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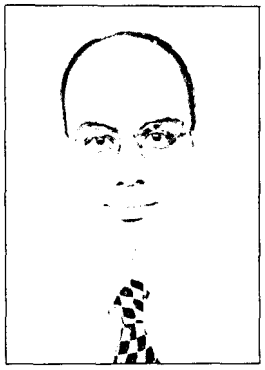

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Abstract: 3'-Azido-2',3'-dideoxythymidine (AZT, **1**, zidovudine, Retrovir™) is used to treat patients with human immunodeficiency virus (HIV) infection. AZT, after conversion to AZT-5'-triphosphate (AZT-TP) by cellular enzymes, inhibits HIV-reverse transcriptase (HIV-RT). The major clinical limitations of AZT are due to clinical toxicities that include bone marrow suppression, hepatic abnormalities and myopathy, absolute dependence on host cell kinase-mediated activation which leads to low activity, limited brain uptake, a short half-life of about one hour in plasma that dictates frequent administration to maintain therapeutic drug levels, low potential for metabolic activation and/or high susceptibility to catabolism, and the rapid development of resistance by HIV-1. These limitations have prompted the development of strategies for designing prodrugs of AZT. A variety of 5'-*O*-substituted prodrugs of AZT constitute the subject of this review. The drug-design rationale on which these approaches are based is that the ester conjugate will be converted by hydrolysis and/or enzymatic cleavage to AZT or its 5'-monophosphate (AZT-MP). Most prodrug derivatives of AZT have been prepared by derivatization of AZT at its 5'-*O* position to provide two prominent classes of compounds that encompass: A) 5'-*O*-carboxylic esters derived from 1) cyclic 5'-*O*-carboxylic acids such as steroidal 17β-carboxylic acids, 1-adamantanecarboxylic acid, bicyclam carboxylic acid derivatives, *O*-acetylsalicylic acid, and carbohydrate derivatives, 2) amino acids, 3) 1,4-dihydro-1-methyl-3-pyridinylcarboxylic acid, 4) aliphatic fatty acid analogs such as myristic acid containing a heteroatom, or without a heteroatom such as stearic acid, and 5) long chain polyunsaturated fatty acid analogs such as retinoic acid, and B) masked phosphates such as 1) phosphodiesters that include monoalkyl or monoaryl phosphate, carbohydrate, ether lipid, ester lipid, and foscarnet derivatives, 2) a variety of phosphotriesters that include dialkylphosphotriesters, diarylphosphotriesters, glycolate and lactate phosphotriesters, phosphotriester approaches using simultaneous enzymatic and chemical hydrolysis of bis(4-acyloxybenzyl) esters, bis(*S*-acyl-2-thioethyl) (SATE) esters, cyclosaligenyl prodrugs, glycosyl phosphotriesters, and steroidal phosphotriesters, 3) phosphoramidate derivatives, 4) dinucleoside phosphate derivatives that possess a second anti-HIV moiety such as AZT-P-ddA, AZT-P-ddI, AZTP2AZT, AZTP2ACV), and 5) 5'-hydrogen phosphonate and 5'-methylene phosphonate derivatives of AZT. In these prodrugs, the conjugating moiety is linked to AZT via a 5'-*O*-ester or 5'-*O*-phosphate group. 5'-*O*-Substituted AZT prodrugs have been designed with the objectives of improving anti-HIV activity, enhancing blood-brain barrier penetration, modifying pharmacokinetic properties to increase plasma half-life and improving drug delivery with respect to site-specific targeting or drug localization. Bypassing the first phosphorylation step, regulating transport and conferring sustained release of AZT prolong its duration of action, decrease toxicity and improve patient acceptability. The properties of these prodrugs and their anti-HIV activities are now reviewed.

Introduction

The development of effective antiviral therapy for the treatment of individuals infected with human immunodeficiency virus (HIV) presents a unique challenge. The integration of the virus into host cell deoxyribonucleic acid (DNA), the dependence of the virus on host cell machinery, the high mutation rate of HIV RT, and the chronicity of viral replication represent formidable obstacles to the development of specific and effective antiretroviral therapies.

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A number of specific events in the replicative cycle of HIV can be considered as potential targets for chemotherapeutic intervention. The steps in viral replication include: attachment or absorption of the viral particle, penetration, uncoating, reverse transcription, RNaseH degradation, DNA synthesis of the second strand, migration to the nucleus, integration, transcription or ribonucleic acid (RNA) processing, protein synthesis (translation), protein glycosylation, myristoylation, assembly of virus, release of virus, and maturation [1-3]. Several review articles have discussed different strategies for HIV chemotherapy [4-13].

Most studies have focused on inhibitors of viral reverse transcriptase (RT), a key enzyme in the replicative cycle of HIV, which is an attractive target for chemotherapy. Viral RT is essential for the transcription of viral RNA into proviral DNA in the cytoplasm.

2',3'-Dideoxynucleosides (ddNs) are targeted at the viral reverse transcriptase (RT) and exhibit potent anti-HIV-1 activity. ddNs must be phosphorylated intracellularly to their active triphosphate form before acting as competitive inhibitors or alternate substrates (chain terminators) of HIV RT. ddNs interact at the substrate binding site of the enzyme where the deoxynucleoside triphosphates (dNTPs) normally bind [7]. There are currently five RT inhibitors approved to treat HIV disease. Accordingly, 2',3'-dideoxynucleosides such as 3'-azido-2',3'-dideoxythymidine (AZT, **1**, zidovudine, Retrovir™), 2',3'-dideoxy-3'-thiacytidine (3TC, **2**, lamivudine, Epivar™) [14], 2',3'-dideoxycytidine (ddC, **3**, zalcitabine, Hivid™) [15-17], 2',3'-didehydro-3'-deoxythymidine (d4T, **4**, stavudine, Zerit™) [18] and 2',3'-dideoxyinosine (ddI, **5**, didanosine, Videx™) [19] are used clinically to treat patients with human immunodeficiency virus (HIV) infection (Fig. **1**).

Among the available drugs for anti-HIV therapy, AZT is still one of the most effective. AZT, the first RT inhibitor approved for clinical use to treat human immunodeficiency virus (HIV) infection [16], is a synthetic pyrimidine analog that differs from thymidine (dThd, **6**) in having an azido substituent in place of a hydroxyl group at the 3' position of the deoxyribose ring. AZT is the therapy of choice for prevention and management of cognitive impairment in symptomatic HIV-infected patients and patients with AIDS. Treatment using AZT delays HIV progression and improves survival in patients with advanced disease. The pharmacodynamics, pharmacokinetics and therapeutic efficacy of AZT have been previously reviewed [20]. Transmission of HIV-1 from mother to newborn can be reduced by AZT treatment [21], and AZT improves neuropsychologic performance in adult patients with HIV-1 infection. Patients receiving AZT show general improvement of neurological functions including attention, memory, motor function, and general cognitive ability [22].

Dramatic reductions of HIV viral load have recently been achieved by the introduction of combination drug therapy. Recent studies have generally employed

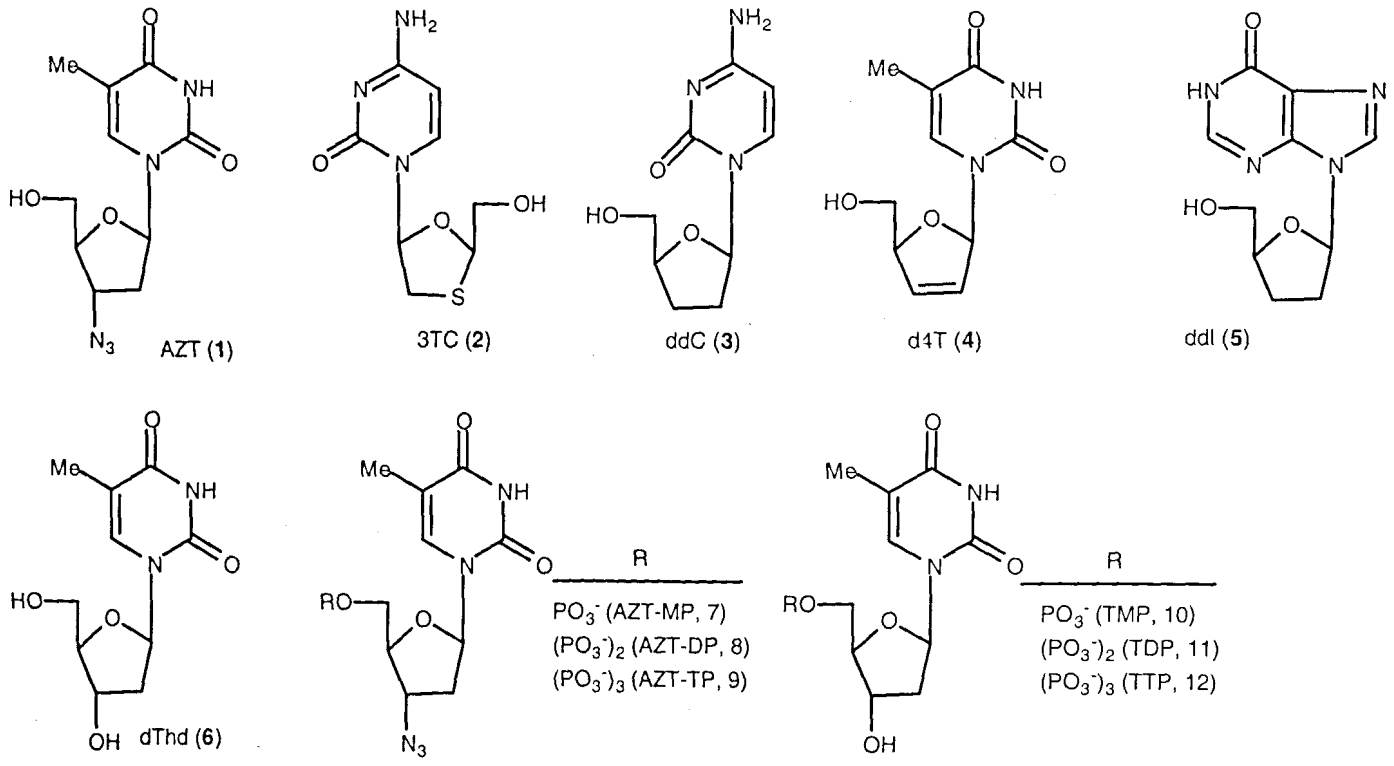


Fig. (1). The chemical structures for AZT (**1**), 3TC (**2**), ddC (**3**), d4T (**4**), ddI (**5**), dThd (**6**), AZT-MP (**7**), AZT-DP (**8**), AZT-TP (**9**), TMP (**10**), TDP (**11**) and TTP (**12**).

three drug regimens that include two nucleoside RT inhibitors plus a third drug that is either a potent protease inhibitor or a nonnucleoside RT inhibitor [23-26]. Most combination therapies for HIV include AZT. The combined use of AZT, another RT inhibitor (ddC, or 3TC), and a protease inhibitor is most effective. Examples include (indinavir, zidovudine, and lamivudine), (ritonavir, zidovudine, and lamivudine) and (zidovudine, didanosine and nevirapine) [23-27]. These drug combinations reduce viral load in plasma, in some cases to undetectable levels, resulting in sharp rebounds in immune function. While cocktails of expensive drugs have helped lower the AIDS death rate in western countries, such treatments are generally not available in the developing world. However, long-term treatment might ultimately result in multi-drug resistant virus, leaving few options for salvage therapy [27]. Zidovudine monotherapy is still advocated to prevent vertical transmission [28], as it does not appear to increase the risk of birth defects [29] while reducing viral load in the mother, thus resulting in a greatly reduced risk of vertical transmission.

There are numerous reasons to utilize an AZT prodrug strategy in drug design. Some major problems associated with AZT chemotherapy include bone marrow toxicity and suppression, low therapeutic index owing to inhibition of cellular polymerases, low localization in brain, and a short half-life in blood (plasma AZT $t_{1/2}$ is approximately 1 h) [30-32] which necessitates frequent AZT administration to maintain a therapeutic drug concentration. The beneficial effect of AZT appears to diminish over time. Although AZT effectively inhibits the replication of HIV-1, it does not inhibit virus production by chronically infected cells or transmission of the infection to uninfected cells by synctium formation [30, 31]. In the design of a prodrug for AZT, the objective is to improve pharmacological properties that detract from its optimal clinical efficacy.

AZT has significant drug-specific, dose-limiting toxicities, that result in a small therapeutic window between the minimum effective dose and the maximum tolerated dose. The more serious of these adverse effects include bone marrow toxicity, myopathy, and hepatic abnormalities [33]. The hematologic toxicities of AZT are reversible, dose or concentration dependent, occur within 1 month of starting therapy, and gradually progress during advanced stages of HIV disease [34]. Resistant viruses can be detected soon after treatment with AZT. Multiple RT mutations have been shown to confer resistance to AZT due to mutations at amino acids 41, 67, 70, 215, and 219 [35]. It is now clear that HIV-1 burdens are substantial, and high level virus replication occurs at all stages of infection. Even during asymptomatic and clinically quiescent periods, replication in lymphoid organs is extensive. High virus turnover drives the pathogenic process and the development of genetic variation. Under the selection pressure of drug therapy, viruses with resistance mutations accumulate, sometimes with complete replacement of wild-type virus by drug resistant mutants in plasma after only 2 to 4 weeks of therapy [36-38].

Since the infected brain may serve as a sanctuary for HIV, from which the periphery may continuously be reinfected, it is essential that an anti-HIV agent crosses the BBB readily to deliver an effective therapeutic concentration in brain without increasing toxicity [39]. AZT is one of the more lipophilic (log P = 0.08 in *n*-octanol: phosphate buffer pH = 7.4) anti-HIV compounds investigated clinically, and it readily enters the cerebrospinal fluid (CSF), possibly by both passive diffusion and active transport. However, the entry of AZT into brain tissue from the CSF is likely not sufficient to provide a therapeutic concentration that suppresses viral replication in the brain [40]. Since the ability of AZT to cross the blood-brain-barrier (BBB) is less than optimal, it does not effectively suppress viral replication in the brain. Moreover, it has been shown that bulk efflux of AZT from CSF and the brain via the organic anion transport system plays an important role in reducing the AZT levels in CSF and brain. Therefore, the low brain concentrations observed are not necessarily due to poor penetration, but rather to active AZT egress from the brain [41-43]. Dykstra *et al.* [42] reported that the low level of AZT in rat brain is related to its efficient elimination from brain rather than to its low uptake into brain. These data suggest that delivery of higher levels of AZT to brain may saturate the active elimination mechanism, thereby reducing efflux of AZT from brain [44]. The peak level of AZT in brain following systemic administration of 200 mg oral or 2.5 mg/kg intravenous doses of AZT would be expected to be five times less than the lowest concentration needed to effectively inhibit HIV-1 replication [39]. Theoretically, a higher concentration in the central nervous system could be achieved by raising the plasma levels of AZT, but AZT's dose-related bone marrow toxicity mitigates against increasing the administered dose [45]. Therefore, prodrugs which improve brain uptake of AZT, have a longer residence time and provide a sustained release of AZT at concentrations adequate to provide therapeutic anti-HIV efficacy, but not cause toxicity, are needed.

Mechanism of Action

To gain a better understanding regarding the mechanism of action of AZT prodrugs, it is necessary to discuss AZT's mechanism of action, metabolism and catabolism briefly. Since AZT was the first drug used to treat HIV infection, its intracellular metabolism has been thoroughly studied. AZT is phosphorylated

intracellularly to AZT monophosphate (AZT-MP, **7**) by the same cellular kinase (thymidine kinase) which phophorylates thymidine (**6**). AZT-MP (**7**) is then phosphorylated to the diphospate (**8**) and triphosphate (**9**) forms by other cellular kinases. AZT-MP (**7**) accumulates in cells because of its slow phosphorylation to AZT diphosphate (AZT-DP, **8**) by host cell thymidylate kinase, the rate-limiting step in AZT triphosphate (AZT-TP, **9**) formation. Based on *in vitro* studies, increasing extracellular concentrations of AZT from the picomolar to micromolar range results in a proportional increase in AZT-MP, but relatively small or no increase in AZT-TP, concentrations. AZT-MP is a competitive inhibitor of thymidylate kinase and reduces the conversion of thymidine monophosphate (TMP, **10**) to thymidine diphosphate (TDP, **11**), leading to decreased formation of thymidine triphosphate (TTP, **12**). The rate of AZT-MP phosphorylation to AZT-TP, was reported to be only 0.3% that of TMP. On the other hand, AZT-TP is believed to interfere with retroviral RNA dependent DNA polymerase (RT), thereby inhibiting viral replication. AZT-TP may inhibit HIV-1 reverse transcription via two mechanisms: 1) competition with thymidine triphosphate (TTP) for RT and 2) competitive inhibition of RT and chain termination of viral DNA due to the absence of a 3'-hydroxyl group. The elongating DNA chains which incorporate AZT-TP are thereby prematurely terminated because AZT cannot accept an additional nucleotide at the 3'-azido position, which is blocked. Both mechanisms are thought to be required for inhibition of HIV in host cells [6, 46-50]. Some studies have suggested that high levels of AZT-MP may impair HIV-1 RT RNase H activity [51] and the 3'-exonuclease [52] that would otherwise cleave AZT-MP from the DNA 3'-terminal end. Furthermore, AZT-MP inhibits protein glycosylation which may explain, at least in part, the cytotoxicity of AZT [53]. Metabolites of AZT can inhibit HIV-1 integrase at concentrations achievable *in vitro*, suggesting the possibility that inhibition of viral integration may contribute to the *in vivo* potency of AZT [54]. AZT-TP has a selective affinity for HIV RT. Cellular DNA polymerase α is 100-fold less sensitive to zidovudine triphosphate but mitochondrial DNA γ polymerase is affected [8 and references cited there]. 3'-Amino-3'-deoxythymidine (AMT), an amino metabolite of AZT, is toxic to granulocyte-macrophage and erythroid progenitor cells [55].

The efficiency of AZT inhibition of HIV-1 replication is dependent on four major factors: 1) cellular uptake of AZT, 2) phosphorylation of AZT by cellular kinases, 3) competitive inhibition of enzymes in the nucleic acid pathways, and 4) AZT-TP binding to RT and its subsequent incorporation into elongating viral DNA [46].

AZT Prodrugs

Prodrugs are pharmacologically inactive derivatives of active agents, which undergo chemical or enzymatic biotransformation resulting in the release of the active drug after administration. The metabolic product (e.g. parent drug) subsequently elicits the desired pharmacological response [56, 57]. The prodrug's design enables it to by-pass a membrane or metabolic barrier and, once past the barrier, to revert to the parent compound by post-barrier enzymatic or nonenzymatic processes. Since penetration through membranes is considered an important prodrug attribute, the AZT prodrug should be designed to ensure it possesses the required lipophilicity. In the case of AZT, however, it should also be selectively released at the targeted site (organ) to prevent undesirable effects. In addition to selectivity for the target tissue, the carrier should protect AZT from the harsh physiological environment, and reduce or abolish its side-effects. Several strategies, to be discussed later, have been employed to design prodrugs to deliver AZT intracellularly, to improve anti-HIV efficacy, to reduce toxicity, to increase plasma half-life and lipophilicity, and to improve delivery to the brain [58-71].

Most prodrugs of AZT have been prepared by derivatization at the 5'-*O* position. However, other approaches encompass 5-halo-6-alkoxy-5,6-dihydro derivatives of AZT [72-75] and 5'-ether prodrugs of AZT [76]. A discussion of latter is beyond the scope of this review.

5'-*O*-Ester Prodrugs

Esters have dominated prodrug research because they have ideal characteristics, exhibiting reasonable chemical stability *in vitro* which allows them to be formulated with adequate shelf lives. In addition, by virtue of their ability to function as esterase substrates, esters are suitably labile *in vivo* [77,78].

Esterification of the 5'-hydroxyl group is a common approach to enhance brain uptake and *in vivo* efficacy of AZT and other anti-HIV nucleoside derivatives. The drug-design rational for this approach is that the conjugate will be hydrolyzed to AZT or AZT-MP. Several groups have used the ester prodrug approach to prepare 5'-*O*-esters of AZT. In these prodrugs, the conjugating moiety is linked to AZT via either a 5'-*O*-carboxylic ester, or a 5'-*O*-phosphate ester group. 5'-*O*-Ester prodrugs of AZT can be structurally categorized into two main groups: A) 5'-*O*-carboxylic ester AZT prodrugs, and B) masked AZT 5'-monophosphate prodrugs.

In the following sections, 5'-*O*-ester derivatives of AZT will be designated as ROAZT. A variety of R-substituents at the 5'-position will be introduced in each section.

A. Carboxylic Esters (13-64)

Carboxylic ester prodrugs of AZT have been synthesized by conjugating AZT with various compounds including:

1) cyclic carboxylic acids, e.g. steroidal 17β-carboxylic acids [79], 1-adamantanecarboxylic acid [80], bicyclam carboxylic acids [81], *O*-acetylsalicylic acid [82] and carbohydrate carboxylic acids [83],

2) amino acids [61],

3) 1,4-dihydro-1-methyl-3-pyridinylcarboxylic acid (chemical delivery system, CDS) at the 5'-position [84],

4) aliphatic acids, e.g. acetic, butyric, hexanoic, octanoic, decanoic [60, 85] and myristic acid analogs [86],

5) long-chain (C:16 upwards) polyunsaturated fatty acids such as linoleic acid (18:2, Δ-9,12), gamma-linoleic acid (GLA) (18:3, Δ-6, 9, 12) [87] and retinoic acid [61].

The utility of 5'-*O*-esters of AZT as potential prodrugs is dependent upon their pharmacokinetic properties, tissue distribution, and rate of bioconversion to AZT. These esters are hydrolyzed *in vivo* by esterases, which are ubiquitous in tissue. Therefore, *in vitro* metabolic regeneration of AZT should occur readily. Since esters are substrates for esterases present in plasma, the design of esters which are stable to plasma esterases, but are cleaved by target intracellular enzymes is a challenge for developing prodrugs of AZT. Esters showing a range of stabilities can be designed by appropriate manipulation of electronic and steric factors which will influence ester stability and/or hydrolysis rate. Therefore, a multitude of ester prodrugs can be prepared to address a wide variety of problems that can be circumvented using the prodrug approach [88].

The usefulness of AZT prodrugs will depend not only on the stability of the prodrug prior to its passage across the cell membrane and into the CNS, but also upon their intracellular bioconversion to AZT in virally infected cells. It has been suggested that macrophages may concentrate large amounts of lipophilic ester [61]. This targeting to macrophages would make prodrug esters of AZT promising candidate agents, with an opportunity to deliver to the reservoir of the HIV infection (macrophage). Since the intracellular bioavailability of AZT may differ, depending upon the rate of cleavage of the prodrug in T-lymphocytes or macrophages, greater efficacy with reduced systemic toxicity could be anticipated.

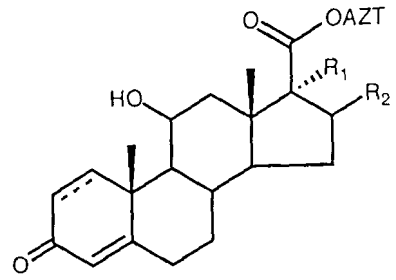
1. Cyclic Carboxylic Acid Derivatives (13-27)

1.1. Steroid Esters (13-15)

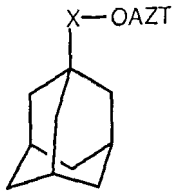
Sharma *et al.* [79] synthesized 5'-*O*-esters of AZT with three different inactive steroidal 17β-carboxylic acids (**13-15**, Fig. **2**). Since the chosen steroidal acids retain some glucocorticoid structural features, such as the 3-keto, 4-ene and 11-hydroxy groups, the conjugates were expected to bind to transcortins, which are plasma glucocorticoid transport proteins. The conjugate-protein complex would be protected from metabolic transformation in plasma, thus prolonging the half-life of the nucleoside. Other rationale for the synthesis of steroidal AZT prodrugs included increased lipophilicty to enhance permeability of the conjugates through cell membranes, and protection of the 5'-OH of AZT from glucuronidation to increase the half-life. These esters were stable in cell culture medium under the conditions of the 6-day anti-HIV activity screening protocol [79]. Two conjugates (**13** and **14**) were active anti-HIV agents that showed activity (EC_{50} = 0.56 and 0.03 μM, respectively) comparable to that of AZT (EC_{50} = 0.01 μM) in T4 lymphocytes (CEM cell line) [89].

