Medicinal Chemistry of Antiviral/Anticancer Prodrugs Subjected to Phosphate Conjugation

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Abstract: Certain xenobiotics are given in the "prodrug" form. Either the human body, or one compartment of the body, or the targeted virus itself metabolizes the prodrug into its active form. The bioprecursor form of drugs is used for a wide variety of reasons, namely: to make drug penetration into the target organ (mainly to the brain through the blood-brain-barrier) possible, eliminate unpleasant taste, alter (either increasing or decreasing) the half life of the active component or supply more than one active components to the body.

Keywords: Anticancer, antiviral, bioprecursor, conjugation, medicinal chemistry, phosphate, prodrug.

INTRODUCTION

The overwhelming majority of antiviral/anticancer drugs gives their medicinal effects as they are taken [1-5]. However, a small percentage of xenobiotics are given in the "prodrug" form. Specific attention to prodrugs was first given by Albert in 1958 [6] when he called a group of compounds prodrugs. Such pharmacologically inactive compounds are transformable into active forms by the mammalian organism. Harper [7] coined the word "drug latentiation" for compounds that were specially designed for bioactivation. Drugs can thereby be divided into two essential classes: (1) hard drugs and (2) soft drugs. Hard drugs are the compounds that possess all structural characteristics of their pharmacological activity in the body and are not susceptible to metabolic transformation before they become active. Alteration of their pharmacological response is thereby avoided, including increasing/decreasing their effect, and production of any toxic metabolites. Hard drugs are especially preferred in the treatment of elderly patients who also take several other drugs that may either induce or inhibit their cytochrome P-450 system. Soft drugs are active compounds and their metabolism is designated to yield non-toxic products.

Prodrugs should be converted into their active forms by metabolic processes such as aliphatic oxidation, N-dealkylation, etc. A usual way of prodrug preparation is linking the drug to a metabolically labile carrier-like compound, these types of compounds are called carrier-linked prodrugs. Carrier-linked prodrugs are used to increase either lipid or water solubility, or even site-directed drug delivery. Carrier-linked prodrugs are designed for one or more of the following, increasing absorption, alleviation of pain at the site of parenteral administration, or elimination of unpleasant taste. An example of this phenomenon is the administration of chloramphenicol in form of chloramphenicol succinate, a water-soluble ester that can be given intravenously (i.v.) but has no antibacterial activity. After i.v. administration of chloramphenicol succinate, it hydrolyses, releasing active chloramphenicol [8]. Another type of prodrugs is called "Prodrugs of functional groups" (PFG) [8]. The most frequently applied PFG-type prodrug construction is when an ester linkage is produced between the real drug entity and the modifying substituent. An essential part of drugs contains either carboxylic or alcoholic or both functional groups, the variety is therefore wide. The metabolic system of the body has an esterase enzyme, which helps in the generation of the active drug. Certain other cases include amine functional groups to be converted either into amide-, or azo-linkage. Amino groups are often incorporated to give a peptide linkage which converts the prodrug into an amide. The lack of amidase enzyme would make amides to be stable components. Moreover, the peptide linkage is easily cleaved by specific peptidase enzymes and uptake of certain peptides can be facilitated by amino acid transporter. The production of a carbonyl compound does occur sometimes but quite uncommon. At the same time, the oxidative transformation of drugs by the help of cytochrome P-450 system is a common metabolic step. Aliphatic or aromatic hydroxylation, N-, O- and S-dealkylation, etc. are also associated with cytochrome P-450.

Based on the functional part of prodrugs, either the active drug is attached to a carrier that generally does not have any real pharmacological function, or a latent part of the prodrug

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is converted into the active form. This conversion is often done by oxidation, reduction and even by phosphorylation.

Phosphorylation is a common function of the body through its own biochemical pathway, however, metabolic phosphorylation is an odd reaction in pharmacology. It is somehow missing from the metabolic alterations enlisted in the books of pharmacology [1-4]. At the same time, there are several prodrugs that are activated by phosphorylation (Table **1**). For the last two decades generations of monophosphates, diphosphates and triphosphates have become evident. The conversion is called anabolic phosphorylation. There are several antiviral and/or anticancer drugs that are activated by the attachment of phosphates to their free hydroxyl groups (abacavir (**1**), acyclovir (**4**), cladribine (**18**), didanosine (**20**), emtricitabine (**22**), ganciclovir (**29**), gemcitabine (**32**), lamivudine (**35**), penciclovir (**37**), ribavirin (**38**), stavudine (**40**), zalcitabine (**50**), zidovudine (**52**) or the existing one phosphate is completed with further phosphates (adefovir (**6**), cidofovir (**15**)). In some cases, hydrolysis should make the hydroxyl group free, which is then subjected to phosphate conjugation, either to the free aliphatic hydroxyl group (famciclovir (**24**), tenofovir (**45**), valaciclovir (**48**), valganciclovir (**49**)) or to the free hydroxyl of the monophosphate (adefovir (**6**)). The activation of fludarabine (**26**) is considered to have a dephosphorylation step first, and then a triple phosphorylation to an active triphosphate.

The aim of this paper is to give details of medicinal chemistry of prodrugs enzymatically phosphorylated by viral enzyme systems and/or cancer cells (Table **1**). This enzymatic attachment of phosphate (phosphate conjugation) is not entirely restricted to either viruses or cancer cells, however, healthy human cells do not have the ability for phosphate conjugation or they have only limited capacity. However, even this limited capacity can cause serious side effects.

EXPERIMENTAL

Calculation of logP was done using the Pallas program of CompuDrug Inc. Since the other fragment set does not contain data for phosphates, only one part of logP (using CDI-Rekker method) could be calculated.

DETAILED DISCUSSION

Abacavir (ABC) (**1**) is considered the most powerful analogue of nucleoside reverse transcriptase inhibitor (NRTI) used for the treatment of HIV and AIDS [1-5]. Abacavir can be used in combination with other antiretroviral agents. Abacavir (**1**) is chemically a carbocyclic synthetic nucleoside analogue, which intracellularly is enzymatically converted to the active metabolite: carbovir-5'-triphosphate (**2**), an analogue of deoxyguanosine-5'-triphosphate (dGTP) (**3**). Carbovir-5'-triphosphate (**2**) inhibits the activity of HIV-1 reverse transcriptase (RT) by competing with the natural substrate dGTP (**3**) in its incorporation into viral DNA. FDA approval for the treatment of HIV infection was in 1998 [5, 55].

Aciclovir (INN) (**4**) or acyclovir (USAN) is indicated in the treatment and management of herpes zoster (shingles), genital herpes, and chickenpox. Acyclovir (**4**) is converted to its triphosphate form in three steps. Conversion of acyclovir to its -5'-monophosphate, -5'-diphosphate and -5'-triphosphate (**5**) formations is carried out by thymidine kinase, cellular guanylate kinase and pyruvate kinase, respectively. The fact that acyclovir-5'- triphosphate is an analogue of deoxyguanosine-5'-triphosphate (dGTP) (**3**), allows both to compete for incorporation into viral DNA. Acyclovir-5'-triphosphate (**5**) has approximately 100 times greater affinity for viral than cellular polymerase. FDA approval for acyclovir (**4**) as an antiviral agent was in 1982 [5, 55].

