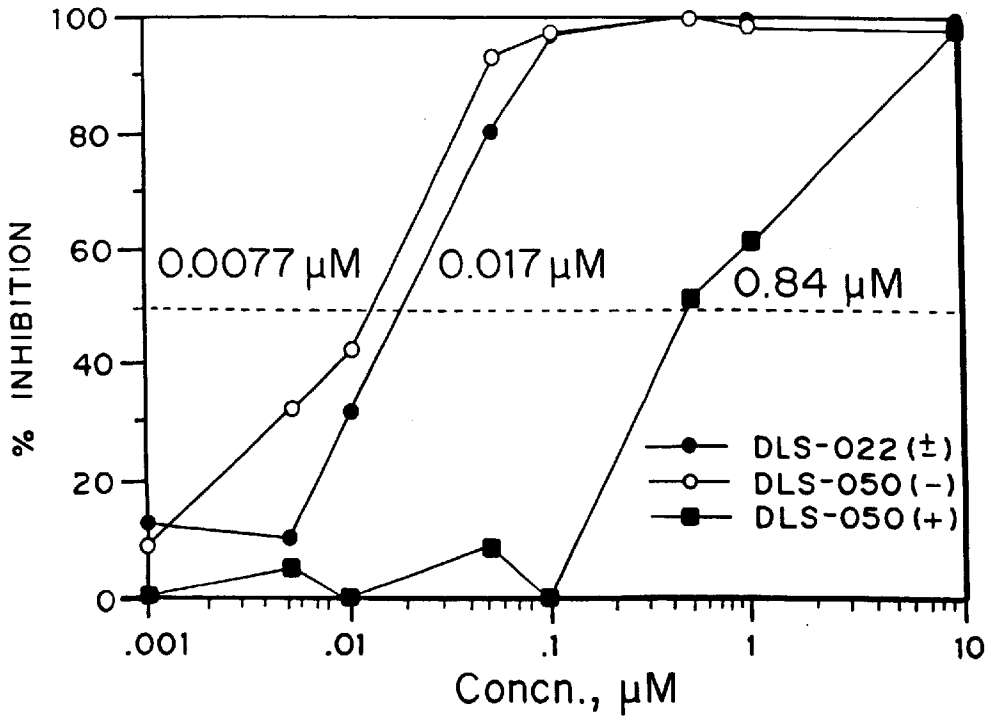

100
80
60
40
20
0
% INHIBITION
0.0077 µM
0.017 µM
0.84 µM
DLS-022 (±)
DLS-050 (-)
DLS-050 (+)
.001
.01
.1
1
10
Concn., µM

FIG. 5

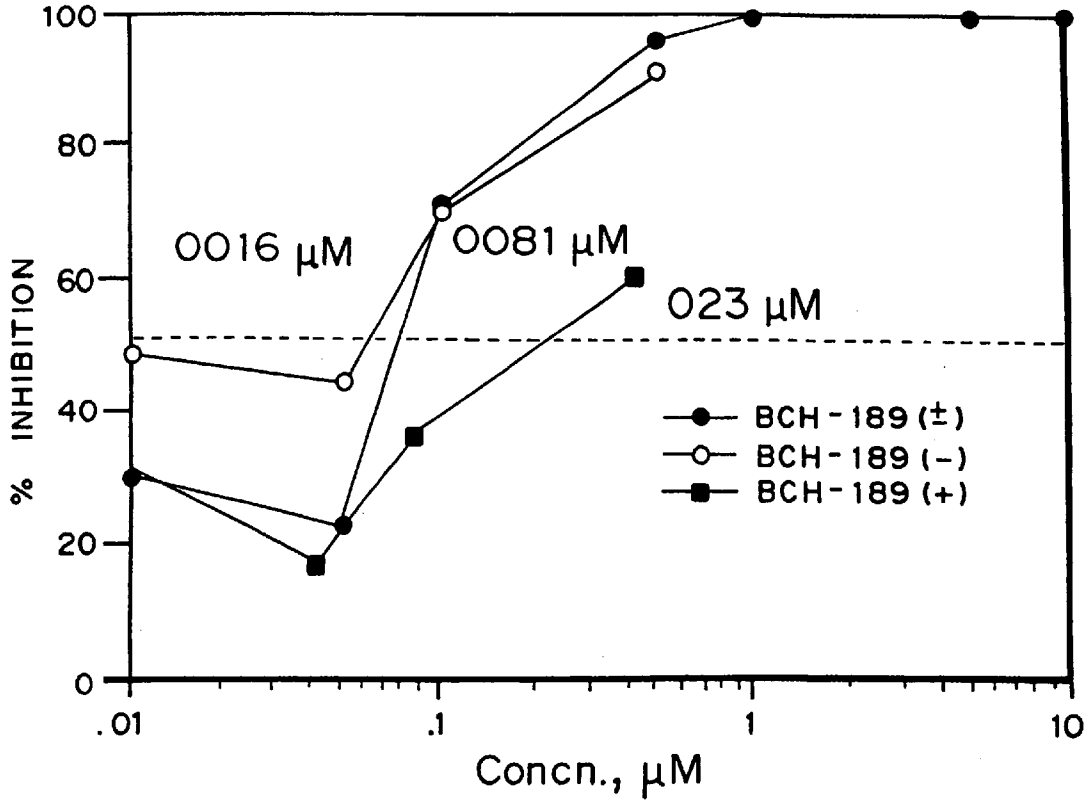

100
80
60
40
20
0
% INHIBITION
0016 µM
0081 µM
023 µM
BCH-189 (±)
BCH-189 (-)
BCH-189 (+)
.01
.1
1
10
Concn., µM

FIG. 6

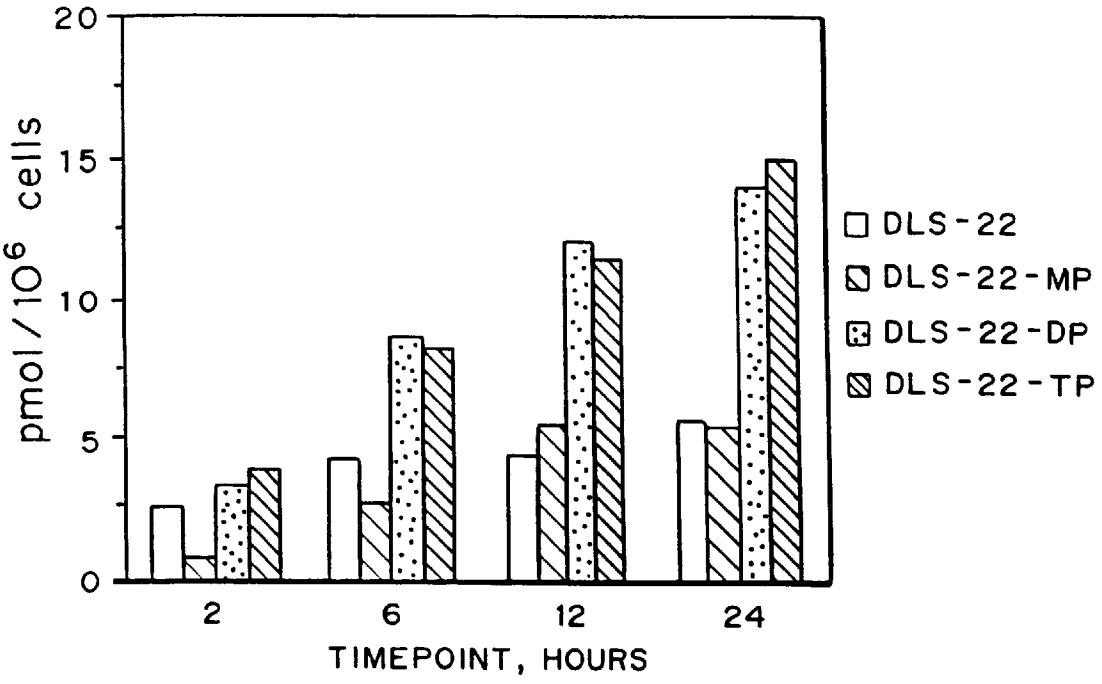

20
15
10
5
0
pmol / 10^6 cells
2
6
12
24
TIMEPOINT, HOURS
DLS-22
DLS-22-MP
DLS-22-DP
DLS-22-TP

FIG. 7

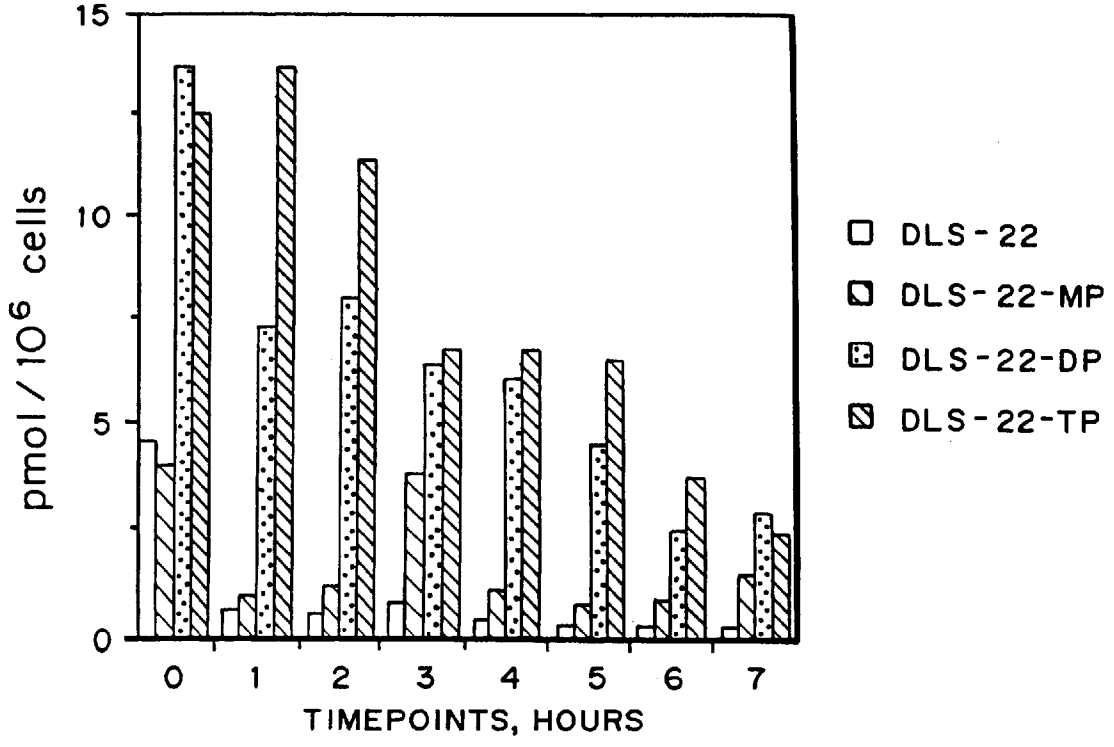

15
10
5
0
pmol / 10^6 cells
0
1
2
3
4
5
6
7
TIMEPOINTS, HOURS
DLS-22
DLS-22-MP
DLS-22-DP
DLS-22-TP

FIG. 8

METHOD OF RESOLUTION AND ANTIVIRAL ACTIVITY OF 1,3-OXATHIOLANE NUCLESOSIDE ENANTIOMERS

This application is a continuation of application U.S. Ser. No. 08/092,248, filed on Jul. 15, 1993, now abandoned, which is a continuation of U.S. Ser. No. 07/736,089, filed on Jul. 26, 1991, now abandoned, which is a continuation-in-part of U.S. Ser. No. 07/659,760, filed on Feb. 22, 1991, now U.S. Pat. No. 5,210,085, which is a continuation-in-part of U.S. Ser. No. 07/473,318, filed on Feb. 1, 1990, now U.S. Pat. No. 5,204,466.

U.S. Government has rights in this invention arising out of the partial funding of work leading to this invention through the National Institutes of Health Grant Nos. NIH 5-21935 and NIH AI-26055, as well as a Veteran's Administration Merit Review Award.

BACKGROUND OF THE INVENTION

This invention is in the area of biologically active nucleosides, and specifically includes a method for the resolution of nucleoside enantiomers, including 1,3-oxathiolane nucleosides, and antiviral compositions that include the enantiomerically enriched 1,3-oxathiolane nucleosides, (−) and (+)-2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane ("FTC").

This application is a continuation-in-part application of U.S. Ser. No. 07/659,760, entitled "Method for the Synthesis, Compositions and Use of 2'-Deoxy-5-Fluoro-3'-Thiacytidine and Related Compounds", filed on Feb. 22, 1991, by Dennis C. Liotta, Raymond Schinazi, and Woo-Baeg Choi, that is a continuation-in-part application of U.S. Ser. No. 07/473,318, entitled "Method and Compositions for the Synthesis of BCH-189 and Related Compounds," filed on Feb. 1, 1990, by Dennis C. Liotta and Woo-Baeg Choi.

In 1981, acquired immune deficiency syndrome (AIDS) was identified as a disease that severely compromises the human immune system, and that almost without exception leads to death. In 1983, the etiological cause of AIDS was determined to be the human immunodeficiency virus (HIV). In December, 1990, the World Health Organization estimated that between 8 and 10 million people worldwide were infected with HIV, and of that number, between 1,000,000 and 1,400,000 were in the U.S.

In 1985, it was reported that the synthetic nucleoside 3'-azido-3'-deoxythymidine (AZT) inhibits the replication of human immunodeficiency virus type 1. Since then, a number of other synthetic nucleosides, including 2',3'-dideoxyinosine (DDI), 2',3'-dideoxycytidine (DDC), 3'-fluoro-3'-deoxythymidine (FLT), 2',3'-dideoxy-2',3'-didehydrothymidine (D4T), and 3'-azido-2',3'-dideoxyuridine (AZDU), have been proven to be effective against HIV. A number of other 2',3'-dideoxynucleosides have been demonstrated to inhibit the growth of a variety of other viruses in vitro. It appears that, after cellular phosphorylation to the 5'-triphosphate by cellular kinases, these synthetic nucleosides are incorporated into a growing strand of viral DNA, causing chain termination due to the absence of the 3'-hydroxyl group.

In its triphosphate form, 3'-azido-3'-deoxythymidine is a potent inhibitor of HIV reverse transcriptase and has been approved by the FDA for the treatment of AIDS. However, the benefits of AZT must be weighed against the severe adverse reactions of bone marrow suppression, nausea, myalgia, insomnia, severe headaches, anemia, peripheral neuropathy, and seizures. These adverse side effects often occur immediately after treatment begins, whereas a minimum of six weeks of therapy is necessary to realize AZT's benefits. DDI, which has recently been approved by an FDA Committee for the treatment of AIDS, is also associated with a number of side effects, including sporadic pancreatis and peripheral neuropathy.

Both DDC and D4T are potent inhibitors of HIV replication with activities comparable (D4T) or superior (DDC) to AZT. However, both DDC and D4T are not efficiently converted to the corresponding 5'-triphosphates in vivo. Both compounds are also toxic and can cause peripheral neuropathies in humans.

The success of various 2',3'-dideoxynucleosides in inhibiting the replication of HIV in vivo or in vitro has led a number of researchers to design and test nucleosides that substitute a heteroatom for the carbon atom at the 3'-position of the nucleoside. Norbeck, et al., disclose that (±)-1-[(2β, 4β)-2-(hydroxymethyl)-4-dioxolanyl]thymine (referred to below as (±)-dioxolane-T) exhibits a modest activity against HIV (EC_{50} of 20 μm in ATH8 cells), and is not toxic to uninfected control cells at a concentration of 200 μm. *Tetrahedron Letters* 30 (46), 6246, (1989).

European Patent Application Publication No. 0 382 526 filed by IAF Biochem International, Inc. discloses a number of substituted 1,3-oxathiolanes with antiviral activity, and specifically reports that the racemic mixture (about the C4'-position) of the C1'-β isomer of 2-hydroxymethyl-5-(cytosin-1-yl)-1,3-oxathiolane (referred to below as (±)-BCH-189) has approximately the same activity against HIV as AZT, and no cellular toxicity at therapeutic levels. (±)-BCH-189 has also been found to inhibit the replication of AZT-resistant, HIV isolates from patients who have been treated with AZT for longer than 36 weeks.

To market a nucleoside for pharmaceutical purposes, it must not only be efficacious with low toxicity, it must also be cost effective to manufacture. An extensive amount of research and development has been directed toward new, low cost processes for large scale nucleoside production. 2',3'-Dideoxynucleosides are currently prepared by either of two routes: derivatization of an intact nucleoside or condensation of a derivatized sugar moiety with a heterocyclic base. Although there are numerous disadvantages associated with obtaining new nucleoside analogues by modifying intact nucleosides, a major advantage of this approach is that the appropriate absolute stereochemistry has already been set by nature. However, this approach cannot be used in the production of nucleosides that contain either nonnaturally occurring bases or nonnaturally occurring carbohydrate moieties (and which therefore are not prepared from intact nucleosides), such as 1,3-oxathiolane nucleosides and 1,3-dioxolane nucleosides.

When condensing a carbohydrate or carbohydrate-like moiety with a heterocyclic base to form a synthetic nucleoside, a nucleoside is produced that has two chiral centers (at the C1' and C4'-positions), and thus exists as a diastereomeric pair. Each diastereomer exists as a set of enantiomers. Therefore, the product is a mixture of four enantiomers.

It is often found that nucleosides with nonnaturally-occurring stereochemistry in either the C1' or the C4'-positions are less active than the same nucleoside with the stereochemistry as set by nature. For example, Carter, et al., have reported that the concentration of the (−)-enantiomer of carbovir (2',3'-didehydro-2',3'-dideoxyguanosine) required to reduce the reverse transcriptase activity by 50% (EC_{50})

is 0.8 μM, whereas the EC_{50} for the (+)-enantiomer of carbovir is greater than 60 μM. *Antimicrobial Agents and Chemotherapy,* 34:6, 1297–1300 (June 1990).

U.S. Ser. No. 07/659,760 discloses that 1,3-oxathiolane and 1,3-dioxolane nucleosides can be prepared with high diastereoselectivity (high percentage of nucleoside with a β configuration of the bond from the C1'-carbon to the heterocyclic base) by careful selection of the Lewis acid used in the condensation process. It was discovered that condensation of a 1,3-oxathiolane nucleoside with a base occurs with almost complete β-stereospecificity when stannic chloride is used as the condensation catalyst, and condensation of 1,3-dioxolane with a base occurs with almost complete β-stereospecificity when various chlorotitanium catalysts are employed. Other Lewis acids provide low (or no) C1'-β selectivity or simply fail to catalyze the reactions.

There remains a strong need to provide a cost effective, commercially viable method to obtain β-stereospecificity of synthetic nucleosides prepared by condensing a carbohydrate-like moiety with a base. This is important because it is likely that many synthetic nucleoside inhibitors of viral replication now emerging from academic and commercial laboratories will require resolution. An economical and facile method for resolving racemic mixtures of nucleosides would greatly facilitate antiviral research and ultimately, commercial manufacture. Further, resolution of racemic mixtures of nucleosides may provide a route to increase the activity of synthetic nucleosides by eliminating or minimizing the undesired enantiomer.

Therefore, it is an object of the present invention to provide a method for the resolution of racemic mixtures of nucleosides.

It is another object of the present invention to provide enantiomerically enriched 1,3-oxathiolane nucleosides.

It is still another object of the present invention to provide enantiomerically enriched 1,3-oxathiolane nucleosides with significant antiviral activity and low toxicity.

SUMMARY OF THE INVENTION

A process for the resolution of a racemic mixture of nucleoside enantiomers or their derivatives is disclosed that includes the step of exposing the racemic mixture to an enzyme that preferentially catalyzes a reaction in one of the enantiomers. The process can be used to resolve a wide variety of nucleosides, including pyrimidine and purine nucleosides that are optionally substituted in the carbohydrate moiety or base moiety. The process can also be used to resolve nucleoside derivatives that contain additional heteroatoms in the carbohydrate moiety, for example, FTC and BCH-189. The resolution of nucleosides can be performed on large scale at moderate cost.

It has been discovered that the nucleoside enantiomer (−) 2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane ("FTC") exhibits significant activity against HIV (EC_{50} of 0.0077 to 0.02 μM), HBV (hepatitis B virus), and other viruses replicating in a similar manner. The (+)-enantiomer of FTC is also active against HIV (EC_{50} of 0.28–0.84 μM).

BRIEF DESCRIPTION OF THE FIGURES

FIG. **1** is an illustration of the chemical structure of 2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane ("FTC").

FIG. **2** is an illustration of a method for the preparation of enantiomerically enriched cytidine and uridine 1,3-oxathiolane nucleosides.

FIG. **3** is a graph indicating the progress of lipase-catalyzed hydrolysis of the 5'-butyryl ester of FTC over time using the enzymes PS800 (-open square-) and PLE (-open circle with dot-).

FIG. **4** is a graph of the effect of concentration (μM) of racemic and enantiomerically enriched FTC (prepared by the method of Example 3) versus the percent inhibition of human PBM cells infected with HIV-1. ((-darkened circle-, (±)-FTC), (-circle-, (−)-FTC), (-darkened square-,(+)-FTC).

FIG. **5** is a graph of the effect of concentration (μM) of racemic and enantiomerically enriched FTC (prepared by method of Example 2) on the percent inhibition of human PBM cells infected with HIV-1. ((-darkened circle-, (±)-FTC), (-circle -,(−)-FTC), (-darkened square-,(+)-FTC).

FIG. **6** is a graph of the effect of concentration (μM) of racemic and enantiomerically enriched BCH-189 (prepared by the method of Example 3) on the percent inhibition of human PBM cells infected with HIV-1. ((-darkened circle-, (±)-BCH-189), (-circle-, (−)-BCH-189), (-darkened square-, (+)-BCH-189).

FIG. **7** is a graph of the uptake of tritiated (±)-FTC in human PBM cells (average of two determinations) in time (hours) versus pmol/10^6 cells.

FIG. **8** is a graph of the egress of radiolabeled (±)-FTC from human PBM cells, measured in hours versus pmol/10^6 cells ((-darkened square-), (±)-FTC; (-///-), (±)-FTC monophosphate; (-// // //-), (±)-FTC diphosphate; and (-/// ///-), (±)-FTC triphosphate).

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the term "enantiomerically enriched nucleoside" refers to a nucleoside composition that includes at least 95% of a single enantiomer of that nucleoside.

As used herein, the term BCH-189 refers to 2-hydroxymethyl-5-(cytosin-1-yl)-1,3-oxathiolane.

As used herein, the term FTC refers to 2-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane.

As used herein, the term "preferential enzyme catalysis" refers to catalysis by an enzyme that favors one substrate over another.

I. Resolution of Racemic Mixtures of Nucleosides During the Hydrolysis

A method is provided for the resolution of racemic mixtures of nucleoside enantiomers. The method involves the use of an enzyme that preferentially catalyzes a reaction of one enantiomer in a racemic mixture. The reacted enantiomer is separated from the unreacted enantiomer on the basis of the new difference in physical structure. Given the disclosure herein, one of skill in the art will be able to choose an enzyme that is selective for the nucleoside enantiomer of choice (or selective for the undesired enantiomer, as a method of eliminating it), by selecting of one of the enzymes discussed below or by systematic evaluation of other known enzymes. Given this disclosure, one of skill in the art will also know how to modify the substrate as necessary to attain the desired resolution. Through the use of either chiral NMR shift reagents, polarimetry, or chiral HPLC, the optical enrichment of the recovered ester can be determined.

The following examples further illustrate the use of enzymes to resolve racemic mixtures of enantiomers. Other known methods of resolution of racemic mixtures can be used in combination with the method of resolution disclosed herein. All of these modifications are considered within the scope of the invention.

Resolution Based on Hydrolysis of C5'-Nucleoside Esters

In one embodiment, the method includes reacting the C5'-hydroxyl group of a mixture of nucleoside racemates with an acyl compound to form C5'-esters in which the nucleoside is in the "carbinol" end of the ester. The racemic mixture of nucleoside C5'-esters is then treated with an enzyme that preferentially cleaves, or hydrolyses, one of the enantiomers and not the other.

An advantage of this method is that it can be used to resolve a wide variety of nucleosides, including pyrimidine and purine nucleosides that are optionally substituted in the carbohydrate moiety or base moiety. The method can also be used to resolve nucleoside derivatives that contain additional heteroatoms in the carbohydrate moiety, for example, FTC and BCH-189. The broad applicability of this method resides in part on the fact that although the carbinol portion of the ester plays a role in the ability of an enzyme to differentiate enantiomers, the major recognition site for these enzymes is in the carboxylic acid portion of the ester. Further, one may be able to successfully extrapolate the results of one enzyme/substrate study to another, seemingly-different system, provided that the carboxylic acid portions of the two substrates are the same or substantially similar.

Another advantage of this method is that it is regioselective. Enzymes that hydrolyse esters typically do not catalyze extraneous reactions in other portions of the molecule. For example, the enzyme lipase catalyses the hydrolysis of the ester of 2-hydroxymethyl-5-oxo-1,3-oxathiolane without hydrolysing the internal lactone. This contrasts markedly with "chemical" approaches to ester hydrolysis.

Still another advantage of this method is that the separation of the unhydrolysed enantiomer and the hydrolysed enantiomer from the reaction mixture is quite simple. The unhydrolysed enantiomer is more lipophilic than the hydrolysed enantiomer and can be efficiently recovered by simple extraction with one of a wide variety of nonpolar organic solvents or solvent mixtures, including hexane and hexane/ether. The less lipophilic, more polar hydrolysed enantiomer can then be obtained by extraction with a more polar organic solvent, for example, ethyl acetate, or by lyophilization, followed by extraction with ethanol or methanol. Alcohol should be avoided during the hydrolysis because it can denature enzymes under certain conditions.

Enzymes and Substrates

With the proper matching of enzyme and substrate, conditions can be established for the isolation of either nucleoside enantiomer. The desired enantiomer can be isolated by treatment of the racemic mixture with an enzyme that hydrolyses the desired enantiomer (followed by extraction of the polar hydrolysate with a polar solvent) or by treatment with an enzyme that hydrolyses the undesired, enantiomer (followed by removal of the undesired enantiomer with a nonpolar solvent).

Enzymes that catalyze the hydrolysis of esters include esterases, for example pig liver esterase, lipases, including porcine pancreatic lipase and Amano PS-800 lipase, substillisin, and α-chymotrypsin.

The most effective acyl group to be used to esterify the C5'-position of the nucleoside can be determined without undue experimentation by evaluation of a number of homologs using the selected enzyme system. For example, when 1,3-oxathiolane nucleosides are esterified with butyric acid, resolutions with both pig liver esterase and Amano PS-800 proceed with high enantioselectivity (94–100 enantiomeric excess) and opposite selectivity. Non-limiting examples of other acyl groups that can be evaluated for use with a particular nucleoside enantiomeric mixture and particular enzyme include alkyl carboxylic acids and substituted alkyl carboxylic acids, including acetic acid, propionic acid, butyric acid, and pentanoic acid. With certain enzymes, it may be preferred to use an acyl compound that is significantly electron-withdrawing to facilitate hydrolysis by weakening the ester bond. Examples of electron-withdrawing acyl groups include α-haloesters such as 2-chloropropionic acid, 2-chlorobutyric acid, and 2-chloropentanoic acid. α-Haloesters are excellent substrates for lipases.

Resolution Conditions

The enzymatic hydrolyses are typically carried out with a catalytic amount of the enzyme in an aqueous buffer that has a pH that is close to the optimum pH for the enzyme in question. As the reaction proceeds, the pH drops as a result of liberated carboxylic acid. Aqueous base should be added to maintain the pH near the optimum value for the enzyme. The progress of the reaction can be easily determined by monitoring the change in pH and the amount of base needed to maintain pH. The hydrophobic ester (the unhydrolysed enantiomer) and the more polar alcohol (the hydrolysed enantiomer) can be sequentially and selectively extracted from the solution by the judicious choice of organic solvents. Alternatively, the material to be resolved can be passed through a column that contains the enzyme immobilized on a solid support.

Enzymatic hydrolyses performed under heterogeneous conditions can suffer from poor reproducibility. Therefore, it is preferred that the hydrolysis be performed under homogeneous conditions. Alcohol solvents are not preferred because they can denature the enzymes. Homogeneity can be achieved through the use of non-ionic surfactants, such as Triton X-100. However, addition of these surfactants not only assists in dissolving the starting material, they also enhance the aqueous solubility of the product. Therefore, although the enzymatic reaction can proceed more effectively with the addition of a non-ionic surfactant than under heterogeneous conditions, the isolation of both the recovered starting material and the product can be made more difficult. The product can be isolated with appropriate chromatographic and chemical (e.g., selective salt formation) techniques. Diacylated nucleosides can be used but are often quite lipophilic and hard to dissolve in the medium used.

EXAMPLE 1

Enantioselective Lipase-Catalyzed Hydrolysis of FTC Esters

A number of 5'-O-acyl derivatives of FTC were prepared by selective O-acylation of the N-hydrochloride salt (see Table 1) of (±)-FTC. The efficiency of the hydrolysis of the derivatives by lipases was investigated. As shown in Table 1, pig liver esterase (PLE) exhibits a high level of selectivity for the hydrolysis of the ester of the (+)-enantiomer of FTC. In contrast, PS-800 hydrolyses the ester of the (−)-enantiomer of FTC preferentially. The rate of the hydrolysis was also found to be dependent on the nature of the acyl group; the acetyl derivative was significantly slower than the butyryl derivative. It has now been discovered that the rate of the hydrolysis of the propionic acid ester of FTC is even faster than that observed for the butyrate derivative. % Recovery and % ee were both determined using HPLC. Although the enantioselectivity is excellent when employing PLE (typically 97% e.e. or higher), additional enrichment can be accomplished by sequential enzymatic hydrolysis

reactions in which the enantiomerically-enriched butyrate from a PLE-catalyzed hydrolysis is subjected to enzymatic hydrolysis by PS-800.

TABLE 1

Comparison of Effect of Ester on Enzyme Hydrolysis.

substrate	% recovery	% e.e. (s.m.)
FTC Esters with PLE:		
acetate	32.68	N.D.
propionate	39.87	N.D.
butyrate	48.00	98
butyrate	45.71	98.6
FTC Esters with PS800:		
acetate	73.17	N.D.
propionate	52.67	N.D.
butyrate	58.34	N.D.
valerate	41.50	94

EXAMPLE 2

Procedure for the Preparation of (+)- and (−)-FTC via Enantioselective, Lipase-Catalyzed Hydrolysis of FTC Butyrate

The 5'-O-butyrate of (±)-FTC (1) (0.47 mmol, 149 mg) was dissolved in 16 mL of a solution of 4:1 pH 8 buffer:CH_3CN. The clear solution was stirred and treated with 26 mg of pig liver esterase (PLE-A). The progress of the reaction was monitored by HPLC (FIG. **3**). After 20 hours (52% conversion), the reaction mixture was extracted with 2×80 mL of $CHCl_3$ and 80 mL of ethyl acetate. The organic layer extracts were combined, dried over anhydrous $MgSO_4$, filtered, and concentrated by rotary evaporation. The resulting residue was eluted on 2×1000 m pTLC plates using ethyl acetate as eluant (double elution) to give, after isolation, 53 mg (36% based on starting material) of FTC butyrate which was determined to have 98% enantiomeric excess (e.e.) by HPLC analysis. The enantiomerically-enriched butyrate was then treated with 1.6 mL of methanol followed by 0.38 mmol (20 mg) of sodium methoxide. The resulting mixture was stirred at room temperature, and the progress of the reaction was monitored by HPLC. The reaction was completed within 30 minutes. The solvent was removed by rotary evaporation to give a crude white solid (76 mg) that was eluted on a 1000 m pTLC using 5:1 ethyl acetate:ethanol. (−)-FTC was isolated as a white solid (33 mg; 82% yield). HPLC analysis of the FTC as its 5'-O-acetate derivative showed 97% e.e.; $[\alpha]({}^{20}{}_{,D})$ −120.5°(c =0.88; abs. ethanol).