1.2. Adamantane Conjugates (16-20)

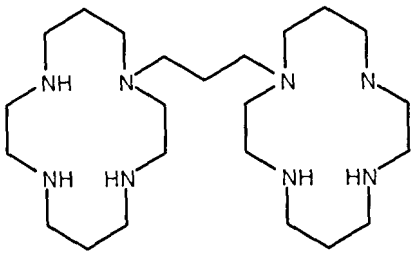
Five derivatives of AZT conjugated via an ester link to a lipophilic adamantane-derived moiety were synthesized by Tsuzuki *et al.* [80] to improve the transport of AZT into the CNS (**16-20**, Fig. **2**). When these prodrugs were administered intravenously to rat, the prodrugs in brain tissue were detected at a 7-18 fold higher concentration than AZT, even though there was a negligible amount of prodrug in the cerebrospinal fluid. The mean brain:plasma drug concentration ratio at 15 min following AZT administration was 0.14. In comparison, the mean brain:plasma ratio for prodrugs **16** and **17** were 0.52 and 3.35, respectively. The improved localization in brain of prodrugs **16** and **17** appear to be due primarily to their greater lipophilicity relative to AZT. However, considering that adamantine has a high affinity for cell membranes [90], the possibility remains that the adamantane moiety might have a tropism for the CNS, and this property might enhance penetration of the BBB by the prodrug compared to AZT. However, the mechanism for the uptake of these prodrugs **16-20** into the CNS is still not clear [80].



13 $R_1 = R_2 = H$
14 Δ^1, $R_1 = H$, $R_2 = CN$
15 Δ^1, $R_1 = OH$, $R_2 = H$



	X
16	CO—
17	CH_2CO—
18	$CONHCH_2CO$—
19	$CONH(CH_2)_2CO$—
20	$CONH(CH_2)_3CO$—



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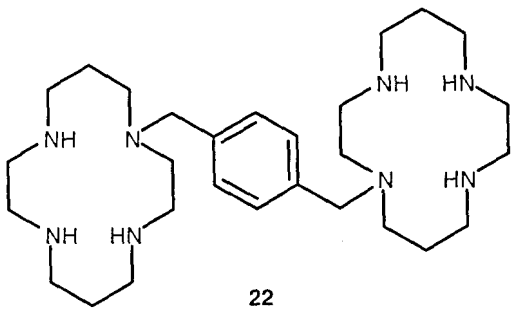

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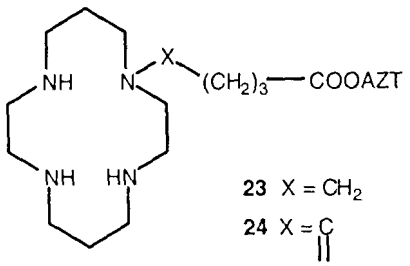
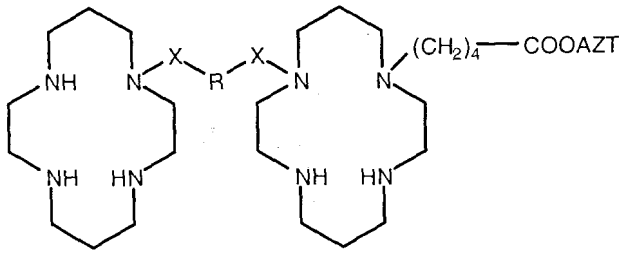


23 $X = CH_2$
24 X = C(=O)



25 X = C(=O) R = $-(CH_2)_3-$
26 $X = CH_2$ R = p-C_6H_4
27 X = C(=O) R = p-C_6H_4

Fig. (2). Cyclic 5'-*O*-carboxylic acid ester derivatives of AZT (**13-27**).

1.3. Bicyclam-AZT Conjugates (23-27)

It has been suggested that bicyclams target the virus uncoating associated process [11], and reported that bicyclams **21** and **22** (Fig. **2**) interfere with the cellular co-receptor for T-cell tropic viruses, thereby preventing cell infection [91-93].

Dessolin *et al.* [81] recently reported the synthesis of mono- and bis-tetraazamacrocycle-AZT conjugates (**23-27**, Fig. **2**). The design of these conjugates was based on the known interaction of the bicyclam moiety with the CXCR-4 coreceptor used by T-tropic viruses to infect target cells [92] in order to achieve a higher AZT concentration in infected cells. All cyclam- or bicyclam-ZT prodrugs that were evaluated elicited anti-HIV activity with EC_{50} values ranging from 0.005 to 0.1 μM. Under the same assay conditions, AZT inhibited *syncytia* formation with an EC_{50} = 0.01-0.05 μM. Analog **26** (EC_{50} = 0.005 μM) appeared to be 1 order of magnitude more active than the parent drug AZT, possibly due to an increase in cellular uptake followed by intracellular release of AZT. Monocyclam-AZT conjugates (**23, 24**) were less active than the corresponding bicyclam-AZT analogs (**25-27**). These N-protected covalent prodrugs were equipotent to AZT as inhibitors of HIV replication, while N-deprotected analogs exhibited both higher activity and selectivity against HIV-infected cells. These AZT-conjugate congeners showed calculated log P values ranging from 1.81 to −1.35. Lipophilicity was likely not the only

factor that determined the antiviral potency of these enzyme-labile prodrugs, since there was no apparent correlation between lipophilicity and antiviral activity. Other factors such as sensitivity to enzymatic hydrolysis and stability of the prodrugs may also be crucial. The half-life for compound **26** in human plasma was found to be approximately 8 h.

Compound **26** and reference compound **22** were evaluated for their ability to bind to the CXCR-4 receptor, using flow cytometry. These compounds were evaluated for their ability to inhibit the fixation of 12G5 mAb, which specifically binds to the CXCR-4 receptor. Compounds **22** and **26** were the most effective with respect to their ability to compete with 12G5 mAb fixation, which was reduced by 81% and 69%, at concentrations of 10.0 and 6.5 μM, respectively. Compound **26**, by targeting the CXCR-4 receptor at the cell surface may selectively facilitate entry of AZT derivatives into cells, thereby increasing their activity. These authors concluded that selective competition for binding to the CXCR-4 receptor requires a prototype molecule to present specific structural parameters where i) the number of free nitrogen atoms in the bicyclam moiety should be maximum, and ii) the preferred linker between the two bicyclams should be a xylyl or alkyl group, since the phthaloyl linker was less effective. The presence of only one bicyclam sidearm bearing the AZT moiety decreased slightly the binding affinity to the CXCR-4 receptor (**26** versus the reference compound **22**).

1.4. Aspirin (O-acetylsalicylic acid) Prodrug of AZT (28)

The mixed anhydride formed from aspirin and AZT, in which the blocked AZT is attached through its 5'-*O*-position to the 2-position of 2-methyl-4*H*-1,3-benzodioxin-4-one (**28**, Fig. **3**), was equipotent to AZT against HIV-1 in MT-4 cells, with EC_{50} values of 0.005 μM and 0.003 μM for **28** and AZT, respectively (Fig. **3**). The prodrug **28** is not a carboxylic ester derivative of AZT, but its hydrolysis metabolite (**29**) is an ester. At 37 °C, in the same growth medium used for determination of antiviral activity, **28** was resistant to hydrolysis for almost 1 h. After 1 h, high pressure liquid chromatography (HPLC) analysis showed the appearance of an intermediate which is believed to be **29**. After 16 h, AZT appeared and within 30 h all of the prodrug **28** was consumed with formation of **29** and AZT. After 46 h, only AZT could be detected by HPLC analysis [82].

1.5. Carbohydrate Ester Derivatives (30-31)

Since De Clercq suggested that polyanions such as dextran sulfate may suppress HIV replication [94], various sulfated saccharides have been reported to exhibit *in vitro* anti-HIV activities [95-97]. Novel AZT prodrugs, such as AZT-bound sulfated laminaripentaose and AZT-bound sulfated alkyl laminaripentaosides (**30**, Fig. **4**) were synthesized by Gao *et al.* [83]. Sulfated laminaripentaose, or sulfated alkyl laminaripentaoside, acts as an AZT carrier as well as an anti-HIV agent in the prodrug. It was observed that AZT-bound sulfated laminaripentaose (**30**) exhibited anti-HIV activity (n = 0, EC_{50} = 0.20 μg/mL) even when AZT was not released from sulfated laminaripentaose, and **30** showed a low cytotoxicity (CC_{50} > 1000 μg/mL). In comparison, AZT exhibited EC_{50} and CC_{50} values of 0.004 μg/mL and 7.6 μg/mL, respectively. This anti-HIV effect is most likely due to introduction of the 5'-Suc-AZT ester moiety, which increases hydrophobicity of the sulfated laminaripentaose, rather than the release of AZT, since released AZT would induce higher cytotoxicity. It was reported that the ester bond connecting AZT to the carrier was moderately stable *in vitro*, and that AZT was not released from the carrier. Therefore, it was assumed that cytotoxic activity measured using the MTT method represents activity due to the prodrug itself. Moreover, an alkyl group attached to the reducing end of AZT-bound sulfated alkylaminaripentaoside increased anti-HIV activity further (n = 8, 12, 16, EC_{50} = 0.04-0.23 μg/mL) against HIV-1 infected MT-4 cells. AZT-bound sulfated alkyl laminaripentaosides exhibited almost no anticoagulant activity [83].

Gao *et al.* [98] also reported that AZT-bound curdlan sulfates (**31**, Fig. **4**) exhibit *in vitro* anti-HIV activities in MT-4 cells (EC_{50} 0.17 to 0.58 μg/mL range), similar to that of the highly active curdlan sulfate (EC_{50} = 0.51 μg/mL). Since compound **31** had low *in vitro* cytotoxicity (CC_{50} > 1000 μg/mL); AZT is not released *in vitro* as free AZT would result in higher toxicity. These authors concluded that the anti-HIV activity of AZT-curdlan sulfate may originate from the curdlan

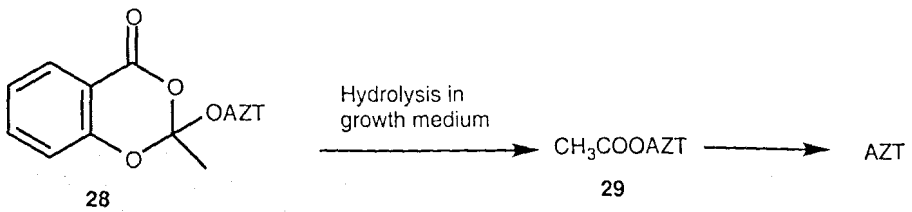
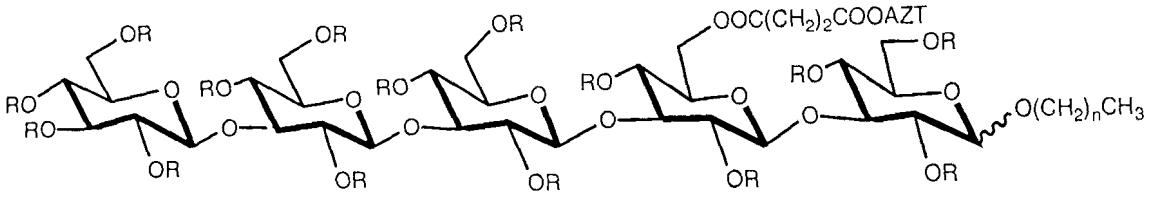
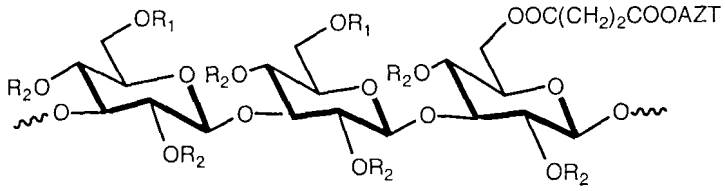


Fig. (3). Hydrolysis of aspirin prodrug of AZT (**28**) in growth medium [82].



R = SO_3Na, 5'-Suc-AZT or H
n = 0,8, 12, 16

ATZ-bound sulfated alkyl laminaripentosides (**30**)



R_1 = SO_3Na, 5'-Suc-AZT
R_2 = SO_3Na or H

ATZ-bound curdlan sulfate (**31**)

Fig. (4). AZT-bound carbohydrates (**30-31**).

sulfate moiety. It was observed that AZT was released gradually from AZT-curdlan sulfate when a AZT-curdlan sulfate solution in a 90% buffer at pH 7.4 and 10% dimethyl sulfoxide mixture was stored in a refrigerator for a few weeks. Since the anti-HIV activity of the AZT-curdlan sulfate preparation increased with longer storage times, AZT must have been released, producing an additive effect. In addition, an increased cytotoxicity attributable to AZT was observed. It was shown that substitution of curdlan sulfate by AZT did not increase the anticoagulant activity, which is regarded as a serious side effect for an anti-HIV agent and that the AZT-curdlan sulfates exhibit low to moderate *in vitro* anticoagulant activities [98]. Due to the high molecular weight of curdlan sulfate and sulfated laminaripentaose derivatives, anti-HIV data was reported as μg/mL. For example, curdlan sulfate possesses an average molecular weight of 7.0×10^4 daltons.

2. Amino Acid Esters (32-37)

L-Amino acid-AZT chimeras can serve as prodrugs to enhance AZT absorption via an active transport system [61, 99, 100]. Moreover, bone marrow progenitor cells may lack the active transport system for amino acids [101], which may lead to reduced toxicity. Amino acid (tyrosinyl, isoleucinyl, lysinyl, phenylalanyl, glutamyl) esters of AZT (**32-36**, Fig. **5**) have been synthesized in an attempt to deliver AZT more efficiently via the active transport system used by amino acids, to target cells at higher concentration than that which can be achieved by AZT alone. The isoleucine (**33**) (log P = 1.01) and phenylalanine (**35**) (log P = 0.52) esters were more lipophilic than the tyrosine (**32**), lysine (**34**), and glutamic acid (**36**) esters (log P = -2.00 to -0.03). All prodrugs were less cytotoxic than AZT, suggesting that the slow release of AZT from prodrugs may be related to the observed reduction in toxicity. The isoleucinyl derivative (**33**), which has the highest partition coefficient (log P = 1.01) and diffused into H9 cells to a greater extent than other analogs at short time intervals, was equipotent to AZT with respect to anti-HIV activity, and showed hydrolysis half-lives of > 240 and 19 minutes in human plasma and rat hepatic microsomes, respectively. These esters of AZT were susceptible to the action of plasma esterases with a $t_{1/2}$ in the range of 20 to 70 minutes, except for the isoleucinyl derivative (**33**) which did not undergo enzymatic hydrolysis for up to 4 h [61].

On the other hand, the inability or insolubility of these lipophilic prodrugs of AZT within the gastrointestinal tract, or slow *in vivo* release of AZT, limits their oral bioavailability. An alternate approach exploits membrane transporters in intestinal epithelial cells that facilitate the transport of polar nutrients such as amino acids and nucleosides. The intestine was 3 fold more permeable to 5'-*O*-valyl AZT (**37**, Fig. **5**) than to AZT. This enhanced dose-dependent permeation was selective for L-amino acid esters. Competitive inhibition studies in rats and in CHO cells transfected with the human peptide transporter, hPEPT1, demonstrated that the membrane transport of L-Val-AZT was mediated predominantly by the PEPT1 H^+/dipeptide cotransporter, even though this prodrug did not possess a peptide bond. The CC_{50} (0.4 mM)

value for L-Val-AZT was lower than for cephradine (15 mM) and enalapril (4.5 mM), which are known substrates for the peptide transporter [102].

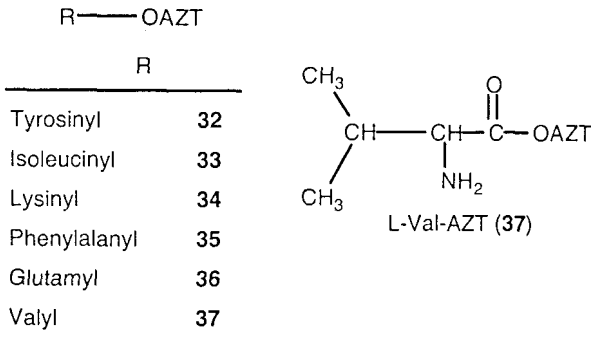


Fig. (5). Amino acid ester prodrugs of AZT (**32-37**).

3. Chemical Delivery System (1,4-Dihydro-1-methyl-3-[(pyridylcarbonyl)oxy]ester) (39)

Increasing the lipophilicity of a compound to an optimal level, using the prodrug approach, may facilitate delivery to the target site of action, but this may not be a specific process since all organs may be exposed to a greater burden. This factor is especially important with respect to cytotoxic agents. While increased membrane permeability may confer higher brain-selectivity, there is almost always a disproportionate rise in systemic toxicity. These two properties of simple antiviral prodrugs, *viz* 1) increased tissue burden with little tissue specificity, and 2) poor CNS retention, illustrate the need for a more sophisticated approach, such as a chemical delivery system, for brain-targeted delivery [103]. Chemically-based drug delivery strategies have been reviewed previously [58, 59, 104-107]. Chemical delivery systems (CDS's), based on a dihydropyridine-quaternary pyridinium ion redox system, can be used for specific delivery of AZT into brain [58, 61, 67, 68, 84, 99, 108-113]. This approach involves the chemical transformation of the quaternary pyridinium derivative of AZT [Q^+] (**38**) (Fig. **6**), which normally does not penetrate the blood-brain barrier, to a reduced lipid soluble dihydropyridine form [HQ] (**39**). After intravenous administration, the lipid-soluble [HQ] (**39**) is readily distributed throughout the body and readily crosses the intact blood-brain barrier. Subsequently, the NAD → NADH redox system regenerates the original impermeable pyridinium species [Q^+] (**38**) from the dihydropyridine compound [HQ] (**39**) in the brain. Oxidation of the CDS carrier moiety **39** to its hydrophilic pyridinium salt **38** (quaternized salt) in the brain slows, or prevents, its egress from brain, while elimination of the hydrophilic pyridinium salt from the general circulation is accelerated. Subsequent ester cleavage of the quaternary carrier of AZT results in sustained retention of AZT in the brain with a reduction in general toxicity due to the rapid elimination of the polar quaternary salt (**38**) (Fig. **6**) from the periphery.

Aggarwal *et al.* [61] reported that the AZT-CDS was a marginally more active anti-HIV agent than AZT, and that it exhibited a long half-life upon incubation with rat hepatic microsomes ($t_{1/2}$ = 110 min) or human plasma ($t_{1/2}$ > 240 min). Uptake of the AZT-CDS derivative (**39**) in H9 cells was approximately 2 times greater than AZT. These results indicated that the increased inhibition of HIV-1 replication by AZT-CDS (**39**) may be due to higher intracellular levels achieved using this prodrug relative to AZT. Furthermore, AZT-CDS (**39**), which is a 2.5-fold more active inhibitor of HIV replication, was approximately half as toxic as AZT.

Torrence *et al.* [84] reported that **39** is active in a MT-4 cell screening system and that it undergoes facile *in vitro* oxidization to a quaternary salt in rat brain cytosol. Gogu *et al.* [110] studied the same prodrug (**39**) in murine bone marrow cells and found it was significantly less toxic than the parent compound AZT. Little *et al.* [112] also evaluated the dihydropyridine derivative of AZT (**39**) in rats and dogs in which it provided significantly higher concentrations in brain and CSF, respectively. Systematic administration of AZT-CDS (**39**) to rats resulted in both higher brain levels of AZT and lower blood levels compared to administration of AZT itself. In this model system, the area under the brain concentration curves (AUC) for AZT after AZT-CDS (**39**) dosing was > double that achieved after AZT administration. These changes resulted in a higher AZT brain/blood ratio. In a canine model, AZT-CDS (**39**) produced significantly higher cerebrospinal fluid (CSF) levels of AZT compared to administration of AZT alone. Initial blood levels of AZT were also much lower after AZT-CDS administration. Their studies indicated that the AZT-CDS can increase the concentration AZT in the CNS and at the same time reduce the peripheral concentration.

Administration of an aqueous formulation of AZT-CDS (**39**) to dogs resulted in rapid tissue uptake and conversion of the CDS to the corresponding quaternary salt (**38**) with the subsequent production of AZT. Delivered in this way, the levels of AZT in brain were 1.75- to 3.3-fold higher than those associated with conventional AZT administration. In addition, the levels of AZT in blood were 46% lower than those associated with AZT administration, resulting in a significantly larger brain:blood ratio [106]. Accordingly, the brain AUC for AZT-CDS was 11.28 μg h/mL compared to the brain AUC for AZT (1.21 μg h/mL) when AZT was administered. The brain exposure to AZT calculated as $(AUC)_{pd\rightarrow p}/(AUC)_p$, where $(AUC)_{pd\rightarrow p}$ equals the area under the AZT brain concentration-time curve following administration of the prodrug **39**, and $(AUC)_p$ is the same area obtained following

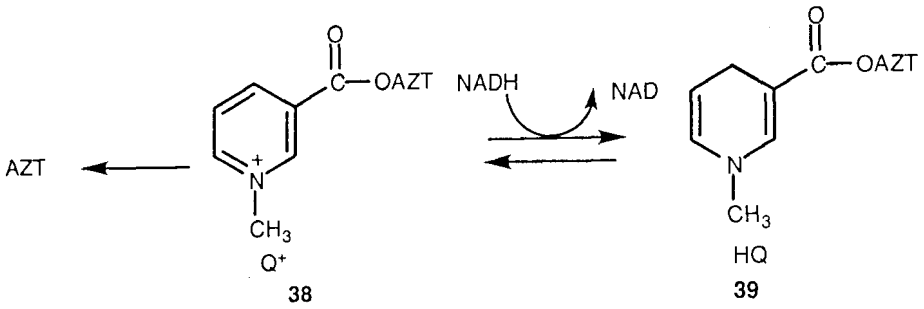


Fig. (6). Central nervous system-enhanced delivery of AZT using the CDS approach.

administration of AZT. A "brain exposure value" of 9.32 for AZT indicated a significant increase in exposure to AZT following prodrug administration. The larger partition coefficient for the AZT-CDS (log P = 1.73), relative to AZT, is consistent with the fact that lipophilic dihydropyridine derivatives cross the BBB more readily than AZT [67].

In vivo studies indicated that a significantly larger amount of AZT could be delivered to the brain following administration of a prodrug than the parent drug. However, consistently lower concentrations of AZT (50% reduction) were observed in plasma following prodrug administration relative to those after AZT dosing [68]. Unfortunately, the dihydropyridine-containing derivatives suffer from stability problems, since even in the dry state they are very sensitive to oxidation as well as to water addition at the C-2 position. Such problems have significantly complicated attempts to commercialize the system. Thus, a different carrier approach to brain-targeted drug delivery which would not include the inherently unstable dihydropyridine system would be desirable [103]. The low pH of the stomach also mitigates against oral administration of the AZT-CDS prodrugs.

4. 5'-*O*-Aliphatic Derivatives of AZT (43-61)

Fatty acid esters of AZT have dramatically different physicochemical properties than AZT. These lipophilic esters have large partition coefficients that lead to enhanced penetration through membranes, and they are rapidly hydrolyzed by a variety of extracellular and cellular esterases. Extensive chemical modification using a variety of 5'-*O*-ester moieties has been used to produce more lipophilic ester prodrugs to enhance brain uptake, to sustain the release of AZT, and to prevent the rapid plasma clearance of AZT.

The advantages to this approach include:

1) The individual components of the ester exhibit anti-HIV activity, so that hydrolysis of the prodrug releases two active species, AZT and the fatty acid analog, each with different target sites, that may result in enhanced efficacy;

2) Development of viral resistance to two active drugs should occur at a slower rate than to either agent alone;

3) This combination of anti-HIV agents may reduce toxicity associated with AZT since the two molecules act by different mechanisms of action and the fatty acids are not associated with bone marrow toxicity observed with AZT;

4) Fatty acid-AZT esters may enable a larger concentration of intact prodrug to enter the cell due to the lipophilic nature of the fatty acid. The prodrug esters are lipophilic compounds that accumulate in the plasma membrane of T-lymphocytes, monocytes/macrophages; their ability to cross the BBB (increased brain uptake) is enhanced. A higher localization of AZT in lymph nodes would be expected, that would prevent HIV infection in macrophages;

5) Hydrolysis of the conjugate by cellular enzymes as esterases could result in the slow release of AZT into treated cells. A sustained release of AZT would prevent spikes in the plasma concentration, thereby reducing bone marrow toxicity since it is known that bone marrow toxicity is dose-related. Decreased delivery to marrow, relative to blood levels, would also elevate the therapeutic index of these drugs. This could result in a requirement for less frequent administration of prodrug compared to the individual prodrug moieties; and

6) Micelles formed from the conjugates could deliver AZT selectively to HIV-1 infected cells.