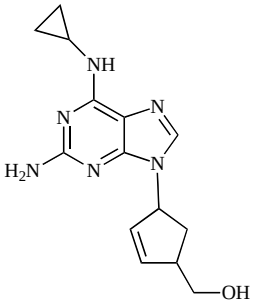
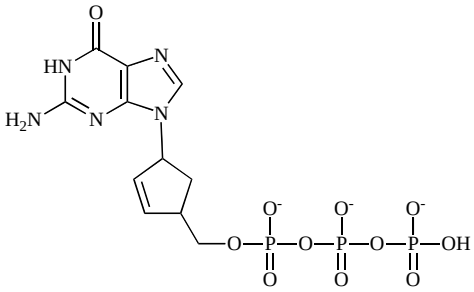
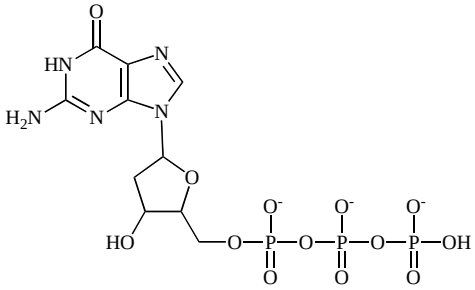
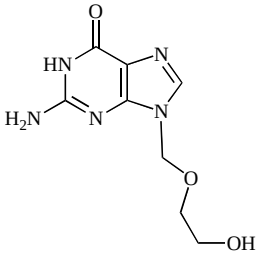
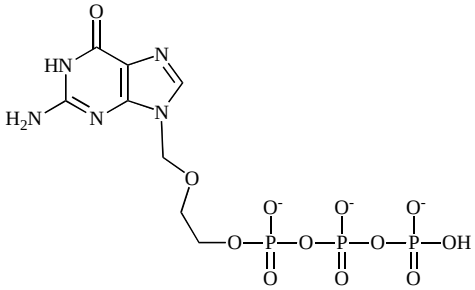
Adefovir dipivoxil (**6**) is a prodrug of adefovir and also of adefovir diphosphate (**7**). The first step in its metabolism is the hydrolysis of dipivoxil and attachment of two phosphate groups by cellular kinases to build up adefovir diphosphate (**7**), which contains a three phosphate moiety, the original one plus two additional phosphate units [5]. Elimination half life of adefovir dipivoxil is 7.5 h. Adefovir diphosphate (**7**) is able to inhibit hepatitis B virus (HBV) DNA polymerase by competition with the natural deoxyadenosine-5'-triphosphate (**8**). The main benefit of adefovir (**6**) (in comparison to lamivudine (**35**)) is that it takes a long period of time before the hepatitis B virus develops resistance to it. FDA approval for use in the treatment of hepatitis B was granted on September 20, 2002.

Capecitabine (**9**) is an orally administered chemotherapeutic agent used in the treatment of metastatic breast and colorectal cancers. Capecitabine is selectively metabolized in tumour cells to 5-fluorouracil (5-FU) (**10**) The first step is that capecitabine is subjected to carboxylesterase to form 5'-deoxy-5-fluorocytidine (5'-DFCR), then 5'-DFCR is subjected to cytidine deaminase to be converted to 5'-deoxy-5-fluorouridine (5'DFUR) which is converted to 5-FU (**10**) by thymidine phosphorylase.

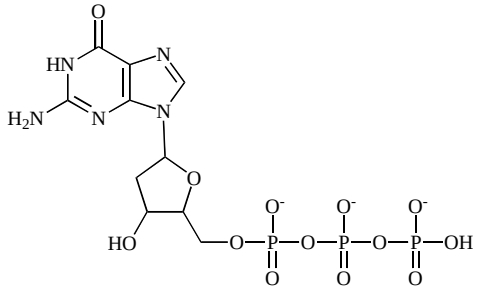
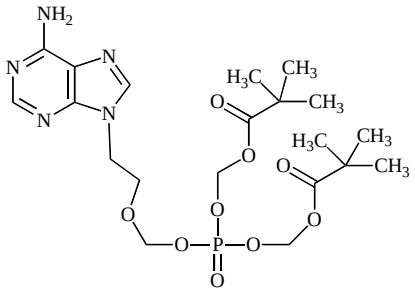
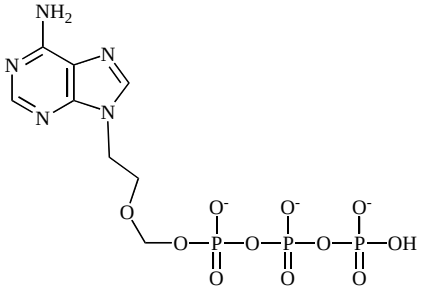
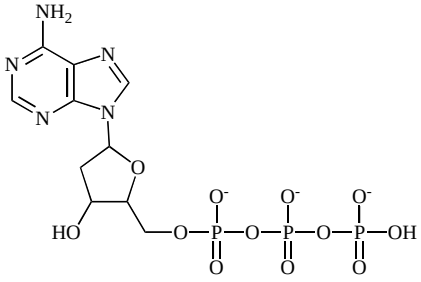
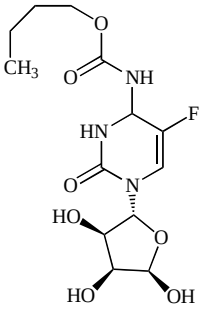
5-Fluorouracil (**10**) injection is indicated in the palliative management of some types of cancer, including that of colon, rectum, breast, stomach and pancreas. 5-Fluorouracil (**10**) is also used topically (as a cream) for treating actinic keratoses and some types of basal cell carcinomas of the skin and warts. The elimination half life of capecitabine (**9**) and 5-fluorouracil (**10**) is 38-45 min and 10-20 min, respectively. 5-fluorouracil (**10**) is further metabolized and gives two active metabolites: 5-fluoro-2-deoxyuridine monophosphate (FdUMP) (**11**) and 5-fluorouridine triphosphate (FUTP) (**13**). FdUMP and the folate cofactor bind to thymidylate synthase inhibiting the formation of thymidylate. As thymidylate is the required precursor of thymidine triphosphate (**44**), essential for the synthesis of DNA, capecitabine (**9**) inhibits cell division. Moreover, nuclear transcriptional enzymes can incorporate FUTP (**13**) at the site of UTP (**14**) during the synthesis of RNA. This mistakenly incorporated FUTP (**13**) makes a metabolic error to interfere with RNA processing and protein synthesis. Capecitabine (**9**) is FDA-approved as adjuvant in stage III colorectal cancer; metastatic colorectal cancer. It is used as a first line monotherapy in metastatic breast cancer or combined with docetaxel, in anthracycline-resistant cases.

Cidofovir (**15**) is a new antiviral drug used in the treatment of herpes and cytomegalovirus (CMV) retinitis in

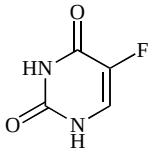
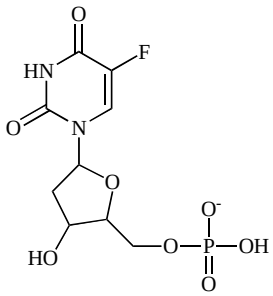
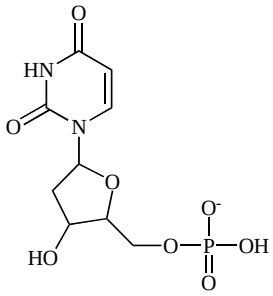
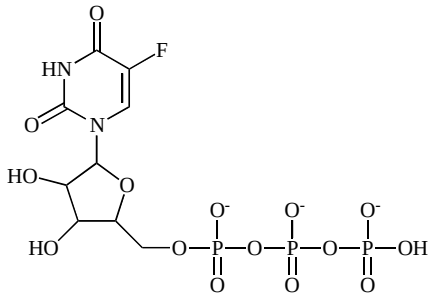
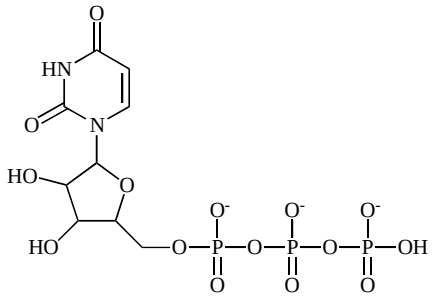
Table 1. Name, Chemical Structure, References to HPLC and that of Capillary Electrophoresis, Calculated Lipophilicity (logP) and Total Polar Surface Area (TPSA, Å^2) of Prodrugs Activated by Phosphate Conjugation. Prodrugs (Bold), Phosphate Conjugates (Regular) and the *nucleotides* (*italic*) to be Substituted/Displaced

Name and Chemical Structure	HPLC	CE	logP (Rekker)	TPSA
Abacavir (1) 	[9-21]	[20, 22]	2.03	101.88
Carbovir-5'-triphosphate (**2**) 	[21-24]		-4.47	222.67
Deoxyguanosine triphosphate (**3**) 			-6.15	252.13
Acyclovir (**4**) 	[25-29]	[26]	-1.47	114.76
Acyclovir-triphosphte (**5**) 	[30]		-5.73	254.35

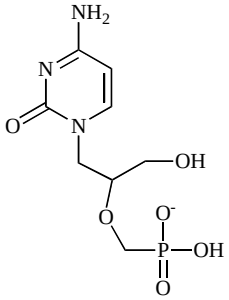
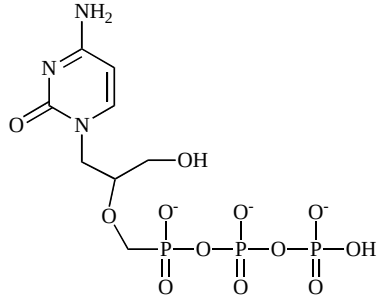
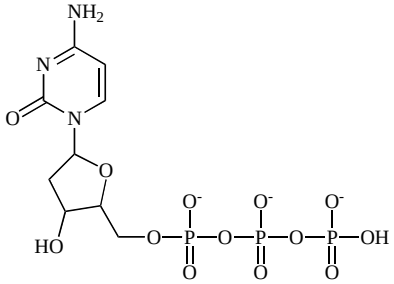
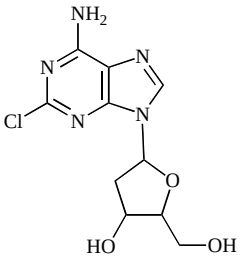
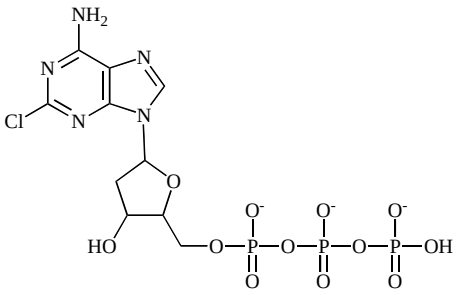
(Table 1). Contd…..

