IN THE MATTER OF THE PATENTS ACT, 1970

and

IN THE MATTER OF THE PATENT RULES, 2003
(as amended by the Patents (Amendment) Rules 2006)

and

IN THE MATTER OF INDIAN PATENT APPLICATION NO. 3658/KOLNP/2009 FILED BY PHARMASSET, INC.

......... the Applicants

and

IN THE MATTER OF A REPRESENTATION BY WAY OF AN OPPOSITION UNDER SECTION 25(1) AND RULE 55 THERETO BY INDIAN PHARMACEUTICALS ALLIANCE

...... the Opponents
REPRESENTATION BY WAY OF OPPOSITION U/S 25(1)

1.0 It is respectfully submitted on behalf of Indian Pharmaceutical Alliance, that a pre-grant Opposition under Section 25(1) of the Patents Act, 1970 and rule 55(1) of the Patents Rules, 2003 (as amended by the Patents (Amendment) Rules 2006), is hereby presented by the “Opponents” against Indian Patent Application No. 3658/KOLNP/2009 (hereinafter also referred to as the “Opposed Application”) in the name of PHARMASSET, INC., (hereinafter referred to as the “Applicants”).

It is respectfully submitted:

2.0 The Opponents are associations of person registered under the SOCIETIES REGISTRATION ACT, XXI OF 1860 in the name and style of “INDIAN PHARMACEUTICAL ALLIANCE” having its registered office 115/11, GROUND FLOOR, WORLD TRADE CENTRE, BABAR ROAD, CONNAUGHT PLACE, NEW DELHI – 110001, the main object of as follows:-

(a) To support the development of international and regional policies, which seek to ensure, access to medical care for all customers.

(b) To promote balanced and generic friendly intellectual property rights in the pharmaceuticals sector to ensure that timely access to markets is guaranteed for new and generic pharmaceutical products.

(c) To promote the global harmonization relating to generic products.

(d) To support the right of all governments to regulate their own pricing, substitution, prescribing and reimbursement policies.

(e) To suggest measures for enhancing pharmaceutical research in India, both in the areas of basic as well as applied research.

(f) To interact with the environmental protection agencies to evolve uniform standards of environmental protection measures across the country and ensure implementation of the same.

(g) To suggest measures to strengthen the pharmaceutical pricing
frame work that ensures an equitable pricing system for industry and consumers.

(h) One of the further object of the society is to promote cause of generic pharmaceutical industry and to provide support for the development of competition on the off Patent pharmaceutical sector and to prepare position papers for representing India at international fora to highlight the problems faced by generic pharmaceutical companies in international market. It also aims at strengthening regulatory agencies for patenting registration and quality assurance of drugs and pharmaceuticals by providing guidance to government and international organization in improving the regulatory and legal expertise relating to registration and marketing of drugs and pharmaceutical. It also further aims at interacting with the regulatory authorities to streamline the guidelines for clinical trials and bio-equivalence studies, to ensure expeditious registration of new as well as existing drugs.

2.1 The opposed patent application is disclosed phosphoramidate prodrugs of nucleoside derivatives for the treatment of viral infections in mammals, which is a compound, its stereoisomer, salt (acid or basic addition salt), hydrate, solvate, or crystalline form thereof, represented by the following structure:

![Chemical Structure](image)

also disclosed are methods of treatment, uses, and processes for preparing each of which utilize the compound represented by formula I.

2.2 Although a representation of Opposition can be made by "any person", "in writing" under Section 25(1) of The Patents Act, 1970; however, the Opponents' interest in opposing this application is substantial and real. The Opponents, therefore, have locus standi in opposing this application.

2.3 It is respectfully submitted that the Opposed Application entitled "NUCLEOSIDE PHOSPHORAMIDATE PRODRUGS" has been filed on 20th

2.4 The Opponents are filing this Representation by way of Opposition against Indian Patent Application No. 3658/KOLNP/2009 (the Opposed Application), along with documentary evidence and facts in support thereof.

2.5 In this representation by way of opposition, the following grounds enumerated in Section 25 (1) of The Patents Act, 1970 are relied upon (hereinafter referred to as the “Act”):

(a) that the applicant for the patent or the person under or through whom he claims, wrongfully obtained the invention or any part thereof from him or from a person under or through whom he claims;

(b) that the invention so far as claimed in any claim of complete specification has been published before the priority date of the claim –

i) in any specification filed in pursuance of an application for a patent made in India on or after the 1st day of January, 1912; or

ii) in India or elsewhere, in any other document

Provided that the ground specified in sub-clause (ii) shall not be available where such publication does not constitute an anticipation of the invention by virtue of sub-section (2) or sub-section (3) of section 29;

(c) that the invention so far as claimed in any claim of the complete specification is claimed in a claim of a complete specification published on or after the priority date of the applicant’s claim and filed in pursuance of an application for a patent in India, being a claim of which the priority date is earlier than that of the applicant’s claim;

(d) that the invention so far as claimed in any claim of the complete specification was publicly known or publicly used in India before the priority date of the claim.

Explanation:- For the purpose of this clause, an invention relating to a process for which a patent is claimed shall be deemed to have been publicly known or publicly used in India before the priority date of the claim if a product made by that process had already been imported into India before that date expect where
such importation has been for the purpose of reasonable trial or experiment only;
(e) That the invention so far as claimed in any claim of the complete specification is obvious and clearly does not involve any inventive step having regard to the matter published as mentioned in clause (b) or having regard what was used in India before the priority date of the applicant’s claim;
(f) that the subject matter of any claim of the complete specification is not invention within the meaning of this Act, or is not patentable under this Act
(g) that the complete specification does not sufficiently and clearly describe the invention or the method by which it is to be performed;
(h) that the applicant has failed to disclose to the Controller the information required by section 8 or has furnished the information which in any material particular was false to his knowledge.
(i) that in the case of convention application, the application was not made within twelve months from the date of the first application for protection for the invention made in a convention country by the applicant or a person from whom he derives title;
(j) that the complete specification does not disclose or wrongly mentions the source or geographical origin of biological material used for the invention;
(k) That the invention so far as claimed in any claim of the complete specification is anticipated having regard to the knowledge, oral or otherwise, available within any local or indigenous community in India or elsewhere.

The present Representation By Way Of Opposition U/S 25(1) takes into consideration the following documents:

**Document 1 [D1]** – WO2005003147 (Published on 13th January, 2005)
**Document 2 [D2]** – WO2004002999 (Published on 8th January, 2004)
**Document 3 [D3]** – WO2005012327 (Published on 10th February, 2005)
**Document 5 [D5]** – Antiviral Research 27, 1-17, (1995)
**Document 7 [D7]** – Molecular Pharmacology, 56, 693–704 (1999)
**Document 8 [D8]** – Amended claims of opposed Patent Application
3. Preliminary Remarks

1. The opposed patent application at present is having 14 claims (amended claim as on 26th December 2011 as obtained from the IPO website). Claims 1 and 8 are independent claims and claims 7 and 14 are product by process claim.

2. The opposed patent application pertains to certain nucleoside phosphoramidates specifically the compound known as Sofosbuvir and one of its stereoisomers and their use as inhibitors of HCV NS5B polymerase, as inhibitors of HCV replication and for the treatment of hepatitis C infection in mammals.

Structure of Sofosbuvir:

![Structure of Sofosbuvir](image)

3. The patent application acknowledges that

3.1 The inhibitors of HCV NS5B as potential therapies for HCV infection have been known.

3.2 Nucleoside inhibitors of NS5B polymerase can act either as a non-natural substrate that results in chain termination or as a competitive inhibitor which competes with the nucleotide binding to the polymerase. To function as a chain terminator the nucleoside analogue must be taken up by the cell and converted \textit{in vivo} to a triphosphate to complete for the polymerase nucleotide binding site have been known.

3.3 The biological activity of a nucleoside is hampered by its poor substrate characteristics for one or more of the kinases needed to convert it to the active triphosphate form. Formation of the monophosphate by nucleoside kinase is generally viewed as the rate limiting step of the three phosphorylation events. To circumvent the need for the initial phosphorylation step in metabolism of a nucleoside to the active
triphosphate analogue, the preparation of stable phosphate pro-drug has been known.

3.4 Nucleoside phosphoramidate pro-drugs have been shown to be precursors of the active nucleoside triphosphates and are known to inhibit viral replication when administered to viral infected whole cells.

3.5 Nucleosides have some limitation for use as a therapeutic agent sometimes due to their poor physicochemical and pharmacokinetic properties. These poor properties can limit the intestinal absorption of an agent and limit uptake into the target tissue or cell. To improve their properties pro-drugs of nucleosides have been employed. It has been known that the preparation of nucleoside phosphoramidates improves the systemic absorption of the nucleoside and furthermore, the phosphoramidate moiety of these pro nucleotides is masked with neutral lipophilic group to obtain suitable partition coefficient to optimize uptake and transport into the cell, dramatically enhancing the intracellular concentration of the nucleoside monophosphate analogue relative to administering the parent nucleoside alone.

2. Independent Compound Claim 1

1. (S)-2-[(2R,3R,4R,5R)-S-(2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl-methoxy]-phenoxyphosphorylamino]-propionic acid isopropyl ester or a stereoisomer thereof.

3. Claims dependent on Claim 1

1. Claim 2. A composition comprising the compound or a stereoisomer thereof as claimed in claim 1 and a pharmaceutically acceptable medium.

2. Claim 3. A composition for treating a hepatitis C virus, which comprises an effective amount of the compound or a stereoisomer thereof as claimed in claim 1 and a pharmaceutically acceptable medium.

3. Claim 4. A method of treating a subject infected by a virus, which comprises: administering to the subject an effective amount of the compound or a stereoisomer thereof as claimed in claim 1; wherein the virus is selected from among hepatitis C virus, West Nile virus, a yellow fever virus, a dengue virus, a rhinovirus, a polio virus, a hepatitis A virus, a bovine viral diarrhea virus, and a Japanese encephalitis virus.
4. **Claim 5.** A method of treating a hepatitis C virus infection in a subject in need thereof, which comprises: administering to the subject an effective amount of the compound or a stereoisomer thereof as claimed in claim 1.

5. **Claim 6.** A process for preparing the compound or a stereoisomer thereof as claimed in claim 1, said process comprising:
   - reacting a compound 4" with a nucleoside analog 5'

4. **Product by process Claim**

1. **Claim 7.** A product comprising the compound or a stereoisomer thereof as claimed in claim 1 obtained by a process comprising:
   - reacting a compound 4" with a nucleoside analog 5'

   ![Chemical Structure](image)

   Wherein X is a leaving group.

5. **Independent Compound Claim 8**

1. **Claim 8.** (S)-isopropyl2-(((S)-(((2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydro pyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl)methoxy (phenoxy)phosphoryl)amino)propanoate. (The diastereomer of Claim 1)

6. **Claims dependent on Claim 8**

1. **Claim 9.** A composition comprising the compound as claimed in claim 8 and a pharmaceutically acceptable medium.

2. **Claim 10.** A composition for treating a hepatitis C virus, which comprises an effective amount of the compound as claimed in claim 8 and a pharmaceutically acceptable medium.

3. **Claim 11.** A method of treating a subject infected by a virus, which comprises: administering to the subject an effective amount of the compound as claimed in claim 8; wherein the virus is selected from among hepatitis C virus, West Nile virus, a yellow fever virus, a dengue virus, a rhinovirus, a polio virus, a hepatitis A virus, a bovine viral diarrhea virus, and a Japanese encephalitis virus.
4. Claim 12. A method of treating a hepatitis C virus infection in a subject in need thereof, which comprises: administering to the subject an effective amount of the compound or a stereoisomer thereof as claimed in claim 8.

5. Claim 13. A process for preparing the compound as claimed in claim 8, said process comprising:
   reacting a compound 4" with a nucleoside analog 5'

7. Product by process Claim

1. Claim 14. A product comprising the compound as claimed in claim 8 obtained by a process comprising:
   reacting a compound 4 with a nucleoside analog 5'
   Wherein X is a leaving group.

It is Opponents humble submission that none of the claims contained in the INDIAN PATENT APPLICATION No. 3658/KOLNP/2009 is novel or inventive or is otherwise patentable under the Act. The Opposed Application concerns a "nucleoside phosphoramidates and their use as inhibitor of HCV NS5B polymerase, as inhibitor of HCV replication and for the treatment of hepatitis C infection in mammals". The Opposed Application does not fulfill the patentability criteria under the Act. The subject matters of the claims lack novelty and inventive step over the prior art. The Opposed Application also does not sufficiently and clearly describes the alleged invention for it to be carried out by a person skilled in the art. The disclosure also falls within those, which are not patentable under the Act. These submissions will be elaborated while dealing with the specific grounds of opposition here under.
4.0 ANTICIPATION BY PRIOR PUBLICATION – SECTION 25 (1) (b)

4.1 Claim 1 and 8 lacks Novelty vis-à-vis WO 200503147 (D1 to Pharmasset)

The alleged application is not novel in the light of WO200503147(D1) which was published on 13th January 2005 (prior publication, earlier than the priority date of the opposed application) and is therefore citable under Section 25(1)(b) of the Act. Opponent would like to draw the attention of Examiner/Controller that:

D1 is an international application of Pharmasset and discloses 2'(R)-2'-deoxy-2'-fluoro-2'-C-methyl nucleoside (β-D or β-L), or their pharmaceutically salts or pro-drug thereof, and the use of such compounds for the treatment of a host infected with a virus belonging to the flaviviridae family, including HCV. Moreover the patent application also discloses that the 2'substitutions of β-D or β-L nucleosides of the invention or their pharmaceutically acceptable salts or prodrugs impart greater specificity for HCV and include a method for treating various viruses included HCV (See page 16 of D1).

More specifically, Document D1 in its seventh embodiment disclosed a chemical compound having formula as below including its monophosphate, diphosphate, triphosphate or a stabilised phosphate pro-drug: (see page 39 of D1)

A seventh embodiment provides a (Z')-2'-deoxy-2'-fluoro-2'-C-methyl nucleoside or its pharmaceutically acceptable salt or prodrug thereof of the structure:

\[
\text{Base} \quad R^1 O \quad R^2 \quad R^3 \quad \text{CH}_3 \quad R^4 O \quad R^7 \quad X \quad \text{R'}
\]

wherein Base is selected from

(a) and (b)

and wherein X, Y, R', R^1, R^3, R^4, R^5, R^7 and R' are as defined above.
wherein Base refers to a naturally occurring or modified purine or pyrimidine base; X is O, S, CH₂, Se, NH, N-alkyl, CHW, C(W)₂, wherein W is F, Cl, Br, or I;

R¹ and R⁷ are independently H, phosphate, including monophosphate, diphosphate, triphosphate, or a stabilized phosphate prodrug, H-phosphonate, including stabilized H-phosphonates, acyl, including optionally substituted phenyl and lower acyl, alkyl, including lower alkyl, O-substituted carboxyalkylamino or its peptide derivatives, sulfonate ester, including alkyl or arylalkyl sulfonyl, including methanesulfonyl and benzyl, wherein the phenyl group is optionally substituted, a lipid, including a phospholipid, an L or D-amino acid, a carbohydrate, a peptide, a cholesterol, or other pharmaceutically acceptable leaving group which when administered in vivo is capable of providing a compound wherein R¹ is H or phosphate; R² is OH or phosphate; R¹ and R² or R⁷ can also be linked with cyclic phosphate group; and

Turning now to the opposed patent application, it discloses and claims (S)-2-([(2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl-methoxy]-phenoxy-phosphoryl)amino}propionic acid isopropyl ester (claim 1) and its stereoisomer in claim 8. Accordingly, the compounds of claims 1 and 8 of the opposed application is directly disclosed in document D1, if the definition of X, Y, R¹, R³, R⁴, R⁷ and base given in D1 are considered as is provided below:

'Wherein Base is;
R³ and R⁴ are independently H, OH;
R⁷ is H;
X is O
R¹ is a Stabilized phosphate Pro-drug;
Definition of Stabilized phosphate Prodrug is given in page 31, line 7-22. The patent document gives the definition: "The term "pharmaceutically acceptable salt or prodrug" is used throughout the specification to describe any acceptable form (such as an ester, phosphate ester, salt of an ester or a related group) of a compound which upon administration to a patient provides the active compound. Typical examples of prodrugs include compounds that have biologically labile protecting groups on a functional moiety of the active compound. Prodrugs include compounds that can be oxidized, reduced, aminated, deaminated, hydroxylated, dehydroxylated, hydrolysed, dehydrolyzed, alkylated, dealkylated, acylated, deacylated, phosphorylated, dephosphorylated to produce the active compound.
Pages 42-43 of D1 also provides that the invention contemplates and includes the 5'-triphosphate tri phosphoric acid ester derivatives of a nucleoside compound and pharmaceutically acceptable salts of the tri phosphate ester, as well as pharmaceutically acceptable salts of 5'disphosphate and 5'monophosphate ester derivatives of the compounds claimed.
Pages 46 of D1 also disclose that 'any of the nucleosides described herein, or any other nucleoside that has anti-hepatitis activity, can be administered as a nucleotide prodrug to increase the activity, bioavailability, stability or otherwise alter the properties of the nucleoside. A number of nucleotide prodrug ligands are known. A nucleotide prodrug, as described herein, refers to a nucleoside that has a phosphate derivative on the 5'-position that is more stable in vivo than the
parent phosphate, and which does not materially adversely affect the anti-hepatitis C activity of the nucleoside.

Moreover, the definition of stereoisomerism given in page 51 of D1 also covers the diastereomer as claimed in claim 8.

Hence, the disclosure & claim to a compound according to claims 1 and 8 of the opposed patent application is found in the document D1.

This means that (S)-2-[(2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl-methoxy]-phenoxy-phosphorylamino]-propionic acid isopropyl ester and diastereomers of claim 1 are anticipated by D1. Hence (S)-2-[(2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl-methoxy]-phenoxy-phosphorylamino]-propionic acid isopropyl ester and its diastereomer as claim in claims 1 & 8 are not novel and is clearly anticipated by D1.

4.2 Claim 1 lacks Novelty vis-à-vis WO 2004/002999 (D2 to Idenix Pharmaceutical Inc.)

The alleged application is not novel in the light of WO 2004/002999 (D2) which was published on 8 Jan 2004 and claiming priority of 28 June 2002 (prior publication, earlier than the priority date of the opposed application) and is therefore citable under Section 25(1)(b) of the Act. The Opponent would like to draw the attention of Examiner/ Controller that D2 is an international application of Idenix Pharmaceutical Inc and discloses modified 2' and 3' nucleotide prodrug for treating Flaviviridae infections.

More specifically, Document D2 discloses a chemical compound having formula IV (pl. see page 19 of D2):

![Formula IV](image)

R\(^1\) is selected from phosphate (including a stabilized phosphate prodrug);

R\(^2\) is H.
$X$ is -O-

$R^6$ is methyl (-CH$_3$); $R^7$ is Fluoro (-F);

Base is of the formula (F) (see page 20 (F))

![](image)

$W^1$ is N;

$W^4$ is CH;

$X^2$ is H;

$Y^1$ is OH;

Definition of Stabilized phosphate prodrug is given in Page 107 of D2.

Nucleotide Prodrug Formulations:

The nucleosides described herein can be administered as a nucleotide prodrug to increase the activity, bioavailability, stability or otherwise alter the properties of the nucleoside. A number of nucleotide prodrug ligands are known. In general, alkylation, acylation or other lipophilic modification of the mono-, di-or triphosphate of the nucleoside reduces polarity and allows passage into cells. Examples of substituent groups that can replace one or more hydrogens on the phosphate moiety are alkyl, aryl, steroids, carbohydrates, including sugars, 1,2-diacylglycerol and alcohols.

Many are described in R. Jones and N. Bischoferger, Antiviral Research, 1995, 27: 1-17. Any of these can be used in combination with the disclosed nucleosides to achieve a desired effect.

As a result, (S)-2-[(2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl-methoxy]-phenoxy-phosphorylamino]-propionic acid isopropyl ester according to opposed claim 1 is disclosed in the embodiments of the document D2, which therefore anticipates the claim 1 of the opposed patent application. Therefore, the Opponents contend that claim 1 is anticipated by D2.
Claim 1 lacks Novelty vis-à-vis WO2005012327 (D3 to University College Cardiff Consultant Limited)

The alleged application is not novel in the light of WO2005012327 (D3) which was published on 10 Feb 2005 (prior publication, earlier than the priority date of the opposed application) and is therefore citable under Section 25(1)(b) of the Act. The Opponents would like to draw the attention of Examiner/Controller that-

D3 is an international application of University College Cardiff Consultant Limited and discloses certain phosphoramidate compounds.

More specifically, Document D3 discloses a chemical compound having formula 1:

Wherein \( R \) is selected from the group comprising alkyl, aryl and alkylaryl;
\( R' \) and \( R'' \) are independently selected from the group comprising \( H \), alkyl and alkylaryl, or \( R' \) and \( R'' \) together form an alkylene chain so as to provide, together with the \( C \) atom to which they are attached, a cyclic system;
\( Q \) is selected from the group comprising \(-O-\) and \(-CH_2-\);
\( X \) and \( Y \) are independently selected from the group comprising \( H \), \( F \), \( Cl \), \( Br \), \( I \), \( OH \) and methyl (\(-CH_3\));
\( Ar \) is a monocyclic aromatic ring moiety or a fused bicyclic aromatic ring moiety, either of which said ring moieties is carbocyclic or heterocyclic and is optionally substituted;
\( Z \) is selected from the group comprising \( H \), alkyl and halogen; and \( n \) is 0 or 1, wherein when \( n \) is 0, \( Z'\) is \( -NH_2 \) and a double bond exists between position 3 and position 4, and when \( n \) is 1, \( Z'\) is \( =O \); or a pharmaceutically acceptable derivative.
or metabolite of a compound of formula I; with the proviso that, except where R is 2-Bu (\(-\text{CH}_2\text{-CH}(\text{CH}_3)\)) and one of \(R'\) and \(R''\) is H and one of \(R'\) and \(R''\) is methyl (\(-\text{CH}_3\)), when \(n\) is 1 and \(X\) and \(Y\) are both H, then \(Ar\) is not unsubstituted phenyl (\(-\text{C}_6\text{H}_5\)).

Turning now to the opposed patent application, it discloses and claims (S)-2-{[(2R,3R,4R,SR)-5-(2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl-methoxy]-phenoxy-phosphorylamino}-propionic acid isopropyl ester. Accordingly, the compound of claim 1 of the opposed application is directly disclosed in document D3, when the definition of \(R\), \(R'\), \(R''\), \(Q\), \(X\), \(Y\), \(Ar\) and \(Z\) given in D3 is as per below:

- \(R\) is selected from the group comprising alkyl;
- \(R'\) and \(R''\) are independently selected from the group comprising H, alkyl;
- \(Q\) is selected from the group comprising \(-\text{O}\)-;
- \(X\) and \(Y\) are independently selected from the group comprising F and methyl (\(-\text{CH}_3\));
- \(Ar\) is a monocyclic aromatic ring moiety;
- \(Z\) is selected from when \(n\) is 1, \(Z'\) is=\(O\);

Hence, the disclosure of a compound according to claim 1 of the opposed patent application is found in claim 1 of the document D3.