A knowledge of the antiviral and antifungal activities of fatty acids is required prior to discussion of 5'-*O*-fatty acid derivatives of AZT. Fatty acids exhibit their antiviral and antifungal activity by various mechanisms. In this review only the most important and probable mechanisms are discussed in detail. However, it is proposed that they act by a combination of these mechanisms. Fatty acids, in general, have been reported to inhibit cellular respiration [114-116], carbohydrate metabolism [117], phosphate uptake [118] and phospholipid metabolism [119]. The partial

incorporation of fatty acids via membrane lipid biosynthesis may account for their differential activity [120]. The differential activity of short and long chain fatty acids may be due to variations in solubility, incorporation into membranes, and in the degree and type of oxidation at different concentrations [120].

Protein *N*-myristoylation in viruses and fungi is catalyzed by myristoyl-CoA:protein *N*-myristoyltransferase (NMT) (E.C. 2.3.1.97). This results in the co-translational linkage of myristic acid (C14:0), via an amide bond, to NH_2-terminal glycine (Gly) residues of a variety of viral and fungal proteins following the removal of the initiator methionine residue by methionylpeptidase [121]. NMT is an attractive molecular target to inhibit HIV-1 replication in AIDS patients and HBV replication in individuals infected by the hepatitis B virus, and for anti-fungal therapy to prevent opportunistic fungal infections caused by *Cryptococcus neoformans* and *Candida albicans*. *Aspergillus niger* contains saturated fatty acids such as myristic acid [122]. Accordingly, this fungal infection is also expected to be sensitive to the incorporation of myristic acid analogs. Recently it has been proposed that inhibition of post-translational processing of viral and fungal precursor protein myristoylation, and more specifically NMT, may be a more probable mechanism for the antiviral and antifungal activity of myrsitic acid analogs. It is necessary to emphasize that NMT inhibition by myristic acid analogs may constitute only one of several mechanisms responsible for the antiviral and antifungal activity of these analogs. However, there is substantial evidence supporting the NMT-inhibition mechanism. For example, studies with oxatetradecanoic acid analogs have already indicated that they enter mammalian, viral and fungal cells, are converted to their CoA thioesters by cellular acylCoA synthetases, and are delivered to target proteins by NMT [123].

Myristoylated proteins include $Pr160^{gag\text{-}pol}$, $Pr55^{gag}$, the capsid protein $p17^{gag}$ and $p27^{nef}$ proteins of human immunodeficiency virus I [124-126], PreS1 protein in the membrane envelope of hepatitis B (HBV) and surface (S) protein and presurface (S) protein in the envelope of duck hepatitis B virus (DHBV) [127-132], and the ADP ribosylation factor (Arf) proteins of *C. albicans* and *C. neoformans* [123]. In spite of their low toxicity, their systemic use has been ineffective, possibly because they are metabolized readily by the host via the usual fatty acid pathways such as β-oxidation. This presents a challenge to the medicinal chemist, because, it is difficult to correlate rapid metabolic processing of a compound with potential therapeutic processing and therapeutic efficacy [133].

It has been demonstrated that replication of HIV-1 can be inhibited by heteroatom-containing analogs of myristic acid without accompanying cellular toxicity [134, 135]. Based on these observations, several analogs of fatty acids have been synthesized and evaluated against HIV-1 and HBV [136] and fungi *in vitro* [137]. Replacing one or more methylene groups with oxygen, sulfur, and/or an aromatic ring had a substantial effect on antiviral and antifungal activity. Selectivity with respect to the nature and position of the heteroatom and aromatic groups was observed. 12-Thioethyldodecanoic acid (**40**) (Fig. **7**) was moderately active (EC_{50} = 9.4 μM) against HIV-infected T4 lymphocytes (CEM-SS cell line), and it exhibited *in vitro* activity (EC_{50} = 17.8 μM) against HBV-producing 2.2.15 cell cultures derived from a human hepatoblastoma cell line (Hep G2). 12-Methoxydodecanoic acid (**41**) (Fig. **7**) exhibited *in vitro* activity (EC_{50} = 20-30 μM) against hepatitis B in the HBV DNA-transfected 2.2.15 cell line [136].

Several (±)-2-halotetradecanoic acids including (±)-2-bromotetradecanoic acid (**42**) (Fig. **7**) exhibited potent activity against *C. albicans* (MIC = 39 μM), *C. neoformans* (MIC = 20 μM), *S. cerevisiae* (MIC = 10 μM) and *A. niger* (MIC < 42 μM) in RPMI 1640 media. An optimal pKa value between 2.9 and 3.3, and a log P value between 6.7 and 7.3, appear to be required for potent antifungal activity [137].

Collectively, these observations suggest that myristic acid analogs may represent a useful approach to treating viral and fungal infections.

4.1. 5'-O-Aliphatic Derivatives of AZT Without Heteroatom Substitution (43-51)

Ester prodrugs of AZT have been synthesized using aliphatic acids (sterically hindered acetic acids), and their enzymatic regeneration to zidovudine has been investigated *in vitro* and *in vivo* [60]. According to Kawaguchi *et al.* [60, 138-140], the enzymatic hydrolysis rate of 5'-*O*-esters of AZT in the presence of different rat enzyme preparations, including plasma and brain homogenate, is dependent upon the length of the acyl chain. For ester prodrugs of AZT with normal acyl chains, a parabolic relationship between acyl chain length and susceptibility to enzymatic hydrolysis has been reported. Thus, acyl chains with 8-10 carbons showed the highest rates of enzymatic cleavage. A decrease or increase in the acyl chain length decreased susceptibility to enzymatic hydrolysis.

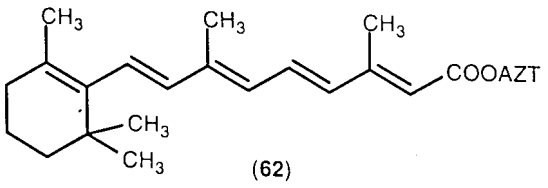
These 5'-aliphatic acid esters exhibited a dramatic increase in lipophilicity with log P values > 5 in comparison to AZT in a chloroform: buffer phosphate system [60]. AZT concentrations in plasma after ip administration of 5'-aliphatic acid-AZTs were shown to be more constant and persistent, especially when the acyl chain was extended to a C18 moiety that results in

a longer retention due to its significantly increased lipophilicity and relatively slow enzymatic hydrolysis. Kawaguchi *et al.* [60, 141] reported that the plasma concentration of caproate-AZT (**43**) and stearate-AZT (**44**) (Fig. **7**), following intraperitoneal administration of the caproate or stearate esters to mice, was below the detection limit. The absence of caproate and stearate in the plasma may be attributable to the high hydrophobicity or favorable tissue distribution of these esters. Similar results were reported after iv administration of other prodrugs to rats [60, 140-142]. Absorption into certain tissues such as erythrocytes or fatty tissues may be responsible for the absence of these esters in plasma, since fatty tissues can act as reservoirs for prodrugs. The AZT concentration in plasma following ip administration of the acetate prodrug (**45**) to mice rapidly decreased with a $t_{1/2}$ of 14.5 min, which is similar to that demonstrated for direct AZT administration ($t_{1/2}$ = 11.7 min). However, the AZT concentrations following caproate (**43**) or stearate (**44**) administration decreased slowly and were maintained for as long as 4 h after dosing (2 h for AZT or AZT acetate) [60]. Their results suggest that AZT concentrations following administration of aliphatic ester prodrugs are dependent upon the length of the acyl chain, which alters the rate of hydrolysis and elimination from the body, and that an optimal sustained release of AZT may be obtained by selecting an appropriate acyl group [60].

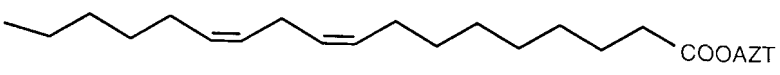
Seki *et al.* [85] synthesized five aliphatic esters (hexanoate **43**, acetate **45**, butyrate **46**, octanoate **47**, and decanoate **48**) (Fig. **7**) of AZT and showed that these esters possessed an enhanced ability to permeate human skin relative to topical application of AZT. Among these esters, AZT-acetate (**45**) and AZT-hexanoate (**43**) showed 2.4 and 4.8-fold enhanced permeation of human skin from an apolar solvent (isopropyl myristate) relative to AZT, respectively. In the case of butyrate (**46**) and decanoate (**48**), only AZT was found in their receptor phase, indicating the efficient enzymatic hydrolysis of these esters during the transport through skin. Acetate (**45**) was present both as intact ester and as AZT in the receptor phase at various sampling times.

Seki and Kawaguchi did not address the potential contribution of lymphatic transport to the overall bioavailability of these prodrugs. The butanoic (**46**), lauric (dodecanoic acid) (**49**) and oleic acid (*cis*-9-octadecenoic acid) (**50**) ester prodrugs of AZT (Fig. **7**) have been investigated by Bibby *et al.* [143], to assess their ability to promote the transport of AZT through the intestinal lymph, which is a major reservoir for HIV. The lymphatic transport of AZT was similar when administered as either AZT alone or as the lipophilic ester prodrugs, where the amount of AZT collected in fistulated mesenteric lymph was approximately 0.1-0.2% of the administered dose (15 mg/kg AZT). After

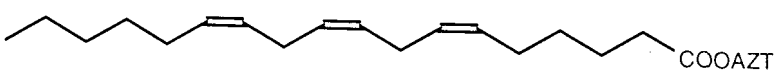
$CH_3CH_2S(CH_2)_{11}COOH$ (**40**)
$CH_3O(CH_2)_{11}COOH$ (**41**)
$CH_3(CH_2)_{11}CH(Br)COOH$ (**42**)
$CH_3(CH_2)_4COOAZT$ (**43**)
$CH_3(CH_2)_{16}COOAZT$ (**44**)
$CH_3COOAZT$ (**45**)
$CH_3(CH_2)_2COOAZT$ (**46**)
$CH_3(CH_2)_6COOAZT$ (**47**)
$CH_3(CH_2)_8COOAZT$ (**48**)
$CH_3(CH_2)_{10}COOAZT$ (**49**)
$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOAZT$ (**50**)
$CH_3(CH_2)_{14}COOAZT$ (**51**)
$CH_3(CH_2)_9O(CH_2)_2COOAZT$ (**52**)
$Br(CH_2)_{11}COOAZT$ (**53**)
$CH_3(CH_2)_{12}COOAZT$ (**54**)
$Ph(CH_2)_{11}COOAZT$ (**55**)
$PhO(CH_2)_{11}COOAZT$ (**56**)
4-I-$PhO(CH_2)_{10}COOAZT$ (**57**)
$PhO(CH_2)_{10}COOAZT$ (**58**)
$CH_3(CH_2)_{11}CHBrCOOAZT$ (**59**)
$CH_3O(CH_2)_{11}COOAZT$ (**60**)
$CH_3(CH_2)_{11}CHFCOOAZT$ (**61**)



(**62**)



Linoleic acid (**63**)



GLA-AZT (**64**)

Fig. (7). Myristic acid analogs and 5'-*O*-fatty acyl derivatives of AZT (**40**-**64**).

administered. The concentration of AZT arising from the ester **59** was 25.7 ± 2.4 nmol/g in brain at 1 minute is higher than AZT detected following AZT administration (9.8 ± 0.8 nmol/g). The highest brain/blood ratios of 0.087 and 0.23 were observed for AZT and prodrug ester (**59**), respectively, at 256 min post-dosing. Experimental studies with this system suggested that the 2-bromoester prodrug to AZT (**59**) has a pharmacokinetic advantage over AZT [148].

AZT was detected and quantified as a primary metabolite of prodrug **59**. (±)-3'-Azido-2',3'-dideoxy-5'-*O*-(2-bromomyristoyl)thymidine (**59**) converted rapidly to AZT after iv injection and afforded a high concentration of AZT in blood samples. The AUC for AZT, as a metabolite of the prodrug ester (**59**), was lower (12.3 ± 2.9 μmol.min/g) than that observed when an equimolar dose of AZT was injected (29.1 ± 2.9 μmol.min/g) [148].

There are different factors involved in the stability of these esters. Differences in their rates of ester hydrolysis are dependent upon the nature, lipophilicity, and steric size of the acyl group, the nature of the nucleoside moiety and the type or concentration of the esterase in the medium. More specifically, the ester cleavage half-lives ($t_{1/2}$) are dependent on the steric bulk, and electronegative inductive effect of the α-substituent (H, Br, F) of the 5'-*O*-myristoyl analog moiety, and/or medium effects. The stability of these 5'-*O*-esters of AZT was determined by *in vitro* incubation in several media including porcine liver esterase, rat plasma and rat brain homogenate at 37 °C. A relatively high extent of ester cleavage, particularly in plasma, was observed. The susceptibility of prodrugs to rapid degradation by plasma esterases constitutes a potential weakness of this prodrug approach. However, more sterically hindered esters were hydrolyzed more slowly. The 2-bromo analog (**59**) ($t_{1/2}$ = 16.8 min) is more stable toward plasma esterase than the 2-fluoromyristoyl analog (**61**) ($t_{1/2}$ = 0.3 min) due to the larger steric size of bromine at the α-position, which partially blocks binding to the esterolytic site of the enzyme. The smaller size of the α-F substituent (**61**), and its electronegative inductive effect, make it more sensitive to the nucleophilic attack by the esterolytic site of the enzyme [86].

The ester cleavage half-life is highly dependent upon the type or concentration of esterase in tissue or plasma. The majority of esters undergo rapid enzymatic hydrolysis to AZT upon incubation with rat plasma due to the high concentration of esterase in plasma. Generally, these ester analogs showed less ester cleavage upon incubation with brain homogenate than rat plasma and porcine liver esterase, possibly due to a lower esterase concentration in rat brain homogenate [86] as illustrated for **59** (Fig. **9**).

5. Polyunsaturated Aliphatic Acids (62-64)

Administration of vitamin A to retrovirus infected mice increased the number of activated macrophages and decreased mortality. A retinoic acid ester (**62**) (Fig. **7**), prepared by Aggarwal [61], showed a substantial 4-fold improvement in cellular uptake by H9 cells relative to the cellular uptake of AZT, probably due to the higher lipophilicity of the retinoate ester (log P = 1.24) compared to AZT (log P = 0.08 in *n*-octanol: phosphate buffer pH 7.4). Although, the retinoyl derivative (**62**) had a higher cellular uptake than AZT, it had approximately equipotent antiviral activity (EC_{50} = 0.2 μM, nontoxic concentration of the drug required to produce 50% inhibition of p24 antigen), and was 6-fold more cytotoxic to H9 cells than AZT. This lower than expected increase in antiviral activity may be due to incomplete intracellular hydrolysis of the prodrug under the *in vitro* conditions. The partial release of retinoic acid from the prodrug did not produce a synergistic or

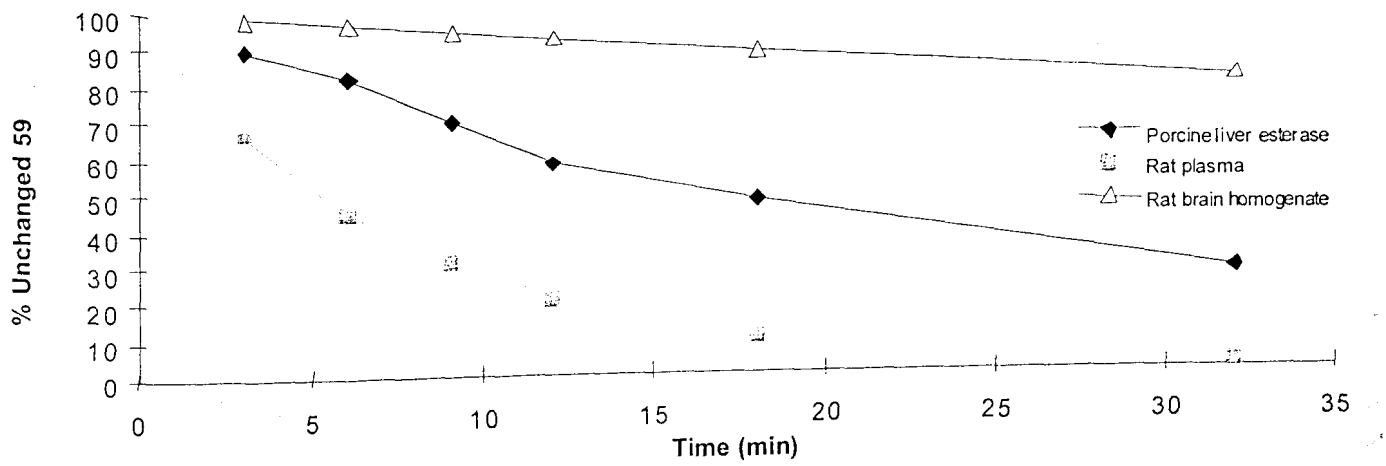


Fig. (9). Stability of (±)-3'-azido-2',3'-dideoxy-5'-*O*-(2-bromomyristoyl)thymidine (**59**) upon *in vitro* incubation in several media at 37 °C.

additive effect. The higher cytotoxicity of prodrug **62** was attributed to partial release of retinoic acid that is relatively more toxic to cells than AZT. Incubation of the ester **62** with human plasma and hepatic microsomes showed relatively long half-lives of 120 and 60 minutes, respectively.

Horrobin *et al.* [87, 152] evaluated 5'-*O*-esters of AZT with long chain (C:16 upwards) unsaturated fatty acids like linoleic acid (18:2, Δ-9,12, **63**) and gamma-linoleic acid (GLA) (18:3, Δ-6,9,12, **64**) (Fig. **7**) to enhance the transport of AZT across lipid barriers, particularly from the gut into the lymph system, into cells from the extra-cellular fluid, or across the blood brain barrier. Compounds **63-64** may be used to improve percutaneous delivery of topical preparations. Gamma linoleic acid-AZT (GLA-AZT, **64**) inhibited herpes virus replication, a property not found with either GLA or AZT individually [87].

Although *in vitro* and *in vivo* evaluations of ester prodrugs have shown improved site-specific delivery and/or pharmacokinetics for the parent nucleoside, these modifications have not produced clear-cut improvements in therapeutic efficacy. Prodrugs which have a longer residence time in the body at concentrations adequate for therapeutic anti-HIV efficacy, but not causing toxicity, are still needed.

B. AZT Masked Phosphate Prodrugs (Nucleotide Delivery Forms) (65-214)

Considerable interest exists in designing prodrugs of AZT that efficiently deliver AZT-MP into intact HIV-infected cells. With the aim of increasing AZT antiviral activity, several investigators have elaborated AZT to a variety of lipophilic phospho derivatives. The mechanism and biological results related to the various strategies reported to date are elaborated in this review. Several masked AZT 5'-monophosphate prodrugs have been synthesized, including: 1) phosphodiesters [monoalkyl or monoarylphosphate derivatives [153-156], glycosyl phosphotriesters [157, 158], ether lipids [159-170], ester lipids [30, 63, 64, 159, 160, 167-169, 171-175], and foscarnet derivatives [176-177]]; 2) phosphotriesters that include the majority of AZT prodrugs [dialkylphosphotriesters [70, 178-180], diarylphosphotriesters [181] glycolate and lactate phosphotriesters [178, 179], (acyloxy)alkyl or (acyloxy)aryl phosphotriesters [182-184], bis(*S*-acyl-2-thioethyl) (SATE) esters [162, 163, 185, 186], cyclosaligenyl prodrugs [187], glycosyl phosphotriesters [157, 158, 188], and steroidal phosphotriester conjugates [89, 189]]; 3) phosphoramidates [190-197]; 4) dinucleoside phosphate derivatives of AZT such as AZT-P-ddA, AZT-P-ddI, AZTP2AZT, AZTP2ACV [6, 198 and references cited therein, 199-205]; and 5) hydrogen and methylene phosphonates of AZT [206-208].

As a general concept, uncharged nucleotides can serve as membrane permeating nucleotide precursors. The major differences of these masked nucleotide approaches are the delivery mechanisms, which involve chemical or enzymatic activation of AZT.

Nucleotides cannot be used as chemotherapeutic agents because they are unable to cross cell membranes efficiently, and they are readily dephosphorylated on cell surfaces and in extracellular fluids by non-specific phosphohydrolases. On the other hand, AZT does not reduce the HIV viral load in peripheral mononuclear cells. Failure to clear the infectious virus from these cells may be due to inadequate inhibition of virus production in macrophages, a major reservoir of HIV infection [64, 162, 163]. To overcome these problems, various neutral 5'-nucleotide esters have been prepared in the hope that they penetrate cells by passive diffusion. Masking the phosphate group is necessary to overcome inadequate penetration of cell membrane by polar (charged) free nucleotides. In attempts to bypass the first phosphorylation step in the metabolic conversion of AZT to AZT-TP, numerous types of prodrugs to AZT-MP have been proposed, with the expectation that these prodrugs would release AZT-MP intracellularly.

The problem of glucuronidation and initial phosphorylation may be avoided by using a prodrug to AZT-MP. However, since AZT-MP is di-anionic at physiologic pH, it is unable to diffuse across cell membranes. Furthermore, AZT-MP is thought to be responsible for toxicity associated with AZT through its effect on the nucleoside pool [209]. One approach to circumvent this toxicity would be to prepare a lipophilic derivative of AZT-MP which will slowly release AZT-MP in a controlled manner.

Phosphate derivatives of AZT often exhibit similar *in vitro* antiviral potency to that of AZT, although in some cases, there may be advantages [178]. Examples of AZT derivatives that do indeed display *in vitro* anti-HIV activity include 5'-alkylamido ether phospholipid [62], the CF_3CH_2– and CCl_3CH_2– 5'-trialkyl phosphates [210], a 5'-dimyristoylphosphatidyl derivative [171], the 5'-monophosphate [211], a 5'-monophosphate diglyceride (phosphatidic acid conjugate) [65], and a sugar *n*-$C_{16}H_{33}$ 5'-phosphate triester [158]. Analog diester conjugates with ddI (**5**), or with another molecule of AZT [212] also showed activity.

AZT is monophosphorylated by cellular deoxynucleoside kinases. It is important to recognize the fundamental role of the initial phosphorylation step,

which is the rate-limiting step for AZT-TP, as subsequent phosphorylation to the di- and triphosphates are accomplished by relatively less specific and less stringently regulated nucleotide kinases [213]. Despite notable antiviral potencies in some cases, it has often been difficult to establish that nucleotide prodrugs do indeed function as phosphate delivery motifs, rather than simply releasing AZT. The use of thymidine kinase-deficient cell lines (TK^- cell lines), which are resistant to the antiviral effect of AZT but sensitive to the intracellular release of free nucleotides, has greatly assisted clarification of this issue, since cleavage of the prodrug may occur in culture medium to release AZT extracellularly [191, 192, 213-215].

The extent of nucleoside phosphorylation varies considerably among cell lines [48, 216]. The presence and activity of these activating intracellular enzymes are highly dependent on host species, cell type, and stage of the cell cycle. For example, thymidine kinase is an S phase-specific enzyme, whose activity is increased by several orders of magnitude during cell division [216].

The major differences among the various nucleotide prodrug concepts are the nucleotide delivery mechanisms. While almost all prodrug strategies described to date are based on chemical hydrolysis, these strategies do not deliver the nucleotide selectively and consequently serve only as nucleoside depots. In contrast, strategies based on enzymatic activation as in the bis-POM phosphotriesters [217], the bis-SATE phosphotriesters [162, 186, 218, 219] and the aryloxyphosphoramidates [192, 220-222] provided successful intracellular delivery of the free nucleotides from highly lipophilic precursors [187].

1. Phosphodiesters of AZT (65-101)

Phosphodiesters are unlikely to cross cell membranes due to the presence of a negative charge on the molecule. In spite of this, several groups have determined the anti-HIV activities and other properties of these derivatives.