Name and Chemical Structure	HPLC	CE	logP (Rekker)	TPSA
Deoxyguanosine triphosphate (**3**) 			-6.15	252.13
Adefovir dipivoxil (**6**) 	[31]		1.48	166.98
Adefovir diphosphate (**7**) 	[32]		-4.53	229.44
Deoxyadenosine triphosphate (dATP) (**8**) 			**-5.96**	**258.90**
Capecitabine (**9**) 	[34]		**-0.01**	**120.69**

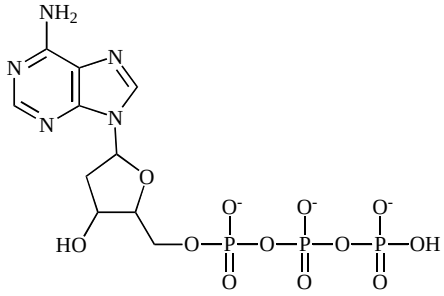
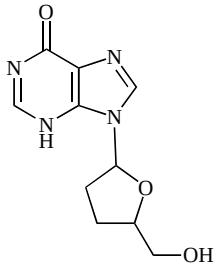
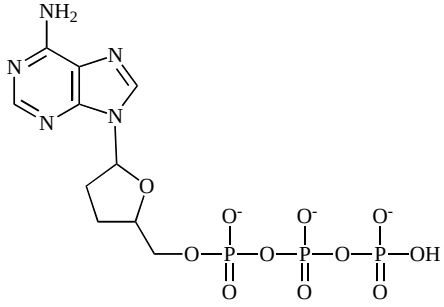
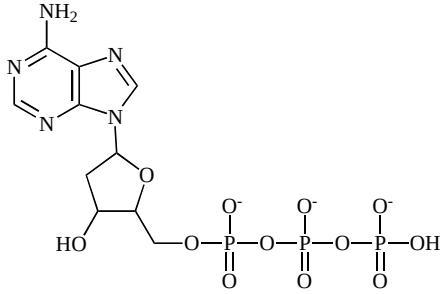
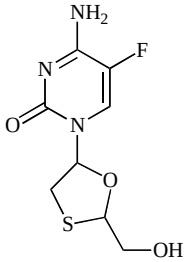
(Table 1). Contd…..

Name and Chemical Structure	HPLC	CE	logP (Rekker)	TPSA
5-Fluorouracil (**10**) 	[35]		-0.89	58.20
5-Fluoro-2'deoxyuridine-5'-monophosphate (**11**) 	[35,36]		-3.05	145.63
2'-Deoxyuridine-5'-monophosphate (**12**) 			-3.28	145.63
5-Fluorouridine triphosphate (FUTP) (**13**) 	[37]		-6.62	258.92
Uridine-triphosphate (UTP) (**14**) 			-6.85	258.92

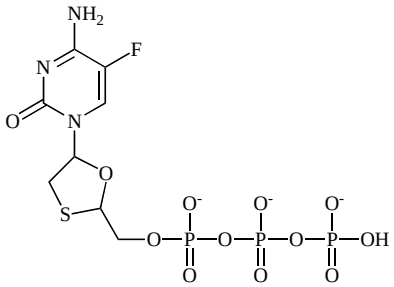
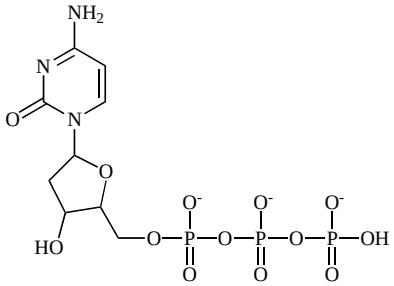
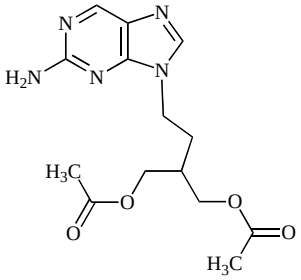
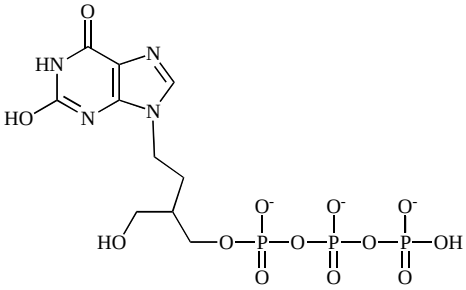
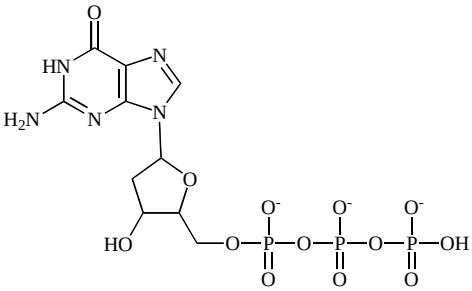
(Table 1). Contd…..

Name and Chemical Structure	HPLC	CE	logP (Rekker)	TPSA
Cidofovir (**15**) 	[38]		-4.46	145.68
Cidofovir-diphosphate (**16**) 	[39]		-7.52	238.74
Deoxycitidine-5'-triphosphate (**17**) 			-7.15	247.97
Cladribine (**18**) 	[40,41]		-0.96	119.31
Cladribine triphosphate (CdATP) (**19**) 	[40]		-5.22	258.90

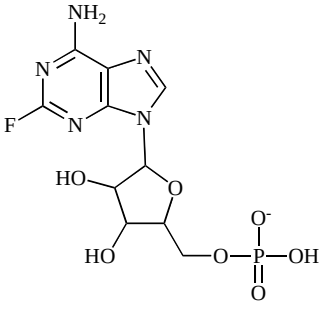
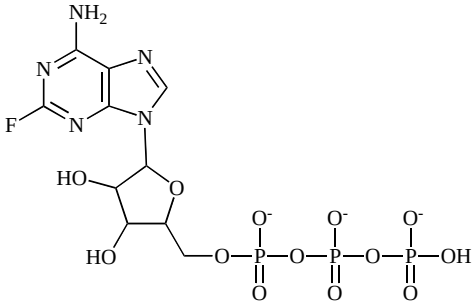
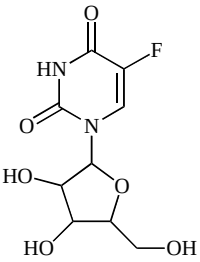
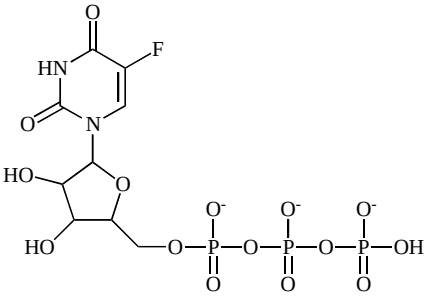
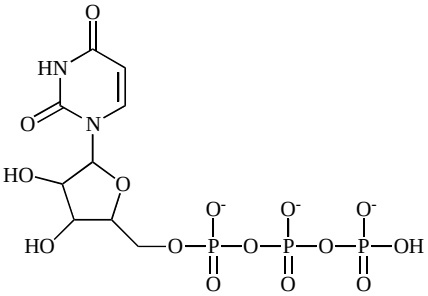
(Table 1). Contd…..