This means that (S)-2-{[(2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl-methoxy]-phenoxy-phosphorylamino}-propionic acid isopropyl ester is anticipated by D3. Hence, the claim to (S)-2-{[(2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl-methoxy]-phenoxy-phosphorylamino}-propionic acid isopropyl ester in claims 1 and its diastereomer claim in claim 8 are not novel and is anticipated by D3.

### 4.4  Dependent Claim 2 and 3 lacks Novelty vis-à-vis WO20050000 (D1 to Pharmasset)

1. **Claim 2.** A composition comprising the compound or a stereoisomer thereof as claimed in claim 1 and a pharmaceutically acceptable medium.
2. **Claim 3.** A composition for treating a hepatitis C virus, which comprises an effective amount of the compound or a stereoisomer thereof as claimed in claim 1 and a pharmaceutically acceptable medium.
3. **Claim 9.** A composition comprising the compound as claimed in claim 8 and a pharmaceutically acceptable medium.

4. **Claim 10.** A composition for treating a hepatitis C virus, which comprises an effective amount of the compound as claimed in claim 8 and a pharmaceutically acceptable medium.

Claims 2, 3, 9 and 10 of opposed patent application which are for pharmaceutical composition of compound of claim 1 and claim 8 with a pharmaceutical acceptable medium is anticipated by D1.

**Claim 21 of Document D1**

21. A pharmaceutical composition comprising a (2'R)-2'-deoxy-2'-fluoro-2'-C-methyl nucleoside (β-D or β-L) or its pharmaceutically acceptable salt or prodrug thereof, in a pharmaceutically acceptable carrier, of the structure:

![Chemical Structure]

wherein Base is a purine or pyrimidine base;

X is O, S, CH₂, Se, NH, N-alkyl, CH₃ (R, S, or racemic), C(W)₂;

wherein W is F, Cl, Br, or I; and,

R¹ and R⁷ are independently H, phosphate, including monophosphate, diphosphate, triphosphate, or a stabilized phosphate prodrug, H-phosphonate, including stabilized H-phosphonates, acyl, including optionally substituted phenyl and lower acyl, alkyl, including lower alkyl, O-substituted carboxyalkylamino or its peptide derivatives, sulfonate ester, including alkyl or arylalkyl sulfonyl, including methanesulfonyl and benzyl, wherein the phenyl group is optionally substituted, a lipid, including a phospholipid, an L or D-amino acid, a carbohydrate, a peptide, a cholesterol, or other pharmaceutically acceptable leaving group which when administered in vivo is capable of providing a compound wherein R¹ or R⁷ is independently H or phosphate; R¹ and R⁷ can also be linked with cyclic phosphate group.

As the discussion above already demonstrates the disclosure and claims made in D1 include the structure of the base compound corresponding to the pro-drug claimed in claims 1 & 8. D1 also teaches the phosphoramide-stabilized pro-
drug of claims 1 & 8. Additionally claim 21 disclosed pharmaceutical composition of the same compound, which are claimed in claims 2, 3, 9 and 10 of the opposed application. Hence, claims 2, 3, 9 and 10 are anticipated by D1.

Dependent Claims 4, 5, 11 and 12 lacks Novelty vis-à-vis WO 2004/002999 (D2 to Idenix Pharmaceutical Inc.)

Claims 4, 5, 11 and 12: Drawn to a method of treating by using the compound of claims 1 or 8.

1. Claim 4. A method of treating a subject infected by a virus, which comprises: administering to the subject an effective amount of the compound or a stereoisomer thereof as claimed in claim 1; wherein the virus is selected from among hepatitis C virus, West Nile virus, a yellow fever virus, a dengue virus, a rhinovirus, a polio virus, a hepatitis A virus, a bovine viral diarrhea virus, and a Japanese encephalitis virus.

2. Claim 5. A method of treating a hepatitis C virus infection in a subject in need thereof, which comprises: administering to the subject an effective amount of the compound or a stereoisomer thereof as claimed in claim 1.

3. Claim 11. A method of treating a subject infected by a virus, which comprises: administering to the subject an effective amount of the compound as claimed in claim 8; wherein the virus is selected from among hepatitis C virus, West Nile virus, a yellow fever virus, a dengue virus, a rhinovirus, a polio virus, a hepatitis A virus, a bovine viral diarrhea virus, and a Japanese encephalitis virus.

4. Claim 12. A method of treating a hepatitis C virus infection in a subject in need thereof, which comprises: administering to the subject an effective amount of the compound or a stereoisomer thereof as claimed in claim 8.

Claims 4, 5, 11 and 12 of alleged invention is for method of treating a hepatitis C or certain other virus infections in a subject comprising the step of administering to the subject an effective amount of the compound or a stereoisomer thereof as claimed in claim 1 is anticipated by D2. The document D2 claim 12 "A method of treatment of a host infected with a Flaviviridar virus, comprising administering an effective treatment amount of a compound
as claimed in any one of claims 1-11, or pharmaceutically acceptable salt thereof.”

Claim 13 “The method of claim 12, wherein the virus is hepatitis C.”

Claim 1-11 of the document D2 generically claims certain compounds and their pharmaceutically acceptable derivatives which discloses the compound (S)-2-([(2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl-methoxy]-phenoxy-phosphorylamino)-propionic acid isopropyl ester (as discussed earlier). Since the method of treating a hepatitis C virus infection using compound of claim 1-11 is disclosed sufficiently in D2 and claimed in claim 12 & 13 of D2, Claims 4, 5, 11 and 12 lacks novelty over D2. Therefore, claims 4, 5, 11 and 12 are anticipated by D2.

4.5 Dependent Claims 6, 13 and product by process claims 7, 14 lacks novelty vis-à-vis WO2005012327 (D3 to University College Cardiff Consultant Limited)

1. Claim 6. A process for preparing the compound or a stereoisomer thereof as claimed in claim 1, said process comprising:
   reacting a compound 4" with a nucleoside analog 5'

2. Claim 7. A product comprising the compound or a stereoisomer thereof as claimed in claim 1 obtained by a process comprising:
   reacting a compound 4" with a nucleoside analog 5"
Wherein X is a leaving group.

3. **Claim 13.** A process for preparing the compound as claimed in claim 8, said process comprising:
   reacting a compound 4" with a nucleoside analog 5'

   ![Chemical Structure 4']

4. **Claim 14.** A product comprising the compound as claimed in claim 8 obtained by a process comprising:
   reacting a compound 4 with a nucleoside analog 5'

   ![Chemical Structure 4']

Wherein X is a leaving group.

**Claim 26 of Document D3**

26. A process for the preparation of a compound of formula I according to claim 1, the process comprising reacting of a compound of formula III with compound of formula IV;

   ![Chemical Structure III]
The compound of claim 1 of the opposed application is directly disclosed in document D3, when the definition of R, R', R", Q, X, Y, Ar and Z given in D3 is as per below:
- R is selected from the group comprising alkyl;
- R' and R" are independently selected from the group comprising H, alkyl;
- Q is selected from the group comprising -O-;
- X and Y are independently selected from the group comprising F and methyl (-CH3);
- Aryl is a monocyclic aromatic ring moiety;
- Z is H when n is 1, Z is=O;

Hence, the disclosure of a compound according to claim 1 and 8 of the opposed patent application is found in claim 1 of the document D3. And also the process for the preparation of compound 1 and 8 as claimed in claims 6, 13 and 7, 14 of the opposed patent application is anticipated by claim 26 of the D3. Therefore, claims 6, 7, 13 & 14 are not novel over D3.

5.0 OBVIOUSNESS AND LACK OF INVENTIVE STEP – SECTION 25 (I)

According to Indian patent Act, Section 2 (1a), "inventive step" means a feature of an invention that involves technical advance as compared to the existing knowledge or having economic significance or both and that makes the invention not obvious to a person skilled in the art;

Without prejudice to what has been submitted in the paragraphs above, it is respectfully submitted that the alleged invention as described and claimed in the opposed specification lacks in inventiveness and is obvious to a person skilled in the art. In other words, even if it is assumed without admitting that the
alleged invention is novel, it is still obvious and lacks in inventive step in view of the teachings contained in the following documents:

As the discussion above already demonstrates the disclosure and claims made in D1 include the structure of the base compound corresponding to the pro-drug claimed in claims 1 & 8. D1 also teaches the phosphoramidate-stabilized pro-drug of claims 1 & 8.

Even if it is considered for the sake of argument that D1 does not teach the specific compounds claimed in claims 1 & 8 of the opposed patent application, one has to determine whether making of such a pro-drug from those known in the art involves an inventive step or not. Nucleoside compounds and their derivatives, in particular uridine derivatives, have a long history of use for antiviral drugs. The base compound (The Base, below) corresponding to the compounds claimed in claims 1 & 8 and its anti HCV activity is well known before the filing date of the opposed application.

![The Base](image)

A numbers of pharmaceutical drugs are available in the market in its prodrug form. The opposed patent itself acknowledge that the biological activity of a nucleoside is hampered by its poor substrate characteristics for one or more of the kinases needed to convert it to the active triphosphate form. Formation of the monophosphate by nucleoside kinase is generally viewed as the rate limiting step of the three phosphorylation events. To circumvent the need for the initial phosphorylation step in metabolism of a nucleoside to the active triphosphate analogue, the preparation of stable phosphate drugs has been known. Nucleoside phosphoramidate prodrug has been shown to be precursors of the active nucleoside triphosphate and to inhibit viral replication when administered to viral infected whole cells have been known.

The opposed patent also acknowledge that nucleosides have some limitations for use as a therapeutic agent sometimes due to their poor physicochemical and pharmacokinetic properties. These poor properties can limit the intestinal absorption of an agent and limit uptake into the target tissue or cell. To
improve on their properties prodrug of nucleoside has been employed. It
has been known that the preparation of nucleoside phosphoramidates improves
the systemic absorption of nucleoside and furthermore, the phosphoramidates
moiety of these pro nucleotides is masked with neutral lipophilic group to
obtain suitable partition coefficient to optimize uptake and transport into the cell
dramatically enhancing the intracellular concentration of the nucleoside
monophosphate analogue relative to administering the parent nucleoside alone.

5.1 Claim 1 and 8 are obvious over D1 and general art D4

Claim 1 of the opposed patent application claims (S)-2-\{(2R,3R,4R,5R)-5-(2,4-
dioxo-3,4-dihydro-2H-pyrimidin-1-yl)-4-fluoro-3-hydroxy-4-
methyltetrahydrofuran-2-yl-methoxy]-phenoxy-phosphorylamino\}-propionic
acid isopropyl ester or a stereoisomer thereof and claim 8 is to a diastereomer of
claim 1 and are obvious over the combination of D1 and D4 for the following
reasons.

As the discussion above already demonstrates that the disclosure and claims
made in D1 include the structure of the base compound (The Base) as well as
the phosphoramidate-stabilized prodrug.

Document D4 discloses how the use of an aryloxy phosphoramidate ProTide
pro-drug approach for a ribonucleoside 4’-azidouridinederivative (a uridine
based nucleoside) was able to deliver themonophosphates to HCV replicon cells
and unleash the antiviral potential of the triphosphate in a manner that vastly
improved the antiviral activity over the parent compound. Moreover, the
ProTide prodrugs tested by the authors included the alanine isopropyl ester as
the phosphoramidate (Table 1, compound 15 on page 4), the same prodrug as
claimed in the opposed patent application.

Thus, the document D4 clearly teaches that the introduction of an aryloxy
phosphoramidate ProTide prodrug approach at R1 position of D1 would
increase the efficacy of the compounds of D1. Hence, the compounds of claims
1 & 8 of the opposed patent is clearly obvious over the teachings of D1 alone as
well as the combination of D1 and D4.
5.2 Claim 1 and 8 are obvious over D1 and general art D5

The document D5 discloses that the phosphoroamidate formed between AZT and alanine methyl ester was found to be active against HIV in a cell line restrictive to the activity of AZT due to poor phosphorylation. Because of their negative charge(s) nucleotides suffer from some disadvantages which can be successfully overcome by the utilization of nucleotide prodrugs. Nucleotide prodrugs were successfully used to increase oral absorption of the nucleotides in vivo.

As the base compound (The Base) in the opposed patent is a nucleotide it is expected that it can easily form a pro-drug. Moreover, one skilled in the art, starting from the base compound of D1, if he wishes to make a more bioavailable (oral absorption of the nucleotides in vivo) drug and also overcome some of the drawbacks of the base compound (The Base), he will be motivated to prepare a pro-drug as taught in D5 and arrive at the present invention.

The present application is rendered obvious in light of D5: Antiviral research 1995 when read in combination of D1.

5.3 Claim 1 and 8 are obvious over D1 and general art D6

Document D6 disclose the success of using the ProTide prodrug strategy to activate the active triphosphates of an inactive HIV compound ddU. On page 13, column 2 of D6, the authors state how the aryloxyphosphoramidate (the prodrug used in opposed patent application) is a potent agent being 50 times more active than the parent nucleoside.

As the base compound (The Base) in the opposed patent is a nucleotide it is expected that it can easily form a pro-drug. Moreover, one skilled in the art, starting from the base compound of D1, wishes to make a more bioavailable drug and also overcome some of the drawbacks of the base compound (The Base), would be motivated to prepare a pro-drug as taught in D6 and arrive at the present invention. The teachings of D6 would motivate him to try an aryloxy phosphoramidate. There is a clear suggestion, motivation to prepare the aryloxyphosphoramidate pro-drug of the base compound as is disclosed in D1.

Thus, the compounds of the Opposed patent application which is an aryloxyphosphoramidate pro-drug of base compound (The base)
is rendered obvious from the teachings of D1, in combination with D6.

5.4 **Claim 1 and claim 8 are obvious over D1 and general art D7**

Document D7 discloses the activation pathway of a series of phosphoramidate prodrugs of d4TMP and AZTMP. The stability in human serum of the L-alaninyl-containing phosphoramidates of d4TMP proved to be highly dependent on the nature of the alkyl ester group and amino acid used. The document also state that superior antiviral activity is found with the L-alaninyl-containing phosphoramidate triesters.

Thus, the document D7 clearly teaches that the introduction of an L-alaninyl-containing phosphoramidate at R1 of the base compound (The Base) of D1 is expected to give superior antiviral activity as compared to the base compounds of D1. A person skilled in the art, when reading D1 in combination with D7, will be motivated to combine the L-alaninyl-containing phosphoramidates of D7 with the base compound of D1 and arrive at the compounds of the opposed application in an obvious way.

Since, all the documents referred to above specifically teaches the possibility of using the phosphoramidate type pro-drugs at R1 position of the base compound of D1 with expected benefits, the same may be quite easily inferred to by a person skilled in the art. Thus, the compounds of the opposed document which represents certain phosphoramidate pro-drugs are rendered obvious by the document D1 in combination with documents D4-D7.

From the above, it becomes evident that in absence of any superior technical effect, the subject-matter of the opposed claim 1 and 8 involves no inventive step. One could arrive at alleged application without the aid of any inventive genius but purely by routine skill hence the compounds claimed in the present application is clearly obvious and lacks in inventive step.

Furthermore the compounds of the alleged invention possess anti HCV properties (claim 3 and 10 of the Opposed application). The document D1 also
discloses "The present invention is directed to compounds which are useful in the treatment or prevention of diseases in which Nucleoside inhibitor of NS5B polymerase is involved, such as HCV. The invention is also directed to pharmaceutical compositions comprising these compounds and the use of these compounds and compositions in the prevention or treatment of such diseases in which Nucleoside inhibitor of NS5B polymerase is involved."

Therefore claimed compound possess the same anti HCV activity. Document D1 clearly state at several places that anti HCV properties are expected of the compound disclosed therein. Therefore the alleged invention is a blatant attempt to again claim compounds already disclosed in the prior art and create monopoly over these compounds, which are already in the public domain.

Therefore, from the above it is clear that the claimed compounds do not possess any surprising properties compared to the properties of the closest prior art. Hence, the opposed patent application directed to certain compounds of claims 1 and claim 8 and their use are obvious and does not involve inventive merit and therefore do not qualify as a patentable invention as per the provisions of the Indian Patent Act.

Additionally, the invention as claimed in the opposed application does not provide any comparative data with the closest prior art to overcome the problem of obviousness by showing either superior or surprising effects. Also the opposed application does not provide any technical advancement over the existing knowledge in the same field. Therefore, in the absence of any comparative data showing the compounds of claim 1 and claim 8 are better in some way to the known compounds of D1 (either the base compound or the corresponding phosphoramidate pro-drugs) there is no technical advancement over the prior art. As per the Indian Patent Act, patent cannot be granted for compounds in absence of clear proof of beneficial effects or technical improvements if the compounds are per se obvious.
6.0 THE COMPOUNDS OF THE OPPOSED APPLICATION DOES NOT CONSTITUTE AN INVENTION UNDER – SECTION 3(d) OF THE ACT

6.0.1 Derivatives of known substance

Even if it is assumed [without admitting] that the subject matter of the product claims of 1 & 8 and their dependent claims constituted an invention, they would still be un-patentable under Chapter II of the Act. In this connection, we would respectfully refer to Section 3[d] of the Act, which precludes from patentability ester, metabolite and derivative, combination of known substances etc., unless these substance show significant improvement in efficacy. It is to be noted that the compounds of claims 1 & 8 constitute certain phosphoramidate pro-drugs which are essentially phosphate esters. As discussed earlier, the Opposed application does not provide any superior beneficial properties over the closest prior art D1 is provided.

It is worth mentioning in this context that according to established jurisprudence of IPAB, that the efficacy for such derivatives can be only ascertained from therapeutic point of view such as how it binds a particular site in the body etc. In the absence of any superior beneficial effects of the compounds of claims 1 and 8 over the base compound (The BASE) as disclosed in D1, in term of therapeutic efficacy, the present application is not patentable under section 3 (d) of the Indian Patent Act.

The opposed application discloses a prodrug which is a derivative of the base form (THE Base) disclosed in D1 and the specification mentions that the said derivative avoids the known disadvantages of the nucleoside. However, such a beneficial effect cannot be considered to enhance the therapeutics efficacy of the base compound.

Hence the alleged application is not an invention under section 3 (d) of Indian Patent Act.

6.0.2 New use for a known substance

D3 is an international application of University College Cardiff Consultant Limited and discloses certain phosphoramidate compounds having antitumor activity.
More specifically, Document D3 discloses a chemical compound having formula I:

Wherein R is selected from the group comprising alkyl, aryl and alkylaryl;
R' and R'' are independently selected from the group comprising H, alkyl and alkylaryl, or R' and R'' together form an alkylene chain so as to provide, together with the C atom to which they are attached, a cyclic system;
Q is selected from the group comprising -O- and -CH2-;
X and Y are independently selected from the group comprising H, F, Cl, Br, I, OH and methyl (-CH3);
Ar is a monocyclic aromatic ring moiety or a fused bicyclic aromatic ring moiety, either of which said ring moieties is carbocyclic or heterocyclic and is optionally substituted;
Z is selected from the group comprising H, alkyl and halogen; and n is 0 or 1, wherein when n is 0, Z' is NH2 and a double bond exists between position 3 and position 4, and when n is 1, Z' is O;
or a pharmaceutically acceptable derivative or metabolite of a compound of formula I; with the proviso that, except where R is 2-Bu (-CH2-CH (CH3) 2) and one of R' and R'' is H and one of R' and R'' is methyl (-CH3), when n is 1 and X and Y are both H, then Ar is not un-substituted phenyl (-C6H5).

Turning now to the opposed patent application, it discloses and claims (S)-2-{

(S)-2-

Turning now to the opposed patent application, it discloses and claims (S)-2-

Turning now to the opposed patent application, it discloses and claims (S)-2-
R' and R" are independently selected from the group comprising H, alkyl;
Q is selected from the group comprising -O-;
X and Y are independently selected from the group comprising F and methyl (-CH₃);
Ar is a monocyclic aromatic ring moiety;
Z is selected from when n is 1, Z' is =O:

Hence, the disclosure of a compound according to claim 1 of the opposed patent application is found in claim 1 of the document D3.

The applicant of the opposed patent application have now tried to use the compounds disclosed in D3 (Compounds of claims 1 and 8 of the opposed patent application) for HCV. However this type of invention is not patentable under Sec-3(d) of Indian Patent Act because the compounds are already known for treating cancer like diseases and the opposed patent application essentially claims the same compounds for anti HCV activity. Hence the alleged application is not a patentable invention under section 3 (d) of Indian Patent Act.

6.1 NOT AN INVENTION OR PATENTABLE INVENTION – SECTION 3 (e)

According to section 3 (e) of the Indian Patent Act 1970: "A substance obtained by a mere admixture resulting only in the aggregation of the properties of the components thereof or a process for producing such substance’’;

The compounds of claims 1 and 8 are obtained by combining the disclosers in documents D1 and D6 i.e.by combining the base form (The Base) disclosed in D1 and an aryloxyphosphoramidates as is disclosed in document D6. We would like to bring to the attention of Examiner that all the features of the claimed compounds were disclosed and already taught in the state of the art documents D1 and D6. These documents suggest explicitly to a person skilled in the art about how and why to combine the base form of D1 with the aryloxyphosphoramidates to obtain the compounds of claim 1 and 8.

Hence compounds of claims 1and 8 of the alleged application constitutes mere admixture wherein the earlier known base form and the aryloxyphosphoramidates (already taught in D1 and D6 respectively). The Applicant has merely picked the component substances from above cited applications and created a combination thereof with knowledge about their
property, known in the art, through a known reaction that was expected to lead to the desired product. Therefore the alleged application is not patentable under section 3(e) of Indian Patent Act.

It is respectfully submitted that the claims of the opposed patent are therefore clearly hit by Section 3(d) and Section 3(e) of the Patents Act, 1970 and do not form a patentable invention under the Patents Act, 1970 as amended in 2005, and should not be granted on these grounds alone.

6.2 **NOT AN INVENTION OR PATENTABLE INVENTION – SECTION 3(i)**

According to Section 3(i) of the Indian Patent Act, "any process for medicinal, surgical, curative, prophylactic [diagnostic, therapeutic] or other treatment of human beings or any process for a similar treatment of animals to render them free of disease or to increase their economic value or that of their products;"

Claim 4 of the opposed patent application is “A method of treating a subject infected by a virus, which comprises: administering to the subject an effective amount of the compound or a stereoisomer thereof as claimed in claim 1; wherein the virus is selected from among hepatitis C virus, West Nile virus, a yellow fever virus, a dengue virus, a rhinovirus, a polio virus, a hepatitis A virus, a bovine viral diarrhea virus, and a Japanese encephalitis virus”.