1.1. Monoalkyl or Monoarylphosphate Derivatives of AZT (65-72)

A number of dialkyl diphosphate diesters have been synthesized by various groups, but the anti-HIV activity and metabolism of these conjugates has not been reported. Chilemmi *et al.* [154] prepared the 5'-*O*-glycerophosphoryl derivative of AZT (**65**), (Fig. **10**). Subsequent lipase-catalyzed acylation with trifluoroethyl palmitate afforded the target 5'-*O*-(1-*O*-

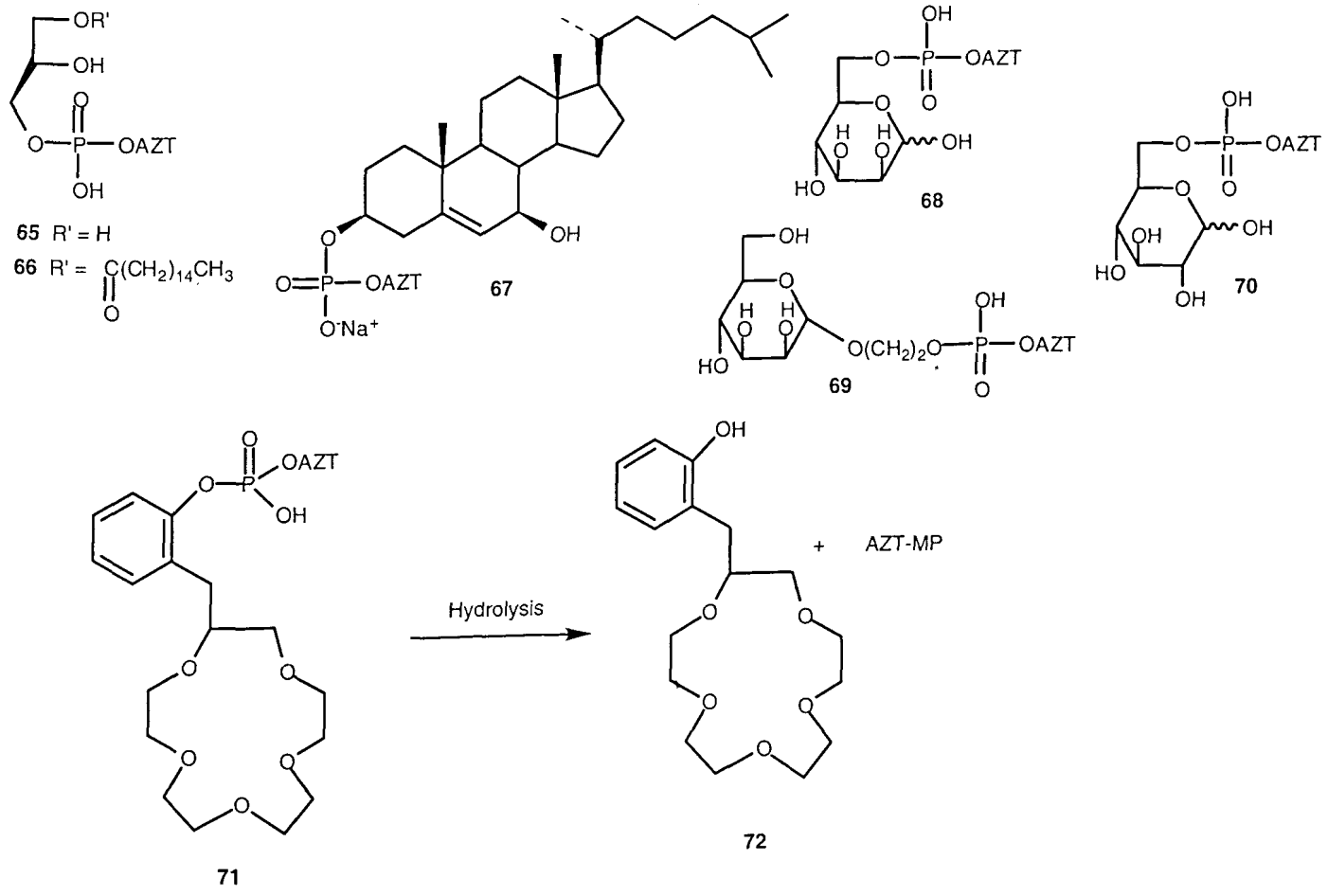


Fig. (10). Phosphodiester derivatives of AZT (monoalkyl, monoaryl and carbohydrate derivatives) (**65-71**).

palmitoyl-*sn*-glycero-3-phosphoryl)AZT (**66**). Synthesis of the monophosphoric acid diester of 3β(7β)-hydroxycholesterol) and AZT (**67**, Fig. **10**) has been reported by Pannecoucke *et al.* [156] with no anti-HIV data reported. Kuipers *et al.* [155] reported optimization of the reaction conditions to prepare the monophosphate derivative of AZT coupled to the neoglycoprotein $Lactose_{28}$-HAS and other conjugates.

Several strategies have utilized AZT-monophosphate-linked to other carriers that could induce internalization of the ligand via cell surface carbohydrate-specific receptors. Examples of glycosylated carriers include carbohydrates such as D-glucose and ethyl D-mannopyranoside (**68-70**, Fig. **10**) [157, 158]. These phosphodiester derivatives were equiactive (μM range) on day 22 postinfection to AZT, although compounds **69** and **70** were less toxic. The reference compound AZT displayed a CC_{50} of 22 μM and an EC_{50} of 0.45 μM at day 22 postinfection of CEM-C113 cells with HIV-1. Under similar conditions the CC_{50} and EC_{50} values were 35 and 0.9 μM, respectively for compound **70** (Fig. **10**). The mannosides phosphodiester (**69**) showed a selectivity index comparable to AZT. The selectivity indices were 49 for AZT, 40 for the mannoside 6-phosphate **68**, 47 for the ethyl mannoside phosphate **69**, and 39 for the glucose-6-phosphate **70**.

An ionophore-nucleotide conjugate has been proposed by Morin *et al.* [153] as a new class of nucleoside prodrug. 5'-Phosphate diester derivatives of AZT were prepared using a crown ether as the phosphate masking group. Because of its anionic charge, the conjugate is quite hydrophilic, but association with a metal cation produces a lipophilic ion-pair that is capable of diffusion through a membrane bilayer. Subsequent intracellular cleavage of the phosphate diester releases AZT-MP which can be converted enzymatically into the bioactive AZT-TP. It has been proposed that at physiologic pH, compound **71** (Fig. **10**) will be anionic and water soluble, but upon association with an alkali metal cation, a neutral and lipophilic complex will be formed that should be capable of diffusing across cell membranes. The phosphate aryl ester in **71** ensures that the preferred cleavage pathway produces AZT-MP, together with the crown ether **72** (Fig. **10**). The prodrug **71**, when tested as an anti-HIV agent in infected CEM-SS cells, showed EC_{50} and CC_{50} values of 70 and > 2 nM, respectively. The anti-HIV activity of **71** is approximately 14-fold less than AZT (EC_{50} = 5 nM).

1.2. Ether Lipids (Els) (73-88)

A number of different justifications have been proposed for synthesizing ether lipids of AZT: 1) ether lipid-nucleoside conjugates may allow a larger concentration of drug to enter the cell due to the lipophilic nature of the ether lipid, 2) hydrolysis of the conjugate by cellular enzymes such as phosphatases could result in a slow release of nucleoside 5'-monophosphate in treated cells, possibly reducing resistance development to the nucleoside, 3) degradative metabolism of the conjugate may release two active species (nucleoside 5'-monophosphate and ether lipid analog) that act at different stages of the viral replication cycle. Ether lipids (Els) are membrane interactive; the mechanisms for their action involves a shift in virus assembly from the plasma membrane to intracytoplasmic vacuoles and production of defective virus. This combination of actions result in a synergistic effect [159, 168, 223], 4) conjugates may provide cells with a depot form from which the antiviral drug can be released as the 5'-monophosphate species to bypass the required initial phosphorylation of the parent anti-HIV nucleoside [168], 5) ELs do not induce the myelosuppression or neuropathy shown by AZT. ELs are lipophilic compounds that accumulate in the plasma membrane of T-lymphocytes and monocytes/macrophages and cross the host blood-brain-barrier [62, 168].

A series of ether lipid-AZT conjugates (**73-80**) (Fig. **11**) were synthesized and evaluated for anti-HIV activity by Tsotinis *et al.* [164] and Calogeropoulou *et al.* [224]. Analogs with a methyl group α to the phosphate moiety (**76, 77**) exhibit a marked degree of stereoselectivity with regard to their anti-HIV activity, since **77** (EC_{50} = 0.2 μM) was 10 fold more active than **76** (EC_{50} = 2.2 μM) against a HIV-1 infected CEM cell line. Also, replacement of the long alkyl chain with aromatic groups in oxyalkyl ether phospholipid-AZT conjugates led to substantially more potent compounds (**78-80**) (EC_{50} = 0.013-0.020 μM) with anti-HIV-1 activity comparable to that of AZT (EC_{50} = 0.006 μM). The presence of a methyl group α or β to the phosphodiester moiety in the oxyalkyl ether phospholipid-AZT conjugates (**74-77**) leads to decreased anti-HIV activity (EC_{50} = 0.2-2.2 μM). These compounds were inactive against CEM/TK^- cells (EC_{50} > 50-125 μM), suggesting that they do not efficiently deliver AZT-MP into cells, but undergo rapid conversion to AZT. These results indicate that the conjugates are partially hydrolyzed only in serum-containing medium, probably via the action of phosphodiesterases and/or phosphatases present in fetal calf serum (FCS). After 9 days of incubation in RPMI-1640 containing 10% FCS only 50% of the conjugate was hydrolyzed to afford AZT. AZT-MP was not detected during the experiment [164].

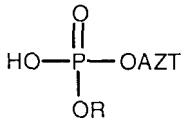
Several groups have prepared other types of ether lipids [62, 162, 163, 165-168, 170, 184, 218]. Liponucleotides containing an ether linkage at the 2-position, and either a thioether, sulfoxide, or sulfone

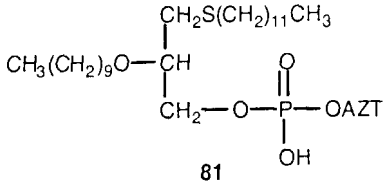
linkage at the 3-position, to the nucleoside component of AZT provided another approach described by Zilch *et al.* [225] and Piantadosi *et al.*, [62, 169] (**81** and **82**, Fig. **11**). AZT conjugates of amidoalkyl, oxyalkyl, and thioalkyl ether lipids (**82**) coupled to AZT via phosphate linkages showed *in vitro* activity (EC_{50} = 0.02-0.03 mM) and a 5-10 fold reduction in cell toxicity (CC_{50} = 25.2-53.8 mM) compared to AZT (EC_{50} = 0.004 mM, CC_{50} = 5.1 mM) in a CEM-SS cell line infected with HIV-1. It was not determined whether the greater efficacy of these ether lipids, relative to AZT, was due to direct action of the conjugate, or the release of AZT-MP [62].

AZT at concentrations up to 100 μM caused only slight inhibition of HBV replication in human hepatoma (2.2.15) cells (EC_{50} > 100 μM). 1-*O*-octadecyl-*sn*-glycero-3-phospho-azidothymidine (ODG-P-AZT) (**83**, Fig. **11**) showed increased antiviral activity, with a 50% reduction in HBV replication at 2.1 μM. Based on EC_{50} values, ODG-P-AZT was > 48 times more active than AZT in reducing replication in 2.2.15 cells. It was assumed that increased uptake of the AZT-lipid prodrug and direct enzymatic conversion to AZT-MP may explain the higher *in vitro* antiviral activity of ODG-P-AZT relative to AZT [226].

Hong *et al.* [168] reported that conjugate **84** (Fig. 11) protected 80% of HIV-infected CEM cells at concentrations as low as 0.58 μM, (EC_{50} < 0.58 μM), whereas cytotoxicity was not observed at < 100 μM. AZT exhibited EC_{50} and CC_{50} values of 0.186 μM and 191 μM, respectively under similar conditions. Pharmacokinetic studies in mice after ip administration of **84** showed a significant increase for the half life of AZT ($t_{1/2}$ = 5.69 h) compared to that for AZT administration ($t_{1/2}$ = 0.28 h) [168].

Kucera *et al.* [159, 160] reported two novel classes of complex synthetic lipids (alkylamidopropyl or alkylthioglycerol phosphocholines and phosphate ester-linked lipid-AZT conjugates) that have selective and potent activity against infectious HIV replication and pathogenesis *in vitro*. Among the reported alkylamidopropyl phosphocholine (PC lipid) analogs evaluated as anti-HIV-1 agents, the most selective compound was 1-octadecanamido-2-ethoxypropyl-3-phosphocholine (CP-51, **85**, Fig. **11**) which exhibited an EC_{50} of 0.11 μM and a SI of 130 [159]. Recently this group synthesized a series of CP-51 analogues to acquire their structure-activity relationships and to ascertain whether a long hydrocarbon chain at position 1 was essential for optimum anti-HIV-1 selectivity. In the case of phospholipid-AZT conjugates, INK-14 [3'-azido-3'-deoxy-5'-(3-dodecylamido-2-octyloxypropyl)-phosphothymidine, **87**] where AZT replaced the choline in PC lipid INK3 (**86**), and CP-92 [3'-azido-3'-deoxy-5'-(3-octadecylamido-2-ethoxypropyl)-phospho-





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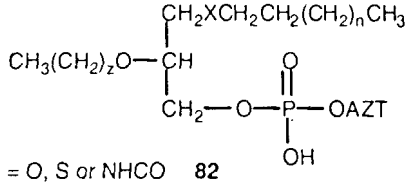
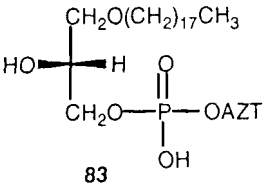


X = O, S or NHCO **82**
n = 13 or 14
z = 0 or 1

73: R = $CH_3(CH_2)_{11}O(CH_2)_2$
74: R = $CH_3(CH_2)_{11}OCH(CH_3)CH_2$ (R)
75: R = $CH_3(CH_2)_{11}OCH(CH_3)CH_2$ (S)
76: R = $CH_3(CH_2)_{11}OCH_2CH(CH_3)$ (R)
77: R = $CH_3(CH_2)_{11}OCH_2CH(CH_3)$ (S)
78: R = $PhOCH_2CH_2$
79: R = 2-naphtyl-OCH_2CH_2
80: R = 1-naphtyl-OCH_2CH_2



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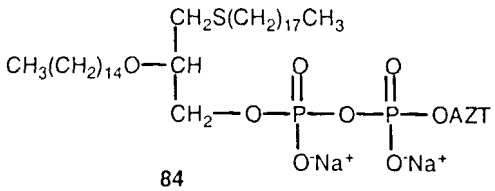
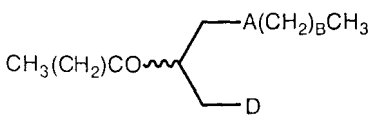
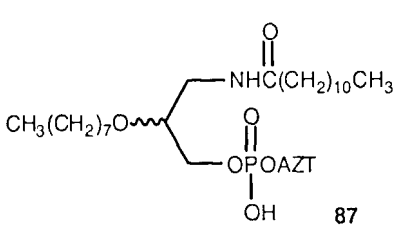


84



Compound	A	B	C	D
85	NHCO	16	1	PC
86	NHCO	10	7	PC

PC = $O-PO_3-CH_2CH_2N^+(CH_3)_3$



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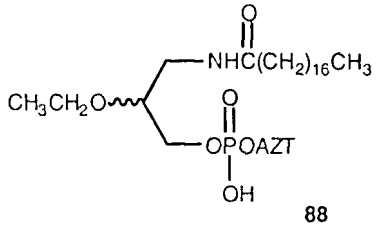


88

Fig. (11). Phosphodiester derivatives of AZT (ether lipids) (**73-88**).

thymidine, **88**], displayed comparable SI values > 1250 and 1793 [62], respectively. Both compounds have a higher SI than AZT alone (SI = 411) and they are 3-to-4 fold more selective than AZT [227]. Their experiments using CEM-SS cells indicated that CC_{50} and EC_{50} values ranged from >100 μM and < 0.08 μM for INK-14 (**87**) and 3.7 μM and 0.009 μM for AZT, respectively. These authors suggested that the most active anti-HIV-1 component in the lipid-AZT conjugate is probably AZT. In the case of the conjugate INK-14 (**87**), the hypothesis is that the lipid component acts as a carrier of AZT; the lipid anchors the conjugate to the cell membrane and the conjugate is metabolized to slowly release a lower concentration of AZT into cells compared to AZT treatment alone. The consequence is reduced toxicity for AZT. Evidence in support of this hypothesis is the lower CC_{50} and higher SI values for INK-14 (**87**) compared to AZT [227].

1.3. AZT Monophosphate, Diphosphate and Triphosphate Diacylglycerols (Ester Lipids) (89-93)

The antiviral activity for mono and polyunsaturated fatty acids has been known for over a decade. Polyunsaturated fatty acids, or 12-methoxydodecanoic acid, have been incorporated into anti-HIV nucleoside conjugates of ester lipids [62, 167, 168]. Nucleoside monophosphate, diphosphate and triphosphate diacylglycerols have been designed as prodrugs to antineoplastic and antiviral nucleosides [64, 174].

Mono- and diphosphate diglyceride conjugates of AZT are capable of inhibiting HIV replication *in vitro*, presumably as a result of releasing the respective nucleoside/nucleoside monophosphate (from the lipid pro-drug by the action of intracellular-metabolizing enzymes) that is subsequently converted to the active triphosphate. Addition of the diacylglycerol diphosphate moiety to AZT restored the activity in TK^--CEM cells, suggesting direct intracellular metabolism to the respective monophosphate, bypassing the normally required thymidine kinase that is absent in TK^- cells [175]. In thymidine kinase deficient mutant CEM cells, and in other cell lines infected with human immunodeficiency virus (HIV), liponucleotide derivatives of AZT are substantially more active than AZT [228].

A sequence of catabolic activating reactions could take place, starting with phospholipase hydrolysis of the fatty acid ester and phosphodiesterase degradation of glycero-3-phospho-5'-AZT to AZT and/or AZT-MP, followed by anabolic phosphorylation to AZT-TP. In this regard, dimyristoylphosphatidylazidothymidine (phosphatidyl-AZT) (**89**) (Fig. **12**) and its metabolites, lysophosphatidyl-AZT and glycero-3-phospho-5'-AZT, lacked the ability to directly inhibit HIV recombinant RT *in vitro*, relative to AZT-TP which was a > 10,000-fold more potent inhibitor of RT. It was therefore concluded that phosphatidyl-AZT (**89**) exerts anti-HIV activity by metabolic conversion to AZT-TP [171].

Hostetler and coworkers formulated the phospholipid analog of AZT (**89**) as a liposome preparation which exhibited *in vitro* anti-HIV-1 activity [171], in an attempt to improve AZT treatment of HIV-1-infected human peripheral blood mononuclear cells (macrophages), and to facilitate transport of AZT into the lymphatic system and into cells, especially HIV-infected cells [64, 171]. Hostetler *et al.* [64] proposed that **89** and **90** have the potential to target macrophages *in vivo* since macrophages take up large quantities of parenterally administered liposomes. Phosphatidyl-AZT (**89**), AZT diphosphate dipalmitin (**90a**) and AZT had EC_{50} values of 1.7, 7.0 and 0.2 μM in CEM cells infected with HIV. AZT can be liberated from these lipid prodrugs by enzymes responsible for phospholipid metabolism. A subsequent *in vitro* incubation study with CEM cells showed that phosphatidyl-AZT conjugates (**89** and **90a**) are converted to lysophosphatidyl-AZT by phospholipase A, which is then further deacylated to glycerophospho-AZT by cellular lysophospholipase. Phosphodiesterase cleavage of glycerophospho-AZT to AZT or AZT-MP then occurs, followed by anabolic phosphorylation to AZT-TP. Although neither prodrug or its lipophilic metabolites inhibited HIV RT, they do serve as a depot-form and precursor of AZT [171]. Furthermore, administration of phospholipid-[^{3}H]AZT (**89**) as a liposomal formulation to mice resulted in a 100-fold increase in the area under the plasma radioactivity concentration versus time curve (AUC) relative to that of AZT. Following administration of liposome formulations incorporating tritiated DPP-AZT (**89**), exposure to lymph nodes, as assessed by the area under the lymph node versus time curves of total radioactivity, was four- to five- fold greater when compared to that after AZT administration [229]. The extent of lymphatic transport after oral administration of prodrug was not assessed.

Administration of the phospholipid prodrug of dipalmitoylphosphatidyl-AZT (**90b**, DPP-AZT) (Fig. **12**) to mice produced improved lymph node concentration of AZT. Pharmacokinetic studies of DPP-AZT (**90b**) revealed that this prodrug produced a lower maximum, but more sustained concentration of AZT in blood and lymph. The serum-AZT AUCs were relatively unchanged by prodrug administration. While the phospholipid prodrug of AZT did not increase AZT concentrations in the lymph nodes, it did provide an extended release of the parent nucleoside, resulting in a sustained concentration of AZT [31, 230].

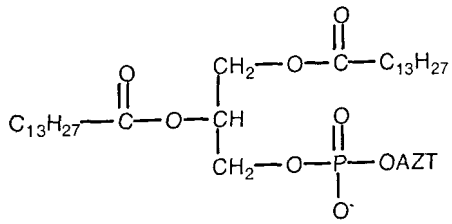
A comparison of the antiviral activities of the mono-, di-, and triphosphate distearoylglycerol conjugates of AZT showed that the anti-HIV potency order was monophosphate (**91**) < triphosphate (**93**) < diphosphate (**92**) (Fig. **12**) [175] in both CEM and HT4-6C cells. The EC_{50} values for AZTTP-DG (**93**) were 0.33 and 0.79 μM in these two cell lines, respectively. Compounds **92** and **91** also exhibited anti-HIV activity: AZTDP-DG (**92**, EC_{50} = 0.29 μM) and AZTMP-DG (**91**, EC_{50} = 2.9 μM). In addition, AZTTP-DG (**93**) was less toxic to CEM cells *in vitro* than other AZT liponucleotides and reduced viable cell numbers in this cell type by 50% at 1000 μM. Preliminary studies on the metabolism of AZTTP-DG (**93**) revealed that both AZT and AZT-MP were released from the lipid prodrug by a rat liver mitochondrial enzyme preparation [175].

Cytidine diphosphate diglyceride (CDP-DG) is an intermediate in the biosynthesis of acidic phospholipids such as phosphatidylinositol (PI), phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG, cardiolipin). Van Wijk *et al.* [231] reported that AZT-DP diglyceride (AZTDP-DG) (**92**) (Fig. **12**) showed no detectable activity as a substitute for CDP-PG in the biosynthesis of PI in microsomes and of PG and DPG in mitochondria isolated from rat liver. The formation of PG by rat liver mitochondria with the natural substrate CDP-DG at concentrations from 100 to 400 μM gave an optimum activity of 70 pmol/min/mg. AZTDP-DG (**92**) showed a rate of 0.03 pmol/min/mg at 80 μM liponucleotide. At this concentration, ddCDP-DG gave a rate of PG synthesis of 0.7 pmol/min/mg. In the mitochondrial biosynthesis of PG and DPG, low activities were found for AZTDP-DG (**92**). For example, DPG produced 4.39 and 0.36 pmol/min/mg with CDP-DG and AZTDP-DG (**92**), respectively.

1.4. Foscarnet Derivatives of AZT (95-101)

Trisoduim phosphonoformate (foscarnet, Foscavir™, **94**) (Fig. **12**) is a broad spectrum antiviral agent with activity against HIV, herpes simplex virus (HSV), and cytomegalovirus (CMV), which are prevalent opportunistic infections in AIDS patients [232]. However, due its highly ionic nature, phosphonoformate shows poor penetration into cells [233]. As a result, the foscarnet concentration needed to block viral replication in an intact cell, *in vitro* or *in vivo*, is orders of magnitude greater than the concentration needed to inhibit viral enzymes in a cell-free assay. In addition, *in vivo* clearance of foscarnet is very rapid, which makes it difficult to achieve long-lasting control of viral infections.

The ammonium salt of 3'-azido-5'-(*O*-ethoxycarbonylphosphonyl)-3'-deoxythymidine (ECP-AZT) (**95**, Fig. **12**), the prototype of a novel class of compounds incorporating the two active antiretroviral agents AZT and phosphoformic acid (PFA) within one structure was found to block viral replication at a 50% inhibitory concentration of 1 μM. The rationale for the synthesis of ECP-AZT (**95**) was that it may be cleaved intracellularly to AZT and PFA via hydrolysis of the phosphate ester bond, or to AZT-MP by oxidative cleavage of the carbon-phosphorous bond. Inhibition



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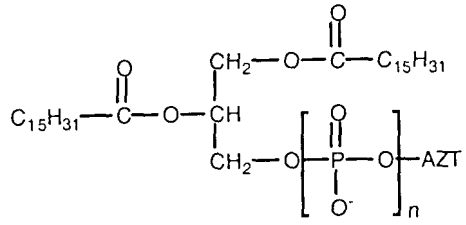
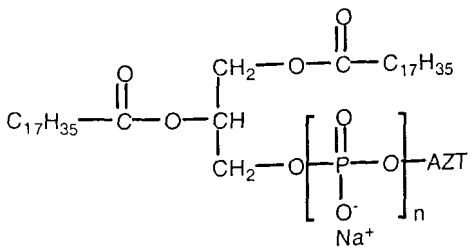
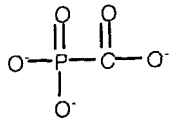


n = 2 **90a**
n = 1 **90b**



n = 1 (**91**, AZT-MP-DG)
n = 2 (**92**, AZT-DP-DG)
n = 3 (**93**, AZT-TP-DG)



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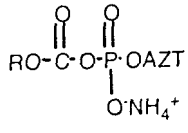
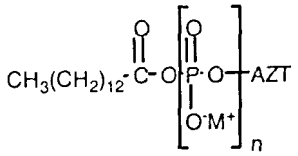


95 R = Et
96 R = *n*-$C_{18}H_{34}$
97 R = *n*-$C_{20}H_{41}$
98 R = *n*-$C_{22}H_{45}$
99 R = 3β -cholest-5-enyl



100 n = 1
101 n = 2

Fig. (12). Phosphodiester derivatives of AZT (ester lipids, foscarnet derivatives **89-101**).

of virus replication in the presence of 10^{-4} M ECP-AZT was virtually complete. By comparison, AZT under identical conditions produced about 90% inhibition of viral replication at 10^{-7} M, but there was 20% inhibition of cell growth at 10^{-6} M and 40% inhibition at 10^{-4} M. ECP-AZT was therefore less potent than AZT. The reason for the lower potency of ECP-AZT may reflect slower uptake into cells due to a negative charge that resides on the phosphorous oxygen, and its slow enzymatic metabolism [176].