Name and Chemical Structure	HPLC	CE	logP (Rekker)	TPSA
Deoxyadenosine-5'-triphosphate, dATP (**8**) 			**-5.96**	**258.90**
Didanosine (**20**) 	[13, 14, 16, 19, 20, 42-57]	[20, 46, 58-64],	-1.80	88.74
Dideoxyadenosine triphosphate (**21**) 	[42, 43, 51]	[46]	-4.86	228.33
Deoxyadenosine-5'-triphosphate (**8**) 			**-5.96**	**258.90**
Emtricitabine (**22**) 	[18, 19, 56, 57, 65]		-1.79	71.08

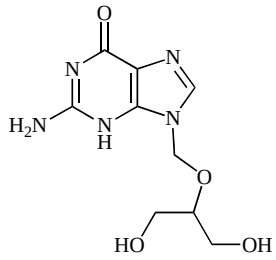
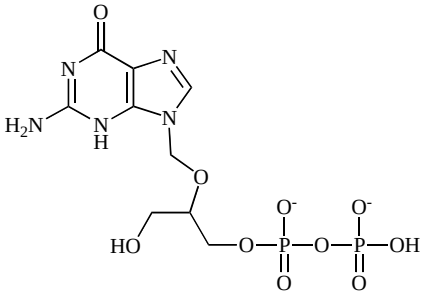
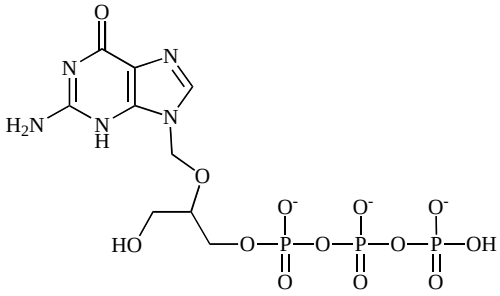
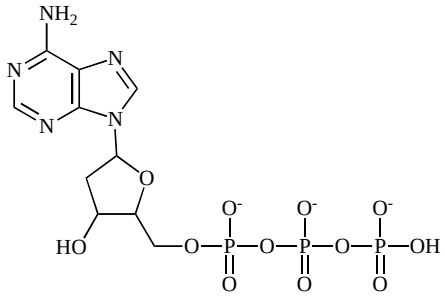
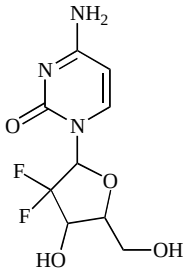
(Table 1). Contd…..

Name and Chemical Structure	HPLC	CE	logP (Rekker)	TPSA
Emtricitrabine-5'-triphosphate (**23**) 	[18, 65]		-2.94	192.21
Deoxycitidine-5'-triphosphate (**17**) 			-6.56	247.64
Famciclovir (**24**) 	[26, 66]		0.34	118.94
Penciclovir-triphosphate (**25**) 	[25, 66]		-7.36	257.67
Deoxyguanosine triphosphate (**3**) 			-6.15	252.13

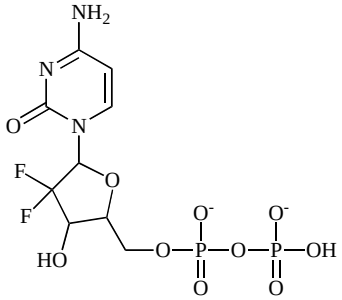
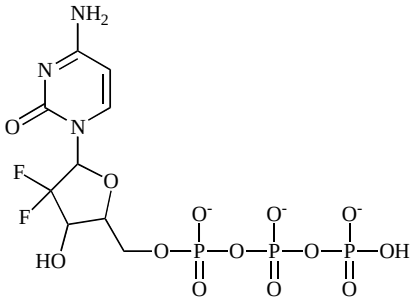
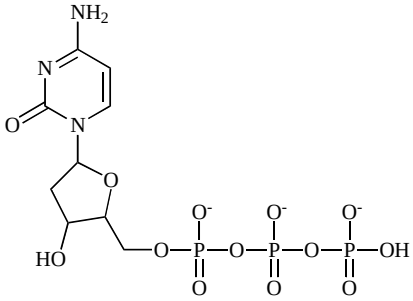
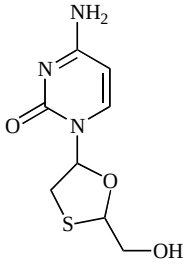
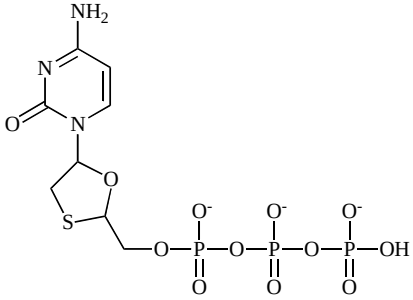
(Table 1). Contd…..

Name and Chemical Structure	HPLC	CE	logP (Rekker)	TPSA
Fludarabine (**26**) 	[67]		-2.78	176.84
2-Fluoroarabine-5'-triphosphate (**27**) 	[67]		-5.83	269.90
5-Fluorouridine (**28**) 	[67]		-2.37	119.33
5-Fluorouridine-5'-triphosphate (**13**) 	[37, 67]		-6.62	258.92
Uridine-5'-triphosphate (**14**) 			-6.85	258.92

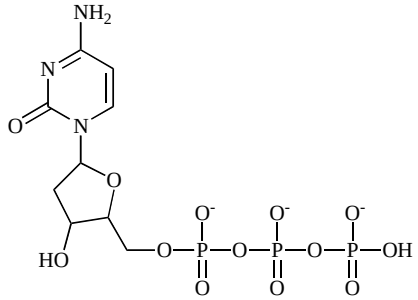
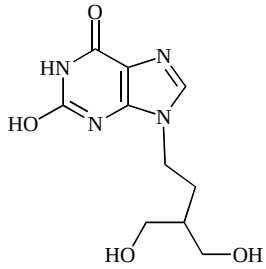
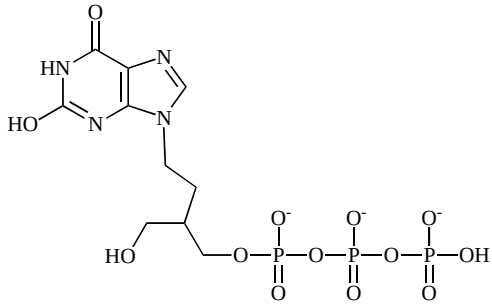
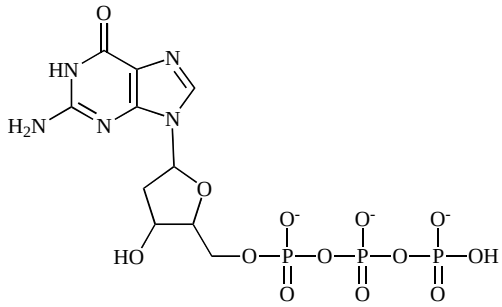
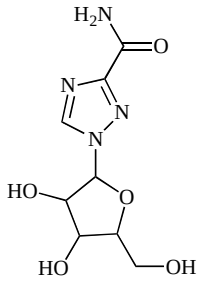
(Table 1). Contd…..

Name and Chemical Structure	HPLC	CE	logP (Rekker)	TPSA
Gancyclovir (**29**) 	[25-27, 29, 68]		-3.79	134.99
Gancyclovir-diphosphate (**30**) 	[68]		-6.84	228.05
Ganciclovir- triphosphate (**31**) 	[68]		-8.05	274.58
Deoxyadenosine-5'-triphosphate (**8**) 			**-5.96**	**258.90**
Gemcitabine (**32**) 	[23, 70]		-3.08	108.38

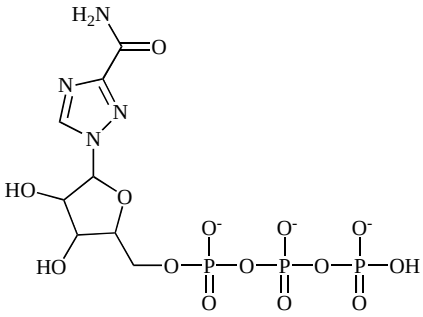
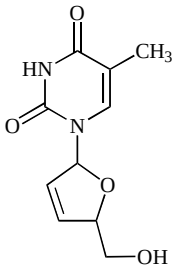
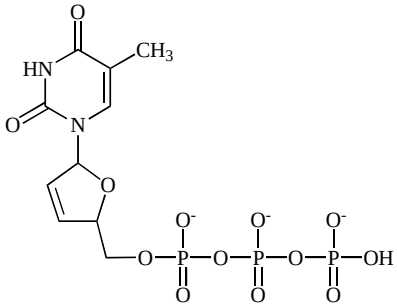
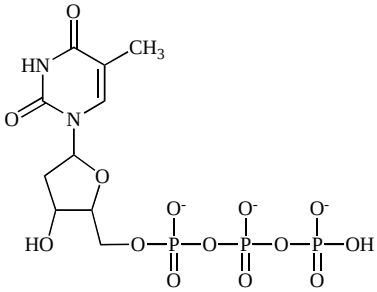
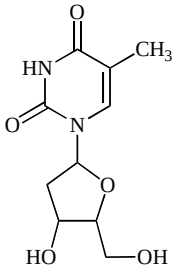
(Table 1). Contd…..