Similarly, 1.2 mmol (375 mg) of the 5'-O-butyrate of (+)-FTC was dissolved in 40 mL of 4:1 pH 8 buffer-CH_3CN. The clear solution was stirred and treated with 58 mg of pig liver esterase (PLE-A). The progress of the reaction was monitored by HPLC. After 90 minutes (38% conversion), the reaction mixture was added to 150 mL of CHCl. The layers were separated and the aqueous layer lyophilized to remove solvent. The white residue from the lyophilization was extracted with 3×10 mL of absolute ethanol. The extracts were filtered, combined, and concentrated in vacuo to yield 179 mg of crude oil. The crude material was eluted on a 45×30 mm column of silica gel using 3×75 mL of ethyl acetate followed by 5:1 ethyl acetate-ethanol. (+)-FTC (a) was isolated as a white solid (109 mg; 37% based on starting butyrate). HPLC analysis of the (+)-FTC as its 5'-O-acetate derivative showed 97.4% e.e.; [a]O($^{20}{}_{,D}$) +113.4° (c=2.53; absolute ethanol)

A similar reaction was performed using 0.12 mmol (37 mg) of the 5'-O-butyrate of FTC and 7 mg of PS-800 in 4.0 mL of 4:1 pH 8 buffer:CH_3CN. The reaction was considerably slower than that with PLE-A and required 74 hours for 59% conversion. The recovered butyrate (11.4 mg; 31% of the initial amount) was found to be 94% e.e. by HPLC.

Resolution of Nucleoside Enantiomers with Cytidine-Deoxycytidine Deaminase

In an alternative embodiment, cytidine-deoxycytidine deaminase is used to resolve racemic mixtures of 2-hydroxymethyl-5-(cytosin-1-yl)-1,3-oxathiolane and its derivatives, including 2-hydroxymethyl-5-(5-fluoro-cytosin-1-yl)-1,3-oxathiolane. The enzyme catalyses the deamination of the cytosine moiety to a uridine. It has been discovered that one of the enantiomers of 1,3-oxathiolane nucleosides is a preferred substrate for cytidine-deoxycytidine deaminase. The enantiomer that is not converted to a uridine (and therefore is still basic) is extracted from the solution with an acidic solution. Care should be taken to avoid strong acidic solutions (pH below 3.0), that may cleave the oxathiolane ring.

Cytidine-deoxycytidine deaminase can be isolated from rat liver or human liver, or expressed from recombinant sequences in procaryotic system such as in *E. coli*.

The method of resolution of cytidine nucleoside enantiomers using cytidine-deoxycytidine deaminase can be used as the sole method of resolution or can be used in combination with other methods of resolution, including resolution by enzymatic hydrolysis of 5'-O-nucleoside esters as described above.

Combination of Enzymatic Resolution with Classical Resolution Methods

The process described above for resolving racemic mixtures of nucleoside enantiomers can be combined with other classical methods of enantiomeric resolution to increase the optical purity of the final product.

Classical methods of resolution include a variety of physical and chemical techniques. Often the simplest and most efficient technique is recrystallization, based on the principle that racemates are often more soluble than the corresponding individual enantiomers. Recrystallization can be performed at any stage, including on the acylated compounds and or the final enantiomeric product. If successful, this simple approach represents a method of choice.

When recrystallization fails to provide material of acceptable optical purity, other methods can be evaluated. If the nucleoside is basic (for example, a cytidine) one can use chiral acids that form diastereomeric mixtures that may possess significantly different solubility properties. Nonlimiting examples of chiral acids include malic acid, mandelic acid, dibenzoyl tartaric acid, 3-bromocamphor-8-sulfonic acid, 10-camphorsulfonic acid, and di-p-toluoyltartaric acid. Similarly, acylation of the free hydroxyl group with a chiral acid derivative also results in the formation of diastereomeric mixtures whose physical properties may differ sufficiently to permit separation.

Small amounts of enantiomerically enriched nucleosides can be obtained or purified by passing the racemic mixture through an HPLC column that has been designed for chiral separations, including cyclodextrin bonded columns marketed by Rainin Corporation.

EXAMPLE 3

Separation of Racemic Mixtures of Nucleosides by HPLC

The resolutions of the C4'-enantiomers of (±)-BCH-189 and (±)-FTC were performed using a chiral cyclodextrin bonded (cyclobond AC-I) column obtained from Rainin Corporation (Woburn, Mass.). The conditions were as follows: Isocratic 0.5% methanol in water; flow rate 1 ml/min., UV detection at 262 nm. HPLC grade methanol was obtained from J. T. Baker (Phillipsburg, N.J.). The racemic mixtures were injected and fractions were collected. Fractions containing each of the enantiomers were pooled, frozen, and then lyophilized. The compounds were characterized by UV spectroscopy and by their retention times on HPLC. In general, the (−)-enantiomers have lower retention times than the (+)-enantiomers (see *J. Liquid Chromatography* 7:353–376, 1984). The concentrations of the compounds were determined by UV spectroscopy, using a stock solution of known concentration (15 μM) prepared in water for biological evaluation. The retention times for the separated enantiomers are provided in Table 2.

TABLE 2

Retention Times of Enantiomers of BCH-189 and FTC

Compound	R_t (min)
(−)-BCH-189	9.0
(+)-BCH-189	10.0
(−)-FTC	8.3
(+)-FTC	8.7

II. Antiviral Activity of 2-Hydroxymethyl-5-(5-Fluorocytosin-1-yl)-1,3-oxathiolane ("FTC")

It is often desirable to screen a number of racemic mixtures of nucleosides as a preliminary step to determine which warrant further resolution into enantiomerically enriched components and evaluation of antiviral activity. The ability of nucleosides to inhibit HIV can be measured by various experimental techniques. The technique used herein, and described in detail below, measures the inhibition of viral replication in phytohemagglutinin (PHA) stimulated human peripheral blood mononuclear (PBM) cells infected with HIV-1 (strain LAV). The amount of virus produced is determined by measuring the virus-coded reverse transcriptase enzyme. The amount of enzyme produced is proportional to the amount of virus produced. Table 4 provides the EC_{50} values (concentration of nucleoside that inhibits the replication of the virus by 50% in PBM cells) and IC_{50} values (concentration of nucleoside that inhibits 50% of the growth of mitogen-stimulated uninfected human PBM cells) of a number of (±)-1,3-oxathiolane and nucleosides.

EXAMPLE 4

Anti-HIV activity of (±)-1,3-Oxathiolane Nucleosides

A. Three-day-old phytohemagglutinin-stimulated PBM cells (10^6 cells/ml) from hepatitis B and HIV-1 seronegative healthy donors were infected with HIV-1 (strain LAV) at a concentration of about 100 times the 50% tissue culture infectious dose (TICD 50) per ml and cultured in the presence and absence of various concentrations of antiviral compounds.

B. Approximately one hour after infection, the medium, with the compound to be tested (2 times the final concentration in medium) or without compound, was added to the flasks (5 ml; final volume 10 ml). AZT was used as a positive control.

C. The cells were exposed to the virus (about 2×10^5 dpm/ml, as determined by reverse transcriptase assay) and then placed in a CO_2 incubator. HIV-1 (strain LAV) was obtained from the Center for Disease Control, Atlanta, Ga. The methods used for culturing the PBM cells, harvesting the virus and determining the reverse transcriptase activity were those described by McDougal et al. (*J. Immun. Meth.* 76, 171–183, 1985) and Spira et al. (*J. Clin. Meth.* 25, 97–99, 1987), except that fungizone was not included in the medium (see Schinazi, et al., *Antimicrob. Agents Chemother.* 32, 1784–1787 (1988); Id., 34:1061–1067 (1990)).

D. On day 6, the cells and supernatant were transferred to a 15 ml tube and centrifuged at about 900 g for 10 minutes. Five ml of supernatant were removed and the virus was concentrated by centrifugation at 40,000 rpm for 30 minutes (Beckman 70.1 Ti rotor). The solubilized virus pellet was processed for determination of the levels of reverse transcriptase. Results are expressed in dpm/ml of sampled supernatant. Virus from smaller volumes of supernatant (1 ml) can also be concentrated by centrifugation prior to solubilization and determination of reverse transcriptase levels.

The median effective (EC_{50}) concentration was determined by the median effect method (*Antimicrob. Agents Chemother.* 30, 491–498 (1986). Briefly, the percent inhibition of virus, as determined from measurements of reverse transcriptase, is plotted versus the micromolar concentration of compound. The EC_{50} is the concentration of compound at which there is a 50% inhibition of viral growth.

E. Mitogen stimulated uninfected human PBM cells (3.8×10^5 cells/ml) were cultured in the presence and absence of drug under similar conditions as those used for the antiviral assay described above. The cells were counted after 6 days using a hemacytometer and the trypan blue exclusion method, as described by Schinazi et al., *Antimicrobial Agents and Chemotherapy,* 22(3), 499 (1982). The IC_{50} is the concentration of compound which inhibits 50% of normal cell growth.

TABLE 3

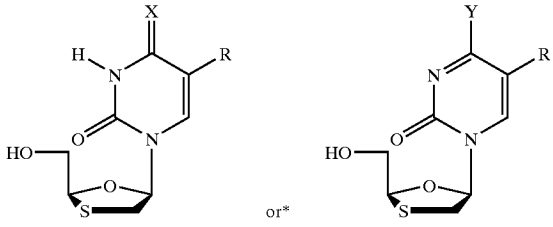
EC_{50} and IC_{50} of Various Analogues of 1,3-Oxathiolane Nucleosides in human PBM cells.

Code	X or Y	R	Antiviral EC_{50}, μM	Cytotoxicity IC_{50}, μM
DLS-009	X = O	H	>100	>100
DLS-010	X = O	Me	64.4	>100
DLS-027	X = O	F	>100	>100
DLS-028	X = O	Cl	60.8	>100
DLS-044	X = O	Br	>100	>100
DLS-029	X = O	I	>100	>100
DLS-020	Y = NH_2	H	0.02	>100
DLS-011	Y = NH_2	Me	>10	>100
DLS-022	Y = NH_2	F	0.011	>100
DLS-023	Y = NH_2	Cl	38.7	>100
DLS-021	Y = NH_2	Br	77.4	>100
DLS-026	Y = NH_2	I	0.72	>100
DLS-058(−)	Y = NH_2	F	0.0077	>100
DLS-059(+)	Y = NH2	F	0.84	>100
DLS-053	Y = NH_2	CF_3	60.7	>100

TABLE 3-continued

EC_{50} and IC_{50} of Various Analogues of 1,3-Oxathiolane Nucleosides in human PBM cells.

Code	X or Y	R	Antiviral EC_{50}, μM	Cytotoxicity IC_{50}, μM



As indicated in Table 3, in general, the substituted cytosine 1,3-oxathiolane nucleosides are more active than the corresponding uracil nucleosides. One of the compounds, (±)-FTC, (referred to as "DLS-022", compound 8) not only exhibited exceptional activity (approximately 10 nM in PBM cells), but also quite low toxicity (>100 μM in PBM, Vero and CEM cells). This activity compares quite favorably with 2',3'-dideoxyadenosine (DDA, EC_{50}=0.91 μM), 3'-azido-2',3'-dideoxyuridine (AZDU, EC_{50}= 0.18–0.46 μM), 3'-dideoxythymidine (DDT, EC_{50}=0.17 μM), and dideoxycytidine (DDC, EC_{50}=0.011 μM).

The IC_{50} of (±)-FTC was measured as over 100 μM, indicating that the compound was not toxic in uninfected PBM cells evaluated up to 100 μM.

EXAMPLE 5

Antiviral Activity of the Enantiomers of FTC Resolved by HPLC

The enantiomers of FTC were isolated by the method of Example 3, and the antiviral activity evaluated by the method of Example 4. The results are provided in Table 4, and illustrated in FIG. **4**.

TABLE 4

Antiviral Activity of the (+) and (−) Enantiomers of FTC

Treatment	Concn., μM	DPM/ml	% Inhibition (Corrected)	EC_{50}:μM
FTC (±)	0.0001	73,755	26.6	0.018
	0.005	83,005	16.3	
	0.01	60,465	41.3	
	0.05	34,120	70.4	
	0.1	14,160	92.4	
	0.5	18,095	88.1	
	1	7,555	99.7	
	5	7,940	99.3	
	10	5,810	101.7	
FTC (−)	0.001	76,275	23.8	0.02
	0.005	58,590	43.3	
	0.01	75,350	24.8	
	0.05	28,890	76.2	
	0.1	13,175	93.5	
	0.5	9,485	97.6	
FTC (+)	0.001	94,340	3.8	0.28
	0.005	107,430	−10.6	
	0.01	99,465	−1.8	
	0.05	87,120	11.8	
	0.1	86,340	12.7	
	0.5	33,225	71.4	

As indicated in Table 4, the (−)-enantiomer of FTC is approximately one order of magnitude more potent than the (+)-FTC enantiomer, and has approximately the same anti-HIV activity as the racemic mixture. Neither the enantiomers nor the racemic mixture is toxic up to 100 μM as measured by Trypan Blue exclusion in human PBM cels.

EXAMPLE 6

Antiviral Activity of Enantiomers Resolved by Method of Example 2

The enantiomers of (±)-FTC were also resolved by the method of Example 2, and the antiviral activity evaluated by the method of Example 4. The results are illustrated in FIG. **5**. As indicated in FIG. **5**, the EC_{50} of the racemic mixture of FTC was measured at 0.017 μM, the EC_{50} of (−)-FTC at 0.0077 μM, and the EC_{50} of (+)-FTC at 0.84 μM.

The differences in EC_{50}s as measured in Examples 5 and 6 may be due to a number of factors, including differences in donor PBM cells, the inherent error of the anti-HIV screening procedure (estimated at approximately 10%), and differences in the measurement of concentration of the nucleosides as resolved in the methods of Examples 2 and 3. In the method of Example 2, the FTC enantiomers were isolated as solids and weighed to prepare the testing solution. In the method of Example 3, the concentration of the FTC enantiomers was estimated from UV absorption measurements.

The data indicates that the (+) enantiomer is significantly less potent than the (−) entantiomer or the racemic mixture.

EXAMPLE 7

Antiviral Activity of the Enantiomers of BCH-189 Resolved by HPLC

The enantiomers of BCH-189 were isolated by the method of Example 3, and the antiviral activity evaluated by the method of Example 4. The results are provided in Table 5, and illustrated in FIG. **6**.

TABLE 5

Antiviral Activity of the (+) and (−) Enantiomers of BCH-189

Treatment	Concn., μM	DPM/ml	% Inhibition (Corrected)	EC_{50}:μM
Blanks	mean	762		
HIV Std.		158,705		
Uninfected	mean	7,320		
Control	±SD	4,520		
Infected	mean	97,795		
Control	±SD	6,790		
BCH-189 (±)	0.001	65,170	36.1	0.081
	0.005	62,595	38.9	
	0.01	70,875	29.8	
	0.05	77,650	22.3	
	0.1	33,165	71.4	
	0.5	10,765	96.2	
	1	7,745	99.5	
	5	6,800	100.6	
	10	4,470	103.2	
BCH-189 (−)	0.001	76,400	23.6	0.016
	0.005	66,875	34.2	
	0.01	54,170	48.2	
	0.05	57,615	44.4	
	0.1	34,705	69.7	
	0.5	15,250	91.2	
BCH-189 (+)	0.00085	71,795	28.7	0.23
	0.00425	99,710	−2.1	
	0.0085	68,355	32.5	
	0.0415	82,845	16.5	
	0.0825	65,100	36.1	
	0.412	43,260	60.3	

As indicated in Table 6, the (−)-enantiomer of BCH-189 is approximately one order of magnitude more potent than

the (+)-FTC enantiomer, and has approximately the same anti-HIV activity as the racemic mixture. Neither enantiomer exhibited any toxicity in a concentration up to 100 μM as measured by Trypan Blue exclusion in human PBM cells.

EXAMPLE 8

Uptake of (±)-FTC Into Human PBM Cells

Studies were undertaken using radiolabeled agent in order to follow the intracellular profiles of the parent drug and metabolites detected within the cell. All studies were conducted in duplicate. Human peripheral blood mononuclear cells (PBM cells) are suspended in RPMI 1640 medium containing 10% getal calf serum and antibiotics (2×10"cells/ml), 10 ml per timepoint) and incubated with addition of 10 μM FTC (specific ctivity about 700 dpm/pmol). Cells are exposed to the drug for 2, 6, 12 and 24 hours. At these timepoints, the medium is removed and the cells are washed two times with cold Hank's balanced salt solution. Extraction is performed with addition of 0.2 ml of 60% cold methanol/water and stored overnight at −70° C. The following morning, the suspensions are centrifuged and extractions are repeated two times for 0.5 hours at −70° C. The total supernatants (0.6 ml) are lyophilized to dryness. The residues are resuspended in 250 μl of water and aliquots comprising between 50 and 100 μl are analyzed by HPLC. Quantitation of intracellular parent drug and metabolic derivatives are conducted by HPLC. Because of the potential acid lability of some compounds, a buffer system close to physiological pH is used for the separation of the metabolites.

FIG. **7** is a graph of the uptake of tritiated (±)-FTC in human PBM cells (average of two determinations) in time (hours) versus pmol/10^6 cells. The uptake studies indicate that radiolabeled FTC is readily taken up in human lymphocytes, that produce very large amounts of 5'-triphosphate.

EXAMPLE 9

Uptake of (±)-FTC into Human Liver Cells; HVB Activity of FTC

The same procedure was used with human liver cells as with PBM cells to determine uptake of FTC.

The (±)-FTC is taken up by hepG2 cells in large amounts. These human liver cells metabolize a large percentage of the (±)-FTC to (±)-FTC triphosphate.

These data in conjunction with other data indicate that (±)-FTC, as well as its (−) and (+) enantiomers, are effective as antiviral agents against HBV (hepatitis B virus).

EXAMPLE 10

Egress of (±)-FTC from Human PBM Cells

Studies were performed using radiolabeled FTC in order to follow the intracellular profiles of the parent drug and metabolites detected within the cell after removal of drug at different times after pulsing for 24 hours, the time needed for high levels of triphosphates to accumulate. Studies are conducted in duplicate. Uninfected cells (2–106 ml) are suspended in the appropriate medium supplemented with serum (10 ml per timepoint) and incubated at 37° C. in a 5% CO_2 incubator. Radiolabeled FTC concentration is 10 μM. After pulsing the cells with the labeled compound for the desired time, the cells are thoroughly washed and then replenished with fresh medium without the antiviral drugs (0 hr). At 0, 2, 4, 6, 12, 24, and 48 hours (second incubation time), the cells are removed, and immediately extracted with 60% cold methanol/water. The extract is obtained by centrifugation and removal of the cell pellet. The extracts are lyophilized and then stored at −70° C. Prior to analysis, the material is resuspended in 250 τl of HPLC buffer and immediately analyzed. Quantitation of intracellular parent drug and metabolic derivatives are conducted by HPLC, as follows.

Either a Micromeritics or Hewlett-Packard model 1090 PHLC system is used with an anion exchange Partisil 10 SAX column (Whatman, Inc.), at a flow rate of 1 ml/min, 1 kpsi pressure, using uv detection at 262 nm.

The mobile phase consists of:

a. deionized water

b. 2 mM NaH_2PO_4/16 mM NaOAc (pH 6.6)

c. 15 mM NaH_2PO_4/120.2 mM NaOAc (pH 6.6)

d. 100 mM NaH_2PO_4/800 mM NaOAc (pH 6.6)

Separation method: isocratic for 5 min with A, followed by a 15 min linear gradient to 100% B, followed by a 20 min linear gradient to 100% C, followed by 10 min linear gradient to 100% D, followed by 30 min isocratic with 100% D.

Retention times (minutes) in human cells:

Compound	Unchanged	Mono-phosphate	Diphosphate	Triphosphate
DLS-022	5.0	39.0	55.0	68.0
BCH-189	3.5	40.0	55.0	69.0

FIG. **8** is a graph of the egress of radiolabeled (±)-FTC from human PBM cells, measured in hours after drug removal versus concentration (pmol/10^6 cells). As indicated in the Figure, FTC-triphosphate has an intracellular half-life of approximately 12 hours and can be easily detected intracellularly at concentrations of 1–5 μM 48 hours after the removal of the extracellular drug, which is well above the EC_{50} for the compound. Further, the affinity (K') for (±)-FTC triphosphate against HIV RT is 0.2 μM, below the 48 hour concentration level.

III. Preparation of Pharmaceutical Compositions

Humans suffering from diseases caused by HIV infection can be treated by administering to the patient an effective amount of (±)-FTC, or its (−) or (+) enantiomer or a pharmaceutically acceptable salt thereof in the presence of a pharmaceutically acceptable carrier or diluent. The active materials can be administered by any appropriate route, for example, orally, parenterally, intravenously, intradermally, subcutaneously, or topically, in liquid or solid form.

Pharmaceutically acceptable salts are known to those in the art and include those derived from pharmaceutically acceptable inorganic and organic acids and bases. Examples of suitable acids include hydrochloric, hydrobromic, sulfuric, nitric, perchloric, fumaric, maleic, phosphoric, glycollic, lactic, salicyclic, succinic, toluene-p-sulfonic, tartaric, acetic, citric, methanesulfonic, formic, benzoic, malonic, naphthalene-2-sulfonic and benzenesulfonic acids. Salts derived from appropriate bases include alkali metal (e.g., sodium), alkaline earth metal (e.g., magnesium), ammonium and quaternary amine.

The active compound is included in the pharmaceutically acceptable carrier or diluent in an amount sufficient to deliver to a patient a therapeutically effective amount of

compound to inhibit viral replication in vivo, especially HIV and HBV replication, without causing serious toxic effects in the patient treated. By "HIV inhibitory amount" is meant an amount of active ingredient sufficient to exert an HIV inhibitory effect as measured by, for example, an assay such as the ones described herein.

A preferred dose of (−) or (±)-FTC will be in the range from about 1 to 20 mg/kg of bodyweight per day, more generally 0.1 to about 100 mg per kilogram body weight of the recipient per day.

The compound is conveniently administered in unit dosage form: for example containing 7 to 7000 mg, preferably 70 to 1400 mg of active ingredient per unit dosage form.

Ideally the active ingredient should be administered to achieve peak plasma concentrations of the active compound of from about 0.2 to 70 μM, preferably about 1.0 to 10 μM. This may be achieved, for example, by the intravenous injection of a 0.1 to 5% solution of the active ingredient, optionally in saline, or administered as a bolus of the active ingredient.

The concentration of active compound in the drug composition will depend on absorption, inactivation, and excretion rates of the drug as well as other factors known to those of skill in the art. It is to be noted that dosage values will also vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition. The active ingredient may be administered at once, or may be divided into a number of smaller doses to be administered at varying intervals of time.

A preferred mode of administration of the active compound is oral. Oral compositions will generally include an inert diluent or an edible carrier. They may be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition.

The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring. When the dosage unit form is a capsule, it can contain, in addition to material of the above type, a liquid carrier such as a fatty oil. In addition, dosage unit forms can contain various other materials which modify the physical form of the dosage unit, for example, coatings of sugar, shellac, or other enteric agents. (±)-FTC, or its (−) or (+)-enantiomer or pharmaceutically acceptable salts thereof can be administered as a component of an elixir, suspension, syrup, wafer, chewing gum or the like. A syrup may contain, in addition to the active compounds, sucrose as a sweetening agent and certain preservatives, dyes and colorings and flavors. (±)-FTC, or its (−) or (+)-enantiomers, or pharmaceutically acceptable salts thereof can also be mixed with other active materials that do not impair the desired action, or with materials that supplement the desired action, such as antibiotics, antifungals, antiinflamnatories, or other antivirals, including other nucleoside anti-HIV compounds.

Solutions or suspensions used for parenteral, intradermal, subcutaneous, or topical application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The parental preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

The pharmaceutical composition can also include antifungal agents, chemotherapeutic agents, and other antiviral agents such as interferon, including α, β, and gamma interferon.

If administered intravenously, preferred carriers are physiological saline or phosphate buffered saline (PBS).

In a preferred embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc.

Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) are also preferred as pharmaceutically acceptable carriers. These may be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811 (which is incorporated herein by reference in its entirety). For example, liposome formulations may be prepared by dissolving appropriate lipid(s) (such as stearoyl phosphatidyl ethanolamine, stearoyl phosphatidyl choline, aractiadoyl phosphatidyl choline, and cholesterol) in an inorganic solvent that is then evaporated, leaving behind a thin film of dried lipid on the surface of the container. An aqueous solution of the active compound or its monophosphate, diphosphate, and/or triphosphate derivatives are then introduced into the container. The container is then swirled by hand to free lipid material from the sides of the container and to disperse. lipid aggregates, thereby forming the liposomal suspension.

IV. Preparation of Phosphate Derivatives of FTC

Mono, di, and triphosphate derivative of FTC can be prepared as described below.

The monophosphate can be prepared according to the procedure of Imai et al., *J. Org. Chem.,* 34(6), 1547–1550 (June 1969). For example, about 100 mg of FTC and about 280 μl of phosphoryl chloride are reacted with stirring in about 8 ml of dry ethyl acetate at about 0° C. for about four hours. The reaction is quenched with ice. The aqueous phase is purified on an activated charcoal column, eluting with 5% ammonium hydroxide in a 1:1 mixture of ethanol and water. Evaporation of the eluant gives ammonium FTC-5'-monophosphate.

The diphosphate can be prepared according to the procedure of Davisson et al., *J. Orq. Chem.,* 52(9), 1794–1801 (1987). FTC diphosphate can be prepared from the corresponding tosylate, that can be prepared, for example, by

reacting the nucleoside with tosyl chloride in pyridine at room temperature for about 24 hours, working up the product in the usual manner (e.g., by washing, drying, and crystallizing it).

The triphosphate can be prepared according to the procedure of Hoard et al., *J. Am. Chem. Soc.,* 87(8), 1785–1788 (1965). For FTC is activated (by making a imidazolide, according to methods known to those skilled in the art) and treating with tributyl ammonium pyrophosphate in DMF. The reaction gives primarily the triphosphate of the nucleoside, with some unreacted monophosphate and some diphosphate. Purification by anion exchange chromatography of a DEAE column is followed by isolation of the triphosphate, e.g., as the tetrasodium salt.

This invention has been described with reference to its preferred embodiments. Variations and modifications of the invention, a method of resolution and antiviral activity of nucleoside enantiomers, will be obvious to those skilled in the art from the foregoing detailed description of the invention. It is intended that all of these variations and modifications be included within the scope of the appended claims.

We claim:

1. The (−)-enantiomer of cis-4-amino-5-fluoro-1-(2-hydroxymethyl-1,3-oxathiolane-5-yl)-(1H)-pyrimidin-2-one that is at least 95% free of the corresponding (+)-enantiomer.

2. (−)-Cis-4-amino-5-fluoro-1-(2-hydroxymethyl-1,3-oxathiolane-5-yl)-(1H)-pyrimidin-2-one or a pharmaceutically acceptable salt, ester or salt of an ester thereof.

3. The substantially pure (−)-enantiomer of cis-4-amino-5-fluoro-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one or a pharmaceutically acceptable salt, ester, or salt of an ester thereof, wherein the (+) enantiomer is present in an amount of no more than 5% w/w.

4. The compound of claim **3** wherein the (+)-enantiomer is present in an amount of no more than about 2% w/w.

5. The compound of claim **3** wherein the (+)-enantiomer is present in an amount of less than 1% w/w.

6. A pharmaceutical composition comprising a compound as claimed in any one of claims **2**–**5** in combination with a pharmaceutically acceptable carrier.

7. (−)-Cis-4-amino-5-fluoro-1-(2-hydroxymethyl-1,3-oxathiolane-5-yl)-(1H)-pyrimidin-2-one or a pharmaceutically acceptable salt thereof.

8. The 5'-O-alkyl derivative of the (−)-enantiomer of cis-4-amino-5-fluoro-1-(2-hydroxymethyl-1,3-oxathiolane-5-yl)-(1H)-pyrimidin-2-one.

9. The 5'-O-alkylC(O)-derivative of the (−)-enantiomer of cis-4-amino-5-fluoro-1-(2-hydroxymethyl-1,3-oxathiolane-5-yl)-(1H)-pyrimidin-2-one.

10. The derivative of claim **9**, wherein alkylC(O)— is selected from the group consisting of acetic, propionic, butyric, and pentanoic.

11. The monophosphate, diphosphate, or triphosphate of the (−)-enantiomer of cis-4-amino-5-fluoro-1-(2-hydroxymethyl-1,3-oxathiolane-5-yl)-(1H)-pyrimidin-2-one.

12. A pharmaceutically acceptable salt of the (−)-enantiomer of cis-4-amino-5-fluoro-1-(2-hydroxymethyl-1,3-oxathiolane-5-yl)-(1H)-pyrimidin-2-one that is at least 95% free of the corresponding (+)-enantiomer.

13. A pharmaceutical composition comprising an effective HIV treatment amount for humans of the (−)-enantiomer of cis-4-amino-5-fluoro-1-(2-hydroxymethyl-1,3-oxathiolane-5-yl)-(1H)-pyrimidin-2-one that is at least 95% free of the corresponding (+)-enantiomer, in combination with a pharmaceutically acceptable carrier or diluent.

14. A pharmaceutical composition comprising an effective HIV treatment amount for humans of the (−)-enantiomer of a pharmaceutically acceptable salt of a compound of cis-4-amino-5-fluoro-1-(2-hydroxymethyl-1,3-oxathiolane-5-yl)-(1H)-pyrimdin-2-one that is at least 95% free of the corresponding (+)-enantiomer, in combination with a pharmaceutically acceptable carrier or diluent.

15. The pharmaceutical composition of claim **13**, in a form for oral administration.

16. The pharmaceutical composition of claim **15**, wherein the composition is in tablet form.

17. The pharmaceutical composition of claim **15**, wherein the composition is in capsule form.

18. The pharmaceutical composition of claim **13**, wherein the composition is a liquid.

19. The pharmaceutical composition of claim **13**, in a form for intravenous administration.

20. The pharmaceutical composition of claim **19**, wherein the carrier comprises a sterile diluent for injection.

21. The pharmaceutical composition of claim **13**, in a form for topical administration.

22. The pharmaceutical composition of claim **14**, in a form for oral administration.

23. The pharmaceutical composition of claim **22**, wherein the composition is in tablet form.

24. The pharmaceutical composition of claim **22**, wherein the composition is in capsule form.

25. The pharmaceutical composition of claim **14**, wherein the composition is a liquid.

26. The pharmaceutical composition of claim **14**, in a form for intravenous administration.

27. The pharmaceutical composition of claim **17**, wherein the carrier comprises a sterile diluent for injection.

28. The pharmaceutical composition of claim **14**, in a form for topical administration.

* * * * *

Exhibit C

US008592397B2

(12) **United States Patent**
Dahl et al.

(10) **Patent No.: US 8,592,397 B2**
(45) **Date of Patent: Nov. 26, 2013**

(54) **COMPOSITIONS AND METHODS FOR COMBINATION ANTIVIRAL THERAPY**

(75) Inventors: **Terrence C. Dahl**, Sunnyvale, CA (US); **Mark M. Menning**, San Francisco, CA (US); **Reza Oliyai**, San Carlos, CA (US)

(73) Assignee: **Gilead Sciences, Inc.**, Foster City, CA (US)

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(52) **U.S. Cl.**
USPC **514/81**

(58) **Field of Classification Search**
USPC 514/45; 424/400, 408
See application file for complete search history.

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(Continued)

Primary Examiner — Alton Pryor

(57) **ABSTRACT**

The present invention relates to therapeutic combinations of [2-(6-amino-purin-9-yl)-1-methyl-ethoxymethyl]-phosphonic acid diisopropoxycarbonyloxymethyl ester (tenofovir disoproxil fumarate, Viread®) and (2R, 5S, cis)-4-amino-5-fluoro-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one(emtricitabine, Emtriva™, (-)-cis FTC) and their physiologically functional derivatives. The combinations may be useful in the treatment of HIV infections, including infections with HIV mutants bearing resistance to nucleoside and/or non-nucleoside inhibitors. The present invention is also concerned with pharmaceutical compositions and formulations of said combinations of tenofovir disoproxil fumarate and emtricitabine, and their physiologically functional derivatives, as well as therapeutic methods of use of those compositions and formulations.