Rosowsky *et al.* [177] prepared lipophilic esters of 3'-azido-3'-deoxy-5'-*O*-(carboxyphosphinyl)thymidine (PFA-AZT, **96-99**) (Fig. **12**) as a method to generate sustained levels of the parent drug in tissues, but more specifically in virus-infected cells. In these conjugates, the carboxyl group of PFA is linked via a C-ester bond to a 1-octadecyl (stearyl, *n*-$C_{18}H_{37}$, **96**), 1-eicosanyl (arachidyl, n-$C_{20}H_{41}$, **97**), 1-docosanyl (behenyl, *n*-$C_{22}H_{45}$, **98**), or 3-β-cholest-5-enyl (**99**), alcohol and the phosphonyl moiety is linked via a P-ester bond to the 5'-hydroxyl group of AZT. The presence of a hydrophobic C18-22 side chain together with a single, weakly acidic OH group on the phosphorous atom was expected to facilitate transport of the prodrug across the cell membrane, ultimately yielding an intracellular PFA level not normally attainable except at extracellular PFA concentrations of 100 μM or more. The resulting diesters, which have the potential to act as either PFA prodrugs, AZT prodrugs, or both, were found to inhibit the replication of wild-type HIV-1LAI in human HT4-6C cells, as well as that of a PFA-resistant strain produced by site-specific mutagenesis (R89K), and of an AZT-resistant strain originating from a patient treated with AZT. Concentrations of **96-99** found to inhibit replication of wild-type HIV-1LAI by 50% (EC_{50} values), as measured in a plaque reduction assay, were in the 0.1-0.3 μM range as compared with 0.013 μM for AZT and 133 μM for PFA. Indeed, even wild-type HIV-1LAI was at least 1000 times more sensitive to **97** than to PFA. A notable feature was that, in addition to being > 1000-fold more potent than PFA against the PFA-resistant mutant, the lipophilic PFA-AZT conjugates were more potent than PFA or AZT, against AZT-resistant HIV-1. Although the mechanism of action of these esterified PFA-AZT conjugates was not elucidated, the fact that **96-99** were more active than either PFA or AZT against the PFA-resistant mutant E89K, suggests that they are taken up by host cells without prior cleavage to AZT and PFA in the medium, and that they are subsequently metabolized to PFA and AZT inside the cell, thus serving as dual prodrugs. The fact that **96-99**, with EC_{50} values in the 0.1-0.5 μM range, were 10-100 times more active against AZT-resistant HIV-1 suggests that the observed antiviral effect was not due to AZT alone and that at least some intracellular PFA must be formed [177].

Bonnaffé *et al.* [234] reported a new type of acyl nucleotides (**100, 101**) (Fig. **12**) as potential nucleotide lipophilic prodrugs, where a fatty acid moiety is linked to a nucleotide phosphate bond. In these compounds the lipophilic acyl moiety should enhance passive diffusion of the charged nucleotide through cell membranes. This mixed carboxylic phosphoric anhydride was expected to be hydrolyzed faster than the symmetrical phosphoric anhydride, thereby liberating the corresponding free nucleotide. When activities of these acyl nucleotides were compared with their parent nucleotide, no differences in antiretroviral activity could be detected between acyl nucleotides and their corresponding nucleotides at either 6 or 10 days after infection. The EC_{50} values at day 6 postinfection for nucleotide compounds (**100-101**) were in the same range as the free nucleoside (10-30 nM). These results were confirmed by a HIV-1 RT inhibition assay and are clearly illustrated by the selectivity index which showed no significant differences between acyl nucleotides and their corresponding nucleotides. These observation may be due to rapid hydrolysis of the phosphate bond in RPMI culture medium containing aminated compounds and proteins [234, 235].

2. Phosphotriesters (102-163)

The use of phosphotriesters has been the most popular approach in designing AZT prodrugs. AZT phosphotriester prodrugs have been investigated extensively for AZT-MP delivery. Such neutral species are expected to cross cell membranes readily, but their selective conversion to AZT-MP could be a limitation. Phosphotriesters must first be transformed to the corresponding phosphodiesters before considering any transformation to the desired AZT-MP. Since phosphotriesterase activity in serum or inside the cells has not been reported, this hydrolysis must be selective. This will dictate the success, at least in part, of the phosphotriester prodrug approach [213]. On the other hand, the phosphotriester must have acceptable stability in cell culture medium prior to cellular uptake and selective intracellular biotransformation of the active species.

2.1. Simple Dialkyl and Diaryl Phosphotriester Derivatives of AZT (102-116)

McGuigan *et al.* have reported a variety of transitory alkyl, aryl, and aminoacid phosphate protecting groups with the aim of deriving structure-activity relationships. All phosphotriesters investigated were less potent than AZT in various cell lines. The absence of stability studies makes interpretation of the data, in terms of metabolic pathways, difficult [213]. Although simple

dialkyl phosphotriester derivatives of AZT are inactive [70], some haloalkyl and other lipophilic long chain phosphotriester derivatives of AZT showed weak to moderate activity relative to that of AZT. For example, the 5'-*O*-bis(2,2,2-trichloroethyl)phosphate derivatives of AZT (**102**, EC_{50} = 0.7 μM) (Fig. **13**) exhibited weak anti-HIV-1 activity relative to AZT (EC_{50} = 0.004 μM) [70, 210]. Introduction of halogen atoms into the alkyl (phosphate) chains increased anti-HIV activity. The potential of the phosphate to undergo alkyl cleavage to release the free nucleotide appeared to correlate with antiviral activity. A halogen substituent in just one of the alkyl (phosphate) chains improves anti-HIV activity, and a halogen atom in the second alkyl chain further enhances activity. The bis(trihaloethyl) compounds (**102-103**) were the most active in this group, whereas compounds with only one trichloroethyl chain (**104-105**) are 4-to-10 fold less active. In contrast, analogs with halogen substitution in neither chain (**106-108**) were inactive. Enhanced activity was attributed to the higher hydrolytic lability of the haloalkyl-phosphate moiety. Anti-HIV activity does not appear to correlate with hydrolytic lability in serum, and no AZT was formed by hydrolysis under a variety of conditions [70, 180].

McGuigan [181, 236] also investigated diaryl triester analogs of AZT (**109-115**). The antiviral effect of the diaryl phosphates (**109-115**), which varied over three orders of magnitude, was dependent on the nature of the aryl substituent. In particular, the effect of strongly electron withdrawing aryl substituents correlated with high anti-HIV potency in C8166 cells. Compounds **110** and **111** were potent (EC_{50} ≈ 0.0032 μM) in C8166 cells compared to AZT (EC_{50} = 0.008 μM). Activity is inversely proportional to the pKa of the phenol, with an apparent relationship between the log anti-viral activity and the pKa of the phenol. A plausible explanation for this correlation is that phenol acidity may correlate with leaving group ability and/or P-O-aryl lability, which would control the release of the biologically active AZT phosphate(s) [181, 236]. Diaryl phosphates with electron withdrawing groups are more potent than unsubstituted diaryl phosphate esters. The most obvious interpretation of these data was that electron withdrawing aryl groups [*para*-NO_2 or –CN] in the diaryl phosphate moiety are susceptible to enhanced intracellular hydrolysis to liberate the free monophosphate [181]. Although the potency of the parent nucleoside was increased about 3-fold by the addition of an appropriately [*para*-NO_2 or –CN] substituted diaryl phosphate moiety, this increase in potency was achieved singly by increased intracellular uptake of the free nucleoside, rather than the delivery of AZT-MP (intended by-pass of the nuceloside kinase). Further evidence for this explanation was gained from incubation of AZT bis(*para*-nitrophenyl)phosphate triester (**110**) with human serum, where **110** underwent negligible change during the first day of incubation, with slow appearance of AZT at longer incubation times. AZT-MP was not detectable at any stage of the incubation [236].

McGuigan *et al.* [236] reported that symmetrical diaryl phosphate derivatives of AZT (**109, 110**) are extremely poor inhibitors of viral replication in a thymidine kinase deficient cell (CEM/TK$^-$, JM cells)

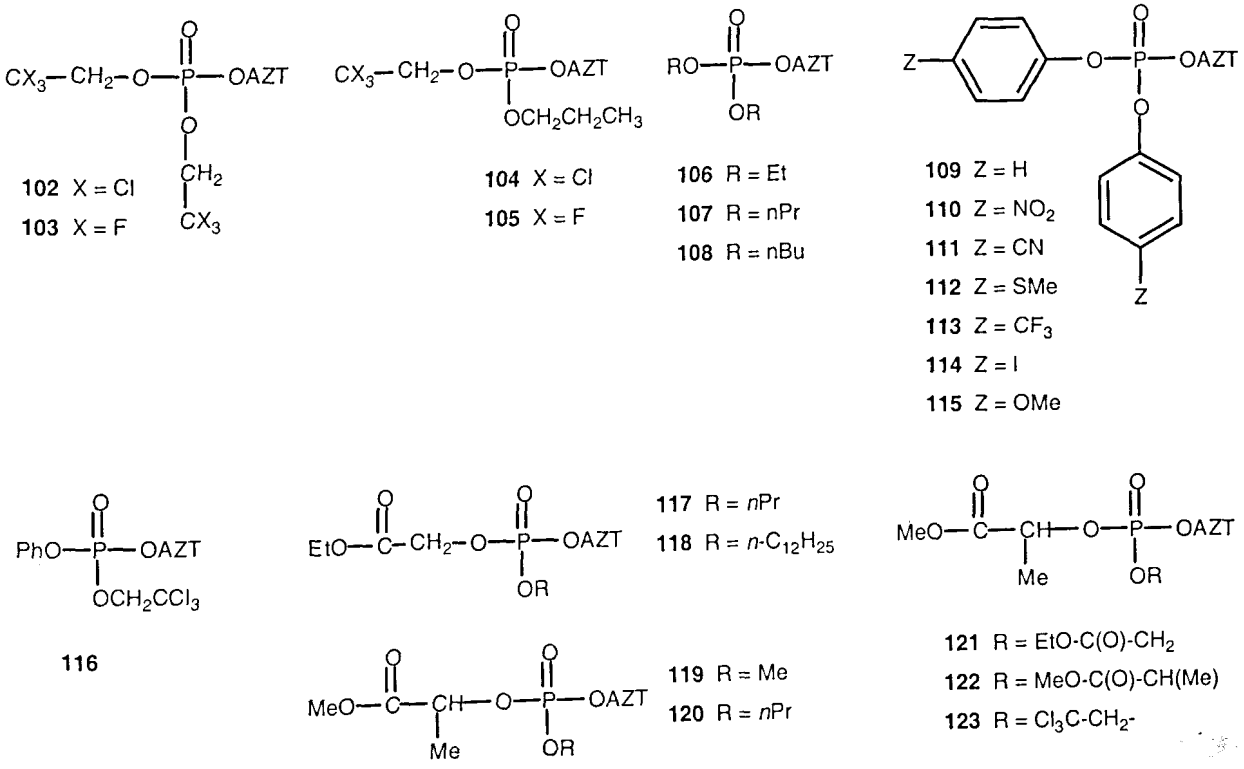


Fig. (13). Phosphotriester derivatives of AZT (dialkyl, diaryl, glycolate and lactate phosphotriesters, **102-123**).

(EC_{50} > 100 μM). One possibility is that the high lability of the aryl group results in rapid *in vitro* hydrolysis to release the nucleotide extracellularly. Thus, it is apparent that diaryl phosphates are only able to act as prodrugs to the free nucleoside. Due to poor membrane penetration by polar nucleotide species such as AZT-MP, the product would only be active following extracellular cleavage to AZT, which would enter cells by passive membrane penetration prior to intracellular re-phosphorylation to the AZT-MP [236].

Compounds **109** (two phenyl substituents) and **116** (Ph and Cl_3CCH_2) exhibit similar antiviral activity in C8166 cells, with EC_{50}s of 0.3 and 0.2 μM, respectively. From a simple additive view, the phenyl and trichloroethyl groups appear to be equally efficacious in their activating effect, relative to simple alkyl groups which have been previously reported to be inactive. Compound **110** was a potent inhibitor of HIV-1 replication in C8166 cells (EC_{50} = 0.0032 μM), being approximately 3-fold more potent than AZT (EC_{50} = 0.008 μM). However, this increase in anti-HIV potency occurred at the expense of increased toxicity in uninfected cells at concentrations as low as 40 μM. The nitrophenyl containing compounds are especially cytotoxic, whereas the amidates tend to be less toxic. In simple additive terms, the order of activating effects would be: *p*-nitrophenyl > phenyl = trichloroethyl = amidate >> alkyl [191].

2.2. Glycolate and Lactate Phosphotriesters (117-123)

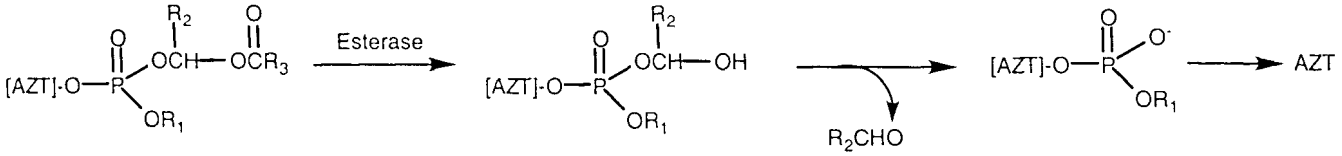
Phosphate triester derivatives of AZT are moderate (**120**, EC_{50} = 10 μM) to potent (**117**, EC_{50} = 0.5 μM) inhibitors of HIV when one of the alkyl chains contains an ester moiety, particularly glycolate or lactate. Intracellular cleavage of these ω-esters by carboxyesterase would yield ω-carboxylates which would be trapped within the cell due to their increased polarity. Phosphate hydrolysis of these ω-carboxylates may be enhanced by intramolecular participation of the free carboxylate function. The activity of these compounds decreased with increasing alkyl phosphate chain length (**117** > **118**; **119** >**120**) (Fig. **13**). In this regard, ethyl glycolate appeared to be more active than methyl lactate. For example, **117** and **120** had EC_{50}s of 0.5 and 10 μM, respectively, against HIV-1 in a C8166 cell line. The origin of the differences in activity for the glycolate/lactate compounds do not appear to correlate with simple hydrolytic lability since incubation in growth medium produced only trace amounts of AZT (0.05%). Thus, nucleoside release (AZT) is not the primary mode of action of these compounds. However, the general level of activity of the ester-substituted compounds (**117-118**) and (**119-120**) is consistent with a mechanism involving intracellular delivery of the free nucleoside or a close derivative thereof, but the structures of these hydrolysis products were not determined [179]. Related compounds with two ester-containing groups such as one ethyl glycolyl group and one methyl lactyl group (**121**) were also evaluated. Comparison of **118** to **121-123** indicated that the presence of two ester-substituted groups enhances activity approximately 100-fold relative to those having only one substituted ester group, although this cannot be precisely defined given the different structures of the esters. The diester compounds **121-122**, which are generally less active than AZT, are also less toxic [178].

2.3. Phosphotriester Approaches Using Simultaneous Enzymatic and Chemical Hydrolysis

For mononucleotide phosphotriester prodrugs, the prodrug must be more stable in the culture medium than inside the cell, even though both environments have the same neutral pH range and the prodrug must undergo selective cytosolic hydrolysis [185]. Phosphodiesters are eventually hydrolyzed to the expected NuMP (nucleotide). For different compounds [**127** in Fig. **15** and **136** in Fig. **17**] this second bioactivation step may follow enzymatic processes that are dependent on the relative affinity of the resulting phosphodiester for carboxyesterases and/or phosphodiesterases [161].

2.3.1. (Acyloxy) Alkyl or Aryl Phosphate Triesters of AZT

(Acyloxy)alkyl or (acyloxy)aryl groups have been studied as a method for carboxylate esterase mediated bioreversible phosphate protection [182, 183, 237,



124 R_1 = C_1-C_8 alkyl; R_2 = C_6-C_{10} aryl; R_3 = C_7-C_{12} aralkyl

125

negatively charged locked-in intermediate

126

Fig. (14). Phosphotriester approach using simultaneous enzymatic and chemical hydrolysis using (acyloxy)alkyl or (acyloxyaryl) esters of AZT-MP [103].

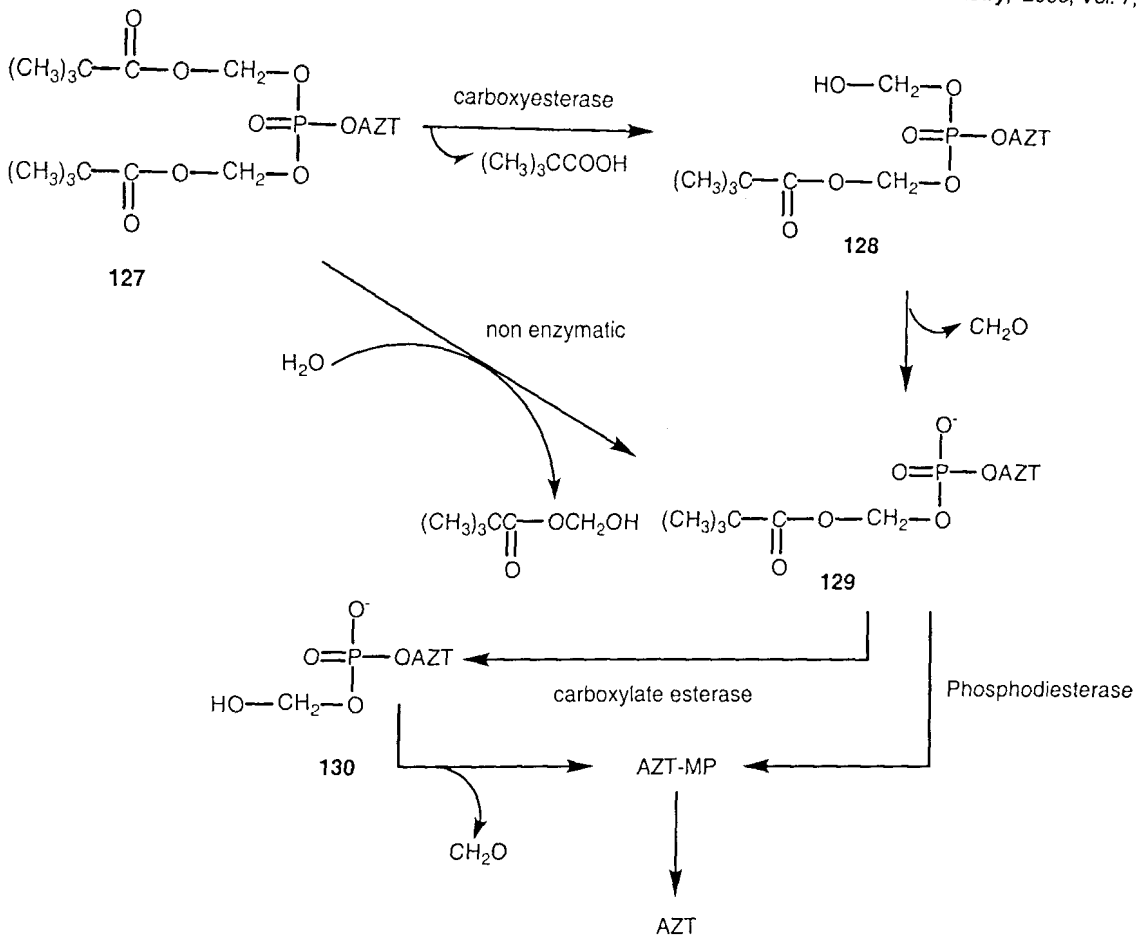


Fig. (15). Mechanism of enzymatic and chemical hydrolysis of (pivaloxy)methyl (POM) phosphotriester of AZT (**127**) [184].

238]. Such transitory protecting groups are preferentially removed by intracellular carboxyesterases with concommitant elimination of formaldehyde [103, 185] (Fig. **14** and **15**).

Novel mixed phosphate derivatives (**124**) were designed by Bodor [103] to undergo *in vivo* cleavage in stages after reaching their desired site of action. The first cleavage of the terminal ester moiety in **124** by an esterase would afford an inherently unstable intermediate **125** which would immediately and spontaneously release R_2CHO and the negatively charged locked in species **126** as depicted in Fig. **14**. Subsequently, a second cleavage catalyzed by alkaline phosphatase would occur to release AZT. In the case of AZT, which is activated by *in vivo* phosphorylation, activation could occur by phosphokinase conversion of the intracellular locked-in intermediate to the active AZT-TP and/or by phosphorylation of AZT after its release from the locked-in intermediate as described above. The nature of the substituents must be carefully controlled so that the respective enzymatic cleavages occur in the proper sequence [103].

The (pivaloxy)methyl (POM) group has been proposed as an efficient transient protecting group for several bioactive nucleoside monophosphate or phosphonate analogs [217]. Pompon *et al.* [184] designed bis(pivaloxymethyl)AZT (piv_2-AZTMP, **127**) (Fig. **15**) as a cell membrane-permeable precursor of AZT-MP. When **127** is incubated with TK^- CEM cells, it gives rise to intracellular AZT-MP, AZT-DP and AZT-TP. Under similar conditions, no intracellular nucleotides were formed with AZT. Kinetics studies using RPMI 1640, containing 10% heat-inactivated fetal calf serum and CEM cell extracts, showed that piv_2-AZTMP (**127**) is slowly hydrolyzed to piv_1-AZTMP (**129**) via the metabolic sequence: piv_2-AZTMP (**127**) → piv_1-AZTMP (**129**) → AZT-MP → AZT. The first step is catalyzed by carboxylate esterase. The mechanism for the second step piv_1-AZTMP (**129**) → AZT-MP is less obvious, although kinetic analysis suggested that it is mediated by phosphodiesterases and that the carboxyesterase does not play a significant role (Fig. **15**) [184].

A related approach, involving the use of acyloxybenzyl bioreversible phosphate protecting groups of AZT-MP, has been reported by Thomson *et al.* [182] (Fig. **16**). The same decomposition pathway as described in Fig. **15** was expected to be operative with esterase catalyzing removal of the 4-acyl group, and the resulting electron-donating 4-hydroxy aryl substituent then promoting cleavage of the benzyl-oxygen bond (selective C-O bond cleavage process) to release the phospho anion (**133**) together with a short-

lived intermediate with 4-hydroxybenzyl carbonium ion character [213]. Thomson *et al.* [182] reported that after bioconversion, bis(4-acyloxybenzyl) esters (**131**) of AZT-MP produce anionic products (**133**, **134** and AZT-MP) that may be locked in the brain. The lipophilicity of the prodrug should facilitate passive diffusion across the blood brain barrier (BBB) into brain. The mechanism for trapping of the phosphate is shown in Fig. **16**. The success of this approach will be dependent upon the design of the ester protecting group, to achieve suitable rates of hydrolysis. The difficulty in achieving this goal is highlighted by the case of simple phospho ester hydrolysis by P-O bond cleavage, where hydrolysis of the second group is typically 1×10^6 times slower than the first. Metabolism of the prodrug to AZT occurred predominantly within the cell (Fig. **16**) [182]. With the exception of the pivaloxy substituted compound, all of the phosphotriester compounds exhibited antiviral activities comparable to that of AZT. However, all compounds were ineffective in preventing HIV-1 infection in a kinase-deficient TK^- cell line. It was therefore suggested that these compounds undergo metabolism to AZT in culture medium [182, 213]. However, toxicities of these compounds, which are cell line dependent, were in several instances substantially greater than that of the parent nucleoside [213].