Name and Chemical Structure	HPLC	CE	logP (Rekker)	TPSA
Gemcitabine-5'-diphosphate (**33**) 	[23]		-6.14	201.44
Gemcitabine-5'-triphosphate (**34**) 	[23, 71]		-7.34	247.97
Deoxycitidine-5'- triphosphate (**17**) 			-7.15	247.97
Lamivudine (**35**) 	[16, 19, 20, 46, 49, 51, 54-57, 72-86]	[20, 87, 88]	-2.31	88.15
Lamivudine-5'-triphosphate (**36**) 	[23, 51, 89, 90]		-6.56	227.74

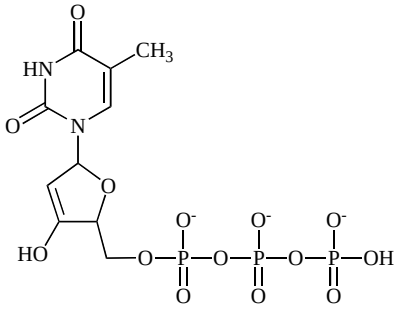
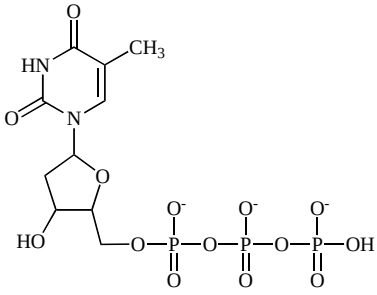
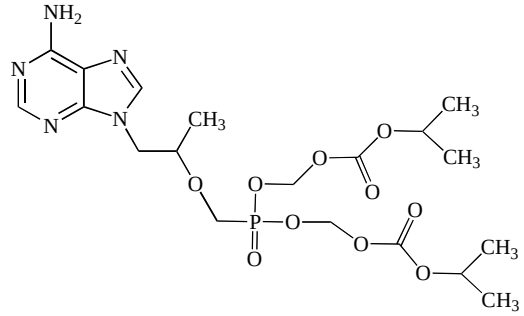
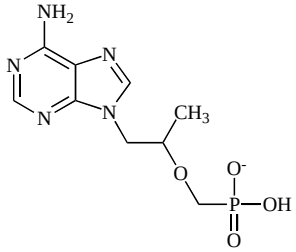
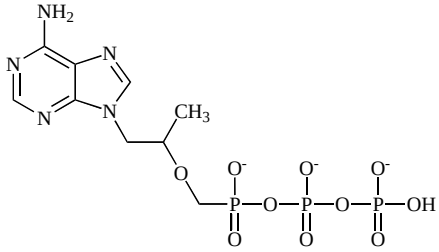
(Table 1). Contd…..

Name and Chemical Structure	HPLC	CE	logP (Rekker)	TPSA
Deoxycitidine-5'-triphosphate (**17**) 			-6.06	227.74
Penciclovir (37) 	[25, 66]		-3.10	118.08
Penciclovir-5'-triphosphate (**38**) 	[25, 66]		-7.36	257.67
Deoxyguanosine-5'-phosphate (**3**) 			-6.15	252.13
Ribavirin (**38**) 	[91, 92]		-4.64	143.72

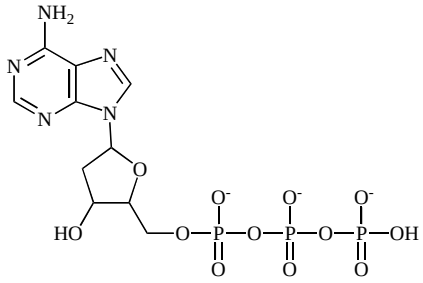
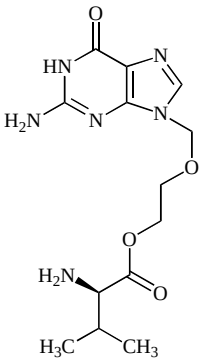
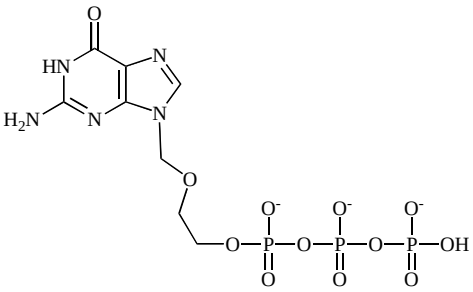
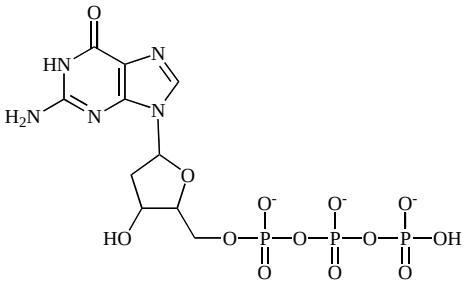
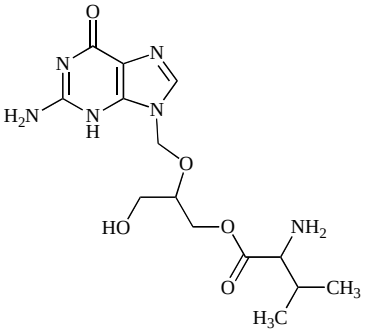
(Table 1). Contd…..

Name and Chemical Structure	HPLC	CE	logP (Rekker)	TPSA
Ribavirin-5'-triphosphate (**39**) 	[91]		-8.90	283.31
Stavudine (**40**) 	[14, 19, 20, 45, 46, 49, 54-56, 75, 77, 78, 80, 85, 86, 93]	[59, 94-96]	-0.82	78.87
Valganciclovir (**50**) 	[51]	[46, 61]	-5.08	218.46
Deoxythymidine-5'-triphosphate (**42**) 			-4.64	218.46
Telbivudin (**43**) 	[97-99]		-1.55	99.10

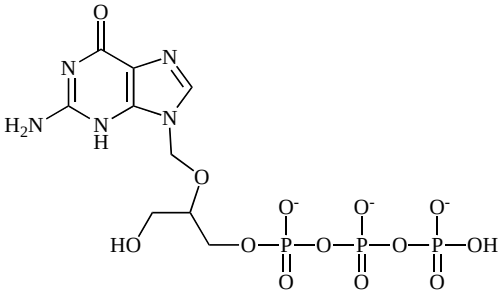
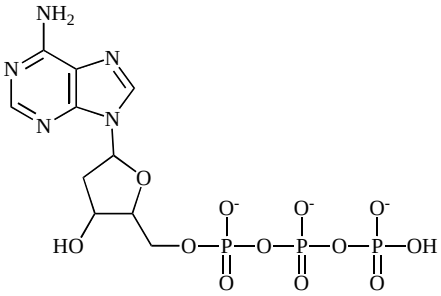
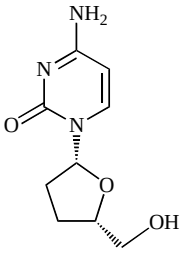
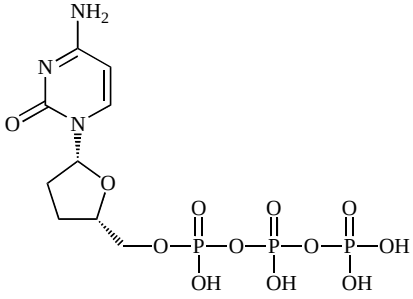
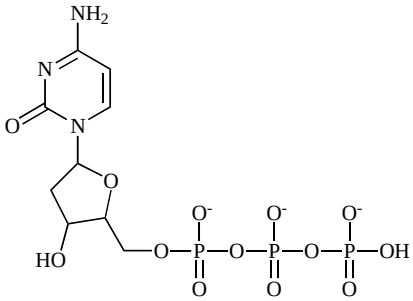
(Table 1). Contd…..