26 Claims, No Drawings

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COMPOSITIONS AND METHODS FOR COMBINATION ANTIVIRAL THERAPY

This non-provisional application is a continuation of U.S. patent application Ser. No. 10/540,794, filed Mar. 20, 2006, which is a national stage entry of PCT/US04/00832, filed Jan. 13, 2004 which claims the benefit of Provisional Application Nos. 60/440,246 and 60/440,308, both filed Jan. 14, 2003, which is incorporated herein by reference.

FIELD OF THE INVENTION

The invention relates generally to combinations of compounds with antiviral activity and more specifically with anti-HIV properties. In particular, it relates to chemically stable combinations of structurally diverse anti-viral agents.

BACKGROUND OF THE INVENTION

Human immunodeficiency virus (HIV) infection and related diseases are a major public health problem worldwide. Human immunodeficiency virus type 1 (HIV-1) encodes at least three enzymes which are required for viral replication: reverse transcriptase (RT), protease (Prt), and integrase (Int). Although drugs targeting reverse transcriptase and protease are in wide use and have shown effectiveness, particularly when employed in combination, toxicity and development of resistant strains have limited their usefulness (Palella, et al *N. Engl. J. Med*. (1998) 338:853-860; Richman, D. D. *Nature* (2001) 410:995-1001). Human immunodeficiency virus type 1 (HIV-1) protease (Prt) is essential for viral replication and is an effective target for approved antiviral drugs. The HIV Prt cleaves the viral Gag and Gag-Pol polyproteins to produce viral structural proteins (p17, p24, p7 and p6) and the three viral enzymes. Combination therapy with RT inhibitors has proven to be highly effective in suppressing viral replication to unquantifiable levels for a sustained period of time. Also, combination therapy with RT and Prt inhibitors (PI) have shown synergistic effects in suppressing HIV replication. Unfortunately, a high percentage, typically 30 to 50% of patients currently fail combination therapy due to the development of drug resistance, non-compliance with complicated dosing regimens, pharmacokinetic interactions, toxicity, and lack of potency. Therefore, there is a need for new HIV-1 inhibitors that are active against mutant HIV strains, have distinct resistance profiles, fewer side effects, less complicated dosing schedules, and are orally active. In particular, there is a need for a less onerous dosage regimen, such as once per day oral dosing, optimally with as few pills as possible.

The use of combinations of compounds can yield an equivalent antiviral effect with reduced toxicity, or an increase in drag efficacy. Lower overall drug doses can reduce the frequency of occurrence of drug-resistant variants of HIV. Many different methods have been used to examine the effects of combinations of compounds acting together in different assay systems (Furman WO 02/068058). Lower doses predict better patient compliance when pill burden decreases, dosing schedules are simplified and, optionally, if synergy between compounds occurs (Loveday, C. "Nucleoside reverse transcriptase inhibitor resistance" (2001) *JAIDS Journal of Acquired Immune Deficiency Syndromes* 26:S10-S24). AZT (Zidovudine™, 3'-azido, 3'-deoxythymidine) demonstrates synergistic antiviral activity in vitro in combination with agents that act at HIV-1 replicative steps other than reverse transcription, including recombinant soluble CD4 castanospermine and recombinant interferon-α. However, it must be noted that combinations of compounds can give rise to increased cytotoxicity. For example, AZT and recombinant interferon-α have an increased cytotoxic effect on normal human bone marrow progenitor cells.

Chemical stability of combinations of antiviral agents is an important aspect of co-formulation success and the present invention provides examples of such combinations.

There is a need for new combinations of orally-active drugs for the treatment of patients infected with certain viruses, e.g. HIV, that provide enhanced therapeutic safety and efficacy, impart lower resistance, and predict higher patient compliance.

SUMMARY OF THE INVENTION

The present invention provides combinations of antiviral compounds, in particular compositions and methods for inhibition of HIV. In an exemplary aspect, the invention includes a composition including tenofovir disoproxil fumarate and emtricitabine which has anti-HIV activity. The composition of tenofovir DF and emtricitabine is both chemically stable and either synergistic and/or reduces the side effects of one or both of tenofovir DF and emtricitabine. Increased patient compliance is likely in view of the lower pill burden and simplified dosing schedule.

The present invention relates to therapeutic combinations of [2-(6-amino-purin-9-yl)-1-methyl-ethoxymethyl]-phosphonic acid diisopropoxycarbonyloxymethyl ester fumarate (tenofovir disoproxil fumarate, tenofovir DF, TDF, Viread®) and (2R, 5S, cis)-4-amino-5-fluoro-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one (emtricitabine, Emtriva™, (-)-cis FTC) and their use in the treatment of HIV infections including infections with HIV mutants bearing resistance to nucleoside and/or non-nucleoside inhibitors. The present invention is also concerned with pharmaceutical compositions and formulations of said combinations of tenofovir disoproxil fumarate and emtricitabine. Another aspect of the invention is a pharmaceutical formulation comprising a physiologically functional derivative of tenofovir disoproxil fumarate or a physiologically functional derivative of emtricitabine.

Therapeutic combinations and pharmaceutical compositions and formulations of the invention include the combination of PMEA or PMPA (tenofovir) compounds with emtricitabine or (2R, 5S, cis)-4-amino-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one (3TC, lamivudine, Epivir™), and their use in the treatment of HIV infections.

One aspect of the invention is a method for the treatment or prevention of the symptoms or effects of an HIV infection in an infected animal which comprises administering to, i.e. treating, said animal with a therapeutically effective amount of a combination comprising [2-(6-ammo-purin-9-yl)-1-methyl-ethoxymethyl]-phosphonic acid diisopropoxycarbonyloxymethyl ester fumarate (tenofovir DF, TDF) or a physiologically functional derivative thereof, and (2R, 5S, cis)-4-amino-5-fluoro-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one (emtricitabine) or a physiologically functional derivative thereof.

Another aspect of the invention is a unit dosage form of a therapeutic combination comprising tenofovir disoproxil fumarate and emtricitabine, or physiological functional derivatives thereof. The unit dosage form may be formulated for administration by oral or other routes and is unexpectedly chemically stable in view of the properties of the structurally diverse components.

Another aspect of the invention is directed to chemically stable combination antiviral compositions comprising tenofovir disoproxil fumarate and emtricitabine. In a further

aspect of the invention, the chemically stable combinations of tenofovir disoproxil fumarate and emtricitabine further comprise a third antiviral agent. In this three-component mixture, the unique chemical stability of tenofovir disoproxil fumarate and emtricitabine is taken advantage of in order to enable the combination with the third antiviral agent. Particularly useful third agents include, by way of example and not limitation, those of Table A. Preferably, the third component is an agent approved for antiviral use in humans, more preferably, it is an NNRTI or a protease inhibitor (PI), more preferably yet, it is an NNRTI. In a particularly preferred embodiment, the invention is directed to a combination of the chemically stable mixture of tenofovir disoproxil fumarate and emtricitabine together with efavirenz.

Another aspect of the invention is a patient pack comprising at least one, typically two, and optionally, three active ingredients and other antiviral agents selected from tenofovir disoproxil fumarate and emtricitabine, and an information insert containing directions on the use of tenofovir disoproxil fumarate and emtricitabine together in combination.

Another aspect of the invention is a process for preparing the combinations hereinbefore described, which comprises bringing into association tenofovir DF and emtricitabine of the combination in a medicament to provide an antiviral effect. In a further aspect of the present invention, there is provided the use of a combination of the present invention in the manufacture of a medicament for the treatment of any of the aforementioned viral infections or conditions.

DETAILED DESCRIPTION OF THE INVENTION

While the invention will be described in conjunction with the enumerated claims, it will be understood that they are not intended to limit the invention to those claims. On the contrary, the invention is intended to cover all alternatives, modifications, and equivalents, which may be included within the scope of the present invention as defined by the claims.

Definitions

Unless stated otherwise, the following terms and phrases as used herein are intended to have the following meanings:

When tradenames are used herein, applicants intend to independently include the tradename product and the active pharmaceutical ingredients) of the tradename product.

The term "chemical stability" means that the two primary antiviral agents in combination are substantially stable to chemical degradation. Preferably, they are sufficiently stable in physical combination to permit commercially useful shelf life of the combination product. Typically, "chemically stable" means that a first component of the mixture does not act to degrade a second component when the two are brought into physical combination to form a pharmaceutical dosage form. More typically, "chemically stable" means that the acidity of a first component does not catalyzes or otherwise accelerate the acid decomposition of a second component. By way of example and not limitation, in one aspect of the invention, "chemically stable" means that tenofovir disoproxil fumarate is not substantially degraded by the acidity of emtricitabine. "Substantially" in this context means at least about less than 10%, preferably less than 1%, more preferably less than 0.1%, more preferably yet, less than 0.01% acid degradation of tenofovir disoproxil fumarate over a 24-hour period when the products are in a pharmaceutical dosage form.

The terms "synergy" and "synergistic" mean that the effect achieved with the compounds used together is greater than the sum of the effects that results from using the compounds separately, i.e. greater than what would be predicted based on the two active ingredients administered separately. A synergistic effect may be attained when the compounds are: (1) co-formulated and administered or delivered simultaneously in a combined formulation; (2) delivered by alternation or in parallel as separate formulations; or (3) by some other regimen. When delivered in alternation therapy, a synergistic effect may be attained when the compounds are administered or delivered sequentially, e.g. in separate tablets, pills or capsules, or by different injections in separate syringes. In general, during alternation therapy, an effective dosage of each active ingredient is administered sequentially, i.e. serially, whereas in combination therapy, effective dosages of two or more active ingredients are administered together. A synergistic antiviral effect denotes an antiviral effect which is greater than the predicted purely additive effects of the individual compounds of the combination.

The term "physiologically functional derivative" means a pharmaceutically active compound with equivalent or near equivalent physiological functionality to tenofovir DF or emtricitabine when administered in combination with another pharmaceutically active compound in a combination of the invention. As used herein, the term "physiologically functional derivative" includes any: physiologically acceptable salt, ether, ester, prodrug, solvate, stereoisomer including enantiomer, diastereomer or stereoisomerically enriched or racemic mixture, and any other compound which upon administration to the recipient, is capable of providing (directly or indirectly) such a compound or an antivirally active metabolite or residue thereof.

"Bioavailability" is the degree to which the pharmaceutically active agent becomes available to the target tissue after the agent's introduction into the body. Enhancement of the bioavailability of a pharmaceutically active agent can provide a more efficient and effective treatment for patients because, for a given dose, more of the pharmaceutically active agent will be available at the targeted tissue sites.

The compounds of the combinations of the invention may be referred to as "active ingredients" or "pharmaceutically active agents."

The term "prodrug" as used herein refers to any compound that when administered to a biological system generates the drug substance, i.e. active ingredient, as a result of spontaneous chemical reaction(s), enzyme catalyzed chemical reaction(s), and/or metabolic chemical reaction(s).

"Prodrug moiety" means a labile functional group which separates from the active inhibitory compound during metabolism, systemically, inside a cell, by hydrolysis, enzymatic cleavage, or by some other process (Bundgaard, Hans, "Design and Application of Prodrugs" in *Textbook of Drug Design and Development* (1991), P. Krogsgaard-Larsen and H. Bundgaard, Eds. Harwood Academic Publishers, pp. 113-191). Prodrug moieties can serve to enhance solubility, absorption and lipophilicity to optimize drug delivery, bioavailability and efficacy. A "prodrug" is thus a covalently modified analog of a therapeutically-active compound.

"Alkyl" means a saturated or unsaturated, branched, straight-chain, branched, or cyclic hydrocarbon radical derived by the removal of one hydrogen atom from a single carbon atom of a parent alkane, alkene, or alkyne. Typical alkyl groups consist of 1-18 saturated and/or unsaturated carbons, such as normal, secondary, tertiary or cyclic carbon atoms. Examples include, but are not limited to: methyl, Me (—CH_3), ethyl, Et (—CH_2CH_3), acetylenic (—C≡CH), ethylene, vinyl (—CH=CH_2), 1-propyl, n-Pr, n-propyl (—$CH_2CH_2CH_3$), 2-propyl, i-Pr, i-propyl (—$CH(CH_3)_2$), allyl (—CH_2CH=CH_2), propargyl (—CH_2C≡CH), cyclopropyl (—C_3H_5), 1-butyl, n-Bu, n-butyl

(—$CH_2CH_2CH_2CH_3$), 2-methyl-1-propyl, i-Bu, i-butyl (—$CH_2CH(CH_3)_2$), 2-butyl, s-Bu, s-butyl (—$CH(CH_3)CH_2CH_3$), 2-methyl-2-propyl, t-Bu, t-butyl (—$C(CH_3)_3$), 1-pentyl, n-pentyl, (—$CH_2CH_2CH_2CH_2CH_3$), 2-pentyl (—$CH(CH_3)CH_2CH_2CH_3$), 3-pentyl (—$CH(CH_2CH_3)_2$), 2-methyl-2-butyl (—$C(CH_3)_2CH_2CH_3$), cyclopentyl (—C_5H_9), 3-methyl-2-butyl (—$CH(CH_3)CH(CH_3)_2$), 3-methyl-1-butyl (—$CH_2CH_2CH(CH_3)_2$), 2-methyl-1-butyl (—$CH_2CH(CH_3)CH_2CH_3$), 1-hexyl (—$CH_2CH_2CH_2CH_2CH_2CH_3$), 5-hexenyl (—$CH_2CH_2CH_2CH_2CH{=}CH_2$) 1-hexyl (—$CH(CH_3)CH_2CH_2CH_2CH_3$), 3-hexyl (—$CH(CH_2CH_3)(CH_2CH_2CH_3)$), cyclohexyl (—$C_6H_{11}$), 2-methyl-2-pentyl (—$C(CH_3)_2CH_2CH_2CH_3$), 3-methyl-2-pentyl (—$CH(CH_3)CH(CH_3)CH_2CH_3$), 4-methyl-2-pentyl (—$CH(CH_3)CH_2CH(CH_3)_2$), 3-methyl-3-pentyl (—$C(CH_3)(CH_2CH_3)_2$), 2-methyl-3-pentyl (—$CH(CH_2CH_3)CH(CH_3)_2$), 2,3-dimethyl-2-butyl (—$C(CH_3)_2CH(CH_3)_2$), and 3,3-dimethyl-2-butyl (—$CH(CH_3)C(CH_3)_3$.

"Aryl" means a monovalent aromatic hydrocarbon radical of 6-20 carbon atoms derived by the removal of one hydrogen atom from a single carbon atom of a parent aromatic ring system. Typical aryl groups include, but are not limited to, radicals derived from benzene, substituted benzene, naphthalene, anthracene, biphenyl, and the like.

"Arylalkyl" refers to an acyclic alkyl radical in which one of the hydrogen atoms bonded to a carbon atom, typically a terminal or sp^3 carbon atom, is replaced with an aryl radical. Typical arylalkyl groups include, but are not limited to, benzyl, 2-phenylethan-1-yl, 2-phenylethen-1-yl, naphthylmethyl, 2-naphthylethan-1-yl, 2-naphthylethen-1-yl, naphthobenzyl, 2-naphthophenylethan-1-yl and the like. The arylalkyl group 6 to 20 carbon atoms e.g., the alkyl moiety, including alkanyl, alkenyl or alkynyl groups, of the arylalkyl group is 1 to 6 carbon atoms and the aryl moiety is 5 to 14 carbon atoms.

"Substituted alkyl", "substituted aryl", and "substituted arylalkyl" mean alkyl, aryl, and arylalkyl respectively, in which one or more hydrogen atoms are each independently replaced with a substituent. Typical substituents include, but are not limited to, —X, —R, —O^-, —OR, —SR, —S^-, —NR_2, —NR_3, ═NR, —CX_3, —CN, —OCN, —SCN, —N═C═O, —NCS, —NO, —NO_2, ═N_2, —N_3, NC(═O)R, —C(═O)R, —C(═O)NRR —$S(═O)_2O^-$, —$S(═O)_2OH$, —$S(═O)_2R$, —$OS(═O)_2OR$, —$S(═O)_2NR$, —S(═O)R, —$OP(═O)O_2RR$, —$P(═O)O_2RR$ —P(═O)$(O^-)_2$, —$P(═O)(OH)_2$, —C(═O)R, —C(═O)X, —C(S)R, —C(O)OR, —$C(O)O^-$, —C(S)OR, —C(O)SR, —C(S)SR, —C(O)NRR, —C(S)NRR, —C(NR)NRR, where each X is independently a halogen: F, Cl, Br, or I; and each R is independently —H, alkyl, aryl, heterocycle, or prodrug moiety.

"Heteroaryl" and "Heterocycle" refer to a ring system in which one or more ring atoms is a heteroatom, e.g. nitrogen, oxygen, and sulfur. Heterocycles are described in: Katritzky, Alan R., Rees, C. W., and Scriven, E. *Comprehensive Heterocyclic Chemistry* (1996) Pergamon Press; Paquette, Leo A.; *Principles of Modern Heterocyclic Chemistry* W. A. Benjamin, New York, (1968), particularly Chapters 1, 3, 4, 6, 7, and 9; "The Chemistry of Heterocyclic Compounds, A series of Monographs" (John Wiley & Sons, New York, 1950 to present), in particular Volumes 13, 14, 16, 19, and 28. Exemplary heterocycles include but are not limited to substituents, i.e. radicals, derived from pyrrole, indole, furan, benzofuran, thiophene, benzothiophene, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-quinolyl, 3-quinolyl, 4-quinolyl, 2-imidazole, 4-imidazole, 3-pyrazole, 4-pyrazole, pyridazine, pyrimidine, pyrazine, purine, cinnoline, pthalazine, quinazoline, quinoxaline, 3-(1,2,4-N)-triazolyl, 5-(1,2,4-N)-triazolyl, 5-tetrazolyl, 4-(1-O 3-N)-oxazole, 5-(1-O, 3-N)-oxazole, 4-(1-S, 3-N)-thiazole, 5-(1-S, 3-N)-thiazole, 2-benzoxazole, 2-benzothiazole, 4-(1,2,3N)-benzotriazole, and benzimidazole.

Stereochemical definitions and conventions used herein generally follow S. P. Parker, Ed., *McGraw-Hill Dictionary of Chemical Terms* (1984) McGraw-Hill Book Company, New York; and Eliel, E. and Wilen, S., *Stereochemistry of Organic Compounds* (1994) John Wiley & Sons, Inc., New York. Many organic compounds exist in optically active forms, i.e., they have the ability to rotate the plane of plane-polarized light. In describing an optically active compound, the prefixes D and L or R and S are used to denote the absolute configuration of the molecule about its chiral center(s). The prefixes d and l or (+) and (−) are employed to designate the sign of rotation of plane-polarized light by the compound, with (−) or l meaning that the compound is levorotatory. A compound prefixed with (+) or d is dextrorotatory. For a given chemical structure, these compounds, called stereoisomers, are identical except that they are mirror images of one another. A specific stereoisomer is also referred to as an enantiomer, and a mixture of such isomers is often called an enantiomeric mixture. A 50:50 mixture of enantiomers is referred to as a racemic mixture or a racemate. The terms "racemic mixture" and "racemate" refer to an equimolar mixture of two enantiomeric species, devoid of optical activity.

The term "chiral" refers to molecules which have the property of non-superimposability of the mirror image partner, while the term "achiral" refers to molecules which are superimposable on their mirror image partner.

The term "stereoisomers" refers to compounds which have identical chemical constitution, but differ with regard to the arrangement of the atoms or groups in space.

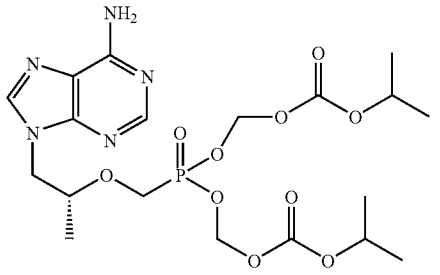
"Diastereomer" refers to a stereoisomer with two or more centers of chirality and whose molecules are not mirror images of one another. Diastereomers have different physical properties, e.g. melting points, boiling points, spectral properties, and reactivities. Mixtures of diastereomers may separate under high resolution analytical procedures such as electrophoresis and chromatography.

"Enantiomers" refer to two stereoisomers of a compound which are non-superimposable mirror images of one another.

Active Ingredients of the Combinations

The present invention provides novel combinations of two or more active ingredients being employed together. In some embodiments, a synergistic antiviral effect is achieved. In other embodiments, a chemically stable combination is obtained. The combinations include at least one active ingredient selected from (1) tenofovir disoproxil fumarate and physiologically functional derivatives, and at least one active ingredient selected from (2) emtricitabine and physiologically functional derivatives. The term "synergistic antiviral effect" is used herein to denote an antiviral effect which is greater than the predicted purely additive effects of the individual components (a) and (b) of the combination.

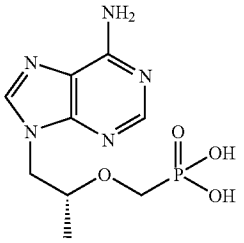
Tenofovir disoproxil fumarate (also known as Viread®, Tenofovir DF, Tenofovir disoproxil, TDF, Bis-POC-PMPA (U.S. Pat. Nos. 5,935,946, 5,922,695, 5,977,089, 6,043,230, 6,069,249) is a prodrug of tenofovir, and has the structure:



and including fumarate salt ($HO_2CCH_2CH_2CO_2^-$).

The chemical names for Tenofovir disoproxil include: [2-(6-amino-purin-9-yl)-1-methyl-ethoxymethyl]-phosphonic acid diisopropoxycarbonyloxymethyl ester; 9-[(R)-2-[[bis [[(isopropoxycarbonyl)oxy]methoxy]phosphinyl]methoxy] propyl]adenine; and 2,4,6,8-tetraoxa-5-phosphanonanedioic acid, 5-[[(1R)-2-(6-amino-9H-purin-9-yl)-1-methylethoxy] methyl]-, bis(1-methylethyl)ester, 5-oxide. The CAS Registry numbers include: 201341-05-1; 202138-50-9; 206184-49-8. It should be noted that the ethoxymethyl unit of tenofovir has a chiral center. The R (rectus, right handed configuration) enantiomer is shown. However, the invention also includes the S isomer. The invention includes all enantiomers, diastereomers, racemates, and enriched stereoisomer mixtures of tenofovir (PMPA) and physiologically functional derivatives thereof.

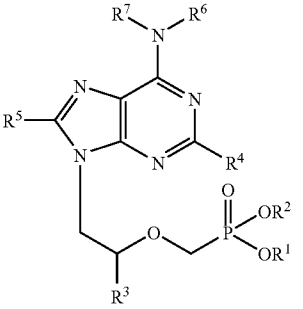
PMPA or tenofovir (U.S. Pat. Nos. 4,808,716, 5,733,788, 6,057,305) has the structure:



The chemical names of PMPA, tenofovir include: (R)-9-(2-phosphonylmethoxypropyl)adenine; and phosphonic acid, [[(1R)-2-(6-amino-9H-purin-9-yl)-1-methylethoxy] methyl]. The CAS Registry number is 147127-20-6.

Tenofovir disoproxil fumarate (DF) is a nucleotide reverse transcriptase inhibitor approved in the United States in 2001 for the treatment of HIV-1 infection in combination with other antiretroviral agents. Tenofovir disoproxil fumarate or Viread® (Gilead Science, Inc.) is the fumarate salt of tenofovir disoproxil. Viread® may be named as: 9-[(R)-2-[[bis [[(isopropoxycarbonyl)oxy]methoxy]phosphinyl]methoxy] propyl]adenine fumarate (1:1); or 2,4,6,8-tetraoxa-5-phosphanonanedioic acid, 5-[[(1R)-2-(6-amino-9H-purin-9-yl)-1-methylethoxy]methyl]-, bis(1-methylethyl) ester, 5-oxide, (2E)-2-butenedioate (1:1). The CAS Registry number is 202138-50-9.

Physiologically functional derivatives of tenofovir disoproxil fumarate include PMEA (adefovir, 9-((R)-2-(phosphonomethoxy)ethyl)adenine) and PMPA compounds. Exemplary combinations include a PMEA or PMPA compound in combination with emtricitabine or 3TC. PMEA and PMPA compounds have the structures:



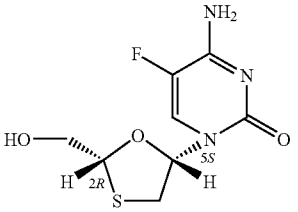
where PMEA (R^3 is H) and PMPA (R^3 is C_1-C_6 alkyl, C_1-C_6 substituted alkyl, or CH_2OR^8 where R^8 is C_1-C_6 alkyl, C_1-C_6 hydroxyalkyl or C_1-C_6 haloalkyl. R^6 and R^7 are independently H or C_1-C_6 alkyl. R^4 and R^5 are independently H, NH_2, NHR or NR_2 where R is C_1-C_6 alkyl. R^1 and R^2 are independently H, C_1-C_6 alkyl, C_1-C_6 substituted alkyl, C_6-C_{20} aryl, C_6-C_{20} substituted aryl, C_6-C_{20} arylalkyl, C_6-C_{20} substituted arylalkyl, acyloxymethyl esters $—CH_2OC(=O)R^9$ (e.g. POM) or acyloxymethyl carbonates $—CH_2OC(=O)OR^9$ (e.g. POC) where R^9 is C_1-C_6 alkyl, C_1-C_6 substituted alkyl, C_6-C_{20} aryl or C_6-C_{20} substituted aryl. For example, R_1 and R_2 may be pivaloyloxymethoxy, POM, $—CH_2OC(=O)C(CH_3)_3$; $—CH_2OC(=O)OC(CH_3)_3$; or POC, $—CH_2OC(=O)OCH(CH_3)_2$. Also for example, tenofovir has the structure where R^3 is CH_3, and R^1, R^2, R^4, R^5, R^6 and R^7 are H. Dialkyl phosphonates may be prepared according to the methods of: Quast et al (1974) *Synthesis* 490; Stowell et al (3990) *Tetrahedron Lett.* 3261; U.S. Pat. No. 5,663,159.

The PMPA compound may be enantiomerically-enriched or purified (single stereoisomer) where the carbon atom bearing R^3 may be the R or S enantiomer. The PMPA compound may be a racemate, i.e. a mixture of R and S stereoisomers.

Adefovir (9-(2-phosphonomethoxyethyl)adenine where R_1-R_7=H) is an exemplary PMEA compound (U.S. Pat. Nos. 4,808,716, 4,724,233). As the bis-pivalate prodrug, Adefovir dipivoxil, also known as bis-POM PMEA, (R_3-R_7=H, R_1 and R_2=$CH_2OC(=O)C(CH_3)_3$, pivoxil, POM, pivaloyloxymethoxy), is effective against HIV and Hepatitis B infections (U.S. Pat. Nos. 5,663,159, 6,451,340). Adefovir dipivoxil has demonstrated minor to moderate synergistic inhibition of HIV replication in combination with other compounds with anti-HIV activity including PMPA, d4T, ddC, nelfinavir, ritonavir, and saquinavir (Mulato et al (1997) *Antiviral Research* 36:91-97).

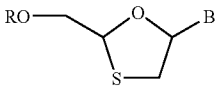
The invention includes all enantiomers, diastereomers, racemates, and enriched stereoisomer mixtures of PMEA and PMPA, and physiologically functional derivatives thereof.

Emtricitabine ((-)-cis-FTC, Emtriva™), a single enantiomer of FTC, is a potent nucleoside reverse transcriptase inhibitor approved for the treatment of HIV (U.S. Pat. Nos. 5,047,407, 5,179,104, 5,204,466, 5,210,085, 5,486,520, 5,538,975, 5,587,480, 5,618,820, 5,763,606, 5,814,639, 5,914,331, 6,114,343, 6,180,639, 6,215,004; WO 02/070518). The single enantiomer emtricitabine has the structure:



The chemical names for emtricitabine include: (-)-cis-FTC; β-L-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane; (2R,5S)-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine; and 4-amino-5-fluoro-1-(2-hydroxymethyl-[1,3]-(2R,5S)-oxathiolan-5-yl)-1H-pyrimidin-2-one. The CAS Registry numbers include: 143491-57-0; 143491-54-7. It should be noted that FTC contains two chiral centers, at the 2 and 5 positions of the oxathiolane ring, and therefore can exist in the form of two pairs of optical isomers (i.e. enantiomers) and mixtures thereof including racemic mixtures. Thus, FTC may be either a cis or a trans isomer or mixtures thereof. Mixtures of cis and trans isomers are diastereomers with different physical properties. Each cis and trans isomer can exist as one of two enantiomers or mixtures thereof including racemic mixtures. The invention includes all enantiomers, diastereomers, racemates, and enriched stereoisomer mixtures of emtricitabine and physiologically functional derivatives thereof. For example, the invention includes physiological functional derivatives such as the 1:1 racemic mixture of the enantiomers (2R, 5S, cis)-4-amino-5-fluoro-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one (emtricitabine) and its mirror image (2S, 5R, cis)-4-amino-5-fluoro-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one, or mixtures of the two enantiomers in any relative amount. The invention also includes mixtures of cis and trans forms of FTC.

Physiologically functional derivatives of emtricitabine include 1,3 oxathiolane nucleosides having the structure:

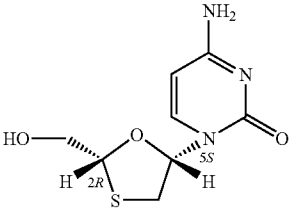


In the 1,3 oxathiolane nucleoside structure above, B is a nucleobase including any nitrogen-containing heterocyclic moiety capable of forming Watson-Crick hydrogen bonds in pairing with a complementary nucleobase or nucleobase analog, e.g. a purine, a 7-deazapurine, or a pyrimidine. Examples of B include the naturally occurring nucleobases: adenine, guanine, cytosine, uracil, thymine, and minor constituents and analogs of the naturally occurring nucleobases, e.g. 7-deazaadenine, 7-deazaguanine, 7-deaza-8-azaguanine, 7-deaza-8-azaadenine, inosine, nebularine, nitropyrrole, nitroindole, 2-aminopurine, 2-amino-6-chloropurine, 2,6-diaminopurine, hypoxanthine, pseudouridine, 5-fluorocytosine, 5-chlorocytosine, 5-bromocytosine, 5-iodocytosine, pseudocytosine, pseudoisocytosine, 5-propynylcytosine, isocytosine, isoguanine, 7-deazaguanine, 2-thiopyrimidine, 6-thioguanine, 4-thiothymine, 4-thiouracil, O^6-methylguanine, N^6-methyladenine, O^4-methylthymine, 5,6-dihydrothymine, 5,6-dihydrouracil, 4-methylindole, pyrazolo[3,4-D]pyrimidines (U.S. Pat. Nos. 6,143,877 and 6,127,121; WO 01/38584), and ethenoadenine (Fasman (1989) in *Practical Handbook of Biochemistry and Molecular Biology*, pp. 385-394, CRC Press, Boca Raton, Fla.).

Nucleobases B may be attached in the configurations of naturally-occurring nucleic acids to the 1,3 oxathiolane moiety through a covalent bond between the N-9 of purines, e.g. adenin-9-yl and guanin-9-yl, or N-1 of pyrimidines, e.g. thymin-1-yl and cytosin-1-yl (Blackburn, G. and Gait, M. Eds. "DNA and RNA structure" in *Nucleic Acids in Chemistry and Biology*, 2nd Edition, (1996) Oxford University Press, pp. 15-81).

Also in the 1,3 oxathiolane nucleoside structure above, R is H, C_1-C_{18} is alkyl, C_1-C_{18} substituted alkyl, C_2-C_{18} alkenyl, C_2-C_{18} substituted alkenyl, C_2-C_{18} alkynyl, C_2-C_{18} substituted alkynyl, C_6-C_{20} aryl, C_6-C_{20} substituted aryl, C_2-C_{20} heterocycle, C_2-C_{20} substituted heterocycle, phosphonate, phosphophosphonate, diphosphophosphonate, phosphate, diphosphate, triphosphate, polyethyleneoxy, or a prodrug moiety.

Physiologically functional derivatives of emtricitabine also include 3TC (lamivudine, Epivir®), a reverse transcriptase inhibitor approved in the United States for the treatment of HIV-1 infection in combination with AZT as Combivir® (GlaxoSmithKline). U.S. Pat. Nos. 5,859,021; 5,905,082; 6,177,435; 5,627,186; 6,417,191. Lamivudine (U.S. Pat. Nos. 5,587,480, 5,696,254, 5,618,820, 5,756,706, 5,744,596, 568,164, 5,466,806, 5,151,426) has the structure:



For example and for some therapeutic uses, 3TC may be a physiologically functional derivative of emtricitabine in combination with tenofovir DF or a physiologically functional derivative of tenofovir DF.