Claim 5 of the opposed patent application is “A method of treating a hepatitis C virus infection in a subject in need thereof, which comprises: administering to the subject an effective amount of the compound or a stereoisomer thereof as claimed in claim 1”.

Both claims are not patentable under Section 3(i) of the Indian Patent Act, 1970 as amended by the Patents (Amendment) Act, 2005 as both the claims 4 and 5 claims the methods for treating an infection.

Claim 11 of the opposed patent application is “A method of treating a subject infected by a virus, which comprises: administering to the subject an effective amount of the compound as claimed in claim 8; wherein the virus is selected from among hepatitis C virus, West Nile virus, a yellow fever virus, a dengue virus, a rhinovirus, a polio virus, a hepatitis A virus, a bovine viral diarrhea virus, and a Japanese encephalitis virus”.

30
Claim 12 of the opposed patent application is "A method of treating a hepatitis C virus infection in a subject in need thereof, which comprises: administering to the subject an effective amount of the compound as claimed in claim 8".

Both claims are not patentable under Section 3(i) of the Indian Patent Act, 1970 as amended by the Patents (Amendment) Act, 2005 as both the claims 11 and 12 claims the methods for treating an infection.

7.0 LACK OF CLARITY AND INSUFFICIENCY OF DESCRIPTION -
SECTION 25(1)(g)

Without prejudice to what is submitted above, it is submitted that the Opposed Application does not contain sufficient information to enable the person skilled in the art to carry out the alleged invention over the entire scope as claimed.

According to section 12(1)(a) read with section 10(4) of the Indian Patent Act during the examination the learned Controller considers whether the complete specification fully and particularly describes the invention.

Whereas, according to section 25(1)(g), in opposition proceedings the Learned controller decides whether the complete specification sufficiently and clearly describes the invention or the method by which it is to be performed.

It is frequently stressed that the monopoly of a patent may only be awarded for subject matter which is re-workable by the skilled person within the entire claimed range. Subject matter which is not accessible based on the provided teaching and by using ordinary skill is to be excluded from the patent protection.

In view of the afore mentioned submissions, it is respectfully submitted that the Opposed Application lacks clarity and sufficiency, i.e. the description of the Opposed Application does not enable a person reasonably skilled in the art to achieve the results of the present invention as claimed, without inventive merit.

7.1 Insufficient disclosure of the subject-matter of claim 2 and claim 9.

Claim 2 - A composition comprising the compound or a stereoisomer thereof as claimed in claim 1 and a pharmaceutically acceptable medium.

Claim 9 - A composition comprising the compound as claimed in claim 8 and a pharmaceutically acceptable medium.
Claims 2 and 9 are directed to the pharmaceutical composition of the compound which is claimed in claim 1 and 8. Claims 2 and 9 are not supported by the body of the specification. The specification gives definition of pharmaceutically acceptable medium which broadly covers excipient, carrier and diluent. But there is no suggestion in the opposed patent how the specific excipient, carrier and diluent is to be used for the specific compounds of claims 1 or 8 and how it is to be produced. Hence, claims 2 and 9 lacks sufficient disclosure for obtaining the composition of compounds claimed in claims 1 and 8. Therefore, a person skilled in the art will not be able to make the specific composition of compounds claimed in claims 1 and 8 from the teaching of the opposed application.

8.0 FAILURE TO DISCLOSE DETAILS OF CORRESPONDING FOREIGN APPLICATIONS - SECTION 25(1)(H)

The controller should verify that the information of corresponding application in other application in other countries have been correctly provided and if not the patent application should be rejected under section 8.

It is also respectfully prayed that the Controller should check whether the Applicant of the opposed application has dutifully informed the status of every other application relating to the same or substantially the same invention. If any, filed in any country outside India subsequently to the filing of the statement referred to in the Section 8(1)(a) as required under Section 8(1)(b) of the Patents Act, 1970 as amended by the Patents (Amendment) Act, 2005. According to the decision of Hon. Delhi High Court on "Erlotinib" (http://lois.nic.in/dhc/MAN/judgement/10-09-2012/MAN07092012S892008.pdf), the applicant has to fulfill all the requirements of Section 8(1)(a) & 8(1)(b) and Section 8(2) (cf. pp 122 to 128) failing to which the application is liable to be rejected on this ground alone. If such information is not provided, it is respectfully submitted that the opposed patent under opposition is liable to be rejected on this ground alone. Therefore, it is our contention that the present application is obvious and constitutes subject matter which is not patentable under the Indian Patent Act. Therefore, this application should not be granted.
9.0 DEPENDENT CLAIMS:

1. Claim 2, 3, 4, 5, 6, 7 are dependent on claim 1. In the view of above discussions it is evident that claim 1 is not fulfilling the patentability criteria as per the provisions of the Indian Patent Act 1970 as amended by the Patents (Amendment) Act, 2005, therefore dependent claims also should not be allowed.

2. Claim 9, 10, 11, 12, 13, 14 are dependent on claim 8. As mentioned above claim 8 is not fulfilling patentability criteria, as per the provisions of the Indian Patent Act 1970 as amended by the Patents (Amendment) Act, 2005, therefore the dependent claims should also not be allowed.

10.0 The Opponents hereby submit that the claim 1-14 contained in the opposed Indian Patent Application No. 3658/KOLNP/2009 is not patentable under the Act.

11.0 The Opponents further submits that the claim 1-14 as contained in the opposed Indian Patent Application No. 3658/KOLNP/2009 is neither inventive nor not patentable under the Act. The Opposed Application concerns "NUCLEOSIDE PHOSPHORAMIDATE PRODRUGS". The Opposed Application does not fulfill the patentability criteria under the Act. The subject matters of the claim lack novelty as well as inventive step over the prior art. The Opposed Application also does not sufficiently and clearly describe the alleged invention for it to be carried out by a person skilled in the art.

12.0 In view of the aforementioned submissions, it is respectfully submitted that the Opposed Application lacks clarity and sufficiency, i.e. the description of the Opposed Application does not enable a person reasonably skilled in the art to achieve the results of the present invention as claimed, without inventive merit.

13.0 Accordingly, it is respectfully submitted that the Opposed Application does not contain sufficient information to enable the person skilled in the art to perform the invention disclosed and claimed in opposed Indian Patent Application No. 3658/KOLNP/2009. Therefore, this ground of opposition has been established and the entire Opposed Application ought to be rejected on this ground alone.
CONCLUSION

14.0 In view of the submissions presented above, we humbly pray that:

i) the Indian Patent Application No. 3658/KOLNP/2009 be dismissed in 
toto;

ii) the opponent be granted leave to file further evidence;

iii) the copy of reply statement and evidence filed by the applicant in the 
response to this opposition be made available to the opponent;

iv) the opponent be permitted to file further response and evidence to the 
reply or evidence produced by the applicant;

v) the opponent be granted leave to make further submissions in case the 
applicant makes any amendments in the claims;

vi) any other relief as the Learned Controller may deem fit be awarded in 
favor of the Opponents.

As a matter of precaution we request the Learned Controller to grant us an oral hearing 
before disposing of this representation.

Dated this the day of 2013

Nayan J. Rawal
Constituted Attorney
for the Opponent
IPA no. 654
<table>
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<tr>
<th>(21) International Application Number:</th>
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**Title:** MODIFIED FLUORINATED NUCLEOSIDE ANALOGUES

**Abstract:** The disclosed invention provides compositions and methods of treating a Flaviviridae infection, including hepatitis C virus, West Nile Virus, yellow fever virus, and a rhinovirus infection in a host, including animals, and especially humans, using a (2'R)-2'-deoxy-2'-fluoro-2'C-methyl nucleosides, or a pharmaceutically acceptable salt or prodrug thereof.
for the evolution of treatment standards, including more effective combination therapies.

The present invention provides a (2'R)-2'-deoxy-2'-fluoro-2'-C-methyl nucleoside (β-D or β-L), or its pharmaceutically acceptable salt or prodrug thereof, and the use of such compounds for the treatment of a host infected with a virus belonging to the Flaviviridae family, including hepatitis C, West Nile Virus and yellow fever virus. In addition, the nucleosides of the present invention show actively against rhinovirus. Rhinoviruses (RVs) are small (30 nm), nonenveloped viruses that contain a single-strand ribonucleic acid (RNA) genome within an icosahedral (20-sided) capsid. RVs belong to the Picornaviridae family, which includes the genera Enterovirus (polioviruses, coxsackieviruses groups A and B, echoviruses, numbered enteroviruses) and Hepatovirus (hepatitis A virus). Approximately 101 serotypes are identified currently. Rhinoviruses are most frequently associated with the common cold, nasopharyngitis, croup, pneumonia, otitis media and asthma exacerbations.

The inventor has made the unexpected discovery that the 2' substitutions on the β-D or β-L nucleosides of the present invention impart greater specificity for hepatitis C virus as well as exhibiting lower toxicity following administration to a host. The invention also includes a method for treating a Flaviviridae infection, including hepatitis C virus, West Nile Virus and yellow fever virus and rhinovirus infection, that includes the administration of an anti-virally effective amount of a β-D or β-L nucleoside disclosed herein, or its pharmaceutically acceptable salt or prodrug, optionally in a pharmaceutically acceptable carrier or diluent, optionally in combination or alternation with another effective antiviral agent.

The nucleosides of the present invention, possess the unique properties of having greater specificity for the hepatitis C virus and lower toxicity in culture or when administered into an animal. One potential, but non-limiting reason for this is the presence of the 2'-fluoro substitution on the ribose ring. For example, U.S. Patent No. 6,348,587 to Schinazi et al., discloses a family of 2'-fluoro nucleoside compounds that are useful in the treatment of hepatitis C virus infection. In contrast, are 2'-methyl substitututions such as found in 2'-C-methylcytidine as shown in WO
and wherein X is defined as above, R\(^1\) is H, R\(^3\) is H, R\(^4\) is NH\(_2\) or OH, R\(^6\) is H, and R\(^7\) is H.

In a twelfth embodiment, a (2'R)-2'-deoxy-2'-fluoro-2'-C-methyl nucleoside or its pharmaceutically acceptable salt or prodrug thereof is provided of the structure:

\[
\begin{align*}
\text{Base} & \quad \text{R}^1 \quad \text{R}^3 \quad \text{R}^4 \\
\text{O} & \quad \text{F} \quad \text{CH}_3 \\
\text{R}^6 \quad \text{R}^7
\end{align*}
\]

wherein Base is:

\[
\begin{align*}
\text{R}^1 \quad \text{R}^3 \quad \text{R}^4 \\
\text{N} \quad \text{O}
\end{align*}
\]

and wherein R\(^1\) is H, R\(^3\) is H, R\(^4\) is NH\(_2\) or OH, and R\(^7\) is H.

A thirteenth embodiment provides a (2'R)-2'-deoxy-2'-fluoro-2'-C-methyl nucleoside or its pharmaceutically acceptable salt or prodrug thereof of the structure:

\[
\begin{align*}
\text{NH}_2 & \quad \text{R}^4 \\
\text{O} \quad \text{F} \quad \text{CH}_3 \\
\text{HO} \quad \text{HO}
\end{align*}
\]

In a fourteenth embodiment, a (2'R)-2'-deoxy-2'-fluoro-2'-C-methyl nucleoside, its pharmaceutically acceptable salt or product thereof is provided by the structure:
genome, whose replication or functions can be altered by the compounds of the present invention. The term host specifically refers to infected cells, cells transfected with all or part of the viral genome, and animals, in particular, primates and humans. In most animal applications of the present invention, the host is a human patient. Veterinary applications, in certain indications, however, are clearly anticipated by the present invention.

The term "pharmaceutically acceptable salt or prodrug" is used throughout the specification to describe any pharmaceutically acceptable form (such as an ester, phosphate ester, salt of an ester or a related group) of a compound which, upon administration to a patient, provides the active compound. Pharmaceutically acceptable salts include those derived from pharmaceutically acceptable inorganic or organic bases and acids. Suitable salts include those derived from alkali metals such as potassium and sodium, alkaline earth metals such as calcium and magnesium, among numerous other acids well known in the pharmaceutical art. Pharmaceutically acceptable prodrugs refer to a compound that is metabolized, for example hydrolyzed or oxidized, in the host to form the compound of the present invention. Typical examples of prodrugs include compounds that have biologically labile protecting groups on a functional moiety of the active compound. Prodrugs include compounds that can be oxidized, reduced, aminated, deaminated, hydroxylated, dehydroxylated, hydrolyzed, dehydrolyzed, alkylated, dealkylated, acylated, deacylated, phosphorylated, dephosphorylated to produce the active compound.

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

A (2'R)-2'-deoxy-2'-fluoro-2'-C-methyl nucleoside or its pharmaceutically acceptable salt or prodrug thereof is provided of the structure:
wherein \( X \) and \( R^1 \) are as defined above.

In a twentieth embodiment, a (2'R)-2'-deoxy-2'-fluoro-2'-C-methyl nucleoside, its pharmaceutically acceptable salt or product thereof is provided by the structure:

![Structure](image1)

The present invention also contemplates 5'-triphosphate triphosphoric acid ester derivates of the 5'-hydroxyl group of a nucleoside compound of the present invention having the following general structural formula:

![Structure](image2)

wherein Base, \( X \), \( R^2 \), \( R^2' \), and \( R^6 \) are as defined as above.

The compounds of the present invention are also intended to include pharmaceutically acceptable salts of the triphosphate ester as well as pharmaceutically acceptable salts of 5'-diphosphate and 5'-monophosphate ester derivatives of the following structural formulas, respectively.

![Structure](image3)
wherein Base, X, R², R²' and R⁶ are as defined above.

Further non-limiting examples of phosphoric acid derivatives are the nucleosides of the present invention are shown below:
acceptable salt may be appropriate. Pharmaceutically acceptable salts include those derived from pharmaceutically acceptable inorganic or organic bases and acids. Suitable salts include those derived from alkali metals such as potassium and sodium, alkaline earth metals such as calcium and magnesium, among numerous other acids well known in the pharmaceutical art. In particular, examples of pharmaceutically acceptable salts are organic acid addition salts formed with acids, which form a physiological acceptable anion, for example, tosylate, methanesulfonate, acetate, citrate, malonate, tartarate, succinate, benzoate, ascorbate, α-ketoglutarate, and α-glycerophosphate. Suitable inorganic salts may also be formed, including, sulfate, nitrate, bicarbonate, and carbonate salts.

Pharmaceutically acceptable salts may be obtained using standard procedures well known in the art, for example by reacting a sufficiently basic compound such as an amine with a suitable acid affording a physiologically acceptable anion. Alkali metal (for example, sodium, potassium or lithium) or alkaline earth metal (for example calcium) salts of carboxylic acids can also be made.

Any of the nucleosides described herein can be administered as a nucleotide prodrug to increase the activity, bioavailability, stability or otherwise alter the properties of the nucleoside. A number of nucleotide prodrug ligands are known. In general, alkylation, acylation or other lipophilic modification of the mono, di or triphosphate of the nucleoside will increase the stability of the nucleotide. Examples of substituent groups that can replace one or more hydrogens on the phosphate moiety are alkyl, aryl, steroids, carbohydrates, including sugars, 1,2-diacylglycerol and alcohols. Many are described in R. Jones and N. Bischofberger, Antiviral Research, 27 (1995) 1-17. Any of these can be used in combination with the disclosed nucleosides to achieve a desired effect.

The active nucleoside can also be provided as a 5'-phosphoroether lipid or a 5'-ether lipid, as disclosed in the following references, which are incorporated by reference herein: Kuca, I.S., N. Iyer, E. Leake, A. Raben, Modest E.K., D.L.W., and C. Piantadosi. 1990. "Novel membrane-active ether lipid analogs that inhibit infectious HIV-1 production and induce defective virus formation." AIDS Res. Hum. Retro Viruses. 6:491-501; Piantadosi, C., J. Marasco C.J., S.L. Morris-
the onset of the viral infection or condition. In the prophylactic treatment according
to the present invention, it is preferred that the antiviral compound utilized should be
low in toxicity and preferably non-toxic to the patient. It is particularly preferred in
this aspect of the present invention that the compound that is used should be
maximally effective against the virus or condition and should exhibit a minimum of
toxicity to the patient. In the case of a Flaviviridae infection, including hepatitis C
virus, West Nile Virus, yellow fever virus, and a rhinovirus infection, compounds
according to the present invention, which may be used to treat these disease states,
may be administered within the same dosage range for therapeutic treatment (i.e.,
about 250 micrograms up to 1 gram or more from one to four times per day for an
oral dosage form) as a prophylactic agent to prevent the proliferation of the viral
infection, or alternatively, to prolong the onset of the viral infection, which
manifests itself in clinical symptoms.

In addition, compounds according to the present invention can be
administered in combination or alternation with one or more antiviral agents,
including other compounds of the present invention. Certain compounds according
to the present invention may be effective for enhancing the biological activity of
certain agents according to the present invention by reducing the metabolism,
catabolism or inactivation of other compounds and as such, are co-administered for
this intended effect.

IV. Stereoisomerism and Polymorphism

It is appreciated that nucleosides of the present invention have several chiral
centers and may exist in and be isolated in optically active and racemic forms. Some
compounds may exhibit polymorphism. It is to be understood that the present
invention encompasses any racemic, optically active, diastereomeric, polymorphic,
or stereoisomeric form, or mixtures thereof, of a compound of the invention, which
possess the useful properties described herein. It being well known in the art how to
prepare optically active forms (for example, by resolution of the racemic form by
recrystallization techniques, by synthesis from optically-active starting materials, by
chiral synthesis, or by chromatographic separation using a chiral stationary phase).
and R² is OH, R² is H, R³ is H, R⁴ is NH₂ or OH, and R⁶ is H.

21. A pharmaceutical composition comprising a (2'R)-2'-deoxy-2'-fluoro-2'-C-methyl nucleoside (β-D or β-L) or its pharmaceutically acceptable salt or prodrug thereof, in a pharmaceutically acceptable carrier, of the structure:

\[
\text{Base} \quad \text{X} \quad \text{CH}_3
\]

wherein Base is a purine or pyrimidine base;

X is O, S, CH₂, Se, NH, N-alkyl, CHW (R, S, or racemic), C(W)₂, wherein W is F, Cl, Br, or I; and,

R¹ and R⁷ are independently H, phosphate, including monophosphate, diphosphate, triphosphate, or a stabilized phosphate prodrug, H-phosphonate, including stabilized H-phosphonates, acyl, including optionally substituted phenyl and lower acyl, alkyl, including lower alkyl, O-substituted carboxyalkylamino or its peptide derivatives, sulfonate ester, including alkyl or arylalkyl sulfonyl, including methanesulfonyl and benzyl, wherein the phenyl group is optionally substituted, a lipid, including a phospholipid, an L or D-amino acid, a carbohydrate, a peptide, a cholesterol, or other pharmaceutically acceptable leaving group which when administered in vivo is capable of providing a compound wherein R¹ or R⁷ is independently H or phosphate; R¹ and R⁷ can also be linked with cyclic phosphate group.
Abstract: 2’ and/or 3’ prodrugs of 1’, 2’, 3’ or 4’-branched nucleosides, and their pharmaceutically acceptable salts and derivatives are described. These prodrugs are useful in the prevention and treatment of Flaviviridae infections, including HCV infection, and other related conditions. Compounds and compositions of the prodrugs of the present invention are described. Methods and uses are also provided that include the administration of an effective amount of the prodrugs of the present invention, or their pharmaceutically acceptable salts or derivatives. These drugs may optionally be administered in combination or alteration with further anti-viral agents to prevent or treat Flaviviridae infections and other related conditions.
In a second principal embodiment, a compound of Formula (II) or a pharmaceutically acceptable salt or prodrug, or a stereoisomeric, tautomeric or polymorphic form thereof, is provided, as well as a method for the treatment of a host infected with a Flaviviridae comprising administering an effective treatment amount of compound of Formula (II):

![Chemical Structure of Formula (II)]

or a pharmaceutically acceptable salt or prodrug, or a stereoisomeric, tautomeric or polymorphic form thereof, wherein:

- R₁, R₂, R₃, R₄, Y₁, Y₂, X₁ and X₂ are as defined above.

In a third principal embodiment, a compound of Formula (III), (IV) or (V), or a pharmaceutically acceptable salt or prodrug, or a stereoisomeric, tautomeric, or polymorphic form thereof, is provided, as well as a method for the treatment of a host infected with a Flaviviridae comprising administering an effective treatment amount of compound of Formula (III), (IV) or (V):

![Chemical Structures of Formulas (III), (IV) and (V)]

or a pharmaceutically acceptable salt or prodrug, or a stereoisomeric, tautomeric or polymorphic form thereof, wherein:

Base is selected from the group consisting of
applications, in certain indications, however, are clearly anticipated by the present invention (such as chimpanzees).

The term "pharmaceutically acceptable salt or prodrug" is used throughout the specification to describe any pharmaceutically acceptable form (such as an ester, phosphate ester, salt of an ester or a related group) of a nucleoside compound which, upon administration to a patient, provides the nucleoside compound. Pharmaceutically acceptable salts include those derived from pharmaceutically acceptable inorganic or organic bases and acids. Suitable salts include those derived from alkali metals such as potassium and sodium, alkaline earth metals such as calcium and magnesium, among numerous other acids well known in the pharmaceutical art. Pharmaceutically acceptable prodrugs refer to a compound that is metabolized, for example hydrolyzed or oxidized, in the host to form the compound of the present invention. Typical examples of prodrugs include compounds that have biologically labile protecting groups on a functional moiety of the active compound. Prodrugs include compounds that can be oxidized, reduced, aminated, deaminated, hydroxylated, dehydroxylated, hydrolyzed, dehydrolyzed, alkylated, dealkylated, acylated, deacylated, phosphorylated, dephosphorylated to produce the active compound. The compounds of this invention possess antiviral activity against a Flaviviridae, or are metabolized to a compound that exhibits such activity.

IV. Prodrugs and Derivatives

The active compound can be administered as any salt or prodrug that upon administration to the recipient is capable of providing directly or indirectly the parent compound, or that exhibits activity itself. Nonlimiting examples are the pharmaceutically acceptable salts (alternatively referred to as "physiologically acceptable salts"), and a compound, which has been alkylated, acylated, or otherwise modified at the 5'-position, or on the purine or pyrimidine base (a type of "pharmaceutically acceptable prodrug"). Further, the modifications can affect the biological activity of the compound, in some cases increasing the activity over the parent compound. This can easily be assessed by preparing the salt or prodrug and testing its antiviral activity according to the methods described herein, or other methods known to those skilled in the art.

A. Pharmaceutically Acceptable Salts

In cases where compounds are sufficiently basic or acidic to form stable nontoxic acid or base salts, administration of the compound as a pharmaceutically acceptable salt may be appropriate. Examples of pharmaceutically acceptable salts are
with the proviso that in bases (g) and (i), R', R'' are not H, OH, or NH₂; and Q, T, V, Q₂, Q₅ and Q₆ are not N.

