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(54) Title: ARYL PHOSPHATE DERIVATIVES WITH SELECTIVE ACTIVITY AGAINST ADENOVIRUS AND HIV

(57) Abstract: Methods and compositions for treating ADV infections and HIV/ADV co-infections by administering an aryl phosphate derivative of d4T having an electron withdrawing substituent on the aryl group and an amino acid substituent on the phosphate group are described. Preferred aryl phosphate derivatives of d4T are d4T-5'-[p-bromophenyl methodyalaninyl phosphate] and d4T-5'-[p-chlorophenyl methoxyalaninyl phosphate].

ARYL PHOSPHATE DERIVATIVES WITH SELECTIVE ACTIVITY AGAINST ADENOVIRUS AND HIV

This application is being filed as a PCT International Patent Application in the name of Parker Hughes Institute, a U.S. national corporation and resident, on 11 June 2003, designating all countries except US, and claiming priority to U.S. Serial No. 60/388,470 filed on 12 June 2002; U.S. Serial No. 60/420,260 filed on 21 October 2002 and U.S. Serial No. 10/281,399 filed on 25 October 2002.

Background of the Invention

Adenovirus (ADV) infection results in significant morbidity and mortality in both immunocompetent and immunosuppressed hosts. Adenoviruses have been recovered from human immunodeficiency virus (HIV) positive patients since the beginning of the AIDS epidemic (Khoo et al. *J. Infect. Dis.*, 1996, 172:629-637). In immunocompromised hosts, such as HIV infected individuals, adenoviruses are responsible for a broad range of clinical diseases that may be associated with high mortality, including pneumonia, hepatitis, encephalitis, hemorrhagic cystitis, nephritis, and gastroenteritis in immunocompromised patients. (Bhanthumkosol, J.C., *J. Med. Assoc. Thai.*, 1998, 81:214-222; Carrigan et al., *Am. J. Med.*, 1997, 102:71-74; De Jong et al., *J. Clin. Microbiol.*, 1999, 37:3940-0945; Dombrowski et al., *Virchows Arch.*, 1997, 431:469-472; Ghez et al., *Am. J. Hematol.*, 2000, 63:32-34; Green et al., *Clin. Infect. Dis.*, 1994, 172:629-637; Khoo et al., *J. Infect. Dis.*, 1995, 172:629-637; Maslo et al., *Am. J. Respir. Crit. Care Med.*, 1997, 156:1263-1264).

Adenovirus colitis is a common cause of diarrhea in HIV-infected patients and may facilitate enteric infection with cytomegalovirus (CMV) (Thomas et al., *HIV Med.*, 1999, 1:19-24). Gastrointestinal adenovirus excretion occurs at an advanced stage of HIV disease (Sabin et al., *J. Med. Virol.*, 1999, 58:280-285). The median survival of HIV-infected patients with adenovirus-positive diarrhea is 1 year compared with 2.4 years for those without adenoviruses (Sabin et al., *J. Med. Virol.*, 1999, 58:280-285). This difference remains significant after accounting for differences in CD4 counts between the groups, suggesting that adenoviruses may contribute to mortality in this population (Sabin et al., *J. Med. Virol.*, 1999, 58:280-285).

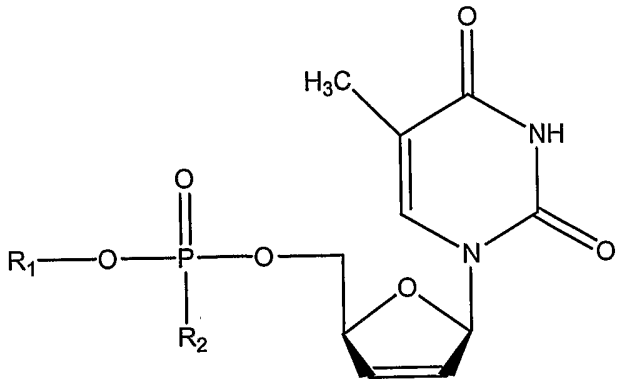
Most nucleoside analogs exhibit broad-spectrum antiviral activity (Allen et al., *J. Med. Chem.*, 1978, 21:742-746; De Clercq, E., *Verh. K. Acad. Geneeskd. Belg.*, 1978, 58:19-47). Several nucleoside analogs, including 2',3'-dideoxynucleoside 5'-triphosphates and 3'-fluoro-2'-deoxythymidine (FTdR), have been discovered as having antiviral activity against adenovirus. These nucleoside analogs inhibit the adenovirus DNA polymerase-mediated DNA replication in adenovirus-infected cells (Mentel et al., *Med. Mircobiol. Immunol.*, 2000, 189:91-95; Mental et al., *Antiviral Res.*, 1997, 34:113-119; Mul et al., *Nucleic Acid Res.*, 1989, 12:8917-8929; Vand der Vliet and Kwant, *Biochemistry*, 1981, 20:2628-2632).

Of the currently available anti-HIV agents, none have been reported to have anti-ADV activity. Moreover, anti-ADV agents such as ribavarin cannot be administered long term in HIV-infected patients because of the associated side effects, such as anemia and severe hematologic toxicity (De Clercq, E., *Verh. K. Acad. Geneeskd. Belg.*, 1996, 58:19-47; Reefschlager et al., *Antiviral Res.*, 1982, 2:41-52). Therefore, there is an urgent need for selective anti-ADV agents with more favorable safety profiles than the available nucleoside analogs as well as dual-function anti-HIV agents with anti-ADV activity.

Summary of the Invention

The present invention is directed to aryl phosphate derivatives of 2',3'-didehydro-2',3'-dideoxythymidine (hereinafter "d4T") that have anti-ADV activity in host cells or anti-ADV and anti-HIV activity in hosts co-infected with ADV and HIV.

One aspect of the invention provides methods for treating ADV infections or ADV/HIV co-infections by administering an aryl phosphate derivative of d4T having an electron withdrawing substituent on the aryl group and an amino acid substituent on the phosphate group as in Formula I:

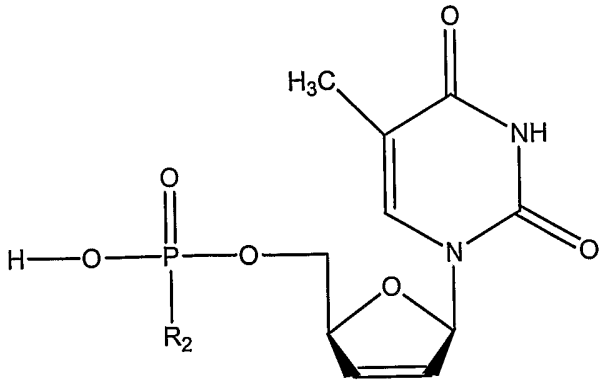


Formula I

where R_1 is an aryl group substituted with an electron withdrawing group and R_2 is an amino acid or an ester of an amino acid. In one embodiment of Formula I, R_1 is a phenyl substituted with an electron withdrawing group and R_2 is an ester of an α-amino acid. Preferably, Formula I is d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] where R_1 is phenyl group substituted with bromo at the para position and R_2 is the methyl ester of alanine or Formula I is d4T-5'-[*p*-chlorophenyl methoxyalaninyl phosphate] where R_1 is phenyl group substituted with chloro at the para position and R_2 is the methyl ester of alanine.

The oral or intravenous administration of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] or d4T-5'-[*p*-chlorophenyl methoxyalaninyl phosphate] results in the formation of two key metabolites: alaninyl-d4T-monophosphate (Ala-d4T-MP) and d4T. The administration of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] or d4T-5'-[*p*-chlorophenyl methoxyalaninyl phosphate] results in more prolonged systemic exposure to Ala-d4T-MP as well as d4T than administration of an equimolar dose of either metabolite. Each metabolite has a significantly longer elimination half life when formed from the administration of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] or d4T-5'-[*p*-chlorophenyl methoxyalaninyl phosphate] than when administered directly.

Another aspect of the invention provides a method for treating ADV infections or ADV/HIV co-infections comprising administering an effective amount of a compound of Formula IV:



Formula IV

where R_2 is an amino acid or amino acid ester residue. In one embodiment, R_2 is the methyl ester of alanine.

d4T-5'-[p-bromophenyl methoxyalaninyl phosphate] and 12 structurally similar d4T derivatives were substantially more potent against ADV than d4T and inhibited ADV-induced plaque formation at nanomolar IC_{50} values. Compounds with halo substitutions in the phenyl ring as well as the unsubstituted compound 607 were more potent against ADV than compounds with methoxy, methyl, or cyano substitutions.

Compound 113, d4T-5'-[p-bromophenyl methoxyalaninyl phosphate], having a 4-Br substitution and compound 609, d4T-5'-[*p*-chlorophenyl methoxyalaninyl phosphate], having a 4-Cl substitution were identified as the most potent lead anti-ADV agents. Both compounds inhibited ADV-induced plaque formation in skin fibroblasts at nanomolar concentrations without cytotoxicity. While the lead compounds 113 and 609 exhibited potent anti-HIV activity, neither compound exhibited any antiviral activity against non-HIV viruses, including Type I or Type II herpes simplex viruses (HSV-1, HSV-2), enterovirus ECHO 30, or respiratory syncytial virus (RSV).

These results establish aryl phosphate derivatives of 2',3'-didehydro-2',3'-dideoxythymidine as a new class of dual-function anti-HIV agents with potent and selective anti-ADV activity. The anti-ADV potency of compounds 113, d4T-5'-[p-bromophenyl methoxyalaninyl phosphate], and compound 609, d4T-5'-[*p*-chlorophenyl methoxyalaninyl phosphate], provides a basis for the design of

effective ADV treatment strategies capable of inhibiting both ADV and HIV in coinfected hosts.

Detailed Description of the Invention

As used herein, the following terms and phrases have the indicated definitions:

The term "administering" refers to providing to a mammal in any manner including: orally, parentally (including subcutaneous injection, intravenous, intramuscular, intrasternal or infusion techniques), by inhalation spray, topically, by absorption through a mucous membrane, or rectally, in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants or vehicles, and other known modes of drug delivery.

The term "amino acid" refers to any of the naturally occurring amino acids, as well as their opposite enantiomers or racemic mixture of both enantiomers, synthetic analogs, and derivatives thereof. The term includes, for example, α-, β-, γ-, δ-, and ω-amino acids. Suitable naturally occurring amino acids include glycine, alanine, valine, leucine, isoleucine, proline, threonine, serine, methionine, cysteine, aspartic acid, asparagine, glutamic acid, glutamine, arginine, lysine, phenylalanine, tryptophan, tyrosine, and histidine. Synthetic, or unnatural, amino acids such as, for example, trifluoroleucine, p-fluorophenylalanine, and 3-triethylalanine can be used. The term amino acid includes esters of the amino acids. Esters include lower alkyl esters in which the alkyl group has one to seven carbon atoms, preferably one to four carbon atom such as, for example, methyl, ethyl, propyl, and butyl. The amino group of the amino acid or ester thereof is attached to the phosphate group in Formula I.

The term "animal" includes, but is not limited to mammals, such as humans.

The term "aryl" includes aromatic groups such as, for example, phenyl, naphthyl, and anthryl.

The term “electron-withdrawing groups” includes groups such as halo (-NO_2, -CN, -SO_3H, -COOH, -CHO, -COR (where R is a (C_1 to C_4) alkyl), and the like.

The term "halo" or "halogen" is used to describe an atom selected from the group of Bromine (Br), Chlorine (Cl), Fluorine (F) and Iodine (I).

The term "protecting" or "preventing" refers to taking advance measures against a possible or probable infection to prevent the morbidity and mortality normally associated with a disease causing agent.

The term "host" in the context of this invention means a mammal, i.e., any class of higher vertebrates that nourish their young with milk secreted by mammary glands, or a cell or cells from a mammal.

The term "cell" in the context of this invention means the smallest structural unit of an organism that is capable of independent functioning, consisting of one or more nuclei, cytoplasm, and various organelles, all surrounded by a semipermeable membrane.