Name and Chemical Structure	HPLC	CE	logP (Rekker)	TPSA
Telbivudine-5'-triphosphate (**44**) 	[98-100]		-5.81	238.69
Thymidine-5'-triphosphate (**45**) 			-5.74	238.69
Tenofovir-disoproxilfumarate (**46**) 	[18, 46, 55, 65]		0.45	185.44
Tenofovir(-mono-phosphate) (**47**) 	[65]		-0.96	136.88
Tenofovir-di(-tri)-phosphate) (**48**) 	[20, 23, 43, 46, 65]		-2.16	182.91

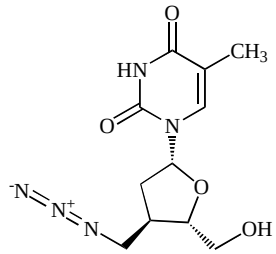
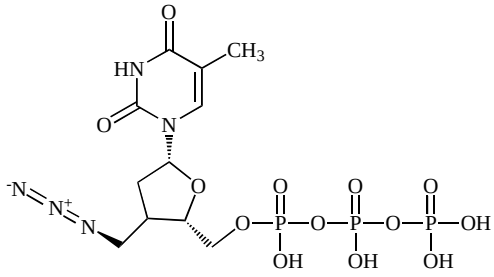
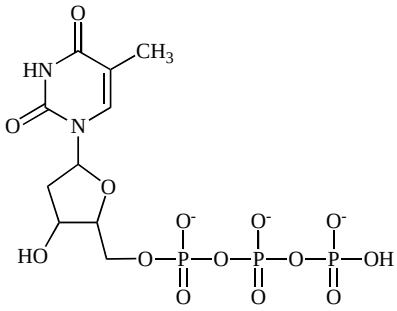
(Table 1). Contd…..

Name and Chemical Structure	HPLC	CE	logP (Rekker)	TPSA
Deoxyadenosine-5'-triphosphate (**8**) 			-5.96	258.90
Valaciclovir (**49**) 	[26, 101]		0.24	146.85
Aciclovir-triphosphate (**5**) 	[30]		-5.73	254.35
Deoxyguanosine-5'-triphosphate (**3**) 			-6.15	252.13
Valganciclovir (**50**) 	[101]		-2.08	167.08

(Table 1). Contd…..

Name and Chemical Structure	HPLC	CE	logP (Rekker)	TPSA
Ganciclovir- triphosphate (**29**) 	[68, 101]		-8.05	274.58
Deoxyadenosine-5'-triphosphate (**8**) 			**-5.96**	**258.90**
Zalcitabine (**51**) 	[16, 19, 55],		-2.72	88.15
Zalcitabine-5'-triphosphate (**52**) 			-6.98	227.74
Deoxycitidine-5'-triphosphate (**17**) 			-6.06	227.74

(Table 1). Contd…..

Name and Chemical Structure	HPLC	CE	logP (Rekker)	TPSA
Zidovudine (AZT) (**53**) 	[10, 12, 14, 18, 20, 46, 49, 51, 54, 55, 57, 69, 79, 81-85, 89, 102-110]	[20, 46, 58, 59, 62, 94-96, 111, 113-115]		115.08
Zidovudine 5'-triphosphate (**54**) 	[16, 20, 89, 112, 117, 118]			254.64
Thymidine-5'-triphosphate (**45**) 			-5.74	238.69

patients with HIV infection, as well as in the treatment of acyclovir-resistant herpes. It is an acyclic nucleoside phosphonate, and is therefore independent of phosphorylation by viral enzymes. Cidofovir (**15**) is metabolized to cidofovir diphosphate (**16**), which is an active intracellular metabolite. Elimination half life of cidofovir is 2.4-3.2 h. Cidofovir triphosphate (**16**) inhibits herpes virus polymerases at concentrations that are 8- to 600-fold lower than those causing inhibition of human cellular DNA polymerase alpha, beta, and gamma. Incorporation of cidofovir triphosphate (**16**) into the growing viral DNA chain results in reductions in the rate of viral DNA synthesis. The FDA has only approved cidofovir (**15**) use for CMV retinitis in AIDS patients.

Cladribine (**18**) is an antineoplastic agent used in the treatment of lymphoproliferative diseases including hairy-cell leukemia (leukemic reticuloendotheliosis) and multiple sclerosis. Elimination half life of cladribine (**18**) is 5.4 h. Although cladribine (**18**) is structurally related to fludarabine and pentostatin, their mode of cytotoxic activity differs. Cladribine (**18**) is phosphorylated to give cladribine triphosphate (CdATP) (**19**), which accumulates and is incorporated into the DNA of cells such as lymphocytes. Unlike the majority of antimetabolites, cladribine (**18**) has cytotoxic effect on both resting and proliferating lymphocytes.

Didanosine (DDI) (**20**) is a prodrug. DDI is effective against HIV and is used in combination with other antiretroviral drug therapies as part of a highly active antiretroviral therapy (HAART). Elimination half life is 1.5 h. DDI (**20**) is metabolized intracellularly into its active form: didanosine triphosphate (**21**), that is dideoxyadenosine triphosphate (ddATP). ddATP inhibits the HIV reverse transcriptase enzyme competitively by displacing natural dATP (**8**). It can also act as a chain terminator by incorporation into viral DNA as the lack of 3'-OH group prevents the formation of 5'- to 3'- phosphodiester linkage. FDA approval for the treatment of HIV infection was in 1991 [5, 55].

Efavirenz is a non-nucleoside reverse transcriptase inhibitor (NNRTI) and (together with lamivudine in combination of either zidovudine or tenofovir) is used as part of highly active antiretroviral therapy (HAART) for the treatment of human immunodeficiency virus (HIV) type 1. Half life is 40-55 h. Antiviral activity of efavirenz depends on its intracellular conversion to a triphosphorylated form. Triphosphorylated efavirenz inhibits the activity of viral

RNA-directed DNA polymerase (i.e. reverse-transcriptase), thereby it interferes with a generation of DNA copies of viral RNA inhibiting the synthesis of new virions. FDA approval of efavirenz as an antiretroviral drug was given in 1998.

Emtricitabine (**22**) is indicated in combination with other antiretroviral agents for the treatment of HIV infection in adults. Half life is 10 h. It is a synthetic nucleoside analogue of citidine, and it is phosphorylated by cellular enzymes to form emtricitabine-5'-triphosphate (**23**). Emtricitabine-5'-triphosphate (**23**) competes with the natural substrate of HIV-reverse transcriptase, with deoxycytidine-5'-triphosphate (**17**) thereby emtricitabine (**22**) treatment helps to lower the amount of HIV or the "viral load" in HIV patient's body, and can indirectly increase the number of immune cells. FDA approval for the treatment of HIV infection was in 2003 [5, 55].

Famciclovir (**24**) is indicated for the treatment of acute herpes zoster (shingles), for the treatment or suppression of recurrent genital herpes in immunocompetent patients and that of recurrent mucocutaneous herpes simplex infections in HIV infected patients. Half life is 2-2.3 h. Orally administered famciclovir is metabolized by ester hydrolysis to penciclovir (**37**). Penciclovir (**37**) is phosphorylated to a monophosphate in herpes simplex virus types 1 or 2 (HSV-1, HSV-2) infected cells by viral thymidine kinase which is then converted to penciclovir-triphosphate (**25**) by cellular kinases. Penciclovir-triphosphate (**25**) selectively inhibits viral DNA polymerase by competing with deoxyguanosine-5'-triphosphate (**3**) (according to *in vitro* experiments). Inhibition of DNA polymerase causes blocking of DNA synthesis of the virus infected cell, i.e. viral replication. In cells not infected with HSV, DNA synthesis remains unaltered. The most commonly found acyclovir resistant HSV mutants are deficient of thymidine kinase, so they do not phosphorylate penciclovir (**37**) to penciclovir-monophosphate resulting in resistance to famciclovir (**24**) (and penciclovir) treatment. FDA approval was in 1998 [5, 55].

Fludarabine (2-fluoro-ara-AMP) (**26**) is used for the treatment of hematological malignancies, such as adult patients with B-cell chronic lymphocytic leukemia (CLL) who have not responded to or whose disease has progressed during treatment with at least one standard alkylating-agent containing regimen. Half life is 20 h. One phosphate moiety is included in fludarabine, which is rapidly hydrolyzed following the treatment to 2-fluoro-ara-A. 2-Fluoro-ara-A is phosphorylated intracellularly by deoxycitidine kinase to the active triphosphate form of fludarabine (2-fluoro-ara-ATP (**27**)), which is considered an inhibitor of DNA polymerase alpha, ribonucleotide reductase and DNA primase. Mechanism of detailed inhibition DNA synthesis by fludarabine has not been completely characterized yet.