9. A compound of Formula (IX):

```
R¹O
      R¹²
X   R¹³
OR²
Base⁺
```

or Table salt thereof, wherein:

- R¹, R² and R³ are independently H; phosphate; straight chained, branched or cyclic alkyl; acyl; CO-alkyl; CO-aryl; CO-alkoxyalkyl; CO-aryloxyalkyl; CO-substituted aryl; sulfonate ester; benzyl, wherein the phenyl group is optionally substituted with one or more substituents; alkylsulfonyl; arylsulfonyl; aralkylsulfonyl; a lipid; an amino acid; a carbohydrate; a peptide; cholesterol; or a pharmaceutically acceptable leaving group which when administered in vivo is capable of providing a compound wherein R¹, R² and/or R³ is independently H or phosphate;
- X is O, S, SO₂ or CH₂;
- Base⁺ is a purine or pyrimidine base;
- R¹² is C(Y³)₃;
- Y³ is independently H, F, Cl, Br or I; and
- R¹³ is fluoro.

10. The compound of claim 9, wherein X is O, and Y³ is H.

11. The compound of claim 10, wherein R¹, R² and R³ are H.

12. A method for the treatment of a host infected with a Flaviviridae virus, comprising administering an effective treatment amount of a compound as claimed in any one of claims 1-11, or a pharmaceutically acceptable salt thereof.

13. The method of claim 12, wherein the virus is hepatitis C.
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(Continued on next page)

(57) Abstract: Phosphoramidate derivatives of nucleotides and their use in the treatment of cancer are described. The base moieties of, for example, each of deoxyuridine, cytarabine, gemcitabine and citidine may be substituted at the 5-position. The phosphoramidate moiety has attached to the P atom an acyl-O moiety and an α-amino acid moiety. The α-amino acid moiety may correspond to or be derived from either a naturally occurring or a non-naturally occurring amino acid.

(58) Figure: Graph showing tumor volume.

(59) Table: Data showing tumor volume.

(60) Figures and Tables: Additional data and figures related to the invention.

(61) Claims: Statements defining the scope of the invention.

(62) Figures: Diagrams illustrating the structure of the compounds.

(63) Experiments: Methods and results of testing the compounds.

(64) References: Literature cited in support of the invention.

(65) Additional Information: Additional details on the background, objectives, and implementation of the invention.

(66) Acknowledgments: Acknowledgments to contributors and sources.

(67) Correspondence: Contact information for further details and inquiries.
26. A process for the preparation of a compound of formula I according to claim 1, the process comprising reacting of a compound of formula (III):

![Chemical structure](image)

with a compound of formula (IV)

![Chemical structure](image)

wherein \( \text{Ar, n, } R, R', R'', X, Y, Z \text{' and } Z'' \) have the meanings described in claim 1.
Application of the Phosphoramidate ProTide Approach to 4'-Azidouridine Confers Sub-micromolar Potency versus Hepatitis C Virus on an Inactive Nucleoside

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We report the application of our phosphoramidate ProTide technology to the ribonucleoside analogue 4'-azidouridine to generate novel antiviral agents for the inhibition of hepatitis C virus (HCV). 4'-Azidocytidine did not inhibit HCV, although 4'-azidocytidine was a potent inhibitor of HCV replication under similar assay conditions. However 4'-azidouridine triphosphate was a potent inhibitor of RNA synthesis by HCV polymerase, raising the question as to whether our phosphoramidate ProTide approach could effectively deliver 4'-azidouridine monophosphate to HCV replicon cells and unleash the antiviral potential of the triphosphate. Twenty-two phosphoramidates were prepared, including variations in the aryl, ester, and amino acid regions. A number of compounds showed sub-micromolar inhibition of HCV in cell culture without detectable cytotoxicity. These results confirm that phosphoramidate ProTides can deliver monophosphates of ribonucleoside analogues and suggest a potential path to the generation of novel antiviral agents against HCV infection. The generic message is that ProTide synthesis from inactive parent nucleosides may be a warranted drug discovery strategy.

Introduction

The hepatitis C virus (HCV) was identified for the first time in 1989 as a single-stranded positive sense RNA virus of the Flaviviridae family.1 According to the World Health Organization (WHO), more than 170 million people are estimated to be clinically infected by this virus, which is a major cause of severe liver disease.2

At present, treatment options comprise immunotherapy using recombinant interferon (often pegylated) in combination with ribavirin. The clinical benefit of this treatment is limited, and a vaccine has not yet been developed. The development of selective inhibitors of essential viral enzymes such as the serine protease NS3 or the RNA-dependent RNA polymerase NS5b are expected to improve the potency and tolerability of future treatment options for HCV infected patients.1,4

Nucleoside analogues have already been validated as an important class of polymerase inhibitors of other viral targets, such as HCMV, HSV, HIV, and HBV.5 All antiviral agents acting via a nucleoside analogue mode of action need to be phosphorylated, most of them to their corresponding 5'-triphosphates, by cellular and/or viral enzymes. The nucleoside triphosphate analogues will then inhibit the requisite polymerase and/or compete with natural nucleotide triphosphates as substrates for incorporation into viral nucleic acid during viral replication.3

Recently, 4'-azidocytidine was discovered as a potent inhibitor of HCV replication in cell culture. The corresponding 5'-azidouridine triphosphate was described as a competitive inhibitor of cytidylate incorporation by HCV polymerase and a potent inhibitor of native, membrane-associated HCV replicase in vitro.6

Interestingly, the corresponding uridine analogue, 4'-azidouridine (1), was inactive as an inhibitor of HCV replication in the cell-based replicon system.7

It was hypothesized that (1) (Figure 1) may be a poor substrate for phosphorylation by cellular enzymes. The first phosphorylation step to produce the 5'-monophosphate has often been found to be the rate-limiting step in the pathway to intracellular nucleotide triphosphate formation, suggesting that nucleoside monophosphate analogues could be useful antiviral agents. However, as unmodified agents, nucleoside monophosphates are unstable in biological media and they also show poor membrane permeation because of the associated negative charges at physiological pH.8,9

Our aryloxy phosphoramidate ProTide approach allows bypass of the initial kinase dependence by intracellular delivery of the monophosphorylated nucleoside analogue as a membrane permeable "ProTide" form.10,11 This technology greatly increases the lipophilicity of the nucleoside monophosphate analogue with a consequent increase of membrane permeation and intracellular availability. Previously we have demonstrated the success of our approach with the aryloxy-phosphoramidate derivatives of ddA,10 dAT,12 LCd4A,13 and d4A.14 These nucleotide monophosphate analogues were shown to exhibit greatly enhanced activity against HIV compared to the parent nucleoside analogues in vitro. In contrast to the parent nucleosides, full antiviral activity of the monophosphates was retained in kinase-deficient cell lines, which was consistent with an efficient bypass of the first phosphorylation step in HIV infected cells. Aryloxy-phosphoramidates are considered to be efficient lipophilic prodrugs of the corresponding 5'-monophosphate species in which the two masking groups are represented by an aryl or acid ester and an amine moiety. After passive diffusion...
Phosphoramidate ProTide Approach to 4'-Azidouridine

Figure 1. Structure of AZU and its corresponding phenyl-phosphoramidate ProTide.

Results and Discussion

Chemistry. The synthesis of 1 has been previously described. To prepare monophosphate prodrugs (2) of 1 we initially followed the previously described phosphoramidate chemistry for the synthesis of ProTides developed in our laboratory, using 1-methylimidazole (NMI) as the coupling agent. Several attempts were performed using different conditions (different amino acid esters, different reaction conditions) without successful isolation of the corresponding arylxy-phosphoramidate. These initial unsuccessful attempts might be explained considering the presence of a bulky group (azon) at the 4'-position adjacent to the coupling site at the S-position; in all previously published ProTide examples the 4'-position was unsubstituted.

The method of Uehyana was investigated next. This approach is based on the treatment of a nucleoside with 1 equiv of a strong organometallic base, such as a solution of tert-butydimagnesium chloride (tbbMgCl), to form the corresponding metal alkoxide. In the case of (1), this reaction was observed to be very rapid and gave yields between 30% and 50% of desired products. In the first instance, we synthesized 4'-azidouridine phosphoramidates starting from unprotected nucleoside. The apparent reactivity at the 2'- and 3'-position was low, suggesting high regioselectivity for the reaction at the S-position. In this way it was also possible to synthesize compounds 13, 21, and 26. In order to achieve higher solubility in the reaction solvent (tetrahydrofuran) and increase reactivity at the S-position, the 2'- and 3'-positions of 1 were protected with a cyclopropyl group. The final synthetic pathway (Scheme 1) involves the coupling of phenyl dichlorophosphate with different amino acid ester salts (5) to give the corresponding phenylxy-phosphoramidates (6), which were purified by flash chromatography and then coupled with the 2',3'-0-cyclopentylidine derivative 7 of (1) in the presence of tbbMgCl (1 M solution in THF).

The deprotection step was performed with a solution of 50% formic acid in water for 4 h at room temperature (Scheme 2). Due to the stereosemistry at the phosphorus center, the final compounds were always isolated as mixtures of two diastereomers. The presence of these diastereomers in the final preparations was confirmed by 31P (two peaks), 1H, and 13C NMR. A total of 22 phenyl phosphoramidates were synthesized as reported in Table I.

We have previously reported extensive structure-activity relationship (SAR) studies of anti-HIV phosphoramidates exploring the amino acid region, including natural amino acid variation, unusual C₂-methyl-tyrotyl, 1,4-dimethylglycine, and amino acid extensions and replacements. In general, L-alanine and the unnatural amino acid 2,4-dimethylglycine showed the best activity for the d₄T parent molecule versus HIV 1.5,22

Using the previously described method (Scheme 1), we synthesized the L-alanine (12), L-4,4-dimethylglycine (18), L-phenylalanine (20), L-lysine (22), L-leucine (27), L-phenylalanine (21), L-2,3'-0,0-cyclopentylidine (23), L-valine (24), and glycine (25). We further compared the importance of the stereocchemistry at the amino acid position by preparing a D-alanine benzyl ester phosphoramidate (26). On the basis of the L-arginine phenylphosphoramidate backbone, we also explored the SAR of different esters including methyl (11), ethyl (12), butyl (13), isopropyl (14), t-buty1 (15), t-buty1 (16), and benzyl (17). In order to have an indirect proof of phenyl phosphoramidate metabolism, we synthesized the 1-1,4-methyleneglycine (30) and phenylalanine (32) analogues, which were considered unfavorable substrates according to the postulated mechanism of activation.3

Recently we noted an increase of in vitro potency of a 1-phenylphosphoramidate analogue compared to the corresponding phenyl derivative while investigating the antiviral activity of WBAU phosphoramidates. Therefore, similar phosphoramidate analogues were also generated for (1). The synthesis of the 1-phenylphosphoramidate (33) was performed by reacting 1-phenylphosphochloridate in a similar reaction to give the corresponding phosphoramidate analogue (Scheme 3), which was then coupled with an amino acid ester and the nucleoside analogue according to our standard procedures. In this case, the separation of the two phosphoramidates (33 and 34) was achieved by using a semi-preparative HPLC purification with elution conditions of 70% water/20% acetonitrile. The 31P NMR spectrum showed the presence of only one peak for the first of the two fractions separated, and the 1H NMR spectrum supported the suggestion of a single diastereomer in this case. The second fraction contained an excess of the second diastereomer together with a minor proportion (estimated at 7%) of the 1H NMR integration of the first diastereomer (see Supporting Information for data).

Antiviral Activity. The phenyl phosphoramidates described above (11–32) were characterized in vitro as inhibitors of HCV replication in a HCV replicon assay as previously reported. Data are presented in Table I as EC₅₀ values (representing the concentration of compounds reducing HCV replication by 50%) and CC₅₀ values (representing the concentration of compounds reducing cell viability by 50%) as determined using the WST assay. All compounds showed CC₅₀ values greater than 100 μM. The parent compound (1) did not inhibit HCV replication significantly in the replicon system (EC₅₀ > 100 μM).
potent inhibition of HCV replication. Assuming that 4'-azido-5'-triphosphate is the active HCV polymerase inhibitor, these results support the notion that the active phosphoramidates successfully delivered 4'-azido nucleoside monophosphate intracellularly, that 4'-azido nucleoside (1) is inefficiently phosphorylated to the monophosphate in replicon cells, and that 4'-azido nucleoside monophosphate can be phosphorylated to the 2'-triphosphate in replicon cells. As shown in Table 2, 4'-azido nucleoside triphosphate not only inhibited recombinant HCV polymerase NS5b in vitro, and did so with sub-micromolar activity, but also in recent contrast to the inactive nucleoside parent (1). The tert-butyl ester (16) was the least active of the series. This was also in agreement with the SARs previously obtained in the d4T series and may relate to the relative stability of tertiary esters to enzyme-mediated hydrolysis. The isopropyl ester (15) showed high potency and represented one of the most active phosphoramidates prepared. Similarly, the 2-butyl ester (14) was also highly active in our assay in contrast to previous observations with other nucleoside analogues. Together with the benzyl analogue (17), these three esters provided the most potent compounds of HCV replication.
Table 1. Anti HCV Activity and Cytotoxicity Data for (1) and Phenyl Phosphoramidate Nucleotide Analogues

<table>
<thead>
<tr>
<th>compound</th>
<th>N at lone arm</th>
<th>EC50 (µM)</th>
<th>CC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>L-Val</td>
<td>3.1</td>
<td>&gt;100</td>
</tr>
<tr>
<td>12</td>
<td>L-Val</td>
<td>1.3</td>
<td>&gt;100</td>
</tr>
<tr>
<td>13</td>
<td>L-Val</td>
<td>1.2</td>
<td>&gt;100</td>
</tr>
<tr>
<td>14</td>
<td>L-Val</td>
<td>&gt;0.63</td>
<td>&gt;100</td>
</tr>
<tr>
<td>15</td>
<td>L-Val</td>
<td>0.74</td>
<td>&gt;100</td>
</tr>
<tr>
<td>16</td>
<td>L-Val</td>
<td>5.1</td>
<td>&gt;100</td>
</tr>
<tr>
<td>17</td>
<td>L-Val</td>
<td>0.61</td>
<td>&gt;100</td>
</tr>
<tr>
<td>18</td>
<td>MeGly</td>
<td>10.3</td>
<td>&gt;100</td>
</tr>
<tr>
<td>19</td>
<td>MeGly</td>
<td>3.4</td>
<td>&gt;100</td>
</tr>
<tr>
<td>20</td>
<td>eProGly</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>21</td>
<td>eProGly</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>22</td>
<td>Pro</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>23</td>
<td>Phe</td>
<td>&gt;100</td>
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<td>24</td>
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<td>&gt;100</td>
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<td>25</td>
<td>Gly</td>
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<td>&gt;100</td>
</tr>
<tr>
<td>26</td>
<td>L-Val</td>
<td>1.2</td>
<td>&gt;100</td>
</tr>
<tr>
<td>27</td>
<td>Leu</td>
<td>2.3</td>
<td>&gt;100</td>
</tr>
<tr>
<td>28</td>
<td>Pro</td>
<td>6.0</td>
<td>&gt;100</td>
</tr>
<tr>
<td>29</td>
<td>Met</td>
<td>1.4</td>
<td>&gt;100</td>
</tr>
<tr>
<td>30</td>
<td>N-MeGly</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>31</td>
<td>BuGly</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>32</td>
<td>L-β-Ala</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

4'-azidouridine (1) - - - - - - 100

Table 2. Inhibition of HCV Polymerase (NS5B) Activity in Vitro

<table>
<thead>
<tr>
<th>enzyme</th>
<th>IC50 [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS5B70-bk</td>
<td>0.29 ± 0.13</td>
</tr>
<tr>
<td>NS5B70-Cam</td>
<td>0.3 ± 0.11</td>
</tr>
</tbody>
</table>

Scheme 3. Synthetic Pathway for the Synthesis of 1-naphthylphosphorodichloridate

inhibitors in the L-alanine series, all having µM inhibitions of HCV. The antiviral activity of these three phosphoramidates was exceptional if compared to the parent compound 1 (EC50 > 100 µM), providing strong support for the notion of ProTide-mediated kinase bypass.

In the benzyl easter family, L-alanine (17) provided the most active compound with L-alanine (26) and glycine (25) being only slightly less potent. These results were striking when compared to the 60–70 fold reduction in anti-HIV potency for ProTides with L-alanine to glycine replacement and a 20–40 fold reduction for the corresponding acyclic ProTides.23-27 This reinforces our earlier conclusion that a separate ProTide motif optimization process is needed for each nucleoside analogue versus a given target. It may be that cell line dependent enzyme expression may determine different phosphoramidate SARs.

The presence of a methyl (26 and L-alanine) or a dimethyl (19) enhanced the activity if compared to larger and hydrophobic amino acid side chain residues such as L-valine (24), L-phenylalanine (23), and cyclopentylglycine (21), which were weakly active in the replicon assay.

An unexpected correlation was found between amino acid and ester function. While the L-phenylalanine derivative was substantially inactive as a benzyl ester (23), the corresponding ethyl ester (22) showed a significantly increased antiviral activity, displaying an EC50 value of 2.4 µM. Therefore, matrix-based optimization of amino acid and ester functionalities may be preferred over stepwise approaches.

The inactivity of the β-alanine (32) and the N-methyl glycine (30) compounds might underline the presence of an α-amino acid and its requirement in the amino acid structure to enable the metabolic activation of acylxy-phosphoramidates. However, the proline compound (with a blocked N) did show modest (38) activity, pointing to a complex amino acid SAR.

In conclusion, ester variation was widely tolerated except for the L-methylglycine which gave a slight reduction in potency in the L-alanine series (16) and the benzyl in the case of the L-phenylalanine derivative (23). L-Alanine retained the most effective amino acid as L-alanine and L-phenylalanine showing only slightly reduced potency. Diaminoglycine, L-methionine, and L-proline also provided compounds with antiviral potencies in a low micromolar range. It therefore appears that the amino acid core could be considerably varied to give antiviral agents with potencies within a 10-fold range in replication cells. Importantly, potency optimization requires consideration of both amino acid and ester moieties as most clearly shown for the methyl and benzyl esters of the L-phenylalanine analogues. Moreover, quite distinct SARs emerged from this family versus HCV as compared to our prior studies in other families.

We also explored the possibility to replace the phenyl substituent on the phosphorodichloridate with 1-naphthyl. Previously, we noted an increase of in vitro potency of naphthyl-phosphoramidates compared to the corresponding phenyl phosphoramidates when investigating BVDU phosphoramidates in an antitarget assay.28 We synthesized 33, the 1-naphthyl analogue of 17 (L-phenyl benzyl ester). As shown in Table 3, compound 33 inhibited HCV replication with an EC50 of 0.22 µM, leading to a further increase in antiviral activity (>450-fold) in comparison to 4'-azidouridine (Table 3). One of the two phosphorus diastereomers could be purified using a C-18 reverse-phase semipreparative HPLC. One of the two main fractions obtained showed only one 1H NMR peak. The second fraction was less pure, although the second diastereomer appeared as the major component of the mixture. We have previously reported a method for the prediction of the phosphorus configuration of such diastereomers based on a different 1H NMR profile of the methylthio protons of the benzyl ester.29 Applying this concept to compounds 34 and 35, we noticed that in one case (more polar, 35) a clear AB-system was observed while, for the other diastereomer (less polar, 34), the two protons displayed an apparent doublet. Conformational studies were performed using the Sybyl 7.0 software package. The lowest energy conformation found for each diastereomer is shown in Figure 2. These differences in proton profiles can be explained by the ability of one, but not the other, diastereomer to form π-π interactions between the naphthyl and the phenyl group of the benzyl ester, resulting in a constrained conformation. This interaction can only occur with the β phosphorus configuration (35) with the two methylene

Table 3. Anti HCV Activity and Cytotoxicity Data for (1) and 1-Phenyl Phosphoramidate Nucleotide Analogues

<table>
<thead>
<tr>
<th>compound</th>
<th>phosphorus configuration</th>
<th>amino acid</th>
<th>ester</th>
<th>IC50 [µM]</th>
<th>CC50 [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>S/R</td>
<td>L-Ala</td>
<td>Bu</td>
<td>0.22</td>
<td>&gt;100</td>
</tr>
<tr>
<td>34</td>
<td>R</td>
<td>L-Ala</td>
<td>Bu</td>
<td>0.39</td>
<td>&gt;100</td>
</tr>
<tr>
<td>35</td>
<td>S</td>
<td>L-Ala</td>
<td>Bu</td>
<td>0.43</td>
<td>&gt;100</td>
</tr>
<tr>
<td>17 (Phenyl ProTide)</td>
<td>S/R</td>
<td>L-Ala</td>
<td>Bu</td>
<td>0.61</td>
<td>&gt;100</td>
</tr>
<tr>
<td>4'-azidouridine (1)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

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Phosphoramidates ProTide Approach to 4'-Azidouridines
protons becoming nonmagnetically equivalent (AB system). For the diastereoisomer with S phosphorus configuration (34), this interaction does not occur and the higher degree of flexibility around the methylene group renders its protons more magnetically similar (apparent doublet). The biological activities of the separated diastereoisomers (34 and 35) were comparable to each other and to the mixture (Table 3).

Interestingly, application of similar ProTide methods to the active 4'-azidouridine gave little or no boost in anti-HCV activity as observed in other assays (Figure 3). Phosphoramidates prepared in the series were the 4'-naphthyl benzyl ester phosphoramidate with an ED_{50} of 0.22 μM in the replication assay. The diastereoisomers of this compound were separated by HPLC and their absolute phosphorus configuration (34), this renders the enantiomers as racemic mixtures.

Conclusion

A series of phosphonamidate ProTides of 4'-azidouridine were prepared and evaluated as inhibitors of HCV replication in vitro. The phosphonamidate approach provided novel compounds with increased potency in the replication assay when compared to the inactive parent compound, corresponding to the enzyme's different conformational state (34). A phosphonamidate tested was nontoxic in the replication assay (CC_{50} = 100 μM). The most active compound prepared in the series was the 1-naphthyl L-alanine benzyl ester phosphoramidate with an ED_{50} of 0.22 μM in the replication assay. The diastereoisomers of this compound were separated by HPLC and their absolute phosphorus configurations predicted by modeling and NMR. However, they did not show any differences in biological activity. This report demonstrates the ability of the ProTide approach to successfully bypass the rate limiting initial phosphorylation of a RNA nucleoside analogue and thus confer significant antiviral activity on an inactive parent nucleoside.

Experimental Section

Biology: HCV replication assay was performed in the stable replication cell line 2209-23 derived from HuH-7 cells strictly transfected with a hypomorphic HCV genotype 1b expressing the results发布会 reporter gene, as described. The RNA synthesis activity of recombinant HCV polymerase proteins was measured by incorporation of radiolabeled UMP into acid-insoluble RNA products using HCV genome derived cRNAs as a template in a primer-dependent RNA synthesis assay. Recombinant proteins used were truncated at amino acid positions 570 and derived from genotype 1a strain BX (NS5B15899-696) or Con1 (NS5B18570-691).