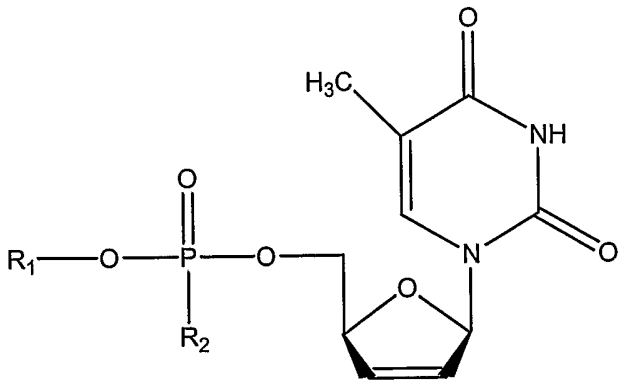
The term "coinfected" in the context of this invention means a host simultaneously infected with both ADV and HIV.

Inhibiting ADV activity in the context of this invention means inhibiting ADV replication, inhibiting ADV induced plaque formation, killing ADV, or inhibiting ADV DNA polymerase.

Inhibiting HIV activity in the context of this invention means inhibiting HIV replication, killing HIV, or inhibiting HIV reverse transcriptase.

Compounds Useful in Methods of the Invention

The invention is directed to methods of using aryl phosphate derivatives of 2',3'-didehydro-2',3'-dideoxythymidine (derivatives of d4T) to inhibit the effects of infection by adenovirus in a cell, in vitro or in vivo. More particularly, the present invention provides methods to inhibit the effects of infection by adenovirus in a cell, in a mammal by administering an aryl phosphate derivative of d4T having an electron withdrawing substituent on the aryl group and an amino acid substituent on the phosphate group as in Formula I:

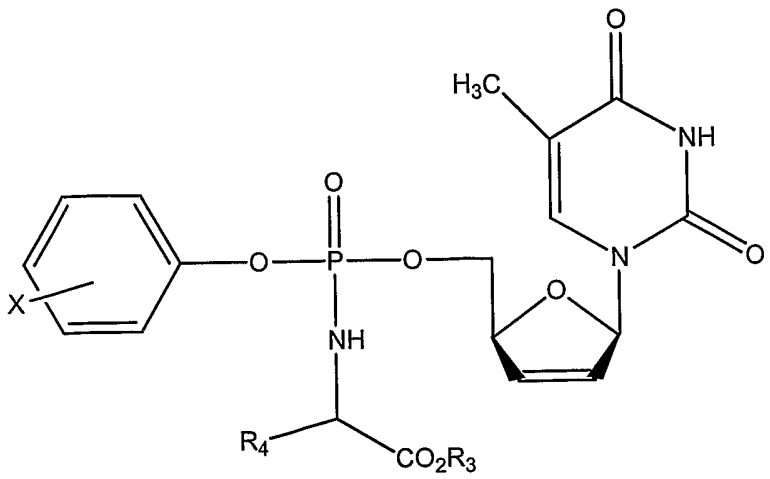


Formula I

where R_1 is an aryl group substituted with an electron withdrawing group and R_2 is an amino acid or an ester of an amino acid.

The compounds of Formula I can also be in the form of pharmaceutically acceptable salts. Pharmaceutically acceptable salts are formed with organic and inorganic acids. Examples of suitable acids for salt formation with the amino group of the amino acid or amino acid ester residue of a compound of Formula I include, but are not limited to hydrochloric, sulfuric, phosphoric, acetic, citric, oxalic, malonic, salicylic, malic, gluconic, fumaric, succinic, asorbic, maleic, methanesulfonic, and the like. The salts are prepared by contacting the free base form with a sufficient amount of the desired acid to produce either a mono or di, etc. salt in the conventional manner. Suitable bases for the formation of a salt with the carboxylate group of the amino acid residue of a compound of Formula I include, for example, sodium hydroxide, sodium carbonate, sodium bicarbonate, potassium hydroxide, potassium carbonate, and potassium bicarbonate.

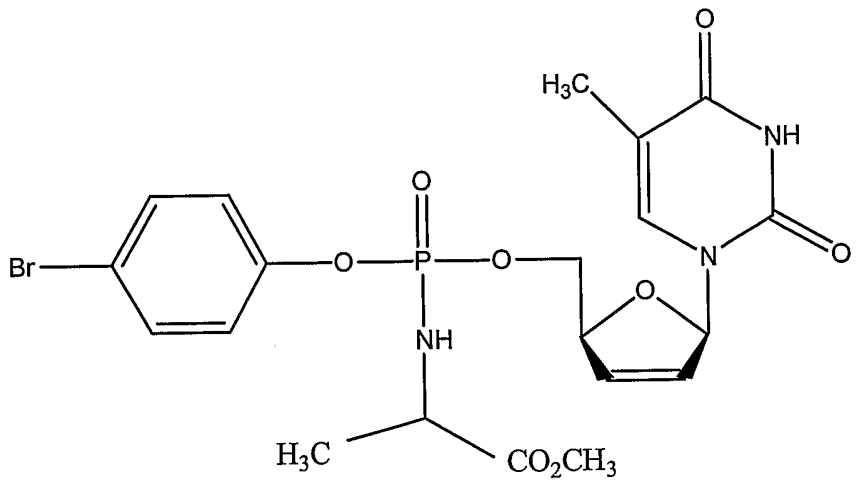
In one embodiment of Formula I, R_1 is a phenyl group substituted with an electron withdrawing group and R_2 is an α-amino acid or ester thereof as shown in Formula II:



Formula II

In Formula II, X is an electron-withdrawing group such as halo -NO_2, -CN, -SO_3H, -COOH, -CHO, -COR (where R is a (C_1 to C_4) alkyl), and the like. R_3 is hydrogen or an alkyl of one to seven carbon atoms, preferably an alkyl of one to four carbon atoms, such as, for example, methyl, ethyl, propyl, and butyl. R_4 is hydrogen (e.g., as in glycine), an alkyl (e.g. as in alanine, valine, leucine, isoleucine, proline), a substituted alkyl (e.g., as in threonine, serine, methionine, cysteine, aspartic acid, asparagine, glutamic acid, glutamine, arginie, and lysine), an arylalkyl (e.g., as in phenylalanine and tryptophan), a substituted arylalkyl (e.g., as in tyrosine), or a heteroalkyl (e.g., as in histidine).

One embodiment, the compound of Formula II is d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate], (d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]) where X is bromo attached to the phenyl group in the *para* position, R_4 is methyl, and R_3 is methyl. The structure of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] is shown in Formula III:

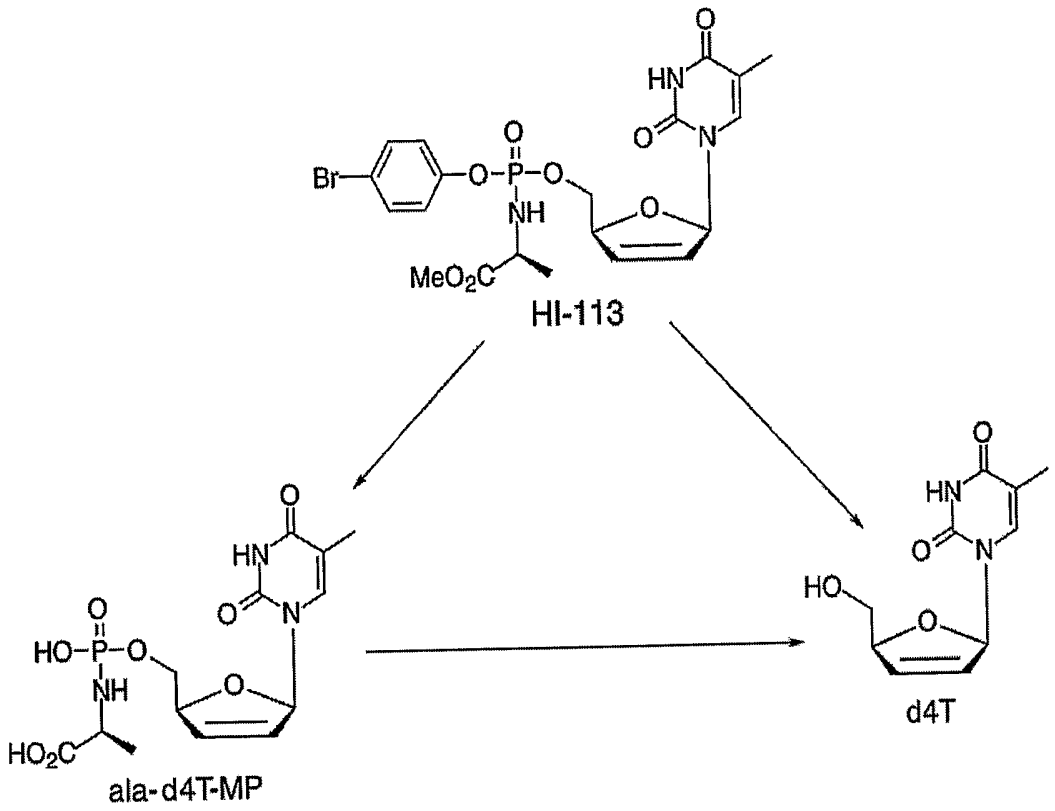


Formula III

Pharmacokinetics

Previous *in vitro* studies have shown that an electron withdrawing group at the para position of the phenyl group enhances the rate of hydrolysis and thereby enhances production of a key metabolite alaninyl-d4T-monophosphate (Ala-d4T-MP) relative to the unsubstituted aryl phosphate derivative (Venkatachalam et al., *Bioorg. Med. Chem. Lett.*, 8, 3121 (1998); Vig et al., *Antiviral Chem. Chemother.*, 9, 445 (1998); and U.S. Pat. No. 6,030,957 (Uckun et al.)).

The anti-viral agent d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] (referred to in Scheme 1 below as HI-113) is quickly metabolized *in vivo* to form two metabolites: 2',3'-didehydro-3'-deoxythymidine (d4T) and alaninyl-d4T-monophosphate (Ala-d4T-MP) as shown in Scheme 1. Ala-d4T-MP can also be metabolized further to yield d4T. The metabolite d4T had not been found in earlier *in vitro* studies with cells.



Scheme 1

d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] readily metabolizes in either plasma or whole blood to form Ala-d4T-MP and a small amount of d4T (see Figure 2). Ala-d4T-MP is stable both in plasma and in whole blood. These results indicate that other enzymes (e.g., liver enzymes) are needed to form d4T by hydrolysis of either Ala-d4T-MP or d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]. This hypothesis is consistent with the formation of a significant amount of d4T after incubation of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] with a liver homogenate (see Figure 4).

Paraoxon, an inhibitor of both cholinesterase and carboxylesterase (Augustinsson, *Ann. N. Y. Acad. Sci.*, 94, 884 (1961); McCracken et al., *Biochem. Pharmacol.*, 46, 1125 (1993); Madhu et al., *J. Pharm. Sci.*, 86, 971 (1997)), significantly prevented the hydrolysis of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] to Ala-d4T -MP and d4T, suggesting that both cholinesterase and carboxylesterase are important for metabolism of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] (see Figure 3A). Physostigmine, an inhibitor of cholinesterase, partially prevented the hydrolysis of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate], which further supports the importance of

cholinesterase in hydrolysis of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] (see Figure 3B). EDTA, an inhibitor of arylesterase, did not affect the hydrolysis of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate], indicating that arylesterase is probably not involved in the hydrolysis of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] (see Figure 3C).

