Pronucleotides: Toward the In Vivo Delivery of Antiviral and Anticancer Nucleotides

Carston R. Wagner, Vidhya V. Iyer, Edward J. McIntee

Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, MN 55455

Abstract: To overcome the many hurdles preventing the use of antiviral and anticancer nucleosides as therapeutics, the development of a prodrug methodology (i.e., pronucleotide) for the *in vivo* delivery of nucleotides has been proposed as a solution. The ideal pronucleotide should be non-toxic, stable in plasma and blood, capable of being i.v. and/or orally dosed, and intracellularly convertible to the corresponding nucleotide. Although this goal has yet to be achieved, many clever and imaginative pronucleotide approaches have been developed, which are likely to be important pharmacological tools. This review will discuss the major advances and future directions of the emerging field of antiviral and anticancer pronucleotide design and development. Med Res Rev, 20, No. 6, 417–451, 2000

Key words: prodrug; pronucleotide; nucleotide; antiviral; anticancer

1. INTRODUCTION

Nucleosides and nucleotides have demonstrated wide-spread utility as antiviral and anti-cancer therapeutics.[1,2] Natural endogenous nucleosides must be phosphorylated to the corresponding 5′-triphosphates (TP) to be incorporated into the DNA strand being synthesized in the cell. The first phosphorylation step leading to the formation of nucleoside 5′-monophosphate (MP), is commonly catalyzed by a nucleoside kinase encoded by the host cell or the virus infecting the host cell (Fig. 1).[3] Conversion of nucleoside-MPs to the corresponding 5′-diphosphates (DP) and triphosphates is carried out by nucleoside, nucleotidyl, and nucleoside diphosphate kinases, respectively. Thus, cellular kinases and virally-encoded kinases play a vital role in the metabolism and replication of cells and viruses.

Based on the metabolite–antimetabolite approach, nucleoside analogs such as 2′,3′-dideoxynucleosides (ddNs) have been developed as competitors of natural 2′-deoxynucleoside 5′-triphosphates (dNTPs). Typically, modifications at the 2′ or the 3′carbon atoms of the glycone (sugar) moiety of nuelcosides are introduced. By virtue of their resemblance to the natural 2′-deoxynucleosides, ddNs are phosphorylated to the corresponding 5′-triphosphates and incorporated

Correspondence to: Carston R. Wagner

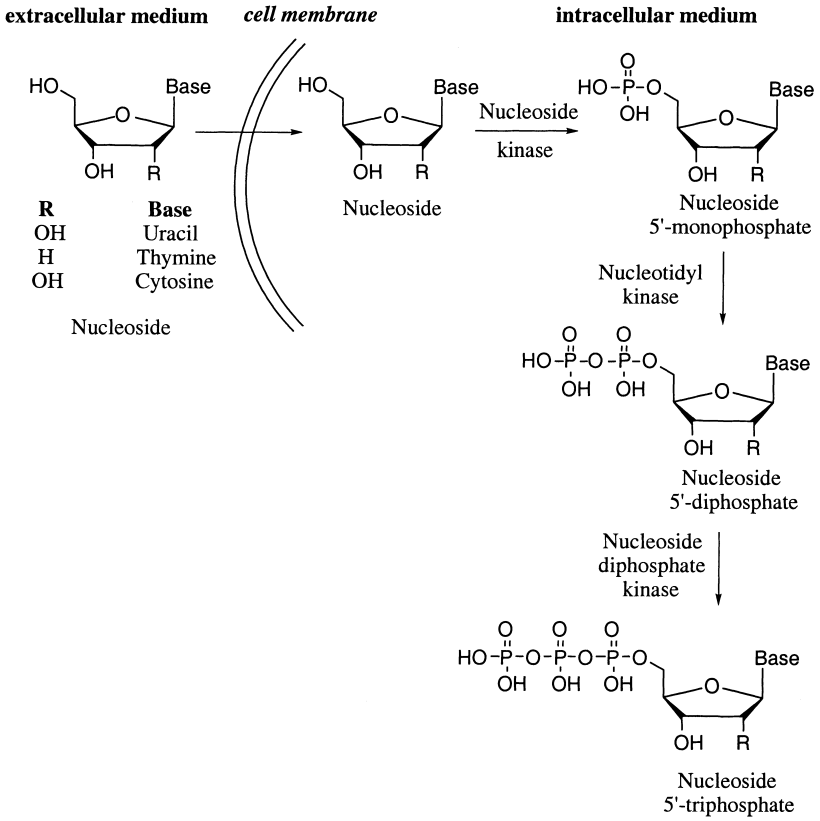


Figure 1. Intracellular metabolism of nucleosides.[3]

into a growing DNA strand by a DNA polymerase, resulting in chain termination. Therefore, ddNs are in essence prodrugs, since they must be phosphorylated intracellularly in order to be biologically active.