It will be appreciated that tenofovir DF and emtricitabine, and their physiologically functional derivatives may exist in keto or enol tautomeric forms and the use of any tautomeric form thereof is within the scope of this invention. Tenofovir DF and emtricitabine will normally be utilized in the combinations of the invention substantially free of the corresponding enantiomer, that is to say no more than about 5% w/w of the corresponding enantiomer will be present.

Prodrugs

The invention includes all prodrugs of tenofovir and emtricitabine. An exemplary prodrug of tenofovir is tenofovir disoproxil fumarate (TDF, Viread®). A large number of structurally-diverse prodrugs have been described for phosphonic acids (Freeman and Ross in *Progress in Medicinal Chemistry* 34:112-147 (1997). A commonly used prodrug class is the acyloxyalkyl ester, which was first used as a prodrug strategy for carboxylic acids and then applied to phosphates and phosphonates by Farquhar et al (1983) *J. Pharm. Sci.* 72:324; also U.S. Pat. Nos. 4,816,570, 4,968,788, 5,663,159 and 5,792,756. Subsequently, the acyloxyalkyl ester was used to deliver phosphonic acids across cell membranes and to enhance oral bioavailability. A close variant of the acyloxyalkyl ester strategy, the alkoxycarbonyloxyalkyl ester, may also enhance oral bioavailability as a prodrug moiety in the compounds of the combinations of the invention. Aryl esters of phosphorus

groups, especially phenyl esters, are reported to enhance oral bioavailability (DeLambert et al (1994) *J. Med. Chem.* 37:498). Phenyl esters containing a carboxylic ester ortho to the phosphate have also been described (Khamnei and Torrence, (1996) *J. Med. Chem.* 39:4109-4115). Benzyl esters are reported to generate the parent phosphonic acid. In some cases, substituents at the ortho- or para-position may accelerate the hydrolysis. Benzyl analogs with an acylated phenol or an alkylated phenol may generate the phenolic compound through the action of enzymes, e.g. esterases, oxidases, etc., which in turn undergoes cleavage at the benzylic C—O bond to generate the phosphoric acid and the quinone methide intermediate. Examples of this class of prodrugs are described by Mitchell et al (1992) *J. Chem. Soc. Perkin Trans. I*2345; Brook et al WO 91/19721. Still other benzylic prodrugs have been described containing a carboxylic ester-containing group attached to the benzylic methylene (Glazier et al WO 91/19721). Thio-containing prodrugs are reported to be useful for the intracellular delivery of phosphonate drugs. These proesters contain an ethylthio group in which the thiol group is either esterified with an acyl group or combined with another thiol group to form a disulfide. Deesterification or reduction of the disulfide generates the free thio intermediate which subsequently breaks down to the phosphoric acid and episulfide (Puech et al (1993) *Antiviral Res.,* 22:155-174; Benzaria et al (1996) *J. Med. Chem.* 39:4958). Cyclic phosphonate esters have also been described as prodrugs of phosphorus-containing compounds.

Prodrug esters in accordance with the invention are independently selected from the following groups: (1) mono-, di-, and tri-phosphate esters of tenofovir or emtricitabine or any other compound which upon administration to a human subject is capable of providing (directly or indirectly) said mono-, di, or triphosphate ester; (2) carboxylic acid esters (3) sulphonate esters, such as alkyl- or aralkylsulphonyl (for example, methanesulphonyl); (4) amino acid esters (for example, alanine, L-valyl or L-isoleucyl); (5) phosphonate; and (6) phosphonamidate esters.

Ester groups (1)-(6) may be substituted with; straight or branched chain C_1-C_{18} alkyl (for example, methyl, n-propyl, t-butyl, or n-butyl); C_3-C_{12} cycloalkyl; alkoxyalkyl (for example, methoxymethyl); arylalkyl (for example, benzyl); aryloxyalkyl (for example, phenoxymethyl); C_5-C_{20} aryl (for example, phenyl optionally substituted by, for example, halogen, C_1-C_4 alkyl, C_1-C_4 alkoxy, or amino; acyloxymethyl esters —$CH_2OC(=O)R^9$ (e.g. POM) or acyloxymethyl carbonates —$CH_2OC(=O)OR^9$ (e.g. POC) where R^9 is C_1-C_6 alkyl, C_1-C_6 substituted alkyl, C_6-C_{20} aryl or C_6-C_{20} substituted aryl. For example, ester groups may be: —$CH_2OC(=O)C(CH_3)_3$, —$CH_2OC(=O)OC(CH_3)_3$ or —$CH_2OC(=O)OCH(CH_3)_2$.

An exemplary aryl moiety present in such esters comprises a phenyl or substituted phenyl group. Many phosphate prodrug moieties are described in U.S. Pat. No. 6,312,662; Jones et al (1995) *Antiviral Research* 27:1-17; Kucera et al (1990) *AIDS Res. Hum. Retro Viruses* 6:491-501; Piantadosi et al (1991) *J. Med. Chem.* 34:1408-14; Hosteller et al (1992) *Antimicrob. Agents Chemother.* 36:2025-29; Hostetler et al (1990) *J. Biol. Chem.* 265:611127; and Siddiqui et al (1999) *J. Med. Chem.* 42:4122-28.

Pharmaceutically acceptable prodrugs refer to a compound that is metabolized in the host, for example hydrolyzed or oxidized, by either enzymatic action or by general acid or base solvolysis, to form an active ingredient. Typical examples of prodrugs of the active ingredients of the combinations of the invention have biologically labile protecting groups on a functional moiety of the active compound. Prodrugs include compounds that can be oxidized, reduced, aminated, deaminated, esterified, deesterified, alkylated, dealkylated, acylated, deacylated, phosphorylated, dephosphorylated, or other functional group change or conversion involving forming or breaking chemical bonds on the prodrug.

Chemical Stability of a Pharmaceutical Formulation

The chemical stability of the active ingredients in a pharmaceutical formulation is of concern to minimize the generation of impurities and ensure adequate shelf-life. The active ingredients, tenofovir disoproxil fumarate and emtricitabine, in the pharmaceutical formulations of the invention have relatively low pKa values, indicative of the potential to cause acidic hydrolysis of the active ingredients. Emtricitabine, with a pKa of 2.65 (Emtriva™ Product Insert, Gilead Sciences, Inc. 2003, available at gilead.com) is subject to hydrolytic deamination of the 5-fluoro cytosine nucleobase to form the 5-fluoro uridine nucleobase. Tenofovir disoproxil fumarate, with a pKa of 3.75 (Yuan L. et al "Degradation Kinetics of Oxycarbonyloxymethyl Prodrugs of Phosphonates in Solution", Pharmaceutical Research (2001) Vol. 18, No. 2, 234-237), is subject also to hydrolytic deamination of the exocyclic amine of the adenine nucleobase, and to hydrolysis of one or both of the POC ester groups (U.S. Pat. No. 5,922,695). It is desirable to formulate a therapeutic combination of tenofovir disoproxil fumarate and emtricitabine, and the physiological functional derivatives thereof, with a minimum of impurities and adequate stability.

The combinations of the present invention provide combination pharmaceutical dosage forms which are chemically stable to acid degradation of: (1) a first component (such as tenofovir disoproxil fumarate, and physiological functional derivatives; (2) a second component (such as emtricitabine, and physiological functional derivatives; and (3) optionally a third component having antiviral activity. The third component includes anti-HIV agents and include: protease inhibitors (PI), nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), and integrase inhibitors. Exemplary third active ingredients to be administered in combination with first and second components are shown in Table A. First and second components are as defined in the above section entitled: ACTIVE INGREDIENTS OF THE COMBINATIONS.

Salts

Any reference to any of the compounds in the compositions of the invention also includes any physiologically acceptable salt thereof. Examples of physiologically acceptable salts of tenofovir DF, emtricitabine and their physiologically functional derivatives include salts derived from an appropriate base, such as an alkali metal (for example, sodium), an alkaline earth (for example, magnesium), ammonium and NX_4^+ (wherein X is C_1-C_4 alkyl), or an organic acid such as fumaric acid, acetic acid, succinic acid. Physiologically acceptable salts of an hydrogen atom or an amino group include salts of organic carboxylic acids such as acetic, benzoic, lactic, fumaric, tartaric, maleic, malonic, malic, isethionic, lactobionic and succinic acids; organic sulfonic acids, such as methanesulfonic, ethanesulfonic, benzenesulfonic and p-toluenesulfonic acids; and inorganic acids, such as hydrochloric, sulfuric, phosphoric and sulfamic acids. Physiologically acceptable salts of a compound of an hydroxy group include the anion of said compound in combination with a suitable cation such as Na^+ and NX_4^+ (wherein X is independently selected from H or a C_1-C_4 alkyl group).

For therapeutic use, salts of active ingredients of the combinations of the invention will be physiologically acceptable, i.e. they will be salts derived from a physiologically accept-

able acid or base. However, salts of acids or bases which are not physiologically acceptable may also find use, for example, in the preparation or purification of a physiologically acceptable compound. All salts, whether or not derived from a physiologically acceptable acid or base, are within the scope of the present invention.

Administration of the Formulations

While it is possible for the active ingredients of the combination to be administered alone and separately as monotherapies, it is preferable to administer them as a pharmaceutical co-formulation. A two-part or three-part combination may be administered simultaneously or sequentially. When administered sequentially, the combination may be administered in one, two, or three administrations.

Preferably, two-part or three-part combinations are administered in a single pharmaceutical dosage form. More preferably, a two-part combination is administered as a single oral dosage form and a three-part combination is administered as two identical oral dosage forms. Examples include a single tablet of tenofovir disoproxil fumarate and emtricitabine, or two tablets of tenofovir disoproxil fumarate, emtricitabine, and efavirenz.

It will be appreciated that the compounds of the combination may be administered: (1) simultaneously by combination of the compounds in a co-formulation or (2) by alternation, i.e. delivering the compounds serially, sequentially, in parallel or simultaneously in separate pharmaceutical formulations. In alternation therapy, the delay in administering the second, and optionally a third active ingredient, should not be such as to lose the benefit of a synergistic therapeutic effect of the combination of the active ingredients. By either method of administration (1) or (2), ideally the combination should be administered to achieve peak plasma concentrations of each of the active ingredients. A one pill once-per-day regimen by administration of a combination co-formulation may be feasible for some HIV-positive patients. Effective peak plasma concentrations of the active ingredients of the combination will be in the range of approximately 0.001 to 100 μM. Optimal peak plasma concentrations may be achieved by a formulation and dosing regimen prescribed for a particular patient. It will also be understood that tenofovir DF and emtricitabine, or the physiologically functional derivatives of either thereof, whether presented simultaneously or sequentially, may be administered individually, in multiples, or in any combination thereof. In general, during alternation therapy (2), an effective dosage of each compound is administered serially, where in co-formulation therapy (1), effective dosages of two or more compounds are administered together.

Formulation of the Combinations

When the individual components of the combination are administered separately they are generally each presented as a pharmaceutical formulation. The references hereinafter to formulations refer unless otherwise stated to formulations containing either the combination or a component compound thereof. It will be understood that the administration of the combination of the invention by means of a single patient pack, or patient packs of each formulation, within a package insert diverting the patient to the correct use of the invention is a desirable additional feature of this invention. The invention also includes a double pack comprising in association for separate administration, formulations of tenofovir disoproxil fumarate and emtricitabine, or a physiologically functional derivative of either or both thereof.

The combination therapies of the invention include: (1) a combination of tenofovir DF and emtricitabine or (2) a combination containing a physiologically functional derivative of either or both thereof.

The combination may be formulated in a unit dosage formulation comprising a fixed amount of each active pharmaceutical ingredient for a periodic, e.g. daily, dose or subdose of the active ingredients.

Pharmaceutical formulations according to the present invention comprise a combination according to the invention together with one or more pharmaceutically acceptable carriers or excipients and optionally other therapeutic agents. Pharmaceutical formulations containing the active ingredient may be in any form suitable for the intended method of administration. When used for oral use for example, tablets, troches, lozenges, aqueous or oil suspensions, dispersible powders or granules, emulsions, hard or soft capsules, syrups or elixirs may be prepared (*Remington's Pharmaceutical Sciences* (Mack Publishing Co., Easton, Pa.). Compositions intended for oral use may be prepared according to any method known to the art for die manufacture of pharmaceutical compositions and such compositions may contain one or more agents including antioxidants, sweetening agents, flavoring agents, coloring agents and preserving agents, in order to provide a palatable preparation. Tablets containing the active ingredient in admixture with non-toxic pharmaceutically acceptable excipient which are suitable for manufacture of tablets are acceptable. These excipients may be, for example, inert diluents, such as calcium or sodium carbonate, lactose, lactose monohydrate, croscarmellose sodium, povidone, calcium or sodium phosphate; granulating and disintegrating agents, such as maize starch, or alginic acid; binding agents, such as cellulose, microcrystalline cellulose, starch, gelatin or acacia; and lubricating agents, such as magnesium stearate, stearic acid or talc. Tablets may be uncoated or may be coated by known techniques including microencapsulation to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monostearate or glyceryl distearate alone or with a wax may be employed.

Formulations for oral use may be also presented as hard gelatin capsules where the active ingredient is mixed with an inert solid diluent, for example pregelatinized starch, calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, such as peanut oil, liquid paraffin or olive oil.

Aqueous suspensions of the invention contain the active materials in admixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients include a suspending agent, such as sodium carboxymethylcellulose, methylcellulose, hydroxypropyl methylcelluose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia, and dispersing or wetting agents such as a naturally occurring phosphatide (e.g., lecithin), a condensation product of an alkylene oxide with a fatty acid (e.g., polyoxyethylene stearate), a condensation product of ethylene oxide with a long chain aliphatic alcohol (e.g., heptadecaethyleneoxycetanol), a condensation product of ethylene oxide with a partial ester derived from a fatty acid and a hexitol anhydride (e.g., polyoxyethylene sorbitan monooleate). The aqueous suspension may also contain one or more preservatives such as ethyl or n-propyl p-hydroxybenzoate, one or more coloring agents, one or more flavoring agents and one or more sweetening agents, such as sucrose, sucralose or saccharin.

Oil suspensions may be formulated by suspending the active ingredient in a vegetable oil, such as arachis oil, olive

oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin. The oral suspensions may contain a thickening agent, such as beeswax, hard paraffin or cetyl alcohol. Sweetening agents, such as those set forth above, and flavoring agents may be added to provide a palatable oral preparation. These compositions may be preserved by the addition of an antioxidant such as ascorbic acid, BHT, etc.

Dispersible powders and granules of the invention suitable for preparation of an aqueous suspension by the addition of water provide the active ingredient in admixture with a dispersing or wetting agent, a suspending agent, and one or more preservatives. Suitable dispersing or wetting agents and suspending agents are exemplified by those disclosed above. Additional excipients, for example sweetening, flavoring and coloring agents, may also be present.

The pharmaceutical compositions of the invention may also be in the form of oil-in-water emulsions or liposome formulations. The oily phase may be a vegetable oil, such as olive oil or arachis oil, a mineral oil, such as liquid paraffin, or a mixture of these. Suitable emulsifying agents include naturally-occurring gums, such as gum acacia and gum tragacanth, naturally occurring phosphatides, such as soybean lecithin, esters or partial esters derived from fatty acids and hexitol anhydrides, such as sorbitan monooleate, and condensation products of these partial esters with ethylene oxide, such as polyoxyethylene sorbitan monooleate. The emulsion may also contain sweetening and flavoring agents. Syrups and elixirs may be formulated with sweetening agents, such as glycerol, sorbitol or sucrose. Such formulations may also contain a demulcent, a preservative, a flavoring or a coloring agent.

The pharmaceutical compositions of the invention may be in the form of a sterile injectable preparation, such as a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated according to the known art using those suitable dispersing or wetting agents and suspending agents which have been mentioned above. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally acceptable diluent or solvent, such as a solution in 1,3-butane-diol or prepared as a lyophilized powder. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile fixed oils may conventionally be employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid may likewise be used in the preparation of injectables.

The pharmaceutical compositions of the invention may be injected parenterally, for example, intravenously, intraperitoneally, intrathecally, intraventricularly, intrasternally, intracranially, intramuscularly or subcutaneously, or they may be administered by infusion techniques. They are best used in the form of a sterile aqueous solution which may contain other substances, for example, enough salts or glucose to make the solution isotonic with blood. The aqueous solutions should be suitably buffered (preferably to a pH of from 3 to 9), if necessary. The preparation of suitable parenteral formulations under sterile conditions is readily accomplished by standard pharmaceutical techniques well known to those skilled in the art.

The pharmaceutical compositions of the invention may also be administered intranasally or by inhalation and are conveniently delivered in the form of a dry powder inhaler or an aerosol spray presentation from a pressurized container or a nebuliser with the use of a suitable propellant, e.g. dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, a hydrofluoroalkane such as 1,1,1,2-tetrafluoroethane (HFC 134a), carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. The pressurized container or nebuliser may contain a solution or suspension of the composition, e.g. using a mixture of ethanol and the propellant as the solvent, which may additional contain a lubricant, e.g. sorbitan trioleate. Capsules and cartridges (made, for example, from gelatin) for use in an inhaler or insufflator may be formulated to contain a powder mix of a compound of the formula (I) and a suitable powder base such as lactose or starch. Aerosol or dry powder formulations are preferably arranged so that each metered dose or "puff" contains from 20 μg to 20 mg of a composition for delivery to the patient. The overall daily dose with an aerosol will be in the range of from 20 μg to 20 mg which may be administered in a single dose or, more usually, in divided doses throughout the day.

The amount of active ingredient that may be combined with the carrier material to produce a single dosage form will vary depending upon the host treated and the particular mode of administration. For example, a time-release formulation intended for oral administration to humans may contain approximately 1 to 1000 mg of active material compounded with an appropriate and convenient amount of carrier material which may vary from about 5 to about 95% of the total compositions (weight:weight). The pharmaceutical composition can be prepared to provide easily measurable amounts for administration. For example, an aqueous solution intended for intravenous infusion may contain from about 3 to 500 μg of the active ingredient per milliliter of solution in order that infusion of a suitable volume at a rate of about 30 mL/hr can occur. As noted above, formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous or non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be administered as a bolus, electuary or paste.

The combinations of the invention may conveniently be presented as a pharmaceutical formulation in a unitary dosage form. A convenient unitary dosage formulation contains the active ingredients in any amount from 1 mg to 1 g each, for example but not limited to, 10 mg to 300 mg. The synergistic effects of tenofovir DF in combination with emtricitabine may be realized over a wide ratio, for example 1:50 to 50:1 (tenofovir DF:emtricitabine). In one embodiment, the ratio may range from about 1:10 to 10:1. In another embodiment, the weight/weight ratio of tenofovir to emtricitabine in a co-formulated combination dosage form, such as a pill, tablet, caplet or capsule will be about 1, i.e. an approximately equal amount of tenofovir DF and emtricitabine. In other exemplary co-formulations, there may be more or less tenofovir than FTC. For example, 300 mg tenofovir DF and 200 mg emtricitabine can be co-formulated in a ratio of 1.5:1 (tenofovir DF:emtricitabine). In one embodiment, each compound will be employed in the combination in an amount at which it exhibits antiviral activity when used alone. Exemplary Formulations A, B, C, D, E, and F (Examples) have ratios of 12:1 to 1:1 (tenofovir DF:emtricitabine). Exemplary Formulations A, B, C, D, E, and F use amounts of tenofovir DF and emtricitabine ranging from 25 mg to 300 mg. Other ratios and amounts of the compounds of said combinations are contemplated within the scope of the invention.

A unitary dosage form may further comprise tenofovir DF and emtricitabine, or physiologically functional derivatives of either thereof, and a pharmaceutically acceptable carrier.

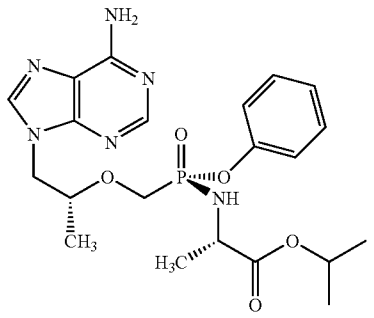
It will be appreciated by those skilled in the art that the amount of active ingredients in the combinations of the invention required for use in treatment will vary according to a variety of factors, including the nature of the condition being treated and the age and condition of the patient, and will ultimately be at the discretion of the attending physician or health care practitioner. The factors to be considered include the route of administration and nature of the formulation, the animal's body weight, age and general condition and the nature and severity of the disease to be treated. For example, in a Phase I/II monotherapy study of emtricitabine, patients received doses ranging from 25 mg to 200 mg twice-a-day for two weeks. At each dose regimen greater or equal to 200 mg, a 98-percent (1.75 log 10) or greater viral suppression was observed. A once-a-day dose of 200 mg of emtricitabine reduced the viral load by an average of 99 percent (1.92 log 10). Viread® (tenofovir DF) has been approved by the FDA for the treatment and prophylaxis of HIV infection as a 300 mg oral tablet. Emtriva™ (emtricitabine) has been approved by the FDA for the treatment of HIV as a 200 mg oral tablet.

It is also possible to combine any two of the active ingredients in a unitary dosage form for simultaneous or sequential administration with a third active ingredient. The three-part combination may be administered simultaneously or sequentially. When administered sequentially, the combination may be administered in two or three administrations. Third active ingredients have anti-HIV activity and include protease inhibitors (PI), nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), and integrase inhibitors. Exemplary third active ingredients to be administered in combination with tenofovir DF, emtricitabine, and their physiological functional derivatives, are shown in Table A.

TABLE A

5,6 dihydro-5-azacytidine
5-aza 2'deoxycytidine
5-azacytidine
5-yl-carbocyclic 2'-deoxyguanosine (BMS200, 475)
9 (arabinofuranosyl)guanine; 9-(2' deoxyribofuranosyl)guanine
9-(2'-deoxy 2'fluororibofuranosyl)-2,6-diaminopurine
9-(2'-deoxy 2'fluororibofuranosyl)guanine
9-(2'-deoxyribofuranosyl)-2,6 diaminopurine
9-(arabinofuranosyl)-2,6 diaminopurine
Abacavir, Ziagen ®
Acyclovir, ACV; 9-(2-hydroxyethoxylmethyl)guanine
Adefovir dipivoxil, Hepsera ®
amdoxivir, DAPD
Amprenavir, Agenerase ®
araA; 9-β-D-arabinofuranosyladenine (Vidarabine)
atazanivir sulfate (Reyataz ®)
AZT; 3'-azido-2',3'-dideoxythymdine, Zidovudine, (Retrovir ®)
BHCG; (.+−.)-(1a,2b,3a)-9-[2,3-bis(hydroxymethyl)cyclobutyl]guanine
BMS200,475; 5-yl-carbocyclic 2'-deoxyguanosine
Buciclovir; (R) 9-(3,4-dihydroxybutyl)guanine
BvaraU; 1-β-D-arabinofuranosyl-E-5-(2-bromovinyl)uracil (Sorivudine)
Calanolide A
Capravirine
CDG; carbocyclic 2'-deoxyguanosine
Cidofovir, HPMPC; (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine
Clevudine, L-FMAU; 2'-Fluoro-5-methyl-β-L-arabino-furanosyluracil
Combivir ® (lamivudine/zidovudine)
Cytallene; [1-(4'-hydroxy-1',2'-butadienyl)cytosine]
d4C; 3'-deoxy-2',3'-didehydrocytidine
DAPD; (−)-β-D-2,6-diaminopurine dioxolane
ddA; 2',3'-dideoxyadenosine
ddAPR; 2,6-diaminopurine-2',3'-dideoxyriboside
ddC; 2',3'-dideoxycytidine (Zalcitabine)
ddI; 2',3'-dideoxyinosine, didanosine, (Videx ®, Videx ® EC)
Delavirdine, Rescriptor ®
Didanosine, ddI, Videx ®; 2',3'-dideoxyinosine
DXG; dioxolane guanosine
E-5-(2-bromovinyl)-2'-deoxyuridine
Efavirenz, Sustiva ®
Enfuvirtide, Fuzeon ®
F-ara-A; fluoroarabinosyladenosine (Fludarabine)
FDOC; (−)-β-D-5-fluoro-1-[2-(hydroxymethyl)-1,3-dioxolane]cytosine
FEAU; 2'-deoxy-2'-fluoro-1-β-D-arabinofuranosyl-5-ethyluracil
FIAC; 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine
FIAU; 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouridine
FLG; 2',3'-dideoxy-3'-fluoroguanosine
FLT; 3'-deoxy-3'-fluorothymidine
Fludarabine; F-ara-A; fluoroarabinosyladenosine
FMAU; 2'-Fluoro-5-methyl-β-L-arabino-furanosyluracil
FMdC
Foscarnet; phosphonoformic acid, PFA
FPMPA; 9-(3-fluoro-2-phosphonylmethoxypropyl)adenine
Gancyclovir, GCV; 9-(1,3-dihydroxy-2-propoxymethyl)guanine
GS-7340; 9-[R-2-[[(S)-[[(S)-1-(isopropoxycarbonyl)ethyl]amino]-phenoxyphosphinyl]methoxy]propyl]adenine
HPMPA; (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine
HPMPC; (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (Cidofovir)
Hydroxyurea, Droxia ®
Indinavir, Crixivan ®
Kaletra ® (lopinavir/ritonavir)
Lamivudine, 3TC, Epivir ™; (2R,5S,cis)-4-amino-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one
L-d4C; L-3'-deoxy-2',3'-didehydrocytidine
L-ddC; L-2',3'-dideoxycytidine
L-Fd4C; L-3'-deoxy-2',3'-didehydro-5-fluorocytidine
L-FddC; L-2',3'-dideoxy-5-fluorocytidine
Lopinavir
Nelfinavir, Viracept ®
Nevirapine, Viramune ®
Oxetanocin A; 9-(2-deoxy-2-hydroxymethyl-β-D-erythro-oxetanosyl)adenine
Oxetanocin G; 9-(2-deoxy-2-hydroxymethyl-β-D-erythro-oxetanosyl)guanine
Penciclovir
PMEDAP; 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine
PMPA, tenofovir; (R)-9-(2-phosphonylmethoxypropyl)adenine
PPA; phosphonoacetic acid
Ribavirin; 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide
Ritonavir, Norvir ®
Saquinavir, Invirase ®, Fortovase ®
Sorivudine, BvaraU; 1-β-D-arabinofuranosyl-E-5-(2-bromovinyl)uracil
Stavudine, d4T, Zerit ®; 2',3'-didehydro-3'-deoxythymidine
Trifluorothymidine, TFT; Trifluorothymidine
Trizivir ® (abacavir sulfate/lamivudine/zidovudine)
Vidarabine, araA; 9-β-D-arabinofuranosyladenine
Zalcitabine, Hivid ®, ddC; 2',3'-dideoxycytidine
Zidovudine, AZT, Retrovir ®; 3'-azido-2',3'-dideoxythymdine
Zonavir; 5-propynyl-1-arabinosyluracil

Another aspect of the present invention is a three-part combination comprising tenofovir DF, FTC, and 9-[(R)-2-[[(S)—[[(S)-1-(isopropoxycarbonyl)ethyl]amino]phenoxy-phosphinyl]methoxy]propyl]adenine, also designated herein as GS-7340, which has the structure:



GS-7340 is a prodrug of tenofovir and the subject of commonly owned, pending application, U.S. Ser. No. 09/909,560, filed Jul. 20, 2001 and Becker et al WO 02/08241.

For example, a ternary unitary dosage may contain 1 mg to 1000 mg of tenofovir disoproxil fumarate, 1 mg to 1000 mg of emtricitabine, and 1 mg to 1000 mg of the third active ingredient. As a further feature of the present invention, a unitary dosage form may further comprise tenofovir DF, emtricitabine, the third active ingredient, or physiologically functional derivatives of the three active ingredients thereof, and a pharmaceutically acceptable carrier.

Combinations of the present invention enable patients greater freedom from multiple dosage medication regimens and ease the needed diligence required in remembering and complying with complex daily dosing times and schedules. By combining tenofovir disoproxil fumarate and emtricitabine into a single dosage form, the desired daily regimen may be presented in a single dose or as two or more sub-doses per day. The combination of co-formulated tenofovir DF and emtricitabine may be administered as a single pill, once per day.

A further aspect of the invention is a patient pack comprising at least one active ingredient: tenofovir disoproxil fumarate, emtricitabine, or a physiologically functional derivative of either of the combination and an information package or product insert containing directions on the use of the combination of the invention.

Segregation of active ingredients in pharmaceutical powders and granulations is a widely recognized problem that can result in inconsistent dispersions of the active ingredients in final dosage forms. Some of the main factors contributing to segregation are particle size, shape and density. Segregation is particularly troublesome when attempting to formulate a single homogenous tablet containing multiple active ingredients having different densities and different particle sizes. Glidants are substances that have traditionally been used to improve the flow characteristics of granulations and powders by reducing interparticulate friction. See Lieberman, *Lachman, & Schwartz, Pharmaceutical Dosage Forms: Tablets*, Volume 1, p. 177-178 (1989), incorporated herein by reference. Glidants are typically added to pharmaceutical compositions immediately prior to tablet compression to facilitate the flow of granular material into the die cavities of tablet presses. Glidants include: colloidal silicon dioxide, asbestos free talc, sodium aluminosilicate, calcium silicate, powdered cellulose, microcrystalline cellulose, corn starch, sodium benzoate, calcium carbonate, magnesium carbonate, metallic stearates, calcium stearate, magnesium stearate, zinc stearate, stearowet C, starch, starch 1500, magnesium lauryl sulfate, and magnesium oxide. Exemplary Tablet Formulation A has colloidal silicon dioxide (Examples). Glidants can be used to increase and aid blend composition homogeneity in formulations of anti-HIV drugs (U.S. Pat. No. 6,113,920). The novel compositions of the present invention may contain glidants to effect and maintain homogeneity of active ingredients during handling prior to tablet compression.

The present invention provides pharmaceutical formulations combining the active ingredients tenofovir DF and emtricitabine, or physiologically functional derivatives thereof, in a sufficiently homogenized form, and a method for using this pharmaceutical formulation. An object of the present invention is to utilize glidants to reduce the segregation of active ingredients in pharmaceutical compositions during pre-compression material handling. Another object of the present invention is to provide a pharmaceutical formulation combining the active ingredients tenofovir DF and emtricitabine, or physiologically functional derivatives thereof, with a pharmaceutically acceptable glidant, resulting in a mixture characterized by a pharmaceutically acceptable measure of homogeneity.

Formulations include those suitable for oral, rectal, nasal, topical (including transdermal, buccal and sublingual), vaginal or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. Such methods represent a further feature of the present invention and include the step of bringing into association the active ingredients with the carrier, which constitutes one or more accessory ingredients, and maintaining chemical stability. In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then if necessary shaping the product.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, caplets, cachets or tablets each containing a predetermined amount of the active ingredients; as a powder or granules; as a solution or a suspension in an aqueous or non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredients in a free-flowing form such as a powder or granules, optionally mixed with a binder (e.g. povidone, gelatin, hydroxypropyl methylcellulose), lubricant, inert diluent, preservative, disintegrant (e.g. sodium starch glycollate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose) surface-active or dispersing agent. Molded tablets may be made by molding a mixture of the powdered compound moistened with an inert liquid diluent in a suitable machine. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredients therein using, for example, cellulose ether derivatives (e.g., hydroxypropyl methylcellulose) or methacrylate derivatives in varying proportions to provide the desired release profile. Tablets may optionally be provided with an enteric coating, to provide release in parts of the gut other than the stomach.

Formulations suitable for topical administration in the mouth include lozenges comprising the active ingredients in a flavored base, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouthwashes comprising the active ingredient in a suitable liquid carrier. Formulations for rectal administration may be presented as a suppository with a suitable base comprising, for example, cocoa butter or a salicylates. Topical administration may also be by means of a transdermal iontophoretic device.

Formulations suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations containing in addition to the active ingredient such carriers as are known in the art to be appropriate.