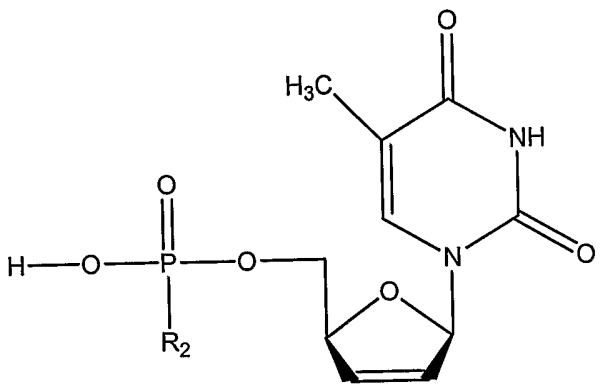
Elimination Half-Life

The elimination half-life of intravenously administered d4T is fairly similar to the elimination half-life of d4T formed after intravenous administration of Ala-d4T-MP ($t_{1/2}$ of 30.3 minutes vs. 34.0 minutes) as shown in the Examples below. In contrast, the elimination half-life for d4T formed after intravenous administration of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] was significantly prolonged ($t_{1/2}$ of 114.8 minutes). Similarly, the elimination half-life for Ala-d4T-MP formed from d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] was significantly longer than the $t_{1/2}$ for Ala-d4T-MP administered intravenously ($t_{1/2}$ of 129.2 minutes vs. 28.5 minutes). The intravenous administration of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] results in prolonged systemic exposure to both Ala-d4T-MP and d4T compared to administration of equimolar dose of Ala-d4T-MP or d4T due to apparently longer elimination half-lives of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]-derived metabolites.

Following intravenous administration, the elimination half-life ($t_{1/2}$) of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] was 3.5 minutes with a systemic clearance (CL) of 160.9 ml/min/kg. Different estimates for systemic clearance (CL) values were obtained for the two diastereomers of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] (d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]A is 208.2 ml/min/kg and d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]B is 123.9 ml/min/kg), but both were completely metabolized within 30 minutes. d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] was converted to the active metabolites Ala-d4T-MP (23%) and d4T (24%). The t_{max} values for Ala-d4T-MP and d4T formed from intravenously administered d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] were 5.9 minutes and 18.7 minutes, respectively.

Intravenous administration of Ala-d4T-MP results in formation of d4T (15%). Ala-d4T-MP can also be used as a d4T prodrug. The invention provides a method for inhibiting the effects of infection by adenovirus in a cell, in vitro or in

vivo by administering an effective amount of a compound of Formula IV:



Formula IV

where R_2 is an amino acid or esterified thereof.

Salts

The compounds of Formula I to IV can also be in the form of pharmaceutically acceptable salts. Pharmaceutically acceptable salts can be formed with organic and inorganic acids. Examples of suitable acids for salt formation with the amino group of the amino acid or amino acid ester residue of Formula IV include, but are not limited to, hydrochloric, sulfuric, phosphoric, acetic, citric, oxalic, malonic, salicylic, malic, gluconic, fumaric, succinic, asorbic, maleic, methanesulfonic, and the like. The salts can be prepared by contacting the free base form with a sufficient amount of the desired acid to produce either a mono or di, etc. salt in the conventional manner. Suitable bases for the formation of a salt with the carboxylate group of the amino acid residue of Formula IV include, for example, sodium hydroxide, sodium carbonate, sodium bicarbonate, potassium hydroxide, potassium carbonate, and potassium bicarbonate.

Bioavailability

Orally administered d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] also yielded Ala-d4T-MP and d4T as the major metabolites. No parent d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] was detectable in the blood after oral administration. Although d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] is

stable in gastric fluid and can be absorbed in the stomach, it can quickly hydrolyze in blood. On the other hand, d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] decomposes readily in intestinal fluid to form Ala-d4T-MP. This metabolite can be absorbed in the intestine and then further metabolized to yield d4T in the blood. The t_{max} and $t_{1/2}$ values for d4T in mice were longer when derived from orally administered d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] (42.4 minutes and 99.0 minutes, respectively) than from orally administered d4T (5 minutes and 18 minutes, respectively). The t_{max} value is higher but the $t_{1/2}$ value is lower for orally administered d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] compared to intravenously administered d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]. The estimated bioavailabilities of Ala-d4T-MP and d4T were approximately 12% and 48%, respectively, after oral administration of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]. However, the bioavailability of d4T metabolized from d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] (48%) was lower than that of orally administered D4T (98%).

The *in vivo* pharmacokinetics, metabolism, toxicity, and antiretroviral activity of d4T-5'-[p-bromophenyl methoxyalaninyl phosphate] in rodent species has been investigated (Uckun et al., *Arzneimittelforschung/Drug Research*, 2002, (*in press*)). In mice and rats, d4T-5'-[p-bromophenyl methoxyalaninyl phosphate] was very well tolerated without any detectable acute or subacute toxicity at single intraperitoneal or oral bolus dose levels as high as 500 mg/kg (Uckun et al., 2002, (*Supra*)). Notably, daily administration of d4T-5'-[p-bromophenyl methoxyalaninyl phosphate] intraperitoneally or orally for up to 8 consecutive weeks was not associated with any detectable toxicity in mice or rats at cumulative dose levels as high as 6.4 g/kg (Uckun et al., 2002, (*Supra*)). In accordance with its safety profile in rodent species, a four-week d4T-5'-[p-bromophenyl methoxyalaninyl phosphate] treatment course with twice daily administration of hard gelatin capsules containing 25 mg/kg – 100 mg/kg d4T-5'-[p-bromophenyl methoxyalaninyl phosphate] was very well tolerated by dogs and cats at cumulative dose levels as high as 8.4 g/kg (Uckun et al., *Antimicrob. Agents Chemother.* (submitted 2002)).

Administration Methods

Compounds of Formulas I to IV can be formulated as pharmaceutical compositions and administered to a mammalian host, including a human patient in a

variety of forms adapted to the chosen route of administration. The compounds are typically administered in combination with a pharmaceutically acceptable carrier, and can be combined with specific delivery agents, including targeting antibodies or cytokines.

Useful Dose

When used *in vivo* to inhibit the effects of infection by adenovirus, the administered dose is that effective to have the desired effect, such as sufficient to reduce or eliminate one or more symptoms of adenovirus. Appropriate amounts can be determined by those skilled in the art, extrapolating using known methods and relationships, from the *in vivo* animal model data provided in the Specification and Examples.

In general, the dose of the aryl phosphate derivatives of d4T effective to achieve therapeutic treatment of adenovirus infection, including reduction of symptoms or effects of adenovirus infection such as increased survival time, is in the approximate range of about 1-500 mg/kg body weight/dose, preferably about 10-100 mg/kg body weight/dose, and approximately 800-1000 mg/kg body weight per week of a cumulative dose.

The effective dose to be administered will vary with conditions specific to each patient. In general, factors such as the viral burden, host age, metabolism, sickness, prior exposure to drugs, and the like, contribute to the expected effectiveness of a drug. One skilled in the art will use standard procedures and patient analysis to calculate the appropriate dose, extrapolating from the data provided in the Examples. In general, a dose which delivers about 1-100 mg/kg body weight is expected to be effective, although more or less may be useful.

In addition, the compositions of the invention may be administered in combination with other therapies. In such combination therapy, the administered dose of the compounds may be less than for single drug therapy.

The compounds can be administered orally, parentally (including subcutaneous injection, intravenous, intramuscular, intrasternal or infusion techniques), by inhalation spray, topically, by absorption through a mucous membrane, or rectally, in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants or vehicles. Pharmaceutical compositions of the invention can be in the form of suspensions or tablets suitable

for oral administration, nasal sprays, creams, sterile injectable preparations, such as sterile injectable aqueous or oleagenous suspensions or suppositories.

For oral administration as a suspension, the compositions can be prepared according to techniques well-known in the art of pharmaceutical formulation. The compositions can contain microcrystalline cellulose for imparting bulk, alginic acid or sodium alginate as a suspending agent, methylcellulose as a viscosity enhancer, and sweeteners or flavoring agents. As immediate release tablets, the compositions can contain microcrystalline cellulose, starch, magnesium stearate and lactose or other excipients, binders, extenders, disintegrants, diluents and lubricants known in the art.

For administration by inhalation or aerosol, the compositions can be prepared according to techniques well-known in the art of pharmaceutical formulation. The compositions can be prepared as solutions in saline, using benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons or other solubilizing or dispersing agents generally known in the art.

For administration as injectable solutions or suspensions, the compositions can be formulated according to techniques well-known in the art, using suitable dispersing or wetting and suspending agents, such as sterile oils, including but not limited to, synthetic mono- or diglycerides, and fatty acids, including oleic acid.

For rectal administration as suppositories, the compositions can be prepared by known methods, for example, by mixing with a suitable non-irritating excipient, such as cocoa butter, synthetic glyceride esters or polyethylene glycols, that are solid at ambient temperatures, but liquefy or dissolve in the rectal cavity to release the drug.

Solutions or suspensions of the compounds can be prepared in water, isotonic saline (PBS), and the like, and optionally can be mixed with a nontoxic surfactant. Dispersions may also be prepared by known methods, for example in glycerol, liquid polyethylene, glycols, DNA, vegetable oils, triacetin, and mixtures thereof. Under ordinary conditions of storage and use, these preparations may contain a preservative, for example, to prevent the growth of microorganisms.

The pharmaceutical dosage form suitable for injection or infusion use can include sterile, aqueous solutions or dispersions, sterile powders comprising an active ingredient, and the like, that are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions. In all cases, the ultimate

dosage form is preferable be sterile, fluid, and stable under the conditions of manufacture and storage. The liquid carrier or vehicle can be a solvent or liquid dispersion medium comprising, for example, water, ethanol, a polyol such as glycerol, propylene glycol, or liquid polyethylene glycols and the like, vegetable oils, nontoxic glyceryl esters, and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the formation of liposomes, by the maintenance of the required particle size, in the case of dispersion, or by the use of nontoxic surfactants. The prevention of the action of microorganisms can be accomplished by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be desirable to include isotonic agents, for example, sugars, buffers, or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the inclusion in the composition of agents delaying absorption--for example, aluminum monosterate hydrogels and gelatin.

Sterile injectable solutions are prepared by incorporating the conjugates in the required amount in the appropriate solvent with various other ingredients as enumerated above and, as required, followed by filter sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying techniques, which yield a powder of the active ingredient plus any additional desired ingredient present in the previously sterile-filtered solutions.

EXAMPLES

The synthetic procedures for the preparation of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate], Ala-d4T-MP and d4T have been previously described in detail (Venkatachalam et al., *Bioorg. Med. Chem. Lett.*, 8, 3121 (1998); Vig et al., *Antiviral Chem. Chemother.*, 9, 445, (1998) the compounds of Formula I to III can also be synthesized as described in U.S. Patent No. 6,030,957 (Uckun et al.)) which patent is` incorporated herein by reference.

Example 1

Quantitative HPLC For Detection of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] and Its Metabolites

The HPLC system used for these studies was a Hewlett Packard (Palo Alto, CA) series 1100 instrument equipped with a quaternary pump, an autosampler, an automatic electronic degasser, an automatic thermostatic column compartment, a diode array detector and a computer with Chemstation software for data analysis (Chen et al., *J. Chromatogr. B.*, 724, 157 (1999); Chen et al., *J. Chromatogr. B.*, 727, 205 (1999); and Chen et al., *J. Liq. Chromatogr.*, 22, 1771 (1999)). The analytical column used was a Zobax SB-Phenyl (5 μm, Hewlett Packard, Inc.) column attached to a guard column (Hewlett Packard, Inc.). The column was equilibrated prior to data collection. The linear gradient mobile phase (flow rate = 1.0 mL/minute) was: 100% A / 0% B at 0 minutes, 88% A / 12% B at 20 minutes, 8% A / 92% B at 30 minutes (A: 10 mM ammonium phosphate buffer, pH 3.7; B: acetonitrile). The detection wavelength was 268 nm, the peakwidth was less than 0.03 minutes, the response time was 0.5 seconds, and the slit was 4 nm.

HPLC-grade reagents and deionized, distilled water were used in this study. Acetonitrile was purchased from Burdick & Jackson (Allied Signal Inc., Muskegon, MI). Acetic acid was purchased from Fisher Chemicals (Fair Lawn, NJ). Ammonium phosphate and phosphoric acid were purchased from Sigma-Aldrich (St. Louis, MO).