2.3.2. Bis (S-Acyl-2-thioethyl)(SATE) Esters of AZT-MP [bis(SATE) Analogs]

The SATE prodrug strategy for AZT involves carboxyesterase or reductase activation, and episulfide elimination [162, 163, 185, 186, 207, 218, 219, 239-245]. The SATE concept is based on a phosphotriester such as **136** that will spontaneously convert to a thioethanol transitory phophotriester (**137**) that will spontaneously eliminate episulfide through a selective C-O bond cleavage mechanism, as shown in Fig. **17**. This approach provides intracellular AZT-MP, [185, 186, 213], and the kinetics of SATE bioconversion in various media fully corroborate the expected mechanism of the phosphotriester biotransformation. It is noteworthy that the overall rate for AZT-MP delivery is dependent upon the first step, which involves competitive hydrolysis of the phosphodiester to AZT-MP via the action of either phosphodiesterase and/or the same mechanism as step 1, depending on the medium [186, 213].

Périgaud *et al.* [185] and Briggs *et al.* [233] have reported different mechanisms by which the unstable thioethyl phosphate ester can be formed following enzymatic bioactivation. Bioactivation of the di[2-(*S*-acetylthio)ethanol] (**136**, SATE) is catalyzed by esterase, giving rise to the thioethyl phosphate triester

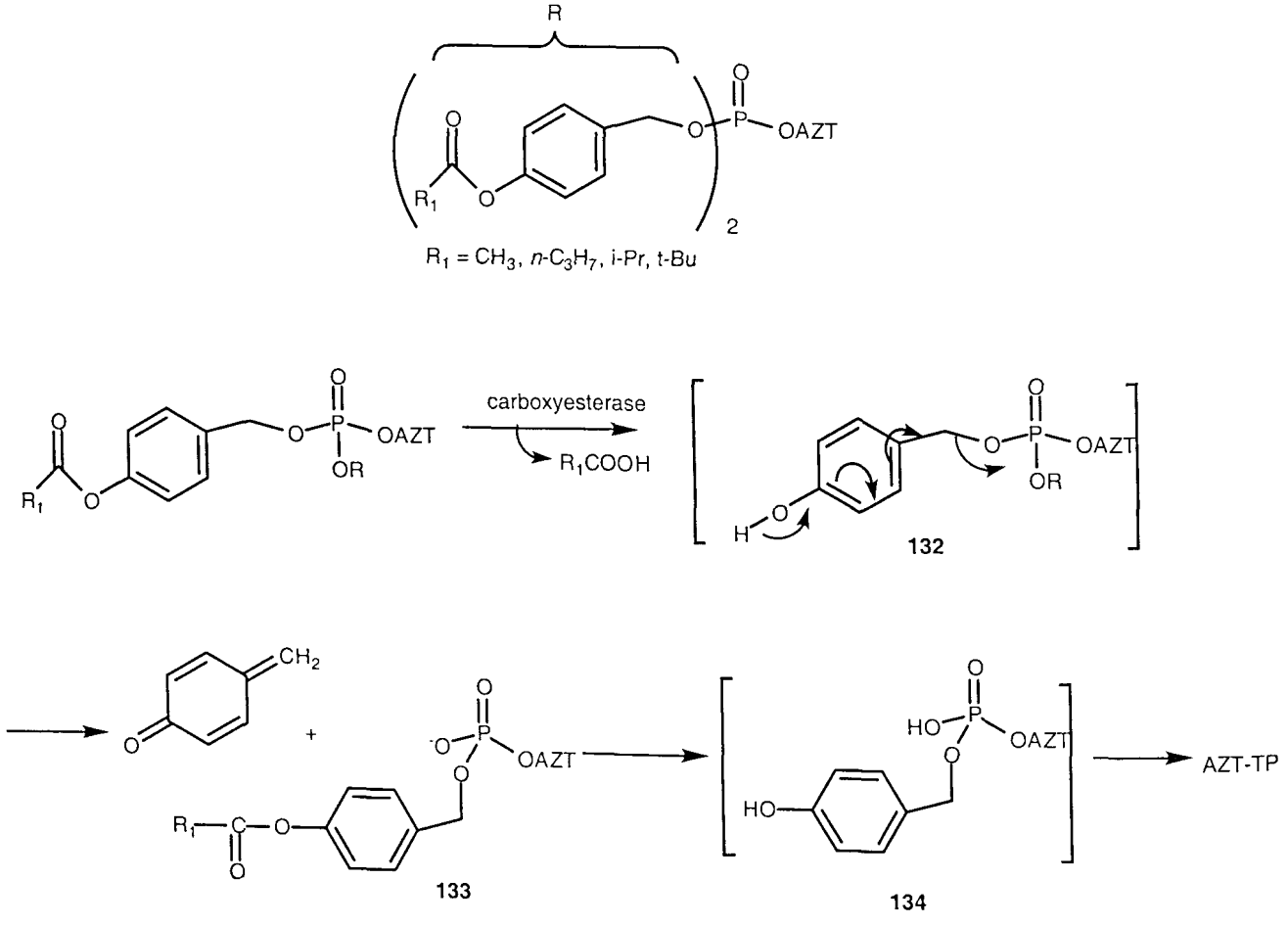


Fig. (16). Prodrug approach using acyloxybenzyl bioreversible phosphate protecting groups of AZT-MP [182].

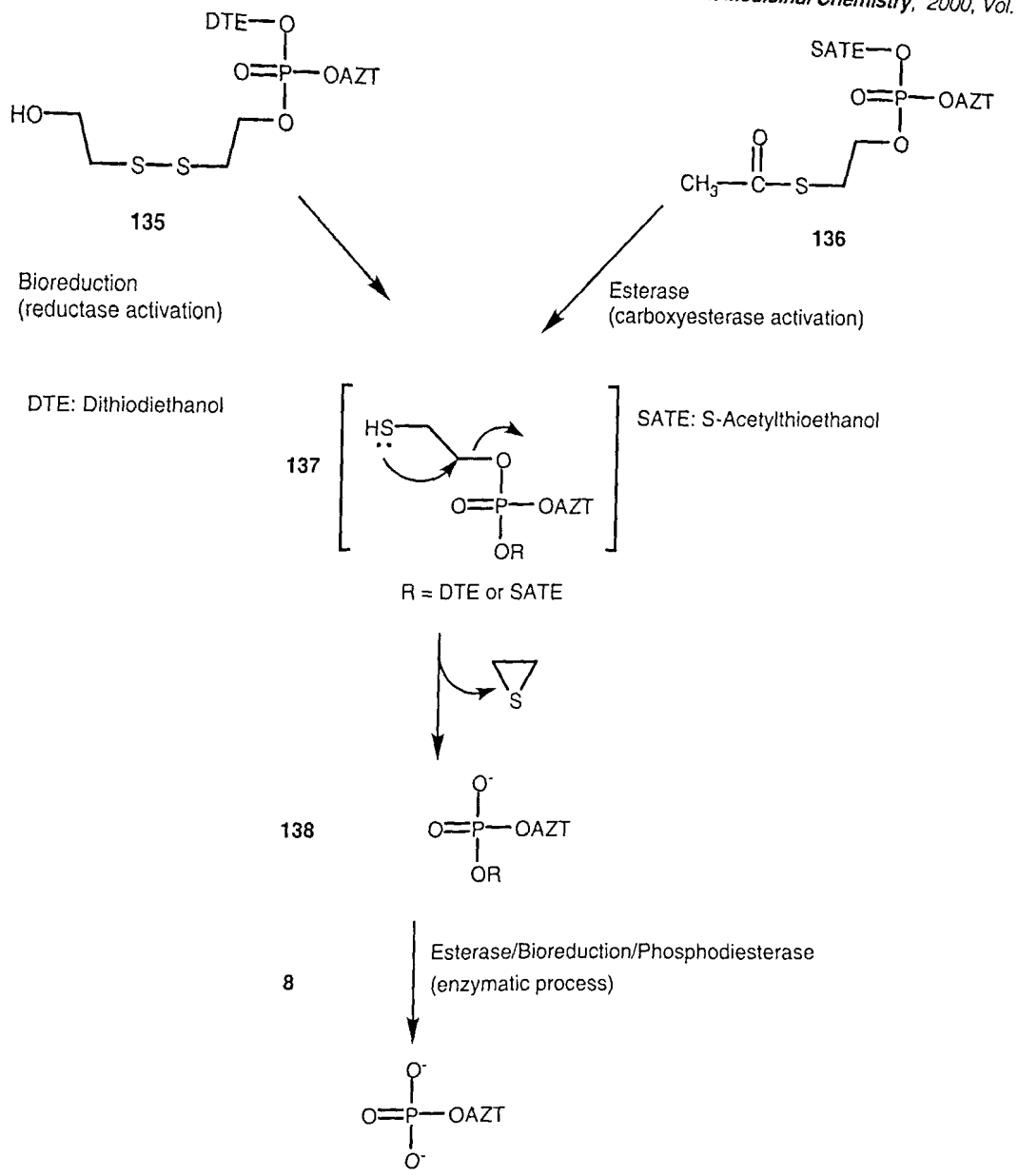


Fig. (17). Bioactivation of SATE and DTE analogues of AZT-MP [185].

137 which is unstable and spontaneously converts to diester **138** and ethylene sulfide. Repetition of these bioactivation reactions with phosphodiesterases affords the Nu-MP (Fig. **17**) [233].

Pronucleotides (**139-142**), as illustrated in Fig. **18**, are protected neutral 5'-monophosphate triesters that can enter cells much more readily than the corresponding unprotected charged 5'-phosphates. After entry into cells, pronucleotides (**139-142**) should be transformed via a sequence of enzymatic thioester cleavage reaction followed by subsequent fragmentation with the concommitant elimination of episulfide to release the 5'-monophosphate (**8**) [162, 246]. To overcome the low level of AZT phosphorylation in monocyte-derived macrophages (MDMs), the SATE approach using 5'-mononucleotide prodrugs (pronucleotides), which bypasses the first anabolic phosphorylation step by enabling the intracellular delivery of AZT-MP, was investigated by Thumann-Schweitzer *et al.* [202]. Bis(SATE) phosphotriester derivatives of AZT were evaluated as anti-HIV agents in MDMs. In comparison of a calculated EC_{50} of 0.5 nM for AZT, the corresponding values for bis(MeSATE)- (**139**), bis(*i*PrSATE)- (**140**) and bis(tBu-SATE)-AZT-MP (**141**) were 0.08, 0.05 and 0.12 nM, respectively, indicating a 4- to -10 fold amelioration of the antiviral effect by delivering the AZT-MP intracellularly. Addition of the SATE promoieties to AZT-MP did not increase toxicity in MDMs since cytotoxicities were virtually unchanged by the SATE moieties, and the calculated SIs were augmented. All pronucleotides with different protecting groups are more active than the parent nucleosides, irrespective of whether the SATE group is a methyl, isopropyl or tert-butyl structure. However, the SATE groups have been shown to influence the stability and the lipophilicity of the pronucleotides [162, 202].

Several lipophilic bis(*S*-acyl-2-thioethyl) (SATE) esters of AZT-MP (**139-142**), designed as prodrugs to AZT-MP, exhibited marked anti-HIV activity in TK$^-$ deficient cell lines. Compound **139** was 5- to 10-fold more effective in inhibiting HIV-1 replication in CEM/TK$^-$ cells than the previously described phosphotriesters **135** [186] and **127** [184], that have the respective DTE and POM enzyme-labile groups. AZT was completely inactive against HIV-1 replication in CEM/TK$^-$ cells at concentrations up to 100 μM. In contrast, the pronucleotides **139-142** are markedly inhibitory to HIV-replication in CEM/TK$^-$ cells with EC_{50}s in the 0.049-0.32 μM range. Compound **139**, the bis(*S*-acetyl-2-thioethyl) ester of AZTMP, emerged as the most potent inhibitor with an EC_{50} of 0.049 μM.

The mechanism by which these SATE derivatives (**139-142**) are converted to AZT-MP involves a carboxyesterase-mediated cleavage process. This is followed by a spontaneous and selective Cα-O bond cleavage to give a phosphodiester which is subsequently transformed to AZT-MP by either a cleavage process similar to the first step and/or by phosphodiesterase mediated cleavage. The second cleavage step may be carboxyesterase mediated, which proceeds at a slower rate than the first cleavage step, presumably because a charged diester has a lower affinity for the enzyme than the starting phosphotriester (Fig. **18**). Biotransformation studies in culture medium and cell extracts using the bis(SATE) phosphotriesters **139-141** confirmed their intracellular conversion to AZT-MP. For example, in RPMI culture medium containing 10% heat-inactivated fetal calf serum, the main pathway (99-100%) was **139-141** → **143-145** → AZT-MP → AZT. In total cell

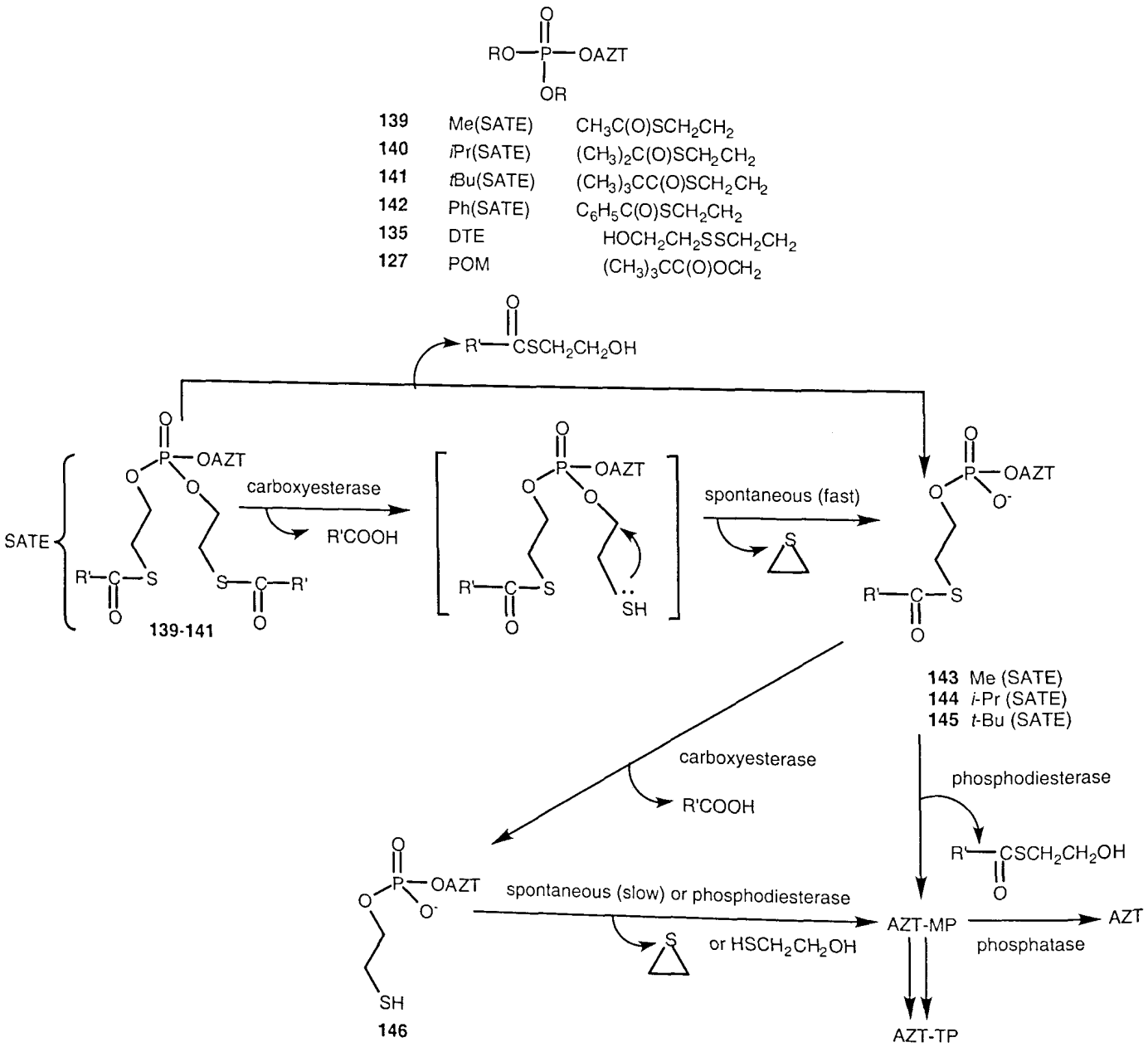


Fig. (18). Lipophilic bis(*S*-acyl-2-thioethyl) (SATE) esters of AZT-MP (**139-142**) (mechanism of action) [162].

extract from CEM-SS cells, the main pathway (90-100%) was **139-141** → **143-145** → **146** → AZT-MP → AZT. These results explain the anti-HIV effect of pronucleotides **139-141** in the CEM/TK$^-$ cell line [162].

Schlienger *et al.* [247, 248] reported synthetic and preliminary stability studies for mononucleoside phosphotriesters of AZT that incorporate a new phosphate protecting group, *viz S*-glycopyranosidyl-2-thioethyl (SGTE) (**147-150**, Fig. **19**). It was predicted that this class of pronucleotides could be hydrolyzed by intracellular glycosidases, and that the carbohydrate groups might serve as site-directing moieties for glycosyl-binding proteins (lectins) on the cell surface of macrophages, or as substrates for monosaccharide facilitated diffusion transport systems at the blood brain barrier [247, 248, and references cited there]. Half-lives for the pronucleotides **148-150** were determined in RPMI and culture medium (RPMI containing 10% heat-inactivated fetal calf serum). Compound **148** showed half-lives of 35 and 30 h in RPMI and culture medium, respectively. However **149** and **150** have half-lives of 4.5 and 8.3 h in both media. The similar half-lives for **149** and **150** in these media indicate that their biotransformation appears to be due to chemical mechanisms involving the participation of the neighboring sulfur atom, giving rise to the corresponding phosphodiester and to an unstable episulfonium ion which is readily converted to the 2-hydroxyethylthioglucopyranoside **151** (Fig. **19**) [247, 248].

Recently Schlienger *et al.* [239] reported monoSATE aryl phosphotriesters of AZT [aryl (*S*-pivaloyl-2-thioethyl) phosphotriesters **152** and **153** of AZT] (Fig. **20**) for intracellular delivery of 5'-AZT-MP. It was postulated that their bioconversion would proceed initially by ester-mediated cleavage of the thioester moiety of the SATE group, followed by spontaneous release of ethylene sulfide and then release of the mono-nucleotide upon cleavage of the aryl ether linkage due to the action of a intracellular phosphodiesterase. The choice of the R group will be of prime importance, in terms of phosphotriester stability and selectivity of the elimination cleavage reactions in intracellular medium. In cell extracts, formation of AZT-MP was observed for **153** and **155**

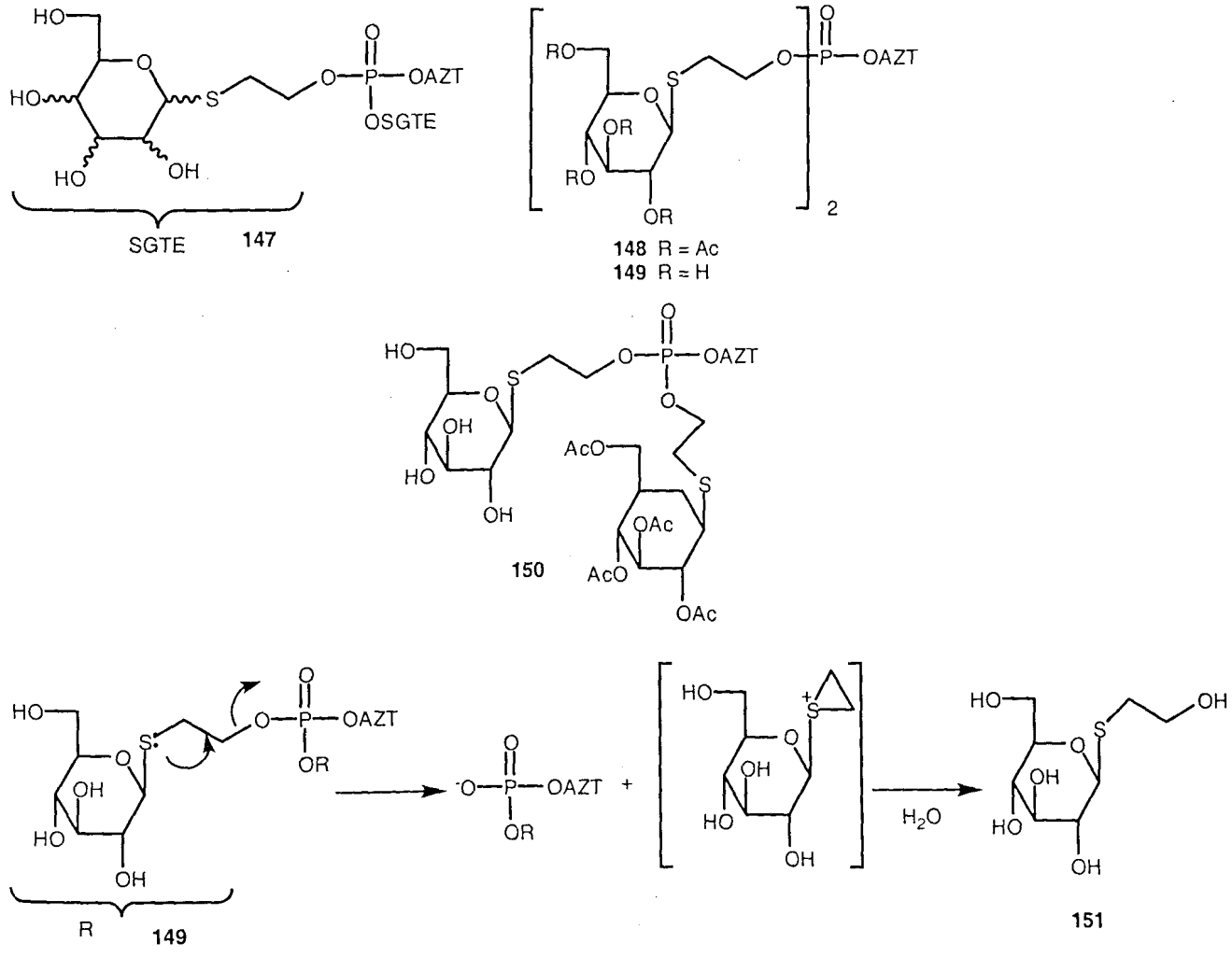


Fig. (19). *S*-glycopyranosidyl-2-thioethyl (SGTE) phosphotriester derivatives of AZT (**147-150**) and hypothetical chemical decomposition mechanism for **149** [247, 248].

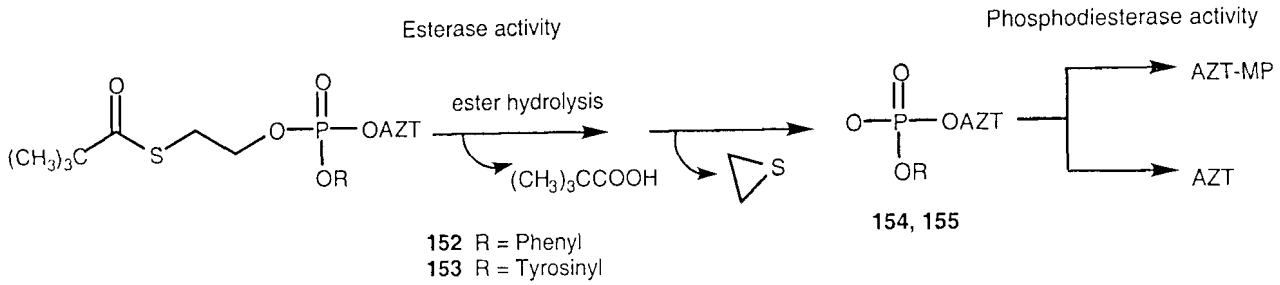


Fig. (20). Expected decomposition pathway for mixed pronucleotides [239].

(R = tyrosinyl), but not for **152** and **154** (R = phenyl). However in both cases, AZT was the final metabolite. For compounds **152** and **154** (R = phenyl), the absence of detectable AZT-MP as an intermediate metabolite may be due to rapid phosphatase hydrolysis of AZT-MP ($t_{1/2}$ = 4 h), compared to the slow biotransformation of **154** ($t_{1/2}$ estimated to be about 4 days). Both **152** and **153** were stable in culture medium ($t_{1/2} \geq 3$ days), whereas they are readily biotransformed in cell extract, which is in agreement with the pronucleotide concept. These aryl phosphotriesters exhibited potent antiviral activity in the same range as AZT with EC_{50} values of 0.001, 0.006 and 0.006 μM for **152**, **153** and AZT, respectively, using CEM-SS cells. The pronucleotides **152** and **153** inhibited HIV-1 replication in CEM/TK$^-$ with EC_{50} values of 3.5 and 29 μM for **152** and **153**, whereas AZT was completely inactive at concentrations up to 100 μM.