Formulations suitable for penile administration for prophylactic or therapeutic use may be presented in condoms, creams, gels, pastes, foams or spray formulations containing in addition to the active ingredient such earners as are known in the art to be appropriate.

Pharmaceutical formulations suitable for rectal administration wherein the carrier is a solid are most preferably presented as unit dose suppositories. Suitable carriers include cocoa butter and other materials commonly used in the art. The suppositories may be conveniently formed by admixture of the active combination with the softened or melted carrier(s) followed by chilling and shaping in moulds.

Formulations suitable for parenteral administration include aqueous and nonaqueous isotonic sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and nonaqueous sterile suspensions which may include suspending agents and thickening agents; and liposomes or other microparticulate systems which are designed to target the compound to blood components or one or more organs. The formulations may be presented in unit-dose or multi-dose sealed containers, for example, ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injection, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Exemplary unit dosage formulations are those containing a daily dose or daily subdose of the active ingredients, as hereinbefore recited, or an appropriate fraction thereof. It should be understood that in addition to the ingredients particularly mentioned above the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example, those suitable for oral administration may include such further agents as sweeteners, thickeners and flavoring agents.

The compounds of the combination of the present invention may be obtained in a conventional manner, known to those skilled in the art. Tenofovir disoproxil fumarate can be prepared, for example, as described in U.S. Pat. No. 5,977,089. Methods for the preparation of FTC are described in WO 92/14743, incorporated herein by reference.

Composition Use

Compositions of the present invention are administered to a human or other mammal in a safe and effective amount as described herein. These safe and effective amounts will vary according to die type and size of mammal being treated and the desired results of the treatment. Any of the various methods known by persons skilled in the art for packaging tablets, caplets, or other solid dosage forms suitable for oral administration, that will not degrade the components of the present invention, are suitable for use in packaging. The combinations may be packaged in glass and plastic bottles. Tablets, caplets, or other solid dosage forms suitable for oral administration may be packaged and contained in various packaging materials optionally including a desiccant, e.g. silica gel. Packaging may be in the form of unit dose blister packaging. For example, a package may contain one blister tray of tenofovir DF and another blister tray of emtricitabine pills, tablets, caplets, or capsule. A patient would take one dose, e.g. a pill, from one tray and one from the other. Alternatively, the package may contain a blister tray of the co-formulated combination of tenofovir DF and emtricitabine in a single pill, tablet, caplet or capsule. As in other combinations and packaging thereof, the combinations of the invention include physiological functional derivatives of tenofovir DF and FTC.

The packaging material may also have labeling and information related to the pharmaceutical composition printed thereon. Additionally, an article of manufacture may contain a brochure, report, notice, pamphlet, or leaflet containing product information. This form of pharmaceutical information is referred to in the pharmaceutical industry as a "package insert." A package insert may be attached to or included with a pharmaceutical article of manufacture. The package insert and any article of manufacture labeling provides information relating to the pharmaceutical composition. The information and labeling provides various forms of information utilized by health-care professionals and patients, describing the composition, its dosage and various other parameters required by regulatory agencies such as the United States Food and Drug Agency.

Assays of the Combinations

The combinations of the inventions may be tested for in vitro activity against HIV and sensitivity, and for cytotoxicity in laboratory adapted cell lines, e.g. MT2 and in peripheral blood mononuclear cells (PBMC) according to standard assays developed for testing anti-HIV compounds, such as WO 02/068058 and U.S. Pat. No. 6,475,491. Combination assays may be performed at varying concentrations of the compounds of the combinations to determine EC_{50} by serial dilutions.

Exemplary Formulations

The following examples further describe and demonstrate particular embodiments within the scope of the present invention. Techniques and formulations generally are found in *Remington's Pharmaceutical Sciences* (Mack Publishing Co., Easton, Pa.). The examples are given solely for illustration and are not to be construed as limitations as many variations are possible without departing from spirit and scope of the Invention. The following examples are intended for illustration only and are not intended to limit the scope of the invention in any way. "Active ingredient" denotes tenofovir disoproxil fumarate, emtricitabine, or a physiologically functional derivative of either thereof.

Tablet Formulation

The following exemplary formulations A, B, C, D, E, and F are prepared by wet granulation of the ingredients with an aqueous solution, addition of extragranular components and then followed by addition of magnesium stearate and compression.

Formulation A:

	mg/tablet
Tenofovir Disoproxil Fumarate	300
emtricitabine	200
Microcrystalline Cellulose	200
Lactose Monohydrate	175
Croscarmellose Sodium	60
Pregelatinized Starch	50
Colloidal silicon dioxide	5
Magnesium Stearate	10
total:	1000

Formulation B:

	mg/tablet
Tenofovir Disoproxil fumarate	300
emtricitabine	100
Microcrystalline Cellulose	200
Lactose Monohydrate	180
Sodium Starch Glycollate	60
Pregelatinized Starch	50
Magnesium Stearate	10
total:	900

Formulation C:

	mg/tablet
Tenofovir Disoproxil fumarate	200
emtricitabine	200
Microcrystalline Cellulose	200
Lactose Monohydrate	180
Sodium Starch Glycollate	60
Pregelatinized Starch	50
Magnesium Stearate	10
total:	900

Formulation D:

	mg/tablet
Tenofovir Disoproxil fumarate	300
emtricitabine	25
Microcrystalline Cellulose	200
Lactose Monohydrate	180
Sodium Starch Glycollate	60
Pregelatinized Starch	50
Magnesium Stearate	10
total:	825

Formulation E:

	mg/tablet
Tenofovir Disoproxil fumarate	200
emtricitabine	25
Microcrystalline Cellulose	200
Lactose Monohydrate	180
Sodium Starch Glycollate	60
Pregelatinized Starch	50
Magnesium Stearate	10
total:	725

Formulation F:

	mg/tablet
Tenofovir Disoproxil fumarate	100
emtricitabine	100
Microcrystalline Cellulose	200
Lactose Monohydrate	180
Sodium Starch Glycollate	60

-continued

	mg/tablet
Pregelatinized Starch	50
Magnesium Stearate	10
total:	700

Formulation G (Controlled Release Formulation):

This formulation is prepared by wet granulation of the ingredients with an aqueous solution, followed by the addition of magnesium stearate and compression.

	mg/tablet
Tenofovir Disoproxil fumarate	300
emtricitabine	200
Hydroxypropyl Methylcellulose	112
Lactose B.P.	53
Pregelatinized Starch B.P.	28
Magnesium Stearate	7
total:	700

Drug release takes place over a period of about 6-8 hours and is complete after 12 hours.

Capsule Formulations

Formulation H:

A capsule formulation is prepared by admixing the ingredients and filling into a two-part hard gelatin or hydroxypropyl methylcellulose capsule.

	mg/capsule
Active Ingredient	500
Microcrystalline Cellulose	143
Sodium Starch Glycollate	25
Magnesium Stearate	2
total:	670

Formulation I (Controlled Release Capsule):

The following controlled release capsule formulation is prepared by extruding ingredients a, b, and c using an extruder, followed by spheronization of the extrudate and drying. The dried pellets are then coated with release-controlling membrane (d) and filled into a two-piece, hard gelatin or hydroxypropyl methylcellulose capsule.

	mg/capsule
(a) Active Ingredient	500
(b) Microcrystalline Cellulose	125
(c) Lactose B.P.	125
(d) Ethyl Cellulose	13
total:	763

Formulation J (Oral Suspension):

The active ingredients are admixed with the ingredients and filling them as dry powder. Purified water is added and mixed well before use.

Active Ingredient	500 mg
Confectioner's Sugar	2000 mg
Simethicone	300 mg
Methylparaben	30 mg
Propylparaben	10 mg
Flavor, Peach	500 mg
Purified Water q.s. to	5.00 ml

Formulation K (Suppository):

One-fifth of the Witepsol H15 is melted in a steam-jacketed pan at 45° C. maximum. The active ingredients are sifted through a 200 micron sieve and added to the molten base with mixing, using a Silverson fitted with a cutting head, until a smooth dispersion is achieved. Maintaining the mixture at 45° C., the remaining Witepsol H15 is added to the suspension and stirred to ensure a homogenous mix. The entire suspension is passed through a 250 micron stainless steel screen and, with continuous stirring, is allowed to cool to 40° C. At a temperature of 38° C. to 40° C., 2.02 g of the mixture is filled into suitable, 2 ml plastic molds. The suppositories are allowed to cool to room temperature.

	mg/Suppository
Active Ingredient	500
Hard Fat, B.P. (Witepsol H15 - Dynamit Nobel)	1770
total	2270

Fixed Dose Combination Tablet

A fixed dose combination tablet of tenofovir disoproxil fumarate (TDF) 300 mg/emtricitabine 200 mg was formulated using a wet granulation/fluid-bed drying process using conventional methods. See: U.S. Pat. No. 5,935,946; L. Young (editor). Tableting Specification Manual 5th ed., American Pharmaceutical Association, Washington, D.C., (2001); L. Lachman, H. Lieberman (editors). Pharmaceutical Dosage Forms: Tablets (Vol 2), Marcel Dekker Inc., New York, 185-202 (1981); J. T. Fell and J. M. Newton, J. Pharm. Pharmacol. 20, 657-659 (1968); US Pharmacopeia 24-National Formulary 19, "Tablet Friability", Chapter <1216>, Page 2148 (2000).

The effects of granulation water level (ranging from 40% to 50% w/w) and wet massing time were studied on the physicochemical properties of the final powder blend and its performance with respect to blend uniformity and compressibility (tablet compactibility). In addition, content uniformity, assay, stability and dissolution performance was evaluated for the TDF/emtricitabine fixed dose combination tablets.

Formulation Equipment

Equipment included a high shear mixer equipped with a pressure tank and spray nozzle tip to add the granulating water, a fluid-bed dryer, a mill, a tumble blender, a rotary tablet press, and a tablet deduster.

Formulation Process

The dried, milled powder was blended with the extragranular microcrystalline cellulose and croscarmellose sodium and then blended with magnesium stearate. Powder samples were removed after mixing with die magnesium stearate. The blend samples were evaluated for, bulk density, mesh analysis and compressibility. The powder blend mixed with the magnesium stearate was compressed into tablets on a press setup.

Materials

The following Table 1 lists the quantitative composition of the TDF/emtricitabine tablet formulation.

TABLE 1

Ingredient	% w/w	Unit Formula for tablet cores (mg/tablet)	Quantity per 12 kg Batch (kg)
Tenofovir Disoproxil Fumarate [a]	30.0	300.0	3.60
Emtricitabine [a]	20.0	200.0	2.40
Pregelatinized Starch, NF/EP	5.0	50.0	0.60
Croscarmellose Sodium, NF/EP	6.0	60.0	0.72
Lactose Monohydrate, NF/EP [a]	8.0	80.0	0.96
Microcrystalline Cellulose, NF/EP [c]	30.0	300.0	3.60
Magnesium Stearate, NF/EP	1.0	10.0	0.12
Purified Water, USP/EP	[b]	[b]	[b]
Totals	100.0	1000.0	12.00

[a] Actual weight is adjusted based on the Drug Content Factor (DCF) of tenofovir disoproxil fumarate and emtricitabine.
[b] Water removed during drying.

Characterization Equipment

Moisture content was measured by loss on drying using a heat lamp/balance system. The powder blend was sampled with a sampling thief fitted with chambers to determine powder blend uniformity. Duplicate samples were removed from each of several locations in the blender. Blend uniformity analysis was performed on one sample from each location.

Particle size analysis of the final powder blend was determined by sifting a multi-gram sample through a screen using a sonic sifter. The quantity of final powder blend retained on each sieve and the fines collector was determined by calculating the difference in weight between the sieves and fines collector before and after the test. The geometric mean diameter particle size was calculated by logarithmic weighting of the sieved distribution.

Bulk density was determined by filling a graduated cylinder with the final powder blend and measuring the weight differential between the empty and filled graduate cylinder per unit volume.

Tablets were characterized for friability using a friabilator, a hardness tester, a thickness micrometer equipped with a printer, and a weighing balance.

Compression characteristics were determined using a rotary tablet press equipped with a flat-faced, beveled edged punch to a target weight of 400 mg. The powder blends were compressed using target upper punch pressures ranging from approximately 100 to 250 MPa. The apparent normalized ejection force was determined and normalized for tablet thickness and diameter.

Tablet hardness was determined using a hardness tester. Tablet thickness was determined using a micrometer, and tablet weights were determined using a top loading balance.

Wet Granulation

The powders were blended in a granulator and then granulated using water. The impeller and chopper speeds were kept constant in the blender at a low setting during the granulation and wet massing operations. After water addition, the impeller and chopper were stopped and the granulator bowl was opened to observe the granulation consistency and texture. The lid was closed and the wet massing phase was performed. Acceptable granules had 40% w/w and 60% w/w water, respectively.

Wet Milling

To facilitate a uniform drying process, each wet granulation was deagglomerated with a mill fitted with a screen and an impeller. The milled wet granules were charged into a fluid-bed dryer immediately following wet milling.

Fluid-Bed Drying

Milled wet granules were dried using an inlet air setpoint temperature of about 70° C. and airflow of approximately 100 cfm. The target LOD was about 1.0% with a range of not more than (NMT) 1.5%. The total fluid-bed drying time ranged from 53 to 75 minutes. Final LOD ranged from 0.4% to 0.7% for all of the batches dried. The final exhaust temperatures for all the batches ranged from 47° C. to 50° C.

Dry Milling

All dried granules were milled through a perforated screen. The mill was equipped with a square impeller and operated. The lots were milled and manually transferred to the V-blender.

Blending

Each lot was blended using the V-blender. In one set of three formulations, starting with 12 kg materials, final powder blend yield available for compression after blending ranged from 10.5 kg (87.5%) to 11.1 kg (92.5%). The final powder blend bulk density ranged from 0.48 to 0.58 g/cc and the geometric mean diameter particle size ranged from 112 to 221 μm. Percent water and wet massing time affect final powder blend particle size and bulk density.

The powder blending for both tenofovir DF and emtricitabine gave a mean (n=10) strength value for tenofovir DF ranged from 100.6% to 102.8% of target strength for the lots and the relative standard deviation (RSD) was from 0.5% to 1.7%. The mean (n=10) strength value for emtricitabine ranged from 101.3% to 104.1% of target strength for the lots with the relative standard deviation (RSD) ranged from 0.6% to 1.7%. The final powder blend moisture level ranged from 0.8% to 1.1% LOD.

Tablet Compression

The final blends were compressed using a rotary tablet press and the tablets were film-coated.

Three 300 gm formulations (Table 2) were granulated in a granulator equipped with a 1-L bowl. The quantities of intragranular components were based on a 300 g total batch size. The formulations in lots 1 and 2 differed in the amount of microcrystalline cellulose 30% vs. 20% w/w, respectively. Lots 2 and 3 were identical except for the type of binder. Lot 2 contained 5% w/w of pregelatinized starch and lot 3 contained 5% w/w povidone as binder.

TABLE 2

Ingredient	Lot 1 % w/w	Lot 2 % w/w	Lot 3 % w/w
Tenofovir Disoproxil Fumarate	30.0	30.0	30.0
Emtricitabine	20.0	20.0	20.0
Pregelatinized Starch, NF/EP	5.0	5.0	N/A
Povidone, USP/NF (C-30)	N/A	N/A	5.0
Croscarmellose Sodium, NF/EP	6.0	6.0	6.0
Lactose Monohydrate, NF/EP	8.0	18.0	18.0
Microcrystalline Cellulose, NF/EP [a]	30.0	20.0	20.0
Magnesium Stearate, NF/EP	1.0	1.0	1.0
Purified Water, USP/EP	[a]	[a]	[a]
Total	100.0	100.0	100.0

[a] Water removed during drying.

After water addition, the impeller and chopper were stopped and the granulator bowl was opened to observe the granulation consistency and texture. To achieve similar granulation consistency, lots 1, 2, and 3 were granulated with 45%, 40%, and 30% w/w water, respectively. The lid was closed and the wet massing phase was performed. All lots had a 30 sec wet massing resulting in acceptable granulations. The wet granulations from all batches were hand screened through a sieve to deagglomerate. The resulting granulations were tray dried in a convection oven set at 60° C. for approximately 20 hours to an LOD<1.0%. The dried granulations from all batches were hand screened through a sieve. In order to fit the granulation into the small scale (300 mL) V-blender, the final blend batch size was adjusted to 100 g. A portion, 81 g of the resulting blend from Lot 1 was blended with 15 g microcrystalline cellulose, 3 g croscarmellose sodium and 1 g magnesium stearate. 86 g of the resulting granulation from Lot 2 and Lot 3 were each blended with 10 g microcrystalline cellulose, 3 g croscarmellose sodium and 1 g magnesium stearate.

Purity analysis was conducted by reverse-phase HPLC (high performance liquid chromatography). Impurities related to tenofovir disoproxil fumarate and emtricitabine were characterized and measured in the bulk API (active pharmaceutical ingredient) before formulation in the three lots of Table 2, and again after formulation in the resulting tablets. The impurities include by-products from hydrolysis of the exocyclic amino groups of tenofovir disoproxil fumarate and emtricitabine, and the hydrolysis of the disoproxil (POC) esters of tenofovir disoproxil fumarate. In each lot, the sum total of impurities related to tenofovir disoproxil fumarate and emtricitabine was less than 1% after formulation and tablet manufacture.

The physicochemical properties of tenofovir disoproxil fumarate and emtricitabine tablets were evaluated by visual appearance, water content, label strength, impurity and degradation product contents, and tablet dissolution. Stability studies were conducted on drug product packaged in container-closure systems that are identical to the intended clinical and commercial container-closure system. There was no sign of discoloration or tablet cracking during the course of the stability study. Film-coated tenofovir disoproxil fumarate and emtricitabine tablets exhibited satisfactory stability at 40° C./75% RH (relative humidity) for up to six months when packaged and stored with silica gel desiccant. No significant loss (defined as ≥ 5% degradation) in % label strength of tenofovir DF or emtricitabine was observed after six months at 40° C./75% RH. when packaged and stored with desiccant. The increase in the total degradation products was 1.5% for tenofovir DF and 0.6-0.7% for emtricitabine after six months at 40° C./75% RH when packaged and stored with 3 grams of desiccant.

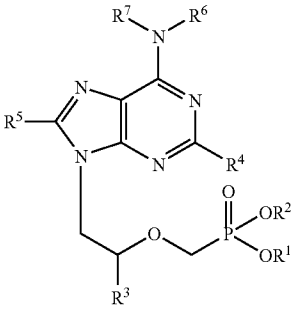
All publications and patent applications cited herein are incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although certain embodiments are described in detail above, those having ordinary skill in the art will clearly understand that many modifications are possible in the claims without departing from the teachings thereof. All such modifications are intended to be encompassed within the claims of the invention.

Embodiments of the Invention

A1. A pharmaceutical composition comprising an effective amount of a compound of the formula:

(1)



wherein R^1 and R^2 are independently selected from H, C_1-C_6 alkyl, C_1-C_6 substituted alkyl, C_6-C_{20} aryl, C_6-C_{20} substituted aryl, C_6-C_{20} arylalkyl, C_6-C_{20} substituted arylalkyl, acyloxymethyl esters $CH_2OC(=O)R^9$ and acyloxymethyl carbonates —$CH_2OC(=O)OR^9$ where R^9 is C_1-C_6 alkyl, C_1-C_6 substituted alkyl, C_6-C_{20} aryl and C_6-C_{20} substituted aryl;

R^3 is selected from H, C_1-C_6 alkyl, C_1-C_6 substituted alkyl, or CH_2OR^8 where R^8 is C_1-C_6 alkyl, C_1-C_6 hydroxyalkyl and C_1-C_6 haloalkyl;

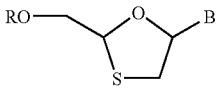
R^4 and R^5 are independently selected from H, NH_2, NHR and NR_2 where R is C_1-C_6 alkyl; and

R^6 and R^7 are independently selected from H and C_1-C_6 alkyl;

or a physiologically functional derivative thereof;

in combination with an effective amount of a compound of the formula

(2)



wherein B is selected from adenine, guanine, cytosine, uracil, thymine, 7-deazaadenine, 7-deazaguanine, 7-deaza-8-azaguanine, 7-deaza-8-azaadenine, inosine, nebularine, nitropyrrole, nitroindole, 2-aminopurine, 2-amino-6-chloropurine, 2,6-diaminopurine, hypoxanthine, pseudouridine, 5-fluorocytosine, 5-chlorocytosine, 5-bromocytosine, 5-iodocytosine, pseudocytosine, pseudoisocytosine, 5-propynylcytosine, isocytosine, isoguanine, 7-deazaguanine, 2-thiopyrimidine, 6-thioguanine, 4-thiothymine, 4-thiouracil, O^6-methylguanine, N^6-methyladenine, O^4-methylthymine, 5,6-dihydrothymine, 5,6-dihydrouracil, 4-methylindole, and a pyrazolo[3,4-D]pyrimidine; and

R is selected from H, C_1-C_{18} alkyl, C_1-C_{18} substituted alkyl, C_2-C_{18} alkenyl, C_2-C_{18} substituted alkenyl, C_2-C_{18} alkynyl, C_2-C_{18} substituted alkynyl, C_6-C_{20} aryl, C_6-C_{20} substituted aryl, C_2-C_{20} heterocycle, C_2-C_{20} substituted heterocycle, phosphonate, phosphophosphonate, diphosphophosphonate, phosphate, diphosphate, triphosphate, polyethyleneoxy or a physiologically functional derivative thereof; and

a pharmaceutically acceptable carrier.

B2. A composition of embodiment A1 wherein, in formula 1, R^1 and R^2 are independently selected from H, C_1-C_6 alkyl, C_1-C_6 substituted alkyl, C_6-C_{20} aryl, C_6-C_{20} substituted aryl, C_6-C_{20} arylalkyl, C_6-C_{20} substituted arylalkyl, acyloxymethyl esters —$CH_2OC(=O)R^9$ and acyloxymethyl carbonates —$CH_2OC(=O)OR^9$ where R^9 is C_1-C_6 alkyl, C_1-C_6 substituted alkyl, C_6-C_{20} aryl and C_6-C_{20} substituted aryl; and R^3, R^4, R^5, R^6 and R^7 are independently H or C_1-C_6 alkyl.

C3. A composition of embodiment A1 wherein, in formula 2, B is cytosine or a 5-halocytosine.

D4. A composition of embodiment A1 wherein, in formula 1, R^1 and R^2 are independently selected from H, C_1-C_6 alkyl, C_1-C_6 substituted alkyl, C_6-C_{20} aryl, C_6-C_{20} substituted aryl, C_6-C_{20} arylalkyl, C_6-C_{20} substituted arylalkyl, acyloxymethyl esters —$CH_2OC(=O)R^9$ and acyloxymethyl carbonates —$CH_2OC(=O)OR^9$ where R^9 is C_1-C_6 alkyl, C_1-C_6 substituted alkyl, C_6-C_{20} aryl and C_1-C_{20} substituted aryl; and R^3, R^4, R^5, R^6 and R^7 are independently H or C_1-C_6 alkyl; and, in formula 2, B is cytosine or a 5-halocytosine.

E5. A composition of embodiment D 4 wherein, in formula 1, R^1 and R^2 are independently selected from H, acyloxymethyl esters —$CH_2OC(=O)R^9$ and acyloxymethyl carbonates —$CH_2OC(=O)OR^9$ where R^9 is C_1-C_6 alkyl; and R^3, R^4, R^5, R^6 and R^7 are independently H or C_1-C_6 alkyl; and, in formula 2, B is cytosine or a 5-halocytosine and R is H.

F6. A composition of embodiment E5 wherein, in formula 1, R^1 and R^2 are independently selected from H and —$CH_2OC(=O)OCH(CH_3)_2$; R^3 is —CH_3; and R^4, R^5, R^6 and R^7 are H; and, in formula 2, B is 5-fluorocytosine and R is H.

G7. A pharmaceutical composition comprising a pharmaceutically effective amount of [2-(6-amino-purin-9-yl)-1-methyl-ethoxymethyl]-phosphonic acid diisopropoxycarbonyloxymethyl ester fumarate (tenofovir disoproxil fumarate) or a physiologically functional derivative thereof and a pharmaceutically effective amount of (2R, 5S,)-4-amino-5-fluoro-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one (emtricitabine) or a physiologically functional derivative thereof; and a pharmaceutically acceptable carrier.

H8. A pharmaceutical formulation of embodiment A1 to G7 further comprising a third active ingredient selected from the group consisting of a protease inhibitor, a nucleoside or nucleotide reverse transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor, and an integrase inhibitor.

I9. A pharmaceutical formulation of embodiments A1 to H8 in unit dosage form.

J10. A method for the treatment or prevention of the symptoms or effects of an HIV infection in an infected animal which comprises administering to said animal a pharmaceutical composition of embodiments claims A1 to I9.

We claim:

1. A chemically stable fixed dose combination pharmaceutical dosage form comprising 300 mg tenofovir disoproxil fumarate and 200 mg emtricitabine; a binder selected from the group consisting of povidone, gelatin, hydroxypropyl methylcellulose, cellulose, microcrystalline cellulose, starch, and acacia; a disintegrant selected from sodium starch glycolate, crosslinked-povidone, cross-linked sodium carboxymethylcellulose, and alginic acid; and a lubricant selected from the group consisting of magnesium stearate, stearic acid, and talc;

wherein said pharmaceutical dosage form exhibits less than 10% degradation of the tenofovir disoproxil fumarate or emtricitabine after 6 months when packaged and stored with silica gel dessicant at 40° C./75% relative humidity.

2. The pharmaceutical dosage form of claim **1** wherein the dosage form is oral.

3. The pharmaceutical dosage form of claim 1 where there is less than 1% degradation of tenofovir disoproxil fumarate over a 24-hour period.

4. The pharmaceutical dosage form of claim 1 where there is less than 0.1% degradation of tenofovir disoproxil fumarate over a 24-hour period.

5. The pharmaceutical dosage form of claim 1 where there is less than 0.01% degradation of tenofovir disoproxil fumarate over a 24-hour period.

6. The pharmaceutical dosage form of claim 1 wherein less than 5% degradation of the tenofovir disoproxil fumarate or emtricitabine occurs after six months at 40° C./75% relative humidity when packaged and stored with desiccant.

7. The pharmaceutical dosage form of claim 1 comprising 300 mg tenofovir disoproxil fumarate, 200 mg emtricitabine, pregelatinized starch, croscarmellose sodium, lactose monohydrate, microcrystalline cellulose, and magnesium stearate.

8. The pharmaceutical dosage form of claim 7 comprising 300 mg tenofovir disoproxil fumarate, 200 mg emtricitabine, 50 mg pregelatinized starch, 60 mg croscarmellose sodium, 80 mg lactose monohydrate, 300 mg microcrystalline cellulose, and 10 mg magnesium stearate.

9. The pharmaceutical dosage form of claim 7 comprising 300 mg tenofovir disoproxil fumarate, 200 mg emtricitabine, 50 mg pregelatinized starch, 60 mg croscarmellose sodium, lactose monohydrate, 200 mg microcrystalline cellulose, and 10 mg magnesium stearate.

10. The pharmaceutical dosage form of claim 1 comprising 300 mg tenofovir disoproxil fumarate, 200 mg emtricitabine, pregelatinized starch, croscarmellose sodium, lactose monohydrate, microcrystalline cellulose, magnesium stearate, and colloidal silicon dioxide.

11. The pharmaceutical dosage form of claim 10 comprising 300 mg tenofovir disoproxil fumarate, 200 mg emtricitabine, 50 mg pregelatinized starch, 60 mg croscarmellose sodium, 175 mg lactose monohydrate, 200 mg microcrystalline cellulose, 10 mg magnesium stearate, and 5 mg colloidal silicon dioxide.

12. The pharmaceutical dosage form of claim 10 comprising 300 mg tenofovir disoproxil fumarate, 200 mg emtricitabine, hydroxypropyl methylcellulose, lactose, pregelatinized starch, and magnesium stearate.

13. The pharmaceutical dosage form of claim 10 comprising 300 mg tenofovir disoproxil fumarate, 200 mg emtricitabine, 112 mg hydroxypropyl methylcellulose, lactose, pregelatinized starch, and 7 mg magnesium stearate.

14. The pharmaceutical dosage form of claim 1 comprising less than 1% of impurities related to tenofovir disoproxil fumarate and emtricitabine.

15. A method for the treatment of the symptoms or effects of an HIV infection in an infected animal which comprises administering to said animal the pharmaceutical dosage form of claim 1.

16. A method for the treatment of the symptoms or effects of an HIV infection in an infected animal which comprises administering to said animal the pharmaceutical dosage form of claim 6.

17. A method for the treatment of the symptoms or effects of an HIV infection in an infected animal which comprises administering to said animal the pharmaceutical dosage form of claim 10.

18. The pharmaceutical dosage form of claim 1, wherein the starch is pregelatinized starch.

19. A chemically stable fixed dose combination pharmaceutical dosage form comprising 300 mg tenofovir disoproxil fumarate and 200 mg emtricitabine; a binder selected from the group consisting of povidone, gelatin, hydroxypropyl methylcellulose, cellulose, microcrystalline cellulose, pregelatinized starch, and acacia; a disintegrant selected from sodium starch glycolate, crosslinked-povidone, cross-linked sodium carboxymethylcellulose, maize starch, and alginic acid; and a lubricant selected from the group consisting of magnesium stearate, stearic acid, and talc;

wherein said pharmaceutical dosage form exhibits less than 10% degradation of the tenofovir disoproxil fumarate or emtricitabine after 6 months when packaged and stored with silica gel dessicant at 40° C./75% relative humidity.

20. A chemically stable fixed dose combination pharmaceutical dosage form comprising 300 mg tenofovir disoproxil fumarate and 200 mg emtricitabine; a binder selected from the group consisting of povidone, gelatin, hydroxypropyl methylcellulose, cellulose, microcrystalline cellulose, starch, and acacia; a disintegrant selected from sodium starch glycolate, crosslinked-povidone, cross-linked sodium carboxymethylcellulose, and alginic acid; and a lubricant selected from the group consisting of magnesium stearate, stearic acid, and talc;

wherein said pharmaceutical dosage form exhibits less than 1% degradation of the tenofovir disoproxil fumarate over a 24-hour period.

21. The pharmaceutical dosage form of claim 20, wherein there is less than 0.1% degradation of tenofovir disoproxil fumarate over a 24-hour period.

22. The pharmaceutical dosage form of claim 20, wherein there is less than 0.01% degradation of tenofovir disoproxil fumarate over a 24-hour period.

23. The pharmaceutical dosage form of claim 20, wherein the starch is pregelatinized starch.

24. A chemically stable fixed dose combination pharmaceutical dosage form comprising 300 mg tenofovir disoproxil fumarate and 200 mg emtricitabine; a binder selected from the group consisting of povidone, gelatin, hydroxypropyl methylcellulose, cellulose, microcrystalline cellulose, pregelatinized starch, and acacia; a disintegrant selected from sodium starch glycolate, crosslinked-povidone, cross-linked sodium carboxymethylcellulose, maize starch, and alginic acid; and a lubricant selected from the group consisting of magnesium stearate, stearic acid, and talc; wherein said pharmaceutical dosage form exhibits less than 1% degradation of the tenofovir disoproxil fumarate over a 24-hour period.

25. The pharmaceutical dosage form of claim 24, wherein there is less than 0.1% degradation of tenofovir disoproxil fumarate over a 24-hour period.

26. The pharmaceutical dosage form of claim 24, wherein there is less than 0.01% degradation of tenofovir disoproxil fumarate over a 24-hour period.

* * * * *

Exhibit D

US008716264B2

(12) **United States Patent**
Dahl et al.

(10) **Patent No.: US 8,716,264 B2**
(45) **Date of Patent: *May 6, 2014**

(54) **COMPOSITIONS AND METHODS FOR COMBINATION ANTIVIRAL THERAPY**

(75) Inventors: **Terrence C. Dahl**, Sunnyvale, CA (US); **Mark M. Menning**, San Francisco, CA (US); **Reza Oliyai**, San Carlos, CA (US)

(73) Assignee: **Gilead Sciences, Inc.**, Foster City, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

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USPC **514/81**

(58) **Field of Classification Search**
USPC 514/45; 424/400, 408
See application file for complete search history.