Chemistry: General Procedures. All experiments involving water-sensitive compounds were conducted under anhydrous dry conditions. Anhydrous tetrahydrofuran and dichloromethane were purchased from Aldrich, Proton, carbon, and phosphorus NMR Spectrometric Magnetic Resonance (1H, 13C, 31P NMR) spectra were recorded on a Bruker Advance spectrometer operating at 500, 125, and 202 MHz, respectively. All 1H and 13C spectra were recorded proton-decoupled. All NMR spectra were recorded in CD_{3}OD at room temperature (25°C ± 0.5°C). Chemical shifts for 1H and 13C spectra are quoted in parts per million downfield from tetramethylsilane. Coupling constants are reported as J values. Signal splitting patterns are described as singlet (s), doublet (d), triplet (t), quartet (q), broad signal (br), doublet of doublet (dd), doublet of triplet (dt), or multiplet (m). Chemical shifts for 31P spectra are quoted in parts per million relative to an external phosphoric acid standard.

Preparation and spectroscopic data on individual precursors and derivatives of ProTides. Variations from these procedures and individual purification methods are given in the main text. Preparative and spectroscopic data on individual precursors, blocked nucleosides were given as Supporting Information only (see below), excluding only the first example.

Standard Procedure 1: Preparation of 2',3'-O-O-Cyclopentylidene-4'-azidouridine Phosphoramidates. BuBuGCl (2.0 mol equiv) and 2',3'-O-Cyclopentylidene-4'-azidouridine (1.0 mol equiv) were dissolved in dry THF (31 mol equiv) and stirred for 15 min. Then a 1 M solution of the appropriate phosphonochloridate (2.0 mol equiv) in dry THF was added dropwise and then stirred overnight. A saturated solution of NH_{4}Cl was added, and the solvent was removed under reduced pressure to give a yellow solid, which was consequently purified by chromatography.

Standard Procedure 2: Deprotection of 2',3'-Protected 4'-Azidouridine Phosphoramidates. The protected 2',3'-O-Cyclopentylidene-4'-azidouridine phosphoramidate was added to a solution 80% of formic acid in water. The reaction was stirred at room temperature for 4 h. Then the solvent was reduced under reduced pressure to give a yellow solid, which was purified by chromatography.

HPLC Method Used for the Separation of Compounds 34 and 35. Varian ProStar instrument using a Polaris C18-4.60 mm column; elution was performed using a mobile phase consisting of water/acetonitrile 70:30 (0.1% TFA) and 17 min elution time with a flow of 20 mL/min. Optimal loading on column: 8 mg of phosphonamide per run.

Synthesis of 2',3'-O-Cyclopentylidene-4'-azidouridine 5'-O-[Phenylmethyloxoy-L-alanine)] Phosphate (Methyl N(1-[3S,5R,5R,5aS]-4-Azido-2-tetrahydro-4-(hydroxymethyl)-2,2-dicyclopentylidene-4'-azidouridine phosphoramidate was added to a solution 80% of formic acid in water.

The reaction was stirred at room temperature for 4 h. Then the solvent was reduced under reduced pressure to give a yellow solid, which was purified by chromatography.
Synthesis of 5'-0-(Phenyl(methoxy-lys-yl)alaninate) Phosphate (2-Propyl N-[1-(3AR,4S,5R)-5-Azido-tetrahydro-3,4-dihydroxy-5-(hydroxymethyl)furran-2-yl]pyrimidine-2,4(1H,3H)-dione} (Phenox)-phosphoryl-α-alaninate). Supporting Information for preparative and spectroscopic data.

Synthesis of 4'-Azidouridine 5'-O-[Phenyl(methoxy-lys-yl)alaninate] Phosphate (2-Propyl N-[1-(3AR,4R,5R)-5-Azido-tetrahydro-3,4-dihydroxy-5-(hydroxymethyl)furran-2-ylypyrimidine-2,4(1H,3H)-dione} (Phenox)-phosphoryl-α-alaninate). See Supporting Information for preparative and spectroscopic data.

Synthesis of 3-Hydroxy-5-(hydroxymethyl)furran-2-ylphosphorochloridate (2.46 mL). The obtained pure product was a white solid (16.8 mg, 0.030 mmol, 94%). 

Synthesis of O-[(phenyl(methoxy-lys-yl)alaninate)] Phosphate (tert-Butyl N-[1-(2S,3R,4R,5R)-5-Azido-tetrahydro-3,4-dihydroxy-5-(hydroxymethyl)furran-2-ylypyrimidine-2,4(1H,3H)-dione} (Phenylox)-phosphoryl-α-alaninate) (13). Prepared according to the standard procedure 2, from 2'-3'-0,0-Cyclopentyliden-4'-azidouridine 5'-0-(Phenyl(methoxy-lys-yl)alaninate) Phosphate (Benzyl :N-tet-butyloxy-L-alanine). See Supporting Information for preparative and spectroscopic data.
Synthesis of 3′-O-Phenyl(ethoxy-dimethylglycinyl) Phosphate (Ethyl N-[[(3R,5S,6R,6aS)-5-Azido-tetrahydro-3,4-dihydroxy-5-(hydroxymethyl)furan-2-yl]phosphoryl]-diphenylglycine) (18). Prepared according to the standard procedure 2, from 3′,3′-O-Cycloproplylene-3′-O-phenyl(ethoxy-dimethylglycinyl) Phosphate (Ethyl N-[[[(3R,3′R,4′R,6aS)-4-Azido-tetrahydro-4-(hydroxymethyl)-2,2′-cyclopropano[3′,4′:3,4]-dioxid]-4′-yl]phosphoryl]-2′-deoxy-2′-0-phenylglycinyl)-1′H,3′H-diene (Phosphoryl)-diphenylglycinate (17). The crude product was purified by column chromatography, using as eluent CHCl₃/Methanol (92:8). The obtained pure product was a white solid (145 mg, 0.226 mmol). Anal. (C₃₁H₃₈N₁₀O₂P) requires 577.672. Found: 577.456.  

Synthesis of 4′-Azidouridine 5′-0-[Phenyl(ethoxy-dimethylglycinyl)] Phosphate (Benzyl N-[[(3R,3′R,4′R,6aS)-4-Azido-tetrahydro-3,4-dihydroxy-5-(hydroxymethyl)furan-2-yl]phosphoryl]-diphenylglycine) (31). Prepared according to the standard procedure 3, from 4′-azidouridine (300 mg, 1.026 mmol), 2YmMgCl₂ (2.10 mL of solution 1 M in THF, 2.10 mmol), and phenyl(ethoxy-dimethylglycinyl)-1′H,3′H-diene (97). The crude product was purified by column chromatography, using as eluent CHCl₃/Methanol (92:8) and then a preparative TLC using as eluent CHCl₃/Methanol (94:6). The obtained pure product was a white solid (130 mg, 0.202 mmol, 20%). Anal. (C₅₃H₅₈N₁₀O₄P) requires 816.105. Found: 816.234.  

Synthesis of 2′,3′,3′-O-Cycloproplylene-3′-O-phenyl(ethoxy-dimethylglycinyl) Phosphate (Ethyl N-[[(3R,5S,6R,6aS)-5-Azido-tetrahydro-3,4-dihydroxy-5-(hydroxymethyl)furan-2-yl]phosphoryl]-2′-deoxy-2′-0-phenylglycinyl)-1′H,3′H-diene (Phosphoryl)-diphenylglycinate (33). Prepared according to the standard procedure 1, from 2′,2′-O-Cycloproplylene-3′-O-phenyl(ethoxy-dimethylglycinyl) Phosphate (Ethyl N-[[[(3R,5S,6R,6aS)-5-Azido-tetrahydro-3,4-dihydroxy-5-(hydroxymethyl)furan-2-yl]phosphoryl]-2′-deoxy-2′-0-phenylglycinyl)-1′H,3′H-diene (Phosphoryl)-diphenylglycinate (18). The obtained pure product was a white solid (154 mg, 0.226 mmol), and a solution 80% of HCOOH in water (10 mL). The crude product was purified by column chromatography, using as eluent CHCl₃/Methanol (92:8). The obtained pure product was a white solid (145 mg, 0.226 mmol, 82%). Anal. (C₃₁H₃₈N₁₀O₂P) requires 577.672. Found: 577.456.  

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Synthesis of 4′-Azidouridine 5′-0-[Phenyl(ethoxy-dimethylglycinyl)] Phosphate (Benzyl N-[[(3R,5S,6R,6aS)-5-Azido-tetrahydro-3,4-dihydroxy-5-(hydroxymethyl)furan-2-yl]phosphoryl]-diphenylglycine) (31). Prepared according to the standard procedure 3, from 4′-azidouridine (300 mg, 1.026 mmol), 2YmMgCl₂ (2.10 mL of solution 1 M in THF, 2.10 mmol), and phenyl(ethoxy-dimethylglycinyl)-1′H,3′H-diene (97). The crude product was purified by column chromatography, using as eluent CHCl₃/Methanol (92:8) and then a preparative TLC using as eluent CHCl₃/Methanol (94:6). The obtained pure product was a white solid (130 mg, 0.202 mmol, 20%). Anal. (C₅₃H₅₈N₁₀O₄P) requires 816.105. Found: 816.234.
Phosphorimidazid ProTide Approach to 4'-Azidouridine

Azido-tetrahydro-3,4-dihydroxy-5-(hydromethyl)furan-2-(3H)-dione (Phenyx)-phosphoryl-1-phenylalanine (Phenyx) (20). Prepared according to the standard procedure 2, from 2\(\beta\)-O-C-phenylcyclcopentyle-4-azidouridine-5'-0-[phenyl(benzyloxy-L-valinate)]. Synthesis of 4'-Azidouridine 5'-0-[Phenyx(benzyloxy-L-valinate)]. 

Phenyx (N\(\|\)-(3 RaR,4R,6R)-4-Azido-tetrahydro-4-(hydromethyl)-2-cyclopentylfuro[3,4-d]1,4-dioxol-6-yl)phosphono-1,1-diphenylmethane (Phenyx). 

Prepared according to the standard procedure 2, from 2\(\beta\)-O-C-phenylcyclcopentyle-4-azidouridine-5'-0-[phenyl(benzyloxy-L-valinate)]. Synthesis of 4'-Azidouridine 5'-0-[Phenyx(benzyloxy-L-valinate)]. 

Phenyx (N\(\|\)-(3 RaR,4R,6R)-4-Azido-tetrahydro-4-(hydromethyl)-2-cyclopentylfuro[3,4-d]1,4-dioxol-6-yl)phosphono-1,1-diphenylmethane (Phenyx). 

Prepared according to the standard procedure 2, from 2\(\beta\)-O-C-phenylcyclcopentyle-4-azidouridine-5'-0-[phenyl(benzyloxy-L-valinate)]. Synthesis of 4'-Azidouridine 5'-0-[Phenyx(benzyloxy-L-valinate)]. 

Phenyx (N\(\|\)-(3 RaR,4R,6R)-4-Azido-tetrahydro-4-(hydromethyl)-2-cyclopentylfuro[3,4-d]1,4-dioxol-6-yl)phosphono-1,1-diphenylmethane (Phenyx).
CH_2(OH), 1.28 (3H, CH_3-ethyl), MS (EI) 589.1416 (M^+). C_{23}H_{22}N_{6}O_{6}P requires 589.1426. Anal. C_{23}H_{22}N_{6}O_{6}P, C, H, N.

Synthesis of 2,3-O-Cyclopentylidene-4'-azidouridine 5'-O-[(Phenylethoxy-ethyl)methanethiol] Phosphate (Ethyl N-[1-(3aR,4R,6R,6aS)-4-Azido-tetrahydro-4-(hydroxymethyl)2,2-cyclopentylidene-3,4-diol]-6-yl-3-[(2H,3H)-dione] (Phenoxy)-phosphoryl]-methanethiolate). See Supporting Information for preparative and spectroscopic data.

Synthesis of 4'-Azidouridine 5'-O-[Phenethyl(ethyloxy-methanethiol)] Phosphate (Phosphate N-[1-(3aR,4R,6R,6aS)-4-Azido-tetrahydro-4-(hydroxymethyl)2,2-cyclopentylidene-3,4-diol]-6-yl-[(2H,3H)-dione] (Phenoxy)-phosphoryl]-methanethiolate). See Supporting Information for preparative and spectroscopic data.

Synthesis of 4'-Azidouridine 5'-O-[Phenethyl(ethyloxy-ethyl-glycinyl)] Phosphate (Ethyl N-[1-(3aR,4R,6R,6aS)-4-Azido-tetrahydro-4-(hydroxymethyl)2,2-cyclopentylidene-3,4-diol]-6-yl-[(2H,3H)-dione] (Phenoxy)-phosphoryl]-ethyl-glycinylate). See Supporting Information for preparative and spectroscopic data.

Synthesis of 4'-Azidouridine 5'-O-[Phenethyl(ethyloxy-ethyl-methyl-glutamyl)] Phosphate (Ethyl N-[1-(3aR,4R,6R,6aS)-4-Azido-tetrahydro-4-(hydroxymethyl)2,2-cyclopentylidene-3,4-diol]-6-yl-[(2H,3H)-dione] (Phenoxy)-phosphoryl]-ethyl-methyl-glutamylate). See Supporting Information for preparative and spectroscopic data.
Phosphoramidate ProTide Approach to 4'-Azidoarbine

dd, H2'-aridine), 4.20–4.21 (1H, m, H2'-aridine, H2'-aradine).
4.12–4.09 (1H, m, CH2-amine).

Acknowledgment. We thank Helen Murphy for secretarial assistance.

Supporting information Available: Analytical data on target compounds, preparative and spectroscopic data on blocked nucleoside intermediates, and figures of HPLCs of separated diastrorormers. This material is available free of charge via the Internet at http://pubs.acs.org.

References

Minireview: nucleotide prodrugs

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Abstract

Nucleotides have shown interesting biological activities in a wide variety of antiviral, antiproliferative, immunomodulatory and other biological assays, and they present promising drug candidates. Because of their negative charge(s), nucleotides suffer from some disadvantages which can be successfully overcome by the utilization of nucleotide prodrugs. Nucleotide prodrugs were successfully used to increase oral absorption of nucleotides in vivo. By taking advantage of intracellular triggers (reducing potential, enzyme activity, pH), nucleotide prodrugs can be used in vitro for the intracellular delivery of the nucleotide resulting in enhanced potency and in some cases enhanced selectivity. Nucleotide prodrugs have also been utilized for tissue specific delivery of the nucleotides in vivo resulting in altered selectivity and reduced toxicity. For nucleotide prodrugs, their ultimate intended use is (in most cases) in vivo for the treatment of a disease. Thus, it is important to incorporate adequate assays and design criteria into any prodrug effort. In vivo systems are complicated because of metabolism, excretion and tissue distribution of the prodrug and the parent. Thus, results of in vitro assays have to be interpreted cautiously because they may be unsuitable predictors of the in vivo situation.

Keywords: Nucleotide prodrug; Intracellular delivery; Increased absorption; Enhanced potency; Enhanced selectivity; Reduced toxicity

Abbreviations and acronyms: ara-C = β-D-arabinofuranosylcytosine; ara-A = β-D-arabinofuranosyladenine; dd = deoxy; U = uridine; T = thymidine; TK = thymidine kinase deficient; p = phosphate; AZT = 3'-azido-3'-deoxythymidine; ACV = acyclovir; FU = 5-fluorouracil; FdU = 5-fluoro-2'-deoxyuridine; GCV = ganciclovir; HSV = herpes simplex virus; HPV = human papilloma virus; cAMP = cyclic adenosine monophosphate; cGMP = cyclic guanosine monophosphate; i.p. = intraperitoneal; HIV = human immunodeficiency virus; SIV = simian immunodeficiency virus; MSV = Moloney sarcoma virus; FIV = feline immunodeficiency virus; CMV = cytomegalovirus; HBV = hepatitis B virus.

Corresponding author.
1. Introduction

Nucleoside and nucleotide analogues have great therapeutic potential for the treatment of viral diseases and cancer (Robins, 1984; De Clercq, 1990; Herdewijn, 1992; Chu and Baker, 1993). The area of nucleotide analogues has received a lot of attention recently due to the discovery of nucleotides with potent antiviral activities (Holy, 1993). Since the negative charge(s) on the phosphorous entail(s) nucleotides with short comings (low permeability and bioavailability), increasing work in the literature is focusing on overcoming these difficulties with nucleotide prodrugs, an approach which temporarily masks the negative charges and liberates the parent nucleotide at a specific site.

It is not the intention of the authors to give an exhaustive survey of the field of nucleotide prodrugs, but the current review is intended to summarize and critically survey the work which has been done in the area with specific emphasis on the rationale and mechanism. The aim of this article is to direct the attention of the reader to the field of nucleotide prodrugs, give a flavor of the types and classes of compounds in the literature, and perhaps stimulate further drug design in this area. This review is generally restricted to compounds containing one nucleoside and one phosphorous moiety. In most cases, the full details of the compounds discussed in this review, such as chemical synthesis and biological evaluation, may be gleaned from the original literature.

2. Nucleosides and nucleotides as drug candidates

A vital step in the mode of action of many purine and pyrimidine nucleoside analogues against viral and neoplastic diseases is their metabolic activation by cellular and viral kinases. Such activation is generally mediated through kinases or transferases to yield the mono-, di-, and triphosphate analogues. The biologically active species of many nucleosides is the triphosphate analogue, which inhibits viral or cellular polymerases. Some nucleoside analogues fail to undergo the necessary phosphorylation to the active nucleotide form either because they are poor substrates for the phosphorylating enzymes, e.g. ddU (Hao et al., 1990), or due to the absence of a phosphorylating enzyme. Such an absence can occur with the development of viral resistance, i.e. TK−HSV mutant is ACV resistant because it lacks the enzyme which converts ACV to ACVp. Another possibility is the temporal or tissue specific absence of the phosphorylating enzyme as in the case of AZT, which is poorly phosphorylated to AZTp in resting cells due to the low level of cellular TK in such cells (Zhu et al., 1990; Gao et al., 1993). In contrast to nucleosides, nucleotides are phosphorylated species and do not require that first step in their metabolic activation. Thus, nucleotides could be expected to show biological activity where the corresponding nucleoside is inactive due to lack of intracellular phosphorylation. Nucleotides will also retain activity against certain nucleoside resistant viral mutants (e.g. TK−HSV) and finally, nucleotides could show an altered biological profile from nucleosides due to different tissue distribution of the activated metabolite. Another rationale for utilizing nucleotides is in cases where nucleosides are metabolically deactivated in vivo due to the actions of certain enzymes, as in the case of ara-C and ara-A, which are rapidly deaminated in vivo by cytidine
deaminase (Caminer and Smith, 1965; Ho, 1973) and adenosine deaminase (Cohen and Plunkett, 1975), respectively. Also, nucleoside analogues are often substrates for phosphorlyase-induced glycosidic bond cleavage (Birnie et al., 1963; Saffhill and Hume, 1986).

Cohen and Plunkett were the first to unequivocally show that the nucleotide ara-Ap could enter cells directly (Cohen and Plunkett, 1975). The field of nucleotides started to flourish with the discovery by Holy and De Clercq of phosphonomethylether nucleosides, a class of nucleotide analogues with interesting biological activities. Two prototype examples in this series are PMEA (I) and HPMPC (2) (Fig. 1) (Holy, 1993). PMEA has antiretroviral and antith ESV virus activity and has shown efficacy in retroviral animal models (MSV, FIV, SIV) (Tsai et al., 1994) and as a parenteral agent in human clinical trials against HIV (Collier et al., 1993; Walker et al., 1993). HPMPC is a broad spectrum antith ESV virus agent and is currently undergoing evaluation as a treatment for CMV, HSV and HPV infections in humans (De Clercq, 1993).

Another nucleotide analogue, the 5'-methylene phosphonate of GCV (3) was found to have activity against CMV and it is currently undergoing preclinical evaluation (Smee et al., 1994; Prisbe et al., 1986).
Nucleotides, however, have some disadvantages over nucleosides. Because of their charge, they enter cells very poorly and show generally low or no activity in vitro. For example, only ~0.01% of exogenously supplied ara-Ap was found to enter cells as the intact nucleotide after 4 h of incubation (Cohen and Plunkett, 1975). Similarly, radiolabeled uptake experiments showed that only 1% of extracellularly applied HPMPC permeated into the cell (Connelly et al., 1993). The charges on the nucleotide are also responsible for their low oral bioavailability. It was shown that the oral bioavailability of both PMEA and HPMPC in rats was less than 5% (Bischotberger et al., 1993). Another disadvantage which pertains specifically to nucleoside phosphate analogues is their low stability in biological media, due to rapid dephosphorylation by phosphatases (Cohen and Plunkett, 1975).

Nucleotide prodrugs can potentially overcome these difficulties. Masking the negative charge(s) on the phosphorous by suitable functionalities, which can be converted chemically or biologically to the parent nucleotide, can make nucleotides orally bioavailable, increase intracellular delivery, and alter pharmacokinetics/tissue distribution/metabolism resulting in improved efficacy and target organ specificity.

3. Rationale for nucleotide prodrugs

3.1. Oral bioavailability

In order for a prodrug to fulfill the requirements necessary to deliver the parent nucleotide D into the systemic circulation, the prodrug PD should be stable to the intestinal environment, it should be permeable to cross the intestinal wall and finally, once in the systemic circulation, has to be labile to be converted back to the parent nucleotide (Fig. 2). Because of these seemingly contradictory properties (intestinal stability/permeability/systemic lability) the choice of functionalities to achieve optimized properties is very limited.

Prodrugs of PMEA (1) (Fig. 1) were evaluated in an attempt to increase the oral bioavailability of the parent PMEA (Shaw et al., 1994; Starrett et al., 1994). Promising candidates were preselected utilizing a number of assays, including chemical stability and stability in biological media (rat intestinal wash, rat and human intestinal homogenate, rat and human plasma and human liver homogenate). As a measure for the

Fig. 2. Prodrug (PD) utilized to increase oral bioavailability of parent drug (D). PD should be stable in the gut, permeate across the intestinal wall, and, once in the systemic circulation, should be converted efficiently to D.
permeability of the prodrugs, the permeation across a cell monolayer (Caco-2 cell culture) was determined in vitro. PMEA prodrugs were also administered orally to rats, and the systemic levels of PMEA were determined by measuring the concentrations of PMEA in the urine. Monoesters exhibited low bioavailability, dialkyl esters were absorbed efficiently; however, their conversion to PMEA was very low; administration of diethyl PMEA (4) resulted in the appearance of 40% of 4 in the urine with no parent PMEA being detectable. Bis(acyloxyethyl) esters, first utilized by Farquhar as phosphate prodrugs (Farquhar et al., 1983, 1991), demonstrated improved bioavailability. Particularly, bis(pivaloxyethyl) PMEA (bis(POM)PMEA (5)) (Fig. 1) showed oral bioavailability of 18% in rats. Subsequent studies showed that 5 is chemically relatively stable (half life \( t_\frac{1}{2} \) at 40°C/\( pH \ 5 = 33 \) h), but labile in biological media (\( t_\frac{1}{2} \) in human plasma = 3 min). More detailed pharmacokinetic studies in monkeys showed that 5 has an oral bioavailability of 22–27% in various formulations and that no intact 5 could be detected in plasma (Cundy et al., 1994). Compound 5 is currently undergoing clinical evaluation as an anti-HIV agent in humans.