2.4. Cyclosaligenyl-AZT Prodrugs (156a-h)

Meier *et al.* reported a new pro-nucleotide approach, employing cyclosaligenyl nucleoside monophosphate (cycloSal-NMP) derivatives, that is based on a pH-driven selective chemical hydrolysis [249]. This class of cycloSal-nucleotide compounds is the first reported pronucleotide system that delivers AZT-MP by a pH driven, chemically activated, tandem reaction without the requirement for an enzymatic contribution. This pronucleotide concept was designed by Meier *et al.* [187] to deliver AZT-MP by selective chemical hydrolysis of the lipophilic cycloSal-AZTMP precursors **156a-h** (Fig. **21**). The experimental partition coefficient values for **156a-h** were higher (log P = 0.8-1.8) compared to the value for AZT (*n*-octanol:aqueous buffer, pH = 6.8). In hydrolysis studies, compounds **156** decomposed selectively releasing AZT-MP and the salicyl alcohols **158** according to a controlled, chemically-induced tandem

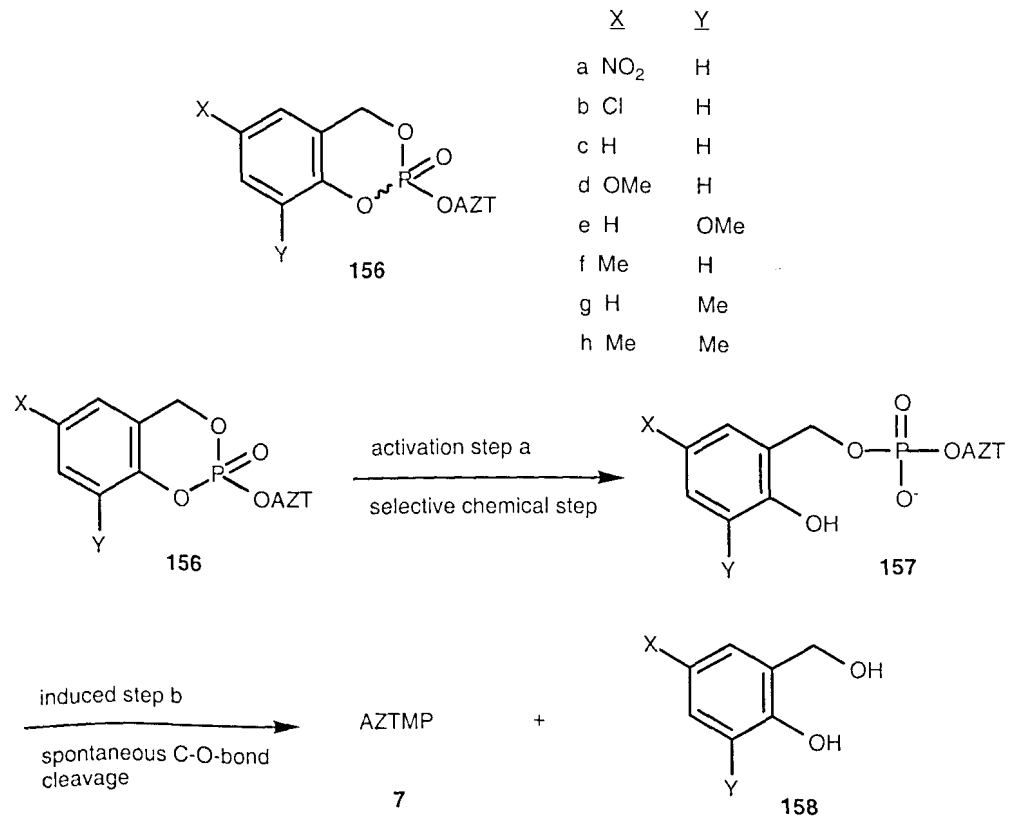


Fig. (21). The hydrolysis pathway of cycloSal-AZTMP phosphotriesters **156a-h** [187].

reaction involving successive, coupled cleavage of the phenyl and the benzyl ester groups of the cycloSal-phosphotriester. In this cycloSal pronucleotide concept, the lower stability of the phenyl as compared to the benzyl phosphate ester allows one to discriminate between these two phosphate ester bonds in the initial step that yields a benzylphosphodiester (**157**) from the cycloSalphosphotriester (**156**). CycloSal-triesters **156a-g** undergo hydrolysis following pseudo-first-order kinetics to yield only AZT-MP in all media that were examined (various aqueous buffers, RPMI-1640 culture medium) and the results confirmed the anticipated pH dependence for the hydrolysis reaction. These results are consistent with a hydrolysis pathway involving selective cleavage of the phenyl ester to give a 2-hydroxybenzylphosphodiester **157** (step a) and a subsequent spontaneous cleavage of **157**, presumably via C-O bond cleavage according to the Grob fragmentation mechanism, followed by a water quench [182, 250] of the remaining masking group to afford AZT-MP and the salicyl alcohol **158** (step b) (Fig. **21**). Phosphotriesters **156** exhibit considerable biological activity in HIV-1 and HIV-2 infected wild-type human T-lymphocyte (CEM/O) cells, whereas nearly all activity was lost in HIV-2 infected CEM/TK$^-$ cells. All cycloSal-AZTMPs **156a-h** proved to be as active as AZT against HIV-1 or HIV-2 induced cytopathicity in a wild-type cell line. Nevertheless, no correlation was observed between antiviral activity and either hydrolytic stability in the aqueous buffers or in RPMI-1640 culture medium, or with the lipophilicity of **156a-h**. Only donor-substituted triesters (**156f-h**) exhibited weak antiviral activity against TK$^-$ cells (5 to 14-times more active than AZT). No clear explanation was given to explain the weak activity exhibited by the cycloSal-AZTMP derivatives in the CEM/ TK$^-$ anti-HIV assay [187].

2.5. Carbohydrate Derivatives (159-161)

A group of AZT phosphotriesters (Fig. **22**) structurally related to the dolichylphosphate structure have been investigated as a transport system to deliver AZT-MP [157, 158, 188]. A variety of phosphate derivatives of AZT coupled to a carbohydrate (D-glucose, D-mannose, and ethyl D-mannopyranoside) and a hexadecyl chain were prepared to investigate this approach. This concept may have additional advantages relative to other esters (aliphatic and amino acid esters), *viz* 1) hydrolysis of the prodrug yields the 5'-monophosphate which is the first metabolite of AZT anabolism. This could be very important for macrophages which have lower levels of kinases, and for reducing the toxicity associated with AZT since thymidine kinase is not needed to form AZT-MP; 2) the bioconversion products, a carbohydrate (glucose or mannose) and the lipidic alcohol (hexadecanol), do not contribute to toxicity; and 3) increased lipophilicity is provided by the hexadecyl group. The solubility of the prodrugs in water allows oral administration due to the presence of the glycosyl moiety.

AZT derivatives containing a polar carbohydrate and a hydrophobic hexadecyl chain retain their anti-HIV-1 activity and display high affinity for lipid membranes [158, 251]. The reference compound AZT displayed a CC_{50} of 22 μM and an EC_{50} of 0.45 μM at day 22 postinfection of CEM-C113 cells with HIV-1. The *in vitro* antiviral results revealed that all phosphotriester derivatives were less active than AZT. Moreover, they were found to be more toxic against CEM-C113 cells. For example, **160** showed EC_{50} and CC_{50} values of 1.3 and 3.4 μM, respectively, in CEM-C113 cells at day 22 postinfection with HIV-1. These two facts may be related to a delayed hydrolysis in cells which prompted studies to measure their degradation under various conditions. On day 22 postinfection, **159** showed a EC_{50} of ≈ 0.4 μM. A NMR study indicated that interaction of these compounds with unilamellar vesicles was determined by the carbohydrate moiety and that a phosphotriester could be detected in the intravesicular water membrane interface, implying the crossing of the membrane bilayer by a flip-flop process [158].

Glycosyl phosphotriesters (**159-160**) were evaluated as lipophilic prodrugs to AZT which could provide increased brain uptake (Fig. **22**) [157, 158]. These studies suggested that glycosyl phosphotriesters provide a significantly improved pharmacokinetic profile for AZT-MP in brain compared to that observed after AZT dosing. Namane *et al.* [157] prepared a glycosyl phosphotriester of AZT (**160**) to deliver a high cerebral concentration of AZT-MP. The prodrug (**160**) was completely stable in plasma *in vitro*, and was slowly released *in vivo* from its depot form or from binding to lipoproteins or interfacial membranes, to give the alkyl ether cleavage product of **160** (0.3-8 nmol/g), AZT-MP (36-147 nmol/g) and AZT (1-3 nmol/g). The total amount of AZT derivatives detected from phosphotriester **160** ranged from 38-156 nmol/g of brain tissue, with an area under the curve (AUC) of 4366 nmol h/g as compared to 4 nmol h/g for AZT. Thus, the lipophilic character of **160** facilitate its efficient transfer across the BBB and subsequent metabolism to AZT and especially AZT-MP. At 48 h post-injection of **160**, the cerebral concentration of these AZT derivatives were still 50-fold higher than the minimum concentration needed for anti-HIV-1 activity. These data indicate that the glycosyl phosphotriester prodrug approach is a very effective system for brain targeting of AZT [157].

Desseaux *et al.* [252] synthesized lipophilic glycosyl phosphotriester derivatives of AZT such as **161** (Fig.

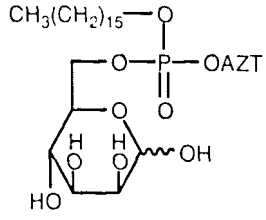
22), which undergoes favorable transport across membranes. Compounds such as **161** could be hydrolyzed *in vivo* releasing the free nucleotide, that is the precursor to the active AZT-TP. Compounds of this type that are tethered to a compound like the *N,N,N'*-trimethyldiamine (**161**) were able to promote the degradation of RNA viruses. Preliminary data indicated that the Cu(II) complex of phosphodiester **161** with *N,N,N'*-trimethylethylenediamine (TMED) was able to promote the hydrolysis of a RNA fragment at neutral pH after a 48 h incubation at 37 °C.

Molema *et al.* [253] reported the synthesis of some neoglycoproteins conjugated with the 5'-monophosphate of AZT. These compounds were prepared in order to investigate whether neoglycoproteins can act as potential carriers of AZT to HIV-infected T4 lymphocytes, depending on the nature (glucose, mannose) and number of coupled sugars. AZT-neoglycoprotein conjugates appeared to be fairly stable during storage, in lyophilized form, at -20 °C. Anti-HIV-1 activity for neoglycoprotein-AZT conjugates, determined *in vitro* using MT-4 cells, was shown to be dependent on the glycosylation of albumin and also on the specific sugar present in the neoglycoprotein. For example, the anti-HIV-1 activity exhibited by the AZT-MP-mannose-albumin conjugate (Man_{22}HAS-AZT-MP) (EC_{50} = 0.6 ± 0.2 nM) was more active than the parent drug AZT (EC_{50} = 5 nM). Glu_{26}HAS (EC_{50} = 1.8 ± 1.5 nM) and HAS (EC_{50} = 5.9 ± 1.8 nM) conjugates exhibited much lower anti-HIV activity. It remains to be established whether the marked potency shown by the Man_{22}HAS-AZT-MP conjugate can be explained by sugar-specific recognition and subsequent endocytosis of the conjugate into MT-4 cells, or alternatively by some synergistic action of the glycoprotein carrier and the AZT-MP molecules [253].

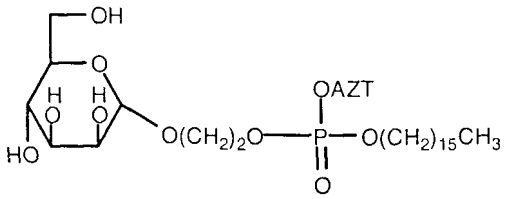
2.6. Steroidal Phosphotriester Derivatives (162, 163)

Steroidal phosphotriester conjugates of AZT have been reported by Balagopala [89, 189]. The anti-HIV activity for the phosphotriester conjugates **162-163** (EC_{50} = 1.65-15.2 μM) is lower than that for AZT (EC_{50} = 0.011 μM. Differences in configuration at the chiral P centre of the phosphotriesters diastereomers **162** showed a ten-fold difference in the activity, whereas a similar difference was not observed for **163** (Fig. 22).

While the nucleotide phosphotriester prodrug strategy remains promising there are a number of associated problems that have hindered progress. The initial prodrug is often a neutral phosphate triester, which is hydrolytically labile. The subsequent diester, however, is up to a million times less reactive, and most strategies are dependent on this cleavage step being achieved by cellular phosphatases. When an anionic nucleoside 5'-phosphate diester diffuses through a lipophilic membrane, it must presumably be accompanied by a metal cation, which is an energetically demanding process. Prodrug strategies have attempted to overcome this undesired requirement by making very lipophilic prodrugs. However, extreme lipophilicity is not always desirable since it may result in poor bioavailability [153].



159



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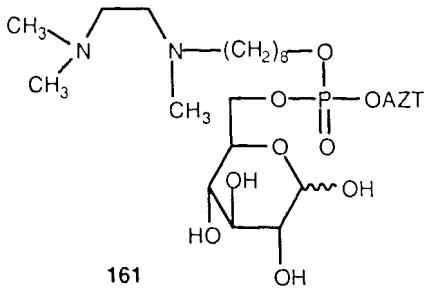

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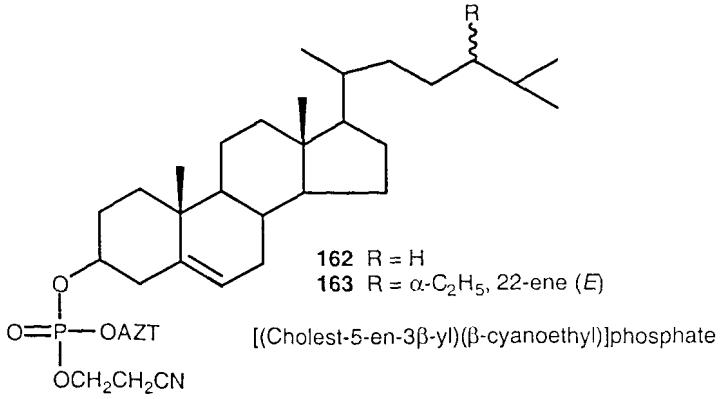


162 R = H
163 R = α-C_2H_5, 22-ene (*E*)

[(Cholest-5-en-3β-yl)(β-cyanoethyl)]phosphate

Fig. (22). Carbohydrate and steroidal phosphotriester derivatives of AZT (159-163).

3. Phosphoramidate Derivatives (164-193)

Many phosphoramidate derivatives of AZT have been synthesized [243, 254-259]. The initial rational for the synthesis of phosphoramidates was that HIV aspartate proteinase [260] may effect a specific hydrolysis of these membrane-soluble prodrugs. The resulting phosphate diesters would be trapped inside the (infected) cell, where subsequent phosphodiesterase action may yield either AZT and/or AZT-MP [190].

Curley *et al.* [257] prepared a series of phosphoramidate derivatives of AZT (**164-169**) (Fig. **23**) which exhibited EC_{50} values in the 10-100 μM range against HIV-1 infected H9 cells, relative to AZT (EC_{50} of 0.005 μM). Compounds having an *N*-alkyl substituent have potent anti-HIV activity which decreases with increasing length of the alkyl spacer. Thus, **164**, having a methylene spacer, was the most active of the series, showing a 50% inhibition of viral replication at a concentration of 10 μM. Accordingly, compounds **166-168** were active only at high concentrations (EC_{50} = 100 μM). It is clear that the anti-HIV activity exhibited by these phosphoramidate derivatives (**164-168**) declines with increasing spatial separation between the phosphoramidate and the terminal ester moieties. This is consistent with a mechanism of action involving intracellular cleavage of the P-N bond, where increased separation of the terminal carboxy ester might be expected to stabilize the P-N bond and thus reduce activity. The presence of a distant ester group must not make a contribution to the activity exhibited by these phosphoramidates since the unsubstituted propylamino analog (**169**) which lacks the terminal ester moiety, displayed anti-HIV activity (EC_{50} = 50 μM) intermediate in magnitude between that of **165** (EC_{50} = 20 μM), and **166-168** (EC_{50} = 100 μM).

The synthesis and anti-HIV evaluation of some aryl phosphoramidate derivatives of AZT have also been reported [191, 192]. Some phosphoramidate derivatives of AZT (Fig. **23**) have been shown to exhibit anti-HIV activity in TK^- cell lines [192-196]. These data clearly show that aryl phosphoramidate derivatives of AZT are efficient AZT-MP prodrugs that partially circumvent the first activation step (phosphorylation of AZT to AZT-MP) by thymidine kinase. These data support the hypothesis that these phosphoramidate derivatives may exert their biological effects via intracellular release of AZT-MP [213]. Phosphoramidates are efficient phosphate delivery systems, particularly when the amine used is an amino acid. Thus, amino acid-derived phosphoramidates are potent anti-HIV agents when coupled to AZT [191, 192]. Compound **170** (Fig. **23**) showed potent activity against HIV-infected JM cells (EC_{50} = 0.32 μM). Moreover, **170** is relatively non-toxic to uninfected JM cells (CC_{50} = 500 μM) and it has a selectivity index of 1500, substantially greater than that for AZT (EC_{50} and CC_{50} in JM cells are 100 and > 1000 μM, respectively) [191].

The lipophilic masked aryloxyaminoacylphosphoramidate derivative of AZT (**170**) was evaluated against visna virus (VV) in sheep choroid plexus (SCP) cells, where it exhibited a similar antiviral activity (EC_{50} = 0.9 μM) to AZT (EC_{50} = 1.5 μM). The prodrug **170** was also markedly more active against HIV-1 (EC_{50} = 0.06 μM) than against VV (EC_{50} = 0.9 μM) infected cells. The aryloxyalaninylphosphoramidate prodrug was stable in lamb serum for prolonged incubation times. The marked differences in antiviral efficacy of **170** against HIV and VV may result from a different metabolic capacity of cells to convert it to active metabolites. Prodrug **170** did not release any detectable amount (HPLC analysis) of AZT, AZT-MP, or any other metabolite on incubation with lamb serum for 15, 60, or 120 minutes at 37 °C [261]. The mechanisms responsible for the antiviral activity exhibited by these compounds remains unknown, although Valette *et al.* [161] has proposed that carboxyesterase and phosphodiesterase activation are involved in the activation process.

An amino acid (or close analogue) is necessary for activity, since simple amine substitution leads to a complete loss of antiviral activity. Simple phenyl alkylamino phosphate derivatives of AZT (**171-173**) (Fig. **23**) inhibit HIV replication at cytotoxic concentrations and have no detectable selectivity (EC_{50} = 7 to > 42 μM) in an HIV-1 infected CEM cell line, with CC_{50}s in the 7- 210 μM range [194].

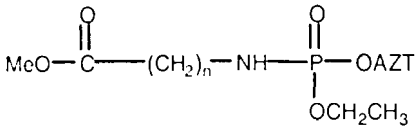
Indeed, relatively small changes in the amino acid, such as inversion of the natural (L) configuration of an alanine substituent to the (D) configuration, appear to result in a significant reduction in potency [195]. McGuigan *et al.* [192] examined several different (L) amino acids attached to AZT, in which the alanine analog was found to be the most potent inhibitor in MT-4 cells, (EC_{50} was only 3-4-fold higher than that of AZT). A number of these amino acid phosphoramidates retained marked antiviral activity against TK^- mutant CEM cells in which AZT was virtually inactive. Therefore, these AZT-MP derivatives should be considered as efficient prodrugs to AZT-MP that circumvent the first phosphorylation step in cells.

As previously mentioned, bis(trihaloethyl) phosphate derivatives are potent anti-HIV agents [210]. The combination of a haloethyl and an amino acid moiety on the phosphorous of AZT-MP was also evaluated. Compounds (**174-180**) (Fig. **23**) displayed anti-HIV activity in C8166 cells (EC_{50}s = 0.08-10 μM

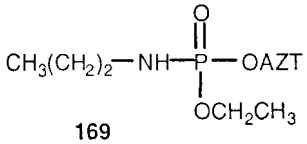
range), but surprisingly, activity did not increase on the introduction of a haloalkyl moiety. This suggests that different mechanisms are operative for intracellular activation of phosphate and phosphoramidate compounds. The trichloroethyl methoxyalaninyl compound (**177**) was 50-fold more active (EC_{50} = 0.08 μM) than the ethyl (**180**) (EC_{50} = 3 μM) or trifluoroethyl (**174**) (EC_{50} = 3 μM) analogs. No reason for this anomaly was given.

McIntee *et al.* [197] reported that the AZT phosphomonoester (**184**) (Fig. **23**) displayed comparable antiviral activity to AZT. Compound **184** was an 8-fold more active inhibitor (EC_{50} = 0.01 μM) of HIV-1 replication in human peripheral blood mononuclear cells (PBMCs) than AZT (EC_{50} = 0.08 μM) and **184** was at least 10-fold less cytotoxic than AZT. AZT phosphoramidates (**181-186**) exhibited no cytotoxicity toward CEM and PBM cells at concentrations up to 100 μM. In lymphocytes, the amino acid carbomethoxy ester phosphomonoester amidates of AZT are not significantly metabolized to either AZT or the mono-, di-, or triphosphate of AZT. The reduced toxicity of the AZT phosphoramidates, relative to that of AZT, is in accord with the lack of detectable monophosphate production. AZT phosphomonoester amidates are internalized to the same extent as AZT by a non-saturable process. The amount of active anabolite, AZT-TP, formed in PBMCs incubated with the AZT phosphomonoester amidates (**183**) and (**184**) was 2- and 3-fold lower than that observed after treatment with AZT, respectively.

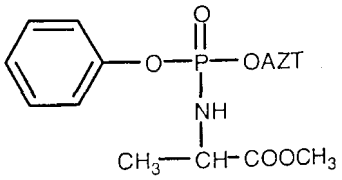
Nilroth *et al.* [262] showed that **187** (Fig. **23**) has anti-HIV activity in MT4 cells (EC_{50} and therapeutic ratio of 0.01 μM and 10, respectively), relative to AZT (EC_{50} and therapeutic ratio of 0.01 μM and 3500, respectively). In cell culture, the inhibition exhibited by **187** was reversed by thymidine, but **187** was not a direct inhibitor of HIV RT in a cell-free assay. No



164 n = 1
165 n = 2
166 n = 3
167 n = 4
168 n = 5



169



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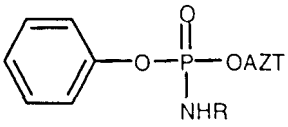
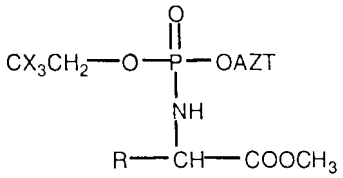
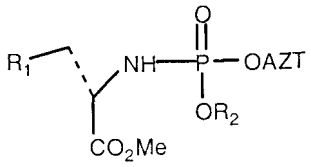


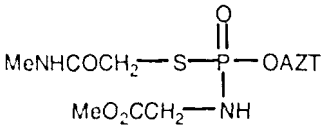
171 R = C_4H_9
172 R = C_6H_{13}
173 R = $C_{12}H_{25}$



174 R = Me, X = F
175 R = *i*Pr, X = F
176 R = H, X = F
177 R = Me, X = Cl
178 R = *i*Pr, X = Cl
179 R = H, X = Cl
180 R = Me, X = H



	R_1	R_2
181	Phenyl	cyanoethyl
182	3-indolyl	cyanoethyl
183	phenyl	H
184	3-indolyl	H
185	phenyl	methyl
186	3-indolyl	methyl



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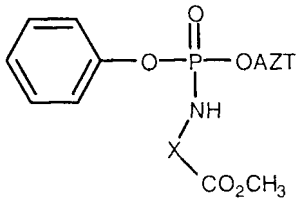
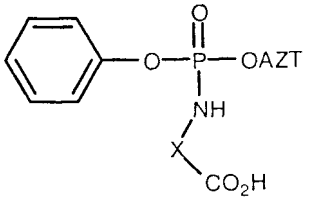


188 X = $CH(CH_3)$ (*L*-alanninyl)
189 X = CH_2 (glycinyl)
190 X = CH_2CH_2 (β-alaninyl)



191 X = $CH(CH_3)$ (*L*-alanninyl)
192 X = CH_2 (glycinyl)
193 X = CH_2CH_2 (β-alaninyl)

Expected metabolites

Fig. (23). Phosphoramidate derivatives of AZT (**164-193**).

definitive mechanism was proposed regarding the activation of the prodrug by hydrolysis releasing free nucleoside or nucleotide.