Plasma samples (200 μL) were mixed 1:4 with acetone (800 μL) and vortexed for at least 30 seconds. Following centrifugation, the supernatant was transferred into a clean tube and was dried under nitrogen. A 50 μL solution of 50% methanol in 200 mM HCl was used to reconstitute the extraction residue, and 40 μL was injected into the HPLC.

The chromatographic retention times (R_T) measured for d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] and its metabolites in spiked samples were 28.7±0.02 minutes (d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]A; n=13; Figure 1B), 28.9±0.02 minutes (d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]B; n=13; Figure 1C), 15.3±0.2 minutes (Ala-d4T-MP; n=30) and 18.5±0.1 minutes (d4T; n=30). d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]A and d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]B are

diastereomers of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]. At these retention times, no significant interference peaks from the blank plasma were observed (Figures 1A and 1B).

The hydrochloric acid component of the reconstituted solutions played a role in the chromatography of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] and its metabolites; the acid protonated Ala-d4T-MP. No peak appeared in the chromatogram for this metabolite in the absence of hydrocholoric acid in the reconstituted solution. The acidic solution decreased the stability of Ala-d4T-MP, however. Therefore, all of the extracted samples were analyzed immediately after reconstitution.

Example 2

Stability of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] and Ala-d4T-MP in Whole Blood and Plasma

Whole blood and plasma samples were spiked with d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] and Ala-d4T-MP to yield final concentrations of 250 μM d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] and 100 μM Ala-d4T-MP, respectively. The whole blood samples were placed in a 37°C water bath, while plasma samples were stored at -20°C. At a predetermined time, an aliquot (100 μl) of spiked whole blood or plasma was extracted by adding 400 μl of acetone to induce precipitation of proteins, as described above. The absolute peak area was used to evaluate the stability of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] and Ala-d4T-MP.

The results shown in Figures 2A and 2B indicate that d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] is very unstable in plasma and in whole blood. Following incubation with plasma, over 95% of the d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] decomposed after 5 minutes. In the whole blood samples, 68%, 87%, and 92% of the d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] decomposed in samples taken at 5, 10, and 15 minutes, respectively. In both the plasma and whole blood samples, decomposition of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] was complete within 30 minutes. (see Table 1 for plasma data). Thus, samples were extracted immediately after the

samples were obtained. In contrast, Ala-d4T-MP was stable in both whole blood and plasma for 1 day.

Example 3

Stability of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] in Plasma in the Presence of Selective Esterase Inhibitors

Plasma samples were pre-incubated with the esterase inhibitors paraoxon (final concentration of 0.1 mM), physostigmine (final concentration of 0.1 mM), and EDTA (final concentration of 1M) at 37°C for 30 minutes. Then d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] was added to yield final concentrations of 250 μM. At a predetermined time, an aliquot (100 μl) of spiked plasma was extracted by adding 400 μl of acetone to induce precipitation of proteins, as described above. Decomposition of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] in plasma was significantly inhibited by paraoxon, partially inhibited by physostigmine, but not affected by EDTA (see Figures 3A, 3B, and 3C as well as Table 1). The data shown in Table 1 was calculated as mean percent hydrolysis from two experiments.

Table 1

Effect of Selective Esterase Inhibitors on Metabolism of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] in Plasma

Specificity	No inhibitor	Paraoxon (0.1 mM) cholinesterase & carboxylesterase	Physostigmine (0.1 mM) cholinesterase	EDTA (1 mM) arylesterase
5 min	95%	0%	43%	99%
10 min	98%	2%	65%	100%
15 min	99%	2%	76%	100%
30 min	100%	2%	89%	100%
60 min	100%	3%	100%	100%
120 min	100%	24%	100%	100%

Example 4

Stability of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] in Murine Liver Homogenates

Fresh mouse liver was obtained from Balb/c mice and homogenated in 1x PBS (1:1, W/V) using a Polytron (PT-MR2000) homogenizer (Kinematical AG, Littau, Switzerland). d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] was added to the liver homogenate to yield a final concentration of 100 μM. At a predetermined time, an aliquot (100 μl) of spiked liver homogenate was extracted by adding 400 μl of acetone to induce precipitation of proteins, as described above.

The compound d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] decomposed after incubation with the liver homogenate within 30 minutes (Figure 4), similar to the data obtained in plasma. However, unlike in plasma, significant amounts of d4T were detected after incubation with the liver homogenate.

Example 5

Stability of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] and Ala-d4T-MP in Gastric and Intestinal Fluids

Simulated gastric and intestinal fluids were prepared following United States Pharmacopia methods and were spiked with d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] and Ala-d4T-MP to yield a solution with a final concentration of 100 μM of each compound. The spiked fluids were then placed in a 37°C water bath. At a predetermined time, 100 μl aliquots of the spiked gastric or intestinal fluid were extracted by adding 400 μl of acetone as discussed above.

d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] is relatively stable in gastric fluid for 8 hours, but it is not stable in intestinal fluid (Figures 5A and 5B). d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] quickly decomposed to yield Ala-d4T-MP in intestinal fluid (approximately 94% of the d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] had decomposed within 2 hours). Ala-d4T-MP was stable in intestinal fluid; only a trace amount of d4T was detected in the intestinal fluid.

Example 6

Pharmacokinetic Studies in Mice

Female Balb/c mice (6-8 weeks old) from Taconic (Germantown, NY) were housed in a controlled environment (12-hours of light/12-hours of dark, 22 ± 1°C, 60 ± 10% relative humidity), which is fully accredited by the USDA. All rodents were housed in microisolator cages (Lab Products, Inc., NJ) containing autoclaved bedding. The mice were allowed free access to autoclaved pellet food and tap water throughout the study. All animal care procedures conformed to the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, Washington DC 1996).

A solution (50 µl) of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] (100 mg/kg) dissolved in DMSO was administered intravenously via the tail vein. This volume of DMSO is well-tolerated by mice when administrated by rapid intravenous or extravascular injection (Rosenkrantz et al., *Cancer Chemother. Rep.*, 31, 7 (1963); Wilson et al., *Toxicol. Appl. Pharmacol.*, 7, 104 (1965)). Blood samples (~500 µL) were obtained from the ocular venous plexus by retro-orbital venipuncture at 0, 2, 5, 10, 15, 30, 45, 60, 120, 240 and 360 minutes after intravenous injection. In order to study the pharmacokinetics of Ala-d4T-MP and d4T following systemic administration of these compounds, mice were injected with 75 mg/kg Ala-d4T-MP and 40 mg/kg d4T, respectively (these doses are equimolar to the 100 mg/kg d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]).

In order to determine the pharmacokinetics of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] following oral administration, 12 hour fasted mice were given a bolus dose of 100 mg/kg d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] via gavage using a #21 stainless-steel ball-tipped feeding needle. Sampling time points were 0, 2, 5, 10, 15, 30, 45, 60, 120, 240 and 360 minutes after the gavage.

All collected blood samples were heparinized and centrifuged at 7000 x g for 5 minutes to separate the plasma fraction from the whole blood. The plasma samples were then processed immediately using the extraction procedure described above.

Pharmacokinetic modeling and parameter calculations were carried out using the WinNonlin Professional Version 3.0 (Pharsight, Inc., Mountain, CA) pharmacokinetics software (Chen et al., *Pharm. Res.*, 16, 1003 (1999); Chen et al.,

Pharm. Res., 16, 117 (1999); Chen et al., *J. Clin. Pharmacol.*, 39, 1248 (1999); Uckun et al., *Clin. Cancer Res.*, 5, 2954 (1999); and Uckun et al., *J. Pharmacol. Exp. Ther.*, 291, 1301 (1999)). An appropriate model was chosen on the basis of the lowest sum of weighted squared residuals, the lowest Schwartz Criterion (SC), the lowest Akaike's Information Criterion (AIC) value, the lowest standard errors of the fitted parameters, and the dispersion of the residuals. The elimination half-life was estimated by linear regression analysis of the terminal phase of the plasma concentration-time profile. The area under the concentration-time curve (AUC) was calculated according to the linear trapezoidal rule between the first sampling time (0 hours) and the last sampling time plus C/k, where C is the concentration of the last sampling and k is the elimination rate constant. The systemic clearance (CL) was determined by dividing the dose by the AUC. The metabolic clearance of the parent drug, the formation clearance of the metabolite, the clearance elimination of the metabolite, and the distribution clearance of the metabolite were estimated by simultaneous fitting of the concentration of parent drug and metabolites as a function of time curve to pharmacokinetic models (see Figures 7A & 8A) specified as a system of differential equations (Gabrielsson & Weiner, Phamacokinetic / Phamacodynamic Data Analysis: Concepts and Applications, Swedish Pharmaceutical Press (1997)). The fraction of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] converted to a metabolite (f_m) was calculated as the ratio of the AUC for the metabolite after administration of the parent drug [$(AUC_m)_p$] to the AUC after administration of an equimolar dose of the metabolite [$(AUC_m)_m$] (Gibaldi & Perrier, 1982): $f_m=[(AUC_m)_p/D_p]x[D_m/(AUC_m)_m] = (AUC_m)_p \bullet CL_m/D_p$

Example 7

Metabolism and Pharmacokinetic Profile of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] Following Intravenous Administration

Following intravenous administration, d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] (100 mg/kg) was metabolized to yield d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]-M1 (R_T = 15.3 minutes) and d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]-M2 (R_T = 18.5 minutes) (Figure 1C). d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]-M1 had the same retention

time as Ala-d4T-MP, whereas d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]-M2 had the same retention time as d4T (Figures 1B and 1C). The UV spectra of these two metabolites were identical to those of Ala-d4T-MP and d4T, respectively.

After intravenous administration of 100 mg/kg d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate], the plasma concentration of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] as a function of time was described by a one-compartment model (Figure 6A). The estimated pharmacokinetic parameter values are presented in Table 2. d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] had a C_{max} of 224.2 μM and an AUC of 1142.0 μM•minute. The systemic clearance of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] was moderately fast with a CL of 160.9 mL/minute/kg, which is approximately twice the rate of blood flow to the kidney or the liver (Davies et al., *Pharm. Res.*, 10, 1093 (1993)). d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] had a moderate size volume of distribution with a V_{ss} of 819.9 ml/kg, which is roughly equal to the total volume of water in the body. d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] had a short elimination half-life ($t_{1/2}$ = 3.5 minutes), however, because of its rapid metabolism.

The diastereomers of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] were separated using the HPLC conditions described above (the retention times were 28.7 and 28.9 minutes). One of the diastereomers (d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]-A, retention time = 28.7 minutes) was metabolized more quickly than the other (d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]-B, retention time = 28.9 minutes; Figure 1C). The pharmacokinetic features of these two diastereomers are summarized in Table 2. d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]-B had a higher AUC (741.2 *vs.* 441.5 μM•minute) and C_{max} (125.7 *vs.* 107.9 μM) than d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]-A (Figures 6B and 6C). d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]-B also had a slightly longer elimination half-life than the d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]-A diastereomer (4.1 minutes *vs.* 2.8 minutes), which may be due to faster clearance of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]-A relative to that of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]-B (208.2 *vs.* 123.9 ml/min/kg). However, both d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] diastereomers were completely metabolized within 30 minutes.

Following intravenous injection, d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] was rapidly metabolized to yield Ala-d4T-MP (t_{max} = 5.9 minutes; C_{max} = 67.4 μM; $t_{1/2}$ = 129.2 minutes) and d4T (t_{max} = 18.7 minutes; C_{max} = 15.7 μM; $t_{1/2}$ = 114.8 minutes) as shown in Table 2.