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Primary Examiner — Alton Pryor

(74) *Attorney, Agent, or Firm* — Gilead Sciences, Inc.

(57) **ABSTRACT**

The present invention relates to therapeutic combinations of [2-(6-amino-purin-9-yl)-1-methyl-ethoxymethyl]-phosphonic acid diisopropoxycarbonyloxymethyl ester (tenofovir disoproxil fumarate, Viread®) and (2R,5S,cis)-4-amino-5-fluoro-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one (emtricitabine, Emtriva™, (−)-cis FTC) and their physiologically functional derivatives. The combinations may be useful in the treatment of HIV infections, including infections with HIV mutants bearing resistance to nucleoside and/or non-nucleoside inhibitors. The present invention is also concerned with pharmaceutical compositions and formulations of said combinations of tenofovir disoproxil fumarate and emtricitabine, and their physiologically functional derivatives, as well as therapeutic methods of use of those compositions and formulations.

38 Claims, No Drawings

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COMPOSITIONS AND METHODS FOR COMBINATION ANTIVIRAL THERAPY

This non-provisional application is a continuation of U.S. patent application Ser. No. 10/540,794, filed Mar. 20, 2006, now abandoned which is a national stage entry of PCT/US04/00832, filed Jan. 13, 2004 which claims the benefit of Provisional Application Nos. 60/440,246 and 60/440,308, both filed Jan. 14, 2003, which is incorporated herein by reference.

FIELD OF THE INVENTION

The invention relates generally to combinations of compounds with antiviral activity and more specifically with anti-HIV properties. In particular, it relates to chemically stable combinations of structurally diverse anti-viral agents.

BACKGROUND OF THE INVENTION

Human immunodeficiency virus (HIV) infection and related diseases are a major public health problem worldwide. Human immunodeficiency virus type 1 (HIV-1) encodes at least three enzymes which are required for viral replication: reverse transcriptase (RT), protease (Prt), and integrase (Int). Although drugs targeting reverse transcriptase and protease are in wide use and have shown effectiveness, particularly when employed in combination, toxicity and development of resistant strains have limited their usefulness (Palella, et al N. *Engl. J. Med*. (1998) 338:853-860; Richman, D. D. *Nature* (2001) 410:995-1001). Human immunodeficiency virus type 1 (HIV-1) protease (Prt) is essential for viral replication and is an effective target for approved antiviral drugs. The HIV Pit cleaves the viral Gag and Gag-Pol polyproteins to produce viral structural proteins (p17, p24, p7 and p6) and the three viral enzymes. Combination therapy with RT inhibitors has proven to be highly effective in suppressing viral replication to unquantifiable levels for a sustained period of time. Also, combination therapy with RT and Prt inhibitors (PI) have shown synergistic effects in suppressing HIV replication. Unfortunately, a high percentage, typically 30 to 50% of patients currently fail combination therapy due to the development of drug resistance, non-compliance with complicated dosing regimens, pharmacokinetic interactions, toxicity, and lack of potency. Therefore, there is a need for new HIV-1 inhibitors that are active against mutant HIV strains, have distinct resistance profiles, fewer side effects, less complicated dosing schedules, and are orally active. In particular, there is a need for a less onerous dosage regimen, such as once per day oral dosing, optimally with as few pills as possible.

The use of combinations of compounds can yield an equivalent antiviral effect with reduced toxicity, or an increase in drug efficacy. Lower overall drug doses can reduce the frequency of occurrence of drug-resistant variants of HIV. Many different methods have been used to examine the effects of combinations of compounds acting together in different assay systems (Furman WO 02/068058). Lower doses predict better patient compliance when pill burden decreases, dosing schedules are simplified and, optionally, if synergy between compounds occurs (Loveday, C. "Nucleoside reverse transcriptase inhibitor resistance" (2001) *JAIDS Journal of Acquired Immune Deficiency Syndromes* 26:S10-S24). AZT (Zidovudine™, 3',-azido, 3'-deoxythymidine) demonstrates synergistic antiviral activity in vitro in combination with agents that act at HIV-1 replicative steps other than reverse transcription, including recombinant soluble CD4 castanospermine and recombinant interferon-α. However, it must be noted that combinations of compounds can give rise to increased cytotoxicity. For example, AZT and recombinant interferon-α have an increased cytotoxic effect on normal human bone marrow progenitor cells.

Chemical stability of combinations of antiviral agents is an important aspect of co-formulation success and the present invention provides examples of such combinations.

There is a need for new combinations of orally-active drugs for the treatment of patients infected with certain viruses, e.g. HIV, that provide enhanced therapeutic safety and efficacy, impart lower resistance, and predict higher patient compliance.

SUMMARY OF THE INVENTION

The present invention provides combinations of antiviral compounds, in particular compositions and methods for inhibition of HIV. In an exemplary aspect, the invention includes a composition including tenofovir disoproxil fumarate and emtricitabine which has anti-HIV activity. The composition of tenofovir DF and emtricitabine is both chemically stable and either synergistic and/or reduces the side effects of one or both of tenofovir DF and emtricitabine. Increased patient compliance is likely in view of the lower pill burden and simplified dosing schedule.

The present invention relates to therapeutic combinations of [2-(6-amino-purin-9-yl)-1-methyl-ethoxymethyl]-phosphonic acid diisopropoxycarbonyloxymethyl ester fumarate (tenofovir disoproxil fumarate, tenofovir DF, TDF, Viread®) and (2R,5S,cis)-4-amino-5-fluoro-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one (emtricitabine, Emtriva™, (−)-cis FTC) and their use in the treatment of HIV infections including infections with HIV mutants bearing resistance to nucleoside and/or non-nucleoside inhibitors. The present invention is also concerned with pharmaceutical compositions and formulations of said combinations of tenofovir disoproxil fumarate and emtricitabine. Another aspect of the invention is a pharmaceutical formulation comprising a physiologically functional derivative of tenofovir disoproxil fumarate or a physiologically functional derivative of emtricitabine.

Therapeutic combinations and pharmaceutical compositions and formulations of the invention include the combination of PMEA or PMPA (tenofovir) compounds with emtricitabine or (2R,5S,cis)-4-amino-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one (3TC, lamivudine, Epivir™), and their use in the treatment of HIV infections.

One aspect of the invention is a method for the treatment or prevention of the symptoms or effects of an HIV infection in an infected animal which comprises administering to, i.e. treating, said animal with a therapeutically effective amount of a combination comprising [2-(6-amino-purin-9-yl)-1-methyl-ethoxymethyl]-phosphonic acid diisopropoxycarbonyloxymethyl ester fumarate (tenofovir DF, TDF) or a physiologically functional derivative thereof and (2R,5S,cis)-4-amino-5-fluoro-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one (emtricitabine) or a physiologically functional derivative thereof.

Another aspect of the invention is a unit dosage form of a therapeutic combination comprising tenofovir disoproxil fumarate and emtricitabine, or physiological functional derivatives thereof. The unit dosage form may be formulated for administration by oral or other routes and is unexpectedly chemically stable in view of the properties of the structurally diverse components.

Another aspect of the invention is directed to chemically stable combination antiviral compositions comprising tenofovir disoproxil fumarate and emtricitabine. In a further

aspect of the invention, the chemically stable combinations of tenofovir disoproxil fumarate and emtricitabine further comprise a third antiviral agent. In this three-component mixture, the unique chemical stability of tenofovir disoproxil fumarate and emtricitabine is taken advantage of in order to enable the combination with the third antiviral agent. Particularly useful third agents include, by way of example and not limitation, those of Table A. Preferably, the third component is an agent approved for antiviral use in humans, more preferably, it is an NNRTI or a protease inhibitor (PI), more preferably yet, it is an NNRTI. In a particularly preferred embodiment, the invention is directed to a combination of the chemically stable mixture of tenofovir disoproxil fumarate and emtricitabine together with efavirenz.

Another aspect of the invention is a patient pack comprising at least one, typically two, and optionally, three active ingredients and other antiviral agents selected from tenofovir disoproxil fumarate and emtricitabine, and an information insert containing directions on the use of tenofovir disoproxil fumarate and emtricitabine together in combination.

Another aspect of the invention is a process for preparing the combinations hereinbefore described, which comprises bringing into association tenofovir DF and emtricitabine of the combination in a medicament to provide an antiviral effect. In a further aspect of the present invention, there is provided the use of a combination of the present invention in the manufacture of a medicament for the treatment of any of the aforementioned viral infections or conditions.

DETAILED DESCRIPTION OF THE INVENTION

While the invention will be described in conjunction with the enumerated claims, it will be understood that they are not intended to limit the invention to those claims. On the contrary, the invention is intended to cover all alternatives, modifications, and equivalents, which may be included within the scope of the present invention as defined by the claims.

DEFINITIONS

Unless stated otherwise, the following terms and phrases as used herein are intended to have the following meanings:

When tradenames are used herein, applicants intend to independently include the tradename product and the active pharmaceutical ingredient(s) of the tradename product.

The term "chemical stability" means that the two primary antiviral agents in combination are substantially stable to chemical degradation. Preferably, they are sufficiently stable in physical combination to permit commercially useful shelf life of the combination product. Typically, "chemically stable" means that a first component of the mixture does not act to degrade a second component when the two are brought into physical combination to form a pharmaceutical dosage form. More typically, "chemically stable" means that the acidity of a first component does not catalyzes or otherwise accelerate the acid decomposition of a second component. By way of example and not limitation, in one aspect of the invention, "chemically stable" means that tenofovir disoproxil fumarate is not substantially degraded by the acidity of emtricitabine. "Substantially" in this context means at least about less than 10%, preferably less than 1%, more preferably less than 0.1%, more preferably yet, less than 0.01% acid degradation of tenofovir disoproxil fumarate over a 24-hour period when the products are in a pharmaceutical dosage form.

The terms "synergy" and "synergistic" mean that the effect achieved with the compounds used together is greater than the sum of the effects that results from using the compounds separately, i.e. greater than what would be predicted based on the two active ingredients administered separately. A synergistic effect may be attained when the compounds are: (1) co-formulated and administered or delivered simultaneously in a combined formulation; (2) delivered by alternation or in parallel as separate formulations; or (3) by some other regimen. When delivered in alternation therapy, a synergistic effect may be attained when the compounds are administered or delivered sequentially, e.g. in separate tablets, pills or capsules, or by different injections in separate syringes. In general, during alternation therapy, an effective dosage of each active ingredient is administered sequentially, i.e. serially, whereas in combination therapy, effective dosages of two or more active ingredients are administered together. A synergistic antiviral effect denotes an antiviral effect which is greater than the predicted purely additive effects of the individual compounds of the combination.

The term "physiologically functional derivative" means a pharmaceutically active compound with equivalent or near equivalent physiological functionality to tenofovir DF or emtricitabine when administered in combination with another pharmaceutically active compound in a combination of the invention. As used herein, the term "physiologically functional derivative" includes any: physiologically acceptable salt, ether, ester, prodrug, solvate, stereoisomer including enantiomer, diastereomer or stereoisomerically enriched or racemic mixture, and any other compound which upon administration to the recipient is capable of providing (directly or indirectly) such a compound or an antivirally active metabolite or residue thereof.

"Bioavailability" is the degree to which the pharmaceutically active agent becomes available to the target tissue after the agent's introduction into the body. Enhancement of the bioavailability of a pharmaceutically active agent can provide a more efficient and effective treatment for patients because, for a given dose, more of the pharmaceutically active agent will be available at the targeted tissue sites.

The compounds of the combinations of the invention may be referred to as "active ingredients" or "pharmaceutically active agents."

The term "prodrug" as used herein refers to any compound that when administered to a biological system generates the drug substance, i.e. active ingredient, as a result of spontaneous chemical reaction(s), enzyme catalyzed chemical reaction(s), and/or metabolic chemical reaction(s).

"Prodrug moiety" means a labile functional group which separates from the active inhibitory compound during metabolism, systemically, inside a cell, by hydrolysis, enzymatic cleavage, or by some other process (Bundgaard, Hans, "Design and Application of Prodrugs" in *Textbook of Drug Design and Development* (1991), P. Krogsgaard-Larsen and H. Bundgaard, Eds. Harwood Academic Publishers, pp. 113-191). Prodrug moieties can serve to enhance solubility, absorption and lipophilicity to optimize drug delivery, bioavailability and efficacy. A "prodrug" is thus a covalently modified analog of a therapeutically-active compound.

"Alkyl" means a saturated or unsaturated, branched, straight-chain, branched, or cyclic hydrocarbon radical derived by the removal of one hydrogen atom from a single carbon atom of a parent alkane, alkene, or alkyne. Typical alkyl groups consist of 1-18 saturated and/or unsaturated carbons, such as normal, secondary, tertiary or cyclic carbon atoms. Examples include, but are not limited to: methyl, Me (—CH_3), ethyl, Et (—CH_2CH_3), acetylenic (—C≡CH), ethylene, vinyl (—CH=CH_2), 1-propyl, n-Pr, n-propyl (—$CH_2CH_2CH_3$), 2-propyl, i-Pr, i-propyl (—$CH(CH_3)_2$),

allyl (—$CR_2CH{=}CH_2$), propargyl (—$CH_2C{\equiv}CH$), cyclopropyl (—C_3H_5), 1-butyl, n-Bu, n-butyl (—$CH_2CH_2CH_2CH_3$), 2-methyl-1-propyl, i-Bu, 1-butyl (—$CH_2CH(CH_3)_2$), 2-butyl, s-Bu, s-butyl (—$CH(CH_3)CH^2CH_3$), 2-methyl-2-propyl, t-Bu, t-butyl (—$C(CH_3)_3$), 1-pentyl, n-pentyl, (—$CH_2CH_2CH_2CH_2CH_3$), 2-pentyl (—$CH(CH_3)CH_2CH_2CH_3$), 3-pentyl (—$CH(CH_2CH_3)_2$), 2-methyl-2-butyl (—$C(CH_3)_2CH_2CH_3$), cyclopentyl (—C_5H_9), 3-methyl-2-butyl (—$CH(CH_3)CH(CH_3)_2$), 3-methyl-1-butyl (—$CH_2CH_2CH(CH_3)_2$), 2-methyl-1-butyl (—$CH_2CH(CH_3)CH_2CH_3$), 1-hexyl (—$CH_2CH_2CH_2CH_2CH_2CH_3$), 5-hexenyl (—$CH_2CH_2CH_2CH_2CH{=}CH_2$) 1-hexyl (—$CH(CH_3)CH_2CH_2CH_2CH_3$), 3-hexyl (—$CH(CH_2CH_3)(CH_2CH_2CH_3)$), cyclohexyl (—$C_6H_{11}$), 2-methyl-2-pentyl (—$C(CH_3)_2CH_2CH_2CH_3$), 3-methyl-2-pentyl (—$CH(CH_3)CH(CH_3)CH_2CH_3$), 4-methyl-2-pentyl (—$CH(CH_3)CH_2CH(CH_3)_2$), 3-methyl-3-pentyl (—$C(CH_3)(CH_2CH_3)_2$), 2-methyl-3-pentyl (—$CH(CH_2CH_3)CH(CH_3)_2$), 2,3-dimethyl-2-butyl (—$C(CH_3)_2CH(CH_3)_2$), and 3,3-dimethyl-2-butyl (—$CH(CH_3)C(CH_3)_3$.

"Aryl" means a monovalent aromatic hydrocarbon radical of 6-20 carbon atoms derived by the removal of one hydrogen atom from a single carbon atom of a parent aromatic ring system. Typical aryl groups include, but are not limited to, radicals derived from benzene, substituted benzene, naphthalene, anthracene, biphenyl, and the like.

"Arylalkyl" refers to an acyclic alkyl radical in which one of the hydrogen atoms bonded to a carbon atom, typically a terminal or Sp^3 carbon atom, is replaced with an aryl radical. Typical arylalkyl groups include, but are not limited to, benzyl, 2-phenylethan-1-yl, 2-phenylethen-1-yl, naphthylmethyl, 2-naphthylethan-1-yl, 2-naphthylethen-1-yl, naphthobenzyl, 2-naphthophenylethan-1-yl and the like. The arylalkyl group 6 to 20 carbon atoms e.g., the alkyl moiety, including alkanyl, alkenyl or alkynyl groups, of the arylalkyl group is 1 to 6 carbon atoms and the aryl moiety is 5 to 14 carbon atoms.

"Substituted alkyl", "substituted aryl", and "substituted arylalkyl" mean alkyl, aryl, and arylalkyl respectively, in which one or more hydrogen atoms are each independently replaced with a substituent. Typical substituents include, but are not limited to, —X, —R, —O^-, —OR, —SR, —S^-, —NR_2, —NR_3, ═NR, —CX_3, —CN, —OCN, —SCN, —N═C═O, —NCS, —NO, —NO_2, ═N_2, —N_3, NC(═O)R, —C(═O)R, —C(═O)NRR—$S(═O)_2O^-$, —$S(═O)_2$OH, —$S(═O)_2$R, —OS(═O)OR, —$S(═O)_2$NR, —S(═O)R, —$OP(═O)O_2RR$, —$P(═O)O_2RR$ —$P(═O)(O^-)_2$, —$P(═O)(OH)_2$, —C(═O)R, —C(═O)X, —C(S)R, —C(O)OR, —$C(O)O^-$, —C(S)OR, —C(O)SR, —C(S)SR, —C(O)NRR, —C(S)NRR, —C(NR)NRR, where each X is independently a halogen: F, Cl, Br, or I; and each R is independently —H, alkyl, aryl, heterocycle, or prodrug moiety.

"Heteroaryl" and "Heterocycle" refer to a ring system in which one or more ring atoms is a heteroatom, e.g. nitrogen, oxygen, and sulfur. Heterocycles are described in: Katritzky, Alan R., Rees, C. W., and Scriven, E. *Comprehensive Heterocyclic Chemistry* (1996) Pergamon Press; Paquette, Leo A.; *Principles of Modern Heterocyclic Chemistry* W. A. Benjamin, New York, (1968), particularly Chapters 1, 3, 4, 6, 7, and 9; "The Chemistry of Heterocyclic Compounds, A series of Monographs" (John Wiley & Sons, New York, 1950 to present), in particular Volumes 13, 14, 16, 19, and 28. Exemplary heterocycles include but are not limited to substituents, i.e. radicals, derived from pyrrole, indole, furan, benzofuran, thiophene, benzothiophene, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-quinolyl, 3-quinolyl, 4-quinolyl, 2-imidazole, 4-imidazole, 3-pyrazole, 4-pyrazole, pyridazine, pyrimidine, pyrazine, purine, cinnoline, pthalazine, quinazoline, quinoxaline, 3-(1,2,4-N)-triazolyl, 5-(1,2,4-N)-triazolyl, 5-tetrazolyl, 4-(1-O,3-N)-oxazole, 5-(1-O,3-N)-oxazole, 4-(1-S,3-N)-thiazole, 5-(1-S,3-N)-thiazole, 2-benzoxazole, 2-benzothiazole, 4-(1,2,3-N)-benzotriazole, and benzimidazole.

Stereochemical definitions and conventions used herein generally follow S. P. Parker, Ed., *McGraw-Hill Dictionary of Chemical Terms* (1984) McGraw-Hill Book Company, New York; and Eliel, E. and Wilen, S., *Stereochemistry of Organic Compounds* (1994) John Wiley & Sons, Inc., New York. Many organic compounds exist in optically active forms, i.e., they have the ability to rotate the plane of plane-polarized light. In describing an optically active compound, the prefixes D and L or R and S are used to denote the absolute configuration of the molecule about its chiral center(s). The prefixes d and l or (+) and (−) are employed to designate the sign of rotation of plane-polarized light by the compound, with (−) or l meaning that the compound is levorotatory. A compound prefixed with (+) or d is dextrorotatory. For a given chemical structure, these compounds, called stereoisomers, are identical except that they are mirror images of one another. A specific stereoisomer is also referred to as an enantiomer, and a mixture of such isomers is often called an enantiomeric mixture. A 50:50 mixture of enantiomers is referred to as a racemic mixture or a racemate. The terms "racemic mixture" and "racemate" refer to an equimolar mixture of two enantiomeric species, devoid of optical activity.

The term "chiral" refers to molecules which have the property of non-superimposability of the mirror image partner, while the term "achiral" refers to molecules which are superimposable on their mirror image partner.

The term "stereoisomers" refers to compounds which have identical chemical constitution, but differ with regard to the arrangement of the atoms or groups in space.

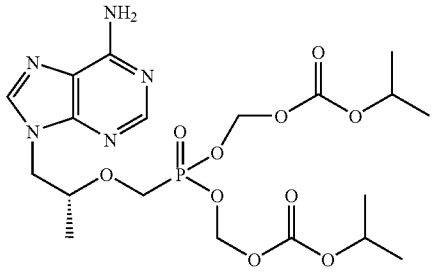
"Diastereomer" refers to a stereoisomer with two or more centers of chirality and whose molecules are not mirror images of one another. Diastereomers have different physical properties, e.g. melting points, boiling points, spectral properties, and reactivities. Mixtures of diastereomers may separate under high resolution analytical procedures such as electrophoresis and chromatography.

"Enantiomers" refer to two stereoisomers of a compound which are non-superimposable mirror images of one another.

Active Ingredients of the Combinations

The present invention provides novel combinations of two or more active ingredients being employed together. In some embodiments, a synergistic antiviral effect is achieved. In other embodiments, a chemically stable combination is obtained. The combinations include at least one active ingredient selected from (1) tenofovir disoproxil fumarate and physiologically functional derivatives, and at least one active ingredient selected from (2) emtricitabine and physiologically functional derivatives. The term "synergistic antiviral effect" is used herein to denote an antiviral effect which is greater than the predicted purely additive effects of the individual components (a) and (b) of the combination.

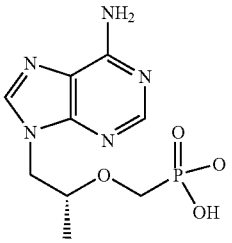
Tenofovir disoproxil fumarate (also known as Viread®, Tenofovir DF, Tenofovir disoproxil, TDF, Bis-POC-PMPA (U.S. Pat. Nos. 5,935,946, 5,922,695, 5,977,089, 6,043,230, 6,069,249) is a prodrug of tenofovir, and has the structure:



and including fumarate salt ($HO_2CCH_2CH_2CO_2^-$).

The chemical names for Tenofovir disoproxil include: [2-(6-amino-purin-9-yl)-1-methyl-ethoxymethyl]-phosphonic acid diisopropoxycarbonyloxymethyl ester; 9-[(R)-2-[[bis [[(isopropoxycarbonyl)oxy]methoxy]phosphinyl]methoxy] propyl]adenine; and 2,4,6,8-tetraoxa-5-phosphanonanedioic acid, 5-[[(1R)-2-(6-amino-9H-purin-9-yl)-1-methylethoxy] methyl]-, bis(1-methylethyl)ester, 5-oxide. The CAS Registry numbers include: 201341-05-1; 202138-50-9; 206184-49-8. It should be noted that the ethoxymethyl unit of tenofovir has a chiral center. The R (rectus, right handed configuration) enantiomer is shown. However, the invention also includes the S isomer. The invention includes all enantiomers, diastereomers, racemates, and enriched stereoisomer mixtures of tenofovir (PMPA) and physiologically functional derivatives thereof.

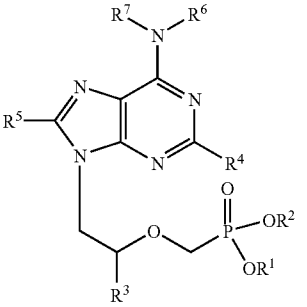
PMPA or tenofovir (U.S. Pat. Nos. 4,808,716, 5,733,788, 6,057,305) has the structure:



The chemical names of PMPA, tenofovir include: (R)-9-(2-phosphonylmethoxypropyl)adenine; and phosphonic acid, [[(1R)-2-(6-amino-9H-purin-9-yl)-1-methylethoxy] methyl]. The CAS Registry number is 147127-20-6.

Tenofovir disoproxil fumarate (DF) is a nucleotide reverse transcriptase inhibitor approved in the United States in 2001 for the treatment of HIV-1 infection in combination with other antiretroviral agents. Tenofovir disoproxil fumarate or Viread® (Gilead Science, Inc.) is the fumarate salt of tenofovir disoproxil. Viread® may be named as: 9-[; R)-2-[[bis [[(isopropoxycarbonyl)oxy]methoxy]phosphinyl]methoxy] propyl]adenine fumarate (1:1); or 2,4,6,8-tetraoxa-5-phosphanonanedioic acid, 5-[[(1R)-2-(6-amino-9H-purin-9-yl)-1-methylethoxy]methyl]-, bis(1-methylethyl) ester, 5-oxide, (2E)-2-butenedioate (1:1). The CAS Registry number is 202138-50-9.

Physiologically functional derivatives of tenofovir disoproxil fumarate include PMEA (adefovir, 9-((R)-2-(phosphonomethoxy)ethyl)adenine) and PMPA compounds. Exemplary combinations include a PMEA or PMPA compound in combination with emtricitabine or 3TC. PMEA and PMPA compounds have the structures:



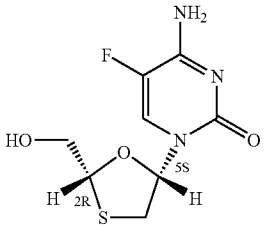
where PMEA (R^3 is H) and PMPA (R^3 is C_1-C_6 alkyl, C_1-C_6 substituted alkyl, or CH_2OR^8 where R^8 is C_1-C_6 alkyl, C_1-C_6 hydroxyalkyl or C_1-C_6 haloalkyl. R^6 and R^7 are independently H or C_1-C_6 alkyl. R^4 and R^5 are independently H, NH_2, NHR or NR_2 where R is C_1-C_6 alkyl. R^1 and R^2 are independently H, C_1-C_6 alkyl, C_1-C_6 substituted alkyl, C_6-C_{20} aryl, C_6-C_{20} substituted aryl, C_6-C_{20} arylalkyl, C_6-C_{20} substituted arylalkyl, acyloxymethyl esters $—CH_2C(=O)R^9$ (e.g. POM) or acyloxymethyl carbonates $—CH_2OC(=O)OR^9$ (e.g. POC) where R^9 is C_1-C_6 alkyl, C_1-C_6 substituted alkyl, C_6-C_{20} aryl or C_6-C_{20} substituted aryl. For example, R_1 and R_2 may be pivaloyloxymethoxy, POM, $—CH_2C(=O)C(CH_3)_3$; $CH_2OC(=O)OC(CH_3)_3$; or POC, $—CH_2C(=O)OCH(CH_3)_2$. Also for example, tenofovir has the structure where R^3 is CH_3, and R^1, R^2, R^4, R^5, R^6 and R^7 are H. Dialkyl phosphonates may be prepared according to the methods of: Quast et al (1974) *Synthesis* 490; Stowell et al (1990) *Tetrahedron Lett.* 3261; U.S. Pat. No. 5,663,159.

The PMPA compound may be enantiomerically-enriched or purified (single stereoisomer) where the carbon atom bearing R^3 may be the R or S enantiomer. The PMPA compound may be a racemate, i.e. a mixture of R and S stereoisomers.

Adefovir (9-(2-phosphonomethoxyethyl)adenine where R_1-R_7═H) is an exemplary PMEA compound (U.S. Pat. Nos. 4,808,716, 4,724,233). As the bis-pivalate prodrug, Adefovir dipivoxil, also known as bis-POM PMEA, (R_3-R_7═H, R_1 and R_2=$—CH_2CO(=O)C(CH_3)_3$, pivoxil, POM, pivaloyloxymethoxy), is effective against HIV and Hepatitis B infections (U.S. Pat. Nos. 5,663,159, 6,451,340). Adefovir dipivoxil has demonstrated minor to moderate synergistic inhibition of HIV replication in combination with other compounds with anti-HIV activity including PMPA, d4T, ddC, nelfinavir, ritonavir, and saquinavir (Mulato et al (1997) *Antiviral Research* 36:91-97).

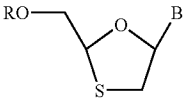
The invention includes all enantiomers, diastereomers, racemates, and enriched stereoisomer mixtures of PMEA and PMPA, and physiologically functional derivatives thereof.

Emtricitabine ((−)-cis-FTC, Emtriva™), a single enantiomer of FTC, is a potent nucleoside reverse transcriptase inhibitor approved for the treatment of HIV (U.S. Pat. Nos. 5,047,407, 5,179,104, 5,204,466, 5,210,085, 5,486,520, 5,538,975, 5,587,480, 5,618,820, 5,763,606, 5,814,639, 5,914,331, 6,114,343, 6,180,639, 6,215,004; WO 02/070518). The single enantiomer emtricitabine has the structure:



The chemical names for emtricitabine include: (−)-cis-FTC; β-L-hydroxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane; (2R,5S)-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine; and 4-amino-5-fluoro-1-(2-hydroxymethyl-[1,3]-(2R,5S)-oxathiolan-5-yl)-1H-pyrimidin-2-one. The CAS Registry numbers include: 143491-57-0; 143491-54-7. It should be noted that FTC contains two chiral centers, at the 2 and 5 positions of the oxathiolane ring, and therefore can exist in the form of two pairs of optical isomers (i.e. enantiomers) and mixtures thereof including racemic mixtures. Thus, FTC may be either a cis or a trans isomer or mixtures thereof. Mixtures of cis and trans isomers are diastereomers with different physical properties. Each cis and trans isomer can exist as one of two enantiomers or mixtures thereof including racemic mixtures. The invention includes all enantiomers, diastereomers, racemates, and enriched stereoisomer mixtures of emtricitabine and physiologically functional derivatives thereof. For example, the invention includes physiological functional derivatives such as the 1:1 racemic mixture of the enantiomers (2R,5S,cis)-4-amino-5-fluoro-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one (emtricitabine) and its mirror image (2S,5R, cis)-4-amino-5-fluoro-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one, or mixtures of the two enantiomers in any relative amount. The invention also includes mixtures of cis and trans forms of FTC.

Physiologically functional derivatives of emtricitabine include 1,3 oxathiolane nucleosides having the structure:

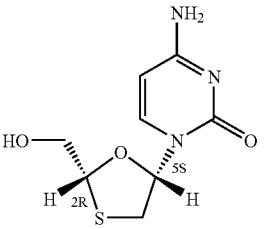


In the 1,3 oxathiolane nucleoside structure above, B is a nucleobase including any nitrogen-containing heterocyclic moiety capable of forming Watson-Crick hydrogen bonds in pairing with a complementary nucleobase or nucleobase analog, e.g. a purine, a 7-deazapurine, or a pyrimidine. Examples of B include the naturally occurring nucleobases: adenine, guanine, cytosine, uracil, thymine, and minor constituents and analogs of the naturally occurring nucleobases, e.g. 7-deazaadenine, 7-deazaguanine, 7-deaza-8-azaguanine, 7-deaza-8-azaadenine, inosine, nebularine, nitropyrrole, nitroindole, 2-aminopurine, 2-amino-6-chloropurine, 2,6-diaminopurine, hypoxanthine, pseudouridine, 5-fluorocytosine, 5-chlorocytosine, 5-bromocytosine, 5-iodocytosine, pseudocytosine, pseudoisocytosine, 5-propynylcytosine, isocytosine, isoguanine, 7-deazaguanine, 2-thiopyrimidine, 6-thioguanine, 4-thiothymine, 4-thiouracil, O^6-methylguanine, N^6-methyladenine, O^4-methylthymine, 5,6-dihydrothymine, 5,6-dihydrouracil, 4-methylindole, pyrazolo[3,4-D]pyrimidines (U.S. Pat. Nos. 6,143,877 and 6,127,121; WO 01/38584), and ethenoadenine (Fasman (1989) in *Practical Handbook of Biochemistry and Molecular Biology*, pp. 385-394, CRC Press, Boca Raton, Fla.).

Nucleobases B may be attached in the configurations of naturally-occurring nucleic acids to the 1,3 oxathiolane moiety through a covalent bond between the N-9 of purines, e.g. adenin-9-yl and guanin-9-yl, or N-1 of pyrimidines, e.g. thymin-1-yl and cytosin-1-yl (Blackburn, G. and Gait, M. Eds. "DNA and RNA structure" in *Nucleic Acids in Chemistry and Biology*, 2nd Edition, (1996) Oxford University Press, pp. 15-81).