Therapies involving long-term administration of ddNs such as 3′-azido-2′3′-dideoxythymidine (AZT) have been reported to lead to decreased activity of the first phosphorylating enzyme, thymidine kinase and thus, resistance.[4] This type of resistance is observed not only in host tissues of the patients undergoing ddN therapy, but also in viruses. This resistance mechanism renders the ddNs less effective since their activation is hindered at the first phosphorylation step. In addition, for antiviral acyclic nucleosides such as acyclovir and penciclovir, the dependence of the nucleosides on activation to the triphosphates by virally encoded thymidine kinase, limits their spectrum of antiviral activity to those viruses such as herpes simplex virus (HSV) and Varicella zoster virus (VZV) which encode their own thymidine kinase.[5] Thus, viruses such as hepatitis B virus (HBV), which do not encode their own nucleoside kinase, do not fall within the purview of activity of these antiviral nucleosides. Moreover, ddNs such as AZT, are associated with myelosuppressive side-effects, such as anemia and neutropenia.[6] Toxic side-effects have been widely reported to lead to the discontinuation of ddN therapy. Certain other ddNs such as 2′,3′-dideoxyuridine (ddU), are poor substrates for thymidine kinase or other cellular kinases, and are, therefore, not converted to the corresponding triphosphates.[7,8]

In principle, administration of the 5′-phosphates would aid in overcoming the drawbacks of ddN therapy posed by resistance mechanisms and inherent biological differences. However, because phosphates are strongly acidic and thus negatively charged at physiological pH (pH = 7.4), they are too hydrophilic to penetrate the lipid-rich cell membranes. In addition, blood and cell surface

phosphohydrolases (acid and alkaline phosphatases, 5′-nucleotidases) rapidly convert the phosphates to the corresponding nucleosides.

In order to overcome the poor cell penetration of nucleoside 5′-phosphates, Montgomery[9] proposed that ''this difficulty might be overcome if one could prepare an ester of a nucleotide which could penetrate the cell wall and then be metabolized to the nucleotide itself.'' Consequently, various prodrug or ''pronucleotide''[8] approaches have been devised and investigated. In general, the goal of these approaches has been to promote passive diffusion through cell membranes and increase the bioavailability of phosphorylated nucleosides. This approach of derivatization has been applied using various protecting groups for the phosphate moiety. Ideally, the attempts have been designed to achieve stability in the extracellular medium and rapid intracellular hydrolysis to release the phosphate. In most cases, to be biologically active, the phosphate has to be activated to the diphosphate and triphosphate. This review will discuss what we believe to be the major advances and future directions of the emerging field of antiviral and anticancer pronucleotide design and development.

2. ALKYL AND ARYL PHOSPHATE DERIVATIVES

A. Alkyl and Aryl Phosphodiesters

A simple solution to the delivery of nucleotide monophosphates to cells is the use of alkyl phosphate esters. For example, aryl phosphodiesters of 6-mercaptopurine (6-MP) riboside were prepared in order to deliver 6-MP to 6-MP resistant neoplasms.[9] The attempt was unsuccessful, probably due to poor cell penetration.[10]

Alkyl and aryl phosphodiesters of β-D-arabinofuranosylcytosine (Ara-C), 2′,3′-didehydro-3′-deoxyadenosine (d4A), 2′,3′-didehydro-3′-deoxycytosine (d4C), and ddU have also been synthesized (Fig. 2). It had been reported that of a series of lipophilic 5′-(alkyl phosphate) esters of Ara-C, only the Ara-C 5′-(glyceryl phosphate) possessed cytotoxicity comparable to that of Ara-C-MP ($CC_{50} = 0.35$ and 0.65 μM, respectively) towards L1210 leukemia cells.[11] The glyceryl phosphate ester also demonstrated *in vivo* activity against a kinase-deficient P388 murine leukemia.[12] These results suggested that the development of β-hydroxyalkyl phosphate esters and other hydroxyalkyl (steroids) substitutions would be a worthwhile endeavor.[11] Hong and co-workers[13] reported that alkyl esters of both Ara-C-MP and Ara-C-DP demonstrated lower growth inhibitory activity than steroidal esters of Ara-C and Ara-C-DP. Nevertheless, the Ara-C-DP steroidal esters were found to possess lower antitumor activity than Ara-C-MP and Ara-C-DP alkyl esters against L1210 lymphoid leukemia in mice *in vivo*.[13] A decrease in anti-leukemic activity was observed with increasing alkyl chain length towards cultured L1210 leukemic cells. However, Ara-C-MP alkyl esters containing alkyl groups of 16–20 carbon atom length were reported to be orally active prodrugs of Ara-C.[14]