Table 2

Estimated Pharmacokinetic Parameter Values for d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] and Its Metabolites in Balb/C Mice

Measured	V_{ss} (ml/kg)	AUC (μM·min)	C_{max} (μM)	$t_{1/2}$ (min)	CL (ml/min/kg)	t_{max} (min)
Total d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]	819.9 (920±127.4)	1142.0 (1071.8±81.8)	224.2 (211.6±29.3)	3.5 (3.6±0.3)	160.9 (174.5±13.2)	ND
d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]-A	852.1 (1005.3±134.0)	441.5 (359.7±43.9)	107.9 (96.5±12.8)	2.8 (2.6±0.1)	208.2 (266.5±30.2)	ND
d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]-B	731.1 (791.8±113.1)	741.2 (730.8±45.7)	125.7 (123.1±16.8)	4.1 (4.3±0.4)	123.9 (127.3±8.2)	ND
Ala-d4T-MP	ND	2854.8 (2795.9±361.2)	67.4 (69.3±4.1)	129.2 (138.8±40.2)	ND	5.9 (5.1±0.7)
d4T	ND	2915.2 (2858.1±182.2)	15.7 (15.6±1.2)	114.8 (116.2±11.9)	ND	18.7 (17.4±2.6)

Pharmacokinetic parameters in Balb/c mice (N = 4 mice per time-point) are presented as the average values estimated from composite plasma concentration-time curves of pooled data. The mean ± S.E.M values are indicated in parentheses. ND means the value was not determined.

The model depicted in Figures 7A and 7B describes the metabolite pharmacokinetics of Ala-d4T-MP and d4T after intravenous injection of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate]. According to this model, d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] is biotransformed to produce Ala-d4T-MP (CL_{m1}) and d4T (CL_{m2}), respectively. Ala-d4T-MP derived from d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] can be further metabolized to form D4T

(CL_{m3}) or distributed to the extravascular compartment (Cl_{m1d}). D4T produced from either d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] or Ala-d4T-MP is finally eliminated from the body (CL_{me2}). The pharmacokinetic parameters estimated for these two metabolites are presented in Table 3.

Table 3

Estimated Metabolite Pharmacokinetic Parameter Values

Phamacokinetic Parameter	ml/min/kg
CL_{m1}	83.9 (21.5%)
CL_{m2}	87.4 (24.4%)
CL_{m3}	36.1 (85.9%)
CL_{m1d}	62.0 (69.8%)
CL_{me2}	47.1 (74.1%)

The values in parenthesis are the C.V. of modeling. The metabolic clearance of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] and the formation clearance of the metabolites were 83.9 ml/minute/kg for Ala-d4T-MP and 87.4 ml/minute/kg for d4T, respectively. The metabolic clearance of Ala-d4T-MP and the formation clearance of its metabolite, d4T, were 36.1 ml/minute/kg, and a small portion of Ala-d4T-MP was distributed to the extravascular compartment with a CL_{m1d} of 47.1 ml/minute/kg. Finally, d4T was eliminated with a CL_{me2} of 62.0 ml/minute/kg.

Example 8

Pharmacokinetic Profile of Ala-d4T-MP Following Intravenous Administration

Following intravenous injection of Ala-d4T-MP (75 mg/kg, a dose equimolar to the 100 mg/kg dose of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] discussed above), Ala-d4T-MP was quickly metabolized to yield d4T (t_{max} = 4.4 minutes; $t_{1/2}$ = 34.0 minutes) (Figures 8A and 8B, Table 4). The concentration of Ala-d4T-MP as a function of time can be described using a two-compartment model, while a one-compartment model best fits the concentration of its metabolite, d4T, as a function of time (Figure 8B). The C_{max} values for Ala-d4T-MP and d4T were 1206.6 μM and 35.2 μM, respectively. The AUC was 11648.7 μM•minute for Ala-d4T-MP and 1888.0 μM•minute for d4T. The systemic clearance of Ala-d4T-MP

was only 15.8 mL/minute/kg (Table 4), which is much less than the blood flow to either the kidney or the liver (Davies et al., *Pharm. Res.*, 10, 1093 (1993)). Ala-d4T-MP also had a small volume of distribution (V_{ss} = 275.5 ml/kg) that is less than the total volume of water in the body. Nevertheless, the elimination half-life of Ala-d4T-MP was short ($t_{1/2}$ = 28.5 minutes), due to its rapid metabolism.

Table 4

Estimated Pharmacokinetic Parameter Values for Ala-d4T-MP and Its Metabolite in Balb/C Mice

Measured	Vss (ml/kg)	AUC (μM·min)	C_{max} (μM)	$t_{1/2}$ (min)	CL (ml/min/kg)	t_{max} (min)
Ala-d4T-MP	275.5 (412.6±126.3)	11648.7 (11761.5±447.2)	1206.6 (1658.1±544.9)	28.5 (91.5±54.5)	15.8 (15.7±0.6)	ND
d4T	ND	1888.0 (1818.2±42.9)	35.2 (35.9±3.7)	34.0 (32.4±2.2)	ND	4.4 (5.0±1.2)

Pharmacokinetic parameters in Balb/c mice (N = 5 mice per time-point) are presented as the average values estimated from composite plasma concentration-time curves of pooled data. The mean ± S.E.M values are indicated in parentheses. ND means the value was not determined.

The model depicted in Figure 8A best described the metabolite pharmacokinetics after intravenous injection of Ala-d4T-MP. According to this model, Ala-d4T-MP can either be metabolized to form d4T (CL_{ml}) or distributed to the extravascular compartment (Cl_{pd}). D4T derived from Ala-d4T-MP is eliminated from the body (CL_{me}). By simultaneous fitting of the parent Ala-d4T-MP and d4T concentration values as a function of time to the described model, the metabolic clearance of Ala-d4T-MP and the formation clearance of d4T (CL_{ml}) were estimated to be 15.6 ml/min/kg as shown in Table 5.

Table 5

Estimated Metabolite Pharmacokinetic Parameter Values

Pharmacokinetic Parameter	ml/min/kg
CL_{ml}	15.6 (16.6%)
CL_{me}	88.4 (13.0%)
CL_{pd}	4.7 (44.7%)

The data in parentheses are the C.V. of modeling. A small portion of Ala-d4T-MP was distributed to extravascular compartment with a CL_{pd} of 4.7 ml/minute/kg and d4T derived from Ala-d4T-MP was finally eliminated with a relatively high CL_{me} of 88.4 ml/minute/kg. The CL_{ml} of 15.6 ml/minute/kg accounts for 99% of the total systemic clearance (CL = 15.8 ml/minute/kg) (see Table 4), indicating that most of Ala-d4T-MP was biotransformed to form d4T.

Example 9

Pharmacokinetic Profile of d4T Following Intravenous Administration

Following intravenous injection at a dose level of 40 mg/kg, a dose equimolar to the 100 mg/kg dose of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate], the concentration of d4T as a function of time was described using a one-compartment model (Figure 9). The estimated pharmacokinetic parameter values are presented in Table 6. The estimated C_{max} and AUC values for D4T were 279.5 μM and 12227.1 μM•minute, respectively. D4T had a short elimination half-life (30.3 minutes). The systemic clearance of d4T was slow with a CL of only 15.0 ml/min/kg, which is much lower than the blood flow to either the kidney or the liver (Davies et al., *Pharm. Res.*, 10, 1093 (1993)). D4T had a moderately large volume of distribution (V_{ss} = 657.8 ml/kg) that is approximately equal to the volume of water in the body.

Table 6

Estimated Pharmacokinetic Parameter Values for D4T in Balb/C Mice

Measured	Vss (ml/kg)	AUC (μM·min)	C_{max} (μM)	$t_{1/2}$ (min)	CL (ml/min/kg)
d4T	657.8 (581.8±62.8)	12227.1 (12173.6±559.5)	279.5 (318.9±15.7)	30.3 (26.6±1.2)	15.0 (15.2 ±0.7)

Pharmacokinetic parameters in Balb/c mice (N = 5 mice per time-point) are presented as the average values estimated from composite plasma concentration-time curves of pooled data. The mean ± S.E.M values are indicated in parentheses.

Example 10

Pharmacokinetic Profile of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] Following Oral Administration

The pharmacokinetic behavior of orally administered d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] (100 mg/kg) was also examined. Both metabolites (Ala-d4T-MP and d4T) were detected, but the concentration of the parent d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] was below the detection limit (0.5 μM). The t_{max} values are 10.3 minutes for Ala-d4T-MP and 42.4 minutes for d4T. A one-compartment pharmacokinetic model was used to describe both the Ala-d4T-MP and the d4T concentration changes as a function of time (Figures 10A and 10B). The estimated values for the pharmacokinetic parameters are presented in Table 7. The maximum concentrations (C_{max}) for Ala-d4T-MP and D4T are 12.7 μM and 30.7 μM, respectively. The elimination half-lives were 66.4 minutes and 99.0 minutes for Ala-d4T-MP and d4T, respectively.

Table 7

Estimated Pharmacokinetic Parameter Values Following Oral Administration of d4T-5'-[*p*-bromophenyl methoxyalaninyl phosphate] in Balb/C Mice

Measured	AUC (μM·min)	C_{max} (μM)	$t_{1/2}$ (min)	t_{max} (min)
Ala-d4T-MP	1350.5 (1355.4±88.2)	12.7 (15.6±4.1)	66.4 (56.1±8.5)	10.3 (9.3±0.9)
d4T	5905.3 (5928.4±294.6)	30.7 (29.5±0.3)	99.0 (102.6±3.8)	42.4 (45.2±5.2)

Pharmacokinetic parameters in Balb/c mice (N = 4 mice per time-point) are presented as the average values estimated from composite plasma concentration-time curves of pooled data. The mean±S.E.M values are indicated in parentheses.

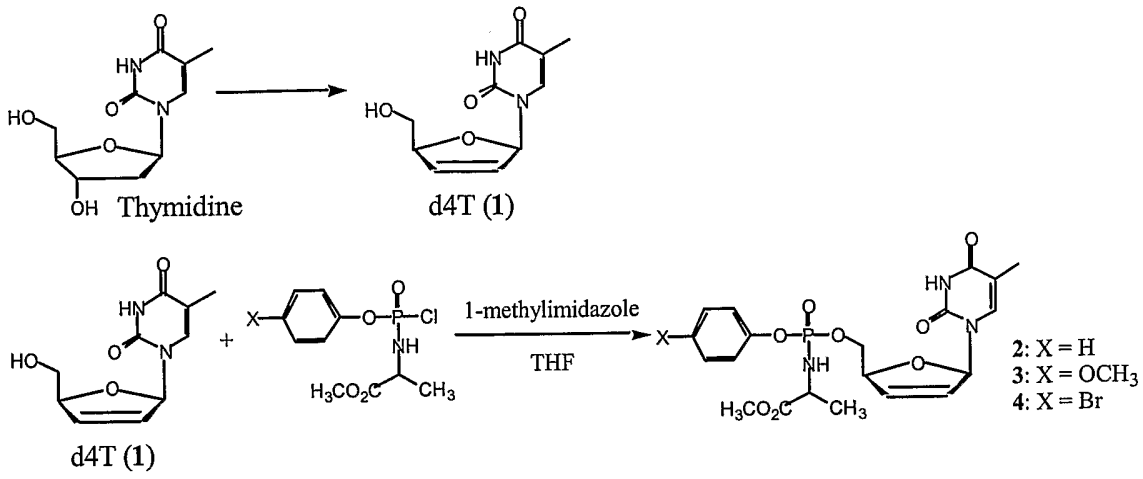
Example 11

Synthesis and Characterization of Stavudine Derivatives

All chemicals were purchased from Aldrich (Milwaukee, WI). All syntheses were performed under a nitrogen atmosphere. ^{1}H, ^{13}C, ^{19}F, and ^{31}P NMR were obtained on a Varian Mercury 300 instrument at ambient temperature in $CDCl_3$ or DMSO-d_6. Chemical shifts were reported as δ values in parts per million (ppm) downfield from tetramethylsilane (δ=0 ppm) as the internal standard. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad peak. FT-IR spectra were recorded on a Nicolet Protege 460 spectrometer. MALDI-TOF mass spectra were obtained using a Finnigan MAT 95 system. UV spectra were recorded using a Beckman UV-VIS spectrophotometer (Model 3DU 74000) with a cell path length of 1 cm.