Ganciclovir (**29**) is used for the treatment of complications from AIDS-associated cytomegalovirus infections. Half life is 2.5-5 h. Ganciclovir (**29**) is phosphorylated into its highly selective antiviral form by subsequent reactions: Thymidine kinase converts it to ganciclovir monophosphate, cellular guanylate kinase further metabolizes to ganciclovir-diphosphate (**30**), and the -triphosphate (**31**) is produced by a number of cellular enzymes. Ganciclovir-triphosphate (**31**) is a substrate for viral DNA polymerase, faulty DNA is produced by replacing much of the deoxyadenosine-5'-triphosphate (**8**). This replacement results in the prevention of DNA synthesis by destabilizing the strand. Ganciclovir inhibits viral DNA polymerase more effectively than cellular polymerase.

Gemcitabine (**32**) is indicated as the first-line treatment of patients with metastatic breast cancer, locally advanced (Stage IIIA or IIIB), or metastatic (Stage IV) non-small cell lung cancer and as a first-line treatment for patients with adenocarcinoma of the pancreas. Its half life depends on the rate of infusion. Gemcitabine (**32**) is considered as a prodrug; its active drug form is generated by deoxycitidine kinase by the formation of gemcitabine-5'-diphosphate (**33**) and gemcitabine-5'-triphosphate (**34**). Both gemcitabine-5'-diphosphate (**33**) and gemcitabine-5'-triphosphate (**34**) act in their specific ways. Gemcitabine-5'-diphosphate (**33**) inhibits ribonucleotide reductase, an enzyme responsible for the catalysis of synthesis of deoxynucleoside triphosphates. Inhibition of ribonucleotide reductase essentially diminishes DNA synthesis. Gemcitabine-5'-triphosphate (**34**) (that is difluorodeoxycytidine triphosphate) competes with endogeneous deoxynucleosides-5'-triphosphates (e.g. deoxycitidine-5'-triphosphate (**17**)) for incorporation into DNA.

Lamivudine (**35**) indications include treatments against Human Immunodeficiency Virus Type 1 (HIV-1) and hepatitis B (HBV). Lamivudine (**35**) is an analogue of cytidine. Lamivudine (**35**) is intracellularly phosphorylated to its active triphosphate metabolite, lamivudine-5'-triphosphate (**36**) (L-TP). L-TP (**36**) is incorporated into viral DNA by HIV reverse transcriptase and HBV polymerase, resulting in the termination of the DNA chain. Its half life is 5-7 h. Lamivudine (**35**) was approved by the Food and Drug Administration in 1995 for use with zidovudine (**53**) and in 2002 as a once-a-day dosed medication [5, 55].

Penciclovir (**37**) is a guanine analogue antiviral drug used for the treatment of various herpes virus infections. Half life is 2.2-2.3 h. Viral thymidine kinase phosphorylates penciclovir (**37**) to penciclovir monophosphate in herpes simplex virus (HSV) infected cells. Penciclovir monophosphate is metabolized to penciclovir-triphosphate (**25**) by cellular kinases. Penciclovir-triphosphate (**25**) selectively inhibits viral DNA polymerase by competing with deoxyguanosine-5'-triphosphate (**3**) and the inhibition of DNA synthesis of virus-infected cells inhibits viral replication. If cells are not infected with HSV, their DNA synthesis remains unaltered.

Ribavirin (**38**) is indicated for the treatment of chronic hepatitis C and for the treatment of respiratory syncytial virus (RSV) infection. Half life of a single dose is 120-170 h. Ribavirin (**38**) is intracellularly phosphorylated to its mono-, di- and triphosphate derivatives by adenosine kinase. Ribavirin-5'-triphosphate (**39**) is a competitive inhibitor of three important viral enzymes, such as inosine monophoshate dehydrogenase, viral RNA polymerase and messenger RNA guanyltransferase. The inhibition of guanyltransferase stops the capping of mRNA. The multiple inhibition results in a reduction of the intracellular guanosine-5'-

triphosphate pool, in an inhibition of viral RNA as well as protein synthesis.

Stavudine (**40**) is indicated for the treatment of human immunovirus (HIV) infections. Stavudine (**40**) is a nucleoside reverse transcriptase inhibitor with definite activity against HIV-1. Half life is 0.8-1.5 h. Stavudine (**40**) is an analogue of thymidine, but lacks the 3'-OH group. Stavudine is phosphorylated into active stavudine-5'-triphosphate (**41**) that inhibits the HIV reverse transcriptase by competing with deoxythymidine-5'-triphosphate (**3**). The lack of 3'-hydroxyl group in the incorporated stavudine-5'-triphosphate (**41**) prevents the formation of the 5' to 3' phosphodiesterase linkage essential for DNA elongation. Therefore, the DNA growth is terminated. Simultaneous use of stavudine (**40**) and azidothymidine (**52**) (AZT) is not recommended as AZT (**52**) can inhibit the intracellular phosphorylation of stavudine (**40**). Other anti-HIV drugs do not possess this property. FDA approval for the treatment of HIV infection was given in 1994 [5, 55].

Tenofovir is marketed in a diester form. The diester requires initial hydrolysis to yield tenofovir, an acyclic nucleoside phosphonate diester, an analogue of adenosine monophosphate. Its indication means its use in combination with other antiretroviral agents for the treatment of HIV-1 (Tenofovir-disoproxilfumarate (**45**)). Subsequent phosphorylation by cellular enzymes forms tenofovir-monophosphate (**46**) and tenofovir-diphosphate (**47**) which blocks reverse transcriptase, an enzyme crucial for viral production of HIV infected people. FDA approval for the treatment of HIV infection was procured in 2001 and for chronic hepatitis in 2008 [5, 55].

Valaciclovir (**48**) is indicated in the treatment or suppression of cold sores (herpes labialis), herpes zoster (shingles), genital herpes in immunocompetent individuals, and recurrent genital herpes in HIV-infected individuals. Valaciclovir (**48**) is a prodrug of acyclovir (**4**) and acyclovir-5'-triphosphate (**5**), as it is almost completely hydrolyzed to L-valine and acyclovir (**4**) (see the metabolic fate and phosphorylation of valacyclovir/acyclovir). Half life is less than 30 min (for valaciclovir) and 2.5-3.6 h (for aciclovir)

Valganciclovir (**49**) is used for the treatment of cytomegalovirus infections. Valganciclovir (**49**) is the L-valyl ester of ganciclovir (**29**), it is actually a prodrug for ganciclovir (**29**). Half life is 4 h. After oral administration, it is rapidly hydrolyzed to ganciclovir (**29**) by intestinal and hepatic esterases (details of phosphorylations and effects are given at ganciclovir).

Zalcitabine (**50**) is indicated in the treatment of human immunodeficiency virus (HIV) infections. Zalcitabine (**50**) inhibits the activity of HIV-1 reverse transcriptase (RT) both by competing with the natural substrate dGTP and by its incorporation into viral DNA. Half life is 2 h. Zalcitabine (**50**) is phosphorylated into zalciatbine-5'-triphosphate (**51**), that competes with dGTP for incorporation into viral DNA. Zalcitabine-5'-triphosphate (**51**) inhibits the HIV reverse transcriptase enzyme competitively, and acts as chain terminator of DNA synthesis, as lack of 3'-OH group in the incorporated nucleoside analogue prevents the formation of 5'- to 3'-linkage. FDA approval for the treatment of HIV infection was achieved in 1992 [55] in 1996 in combination with zidovudine (**52**). Sales and distributions were discontinued from 2006 because of other NRTI drugs were on the market having more favorable risk/benefit profile.

Zidovudine (**52**) (ZDV) or azidothymidine (AZT) is a type of antiretroviral drug, and acts as nucleoside reverse transcriptase inhibitor (NRTI). The presence of azido group in the chemical structure increases the lipophilic nature of zidovudine (**52**), which can easily cross cell membranes by diffusion. Zidovudine (**52**) can penetrate through the blood-brain barrier. Zidovudine (**52**) is phosphorylated to its active metabolites. The lack of a 3'-OH group in the incorporated zidovudine-5'-triphosphate (**53**) prevents the formation of a 5' to 3' linkage, which is essential for DNA elongation. Zidovudine-5'-triphosphate (**53**) has about 100-fold less ability to inhibit the human cellular DNA polymerase than that of HIV reverse transcriptase. Certain toxic effects (possible damage of cardiac and other muscles) of ZDV (**52**) treatment can account for the relatively high sensitivity of cellular DNA polymerase of the mitochondria. ZDV (**52**) (i.e. AZT) was the first drug to be approved for treatment for HIV. The use of ZDV (**52**) made a major breakthrough in AIDS therapy in the 1990s, which significantly altered the course of the disease and helped destroy the notion that having HIV/AIDS means an instant death sentence. ZDV (**52**) slows HIV spread significantly, but does not stop it entirely. ZDV (**52**) was approved by the FDA for use against HIV-induced AIDS, and AIDS Related Complex in 1987 [5, 55].