Mullah and co-workers[15] reported that 5′-phenyl- and 5′-methyl-phosphate diesters of d4A and d4C demonstrated *in vitro* anti-HIV activity and cytotoxicity comparable to the corresponding parent nucleosides (Fig. 2). The compounds were cleaved under test conditions and released the parent nucleosides or the nucleoside 5′-monophoshates in medium containing 10% serum.[15] Synthesis of ionophore–nucleotide conjugates of 15 crown ether-AZT and 15 crown ether-ddU 5′-phosphate diesters has also been reported (Fig. 2).[16] After association with a metal cation, these conjugates likely form a lipophilic ion-pair which could diffuse through a bilayer membrane. Once inside the membrane, the aryl phosphate ester linkage of the conjugate could be cleaved to release the nucleoside 5′-monophosphate. Although AZT was found to be approximately 14-fold more potent than the AZT–ionophore conjugate, the ddU–ionophore conjugate had approximately 11

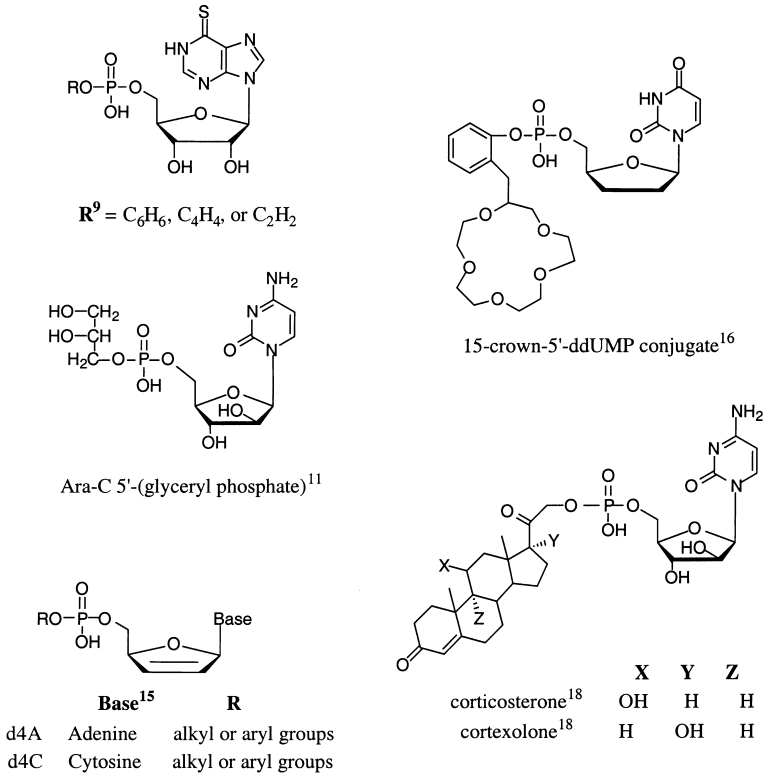


Figure 2. Alkyl and aryl phosphodiesters.[9,11,15,16,18]

times the activity of ddU against HIV-1 infected CEM-SS cells, suggesting nucleoside 5′-monophosphate delivery.[16]