Similarly, oxo-PMEA 6 (Fig. 1), a compound closely related to PMEA, has low bioavailability in mice (1–2%). Following evaluation of a large number of prodrugs, the diphenyl ester 7 was selected as the preferred prodrug. It showed 50% oral bioavailability and corresponding efficacy on oral administration in mice (Perkins et al., 1993).

Interestingly, in the case of PMEA, the corresponding diphenylester 8 was promising based on in vitro assays (stability, permeability), and it also showed high absorption on oral administration in rats. However, PMEA was only found at low levels in plasma (3%), the major product was an unknown metabolite (Shaw et al., 1994).

The octadecanolester of ara-Cp 9 was shown to be orally active in mice with a minimum effective dose only twice the i.p. dose. The prodrug was found in the plasma of the rats with a \( t_{\text{max}} \) of 1 h. Additionally, this prodrug was not hydrolyzed by cytidine deaminase after 2 h in plasma (Saneyoshi et al., 1980).

### 3.2. Intracellular delivery

As mentioned, nucleotides cross cellular membranes very inefficiently and are generally not very potent in vitro (Cohen and Plunkett, 1975). Thus, increasing the rate of cellular permeation by the prodrug approach and delivering the nucleotide to the inside of a cell would increase the potency of nucleotides. Intracellular delivery of the nucleotide is especially important for nucleotide phosphates where the phosphate is required to overcome the lack of phosphorylation of the nucleoside by cellular or viral kinases. In this case, the extracellular metabolism of the prodrug or the nucleotide to the nucleosides competes with the uptake into the cell (Lichtenstein et al., 1960).

The efficient intracellular delivery of nucleotides using a prodrug approach necessitates the existence of triggers or different rates of conversion of the prodrug to the drug intracellularly vs extracellularly \( (k_2 \text{ has to be bigger than } k_1) \) (Fig. 3).

The intracellular triggers that have been utilized and exploited are the differences in reducing potential, enzyme activity (especially esterases), and pH. One class of compounds are dithiocetyl- and S-acyl-2-thioethyl (SATE) esters (Fig. 4). The disulfide group takes advantage of the greater reducing potential within the cell to target the
Fig. 3. Prodrug (PD) utilized to deliver the parent drug (D) to the inside of a cell. The conversion of PD to D has to be faster intracellularly than extracellularly ($k_2 > k_1$).

cytosol. Dithiocarbonyl esters of ddUp and AZTp were hydrolyzed 30 times faster in cell extracts than in culture medium. The ddUp ester derivative was active in vitro whereas ddU and ddUp were not. The AZTp ester derivative was active in TK- cells in which AZT itself was inactive (Puech et al., 1993).

S-Acetyl-2-thioethyl derivatives can be hydrolyzed enzymatically by carboxylesterases which may be more prevalent intracellularly. The 2-thioethyl moiety collapses to episulfide and the nucleotide. However, episulfide is reported to be toxic on acute and chronic exposure in mice and rats (Pugaeva et al., 1969) and toxic and mutagenic in vitro (Luethy et al., 1981) which may limit the practical applications of this approach. The bis(SATE) ester of ddAp (Fig. 4) is reported to be more stable in serum complemented culture medium ($t_1 = 9$ h) than in CEM cell extracts ($t_1 = 10$ min), and displays little or no drop off in activity in CEM TK- cells (Benzaria et al., 1994).

Similarly, the acyloxymethyl group has been attached to both nucleoside phosphates and phosphonates with interesting biological activity in the resulting compounds. Specifically, bis(POM)PMEA (5) (Fig. 1) was found to be ~100-fold more potent in vitro due to increased cellular permeation (Starrett et al., 1992). Once inside the cell, the molecule is converted by esterases to PMEA. Radiolabel uptake experiments also showed that the intracellular levels of PMEA and its metabolites were 100 X higher when 5 was added to cells than when PMEA was applied (Srinivas et al., 1993).

Similarly, the bis(POM) derivatives of AZTp 10 (Pompon et al., 1994), ddUp 11 (Sastry et al., 1992) and FdUP 12 (Farquhar et al., 1994) were synthesized (Fig. 1).

![Chemical structures](image)

Fig. 4. Decomposition pathways of S-acyl-2-thioethyl (SATE) and dithioethyl esters.
Incubation of TK− CEM cells with 10 gave rise to intracellular AZTp, AZTpp and AZTppp, whereas no intracellular nucleotides were formed with AZT. From kinetic studies it was shown that the first step in the metabolism of 10 was catalyzed by a carboxyesterase. Compound 11 was effective in vitro against HIV in wild-type and CEM TK− cells, whereas the parent molecule dU, which is not phosphorylated by TK (Hao et al., 1990), was not. Compound 12 was found to have growth inhibitory potency in vitro against a cell line which was resistant to FU. It also retained therapeutic activity in vivo when dosed i.p. in mice against a P 388 leukemia line resistant to FU, indicating that 12 was an effective membrane permeable prodrug of 5-fluoro-dU.

The acyloxybenzyl derivatives A (Fig. 5) also rely on in vivo activation for activity. After chemical or enzymatic deacylation, the phenolic moiety B is oxidized or hydrolyzed to the p-quinonemethide to release the nucleotide (Thomson et al., 1993). However, the p-quinonemethides are very reactive alkylating agents (Filar and Weinstein, 1960). Acyloxybenzyl prodrugs of AZTp were prepared which showed comparable in vitro antiviral activity against HIV-1 and SIV to AZT, although they were more toxic than the parent (Thomson et al., 1993; Glazier et al., 1992). Similarly, acyloxybenzyl esters of PMEA were prepared and were found to be more potent in vitro than PMEA itself (Glazier et al., 1994).

Another class of nucleotide prodrugs are phosphoramidates which have one or two nitrogens attached to the phosphorous. The chemical hydrolysis of phosphoramidates proceeds rapidly at lower pH and, therefore, the compounds may utilize an intracellular pH trigger to target endosomes, lysosomes, or some tumor cells where the pH is lower than the normal physiological pH (Ross, 1961). Amino acid ester phosphoramidates C (Fig. 5) can also be hydrolyzed by esterases to form the free amino acid phosphoramide D which is unstable and undergoes hydrolysis to yield the free nucleotide (Judoka and Smrt, 1974). A wide variety of amines and amino acids have been utilized as prodrug moieties (McGuigan et al., 1990a, 1991, 1992; Gabrielsen et al., 1994). The phosphoramidate formed between AZTp and alanine methyl ester (13) (Fig. 6) was found to be active against HIV in a cell line restrictive to the activity of AZT due to
poor phosphorylation (McGuigan et al., 1992). The two substituents on the phosphorous have been connected by linkers to give cyclic phosphoramidates (Farquhar et al., 1983; Jones et al., 1984; Kumar et al., 1990). Amidates of cAMP and cGMP (A,B) (Fig. 6) have been prepared as diastereomeric mixtures (Russell and Moffatt, 1969; Meyer et al., 1973; Bentrude and Tomasz, 1984; Beres et al., 1985a,b; Bottka and Tomasz, 1985, 1988; Beres et al., 1986; Baraniak and Stec, 1987; Tomasz et al., 1987; Curley et al., 1990; Kinchington et al., 1992). The amidate prodrug (R=NH₂) (Fig. 6) is reported to hydrolyze under acidic conditions to a mixture of products, while the dialkylamidate (R=NMe₂) yields mainly cAMP (Meyer et al., 1973; Bottka and Tomasz, 1985; Tomasz et al., 1987). Therefore, the N,N-dialkyl phosphoroamidates may be useful prodrug moieties for cAMP although the behavior of these compounds in biological systems has not been investigated.

In order to completely mask the negative charge on phosphorous, a wide variety of triester prodrugs have been made of ara-Cp (Colin et al., 1989), ara-Ap (McGuigan et al., 1989), AZTp (Gouyette et al., 1989; Devine et al., 1990; McGuigan et al., 1990b; 1992; 1993a; Le Bec and Huynh-Dinh, 1991), ddCp (McGuigan et al., 1990b), Tp (Chawla et al., 1984), 3'-substituted Tp (Russell and Moffatt, 1969; McGuigan et al., 1990c), and 5-substituted dUp (Shuto et al., 1987; Shuto et al., 1988; Hostetter et al., 1990; Henin et al., 1991). The trihaloethyl and aryl esters have shown some good in vitro activity in CEM TK⁺ cells (McGuigan et al., 1991, 1992, 1993b). The R groups are sometimes connected by linkers to give cyclic derivatives (i.e. substituted 1,3-propanediols) (Farquhar et al., 1983; Hunston et al., 1984). In general, simple alkyl triesters are too stable to be useful as prodrugs and the resulting phosphorous esters are inactive; however, aryl esters and activated alkyl esters are capable of functioning as nucleotide prodrugs.
Alkylesters of AZT-5'-hydrogen phosphonates were evaluated as potential prodrugs of AZTp; however, they appear to act as depot forms of AZT rather than AZTp (McGuigan et al., 1994).

Phosphate esters of cAMP and cGMP have also been prepared (Nagyvary et al., 1973; Gohil et al., 1974; Engels and Pfleiderer, 1975a, b; Engels and Schlaeger, 1977; Engels and Reidys, 1978; Engels, 1979; Nargeot et al., 1983; Nerbonne et al., 1984; Kataoka et al., 1986, 1989, 1991; Nelson et al., 1987). These compounds were obtained as a diastereomeric mixture of axial (A) and equitorial (B) isomers at phosphorous (Fig. 6), with the axial isomer being thermodynamically favored (Engels and Schlaeger, 1977). Nucleophilic attack by thiol on the benzyl or methyl esters gives cAMP exclusively while alkaline hydrolysis predominantly affords 5'-AMP via ring opening (Gohil et al., 1974). The benzyl esters of cAMP and cGMP, especially the o-nitrobenzyl esters (Nargeot et al., 1983) are useful tools for studying the various intracellular roles these secondary messengers play (Postemak, 1974). The benzyl esters were shown in vitro to penetrate into cells and induce cAMP responses in guinea pig myocardium. The photolabile o-nitrobenzyl esters upon irradiation produce intracellular 'concentration jumps' of cAMP or cGMP, leading to cellular responses and changes in the strength and frequency of heartbeats in isolated bullfrog hearts (Korth and Engels, 1979; Nargeot et al., 1983; Nerbonne et al., 1984).

The intracellular delivery of nucleotides through prodrugs in vivo is complicated by differences in distribution, (e.g. uptake of the prodrug preferentially into certain tissues and organs), metabolism (e.g. preferential conversion of the prodrug to the nucleotide in the liver) and excretion (e.g. rapid excretion of the prodrug through the kidney). Such differences would be expected to lead to a different toxicity/efficacy profile of the prodrug. An example of the complexity with intracellular delivery of a nucleotide in vivo is the phosphoramidate prodrug of 6-aza-Up (Fig. 6) which exhibited antiviral activity in vitro similar to 6-azaUp except that 300- to 450-fold higher concentrations of 14 were required to attain comparable activity. However, in an in vivo model in mice, 14 showed enhanced activity when administered i.p. along with reduced toxicity. These results could imply that 14 delivered 6-aza-Up intracellularly in vivo, but it is also possible that the observed effects are due to differences in distribution or pharmacokinetics (Gabrielsen et al., 1994). Such differences could also be highly dependent on the corresponding animal model, which make any extrapolation to humans difficult. Another example is 5 which was shown to deliver high levels of the parent PMEA into cells resulting in 100x higher potency in vitro (Srinivas et al., 1993). However, in vivo, no intact 5 could be detected on oral application of 5 to monkeys and thus, 5 apparently in vivo merely delivers the parent PMEA to the blood but not intracellularly (Cundy et al., 1994). Thus, it is clear that tissue culture antiviral assays of prodrugs are poor predictors of prodrug function in vivo.

3.3. Altered pharmacokinetics, target organ specificity, tissue distribution and metabolism

Prodrugs can be utilized to change the pharmacokinetics of a nucleotide. This is warranted, for example, in cases where the nucleotide is excreted rapidly. In such cases, a prodrug can act as a slow release form. Prodrugs can also be utilized to change the
target organ specificity and tissue distribution. The objective of this approach can be to deliver the nucleotide to a site of action (e.g., the brain) or to decrease delivery to a site of toxicity (e.g., the kidney).

Historically, the first major work for nucleotide prodrugs was with ara-Cp. The objective was not only to increase the bioavailability of ara-C, but also to circumvent the deactivating actions of cytidine deaminase (Caminer and Smith, 1965). Numerous negatively charged lipophilic mono-esters of ara-Cp have been prepared with various ester groups attached including: alkyls and aryls (Saneyoshi et al., 1980; Rosowsky et al., 1982), steroids (Hong et al., 1979a, 1979b, 1980, 1985), sugars, 1,2-diacetylglycerol, 1,2-diamidoalkyl, oxyalkyl and thioalkyl ether glycerols, cyclic and long-chain aliphatic alcohols (Ryu et al., 1982). The ester 9 (Fig. 1) has recently been approved in Japan for the treatment of various leukemias via both parental and oral administration, and has been shown to slowly release ara-C in the liver. After a 5-day oral administration of 300 mg/day of the prodrug, ara-C concentrations in the blood corresponded to low-dose (15–20 mg/day) continuous infusion (Ohno et al., 1991; Kodama et al., 1989). Similar compounds have also been prepared of the 2,2'-anhydro ara-Cp 15 (Fig. 7), ara-Ap 16 (Ji et al., 1990), AZT (Hostetler et al., 1991; Piantadosi et al., 1991), as well as other nucleosides (Shuto et al., 1987, 1988; Hostetler et al., 1990; Ji et al., 1990).

Dioleoyl phosphatidyl ddC was shown to be less toxic in human HBV-infected hepatoma cells, and when administered to mice, the levels of ddC in the liver were 40 × greater than when ddC was administered (Hostetler et al., 1994). This may result in an improved therapeutic index for ddC in vivo in the treatment of HBV and this strategy may also be useful to target drugs to lymphoid tissues, important reservoirs of HIV infection (Hostetler et al., 1994b).
Ara-Ap has been conjugated with fetuin and lactosaminated serum albumin to target hepatocytes for the treatment of HBV virus (Fiume et al., 1980, 1981, 1984, 1986). The serum albumin lysosomotropic drug-carrier complex was stable in mouse blood after parental administration, was specifically released in the liver, and did not display any recognizable sign of acute bone marrow toxicity inherent in the unconjugated ara-A, although it was not determined whether ara-Ap or just ara-A was being delivered.

Targeting the blood–brain barrier has been classically addressed by applying Bodor’s nicotinic acid/dihydropyridine prodrug carriers (Bodor and Farag, 1983) to nucleotides (Torrence et al., 1988; Gogu et al., 1989; Palomino et al., 1989; Chu et al., 1990). For nucleotides, a glucosyl phosphothriester prodrug of AZTp (17) (Fig. 7) has been reported to improve the uptake of AZT in mouse brain. Oral dosing of 17 provided a 100-fold increase of AZT derivatives in the plasma and 1000-fold increase in the brain over AZT itself, with AZTp as the primary metabolite (Henin et al., 1991; Namane et al., 1992). This prodrug concept was borrowed from the glucosyl doicichol phosphate (Neumann et al., 1989) and could allow efficient delivery of nucleotides to the systemic circulation and the brain.

An example where the toxicity of the nucleotide was reduced by a prodrug approach is in the case of cyclic HPMPC (cHPMPC) 18 (Fig. 7) which was found to be an intracellular prodrug for HPMPC. Compound 18 is stable chemically and also in biological media, but gets converted intracellularly to the active parent compound, HPMPC. Compound 18 has similar in vitro and in vivo potency to HPMPC, but it displays much reduced nephrotoxicity in the rat. The reason for this reduced nephrotoxicity is presumably the fact that 18, because of its reduced charge, does not get transported into the proximal convoluted tubular cells by the anion-specific transport system in the kidney as efficiently as HPMPC itself (Bischofberger et al., 1994).

References


Chawla, R.R., Freed, J.J. and Hampton, A. (1984) Bis(2-nitrophenyl) and bis(p-nitrophenyl) esters and the bioavailability of antiretroviral agent 9-(2-phosphonylmethoxyethyl)adenine (PMEA) from three formula­tions of the prodrug bis(pivaloyloxymethyl)-PMEA in fasted male cynomolgus monkeys. Pharm. Res. 11, 839-843.


Dried dissolved in 6.1 (1H, J = 8 Hz) gave the product at 34°C. After the product was purified by chromatography on silica using 5% methanol in chloroform as eluant. Pooling and evaporation of appropriate fractions, followed by crystallisation from ethyl alcohol gave the product as a white solid (52.2 g, 86%). Σνh (DMSO) 3.3(2H, d, H4), 4.0(1H, m, H3), 4.5(1H, m, H2'), 4.6(1H, m, H2'), 5.3(H, d, H1, J = 8 Hz), 5.3(1H, d, H5, J = 8 Hz), 5.4(1H, d, H5, J = 8 Hz), 5.7(1H, d, H6, J = 8 Hz), 9.4(1H, s, NH); FAB MS measured 544.1379 (M + H), 299.0739 (M + H) requires 544.1379. 

Thus, 5'-trityl uridine (2.15 mmol) was added to a solution of uridine (3 g, 12.3 mmol) in pyridine (50 ml) and the reaction mixture heated at 100°C for 24 h. The solvent was removed under high vacuum and the residue purified by chromatography on silica using 5% methanol in chloroform as eluant. Pooling and evaporation of appropriate fractions, followed by crystallisation from ethyl alcohol gave the product as a white solid (595 mg, 91%). Σνh (CDCl3) 3.5(2H, m, H5'), 4.5(1H, m, H4'), 5.6(1H, d, H5, J = 9 Hz), 6.0(1H, d, H6, J = 9 Hz), 6.0(1H, d, H6, J = 9 Hz), 7.0(5H, m, HI, H2', H3', H4', H5'). 7.2-7.6(16H, m, H3'). 9.1(1H. s, NH). 10.8(1H, s, NH) requires 544.1379. 

At 34°C the solvent was evaporated and the crude product purified entirely as described for (3a) above, except that the second chromatographic column was necessary, using an eluant of 15% methanol in diethyl ether, in order to obtain pure (3b) (0.051 g, 54%). Σνh (CDCl3) 1.30(4H, m, Ala-Me, POCC), 2.0(3H, m, H2', H3'), 3.4(2H, m, H2'), 4.15(1H, m, H4'), 5.60, 5.65(1H, s, NH); FAB MS measured 544.1379 (M + H), 299.0739 (M + H) requires 544.1379. 

Results and discussion

2.2. Virology

2.2.1. Antiviral assays. The anti-HIV-1 activities and toxicities of compounds were assessed in two cell lines [14]. C8166 (a normal T-cell transformed by co-cultivation with leukemia lymphocytes harbouring HTLV-1) were infected with the IL-2 strain of HIV-1. Similarly, JM, a semi-mature T-cell line derived from a patient with lymphoblastic leukaemia, were infected with HIV-1 strains GIB or HIB. JM cells are relatively resistant to the antiviral effects of AZT and a number of its derivatives.

Cells were grown in RPMI 1640 with 10% calf serum. 4 x 105 cells per microtitre plate well were mixed with 5-fold dilutions of compound prior to addition to the cells. Cell viability was assessed after 12-16 h incubation at 37°C. Bound antigen was detected using an ELISA assay. 

The plates were coated with 5% carboxymethylcellulose and blocked with 10% foetal bovine serum. Virus supernatants in 0.25% Empigen solution was added to the wells and incubated at 37°C for 12-16 h. Bound antigen was detected using an ELISA assay.
allowed to react with bis(2,2,2-trichloroethyl) phosphate (3a) in good yield. This
dield the best route to d4U was via the 5'-trityl protected
phosphate (3c) is a potent agent, being approximately
50-times more active than the parent nucleoside (3e) is a potent
agent, being approximately 50-times more active than the parent
nucleoside analogue. This confirms the importance of data derived from
assays in kinase deficient cells for the interpretation of the
activities of blocked nucleotides. As we have noted [20] only

overall yield by this route was 29%. Thus, in terms of
yield the best route to d4U was via the 5'-trityl protected
2',3'-didehydro compound d4U generated by either of
these routes was hydrogenated to give d4U (2) in 83%
yield, and the structure and purity of this compound was
confirmed by 'H, 13C NMR, mass spectrometry and HPLC.

The nucleoside analogue was then subjected to 5'-
phosphorylation with a range of phosphates. A labile
phosphate blocking group is clearly a prerequisite for
the masked phosphate approach, in order to facilitate
intracellular release of the free nucleotides. Thus, simple
dialkyl phosphate derivatives of AZT are extremely
resistant to phosphate hydrolysis, and they display no
antiviral effect [1]. On the other hand bis(trihaloethyl)
phosphate derivatives are potent inhibitors of viral
proliferation [5]. We have also noted that one of these phosphate
masking groups, the bis(2,2,2-trichloroethyl) phosphate
moiety is successful in the kinase by-pass activation of
certain inactive 3'-modified nucleosides [11-12]. We
therefore chose this phosphate as the first blocking group
for d4U.

Thus, d4U was allowed to react with bis(2,2,2-trichloro-
yethyl) phosphorochloridate in tetrahydrofuran con-
taining N-methyl imidazole at room temperature to give
(3a) in good yield. This was fully characterised by heter-
onuclear NMR, FAB mass spectrometry, and HPLC, all
data being consistent with its structure and purity. We
have also noted that certain phosphoramidate deri-
atives of AZT are potent and selective inhibitors of HIV
[4]. Thus, ethyl methoxyalaninyl phosphorochloridate
was prepared by methods we have reported [4] and was
allowed to react with d4U to give the target compound
(3b) in moderate yield. This compound was recently re-
ported by another group, following our earlier phosphor-
amidate strategy [13]. However, we have found that
aryloxy phosphoramidates are especially potent phos-
phate blocking groups for AZT, and appear to release
the free nucleotides within cells, on the basis of data in
thymidine kinase-deficient cells [6,20]. Thus, phenyl
methoxyalaninyl phosphorochloridate was similarly pre-
pared [20] and was allowed to react with d4U to give (3c)
in good yield. Full carbon-13 and (where appropriate)
phosphorus-31 NMR data for the nucleoside (2) and the
phosphates (3a-c) are given in Table 1.