To complete the medicinal chemistry of this special group of antiviral/anticancer compounds, HPLC and capillary zone electrophoresis (CZE)methods for their quantitative analysis are given in Table **1**.

Analytical determinations are done to monitor the level and also the conversion of these synthetic nucleoside analogues to their phosphate-conjugated form. Table **1** gives an impression, of how often HPLC-UV, HPLC combined with mass spectrometry, CZE-UV combined with mass spectrometric detection (CZE-MS) were used to detect and to quantify nucleosides and the corresponding nucleotide analogues.

Quality Control

Anbazhagan *et al*. [74] compared three simple methods to quantify stavudine (**40**), lamivudine (**35**) (and also nevirapine) in tablets. Both UV spectroscopy, and HPLC and HPTLC (high-performace thin-layer chromatography) gave adequate results for the routine analysis of tablets.

The overwhelming majority of chromatographic and electrophoretic separation deals with nucleosides. First of all, concentration level of nucleosides is higher than that of nucleotides.

Therapeutic drug monitoring (TDM) and pharmacokinetics serve not only the science but also everyday's interest of patients.

Rebiere *et al.* [19] worked out two reliable methods for parallel quantification of several antiretroviral agents using HPLC with UV detection. The drugs belong to either nucleo-

side/nucleotide reverse transcriptase inhibitors (abacavir (**1**), amdoxovir, didanosine (**20**), emtricitabine (**22**), lamivudine (**35**), stavudine (**40**), zalcitabine (**50**), zidovudine (**52**)) or non-nucleoside reverse transcriptase inhibitors or protease inhibitors. They used HPLC on YMC pack ODS-AM (250 x 4.6 mm, 5 μm) column. This method was used to monitor therapeutic drug level even when they are used in combination.

Pereira *et al.* [58] worked out a validated capillary electrophoresis (CZE) method to determine didanosine (**20**), efavirenz and zidovudine (52) in blood, even these antiretroviral drugs were used in combinations. Detection was done using ultraviolet absorbance at 200 nm.

Chong *et al.* [63] analysed famciclovir (**24**) concentration in serum and vitreous fluid to determine serum to vitreous penetration. They found that vitreous concentrations of famciclovir (**24**) are within the inhibitory level for herpes simplex 1, herpes simplex 2, varicella zoster virus. They concluded that oral famciclovir (**24**) maybe a reasonable alternative to i.v. aciclovir (**4**). Determinations were carried out using reversed-phase HPLC and detection of UV absorbance at 254 nm.

A method for reliable quantification of several AIDS drugs was reported by Checa *et al.* [56], including the analytical method for didanosine (**20**), emtricitabine (**22**), lamivudine (**35**), stavudine (**40**), zalcitabine (**50**) and zidovudine (**52**). Optimized chromatographic procedures were given for several triplets of drugs, the limit of detection (LOD) values varied between 3 and 15 ng/mL. Zou *et al.* [30] used LC-MS-MS to establish pharmacokinetic parameters of adefovir (**6**) in healthy Chinese volunteers. The t_{max} was about 1.5, 1.6 and 1.8 h, and the elimination half life values were about 8, 7.5 and 7.5 h after administration of 10, 20 and 30 mg adefovir dipivoxil (**6**), respectively [30].

Bioequivalency studies have also been done on these drugs. For instance, the patent of AZT (**52**) expired in 2005 (placing AZT (**52**) in the public domain), allowing other drug companies to manufacture and market generic AZT (**53**) without having to pay. The U.S. FDA has since approved several generic forms of AZT (**52**). Zou *et al.* [30] and also Sun *et al.* [117] determined adefovir (**6**) using LC/MS/MS. Their validated methods were used to determine adefovir (**6**) in serum and urine, and were proven to be suitable to clinical pharmacokinetic study.

Phosphorylation of antiretroviral agents can be monitored using HPLC. Pioneering works on the analysis of zidovudine (**52**) phosphorylation were published by Peter *et al.* [109, 114] and Brody *et al.* [108]. Peripheral blood mononuclear cells were collected [109, 114], a clean-up was followed by HPLC. Fractions consisting of zidovudine (**52**) and its phosphorylated anabolites were collected, the anabolites were dephosphorylated to zidovudine, and they were quantified by RIA. This method was further developed by the use of radiolabelled zidovudine (**52**) for incubation. Radioactivity of the separated fractions (zidovudine (**52**), zidovudine-5'-monophosphate, zidovudine-5'-diphosphate and zidovudine-5'-triphosphate (**53**) were determined to give intracellular concentration of the parent drug and that of the different phosphate-conjugated metabolites. Non-significant effect of the presence/absence of stavudine (**40**) on the phosphate conjugation was also established. Culture, and the nucleoside anabolism was analyzed after separating them.

Sensitivity of analysis is highly increased by monitoring the separation with on-line coupled tandem mass spectrometry (HPLC/MS/MS). Ray *et al.* [31] followed phosphorylation of adefovir (**6**) using hepatic cell lines and primary human hepatocytes. Separation and quantification of adefovir (**6**), adefovir-monophosphate and adefovir-diphosphate (**7**) were done on Phenomex Prodigy 5 μm stationary phase using gradient elution. Moore *et al.* [82] made simultaneous quantification of the 5'-triphosphate metabolites of lamivudine, stavudine and zidovudine in peripheral mononuclear blood cells (PMBCs) of HIV infected patients. PMBCs are the sites where HIV replication and the possible drug action take place. Moore *et al.* [82] used HPLC/MS/MS with a Phenomex Columbus (100 x 1 mm, 5 μm) C-18 stationary phase. Pharmacokinetics of gemcitabine (**32**) was detected by Losa *et al.* [23], who determined gemcitabine-5'-diphosphate (**33**) and gemcitabine-5'-triphosphate (**34**) in PMBCs of patients treated with i.v. gemcitabine (**32**). Tracer Excel ODSA C18 column (100 x 4.6 mm, 3 μm) stationary phase and diode array UV detection were used.

Fludarabine phosphorylation by human leukemic cell was followed by Kalhorn *et al.* [64] using HPLC/MS. Prodigy C-8 (100 x 2 mm, 5 μm) column was used as the stationary phase.

Carli *et al.* [36] reported the simultaneous quantification of 5-fluorouracil (**10**) and several members of its anabolic pathway (5-fluoro-2'-deoxyuridine, 5-fluoro-2'-deoxyuridine-monophosphate (**11**), etc.) using LC/MS/MS. The separation was done using an Atlantis (100 x 2.1 mm, 3.5 μm) C-18 stationary phase.

Separation of antiviral nucleoside reverse transcriptase inhibitors and their phosphorylated metabolites is not restricted to the reversed-phase stationary phases. Anion exchange liquid chromatography and on-line coupled to tandem mass spectrometer was used by Jansen *et al.* [39, 62] to quantify nucleotides of emtricitabine (**22**) and tenofovir (**46**) in PMBCs. Anion-exchange chromatography was also used by Sparidans *et al.* [68] to analyze gemcitabin-5'-triphosphate (**31**) in human white blood cells. Later on, porous graphitic carbon was used to separate 2′-2′-difluorodeoxycytidine and 2′-2′-difluorodeoxyuridine nucleosides and nucleotides by Jansen *et al.* [116].

Excellent reviews [20, 26, 55] have been published on the clean-up and the separation possibilities of several antiviral compounds, comparing the methods (HPLC versus capillary electrophoresis), conditions of separation, and methods of detections (UV, fluorescence). The sources of samples were also specified, such as bulk drug, serum, urine and many others.

CONCLUSIONS

Two distinct and essential steps are required for the medicinal action of a drug. These are that the drug should reach the site of action, and it should be able to fit the site of ac-

tion. Drug distribution (penetration through the membranes) is generally possible for drugs of lipophilic character. However, drugs used as reverse transcriptase inhibitors should change their lipophilicity and chemical character inside the cell to exert their antivirus or anticancer effects. Enzymatic attachment of two or three phosphates contributes to the vital metabolic/anabolic characters of reverse transcriptase inhibitors.

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