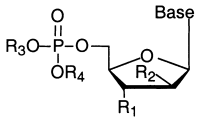
B. Steroid Phosphodiesters

Based on the synergistic effect observed between certain drugs and the steroid prednisolone against human lymphoid leukemias and lymphomas, nucleotide–steroid conjugates were developed and evaluated for their anti-cancer effects. Ara-C-MP-prednisolone and Ara-C-MP-prednisone conjugates had greater anti-cancer effects against L1210 lymphoid leukemia in female mice than Ara-C alone or in combination with the individual steroid components.[17] Ara-C-MP conjugates of corticosterone, cortexolone, 6α-methylprednisolone, and 11-deoxycorticosterone were also found to increase the life-span of mice with L1210 leukemia to a greater extent than Ara-C[18] (Fig. 2) The conjugates were also found to be resistant to human liver cytidine deaminase and alkaline phosphatase, but sensitive to phosphodiesterase I, 5′-nucleotidase, and acid phosphatase. They were shown to hydrolyze slowly in blood ($t_{1/2} = 24–48$ hr) and were able to cross the blood–brain barrier. These nucleotide–steriod conjugates did not deliver Ara-C-MP to the cells, as was evident from their lack of activity against a kinase-deficient L1210 leukemia resistant to Ara-C. However, many of the conjugates did show superior potency, compared to Ara-C, upon intraperitoneal administration against L1210 leukemia implanted intracerebrally.[19,20] Other examples of steroid conjugates include the 3-fluorodeoxyuridine (FUdR)-MP-7β-hydroxycholestrol conjugate, which was found to possess *in vitro* cytotoxic activity against EL-4 murine leukemia cells and *in vivo* antitumor activity in mice with Krebs II ascitic carcinoma.[21] The AZT-MP-3β(7β-hydroxycholesterol) conjugate also demonstrated *in vitro* anti-HIV activity.[22]

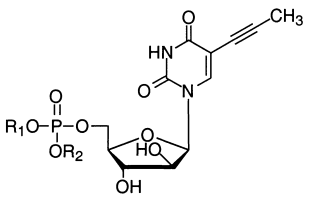
C. Alkyl and Aryl Phosphotriesters

In general, alkyl and aryl phosphodiesters have proven to be unsuitable for the delivery of nucleotides, presumably due to their inherent polarity and sensitivity to phosphodiesterase. Consequently, simple alkyl and aryl phosphotriesters of nucleosides have been developed in order to increase the lipophilicity of the phosphate by the neutralization of negative charges of the phosphate moiety.[9] The increased lipophilicity was postulated to overcome the inactivity of phosphodiesters by promoting passive diffusion of the phosphotriesters into the cells (Fig. 3). Literature reports have documented the synthesis and biological evaluation of alkyl and aryl phosphotriesters of AZT, Ara-C, dideoxycytidine (ddC), β-D-arabinofuranosyladenine (Ara-A), 2′,3′-didehydrodideoxythymidine (d4T), 3′-acylthymidine and netivudine (1-(β-D-arabinofuranosy)-5-prop-1-ynyluracil) and 6-MP. In general, the phosphotriesters were found to be less potent than the corresponding parent nucleosides.[9,10,23–34]

Ara-A and Ara-C are inactivated by the deamination catalyzed by adenosine deaminase and cytidine deaminase, respectively. However, the simple alkyl phosphotriesters of Ara-A and Ara-C were reported to display resistance to the action of deaminases, phosphodiesterase I, and lipase (Fig. 3).[24,25] These phosphotriesters were found to be hydrolyzed slowly by alkaline phosphatase

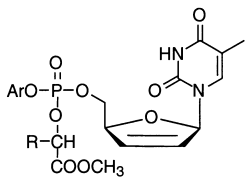


Phosphotriester [24-26, 28, 29]	Base	R_1	R_2	R_3	R_4
ddC bis(2,2,2-trichloroethyl)	Cytosine	H	H	CH_2-CCl_3	CH_2-CCl_3
dT bis(2,2,2-trichloroethyl)	Thymine	OH	H	CH_2-CCl_3	CH_2-CCl_3
AZT bis(2,2,2-trichloroethyl)	Thymine	OH	H	CH_2-CCl_3	CH_2-CCl_3
AZT bis(2,2,2-trifluoroethyl)	Thymine	OH	H	CH_2-CF_3	CH_2-CF_3
Ara-C alkyl trichloroethyl	Cytosine	OH	OH	alkyl	CH_2-CCl_3
Ara-A alkyl	Adenine	OH	OH	alkyl	alkyl
AZT diaryl	Thymine	N_3	H	aryl	aryl



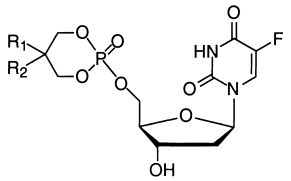
Netivudine phosphotriesters[33]

R_1,R_2 = alkyl, haloalkyl, aryl



Phosphotriester[34]	R	Ar
d4T aryl glycolate	H	aryl
d4T aryl lactate	CH_3	aryl

aryl = *p*-nitrophenyl, phenyl



$R_1 = R_2 = F$[38]

Figure 3. Nucleoside alkyl and aryl phosphotriesters.[24–26,28,29,33,34,38]

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.