The parent nucleoside (2) and the corresponding
masked phosphates (3a-c) were tested for their ability to
inhibit the replication of HIV-1 in C8166 cells, and in
thymidine kinase-deficient [JM] cells, data being pre-
sented in Table 2. As expected, the parent nucleoside (2)
is active only at the highest concentrations tested, and is
especially inactive in JM cells. The bis(trichloroethyl)
phosphate (3a) is approximately 5-10 times more active
in each assay. On the other hand, the simple phospho-
ramidate (3b) is devoid of antiviral activity in this assay,
as has been recently noted by other researchers in a
different assay [13]. However, the aryloxy phosphorami-
date (3e) is a potent agent, being approximately 30-times
more active than the parent nucleoside analogue. This
confirms the importance of data derived from assays in
kinase deficient cells for the interpretation of the activi-
ties of blocked nucleotides. As we have noted [20] only

Table 1
Carbon-13 and phosphorus-31 NMR data for compounds (2) and
(3a-c)

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<td>-</td>
<td>77.3*</td>
<td>-</td>
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<td>P-OAr</td>
<td>POC</td>
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<td>94.7*</td>
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*Multiplie. All spectra were recorded in CDCl 3 , except for (3b) which
were recorded in CDC 6 , plus CD 3 OD. Data are presented as ppm.
Many carbon peaks for (3b) display diastereomeric splitting arising
from mixed stereoisomerism at the phosphate centre.

Fig. 1. The structures of potential anti-HIV nucleoside and nucleotide
anallogues.
such data will allow a clear understanding of the likely mechanism of action of blocked nucleotides, and discriminate between the release of nucleosides and nucleotides.

In conclusion, we report the antiviral activity of certain masked phosphate derivatives of the inactive nucleoside dU. We note that anyloxy phosphoramidates are particularly efficacious, and attribute the introduction of activity to kinase by-pass. Finally we stress the importance of data in kinase-deficient cells for the clear interpretation of results on blocked phosphates.

Acknowledgements: We thank the AIDS Directed Programme of the Medical Research Council and the Biomedical research Programme of the European Community for financial support.

References


Table 2

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The antiviral activity and cytotoxicity of test compounds in two different cell lines. ED₅₀ represents the concentration of compound (in μM) that decreases viral antigen production in infected cells to 50% of control. CC₅₀ represents the concentration of compound (in μM) which causes 50% cytotoxicity to uninfected cells.
Characterization of the Activation Pathway of Phosphoramidate Triester Prodrugs of Stavudine and Zidovudine

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Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium (D.S., L.N., E. De C., J.B.); and Welsh School of Pharmacy, University of Wales, Cardiff, United Kingdom (D.C., A.S., R.P., S.V., C.M.)

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ABSTRACT

The phosphoramidate triester prodrugs of anti-human HIV 2',3'-dideoxynucleoside analogs (ddN) represent a convenient approach to bypass the first phosphorylation to ddoN 5'-monophosphate (ddoNMP), resulting in an improved formation of ddoN 5'-triphosphate and, hence, higher antiviral efficacy. Although phosphoramidate derivatization markedly increases the anti-HIV activity of 2',3'-dideoxynucleoside (d4T) in both wild-type and thymidine kinase-deficient CEM cells, the concept is far less successful for the 5'-azido-2',3'-dideoxythymidine (AZT) triesters. We now investigated the metabolism of triester prodrugs of d4T and AZT using pure enzymes or different biological media. The efficiency of the first activation step, mediated by carboxylesterases, consists of the formation of the amino acyl ddoNMP metabolite. The efficiency of this step was shown to be dependent on the amino acid, acyl ester, and ddoN moiety. Triesters that showed no conversion to the amino acyl ddoNMP accumulated as the phenyl-containing intermediate and had poor, if any, anti-HIV activity. In contrast to the relative stability of the triesters in human serum, carboxylesterase-mediated cleavage of the prodrugs was found to be remarkably high in mouse serum. The subsequent conversion of the amino acyl ddoNMP metabolite to ddoNMP or ddoN was highest in rat liver cytosolic enzyme preparations. Although L-alaninyl-d4TMP was efficiently converted to d4TMP, the main metabolite formed from L-alaninyl-AZTMP was the free nucleoside (AZT), thus explaining why d4T prodrugs, but not AZT prodrugs, retain anti-HIV activity in HIV-infected thymidine kinase-deficient cell cultures. The rat liver phosphoramidase responsible for the formation of ddoNMP was shown to be distinct from creatine kinase, alkaline phosphatase, and phosphodiesterase.

2',3'-Dideoxynucleoside analogs (ddN) that are active against HIV [i.e., zidovudine (AZT), stavudine (d4T), didanosine, zalcitabine, and lamivudine] must be converted after cell penetration to their corresponding 5'-triphosphate metabolites to act as inhibitors of HIV reverse transcriptase (Balzarin and De Clercq, 1999). However, for several ddNs, the first phosphorylation catalysed by cellular kinases (i.e., thymidine kinase [TK] in the case of d4T and AZT) is the rate-limiting step that determines the eventual antiviral activity. In vitro studies on the metabolism of ddN in tumor cell lines or mitogen-stimulated lymphocytes from HIV-infected persons is about 3-fold lower than that seen in seronegative individuals. In addition, the in vitro and ex vivo data of Antonelli et al. (1996) strongly suggest that long-term treatment with ddN may result in a reduction of TK activity and, hence, reduced phosphorylation efficiency of the lymphocytes. Circumvention of this initial activation step is possible by the design of membrane-soluble prodrugs that deliver directly the ddN 5'-monophosphate (ddoNMP) into the HIV-infected cells. Among the several types of nucleoside prodrugs that have already been synthesized, a series of phosphoramidate triesters have emerged as highly promising antiviral agents (Farrov et al., 1996; Val­latt et al., 1996; Winter et al., 1996; Balzarni et al., 1997; Meier et al., 1997). These triesters consist of a ddoNMP for which the phosphate is linked, on one side, to a lipophilic (aryl) group and, on the other side, to an amino acid moiety, via a phosphoramidate (P=N) bond. The L-alaninyl-d4TMP phosphotriester (Fig. 1) can be considered the prototype compound of the phosphoramidate prodrug concept (Balzar­ini et al., 1996a; McGuigan et al., 1996). Our previous metabolism studies with radiolabeled 2 in human lymphocyte CRM cells revealed that this phosphoramidate triester is

ABBREVIATIONS: ddN, 2',3'-dideoxynucleoside analog; ddoNMP, 2',3'-dideoxynucleoside 5'-monophosphate; ddoNYP, 2',3'-dideoxynucleoside 5'-triphosphate; d4T, 2',3'-didehydro-2',3'-dideoxythymidine; PMSF, phenylmethylsulfonyl fluoride; AZT, 3'-azido-2',3'-dideoxythymidine; C020, 50% cytotoxic concentration; HIV, human immunodeficiency virus; IM, intermediate metabolite; AAM, amino acyl metabolite.

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able to deliver d4TMP intracellularly (Balzarini et al., 1996a). Consequently, the independence of this prodrug from cellular TK resulted in a markedly improved antiviral activity in TK-deficient cells (CEM/TK-) compared with the parent nucleoside d4T (Balzarini et al., 1996b; McGuigan et al., 1996).

The phosphoramidate prodrug technology has been used for the synthesis of a series of closely related amino acyl phosphoramidate triester derivatives of d4T, AZT, and lamivudine (Devine et al., 1996; McGuigan et al., 1996; Valette et al., 1996). The antiviral activity of these nucleoside prodrugs is determined by the different structural parts of the molecule (i.e., the nature of nucleoside, the amino acid, and the alkyl group). However, it is not fully understood how the antiviral data can be correlated to the intracellular decomposition pathway followed by the phosphoramidate derivatives.

Several data indicate that the first step in the activation pathway consists of carboxylesterase-mediated hydrolysis of the carboxylic ester function in the amino acid part (McGuigan et al., 1998; Naesens et al., 1998). This esterase cleavage is thought to be followed by an intramolecular nucleophilic attack of the phosphorus by the carboxylester group with spontaneous elimination of phenol after transient formation of a five-membered cyclic intermediate (Fig. 1). This is followed by the conversion of the ddNMP amino acyl metabolite (AAM) to free ddNMP. It has not been clarified whether cleavage of the N–P bond is predominantly catalyzed by one or more less specific phosphatases (that normally use phosphate esters as a substrate) or by a distinct and specific phosphoramidase (Holzer et al., 1966; Holzer et al., 1966; Ferenley, 1971; Snyder and Wilson, 1972; Kelly et al., 1975; Nishino et al., 1994). Phosphoramidases that catalyze the hydrolysis of phosphoramidate compounds have been described in mammalian cells and bacteria (Singer and Fruton, 1957; Stevens-Clark et al., 1968; Parvin and Smith, 1969; Kuba et al., 1995; Abraham et al., 1996) and have been characterized in more detail by Shabarova and coworkers (Shabarova, 1970; Ledeva et al., 1987, 1975, 1971; Duclein et al., 1971a, b; McIntee et al., 1997).

We now investigated the activation pathway of a series of phosphoramidate prodrugs of d4TMP and AZTMP in different biological media (i.e., CEM cell extracts, human serum, mouse serum, and rat liver). The purpose of this study was to reveal the influence of the nature of nucleoside, amino acid, and alkyl moiety on the conversion of the triester to ddNMP, with the aim of optimizing the design of new phosphoramidate derivatives.

**Materials and Methods**

**Cells and Viruses.** Wild-type CEM cells (C93N40) were obtained from the American Type Culture Collection (Rockville, MD). The TK-deficient cell line (CEM/TK-) was kindly provided by Prof. Stefan Eriksson (Swedish University of Agricultural Sciences, Uppsala, Sweden) and Prof. Anna Karlinson (Karolinska Institute, Stockholm, Sweden). CEM/0 and CEM/TK- cells were grown in 75-cm² flasks in RPMI 1640 medium (GIBCO, Paisley, Scotland) supplemented with 10% FCS (GIBCO). 2 mM glutamine (GIBCO), and 0.075% sodium bicarbonate (GIBCO). HIV-1 (strain IIb) was a generous gift from Dr. R. C. Gallo (at that time at the National Cancer Institute, Bethesda, MD). HIV-2 (strain ROD) was kindly provided by Dr. L. Montagnier (Pasteur Institute, Paris, France).

**Enzymes.** Pig liver carboxylesterase (EC 3.1.1.1), 5' nucleotidase (EC 3.1.3.5, from Crocius alaminatus), and phosphodiesterase I Type VI (EC 3.1.4.1, from C. alaminatus) were purchased from Sigma Chemical Co. (St. Louis, MO). Alkaline phosphatase.
Activation of Phosphoramidate Prodrugs of d4T and AZT

Activity of recombinant human carboxylesterase was measured by HPLC analysis. The enzyme was incubated with the prodrug and with substrate to determine the rate of hydrolysis of the prodrug to the active form. The reaction was monitored by measuring the decrease in absorbance at 300 nm due to the formation of the active form. The enzyme activity was expressed as units per milligram of protein.

HPLC analysis was performed using a Waters 510 HPLC system with a Waters 996 photodiode array detector. The chromatographic conditions were as follows: column, Inertsil ODS-3 (5 μm, 4.6 × 250 mm); mobile phase, 0.01 M sodium dihydrogen phosphate (pH 7.0) and acetonitrile (97:3); flow rate, 1 mL/min; detection wavelength, 254 nm; injection volume, 10 μL. The retention times for the prodrug and its active form were 2.5 and 5.0 minutes, respectively.

The reaction mixture was incubated at 37°C for 24 hours, and the reaction was stopped by the addition of acetonitrile. The mixture was then analyzed by HPLC to determine the conversion of the prodrug to the active form. The percentage of conversion was calculated by comparing the peak areas of the prodrug and the active form.

Enzyme Hydrolysis of Phosphoramidate Prodrugs with Pig Liver Carboxylesterase. The 200 μM substrate solution was incubated with pig liver carboxylesterase (10 U/mL) at 37°C for 1 hour. The reaction was monitored by measuring the increase in absorbance at 250 nm due to the formation of the active form. The enzyme activity was expressed as units per milligram of protein.

The reaction mixture was incubated at 37°C for 1 hour, and the reaction was stopped by the addition of acetonitrile. The mixture was then analyzed by HPLC to determine the conversion of the prodrug to the active form. The percentage of conversion was calculated by comparing the peak areas of the prodrug and the active form.

Carboxylesterase. The enzyme was purchased from Sigma and was used as received. The enzyme activity was measured by the release of p-nitrophenol from p-nitrophenyl acetate. The enzyme was incubated with the substrate at 37°C for 30 minutes, and the reaction was stopped by the addition of acetonitrile. The mixture was then analyzed by HPLC to determine the conversion of the prodrug to the active form. The percentage of conversion was calculated by comparing the peak areas of the prodrug and the active form.

Inhibition of Phosphoramidase Activity by Iodobenzene. Partially purified liver enzyme extract was preincubated with several concentrations of iodobenzene (1, 0.1, 0.01, 0.001, and 0.0001 μM) for 30 minutes at 37°C. The substrate (d4T or AZT-P) was then added, and the reaction was monitored by measuring the increase in absorbance at 250 nm due to the formation of the active form. The percentage of inhibition was calculated by comparing the peak areas of the prodrug and the active form in the presence and absence of iodobenzene.
mL/min by a linear gradient from 100% A to 97% B plus 3% acetonitrile (0-20 min) and then to 90% buffer B plus 10% acetonitrile (20-60 min).

The last conditions were maintained isocratically (60-70 min), followed by a linear gradient to 100% buffer A (70-75 min), and ended by a reequilibration step (75-80 min). The peaks were identified based on comparison with synthetic standards. The retention times for the phosphoramidate prodrugs of d4TMP and AZTMP were in the range of 56 to 64 min. For compound 2, the retention times were 56, 52, 49, 31, and 29 min, for the prodrug, the intermediate metabolite (IM), the AAM, d4TMP, and d4T, respectively. For compound 14, the retention times for the prodrug, the IM, the AAM, AZTMP, and AZT were 50, 57, 53, 44, and 43 min, respectively. The other prodrugs and their corresponding metabolites had similar elution patterns.

In addition, the samples were analysed on an anion exchange Partispher SAX column (6 μm, 4.6 × 125 mm; Whatman) to quantify d4TMP and AZTMP and to allow better identification of AAM and IM. The column was equilibrated with 50% buffer A (5 mM ammonium dihydrogen phosphate, pH 5.0) plus 50% water. The samples were separated at a flow rate of 2 mL/min by the following gradient: 50% buffer A and 50% water (0–5 min), linear gradient to 90% buffer A plus 10% buffer B (0.25 M ammonium dihydrogen phosphate, pH 5.0, 5–50 min), then isocratic conditions (50%, 50% buffer A plus 50% water, 5–45 min), and finally reequilibration during 13 min. Under these conditions, the retention times for L-alanyl-d4TMP, t-alaninyd4TMP, and AZT were 21, 26, 15, and 13 min, respectively.

## Results

**Anti-HIV Activity in CEM/0 and CEM/TK cells.** A selection of phosphoramidate triester derivatives of d4TMP and AZTMP, carrying different amino acids (Fig. 2), were evaluated for their antiviral activity against HIV-1 and HIV-2 in CEM/0 and CEM/TK cells (Table 1). For both the d4TMP and AZTMP phosphoramidate derivatives, L-alanine was shown to be the preferred amino acid. Among the d4TMP triesters, the t-alaninyl derivatives 1, 2, and 3, carrying different ester moieties on the alanine part, ranked among the most active compounds, with the EC_{50} value against HIV-1 and HIV-2 in CEM/0 cells being 0.05 to 0.08 μM. Modification of the amino acid moiety resulted in partial or virtually complete loss of antiviral activity compared with the t-alaninyl prodrug derivative. Relatively small structural changes of the amino acids had a marked effect on the eventual antiviral activity. For instance, the t-alanine compound 2 is 40–5000–fold more active than the corresponding L-alanine 5, β-alanine 12, or glycine 6 prodrugs. As a rule

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**Table 1.** Amino acid compositions of d4TMP and AZTMP derivatives

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<td>[18]</td>
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**Fig. 2.** Chemical structures of the aryloxyphosphoramidate esters of d4TMP (left) and AZTMP (right).
for all of the d4TMP triesters tested, the anti-HIV activity was not markedly different in CEM/0 and CEM-TK- cells. By contrast, the anti-HIV activity of the triesters of AZTMP was significantly lower in CEM/TK- than CEM/0 cells, and the effect of the different amino acids on the eventual antiviral activity of the AZTMP prodrugs was less pronounced than that observed for the d4TMP prodrugs. From these data, it can be concluded that the antiviral activity of the phosphoramidate prodrug derivatives is strongly dependent on the nature of the nucleoside moieties (d4T or AZT) and the amino acid substituents.

Concentration by Esterase, CEM Cell Extract, Human Serum, or Mouse Serum. The metabolism of the d4TMP prodrugs to the corresponding AAM was obtained with the compounds containing L-alanine (1, 2, and 3), methyl-L-aspartic acid (4), t-alanine (5), glycine (6), and L-phenylalanine (10). No formation of the AAM was observed with the L-valine derivative 8 (prodrug kept 100% intact) and the β-alanine derivative 12, in which case no IM accumulated. Finally, an incomplete conversion of the triester to AAM was observed with the compounds containing L-leucine (7), t-methionine (9), and methyl-L-glutamic acid (11). For these three compounds, a mixture was obtained containing intact prodrug, AAM, and/or IM.

Similar to what was seen with the d4T prodrugs, complete conversion to AAM was obtained for the AZT derivatives containing L-alanine (14), t-alanine (16), t-phenylalanine (17), and glycine (13). The AZT derivatives containing L-leucine (15) and methyl-L-glutamic acid (18) showed an incomplete conversion to AAM, giving a mixture of AAM and IM. For these two compounds, formation of AAM was found to be somewhat less efficient than that for the corresponding d4T compounds.

Next, we determined the carboxylesterase-mediated metabolism of the d4T and AZT prodrugs in different biological media (i.e., CEM cell extract, human serum, and mouse serum). As can be seen in Table 2, the relative conversion patterns in these biological media were fairly comparable to those observed for pig liver carboxylesterase. In all cases, 8 was found to be fully stable. For 12, no AAM was formed, due to the stability of the IM. For the other compounds, the conversion to AAM was most pronounced in mouse serum and least efficient in human serum, whereas an intermediate enzyme activity was present in CEM cell extract. In all three media, the L-alanine derivatives of d4TMP and AZTMP (2 and 14, respectively) were among the best converters to their AAMs. For all d4T prodrugs except for 11 and 12, no accumulation of the IM was seen.

Interestingly, a few AZTMP prodrugs showed partial accumulation of the IM (i.e., 15 and 17) that was not observed for their corresponding d4TMP derivatives. This is presumably due to a higher chemical stability of the IM.

Finally, when the pig liver carboxylesterase or mouse serum was preincubated during 30 min in the presence of the serine protease inhibitor PMSF (final concentration, 10 mM), which is known to also be an inhibitor of carboxylesterase (Shne and Mitra, 1994), followed by the addition of prototype compounds of the AZTMP prodrug or d4TMP prodrug and overnight incubation, the formation of their AAM was inhibited by more than 90%.

As shown in Table 2, the stability of the L-alanine derivatives of d4T on incubation in CEM cell extracts and in human serum was also found to depend on the alkyl moiety, with the conversion rate to AAM in human serum being 50, 62, and 23% for the benzyl, methyl, and ethyl derivatives, respectively. We therefore extended the stability studies in

Table 2

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* The anti-HIV activity against HIV-1 or HIV-2 was determined in wild-type CEM/0 or TK-deficient CEM-TK- cells and expressed as EC50, or compound concentration that results in a 50% inhibition of virus-induced cytopathy.

* The cytotoxicity of the compounds was determined in CEM/0 cells and expressed as CC50, or compound concentration that suppresses the cell proliferation by 50%.
human serum to a large series of L-alaninyl-d4TMP prodrug derivatives, with variations in the alkyl moiety (Fig. 3). To better discriminate between the compounds, the incubation was performed during 3 h (instead of overnight as in the previous studies). The two most striking extremes in stability were the compounds containing phenyl (least stable) and tert-butyl (most stable). Compared with the prototype compound 2, which showed an intermediate stability in human serum, the ethyl derivative was more stable, whereas the benzyl compound was least stable. These data were confirmed in studies with pig liver carboxylesterase. The percentages of prodrug left after 10 min incubation were 1.5, 31, 66, 82, and 56% for the derivatives containing phenyl, benzyl, methyl, ethyl, and tert-butyl, respectively.

Optimization of Rat Liver Enzyme Preparation. Incubation of the prodrugs in CEM cell extract, human serum, or mouse serum resulted in only trace levels of ddNMP. These data suggest that an enzyme is involved in the cleavage of the phosphoramidate linkage of the AAM d4TMP to ddNMP.

To obtain a partially purified enzyme preparation that is able to convert the AAM to ddNMP (or ddN), we used rat liver as the enzyme source because it is known that liver is rich with hydrolytic enzymes, including amidases (Ledeneva et al., 1967, 1970; Shabarova, 1970). Our procedure was based on the method of Khandwalla and Smith (1997). The enzyme was partially purified by ammonium sulfate precipitation. The fraction between 32% and 42% was used. The purification was about 50-fold. The enzymatic activity was determined by monitoring the formation of ddTMP from L-alaninyl-d4TMP. The L-alaninyl-d4TMP source was obtained from high amounts of 2, exposed to pig liver carboxylesterase. After an extraction and cleaning step with C18 cartridge columns, we obtained an AAM yield of 95% and a compound purity of 98%, as assessed by HPLC.

Six different procedures for partial purification of the rat liver phosphoramidase were performed (see Materials and Methods). The phosphoramidase activity in these enzyme fractions was determined from the percentage of L-alaninyl-d4TMP converted to d4TMP plus d4T. The values for the different enzyme preparations were normalized for an equal amount of total protein. Preparations 1 and 2, corresponding to the microsomal fraction of the liver cells, showed a weak phosphoramidase activity (1.6 and 2.4% conversion, respectively). In contrast, a much higher phosphoramidase activity (6.8% conversion to ddNMP plus ddN) was present in preparation 3, obtained through ammonium sulfate precipitation of the cytosolic fraction. This enzymatic activity could be further increased to 23.1% by the addition of 10 mM β-mercaptoethanol during preparation (4), whereas EDTA had no influence (preparation 5; 25% conversion). However, the highest phosphoramidase activity was recovered from rat liver by omitting the centrifugation step at -80°C and was routinely used in the incubation studies with the phosphoramidate derivatives.

Metabolism of AAM in a Rat Liver Enzyme Preparation. Table 3 shows the metabolism of the triester prodrugs of d4T or AZT after overnight incubation in the rat liver enzyme preparation 6. The conversion of the prodrugs to d4TMP was most pronounced for the derivatives containing L-tyrosine, followed (in decreasing order) by methyl-L-aspartic acid, glycine, and D-alanine.