HPLC purification was achieved using a reverse-phase Lichrospher column (250 x 4 mm, Hewlett-Packard, RP-18, Cat. # 79925) and an isocratic flow (1 ml/minute) consisting of water (70%) and acetonitrile (30%). Melting points were determined using a Melt John's apparatus and are uncorrected. Column chromatography was performed using silica gel obtained from Baker Company. The octanol/water partition coefficient was determined by the shake flask method. The phosphoramidate analogs were added to 2 ml of water and 2 ml of octanol in a glass vial. The mixture was shaken for 4 hours at room temperature. The two phases were carefully separated and filtered through a Millipore filter and analyzed by HPLC. The partition coefficient was calculated using the ratio of the area under the curve for octanol and water, respectively.

Antiviral drugs. The synthetic procedures for preparation of d4T-5' [para-bromophenyl methoxyalanininyl phosphate], have been previously described in detail by Vig et al., 1992 *Antiviral Chem. and Chemother.*, 9:445-447. Zidovudine was obtained from Toronto Research Chemicals Inc, Missassagua, Ontario, Canada. d4T was prepared from thymidine following the procedure of Mansuri et al., 1989 *J. Med.Chem. 32*, 461. Appropriately substituted phenyl methoxyalaninyl phosphorochloridates were also prepared according to the method reported by McGuigan et al., 1992 *Antiviral Res., 17*, 311. Compounds were synthesized as outlined below in Scheme 2.



Scheme 2. Phenylmethoxyalaninyl phosphorochloridate was added to the solution of d4T and 1-methylimidazole in anhydrous THF and the mixture was stirred at room temperature for 5-6 hours. Work up of the reaction mixture furnished the required derivatives in good yields. Column chromatography was applied to obtain pure compounds.

Physical data of the synthesized compounds was determined by HPLC was conducted by using C18 4x250 mm LiChrospher column eluted with 70:30 water/acetonitrile at the flow rate of 1ml/minute. The purity of the following compounds exceeded 96% by HPLC. ^{13}C NMR peaks labeled by stars are split due to diastereomers.

The physicochemical properties of the synthesized compounds were as follows:

2', 3'-Didehydro-3'-deoxy thymidine (d4T): Yield: 57%; mp. 165-166°C; UV (MeOH) λ_{max} 204, 257 nm; IR (KBr): 3463, 3159, 3033, 1691, 1469, 1116, 1093 Cm^{-1}; ^1H NMR (DMSO-d_6) δ 11.29 (br s, 1H), 7.63 (s, 1H), 6.80 (d, 1H, J=1.2Hz); 6.38 (d, 1H, J=5.9Hz), 5.90 (dd, 1H, J=1.1, 4.7Hz), 5.01 (m, 1H), 4.76 (s, 1H), 3.60 (dd, 2H, J=4.8, 3.6Hz), 1.71 (d, 3H, J=1.2Hz), ^{13}C NMR (DMSO-d_6) δ 164.4, 151.3, 137.2, 135.4, 126.4, 109.3, 89.2, 87.6, 62.4, 12.2; mass calculated: 224, found: 225 (M+1); HPLC retention time (t_R): 8.7 minutes.

5'-[4-Bromophenyl methoxylaninylphosphate]-2', 3'-didehydro-3'-deoxy thymidine (Compound 113): Yield: 83%; UV (MeOH) λ_{max}: 209, 218 and 266 nm; IR (Neat): 3203, 3070, 2954, 2887, 2248, 1743, 1693, 1485, 1221, 1153, 1038, 912,

835, 733 cm^{-1}; ^1H NMR ($CDCl_3$) δ 9.60-9.58 (br s, 1H), 7.45-7.42 (m, 2H), 7.30-7.09 (m, 4H), 6.37-6.27 (m, 1H), 5.93-5.88 (m, 1H), 5.04-5.01 (br m, 1H), 4.35-4.33 (m, 2H), 4.27-3.98 (m, 2H), 3.71-3.70 (s, 3H), 1.85-1.81 (s, 3H), 1.37-1.31 (m, 3H); ^{13}C NMR ($CDCl_3$) δ 173.7, 163.8, 150.8, 149.7-149.6, 135.6-135.4, 133.1-132.5, 127.4-127.3, 121.9-121.7, 118.0, 111.2-111.1, 89.7-89.4, 84.4-84.3, 67.8- 66.4, 52.5, 50.0-49.9, 20.7, and 12.3; ^{31}P NMR ($CDCl_3$) δ 3.41, 2.78; MALDI-TOF mass calculated (M+Na) 567.2, found 567.1; HPLC t_R: 12.04 & 12.72 minutes.

5'-[4-Methoxy phenyl methoxylaninylphosphate]-2', 3'-didehydro-3'-deoxy thymidine (Compound 598): Yield: 25%; UV (MeOH) λ_{max}: 223, 229 and 270 nm; IR (Neat): 3223, 3072, 2999, 2953, 2837, 1743, 1693, 1506,1443, 1207, 1153, 1111, 1034, 937, 837 and 756 cm^{-1}; ^1H NMR ($CDCl_3$) δ 9.40 (br s, 1H), 7.30-7.00 (m, 5H), 6.83-6.81 (m, 1H), 6.37-6.27 (m, 1H), 5.91-5.86 (m, 1H), 5.00 (br m, 1H), 4.40-4.30 (m, 2H), 4.20-4.10 (m, 2H), 3.95-3.93 (s, 3H), 3.82-3.80 (s, 3H), 1.85-1.81 (s, 3H) and 1.39-1.29 (m, 3H); ^{13}C NMR ($CDCl_3$) δ 174.0, 163.9, 156.6, 150.8, 143.5, 135.8-135.5, 133.3-132.9, 127.4-127.2, 121.2-120.9, 114.5, 111.2, 89.7-89.4, 84.5, 66.9-66.3, 55.5, 52.5, 50.6-49.9, 20.9, and 12.3; ^{31}P NMR($CDCl_3$) δ 3.82 , 3.20; MALDI-TOF mass calculated (M+Na) 518.2, found 518.2; HPLC t_R: 5.83 & 6.26 minutes.

5'-[3-Dimethylaminophenyl methoxylaninylphosphate]-2', 3'-didehydro-3'-deoxy thymidine (Compound 599): Yield: 18%; mp. 61-62°C; UV (MeOH) λ_{max}: 203, 206, 211 and 258 nm; IR: 3448, 3050, 2952, 1691, 1506, 1450, 1247, 1143 cm^{-1}; ^1H NMR ($CDCl_3$) δ 9.93 (s, 1H), 7.27 (m, 1H), 7.04 (m, 1H), 6.97 (m, 1H), 6.44 (q, 3H), 6.24 (m, 1H), 5.81 (t, 1H), 4.94 (t, 1H), 4.24 (s, 2H), 4.03 (m, 1H), 3.92 (m, 1H), 3.64* (d, 3H), 2.86 (s, 6H), 1.77* (d, 3H), 1.28* (t, 3H); ^{13}C NMR ($CDCl_3$) δ 173.7(d), 163.9(d), 151.3(t), 150.8(t), 135.5(d), 132.9(d), 129.5(d), 126.9(d), 111.0(d), 108.8(d), 107.2(q), 103.7(q), 89.3(d), 84.4(q), 66.7(d), 66.1(d), 52.3(d), 49.9(d), 40.2, 20.7(t), 12.2; ^{31}P NMR($CDCl_3$) δ 3.32, 2.70; HPLC t_R: 3.36 minutes.

5'-[2,6-Dimethoxyphenyl methoxyalaninyl phosphate]-2', 3'-didehydro-3'-deoxy thymidine (Compound 600): Yield: 23%; mp. 51-53°C; UV (MeOH) λ_{max}: 210 and 267 nm; IR: 3432, 3072, 2950, 1691, 1483, 1261, 1112, 931 cm^{-1}; ^1H NMR ($CDCl_3$) δ 9.78 (s, 1H), 6.95 (m, 3H), 6.48 (t, 2H), 6.29 (m, 1H), 5.81 (m, 1H), 4.36 (m, 3H), 4.02 (m, 2H), 3.74 (m, 6H), 3.63* (t, 3H), 1.74* (d, 3H), 1.29*

(m, 3H); ^{13}C NMR ($CDCl_3$) δ 173.7(q), 163.9(d), 151.7(t), 150.8(t), 135.7(d), 133.1(d), 128.4(d), 126.8(d), 125.0(d), 110.9(d), 104.8(t), 89.2(d), 84.6(d), 66.8(t), 55.8(d), 52.2(t), 49.7(d), 49.4(d), 21.0(d), 11.8(d); ^{31}P NMR ($CDCl_3$) δ 4.97, 4.28; HPLC t_R: 6.55 minutes.

5'-[4-Cyanophenyl methoxyalaninyl phosphate]-2', 3'-didehydro-3'-deoxy thymidine (Compound 601): Yield: 20%; mp. 62-63°C; UV (MeOH) λ_{max}: 207, 213, 233, and 267 nm; IR: 3214, 3070, 2954, 2229, 1691, 1502, 1467, 1245, 1035, 925 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 9.92 (s, 1H), 7.60 (m, 2H), 7.28 (m, 2H), 7.16 (m, 1H), 6.96 (m, 1H), 6.28 (m, 1H), 5.86 (t, 1H), 4.99 (m, 1H), 4.32 (m, 3H), 3.92 (m, 1H), 3.65* (m, 3H), 1.75* (m, 3H), 1.29* (m, 3H); ^{13}C NMR ($CDCl_3$) δ 173.5(t), 163.7(d), 153.4(q), 150.7, 135.3(d), 133.7(d), 132.6(d), 127.2(d), 121.0(q), 117.9, 111.0(d), 108.6(d), 89.5(d), 84.2(d), 67.3(t), 52.5(d), 50.0(d), 20.6(t), 12.3(d); ^{31}P NMR ($CDCl_3$) δ 4.15, 3.62; HPLC t_R: 5.02 minutes.

5'-[3-Bromophenyl methoxyalaninyl phosphate]-2', 3'-didehydro-3'-deoxy thymidine (Compound 602): Yield: 15%; mp. 47-48°C; UV (MeOH) λ_{max}: 208, 213, and 267 nm; IR: 3432, 3070, 2954, 1685, 1473, 1247, 941 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 9.65 (s, 1H), 7.34-7.11(m, 5H), 6.97 (m, 1H), 6.26(m, 1H), 5.87 (t, 1H), 4.98 (m, 1H), 4.26 (m, 3H), 3.93 (m, 1H), 3.67* (m, 3H), 1.76* (m, 3H), 1.32* (t, 3H); ^{13}C NMR ($CDCl_3$) δ 173.5(d), 163.8(d), 150.6(d), 135.4(d), 132.8 (d), 130.6(d), 128.0, 127.3(d), 123.3(q),122.3(d), 118.8(q), 111.1(d), 89.5(d), 84.4(q), 67.2(d), 52.6, 50.0(d), 20.7(t), 12.3(d); ^{31}P NMR ($CDCl_3$) δ 3.36, 2.74 ; HPLC t_R: 10.3, 10.7 minutes.

5'-[4-Bromo-2-chlorophenyl methoxyalaninyl phosphate]-2', 3'-didehydro-3'-deoxy thymidine (Compound 603): Yield: 18%; mp. 51-52°C; UV (MeOH) λ_{max}: 215, and 267 nm; IR: 3415, 3222, 3072, 2952, 1691, 1475, 1245, 1085, 1035, 925 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 9.52 (s, 1H), 7.52 (s, 1H), 7.32 (m, 2H), 7.22 (m, 1H), 6.99 (m, 1H), 6.29 (m, 1H), 5.90 (m, 1H), 5.00 (m, 1H), 4.33 (m, 2H), 4.19 (m, 1H), 4.01 (m, 1H), 3.67 (s, 3H), 1.79* (m, 3H), 1.31* (m, 3H); ^{13}C NMR ($CDCl_3$) δ 173.5(q), 163.8(d), 150.8(d), 145.5(t), 135.3 (d), 132.8 (d), 130.9(d), 127.3(d), 126.2(d), 122.7(d), 117.8(d), 113.3(d), 89.6(d), 84.3(d), 67.5(d), 67.1(d), 52.6, 50.1, 20.8(t), 12.3(d); ^{31}P NMR ($CDCl_3$) δ 3.11, 2.54; HPLC t_R: 18.6, 20.6 minutes.