Also in the 1,3 oxathiolane nucleoside structure above, R is H, C_1-C_{18} alkyl, C_1-C_{18} substituted alkyl, C_2-C_{18} alkenyl, C_2-C_{18} substituted alkenyl, C_2-C_{18} alkynyl, C_2-C_{18} substituted alkynyl, C_6-C_{20} aryl, C_6-C_{20} substituted aryl, C_2-C_{20} heterocycle, C_2-C_{20} substituted heterocycle, phosphonate, phosphophosphonate, diphosphophosphonate, phosphate, diphosphate, triphosphate, polyethyleneoxy, or a prodrug moiety.

Physiologically functional derivatives of emtricitabine also include 3TC (lamivudine, Epivir®), a reverse transcriptase inhibitor approved in the United States for the treatment of HIV-1 infection in combination with AZT as Combivir® (GlaxoSmithKline). U.S. Pat. Nos. 5,859,021; 5,905,082; 6,177,435; 5,627,186; 6,417,191. Lamivudine (U.S. Pat. Nos. 5,587,480, 5,696,254, 5,618,820, 5,756,706, 5,744,596, 568,164, 5,466,806, 5,151,426) has the structure:



For example and for some therapeutic uses, 3TC may be a physiologically functional derivative of emtricitabine in combination with tenofovir DF or a physiologically functional derivative of tenofovir DF.

It will be appreciated that tenofovir DF and emtricitabine, and their physiologically functional derivatives may exist in keto or enol tautomeric forms and the use of any tautomeric form thereof is within the scope of this invention. Tenofovir DF and emtricitabine will normally be utilized in the combinations of the invention substantially free of the corresponding enantiomer, that is to say no more than about 5% w/w of the corresponding enantiomer will be present.

Prodrugs

The invention includes all prodrugs of tenofovir and emtricitabine. An exemplary prodrug of tenofovir is tenofovir disoproxil fumarate (TDF, Viread®). A large number of structurally-diverse prodrugs have been described for phosphonic acids (Freeman and Ross in *Progress in Medicinal Chemistry* 34: 112-147 (1997). A commonly used prodrug class is the acyloxyalkyl ester, which was first used as a prodrug strategy for carboxylic acids and then applied to phosphates and phosphonates by Farquhar et al (1983) *J. Pharm. Sci.* 72: 324; also U.S. Pat. Nos. 4,816,570, 4,968,788, 5,663,159 and 5,792,756. Subsequently, the acyloxyalkyl ester was used to deliver phosphonic acids across cell membranes and to enhance oral bioavailability. A close variant of the acyloxyalkyl ester strategy, the alkoxycarbonyloxyalkyl ester, may also enhance oral bioavailability as a prodrug moiety in the compounds of the combinations of the invention. Aryl esters of phosphorus

groups, especially phenyl esters, are reported to enhance oral bioavailability (DeLambert et al (1994) *J. Med. Chem.* 37: 498). Phenyl esters containing a carboxylic ester ortho to the phosphate have also been described (Khamnei and Torrence, (1996) *J. Med. Chem.* 39:4109-4115). Benzyl esters are reported to generate the parent phosphonic acid. In some cases, substituents at the ortho- or para-position may accelerate the hydrolysis. Benzyl analogs with an acylated phenol or an alkylated phenol may generate the phenolic compound through the action of enzymes, e.g. esterases, oxidases, etc., which in turn undergoes cleavage at the benzylic C—O bond to generate the phosphoric acid and the quinone methide intermediate. Examples of this class of prodrugs are described by Mitchell et al (1992) *J. Chem. Soc. Perkin Trans. I*2345; Brook et al WO 91/19721. Still other benzylic prodrugs have been described containing a carboxylic ester-containing group attached to the benzylic methylene (Glazier et al WO 91/19721). Thio-containing prodrugs are reported to be useful for the intracellular delivery of phosphonate drugs. These proesters contain an ethylthio group in which the thiol group is either esterified with an acyl group or combined with another thiol group to form a disulfide. Deesterifecation or reduction of the disulfide generates the free thio intermediate which subsequently breaks down to the phosphoric acid and episulfide (Puech et al (1993) *Antiviral Res.*, 22: 155-174; Benzaria et al (1996) *J. Med. Chem.* 39: 4958). Cyclic phosphonate esters have also been described as prodrugs of phosphorus-containing compounds.

Prodrug esters in accordance with the invention are independently selected from the following groups: (1) mono-, di-, and tri-phosphate esters of tenofovir or emtricitabine or any other compound which upon administration to a human subject is capable of providing (directly or indirectly) said mono-, di, or triphosphate ester; (2) carboxylic acid esters (3) sulphonate esters, such as alkyl- or aralkylsulphonyl (for example, methanesulphonyl); (4) amino acid esters (for example, alanine, L-valyl or L-isoleucyl); (5) phosphonate; and (6) phosphonamidate esters.

Ester groups (1)-(6) may be substituted with; straight or branched chain C_1-C_{18} alkyl (for example, methyl, n-propyl, t-butyl, or n-butyl); C_3-C_{12} cycloalkyl; alkoxyalkyl (for example, methoxymethyl); arylalkyl (for example, benzyl); aryloxyalkyl (for example, phenoxymethyl); C_5-C_{20} aryl (for example, phenyl optionally substituted by, for example, halogen, C_1-C_4 alkyl, C_1-C_4 alkoxy, or amino; acyloxymethyl esters —$CH_2C(=O)R^9$ (e.g. POM) or acyloxymethyl carbonates —$CH_2C(=O)OR^9$ (e.g. POC) where R^9 is C_1-C_6 alkyl, C_1-C_6 substituted alkyl, C_6-C_{20} aryl or C_6-C_{20} substituted aryl. For example, ester groups may be: —$CH_2OC(O)C(CH_3)_3$, —$CH_2OC(O)OC(CH_3)_3$ or —$CH_2C(=O)OCH(CH_3)_2$.

An exemplary aryl moiety present in such esters comprises a phenyl or substituted phenyl group. Many phosphate prodrug moieties are described in U.S. Pat. No. 6,312,662; Jones et al (1995) *Antiviral Research* 27:1-17; Kucera et al (1990) *AIDS Res. Hum. Retro Viruses* 6:491-501; Piantadosi et al (1991) *J. Med. Chem.* 34:1408-14; Hosteller et al (1992) *Antimicrob. Agents Chemother.* 36:2025-29; Hostetler et al (1990) *J. Biol. Chem.* 265:611127; and Siddiqui et al (1999) *J. Med. Chem.* 42:4122-28.

Pharmaceutically acceptable prodrugs refer to a compound that is metabolized in the host, for example hydrolyzed or oxidized, by either enzymatic action or by general acid or base solvolysis, to form an active ingredient. Typical examples of prodrugs of the active ingredients of the combinations of the invention have biologically labile protecting groups on a functional moiety of the active compound. Prodrugs include compounds that can be oxidized, reduced, aminated, deaminated, esterified, deesterified, alkylated, dealkylated, acylated, deacylated, phosphorylated, dephosphorylated, or other functional group change or conversion involving forming or breaking chemical bonds on the prodrug.

Chemical Stability of a Pharmaceutical Formulation

The chemical stability of the active ingredients in a pharmaceutical formulation is of concern to minimize the generation of impurities and ensure adequate shelf-life. The active ingredients, tenofovir disoproxil fumarate and emtricitabine, in the pharmaceutical formulations of the invention have relatively low pKa values, indicative of the potential to cause acidic hydrolysis of the active ingredients. Emtricitabine, with a pKa of 2.65 (Emtriva™ Product Insert, Gilead Sciences, Inc. 2003, available at gilead.com) is subject to hydrolytic deamination of the 5-fluoro cytosine nucleobase to form the 5-fluoro uridine nucleobase. Tenofovir disoproxil fumarate, with a pKa of 3.75 (Yuan L. et al "Degradation Kinetics of Oxycarbonyloxymethyl Prodrugs of Phosphonates in Solution", *Pharmaceutical Research* (2001) Vol. 18, No. 2, 234-237), is subject also to hydrolytic deamination of the exocyclic amine of the adenine nucleobase, and to hydrolysis of one or both of the POC ester groups (U.S. Pat. No. 5,922, 695). It is desirable to formulate a therapeutic combination of tenofovir disoproxil fumarate and emtricitabine, and the physiological functional derivatives thereof, with a minimum of impurities and adequate stability.

The combinations of the present invention provide combination pharmaceutical dosage forms which are chemically stable to acid degradation of: (1) a first component (such as tenofovir disoproxil fumarate, and physiological functional derivatives; (2) a second component (such as emtricitabine, and physiological functional derivatives; and (3) optionally a third component having antiviral activity. The third component includes anti-HIV agents and include: protease inhibitors (PI), nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), and integrase inhibitors. Exemplary third active ingredients to be administered in combination with first and second components are shown in Table A. First and second components are as defined in the above section entitled: *ACTIVE INGREDIENTS OF THE COMBINATIONS.*

Salts

Any reference to any of the compounds in the compositions of the invention also includes any physiologically acceptable salt thereof. Examples of physiologically acceptable salts of tenofovir DF, emtricitabine and their physiologically functional derivatives include salts derived from an appropriate base, such as an alkali metal (for example, sodium), an alkaline earth (for example, magnesium), ammonium and NX_4^+ (wherein X is C_1-C_4 alkyl), or an organic acid such as fumaric acid, acetic acid, succinic acid. Physiologically acceptable salts of an hydrogen atom or an amino group include salts of organic carboxylic acids such as acetic, benzoic, lactic, fumaric, tartaric, maleic, malonic, malic, isethionic, lactobionic and succinic acids; organic sulfonic acids, such as methanesulfonic, ethanesulfonic, benzenesulfonic and p-toluenesulfonic acids; and inorganic acids, such as hydrochloric, sulfuric, phosphoric and sulfamic acids. Physiologically acceptable salts of a compound of an hydroxy group include the anion of said compound in combination with a suitable cation such as Na^+ and NX_4^+ (wherein X is independently selected from H or a C_1-C_4 alkyl group).

For therapeutic use, salts of active ingredients of the combinations of the invention will be physiologically acceptable, i.e. they will be salts derived from a physiologically accept-

able acid or base. However, salts of acids or bases which are not physiologically acceptable may also find use, for example, in the preparation or purification of a physiologically acceptable compound. All salts, whether or not derived from a physiologically acceptable acid or base, are within the scope of the present invention.

Administration of the Formulations

While it is possible for the active ingredients of the combination to be administered alone and separately as monotherapies, it is preferable to administer them as a pharmaceutical co-formulation. A two-part or three-part combination may be administered simultaneously or sequentially. When administered sequentially, the combination may be administered in one, two, or three administrations.

Preferably, two-part or three-part combinations are administered in a single pharmaceutical dosage form. More preferably, a two-part combination is administered as a single oral dosage form and a three-part combination is administered as two identical oral dosage forms. Examples include a single tablet of tenofovir disoproxil fumarate and emtricitabine, or two tablets of tenofovir disoproxil fumarate, emtricitabine, and efavirenz.

It will be appreciated that the compounds of the combination may be administered: (1) simultaneously by combination of the compounds in a co-formulation or (2) by alternation, i.e. delivering the compounds serially, sequentially, in parallel or simultaneously in separate pharmaceutical formulations. In alternation therapy, the delay in administering the second, and optionally a third active ingredient, should not be such as to lose the benefit of a synergistic therapeutic effect of the combination of the active ingredients. By either method of administration (1) or (2), ideally the combination should be administered to achieve peak plasma concentrations of each of the active ingredients. A one pill once-per-day regimen by administration of a combination co-formulation may be feasible for some HIV-positive patients. Effective peak plasma concentrations of the active ingredients of the combination will be in the range of approximately 0.001 to 100 μM. Optimal peak plasma concentrations may be achieved by a formulation and dosing regimen prescribed for a particular patient. It will also be understood that tenofovir DF and emtricitabine, or the physiologically functional derivatives of either thereof, whether presented simultaneously or sequentially, may be administered individually, in multiples, or in any combination thereof. In general, during alternation therapy (2), an effective dosage of each compound is administered serially, where in co-formulation therapy (1), effective dosages of two or more compounds are administered together.

Formulation of the Combinations

When the individual components of the combination are administered separately they are generally each presented as a pharmaceutical formulation. The references hereinafter to formulations refer unless otherwise stated to formulations containing either the combination or a component compound thereof. It will be understood that the administration of the combination of the invention by means of a single patient pack, or patient packs of each formulation, within a package insert diverting the patient to the correct use of the invention is a desirable additional feature of this invention. The invention also includes a double pack comprising in association for separate administration, formulations of tenofovir disoproxil fumarate and emtricitabine, or a physiologically functional derivative of either or both thereof.

The combination therapies of the invention include: (1) a combination of tenofovir DF and emtricitabine or (2) a combination containing a physiologically functional derivative of either or both thereof.

The combination may be formulated in a unit dosage formulation comprising a fixed amount of each active pharmaceutical ingredient for a periodic, e.g. daily, dose or subdose of the active ingredients.

Pharmaceutical formulations according to the present invention comprise a combination according to the invention together with one or more pharmaceutically acceptable carriers or excipients and optionally other therapeutic agents. Pharmaceutical formulations containing the active ingredient may be in any form suitable for the intended method of administration. When used for oral use for example, tablets, troches, lozenges, aqueous or oil suspensions, dispersible powders or granules, emulsions, hard or soft capsules, syrups or elixirs may be prepared (Remington's Pharmaceutical Sciences (Mack Publishing Co., Easton, Pa.). Compositions intended for oral use may be prepared according to any method known to the art for the manufacture of pharmaceutical compositions and such compositions may contain one or more agents including antioxidants, sweetening agents, flavoring agents, coloring agents and preserving agents, in order to provide a palatable preparation. Tablets containing the active ingredient in admixture with non-toxic pharmaceutically acceptable excipient which are suitable for manufacture of tablets are acceptable. These excipients may be, for example, inert diluents, such as calcium or sodium carbonate, lactose, lactose monohydrate, croscarmellose sodium, povidone, calcium or sodium phosphate; granulating and disintegrating agents, such as maize starch, or alginic acid; binding agents, such as cellulose, microcrystalline cellulose, starch, gelatin or acacia; and lubricating agents, such as magnesium stearate, stearic acid or talc. Tablets may be uncoated or may be coated by known techniques including microencapsulation to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monostearate or glyceryl distearate alone or with a wax may be employed.

Formulations for oral use may be also presented as hard gelatin capsules where the active ingredient is mixed with an inert solid diluent, for example pregelatinized starch, calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, such as peanut oil, liquid paraffin or olive oil.

Aqueous suspensions of the invention contain the active materials in admixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients include a suspending agent, such as sodium carboxymethylcellulose, methylcellulose, hydroxypropyl methylcelluose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia, and dispersing or wetting agents such as a naturally occurring phosphatide (e.g., lecithin), a condensation product of an alkylene oxide with a fatty acid (e.g., polyoxyethylene stearate), a condensation product of ethylene oxide with a long chain aliphatic alcohol (e.g., heptadecaethyleneoxycetanol), a condensation product of ethylene oxide with a partial ester derived from a fatty acid and a hexitol anhydride (e.g., polyoxyethylene sorbitan monooleate). The aqueous suspension may also contain one or more preservatives such as ethyl or n-propyl p-hydroxybenzoate, one or more coloring agents, one or more flavoring agents and one or more sweetening agents, such as sucrose, sucralose or saccharin.

Oil suspensions may be formulated by suspending the active ingredient in a vegetable oil, such as arachis oil, olive

oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin. The oral suspensions may contain a thickening agent, such as beeswax, hard paraffin or cetyl alcohol. Sweetening agents, such as those set forth above, and flavoring agents may be added to provide a palatable oral preparation. These compositions may be preserved by the addition of an antioxidant such as ascorbic acid, BHT, etc.

Dispersible powders and granules of the invention suitable for preparation of an aqueous suspension by the addition of water provide the active ingredient in admixture with a dispersing or wetting agent, a suspending agent, and one or more preservatives. Suitable dispersing or wetting agents and suspending agents are exemplified by those disclosed above. Additional excipients, for example sweetening, flavoring and coloring agents, may also be present.

The pharmaceutical compositions of the invention may also be in the form of oil-in-water emulsions or liposome formulations. The oily phase may be a vegetable oil, such as olive oil or arachis oil, a mineral oil, such as liquid paraffin, or a mixture of these. Suitable emulsifying agents include naturally-occurring gums, such as gum acacia and gum tragacanth, naturally occurring phosphatides, such as soybean lecithin, esters or partial esters derived from fatty acids and hexitol anhydrides, such as sorbitan monooleate, and condensation products of these partial esters with ethylene oxide, such as polyoxyethylene sorbitan monooleate. The emulsion may also contain sweetening and flavoring agents. Syrups and elixirs may be formulated with sweetening agents, such as glycerol, sorbitol or sucrose. Such formulations may also contain a demulcent, a preservative, a flavoring or a coloring agent.

The pharmaceutical compositions of the invention may be in the form of a sterile injectable preparation, such as a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated according to the known art using those suitable dispersing or wetting agents and suspending agents which have been mentioned above. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally acceptable diluent or solvent, such as a solution in 1,3-butane-diol or prepared as a lyophilized powder. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile fixed oils may conventionally be employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid may likewise be used in the preparation of injectables.

The pharmaceutical compositions of the invention may be injected parenterally, for example, intravenously, intraperitoneally, intrathecally, intraventricularly, intrasternally, intracranially, intramuscularly or subcutaneously, or they may be administered by infusion techniques. They are best used in the form of a sterile aqueous solution which may contain other substances, for example, enough salts or glucose to make the solution isotonic with blood. The aqueous solutions should be suitably buffered (preferably to a pH of from 3 to 9), if necessary. The preparation of suitable parenteral formulations under sterile conditions is readily accomplished by standard pharmaceutical techniques well known to those skilled in the art.

The pharmaceutical compositions of the invention may also be administered intranasally or by inhalation and are conveniently delivered in the form of a dry powder inhaler or an aerosol spray presentation from a pressurized container or a nebuliser with the use of a suitable propellant, e.g. dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, a hydrofluoroalkane such as 1,1,1,2-tetrafluoroethane (HFC 134a), carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. The pressurized container or nebuliser may contain a solution or suspension of the composition, e.g. using a mixture of ethanol and the propellant as the solvent, which may additional contain a lubricant, e.g. sorbitan trioleate. Capsules and cartridges (made, for example, from gelatin) for use in an inhaler or insufflator may be formulated to contain a powder mix of a compound of the formula (I) and a suitable powder base such as lactose or starch. Aerosol or dry powder formulations are preferably arranged so that each metered dose or "puff" contains from 20 μg to 20 mg of a composition for delivery to the patient. The overall daily dose with an aerosol will be in the range of from 20 μg to 20 mg which may be administered in a single dose or, more usually, in divided doses throughout the day.

The amount of active ingredient that may be combined with the carrier material to produce a single dosage form will vary depending upon the host treated and the particular mode of administration. For example, a time-release formulation intended for oral administration to humans may contain approximately 1 to 1000 mg of active material compounded with an appropriate and convenient amount of carrier material which may vary from about 5 to about 95% of the total compositions (weight:weight). The pharmaceutical composition can be prepared to provide easily measurable amounts for administration. For example, an aqueous solution intended for intravenous infusion may contain from about 3 to 500 μg of the active ingredient per milliliter of solution in order that infusion of a suitable volume at a rate of about 30 mL/hr can occur. As noted above, formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous or non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be administered as a bolus, electuary or paste.

The combinations of the invention may conveniently be presented as a pharmaceutical formulation in a unitary dosage form. A convenient unitary dosage formulation contains the active ingredients in any amount from 1 mg to 1 g each, for example but not limited to, 10 mg to 300 mg. The synergistic effects of tenofovir DF in combination with emtricitabine may be realized over a wide ratio, for example 1:50 to 50:1 (tenofovir DF:emtricitabine). In one embodiment the ratio may range from about 1:10 to 10:1. In another embodiment, the weight/weight ratio of tenofovir to emtricitabine in a co-formulated combination dosage form, such as a pill, tablet, caplet or capsule will be about 1, i.e. an approximately equal amount of tenofovir DF and emtricitabine. In other exemplary co-formulations, there may be more or less tenofovir than FTC. For example, 300 mg tenofovir DF and 200 mg emtricitabine can be co-formulated in a ratio of 1.5:1 (tenofovir DF: emtricitabine). In one embodiment, each compound will be employed in the combination in an amount at which it exhibits antiviral activity when used alone. Exemplary Formulations A, B, C, D, E, and F (Examples) have ratios of 12:1 to 1:1 (tenofovir F: emtricitabine). Exemplary Formulations A, B, C, D, E, and F use amounts of tenofovir DF and emtricitabine ranging from 25 mg to 300 mg. Other ratios and amounts of the compounds of said combinations are contemplated within the scope of the invention.

A unitary dosage form may further comprise tenofovir DF and emtricitabine, or physiologically functional derivatives of either thereof, and a pharmaceutically acceptable carrier.

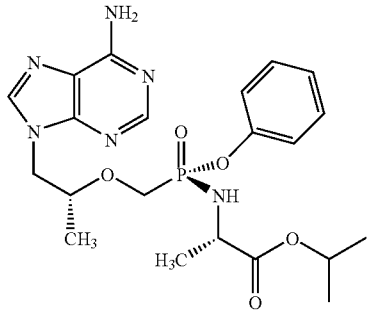
It will be appreciated by those skilled in the art that the amount of active ingredients in the combinations of the invention required for use in treatment will vary according to a variety of factors, including the nature of the condition being treated and the age and condition of the patient, and will ultimately be at the discretion of the attending physician or health care practitioner. The factors to be considered include the route of administration and nature of the formulation, the animal's body weight, age and general condition and the nature and severity of the disease to be treated. For example, in a Phase I/II monotherapy study of emtricitabine, patients received doses ranging from 25 mg to 200 mg twice-a-day for two weeks. At each dose regimen greater or equal to 200 mg, a 98-percent (1.75 log 10) or greater viral suppression was observed. A once-a-day dose of 200 mg of emtricitabine reduced the viral load by an average of 99 percent (1.92 log 10). Viread® (tenofovir DF) has been approved by the FDA for the treatment and prophylaxis of HIV infection as a 300 mg oral tablet. Emtriva™ (emtricitabine) has been approved by the FDA for the treatment of HIV as a 200 mg oral tablet.

It is also possible to combine any two of the active ingredients in a unitary dosage form for simultaneous or sequential administration with a third active ingredient. The three-part combination may be administered simultaneously or sequentially. When administered sequentially, the combination may be administered in two or three administrations. Third active ingredients have anti-HIV activity and include protease inhibitors (PI), nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), and integrase inhibitors. Exemplary third active ingredients to be administered in combination with tenofovir DF, emtricitabine, and their physiological functional derivatives, are shown in Table A.

TABLE A

5,6 dihydro-5-azacytidine
5-aza 2'deoxycytidine
5-azacytidine
5-yl-carbocyclic 2'-deoxyguanosine (BMS200,475)
9 (arabinofuranosyl)guanine; 9-(2' deoxyribofuranosyl)guanine
9-(2'-deoxy 2'fluororibofuranosyl)-2,6-diaminopurine
9-(2'-deoxy 2'fluororibofuranosyl)guanine
9-(2'-deoxyribofuranosyl)-2,6 diaminopurine
9-(arabinofuranosyl)-2,6 diaminopurine
Abacavir, Ziagen ®
Acyclovir, ACV; 9-(2-hydroxyethoxylmethyl)guanine
Adefovir dipivoxil, Hepsera ®
amdoxivir, DAPD
Amprenavir, Agenerase ®
araA; 9-β-D-arabinofuranosyladenine (Vidarabine)
atazanivir sulfate (Reyataz ®)
AZT; 3'-azido-2',3'-dideoxythymdine, Zidovudine, (Retrovir ®)
BHCG; (.+−.)-(1a,2b,3a)-9-[2,3-bis(hydroxymethyl)cyclobutyl]guanine
BMS200,475; 5-yl-carbocyclic 2'-deoxyguanosine
Buciclovir; (R) 9-(3,4-dihydroxybutyl)guanine
BvaraU; 1-β-D-arabinofuranosyl-E-5-(2-bromovinyl)uracil (Sorivudine)
Calanolide A
Capravirine
CDG; carbocyclic 2'-deoxyguanosine
Cidofovir, HPMPC; (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine
Clevudine, L-FMAU; 2'-Fluoro-5-methyl-β-L-arabino-furanosyluracil
Combivir ® (lamivudine/zidovudine)
Cytallene; [1-(4'-hydroxy-1',2'-butadienyl)cytosine]
d4C; 3'-deoxy-2',3'-didehydrocytidine
DAPD; (−)-β-D-2,6-diaminopurine dioxolane
ddA; 2',3'-dideoxyadenosine
ddAPR; 2,6-diaminopurine-2',3'-dideoxyriboside
ddC; 2',3'-dideoxycytidine (Zalcitabine)
ddI; 2',3'-dideoxyinosine, didanosine, (Videx ®, Videx ® EC)
Delavirdine, Rescriptor ®
Didanosine, ddI, Videx ®; 2',3'-dideoxyinosine
DXG; dioxolane guanosine
E-5-(2-bromovinyl)-2'-deoxyuridine
Efavirenz, Sustiva ®
Enfuvirtide, Fuzeon ®
F-ara-A; fluoroarabinosyladenosine (Fludarabine)
FDOC; (−)-β-D-5-fluoro-1-[2-(hydroxymethyl)-1,3-dioxolane]cytosine
FEAU; 2'-deoxy-2'-fluoro-1-β-D-arabinofuranosyl-5-ethyluracil
FIAC; 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine
FIAU; 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouridine
FLG; 2',3'-dideoxy-3'-fluoroguanosine
FLT; 3'-deoxy-3'-fluorothymidine
Fludarabine; F-ara-A; fluoroarabinosyladenosine
FMAU; 2'-Fluoro-5-methyl-β-L-arabino-furanosyluracil
FMdC
Foscarnet; phosphonoformic acid, PFA
FPMPA; 9-(3-fluoro-2-phosphonylmethoxypropyl)adenine
Gancyclovir, GCV; 9-(1,3-dihydroxy-2-propoxymethyl)guanine
GS-7340; 9-[R-2-[[(S)-[[(S)-1-(isopropoxycarbonyl)ethyl]amino]-phenoxyphosphinyl]methoxy]propyl]adenine
HPMPA; (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine
HPMPC; (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (Cidofovir)
Hydroxyurea, Droxia ®
Indinavir, Crixivan ®
Kaletra ® (lopinavir/ritonavir)
Lamivudine, 3TC, Epivir ™; (2R,5S,cis)-4-amino-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one
L-d4C; L-3'-deoxy-2',3'-didehydrocytidine
L-ddC; L-2',3'-dideoxycytidine
L-Fd4C; L-3'-deoxy-2',3'-didehydro-5-fluorocytidine
L-FddC; L-2',3'-dideoxy-5-fluorocytidine
Lopinavir
Nelfinavir, Viracept ®
Nevirapine, Viramune ®
Oxetanocin A; 9-(2-deoxy-2-hydroxymethyl-β-D-erythro-oxetanosyl)adenine
Oxetanocin G; 9-(2-deoxy-2-hydroxymethyl-β-D-erythro-oxetanosyl)guanine
Penciclovir
PMEDAP; 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine
PMPA, tenofovir; (R)-9-(2-phosphonylmethoxypropyl)adenine
PPA; phosphonoacetic acid
Ribavirin; 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide
Ritonavir, Norvir ®
Saquinavir, Invirase ®, Fortovase ®
Sorivudine, BvaraU; 1-β-D-arabinofuranosyl-E-5-(2-bromovinyl)uracil
Stavudine, d4T, Zerit ®; 2',3'-didehydro-3'-deoxythymidine
Trifluorothymidine, TFT; Trifluorothymidine
Trizivir ® (abacavir sulfate/lamivudine/zidovudine)
Vidarabine, araA; 9-β-D-arabinofuranosyladenine
Zalcitabine, Hivid ®, ddC; 2',3'-dideoxycytidine
Zidovudine, AZT, Retrovir ®; 3'-azido-2',3'-dideoxythymdine
Zonavir; 5-propynyl-1-arabinosyluracil

Another aspect of the present invention is a three-part combination comprising tenofovir DF, FTC, and 9-[(R)-2-[[(S)-[[(S)-1-(isopropoxycarbonyl)ethyl]amino]phenoxy-phosphinyl]methoxy]propyl]adenine, also designated herein as GS-7340, which has the structure:



GS-7340 is a prodrug of tenofovir and the subject of commonly owned, pending application, U.S. Ser. No. 09/909,560, filed Jul. 20, 2001 and Becker et al WO 02/08241.

For example, a ternary unitary dosage may contain 1 mg to 1000 mg of tenofovir disoproxil fumarate, 1 mg to 1000 mg of emtricitabine, and 1 mg to 1000 mg of the third active ingredient. As a further feature of the present invention, a unitary dosage form may further comprise tenofovir DF, emtricitabine, the third active ingredient, or physiologically functional derivatives of the three active ingredients thereof, and a pharmaceutically acceptable carrier.

Combinations of the present invention enable patients greater freedom from multiple dosage medication regimens and ease the needed diligence required in remembering and complying with complex daily dosing times and schedules. By combining tenofovir disoproxil fumarate and emtricitabine into a single dosage form, the desired daily regimen may be presented in a single dose or as two or more sub-doses per day. The combination of co-formulated tenofovir DF and emtricitabine may be administered as a single pill, once per day.

A further aspect of the invention is a patient pack comprising at least one active ingredient: tenofovir disoproxil fumarate, emtricitabine, or a physiologically functional derivative of either of the combination and an information package or product insert containing directions on the use of the combination of the invention.

Segregation of active ingredients in pharmaceutical powders and granulations is a widely recognized problem that can result in inconsistent dispersions of the active ingredients in final dosage forms. Some of the main factors contributing to segregation are particle size, shape and density. Segregation is particularly troublesome when attempting to formulate a single homogenous tablet containing multiple active ingredients having different densities and different particle sizes. Glidants are substances that have traditionally been used to improve the flow characteristics of granulations and powders by reducing interparticulate friction. See Lieberman, *Lachman, & Schwartz, Pharmaceutical Dosage Forms: Tablets, Volume* 1, p. 177-178 (1989), incorporated herein by reference. Glidants are typically added to pharmaceutical compositions immediately prior to tablet compression to facilitate the flow of granular material into the die cavities of tablet presses. Glidants include: colloidal silicon dioxide, asbestos free talc, sodium aluminosilicate, calcium silicate, powdered cellulose, microcrystalline cellulose, corn starch, sodium benzoate, calcium carbonate, magnesium carbonate, metallic stearates, calcium stearate, magnesium stearate, zinc stearate, stearowet C, starch, starch 1500, magnesium lauryl sulfate, and magnesium oxide. Exemplary Tablet Formulation A has colloidal silicon dioxide (Examples). Glidants can be used to increase and aid blend composition homogeneity in formulations of anti-HIV drugs (U.S. Pat. No. 6,113,920). The novel compositions of the present invention may contain glidants to effect and maintain homogeneity of active ingredients during handling prior to tablet compression.

The present invention provides pharmaceutical formulations combining the active ingredients tenofovir DF and emtricitabine, or physiologically functional derivatives thereof in a sufficiently homogenized form, and a method for using this pharmaceutical formulation. An object of the present invention is to utilize glidants to reduce the segregation of active ingredients in pharmaceutical compositions during pre-compression material handling. Another object of the present invention is to provide a pharmaceutical formulation combining the active ingredients tenofovir DF and emtricitabine, or physiologically functional derivatives thereof, with a pharmaceutically acceptable glidant, resulting in a mixture characterized by a pharmaceutically acceptable measure of homogeneity.