TABLE 3

<table>
<thead>
<tr>
<th>Number</th>
<th>Amino Acid/Ester</th>
<th>Carboxylesterase</th>
<th>Human Serum</th>
<th>CEM Extract</th>
<th>Mouse Serum</th>
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<tr>
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</table>

| AZT derivatives | | | | | |
|-----------------|------------------|-------------|-------------|-------------|
| 13               | Glycine/Me       | 0 0 100      | 0 0 100    | 0 0 100     |
| 14               | L-Alanine/Me     | 0 0 100      | 0 0 100    | 0 0 100     |
| 15               | L-Leucine/Me     | 0 0 100      | 0 0 100    | 0 0 100     |
| 16               | L-Phenylalanine/Me | 0 0 100 | 0 0 100 | 0 0 100 |
| 17               | Methyl-L-aspartic acid/Me | 0 0 100 | 0 0 100 | 0 0 100 | 0 0 100 |

* The phosphoramidase activities of d4TMP or AZT were incubated overnight with carboxylesterase or with biological media, after which the remaining intact prodrug and the IM and AAM following metabolism were determined by HPLC.

* Due to the slower formation of ddNMP + AAM ~ 75%, the total intact prodrug + IM + AAM was not 100%.
For these prodrugs, metabolism to d4T was low because the d4T levels were 8- to 32-fold lower than those measured for d4T-MP. In contrast, no d4T or d4TMP was formed after incubation of 7, 8, 9, 10, 11, and 12 under our experimental conditions.

Interestingly, d4TMP formation from the benzyl derivative 1 was found to be considerably lower than that from the ethyl 3 or methyl 2 derivatives, although the AAM was in all three cases l-alaninyl-d4TMP. This suggested that the benzyl alcohol that was released from 1 on carboxylesterase-mediated formation of the AAM inhibited the further conversion to d4TMP. To check this hypothesis, an experiment was performed in which 2 or l-alaninyl-d4TMP was incubated with rat liver enzyme preparation in the presence of 10 μM benzyl alcohol. No formation of d4TMP was formed from either 2 or l-alaninyl-d4TMP. In contrast, 10 μM ethanol or methanol had no effect on the conversion of the AAM to ddNMP.

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**Activation of Phosphoramidate Prodrugs of d4T and AZT**

---

**TABLE 3**

<table>
<thead>
<tr>
<th>Number</th>
<th>Amino Acid/Ester</th>
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<th>IM</th>
<th>AAM</th>
<th>ddNMP</th>
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*The prodrug derivatives of d4TMP or AZTMP were incubated overnight with the rat liver enzyme preparation, after which the remaining intact prodrug and the following metabolites were determined by HPLC: IM, d4TMP or AZTMP (ddNMP), and free d4T or AZT (ddN).*
From a comparison of compounds 5 and 2, it is clear that formation of d4TMP from the L-alaninyl-d4TMP metabolite is much less efficient than that from the L-alaninyl-d4TMP metabolite. This is in sharp contrast to what was seen for the corresponding AZT prodrugs 12 and 14 because for these compounds, the total rate of conversion to AZTMP plus AZT was 19%. In fact, a clear difference was visible between the L-alaninyl derivatives of d4T 2 and AZT 14, with the total conversion to ddN plus ddNMP being 71% and 10%, respectively. Finally, all the AZT prodrugs tested showed conversion to free AZT, whereas for the d4T prodrugs, formation of the free nucleoside was much less pronounced or not detectable under our assay conditions.

Conversion of AAMs to d4TMP or AZTMP. Pure L-alaninyl-d4TMP and L-alaninyl-AZTMP (prepared from 2 and 14 by carboxylesterase-mediated hydrolysis) were incubated overnight in the rat liver enzyme preparation. The marked differences in their metabolism are clearly depicted in Fig. 4. After 2-h incubation, 20% of L-alaninyl-d4TMP was converted, with the main metabolite being d4TMP, whereas the d4T formation was negligible at this time point. After 16 h, only 20% of L-alaninyl-d4TMP was left, 59% was present as d4T, and 21% was present as d4T.

In contrast, conversion of L-alaninyl-AZTMP to AZTMP was markedly slower and much less efficient: after 16 h, 84% of L-alaninyl-AZTMP had remained intact, and the percentage of AZTMP and AZT was 6% and 10%, respectively.

To study the enzymatic stability of the ddNMPs under these experimental conditions, an overnight incubation of AZTMP and d4TMP was performed in the rat liver preparation. Enzymatic hydrolysis to free ddN was found to be much more pronounced for AZTMP than for d4TMP. For instance, the percentage hydrolysis of d4TMP was 6% and 35% after 1- and 16-h incubation, respectively, whereas for AZTMP, the percentage hydrolysis was 24% and 70% after 1- and 16-h incubation, respectively.

To further determine whether the enzyme responsible for conversion of AAMs to ddNMP had phosphoramidase activity, the effect of iodo benzene, which is structurally closer related to the phosphoramidase-inhibitor iodochlorhydroxyquin (Fruton, 1957), was determined. A complete inhibition of L-alaninyl-d4TMP conversion to d4TMP in the rat liver preparation was achieved with iodo benzene at a concentration of 1 μM (Fig. 5). At lower concentrations, iodo benzene inhibited the phosphoramidase activity in a concentration-dependent manner, with a 50% inhibitory concentration of 10 μM (defined as the concentration at which the formation of d4TMP plus d4T was inhibited by 50%). It should be mentioned that iodo benzene was inactive as an inhibitor of phosphodiesterase and carboxylesterase at 1 μM (data not shown).

In addition, we examined the effect of phosphocreatine on the metabolism of L-alaninyl-d4TMP during overnight incubation of L-alaninyl-d4TMP with our partially purified rat liver phosphonamidase preparation. Phosphocreatine at a concentration of 10 mM caused a partial inhibition (about 50%) of d4TMP formation. On the other hand, the effect of 2 or L-alaninyl-d4TMP on the creatine phosphokinase-dependent metabolism of phosphocreatine was also determined. Creatine phosphokinase catalyzes the reversible transfer of a phosphate group from phosphocreatine to ADP to form creatine and ATP. An inhibition assay was performed in which phosphocreatine and ADP (both at 1 mM) were incubated with creatine phosphokinase (6 U/ml) in the presence of 2 (5 mM) or L-alaninyl-d4TMP (2 mM). Neither phosphoramidase compound had any influence on ATP formation (as determined by HPLC).

Finally, we investigated the metabolism of 2, 14, and their AAMs by different commercial enzymes, namely, phosphodiesterase I (type VI), alkaline phosphatase, and 5'-nucleotidase. Enzymatic hydrolysis of the AAM was observed only with phosphodiesterase I (0.5 U/ml). After overnight incubation, 40% of L-alaninyl-d4TMP was metabolized, giving 36% d4TMP and 4% d4T, whereas L-alaninyl-AZTMP was 67% converted, giving 14% AZTMP and 53% AZT. This phosphodiesterase activity was not inhibited by 10 μM iodo benzene. Phosphodiesterase I was also able to hydrolyze d4TMP and AZTMP, giving 19 and 100% of d4T and AZT, respectively, pointing to contaminating phosphatase in the enzyme preparation. Alkaline phosphatase (10 U/ml) and 5'-nucleotidase (5 U/ml) caused a complete hydrolysis of d4TMP and AZTMP to the free nucleoside.

Physicochemical Properties of Rat Liver Phosphonamidase. The partially purified rat liver enzyme was found to display a markedly enhanced activity on the addition of MgCl₂ (10 mM), and this cofactor was routinely used in phosphoramidase assays.
The rat liver phosphoramidase showed an optimal enzymatic activity at pH 7.4 that was 8-fold reduced at pH 5.4 and 9.4. Metal-chelating agents such as EDTA had no effect on the phosphoramidase activity of the rat liver enzyme preparation. By using ultrafiltration membranes with different molecular mass cut-off values (from 10 to 100 kDa), we determined that the highest phosphoramidase activity was present in the rat liver enzyme fraction with a molecular mass ranging from 50 to 100 kDa.

**Discussion**

The phosphoramidate derivatives of ddN were designed to act as membrane-soluble nucleotide prodrugs that enable intracellular delivery of the ddNMP, thus bypassing the first activation step by cellular kinases (TK in the case of d4T and AZT; McGuigan et al., 1996). The ddNMP is then further phosphorylated to ddN 5'-triphosphate, the active metabolite that inhibits HIV reverse transcriptase (Balzarini et al., 1998; Balzarini and De Clercq, 1999). In this study, we focused on a series of phosphoramidate triesters of d4TMP and AZTMP, with variations in the amino acid moiety and the attached alkyl group. The antiviral activity in HIV-infected CEM cells was found to be determined by three structural parameters: the nature of the nucleoside (d4T or AZT), the amino acid moiety, and the carboxyl ester group. Most importantly, the phosphoramidate derivatives of d4TMP were found to be equally active in wild-type and TK-deficient CEM/TK− cells, thus proving that the TK bypass concept is initiated by identified enzymes as well as crude and partially purified enzyme preparations. Several groups found to be equally active in wild-type and TK-deficient CEM/TK− cells, thus proving that the TK bypass concept is initiated by identified enzymes as well as crude and partially purified enzyme preparations. Several groups

![Graph](image-url)

**Fig. 6.** Inhibitory effect of iodobenzene on the metabolism of L-alaninyl-d4TMP by rat liver phosphoramidase. L-Alaninyl-d4TMP was incubated with the rat liver enzyme preparation in the presence of different concentrations of iodobenzene. Metabolism was followed by HPLC analysis of the remaining alaninyl-d4TMP and formation of the metabolites (d4TMP plus d4T). The dashed line represents the IC50 value, defined as the iodobenzene concentration at which the conversion of L-alaninyl-d4TMP to d4TMP plus d4T is inhibited by 50%.

Our studies have shown that the phosphoramidate derivatives of d4TMP and AZTMP with high amounts of pig liver carboxylesterase. AAM was very efficiently formed from the L-alanine-containing triesters of d4TMP or AZTMP. The L-valine- and β-alanine-containing d4TMP derivatives did not convert to AAM. This is consistent with the low or marginal antiviral activity of these two compounds. Qualitatively, a similar pattern for AAM formation was observed when the triesters were incubated in biological media (i.e., human serum, CEM cell extract, or mouse serum). Overall, the conversion to AAM proved to be highest in mouse serum, lowest in human serum, and intermediate in CEM cell extract. These data are in agreement with the ubiquitous presence of carboxylesterases in mammalian tissues, albeit at enzyme levels that are highly dependent on tissue type and species (Robbi and Beaufay, 1983; Hosokawa et al., 1990).

Some triesters showed a significant conversion to a metabolite, of which the retention time on HPLC was between those of the triester and the AAM. We hypothesize that this is the IM that is formed after hydrolysis of the carboxyl ester in the amino acid moiety and that may be assumed to have a high chemical instability (Fig. 1). The nature of the side chain of the amino acid and the nature of the sugar moiety, in particular the azido group at the 3' -position in the case of AZT, may play an essential role in the formation of the AAM through the hypothetical cyclic intermediate. The only excep-
formation seen here was the β-alanine triester of d4TMP, of which the IM proved fully stable. The most logical explanation is that due to the extra carbon in the β-alanine chain, this IM is unable to form a six-membered cyclic intermediate to allow further conversion to AAM.

To obtain optimal delivery of the ddNMP inside the target cells (i.e., the HIV-infected lymphocytes), an efficient conversion rate by carboxylesterase may be considered as favorable. However, in the in vivo situation, the prodrugs can reach the target cells only if they are resistant to hydrolysis by extracellular carboxylesterases (such as in serum). If not, partial conversion of the prodrugs to AAM would result in a lower cell penetration and, hence, reduced antiviral response. Thus, a compromise must be reached between the extracellular stability of the prodrugs and their conversion to the AAM once they have been taken up intracellularly.

Our studies have revealed that the carboxyester group linked to the amino acid moiety has pronounced influence on the pharmacokinetics of the triester and its associated stability. Indeed, the stability in human serum of L-alaninyl-containing derivatives proved to be highly higher when a rat liver enzyme preparation was used. Moreover, the more stable ethyl ester derivative showed a higher stability. Indeed, the stability in human serum of L-alaninyl-d4TIVIP and measurement of the triester of d4TMP proved fully stable. The most logical explanation is that due to the extra carbon in the β-alanine chain, this IM is unable to form a six-membered cyclic intermediate to allow further conversion to AAM.

To obtain optimal delivery of the ddNMP inside the target cells (i.e., the HIV-infected lymphocytes), an efficient conversion rate by carboxylesterase may be considered as favorable. However, in the in vivo situation, the prodrugs can reach the target cells only if they are resistant to hydrolysis by extracellular carboxylesterases (such as in serum). If not, partial conversion of the prodrugs to AAM would result in a lower cell penetration and, hence, reduced antiviral response. Thus, a compromise must be reached between the extracellular stability of the prodrugs and their conversion to the AAM once they have been taken up intracellularly.

Our studies have revealed that the carboxyester group linked to the amino acid moiety has pronounced influence on the pharmacokinetics of the triester and its associated stability. Indeed, the stability in human serum of L-alaninyl-containing derivatives proved to be highly higher when a rat liver enzyme preparation was used. Moreover, the more stable ethyl ester derivative showed a slight advantage in antiviral activity.

Next, we investigated the second part of the activation pathway, consisting of the cleavage of AAM to ddNMP or free ddN. Although incubation of the triesters in CEM cell extract, human serum, or mouse serum resulted in limited formation of ddNMP and ddN, this conversion was considerably higher when a rat liver enzyme preparation was used. The metabolism of AAM to ddNMP and ddN was found markedly depending on the amino acid moiety, with β-alanine being the preferred amino acid, thus fully agreeing with the superior antiviral activity of the L-alaninyl-containing phosphoramidate triesters.

Moreover, the d4TMP triesters were found to be superior to the corresponding AZTTP triesters in two aspects: a higher total amount of ddNMP plus ddN release and a markedly higher ratio of ddNMP to ddN (Fig. 6). These results were further confirmed in incubation studies with purified AAM compounds. After overnight incubation, the percentage of AAM left was 20 and 84% for the L-alaninyl-AZTMP, and the ratio of ddNMP to ddN was 2.4 and 0.6, respectively. The latter result can be explained by the higher sensitivity of AZTMP than d4TMP to nonspecific phosphatases and/or 5'-nucleotidases in this preparation. Similar observations were obtained for the prodrug derivatives of d4TMP (3 and 4) and AZTMP (13 and 16). These data are fully consistent with the observation that the d4TMP triesters, not the AZTTP triesters, keep their anti-HIV activity in TK-deficient CEM cells. Clearly, the nature of the nucleoside in the prodrug determines the degree at which the kinase-bypass concept is successful.

The last part of our study was focused on the partition purification and characterization of the enzyme that hydrolyzes the phosphoramidate (P-N) linkage in the AAM. The original definition of phosphoramidase (EC 3.2.1.1) as given by Dixon and Webb (1979) refers to an enzyme that is acting on a phosphorus-nitrogen (P-N) bond. However, different enzymes with phosphoramidase activity have been isolated from various sources (i.e., rat liver, spleen, or kidney), and the enzyme has been associated with both microsomal and cytosolic fractions (Holzer et al., 1966; Snyder and Wilson, 1972; Zuba et al., 1994; Nishino et al., 1994). In these studies, the phosphoramidase activity was determined based on release of free phosphate from the P-N substrate. In our studies, we describe an enzyme activity that hydrolyzes a P-N bond with the release of a substituted phosphate (i.e., a phosphate attached to a nucleoside moiety). After fractionation of a rat liver homogenate by centrifugal separation, the different subcellular fractions (mitochondrial, microsomal, and cytosolic) were evaluated for phosphoramidase activity by incubation with L-alaninyl-d4TIVIP and measurement of the d4TMP formation. The highest enzymatic activity was found in the cytosolic fraction. Reduction in the preparation time and the addition of 2-mercaptoethanol in the isolation buffer and magnesium chloride in the incubation mixture resulted in a significantly higher enzyme yield and activity. Such an enzyme activity has been described by Shabarov and coworkers (Lednova et al., 1997, 1970; Shabarov et al., 1970, 1971; Dudkin et al., 1971a,b) and recently by McIntee et al. (1997). Shabarov (1970) reported on the discovery of a nucleoside 5'-phosphoramidase in some animal tissues (i.e., rabbit liver). This enzyme hydrolyzes the phosphoramidate bond to form the nucleotide and the amino acid. Nucleoside 5'-amidates were the most readily hydrolyzed compounds, and the enzyme preparation proved capable of hydrolyzing both l- and d-amino acid derivatives of nucleotides (Shabarov, 1970). McIntee et al. (1997) recently found that the 3-indolyl aminomethyl phosphoramidate prodrugs of AZT and 3'-fluoro-2',3'-dideoxycytidine were also substrates for phosphoramidase activity in peripheral blood mononuclear cell extracts. In this respect, the 3'-fluoro-2',3'-dideoxyxymidine-MP derivative was a better substrate than the AZTMP derivative. The d4TMP derivative was not included in this study.

The inhibitory effect of isobutane on the phosphoramidase activity is similar to that previously reported for the closely related compound isodesoxobenzene (Singer and Frutos, 1987). At high concentrations, the naturally occurring phos-
phosphoramidate compound phosphoramidase was shown to be able to partially inhibit the phosphoramidase-mediated hydrolysis of t-alaminy-dTTP. However, t-alaminy-dTTP proved not to be a substrate for creatine phosphokinase, the enzyme that catalyzes the phosphorylation of creatine. In addition, we incubated the AAM compounds with phosphodiesterase, alkaline phosphatase, and 5'-nucleotidase. Phosphodiesterase was able to hydrolyze t-alaminy-dTTP and t-alaminy-AZTTP, yet this reaction was not inhibited by iodocobalamin. Taken together, these results suggest that the phosphoramidase enzyme in the rat liver fraction that recognizes the phosphoramidate dNTP prodrug is distinct from known esterases. We also found that 0.1 μM benzylalcohol is able to completely block the conversion of t-alaminy-dTTP to dTTP. However, when benzylalcohol is released in the intact cells on conversion of the t-alaminy-dTTP prodrug to t-alaminy-dTTP, it will immediately be spread over the whole cell content, and it is even expected to diffuse out of the cells to the extracellular medium. Therefore, it is reasonable to assume that the benzylalcohol released from the phosphoramidate prodrug has no chance to efficiently inhibit the phosphoramidase-catalyzed intracellular reesterification of dTTP. The potent antiviral activity of the benzyl prodrug ester derivative is in agreement with this hypothesis. We are currently planning the isolation of the phosphoramidase enzyme by ion-exchange or affinity chromatography to identify its physicochemical properties, substrate specificity, and physiological role. These insights should help to design new phosphoramidate prodrugs with improved biochemical and therapeutic properties.

In Fig. 1, we proposed as the main metabolic pathway of the prodrugs the release of the alkyl (methyl) ester group by carboxylesterases before the release of the aryl part of the molecule. Indeed, we recently revealed that an α-amino acid is necessary for biological action, and an α-amino acid is necessary for the phenyl cleavage (by intramolecular catalysis), and phenyl loss proceeds after ester cleavage. Similarly, in a recent report (McGuigan et al., 1998b), we noted that replacement of methyl by t-buty1 as the carbouxyl ester lead to a significant reduction in antiviral potency. This directly correlated with the high stability of the t-buty1 ester to any esterase-mediated degradation. Because the stability of the phenyl phosphate group per se should be unaffected by such a modification at the carboxyl terminus, the "apparent" stabilization of the phenyl phosphate toward cleavage (and resulting reduction in antiviral potency) can only arise from the carboxylester ester stabilization. Again, these data strongly support the suggestion that carboxylester cleavage is a necessary prerequisite for phenyl loss and for eventual antiviral activity.

Acknowledgments

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References


Balzarini J and De Clercq E (1990) Nucleosides and nucleotides reverse transcrip-


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We claim:

1. (S)-2-\{[(2R,3R,4R,5R)-5-(2,4-Dioxo-3,4-dihydro-2H-pyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyl-tetrahydro-furan-2-y1methoxy]-phenoxy-phosphorylamino\}-propionic acid isopropyl ester or a stereoisomer thereof.

2. A composition comprising the compound or a stereoisomer thereof as claimed in claim 1 and a pharmaceutically acceptable medium.

3. A composition for treating a hepatitis C virus, which comprises an effective amount of the compound or a stereoisomer thereof as claimed in claim 1 and a pharmaceutically acceptable medium.

4. A method of treating a subject infected by a virus, which comprises: administering to the subject an effective amount of the compound or a stereoisomer thereof as claimed in claim 1; wherein the virus is selected from among hepatitis C virus, West Nile virus, a yellow fever virus, a dengue virus, a rhinovirus, a polio virus, a hepatitis A virus, a bovine viral diarrhea virus, and a Japanese encephalitis virus.

5. A method of treating a hepatitis C virus infection in a subject in need thereof, which comprises: administering to the subject an effective amount of the compound or a stereoisomer thereof as claimed in claim 1.

6. A process for preparing the compound or a stereoisomer thereof as claimed in claim 1, said process comprising:

   reacting a compound $4''$ with a nucleoside analog $5'$

   \[
   \begin{align*}
   4'' & \quad \begin{array}{c}
   \text{Phosphorus} \\
   \text{ Leaving Group} X'
   \end{array} \\
   \text{O} & \quad \text{O} \\
   \text{HO} & \quad \text{HO}
   \end{align*}
   \]

   wherein $X'$ is a leaving group.
7. A product comprising the compound or a stereoisomer thereof as claimed in claim 1 obtained by a process comprising:
   reacting a compound 4'' with a nucleoside analog 5' wherein X' is a leaving group.

8. (S)-isopropyl 2-(((S)-(((2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl)(methoxy)(phenoxy)phosphoryl)amino)propanoate.

9. A composition comprising the compound as claimed in claim 8 and a pharmaceutically acceptable medium.

10. A composition for treating a hepatitis C virus, which comprises an effective amount of the compound as claimed in claim 8 and a pharmaceutically acceptable medium.

11. A method of treating a subject infected by a virus, which comprises:
   administering to the subject an effective amount of the compound as claimed in claim 8, wherein the virus is selected from among hepatitis C virus, West Nile virus, a yellow fever virus, a dengue virus, a rhinovirus, a polio virus, a hepatitis A virus, a bovine viral diarrhea virus, and a Japanese encephalitis virus.

12. A method of treating a hepatitis C virus infection in a subject in need thereof, which comprises: administering to the subject an effective amount of the compound as claimed in claim 8.
13. A process for preparing the compound as claimed in claim 8, said process comprising:
reacting a compound $4''$ with a nucleoside analog $5'$

wherein $X'$ is a leaving group.

14. A product comprising the compound as claimed in claim 8 obtained by a process comprising:
reacting a compound $4''$ with a nucleoside analog $5'$

wherein $X'$ is a leaving group.

Dated this 20th day of October 2009.

Abhishek Sen
of S. MAJUMDAR & CO.
Applicants’ Agent