5'-[4-Flurophenyl methoxyalaninyl phosphate]-2', 3'-didehydro-3'-deoxy thymidine (Compound 604): Yield: 46%; mp.42-44°C; UV (MeOH) λ_{max}: 210 and 266 nm; IR: 3423, 3245, 3072, 2954, 1691, 1504, 1247, 1089, 1037, 939 cm^{-1}; ^1H NMR ($CDCl_3$) δ 10.08 (bs, 1H), 7.16 (m, 1H), 7.08 (m, 2H), 6.91 (m, 3H), 6.20 (m, 1H), 5.79 (t, 1H), 4.92 (m, 1H), 4.42 (t, 1H), 4.22 (m, 2H), 3.85 (m, 1H), 3.58* (m, 3H), 1.70* (m, 3H), 1.22* (m, 3H); ^{13}C NMR ($CDCl_3$) δ 173.5(q), 163.8(d), 160.7, 157.5, 150.7(d), 145.7(q), 135.3(d), 132.7(d), 126.9(d), 121.3(t), 115.8(q), 110.8(d), 89.2(d), 84.2(d), 66.8(t), 52.2, 49.8(d), 20.4(d), 12.1(d); ^{31}P NMR ($CDCl_3$) δ 3.80(d), 3.22(d); ^{19}F NMR ($CDCl_3$) δ -42.8(t); HPLC t_R: 6.3, 6.6 minutes.

5'-[2-Bromophenyl methoxyalaninyl phosphate]-2', 3'-didehydro-3'-deoxy thymidine (Compound 605): Yield: 20%; mp. 45-46°C; UV (MeOH) λ_{max}: 207 and 267 nm; IR: 3432, 3072, 2954, 1685, 1475, 1245, 1089, 933 cm^{-1}; ^1H NMR ($CDCl_3$) δ 9.55 (s, 1H), 7.47 (m, 2H), 7.24 (m, 2H), 6.99 (m, 2H), 6.29 (m, 1H), 5.88 (t, 1H), 5.00 (m, 1H), 4.35 (m, 2H), 4.02 (t, 2H), 3.66 (s, 3H), 1.80* (m, 3H), 1.30* (m, 3H); ^{13}C NMR ($CDCl_3$) δ173.6(t), 163.8(d), 150.8(d), 147.3(t), 135. 4(d), 133.0(t), 128.5(d), 127.2(d), 126.1(d), 121.3(q), 114.4(d), 111.3(d), 89.6(d), 84.3(d), 67.2(q), 52.5, 50.1(d), 29.6, 20.8(t), 12.4; ^{31}P NMR ($CDCl_3$) δ 2.98, 2.37; HPLC t_R: 8.4, 9.2 minutes.

5'-[2-Chlorophenyl methoxyalaninyl phosphate]-2', 3'-didehydro-3'-deoxy thymidine (Compound 606): Yield: 47%; mp. 43-45°C; UV (MeOH) λ_{max}: 214, 215, 219 and 267 nm; IR: 3209, 3070, 2952, 1691, 1481, 1245, 1035, 931 cm^{-1}; ^1H NMR ($CDCl_3$) δ 9.80(s, 1H), 7.39(t, 1H), 7.29(m, 1H), 7.20(m, 1H), 7.13(t, 1H), 7.01(t, 1H), 6.92(d, 1H), 6.24(m, 1H), 5.81(m, 1H), 4.94(m, 1H), 4.28(m, 3H), 3.96(m, 1H), 3.59*(m, 3H), 1.72*(m, 3H), 1.25*(m, 3H); ^{13}C NMR ($CDCl_3$) δ 173.5(t), 163.8(d), 150.8(d), 145.9(d), 135. 3(d), 132.7(d), 130.0, 127.5(d), 127.0(d), 124.8(q), 121.2(q), 111.0(d), 89.3(d), 84.3(d), 66.9(d), 52.3, 49.8(d), 20.5(t), 12.1(d); ^{31}P NMR ($CDCl_3$) δ 3.23, 2.67; HPLC t_R: 7.6, 8.3 minutes.

5'-[Phenyl methoxylaninylphosphate]-2', 3'-didehydro-3'-deoxy thymidine: (Compound 607): Yield: 46%; UV (MeOH) λ_{max}: 211 and 264 nm; IR (Neat): 3222, 2985, 2954, 1743, 1693, 1593, 1491, 1456, 1213, 1153, 1039, 931, 769 cm^{-1}; ^1H NMR ($CDCl_3$) δ 9.30 (br s, 1H), 7.30-7.10 (m, 6H), 6.85-6.82 (m, 1H), 6.36-6.26 (m, 1H), 5.91-5.85 (m, 1H), 5.00 (br m, 1H), 4.19-3.68 (m, 4H), 3.72, 3.71

(s, 3H), 1.83, 1.80 (d, 3H), 1.38-1.25 (m, 3H); ^{13}C NMR ($CDCl_3$) δ 173.9, 163.7, 150.7, 149.7, 135.7-135.4, 133.2-132.9, 129.6-129.4, 127.3-127.2, 125.0-124.4, 120.0, 111.1, 89.6-89.4, 84.5-84.4, 66.9- 66.3, 52.5-52.3, 50.0-49.6, 20.9 and 12.3; ^{31}P NMR ($CDCl_3$) δ 2.66 , 3.20; MALDI-TOF mass calculated (M+Na) 488.0, found 487.9; HPLC t_R: 5.54 , 5.85 minutes.

5'-[2,5-Dichlorophenyl methoxyalaninyl phosphate]-2', 3'-didehydro-3'-deoxy thymidine (Compound 608): Yield: 30%; mp. 42-44°C; UV (MeOH) λ_{max}: 211, 216, 220 and 268 nm; IR: 3423, 3205, 3072, 2954, 1691, 1475, 1245, 1093, 946 cm^{-1}; ^1H NMR ($CDCl_3$) δ 9.43(s, 1H), 7.45(m, 1H), 7.25(m, 2H), 7.04(m, 1H), 6.99(q, 1H), 6.32(m, 1H), 5.88 (m, 1H), 4.99 (m, 1H), 4.32 (m, 3H), 4.00 (m, 1H), 3.67 (s, 3H), 1.77* (m, 3H), 1.33* (t, 3H); ^{13}C NMR ($CDCl_3$) δ 173.5(d), 163.8(d), 150.8(d), 146.4(d), 136.3, 132.7(t), 130.7(d), 127.4, 125.8, 123.7(d), 121.7(q), 111.2(d), 89.6(d), 84.3(t), 67.1(d), 52.6, 50.1, 29.6, 20.7(t), 12.3(d); ^{31}P NMR($CDCl_3$) δ 3.24, 2.60; HPLC t_R: 13.2 minutes.

5'-[4-Chlorophenyl methoxyalaninyl phosphate]-2', 3'-didehydro-3'-deoxy thymidine (Compound 609): Yield: 40%; mp. 42-44°C; UV (MeOH) λ_{max}: 202, 204,212, 219 and 267 nm; IR: 3423, 3214, 3068, 2952, 1691, 1488, 1247, 1089, 929 cm^{-1}; ^1H NMR ($CDCl_3$) δ 9.48 (d, 1H), 7.25 (d, 3H), 7.12(t, 1H), 7.00 (m, 1H), 6.30 (m, 1H), 5.89 (t, 1H), 5.01(m, 1H), 4.29 (m, 3H), 4.05 (t, 1H), 3.90 (m, 1H), 3.69* (d, 3H), 1.80* (d, 3H), 1.32* (d, 3H); ^{13}C NMR ($CDCl_3$) δ 173.7(q), 163.7(d), 150.7, 148.6(q), 135.5(d), 132.9(d), 130.3(d), 129.5(d), 127.3(d), 121.4(q), 111.2(d), 89.5(d), 84.4(d), 67.2(d), 66.5(d), 52.6,50.1(d), 20.9(t), 12.4(d); ^{31}P NMR($CDCl_3$) δ 3.57, 2.82; HPLC t_R: 7.6, 8.3 minutes.

Example 12

Antiviral Activity of Stavudine Derivatives

Virus stocks. The HIV-1 strain used in this study was HTLV-IIIB (Uckun et al., 1998 *Antimicro. Agents and Chemother.*, 42:383-388). The non-HIV viruses included in the present study were the gancyclovir-sensitive cytomegalovirus (CMV) strain AD169 (ATCC VR-538); acyclovir-sensitive herpes simplex virus (HSV) Type I strain HF (ATCC VR-260); acyclovir-sensitive HSV Type II strain G (ATCC VR-734); adenovirus strain Type 5, strain adenoid 75 (ATCC VR-5);

enterovirus strain, ECHO 30, strain Bastianni (ATCC VR-322); and respiratory syncytial virus (RSV), strain Long (ATCC VR-26).

In vitro assays of anti-HIV-1 activity. Normal human peripheral blood mononuclear cells (PBMNC) from HIV-negative donors were cultured 72 hours in RPMI 1640 supplemented with 20% (v/v) heat–inactivated fetal bovine serum (FBS), 3% interleukin-2, 2 mM L-glutamine, 25 mM HEPES, 2 g/L $NaHCO_3$, 50 μg/mL gentamicin, and 4 μg/mL phytohemagglutinin prior to exposure to HIV-1 at a multiplicity of infection (MOI) of 0.1 during a 1hour adsorption period at 37°C in a humidified 5% CO_2 atmosphere. Subsequently, cells were cultured in 96-well microtiter plates (100 μL, 2 x 10^6 cells/mL) in the presence of various concentrations of compounds 113 or 609. Aliquots of culture supernatants were removed from the wells on the seventh day after infection for p24 antigen assays, as previously described. The applied p24 enzyme immunoassay (EIA) was the unmodified kinetic assay commercially available from Coulter Corporation/Immunotech, Inc. (Westbrooke, ME), which utilizes a murine mAb against HIV core protein coated onto microwell strips to which the antigen present in the test culture supernatant samples binds. Percent viral inhibition was calculated by comparing the p24 values from untreated infected cells (i.e., virus controls).

Plaque formation assays. Plaque assays were used to examine the activity of the compounds against non-HIV viruses. The skin fibroblast cell line SF (ATCC CRL-2097) was used as a target for AD169, Adenovirus Type 5. The ECHO 30, VERO cell line (ATCC CCL-81) was used as a target for HSV HF and G. The HEP-2 cell line (ATCC CCL-23) was used as a target for the RSV strain Long. These cell lines were cultured at 1 x 10^5 cells/well in 24-well (all but ECHO 30-infected SF) or 6-well (ECHO 30-infected SF) tissue culture plates with 0.9% methylcellulose or 0.4% SeaPlaque agarose semisolid support. Minimum Essential Medium (MEM) with Earle's salts (Gibco), L-glutamine, non-essential amino acids, 2% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin, and 0.05% gentamicin served as the culture medium. The incubation times were 3 days for HF, G, ECHO 30, and Long; and 7 days for AD169 and Adenovirus Type 5. Plaque counting was performed with a 20X dissecting microscope for AD169, Type 5, ECHO 30, and RSV Long, and with a light box for HF and G. The fixative agent was crystal violet for all viruses, except for the CMV strain AD169 for which methylene blue was used. Percent inhibition of plaque formation was calculated by

comparing the plaque numbers from the test substance-treated infected cells with the plaque numbers from untreated infected cells (i.e., virus controls). The IC_{50} values were determined using the Statview statistics program (SAS Institute, Inc., Cary, NC).