Formulations include those suitable for oral, rectal, nasal, topical (including transdermal, buccal and sublingual), vaginal or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. Such methods represent a further feature of the present invention and include the step of bringing into association the active ingredients with the carrier, which constitutes one or more accessory ingredients, and maintaining chemical stability. In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then if necessary shaping the product.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, caplets, cachets or tablets each containing a predetermined amount of the active ingredients; as a powder or granules; as a solution or a suspension in an aqueous or non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredients in a free-flowing form such as a powder or granules, optionally mixed with a binder (e.g. povidone, gelatin, hydroxypropyl methylcellulose), lubricant, inert diluent, preservative, disintegrant (e.g. sodium starch glycollate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose) surface-active or dispersing agent. Molded tablets may be made by molding a mixture of the powdered compound moistened with an inert liquid diluent in a suitable machine. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredients therein using, for example, cellulose ether derivatives (e.g., hydroxypropyl methylcellulose) or methacrylate derivatives in varying proportions to provide the desired release profile. Tablets may optionally be provided with an enteric coating, to provide release in parts of the gut other than the stomach.

Formulations suitable for topical administration in the mouth include lozenges comprising the active ingredients in a flavored base, usually sucrose and acacia or tragacanth; pastiles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouthwashes comprising the active ingredient in a suitable liquid carrier. Formulations for rectal administration may be presented as a suppository with a suitable base comprising, for

example, cocoa butter or a salicylates. Topical administration may also be by means of a transdermal iontophoretic device.

Formulations suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations containing in addition to the active ingredient such carriers as are known in the art to be appropriate.

Formulations suitable for penile administration for prophylactic or therapeutic use may be presented in condoms, creams, gels, pastes, foams or spray formulations containing in addition to the active ingredient such carriers as are known in the art to be appropriate.

Pharmaceutical formulations suitable for rectal administration wherein the carrier is a solid are most preferably presented as unit dose suppositories. Suitable carriers include cocoa butter and other materials commonly used in the art. The suppositories may be conveniently formed by admixture of the active combination with the softened or melted carrier(s) followed by chilling and shaping in moulds.

Formulations suitable for parenteral administration include aqueous and nonaqueous isotonic sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and nonaqueous sterile suspensions which may include suspending agents and thickening agents; and liposomes or other microparticulate systems which are designed to target the compound to blood components or one or more organs. The formulations may be presented in unit-dose or multi-dose sealed containers, for example, ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injection, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Exemplary unit dosage formulations are those containing a daily dose or daily subdose of the active ingredients, as hereinbefore recited, or an appropriate fraction thereof. It should be understood that in addition to the ingredients particularly mentioned above the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example, those suitable for oral administration may include such further agents as sweeteners, thickeners and flavoring agents.

The compounds of the combination of the present invention may be obtained in a conventional manner, known to those skilled in the art. Tenofovir disoproxil fumarate can be prepared, for example, as described in U.S. Pat. No. 5,977,089. Methods for the preparation of FTC are described in WO 92/14743, incorporated herein by reference.

Composition Use

Compositions of the present invention are administered to a human or other mammal in a safe and effective amount as described herein. These safe and effective amounts will vary according to the type and size of mammal being treated and the desired results of the treatment. Any of the various methods known by persons skilled in the art for packaging tablets, caplets, or other solid dosage forms suitable for oral administration, that will not degrade the components of the present invention, are suitable for use in packaging. The combinations may be packaged in glass and plastic bottles. Tablets, caplets, or other solid dosage forms suitable for oral administration may be packaged and contained in various packaging materials optionally including a dessicant e.g. silica gel. Packaging may be in the form of unit dose blister packaging. For example, a package may contain one blister tray of tenofovir DF and another blister tray of emtricitabine pills, tablets, caplets, or capsule. A patient would take one dose, e.g. a pill, from one tray and one from the other. Alternatively, the package may contain a blister tray of the co-formulated combination of tenofovir DF and emtricitabine in a single pill, tablet, caplet or capsule. As in other combinations and packaging thereof, the combinations of the invention include physiological functional derivatives of tenofovir DF and FTC.

The packaging material may also have labeling and information related to the pharmaceutical composition printed thereon. Additionally, an article of manufacture may contain a brochure, report, notice, pamphlet, or leaflet containing product information. This form of pharmaceutical information is referred to in the pharmaceutical industry as a "package insert." A package insert may be attached to or included with a pharmaceutical article of manufacture. The package insert and any article of manufacture labeling provides information relating to the pharmaceutical composition. The information and labeling provides various forms of information utilized by health-care professionals and patients, describing the composition, its dosage and various other parameters required by regulatory agencies such as the United States Food and Drug Agency.

Assays of the Combinations

The combinations of the inventions may be tested for in vitro activity against HIV and sensitivity, and for cytotoxicity in laboratory adapted cell lines, e.g. MT2 and in peripheral blood mononuclear cells (PBMC) according to standard assays developed for testing anti-HIV compounds, such as WO 02/068058 and U.S. Pat. No. 6,475,491. Combination assays may be performed at varying concentrations of the compounds of the combinations to determine EC_{50} by serial dilutions.

EXEMPLARY FORMULATIONS

The following examples further describe and demonstrate particular embodiments within the scope of the present invention. Techniques and formulations generally are found in *Remington's Pharmaceutical Sciences* (Mack Publishing Co., Easton, Pa.). The examples are given solely for illustration and are not to be construed as limitations as many variations are possible without departing from spirit and scope of the Invention. The following examples are intended for illustration only and are not intended to limit the scope of the invention in any way. "Active ingredient" denotes tenofovir disoproxil fumarate, emtricitabine, or a physiologically functional derivative of either thereof.

Tablet Formulation

The following exemplary formulations A, B, C, D, E, and F are prepared by wet granulation of the ingredients with an aqueous solution, addition of extragranular components and then followed by addition of magnesium stearate and compression.

	mg/tablet
Formulation A:	
Tenofovir Disoproxil Fumarate	300
emtricitabine	200
Microcrystalline Cellulose	200
Lactose Monohydrate	175
Croscarmellose Sodium	60
Pregelatinized Starch	50
Colloidal silicon dioxide	5
Magnesium Stearate	10
total:	1000
Formulation B:	
Tenofovir Disoproxil fumarate	300
emtricitabine	100
Microcrystalline Cellulose	200
Lactose Monohydrate	180
Sodium Starch Glycollate	60
Pregelatinized Starch	50
Magnesium Stearate	10
total:	900
Formulation C:	
Tenofovir Disoproxil fumarate	200
emtricitabine	200
Microcrystalline Cellulose	200
Lactose Monohydrate	180
Sodium Starch Glycollate	60
Pregelatinized Starch	50
Magnesium Stearate	10
total:	900
Formulation D:	
Tenofovir Disoproxil fumarate	300
emtricitabine	25
Microcrystalline Cellulose	200
Lactose Monohydrate	180
Sodium Starch Glycollate	60
Pregelatinized Starch	50
Magnesium Stearate	10
total:	825
Formulation E:	
Tenofovir Disoproxil fumarate	200
emtricitabine	25
Microcrystalline Cellulose	200
Lactose Monohydrate	180
Sodium Starch Glycollate	60
Pregelatinized Starch	50
Magnesium Stearate	10
total:	725
Formulation F:	
Tenofovir Disoproxil fumarate	100
emtricitabine	100
Microcrystalline Cellulose	200
Lactose Monohydrate	180
Sodium Starch Glycollate	60
Pregelatinized Starch	50
Magnesium Stearate	10
total:	700

Formulation G (Controlled Release Formulation):

This formulation is prepared by wet granulation of the ingredients with an aqueous solution, followed by the addition of magnesium stearate and compression.

	mg/tablet
Tenofovir Disoproxil fumarate	300
emtricitabine	200
Hydroxypropyl Methylcellulose	112
Lactose B.P.	53
Pregelatinized Starch B.P.	28
Magnesium Stearate	
total:	700

Drug release takes place over a period of about 6-8 hours and is complete after 12 hours.

Capsule Fomulations

Formulation H:

A capsule formulation is prepared by admixing the ingredients and filling into a two-part hard gelatin or hydroxypropyl methylcellulose capsule.

	mg/capsule
Active Ingredient	500
Microcrystalline Cellulose	143
Sodium Starch Glycollate	25
Magnesium Stearate	2
total:	670

Formulation I (Controlled Release Capsule):

The following controlled release capsule formulation is prepared by extruding ingredients a, b, and c using an extruder, followed by spheronization of the extrudate and drying. The dried pellets are then coated with release-controlling membrane (d) and filled into a two-piece, hard gelatin or hydroxypropyl methylcellulose capsule.

	mg/capsule
(a) Active Ingredient	500
(b) Microcrystalline Cellulose	125
(c) Lactose B.P.	125
(d) Ethyl Cellulose	13
total:	763

Formulation J (Oral Suspension):

The active ingredients are admixed with the ingredients and filling them as dry powder.

Purified water is added and mixed well before use.

Active Ingredient	500 mg
Confectioner's Sugar	2000 mg
Simethicone	300 mg
Methylparaben	30 mg
Propylparaben	10 mg
Flavor, Peach	500 mg
Purified Water q.s. to	5.00 ml

Formulation K (Suppository):

One-fifth of the Witepsol H15 is melted in a steam-jacketed pan at 45° C. maximum. The active ingredients are sifted through a 200 micron sieve and added to the molten base with mixing, using a Silverson fitted with a cutting head, until a smooth dispersion is achieved. Maintaining the mixture at

45° C., the remaining Witepsol H15 is added to the suspension and stirred to ensure a homogenous mix. The entire suspension is passed through a 250 micron stainless steel screen and, with continuous stirring, is allowed to cool to 40° C. At a temperature of 38° C. to 40° C., 2.02 g of the mixture is filled into suitable, 2 ml plastic molds. The suppositories are allowed to cool to room temperature.

	mg/Suppository
Active Ingredient	500
Hard Fat, B.P. (Witepsol H15-Dynamit Nobel)	1770
total	2270

Fixed Dose Combination Tablet

A fixed dose combination tablet of tenofovir disoproxil fumarate (TDF) 300 mg/emtricitabine 200 mg was formulated using a wet granulation/fluid-bed drying process using conventional methods. See: U.S. Pat. No. 5,935,946; L. Young (editor). Tableting Specification Manual 5th ed., American Pharmaceutical Association, Washington, D.C., (2001); L. Lachman, H. Lieberman (editors). Pharmaceutical Dosage Forms: Tablets (Vol 2), Marcel Dekker Inc., New York, 185-202 (1981); J. T. Fell and J. M. Newton, J. Pharm. Pharmacol. 20, 657-659 (1968); US Pharmacopeia 24-National Formulary 19, "Tablet Friability", Chapter <1216>, Page 2148 (2000).

The effects of granulation water level (ranging from 40% to 50% w/w) and wet massing time were studied on the physicochemical properties of the final powder blend and its performance with respect to blend uniformity and compressibility (tablet compactibility). In addition, content uniformity, assay, stability and dissolution performance was evaluated for the TDF/emtricitabine fixed dose combination tablets.

Formulation Equipment

Equipment included a high shear mixer equipped with a pressure tank and spray nozzle tip to add the granulating water, a fluid-bed dryer, a mill, a tumble blender, a rotary tablet press, and a tablet deduster.

Formulation Process

The dried, milled powder was blended with the extragranular microcrystalline cellulose and croscarmellose sodium and then blended with magnesium stearate. Powder samples were removed after mixing with the magnesium stearate. The blend samples were evaluated for, bulk density, mesh analysis and compressibility. The powder blend mixed with the magnesium stearate was compressed into tablets on a press setup.

Materials

The following Table 1 lists the quantitative composition of the TDF/emtricitabine tablet formulation.

TABLE 1

Ingredient	% w/w	Unit Formula for tablet cores (mg/tablet)	Quantity per 12 kg Batch (kg)
Tenofovir Disoproxil Fumarate[a]	30.0	300.0	3.60
Emtricitabine[a]	20.0	200.0	2.40
Pregelatinized Starch, NF/EP	5.0	50.0	0.60
Croscarmellose Sodium, NF/EP	6.0	60.0	0.72
Lactose Monohydrate, NF/EP[a]	8.0	80.0	0.96
Microcrystalline Cellulose, NF/EP[c]	30.0	300.0	3.60
Magnesium Stearate, NF/EP	1.0	10.0	0.12
Purified Water, USP/EP	[b]	[b]	[b]
Totals	100.0	1000.0	12.00

[a]Actual weight is adjusted based on the Drug Content Factor (DCF) of tenofovir disoproxil fumarate and emtricitabine.
[b]Water removed during drying.

Characterization Equipment

Moisture content was measured by loss on drying using a heat lamp/balance system. The powder blend was sampled with a sampling thief fitted with chambers to determine powder blend uniformity. Duplicate samples were removed from each of several locations in the blender. Blend uniformity analysis was performed on one sample from each location.

Particle size analysis of the final powder blend was determined by sifting a multi-gram sample through a screen using a sonic sifter. The quantity of final powder blend retained on each sieve and the fines collector was determined by calculating the difference in weight between the sieves and fines collector before and after the test. The geometric mean diameter particle size was calculated by logarithmic weighting of the sieved distribution.

Bulk density was determined by filling a graduated cylinder with the final powder blend and measuring the weight differential between the empty and filled graduate cylinder per unit volume.

Tablets were characterized for friability using a friabilator, a hardness tester, a thickness micrometer equipped with a printer, and a weighing balance.

Compression characteristics were determined using a rotary tablet press equipped with a flat-faced, beveled edged punch to a target weight of 400 mg. The powder blends were compressed using target upper punch pressures ranging from approximately 100 to 250 MPa. The apparent normalized ejection force was determined and normalized for tablet thickness and diameter.

Tablet hardness was determined using a hardness tester. Tablet thickness was determined using a micrometer, and tablet weights were determined using a top loading balance.

Wet Granulation

The powders were blended in a granulator and then granulated using water. The impeller and chopper speeds were kept constant in the blender at a low setting during the granulation and wet massing operations. After water addition, the impeller and chopper were stopped and the granulator bowl was opened to observe the granulation consistency and texture. The lid was closed and the wet massing phase was performed. Acceptable granules had 40% w/w and 60% w/w water, respectively.

Wet Milling

To facilitate a uniform drying process, each wet granulation was deagglomerated with a mill fitted with a screen and an impeller. The milled wet granules were charged into a fluid-bed dryer immediately following wet milling.

Fluid-Bed Drying

Milled wet granules were dried using an inlet air setpoint temperature of about 70° C. and airflow of approximately 100 cfm. The target LOD was about 1.0% with a range of not more than (NMT) 1.5%. The total fluid-bed drying time ranged from 53 to 75 minutes. Final LOD ranged from 0.4% to 0.7% for all of the batches dried. The final exhaust temperatures for all the batches ranged from 47° C. to 50° C.

Dry Milling

All dried granules were milled through a perforated screen. The mill was equipped with a square impeller and operated. The lots were milled and manually transferred to the V-blender.

Blending

Each lot was blended using the V-blender. In one set of three formulations, starting with 12 kg materials, final powder blend yield available for compression after blending ranged from 10.5 kg (87.5%) to 11.1 kg (92.5%). The final powder blend bulk density ranged from 0.48 to 0.58 g/cc and the geometric mean diameter particle size ranged from 112 to 221 μm. Percent water and wet massing time affect final powder blend particle size and bulk density.

The powder blending for both tenofovir DF and emtricitabine gave a mean (n=10) strength value for tenofovir DF ranged from 100.6% to 102.8% of target strength for the lots and the relative standard deviation (RSD) was from 0.5% to 1.7%. The mean (n=10) strength value for emtricitabine ranged from 101.3% to 104.1% of target strength for the lots with the relative standard deviation (RSD) ranged from 0.6% to 1.7%. The final powder blend moisture level ranged from 0.8% to 1.1% LOD.

Tablet Compression

The final blends were compressed using a rotary tablet press and the tablets were film-coated.

Three 300 gm formulations (Table 2) were granulated in a granulator equipped with a 1-L bowl. The quantities of intragranular components were based on a 300 g total batch size. The formulations in lots 1 and 2 differed in the amount of microcrystalline cellulose 30% vs. 20% w/w, respectively. Lots 2 and 3 were identical except for the type of binder. Lot 2 contained 5% w/w of pregelatinized starch and lot 3 contained 5% w/w povidone as binder.

TABLE 2

Ingredient	Lot 1 % w/w	Lot 2 % w/w	Lot 3 % w/w
Tenofovir Disoproxil Fumarate	30.0	30.0	30.0
Emtricitabine	20.0	20.0	20.0
Pregelatinized Starch, NF/EP	5.0	5.0	N/A
Povidone, USP/NF (C-30)	N/A	N/A	5.0
Croscarmellose Sodium, NF/EP	6.0	6.0	6.0
Lactose Monohydrate, NF/EP	8.0	18.0	18.0
Microcrystalline Cellulose, NF/EP[a]	30.0	20.0	20.0
Magnesium Stearate, NF/EP	1.0	1.0	1.0
Purified Water, USP/EP	[a]	[a]	[a]
Total	100.0	100.0	100.0

[a]Water removed during drying.

After water addition, the impeller and chopper were stopped and the granulator bowl was opened to observe the granulation consistency and texture. To achieve similar granulation consistency, lots 1, 2, and 3 were granulated with 45%, 40%, and 30% w/w water, respectively. The lid was closed and the wet massing phase was performed. All lots had a 30 sec wet massing resulting in acceptable granulations. The wet granulations from all batches were hand screened through a sieve to deagglomerate. The resulting granulations were tray dried in a convection oven set at 60° C. for approximately 20 hours to an LOD <1.0%. The dried granulations from all batches were hand screened through a sieve. In order to fit the granulation into the small scale (300 mL) V-blender, the final blend batch size was adjusted to 100 g. A portion, 81 g of the resulting blend from Lot 1 was blended with 15 g microcrystalline cellulose, 3 g croscarmellose sodium and 1 g magnesium stearate. 86 g of the resulting granulation from Lot 2 and Lot 3 were each blended with 10 g microcrystalline cellulose, 3 g croscarmellose sodium and 1 g magnesium stearate.

Purity analysis was conducted by reverse-phase HPLC (high performance liquid chromatography). Impurities related to tenofovir disoproxil fumarate and emtricitabine were characterized and measured in the bulk API (active pharmaceutical ingredient) before formulation in the three lots of Table 2, and again after formulation in the resulting tablets. The impurities include by-products from hydrolysis of the exocyclic amino groups of tenofovir disoproxil fumarate and emtricitabine, and the hydrolysis of the disoproxil (POC) esters of tenofovir disoproxil fumarate. In each lot, the sum total of impurities related to tenofovir disoproxil fumarate and emtricitabine was less than 1% after formulation and tablet manufacture.

The physicochemical properties of tenofovir disoproxil fumarate and emtricitabine tablets were evaluated by visual appearance, water content, label strength, impurity and degradation product contents, and tablet dissolution. Stability studies were conducted on drug product packaged in container-closure systems that are identical to the intended clinical and commercial container-closure system. There was no sign of discoloration or tablet cracking during the course of the stability study. Film-coated tenofovir disoproxil fumarate and emtricitabine tablets exhibited satisfactory stability at 40° C./75% RH (relative humidity) for up to six months when packaged and stored with silica gel desiccant. No significant loss (defined as >5% degradation) in % label strength of tenofovir DF or emtricitabine was observed after six months at 40° C./75% RH, when packaged and stored with desiccant. The increase in the total degradation products was 1.5% for tenofovir DF and 0.6-0.7% for emtricitabine after six months at 40° C./75% RH when packaged and stored with 3 grains of desiccant.

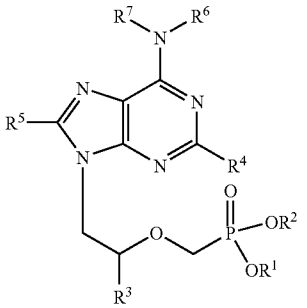
All publications and patent applications cited herein are incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although certain embodiments are described in detail above, those having ordinary skill in the art will clearly understand that many modifications are possible in the claims without departing from the teachings thereof. All such modifications are intended to be encompassed within the claims of the invention.

EMBODIMENTS OF THE INVENTION

A1. A pharmaceutical composition comprising an effective amount of a compound of the formula:

(1)



wherein R^1 and R^2 are independently selected from H, C_1-C_6 alkyl, C_1-C_6 substituted alkyl, C_6-C_{20} aryl, C_6-C_{20} substituted aryl, C_6-C_{20} arylalkyl, C_6-C_{20} substituted arylalkyl, acyloxymethyl esters —$CH_2C(=O)R^9$ and acyloxymethyl carbonates —$CH_2C(=O)OR^9$ where R^9 is C_1-C_6 alkyl, C_1-C_6 substituted alkyl, C_6-C_{20} aryl and C_6-C_{20} substituted aryl;

R^3 is selected from H, C_1-C_6 alkyl, C_1-C_6 substituted alkyl, or CH_2OR^8 where R^8 is C_1-C_6 alkyl, C_1-C_6 hydroxyalkyl and C_1-C_6 haloalkyl;

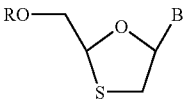
R^4 and R^5 are independently selected from H, NH_2, NHR and NR_2 where R is C_1-C_6 alkyl; and

R^6 and R^7 are independently selected from H and C_1-C_6 alkyl;

or a physiologically functional derivative thereof;

in combination with an effective amount of a compound of the formula

(2)



wherein B is selected from adenine, guanine, cytosine, uracil, thymine, 7-deazaadenine, 7-deazaguanine, 7-deaza-8-azaguanine, 7-deaza-8-azaadenine, inosine, nebularine, nitropyrrole, nitroindole, 2-aminopurine, 2-amino-6-chloropurine, 2,6-diaminopurine, hypoxanthine, pseudouridine, 5-fluorocytosine, 5-chlorocytosine, 5-bromocytosine, 5-iodocytosine, pseudocytosine, pseudoisocytosine, 5-propynylcytosine, isocytosine, isoguanine, 7-deazaguanine, 2-thiopyrimidine, 6-thioguanine, 4-thiothymine, 4-thiouracil, O^6-methylguanine, N^6-methyladenine, O^4-methylthymine, 5,6-dihydrothymine, 5,6-dihydrouracil, 4-methylindole, and a pyrazolo[3,4-D]pyrimidine; and

R is selected from H, C_1-C_{18} alkyl, C_1-C_{18} substituted alkyl, C_2-C_{18} alkenyl, C_2-C_{18} substituted alkenyl, C_1-C_{18} alkynyl, C_2-C_{18} substituted alkynyl, C_6-C_{20} aryl, C_6-C_{20} substituted aryl, C_2-C_{20} heterocycle, C_2-C_{20} substituted heterocycle, phosphonate, phosphophosphonate, diphosphophosphonate, phosphate, diphosphate, triphosphate, polyethyleneoxy or a physiologically functional derivative thereof; and

a pharmaceutically acceptable carrier.

B2. A composition of embodiment A1 wherein, in formula 1, R^1 and R^2 are independently selected from H, C_1-C_6 alkyl, C_1-C_6 substituted alkyl, C_6-C_{20} aryl, C_6-C_{20} substituted aryl, C_6-C_{20} arylalkyl, C_6-C_{20} substituted arylalkyl, acyloxymethyl esters —$CH_2C(=O)R^9$ and acyloxymethyl carbonates —$CH_2C(=O)OR^9$ where R^9 is C_1-C_6 alkyl, C_1-C_6 substituted alkyl, C_6-C_{20} aryl and C_6-C_{20} substituted aryl; and R^3R^4, R^5, R^6 and R^7 are independently H or C_1-C_6 alkyl.

C3. A composition of embodiment A1 wherein, in formula 2, B is cytosine or a 5-halocytosine.

D4. A composition of embodiment A1 wherein, in formula 1, R^1 and R^2 are independently selected from H, C_1-C_6 alkyl, C_1-C_6 substituted alkyl, C_6-C_{20} aryl, C_6-C_{20} substituted aryl, C_6-C_{20} arylalkyl, C_6-C_{20} substituted arylalkyl, acyloxymethyl esters —$CH_2C(=O)R^9$ and acyloxymethyl carbonates $CH_2C(=O)OR^9$ where R^9 is C_1-C_6 alkyl, C_1-C_6 substituted alkyl, C_6-C_{20} aryl and C_6-C_{20} substituted aryl; and R^3 R^4, R^5, R^6 and R^7 are independently H or C_1-C_6 alkyl; and, in formula 2, B is cytosine or a 5-halocytosine.

E5. A composition of embodiment D 4 wherein, in formula 1, R^1 and R^2 are independently selected from H, acyloxymethyl esters —$CH_2C(=O)R^9$ and acyloxymethyl carbonates $CH_2C(=O)OR^9$ where R^9 is C_1-C_6 alkyl; and R^3, R^4, R^5, R^6 and R^7 are independently H or C_1-C_6 alkyl; and, in formula 2, B is cytosine or a 5-halocytosine and R is H.

F6. A composition of embodiment E5 wherein, in formula 1, R^1 and R^2 are independently selected from H and —$CH_2C(=O)OCH(CH_3)_2$; R^3 is —CH_3; and R^4, R^5, R^6 and R^7 are H; and, in formula 2, B is 5-fluorocytosine and R is H.

G7. A pharmaceutical composition comprising a pharmaceutically effective amount of [2-(6-amino-purin-9-yl)-1-methyl-ethoxymethyl]-phosphonic acid diisopropoxycarbonyloxymethyl ester fumarate (tenofovir disoproxil fumarate) or a physiologically functional derivative thereof and a pharmaceutically effective amount of (2R,5S)-4-amino-5-fluoro-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one (emtricitabine) or a physiologically functional derivative thereof; and a pharmaceutically acceptable carrier.

H8. A pharmaceutical formulation of embodiment A1 to G7 further comprising a third active ingredient selected from the group consisting of a protease inhibitor, a nucleoside or nucleotide reverse transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor, and an integrase inhibitor.

I9. A pharmaceutical formulation of embodiments A1 to H8 in unit dosage form.

J10. A method for the treatment or prevention of the symptoms or effects of an HIV infection in an infected animal which comprises administering to said animal a pharmaceutical composition of embodiments claims A1 to 19.

We claim:

1. A chemically stable fixed-dose combination comprising 300 mg of tenofovir disoproxil fumarate and 200 mg of emtricitabine wherein the combination exhibits less than 10% degradation of the tenofovir disoproxil fumarate and emtricitabine after six months at 40° C./75% relative humidity when packaged and stored with silica gel desiccant at 40° C./70% relative humidity.

2. The chemically stable combination of claim **1** in the form of a pharmaceutical dosage form.

3. The chemically stable combination of claim **2** wherein the dosage form is oral.

4. The pharmaceutical dosage form of claim **2** wherein the tenofovir disoproxil fumarate is not substantially degraded.

5. The pharmaceutical dosage form of claim **4** where there is less than 10% degradation of tenofovir disoproxil fumarate over a 24-hour period.

6. The pharmaceutical dosage form of claim **4** where there is less than 1% degradation of tenofovir disoproxil fumarate over a 24-hour period.

7. The pharmaceutical dosage form of claim **4** where there is less than 0.1% degradation of tenofovir disoproxil fumarate over a 24-hour period.

8. The pharmaceutical dosage form of claim **4** where there is less than 0.01% degradation of tenofovir disoproxil fumarate over a 24-hour period.

9. The pharmaceutical dosage form of claim **2** wherein less than 5% degradation of the tenofovir disoproxil fumarate and emtricitabine occurs after six months.

10. A chemically stable fixed-dose combination comprising 300 mg of tenofovir disoproxil fumarate and 200 mg of emtricitabine wherein the combination exhibits less than 10% degradation of tenofovir disoproxil fumarate over a 24-hour period.

11. The chemically stable combination of claim **10**, in the form of a pharmaceutical dosage form.

12. The chemically stable combination of claim **11**, wherein the dosage form is oral.

13. The pharmaceutical dosage form of claim **11**, wherein there is less than 1% degradation of tenofovir disoproxil fumarate.

14. The pharmaceutical dosage form of claim **11**, wherein there is less than 0.1% degradation of tenofovir disoproxil fumarate.

15. The pharmaceutical dosage form of claim **11**, wherein there is less than 0.01% degradation of tenofovir disoproxil fumarate.

16. The pharmaceutical dosage form of claim **11**, wherein the combination exhibits less than 10% degradation of the tenofovir disoproxil fumarate and emtricitabine after six months at 40° C./75% relative humidity when packaged and stored with silica gel desiccant at 40° C./70% relative humidity.

17. The pharmaceutical dosage form of claim **11** wherein the combination exhibits less than 5% degradation of the tenofovir disoproxil fumarate and emtricitabine after six months at 40° C./75% relative humidity when packaged and stored with silica gel desiccant at 40° C./70% relative humidity.

18. The pharmaceutical dosage form of claim **2** or **11** comprising 300 mg tenofovir disoproxil fumarate, 200 mg emtricitabine, pregelatinized starch, croscarmellose sodium, lactose monohydrate, microcrystalline cellulose, and magnesium stearate.

19. The pharmaceutical dosage form of claim **18** comprising 300 mg tenofovir disoproxil fumarate, 200 mg emtricitabine, 50 mg pregelatinized starch, 60 mg croscarmellose sodium, 80 mg lactose monohydrate, 300 mg microcrystalline cellulose, and 10 mg magnesium stearate.

20. The pharmaceutical dosage form of claim **18** comprising 300 mg tenofovir disoproxil fumarate, 200 mg emtricitabine, 50 mg pregelatinized starch, 60 mg croscarmellose sodium, 180 mg lactose monohydrate, 200 mg microcrystalline cellulose, and 10 mg magnesium stearate.

21. The pharmaceutical dosage form of claim **2** or **11** comprising 300 mg tenofovir disoproxil fumarate, 200 mg emtricitabine, pregelatinized starch, croscarmellose sodium, lactose monohydrate, microcrystalline cellulose, magnesium stearate, and colloidal silicon dioxide.

22. The pharmaceutical dosage form of claim **21** comprising 300 mg tenofovir disoproxil fumarate, 200 mg emtricitabine, 50 mg pregelatinized starch, 60 mg croscarmellose sodium, 175 mg lactose monohydrate, 200 mg microcrystalline cellulose, 10 mg magnesium stearate, and 5 mg colloidal silicon dioxide.

23. The pharmaceutical dosage form of claim **21** comprising 300 mg tenofovir disoproxil fumarate, 200 mg emtricitabine, hydroxypropyl methylcellulose, lactose B.P., pregelatinized starch B.P, and magnesium stearate.

24. The pharmaceutical dosage form of claim **21** comprising 300 mg tenofovir disoproxil fumarate, 200 mg emtricitabine, 112 g hydroxypropyl methylcellulose, 53 mg lactose B.P., 28 mg pregelatinized starch B.P, and 7 mg magnesium stearate.

25. The pharmaceutical dosage form of claim **2** or **11** comprising less than 1% of impurities related to tenofovir disoproxil fumarate and emtricitabine.

26. The pharmaceutical dosage form of claim **2** or **11**, further comprising a third anti-viral agent.

27. The pharmaceutical dosage form of claim **26**, wherein the third antiviral agent is selected from the group consisting of protease inhibitors, nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, and integrase inhibitors.

28. The pharmaceutical dosage form of claim **27**, wherein the third antiviral agent is a protease inhibitor.

29. The pharmaceutical dosage form of claim **27**, wherein the third antiviral agent is a nucleoside reverse transcriptase inhibitor.

30. The pharmaceutical dosage form of claim **27**, wherein the third antiviral agent is a non-nucleoside reverse transcriptase inhibitor.

31. The pharmaceutical dosage form of claim **27**, wherein the third antiviral agent is an integrase inhibitor.

32. The pharmaceutical dosage form of claim **30**, wherein the third antiviral agent is efavirenz.

33. A method for the treatment of the symptoms or effects of an HIV infection in an infected animal which comprises administering to said animal the pharmaceutical dosage form of claim **2** or **11**.

34. A method for the treatment of the symptoms or effects of an HIV infection in an infected animal which comprises administering to said animal the pharmaceutical dosage form of claim **9** or **13**.

35. A method for the treatment of the symptoms or effects of an HIV infection in an infected animal which comprises administering to said animal the pharmaceutical dosage form of claim **21**.

36. A method for the treatment of the symptoms or effects of an HIV infection in an infected animal which comprises administering to said animal the pharmaceutical dosage form of claim **26**.

37. A method for the treatment of the symptoms or effects of an HIV infection in an infected animal which comprises administering to said animal the pharmaceutical dosage form of claim **27**.

38. A method for the treatment of the symptoms or effects of an HIV infection in an infected animal which comprises administering to said animal the pharmaceutical dosage form of claim **32**.

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