Statistical analysis. Each drug was tested at 6-7 different concentrations ranging from 0.0001 μM to 100 μM. Each assay was set up in triplicate wells and repeated 1-3 times. An IC_{50} value was calculated from each set of triplicate wells using nonlinear regression modeling of the exponential form of the linearized equation (Statview, SAS Institute, Inc., Cary, NC). Hydrolysis rates were determined by fitting single exponential decay equations to the disappearance of the compound in alkali conditions (all R^2 values greater than 0.85). The IC_{50} values obtained were correlated to the log transformed hydrolysis rate constants by fitting a linear model (JMP Software, SAS Institute Inc., Cary, NC). P-values less than 0.05 were deemed significant.

Identification of compound 113 and compound 609 as dual function anti-HIV agents with potent and selective antiviral activity against ADV. The antiviral activity of stavudine and 13 aryl phosphoramidate derivatives of stavudine against human adenovirus (ADV strain Type 5) were first examined. Stavudine inhibited the cytopathic effects of ADV with an IC_{50} value of 12.3±0.3 μM. All 13 derivatives of stavudine were substantially more potent than stavudine and inhibited ADV-induced plaque formation with nanomolar IC_{50} values (Table 8).

Compounds with halo substitutions in the phenyl ring as well as the unsubstituted compound 607 were more potent than compounds with methoxy, methyl, or cyano substitutions. Compound 113 (d4T-5'-[p-bromophenyl methoxyalaninyl phosphate]) with a 4-Br substitution and compound 609 with a 4-Cl substitution were identified as the most potent lead anti-ADV agents. Compound 113 inhibited ADV-induced plaque formation in skin fibroblasts in a concentration-dependent fashion, with a mean (±SEM) IC_{50} value of 0.022 ± 0.009 μM without any evidence of cytotoxicity even at 100 μM (Table 8). Similarly, compound 609 inhibited ADV-induced plaque formation with an IC_{50} value of 0.0027±0.003 μM (Table 8).

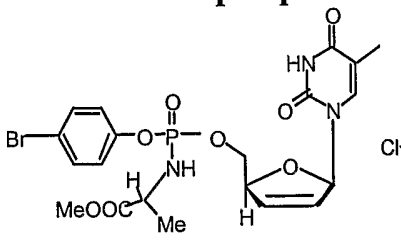
The anti-ADV potency of the aryl phosphate derivatives of stavudine could not be predicted from the lipophilicity, solubility, or alkaline hydrolysis rates for each compound. None of lipophilicity (P=0.08), solubility (P=0.16), or alkaline

hydrolysis rates (P=0.42) of the aryl phosphate derivatives of stavudine could predict their biologic activity against ADV. Compounds with similar or identical partition coefficients, solubility, or hydrolysis rates exhibited a wide range of IC_{50} values (Table 8).

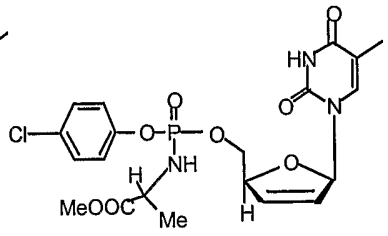
The lead compounds 113 and 609 inhibited ADV and HIV but did not inhibit several other viruses. Both compounds exhibited potent anti-HIV activity, but neither compound exhibited any antiviral activity against Type I or Type II herpes simplex viruses (HSV-1, HSV-2), enterovirus ECHO 30, or respiratory syncytial virus (RSV) (IC_{50}>100 μM) (Table 9).

Unlike other nucleoside analogs with anti-ADV activity, such as ribavarin or HPMC, compound 113 and compound 609 were selectively active against adenovirus only, which suggests a higher susceptibility of adenovirus DNA polymerase to these nucleoside analogs. A selective antiviral activity is not unprecedented for nucleoside antiviral drugs. For example, a number of novel 5-substituted 2'deoxypyrimidine nucleosides exhibited antiviral activity against herpes simplex virus type 1 and type 2 strains V3, but not against adenovirus (Reefschlager et al., 1982 *Antiviral Res.*, 2:41-52). However, compound 113 and compound 609 are the first nucleoside analogs to be identified as dual-function anti-HIV agents with selective anti-ADV activity.

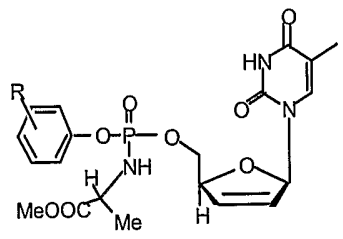
Table 8
Physicochemical properties, hydrolysis rate, and biological activity of phosphoramidate derivatives of Stavudine (d4T)



113



609



Compound #	Substituents (R)	σ-value	Oct/Water (log P)	Solubility (mg/ml)	Hydrolysis Rate (min^{-1})	IC_{50} (μM) (μM ± s.e.)
113	p-Br	0.26	1.21	3.0	0.0210	0.0223 ± 0.009
598	p-OMe	-0.28	0.39	11.9	0.0102	0.5550 ± 0.115
599	m-NMe_2	-0.10	0.76	18.0	0.0058	0.2300 ± 0.020
600	o-diOMe	-0.56	0.40	43.6	0.0029	0.2900 ± 0.120
601	p-CN	0.70	0.05*	4.0	0.1199	0.5750 ± 0.005
602	m-Br	0.37	1.12	5.7	0.0338	0.2350 ± 0.145
603	p-Br,o-Cl	0.50	1.81	1.8	0.1500	0.0800 ± 0.010
604	p-F	0.15	0.54	7.5	0.0117	0.1000 ± 0.040
605	o-Br	0.26	0.95	4.2	0.0336	0.0385 ± 0.022
606	o-Cl	0.24	0.84	7.3	0.0370	0.0285 ± 0.012
607	H	0.00	0.38	44.7	0.0082	0.0575 ± 0.023
608	o,m-diCl	0.61	1.41	3.7	0.1840	0.0330 ± 0.017
609	p-Cl	0.24	0.64	1.4	0.0216	0.0267 ± 0.003
	Stavudine		-0.59	83.0		12.3 ± 0.3

* - The value from the 4-CN substituent was not included in the regression analysis, as clear separation was not obtained between octanol and water.

Table 9

Antiviral Activity Profiles of Compound 113 and Compound 609

Virus	IC_{50}, μM Compound 113	Compound 609	STV
HIV-1, HTLV-IIIB	0.001±0.000	0.001±0.000	0.023±0.008
CMV, Strain AD169	>100	>100	>100
HSV-1, Strain HF	>100	>100	>100
HSV-2, Strain G	>100	>100	>100
Adenovirus, Type 5	0.022±0.009	0.027±0.003	12.3±0.3
Enterovirus, ECHO 30	>100	>100	>100
RSV, Strain Long	>100	>100	>100

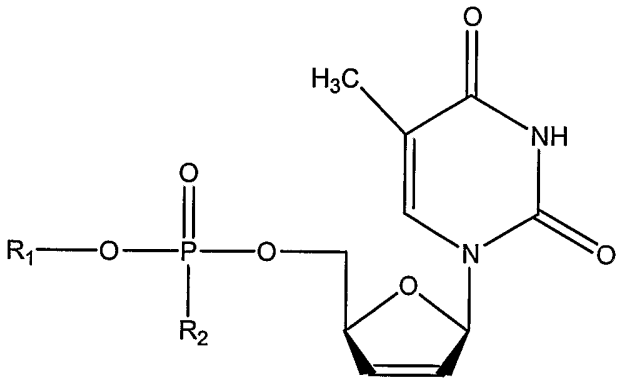
Plaque assays were used to examine the activity of compound 113 and compound 609 against non-HIV viruses. The skin fibroblast cell line SF (ATCC CRL-2097) was used as a target for AD169, Adenovirus Type 5, and ECHO 30; the VERO cell line (ATCC CCL-81) was used as a target for HF and G; and the HEP-2 cell line (ATCC CCL-23) was used as target for the RSV strain Long. Results are expressed as the average IC_{50} values in μM. Stavudine (STV) was included as a control.

While a detailed description of the present invention has been provided above, the invention is not limited thereto. The invention described herein can be modified to include alternative embodiments, as will be apparent to those skilled in the art. All such alternatives should be considered within the spirit and scope of the invention, as claimed below.

The specification includes numerous citations to literature and patent references, each which is hereby incorporated by reference as if fully set forth, for all purposes.

WE CLAIM:

1. Use of a compound or a pharmaceutically acceptable salt of formula I for the preparation of a medicament for inhibiting the effects of infection by adenovirus in a cell, in vitro or in vivo



(I)

where R_1 is an aryl group substituted with an electron withdrawing group or H, and

R_2 is an amino acid residue or an ester of the amino acid residue.

2. The use of claim 1, where R_1 is an aryl group substituted with an electron withdrawing group.

3. The use of claim 2, wherein the aryl group is phenyl, naphthyl, or anthryl.

4. The use of claim 2, wherein the aryl group is phenyl.

5. The use of claim 2, wherein the electron-withdrawing group is a halo.

6. The use of claim 2, wherein R_1 is *para*-bromophenyl.

7. The use of claim 2, wherein R_1 is *para*-chlorophenyl.

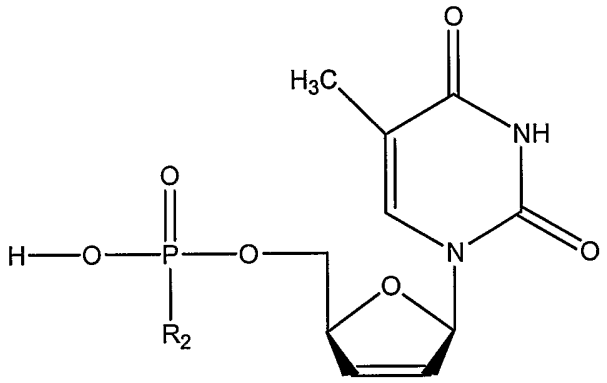
8. The use of claim 1, wherein R_2 is an α-amino acid or ester thereof.

9. The use of claim 1, wherein R_2 is -NHCH(CH_3)$COOCH_3$.

10. The use of claim 2, wherein R_1 is *para*-bromophenyl and R_2 is -NHCH(CH_3)$COOCH_3$.

11. The use of claim 2, wherein R_1 is *para*-chlorophenyl and R_2 is -NHCH(CH_3)$COOCH_3$.

12. The use of claim 1, wherein the compound is a compound of Formula IV:



(IV)

where R_2 is an amino acid residue or an ester of the amino acid residue.

13. The use of claim 1, wherein said medicament is administered to an animal.

14. The use of claim 13, wherein said medicament is administered at a dose of about 1 mg/kg body weight to about 500 mg/kg body weight.

15. The use of claim 14, wherein said medicament is administered at a dose of about 10 mg/kg body weight to about 100 mg/kg body weight.

16. The use of claim 13, wherein said inhibiting comprises reducing one or more symptoms of adenovirus infection.

17. The use of claim 13, wherein said inhibiting comprises preventing or delaying the onset of one or more symptom of adenovirus infection.

18. The use of claim 17, wherein said inhibiting comprises preventing or delaying of both HIV and adenovirus infection.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/18898

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 31/70
US CL : 514/48,49,50,51,52

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/48,49,50,51,52

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CAS ONLINE MEDLINE search term: structural search, adenovirus, HIV, AIDS, antiviral

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 00/00501 A1 (WAYNE HUGHES INSTITUTE) 06 January 2000 (06.01.2000), the entire document, particularly, the abstract, and the claims.	1-18
Y	WO 00/56750 A1 (PARKER HUGHES INSTITUTE) 28 September 2000 (28.09.2000), the entire document, especially, the claims and figures 1-2.	1-18
Y	WO 02/38576 A1 (PARKER HUGHES INSTITUTE) 16 May 2002 (16.05.2002), pages 4-5, the claims.	1-18

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

* Special categories of cited documents:

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