More recently, Egron *et al.* [263] reported biotransformation pathways in CEM cell extracts, for closely related phenyl phosphoramidate diester derivatives of AZT that included the alanine derivative **188** (Fig. **23**) as a reference compound, and the glycine **189** and the β-alanine **190** derivatives. Compounds **188**, **189** and **190** were incubated with a CEM cell extract and their biotrnasformation kinetics, including metabolite identification, were performed using a HPLC/MS technique. The half-lives ($t_{1/2}$) were 2.5 h, 21 h and > 8 days for **188**, **189** and **190**, respectively. The initial esterase-mediated activation step was not accompanied by nucleophilic attack at P or the C atom of the carbonyl function. Formation of **191** was proposed to occur via esterase cleavage followed by internal nucleophilic substitution with the elimination of phenol (transitory formation of a five membered ring intermediate) (Fig. **24**). These data may explain why **189** was less active than the reference compound **188** and why **190** did not exhibit an anti-HIV effect against a CEM TK$^-$ cell line. AZT, **188**, **189** and **190** exhibited EC_{50} values of > 10^{-4}, 4.1 × 10^{-6}, 6.3 × 10^{-5} and >10^{-4}, Molar, respectively in a TK$^-$ CEM cell line infected with HIV-1. It was concluded that the glycine analogue **189**, based on its half-life, was less active than **188** and that the lack of antiviral activity exhibited by **190** was due to the slow rate of the first, or second, bioconversion step.

4. Dinucleoside Phosphate Derivatives (194-205)

The anti-HIV and cytotoxic properties for of a group of homo- and heterodimers, including AZT-P-ddI, AZT-P-ddZ, AZT-P(CyE)-ddA, and AZT-P-AZT, were reported for HIV-infected MT-2 cells [212]. Thus, various homo- and heterodimers of AZT, including AZT-P-ddA, AZT-P-ddI, and AZT-P-AZT, showed enhanced anti-HIV potency relative to monomers. The relative potency was AZT-P-ddA ≥ AZT-P-ddI > AZT > AZT-P-AZT > ddA > ddI [6 and references cited there, 198]. For example, AZT-P-ddI (**194**) (Fig. **25**) was more potent than either AZT, ddI, or AZT and ddI combined, in PBM cells infected with HIV-1. It is not clear if these compounds enter cells intact since they are rapidly cleaved by serum phosphodiesterases. AZT-P-ddI (**194**) is a substrate for phosphodiesterases, but not for alkaline phosphatase or 5'-nucleotidase [6]. In addition, AZT-P-ddI (**194**) was 10 times less toxic than AZT to human granulocyte-macrophage progenitor cells [198].

Zhou *et al.* [264] studied the pharmacokinetics of AZT-P-ddI (**194**), which exhibited enhanced *in vitro* antiviral activity and selectivity compared to either AZT or ddI, in patients with HIV who had CD4$^+$ cell counts higher than 200 cells/mm^3. No monophosphate derivatives were detected in plasma, presumably due to the high levels of phosphatases present in blood. Plasma levels of unchanged AZT-P-ddI (**194**) after intravenous infusion declined rapidly and were not detectable 0.75 h after the end of infusion. The parent compound **194**, which was not detected after oral administration, is indicative of very rapid metabolism (intense gastrointestinal or hepatic first pass effects). The parent compound **194** undergoes *in vivo* enzymatic cleavage to give the two drugs AZT and ddI, which subsequently undergo independent pharmacokinetic and metabolic processes. The β-glucuronide derivative of AZT (GAZT) was the major metabolite of AZT, but there was no detectable level of the toxic metabolite 3'-amino-3'-deoxythymidine (AMT) in plasma or urine after either intravenous or oral administration of the dimer **194**. A major and previously unrecognized *in vivo* metabolite of ddI, referred to as ddI-M [*R*(-)-dihydro-5-(hydroxymethyl)-2-(3*H*)-furanone], was detected in plasma and urine. The formation of AZT and ddI metabolites increased after oral administration of AZT-P-ddI (**194**) compared with intravenous infusion. AZT-P-ddI is primarily responsible

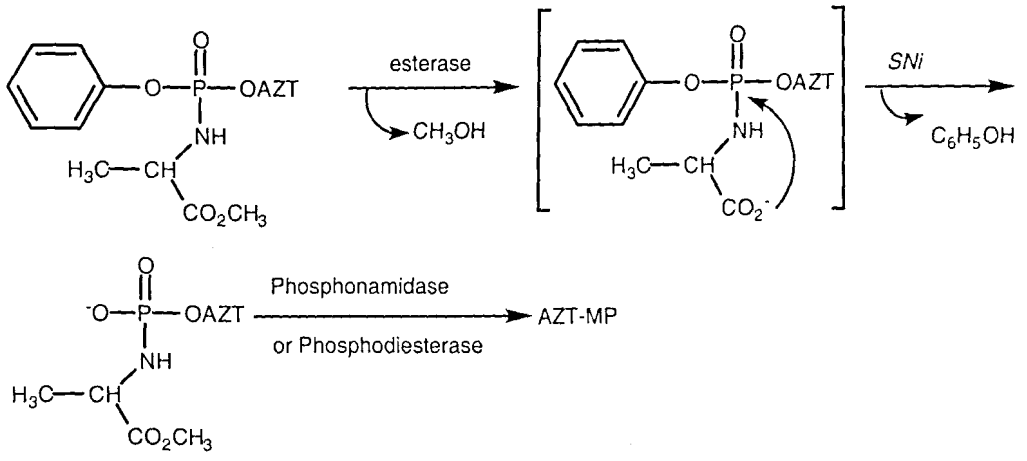


Fig. (24). Proposed mechanism for the decomposition pathway for AZT phenyl phosphoramidate diester [263].

for most the observed pharmacokinetic properties, including bioavailability, plasma kinetics, metabolism, and urine excretion of its component nucleosides AZT and ddI. The dimer **194** appeared to have been cleaved completely before reaching the target cells.

Dinucleoside methylphosphonates of AZT and ddC have also been evaluated *in vitro* as anti-HIV agents where anti-HIV activity was 200-to-450 fold lower than that for the monomeric nucleoside compounds. This decrease in anti-HIV activity may be due to nucleoside resistance for dimeric compounds with a methyl phosphonate linkage [6, 265].

More recently, new lipophilic alkyl/acyl dinucleoside phosphate derivatives of AZT and other nucleosides (**195-197**) have been synthesized (Fig. **25**) [199, 203] which are generally less active than AZT. To overcome the low ability of resting macrophages to phosphorylate antiviral deoxynucleosides, the homodinucleotide di(thymidine-3'-azido-2',3'-dideoxy-D-riboside)-5'-5'-p^1-p^2-pyrophosphate (AZTP2AZT, **195**) was synthesized by Magnani *et al.* [203]. Although **195** is a weak inhibitor of HIV RT, it showed antiviral (EC_{50} = 0.031 μM) and cytotoxic (CC_{50} > 100 μM) activities comparable to those of the parent AZT (EC_{50} = 0.028 μM, CC_{50} = 70 μM) when added to cultures of a HTLV-1 transformed cell line. AZTP2AZT (**195**) encapsulated into erythrocytes was remarkably stable and upon phagocytosis allowed the targeted delivery of this impermeant drug to macrophages, where metabolic activation occurs [203]. The antiretroviral activity of **195** is not directly elicited by the homodinucleotide analog, but most likely by the AZT-TP metabolite.

The anti-HIV activity exhibited by the two lipophilic derivatives of AZT, N^4-palmitoyl-2'-deoxyribocytidylyl-(3'-5')-3'-azido-2',3'-deoxythymidine(N^4-palmitoyldC-AZT, **196**) and N^4-hexadecyl-2'-deoxyribocytydyl-(3'-5')-3'-azido-2',3'-deoxythymidine(N^4-hexadecyl-AZT, **197**) (Fig. **25**) have been evaluated in comparison to AZT. A higher concentration of **196** and **197** was required to obtain a comparable antiviral effect relative to that obtained with AZT. The higher concentration required may be due to their slow uptake into micelles. Formation of other cellular metabolites of unknown structure and activity is conceivable and may further contribute to their delayed antiviral action [199].

Cyclopentyl purine derivatives such as **198** exhibit antiviral activity. A AZT-phosphate ester of these analogs with has been prepared to improve the efficacy of AZT [204] (Fig. **25**).

A new heterodinucleotide (AZTP2ACV, **199**) (Fig. **25**) consisting of both an antiretroviral and antiherpetic drug, connected by a pyrophosphate linker, was reported by Rossi *et al.* [266], which could act as a prodrug for the production of partially phosphorylated antiviral agents. This heterodimer (**199**) is not suitable for direct administered as the parent compound since it

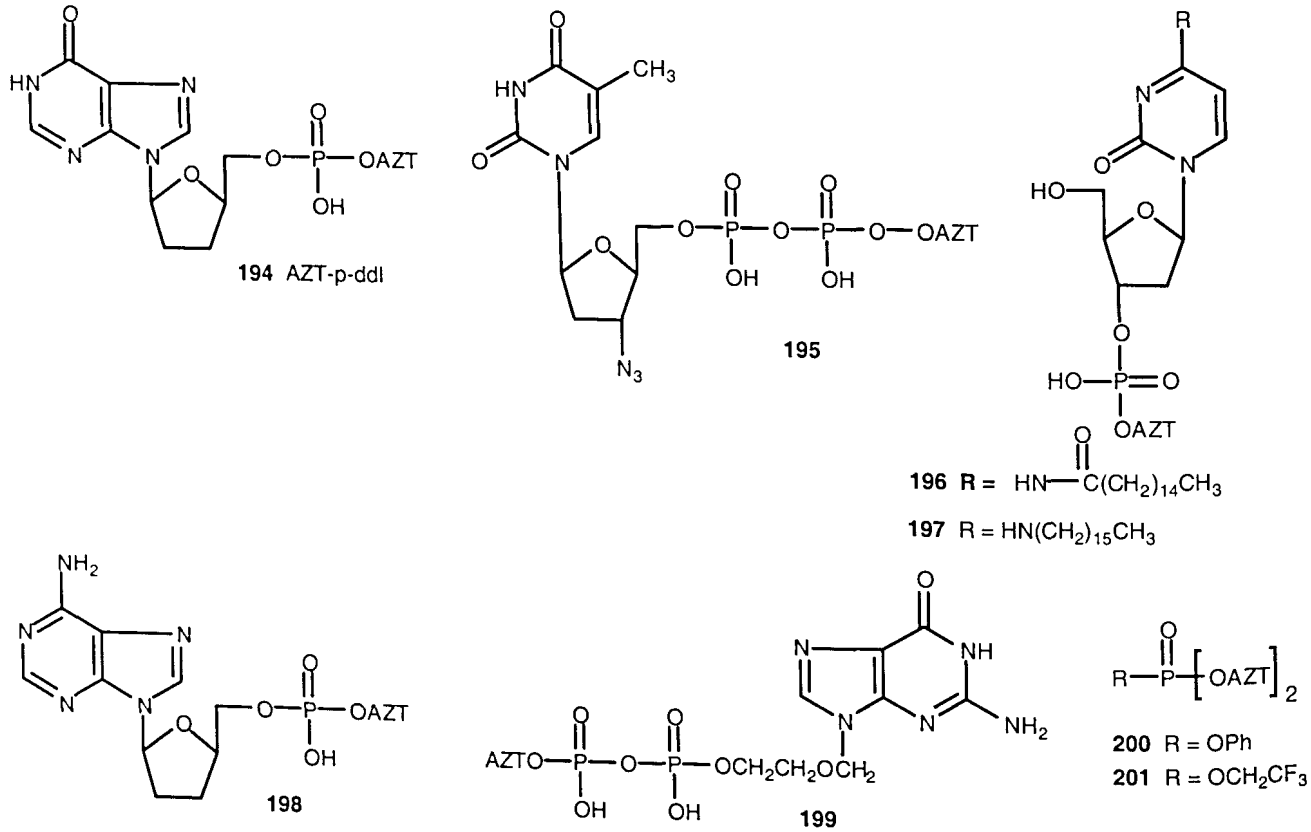


Fig.(25). Dinucleoside phosphate derivatives of AZT (**194-201**).

is not able to permeate cellular membranes and it is degraded by plasma. These limitations were overcome by administering the impermeant AZTP2ACV (**199**) encapsulated in autologous erythrocytes (RBCs) modified to increase their recognition and phagocytosis by human macrophages. After entry into macrophages, metabolic activation of **199** occurred. Human erythrocytes possess a dinucleotide pyrophosphatase able to cleave the pyrophosphate bridge of AZTP2ACV (**199**) with subsequent production of AZT-MP and ACV-MP. The exposure of AZTP2ACV-loaded erythrocytes to human macrophages provided effective and almost complete *in vitro* protection from HIV-1 and HSV-1 replication, respectively.

Novel symmetrical nucleotide-(5',5')-dimers of AZT are poor inhibitors of HIV proliferation. The AZT dimer phosphate derivatives **200** and **201** (Fig. **25**) have marked anti-HIV-1 activity in C8166 cells. Replacing phenyl (**200**), by 2,2,2-trifluoroethyl (**201**), did not result in significant changes in activity (0.8 μM and 0.4 μM, respectively) or cytotoxicity (500 μM and 600 μM, respectively). These compounds (**200-201**) are less potent than AZT. The exact mechanism of action by which phosphate dimer derivatives exhibit their anti-HIV effect is unclear, but there is an indication that AZT phosphate dimers act primarily via the intracellular release of the parent nucleoside, since the AZT-derived compounds (**200** and **201**) were found to be significantly less active in JM cells (EC_{50} > 100 μM) [267]. It was previously noted that AZT is very much less active in JM cells [191].

The hydrolytic behavior of phosphotriesters **202** and **203** (Fig. **26**) in a variety of media including hydroxide, pig liver esterase, human serum and brain cytosol has demonstrated that the essential requirement for potential salicylate-based nucleotide prodrugs is generation of a carboxylate group at a nearby position to facilitate phosphotriester hydrolysis to a dinucleotide via an unstable acyl phosphate intermediate [205]. Nucleotide AZT prodrugs based on salicyl phosphate, methyl carboxylic ester **202** and phenyl carboxylic ester **203**, undergo much more rapid hydrolysis than the triester **204**, most probably due to the formation of an acyl phosphate complex involving attack at phosphorous by the salicylate carboxylate anion (Fig. **26**). In contrast triester **204** has no C-2-carboxylate anion to participate in this type of neighboring group reaction. Incubation of triesters **202** and **203** with pig liver esterase showed $t_{1/2}$s of 20 and 1 h, respectively. Aside from the differences in kinetics, the hydrolytic reaction mediated by pig liver esterase was quite similar to that observed using hydroxide anion mediated hydrolysis since triesters **202** and **203** both gave rise to a labile intermediate (same as with hydroxide) as the final product. Specifically, compounds **202** and **203** gave bis(3'-azido-3'-deoxythymidine-5'-yl)phosphate.

Hydrolysis of derivatives **202** and **203** by human serum also showed a dependence on the nature of the alcohol moiety of the carboxylic ester compared to incubation with pig liver esterase and brain extract, but compounds **202**, **203** still displayed an accelerated hydrolysis rate due to the presence of the esterase-generated neighboring carboxyl group. Triester **203** underwent hydrolysis in rat brain extract with a $t_{1/2}$ of 5 h to yield the 5',5'-dinucleotide bis(3'-azido-3'-deoxythymidien-5'-yl)phosphate via the same labile intermediate observed for hydroxide ion and pig liver esterase hydrolysis. Triester **202** was virtually unchanged after a similar 24 h incubation [205].

Meier *et al.* [268] reported a new prodrug system for AZT that is based on α-hydroxybenzylphosphonates

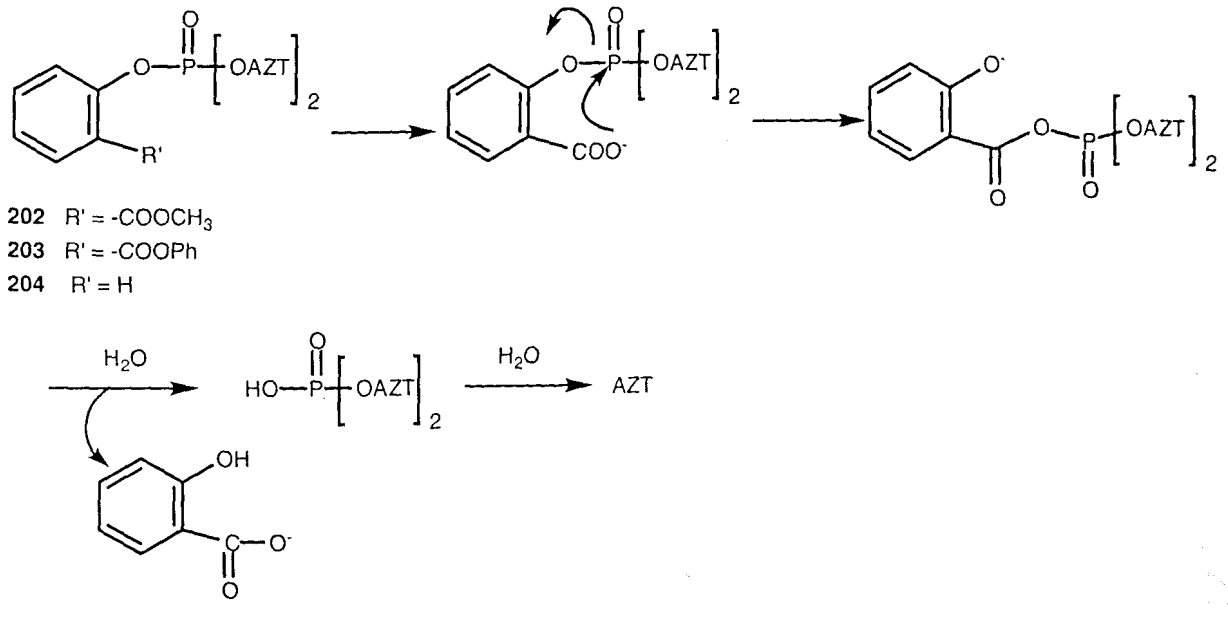


Fig. (26). Mechanism of activation for salicylate based nucleotide prodrugs of AZT (**202-204**) [205].

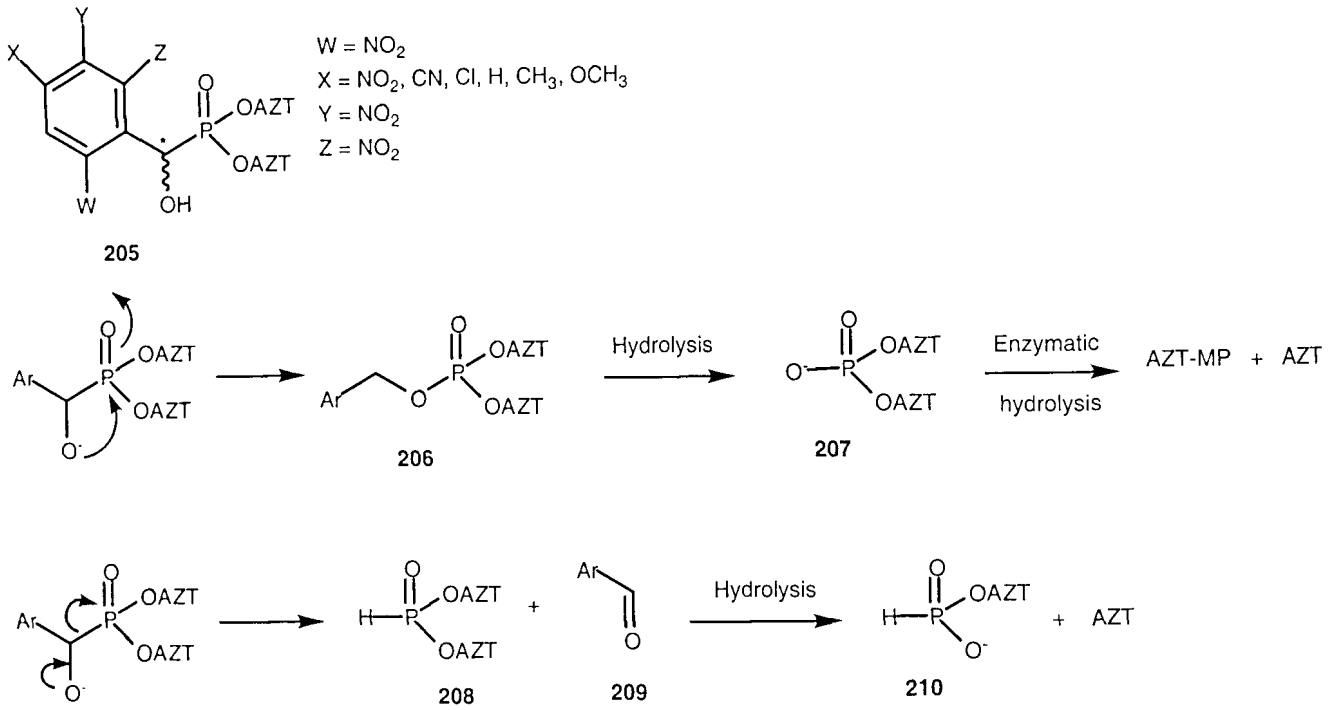


Fig. (27). Two different hydrolysis mechanisms of α-hydroxybenzylphosphonate of AZT [268].

(Fig. **27**). Compounds **205** are hydrolyzed via two different mechanisms (Fig. 27) which give rise to AZT-MP (path a) or a H-phosphonate monoester **210** (path b). The phosphonate-phosphate rearrangement (path a) leads to benzylphosphotriesters **206** which are selectively cleaved to yield a dinucleoside phosphodiester **207**. On the other hand, the direct cleavage reaction (path b) gives a dinucleoside H-phosphonate diester **208** and benzaldehyde **209**. Compound **208** undergoes rapid conversion to afford the nucleoside H-phosphonate monoester **210** and AZT. All compounds of general structure **205** showed higher partition coefficient values (P = 3-28) than AZT. Derivatives of **205** bearing strong electron-withdrawing substituents at the 2- and/or 4- position of the aryl ring undergo conversion to **206** primarily via the rearrangement reaction (path a), whereas derivatives of **205** with electron-donating are converted to **206** via the direct cleavage pathway (path b). Both reactions are spontaneous and are not enzymatically catalyzed.

5. Hydrogen and Methylene Phosphonate Derivatives of AZT (211-214)

The intracellular metabolism of nucleoside-5'-H-phosphonates may be different from that of nucleoside analogs since the catabolic action of pyrophosphate transferase should give rise to pyrophosphorylhydrogenphosphonates, which are analogs of nucleoside triphosphates. On the other hand, 5'-nucleoside H-phosphonates may be oxidized intracellularly to nucleoside monophosphates [268].

Other studies exploring the potential of 5'-hydrogen phosphonate derivatives of anti-HIV nucleosides have been reported, where it was concluded that their antiviral activity is due to release of the parent nucleoside [206, 207, 269]. It was shown that a number of H-phosphonate derivatives of dideoxynucleosides exhibit high antiviral activities with selectivity similar, or higher than, that of the corresponding nucleoside [206, 213, 270]. The 5'-hydrogen phosphonate derivative of AZT (**211**) (Fig. **28**) exhibits potent anti-HIV-1 activity and it has a selectivity index similar to that of AZT. The intracellular metabolism of 5'-AZT H-phosphonates may be different from that of AZT, but this possibility remains to be confirmed. 5'-*O*-Hydrogen phosphonates, unlike highly acidic nucleoside 5'-phosphates, which do not enter the cell, may penetrate the cell membrane due to their weakly acidic undissociated nature. Subsequent conversion to a triphosphate, pyrophosphorylhydrogenphosphonate derivative, which would then inhibit viral DNA synthesis catalyzed by RT, or oxidation to the phosphates and then further conversion to the triphosphate in the cell could occur. However, unlike AZT-MP, these phosphonates do not inhibit dTTP formation prior to their oxidation [206, 207].

Gosselin *et al.* [207] showed that the H-phosphonate of AZT (**211**) was rapidly metabolized to AZT in cell culture medium (24 h < $t_{1/2}$ < 33 h) and even faster in CEM cell extract (41 min < $t_{1/2}$ < 69 min) by an undefined hydrolysis mechanism [213]. Intracellular transformation of **211** was determined by Boal *et al.* [208] using U937 cell lines (8 and 16 h incubations),

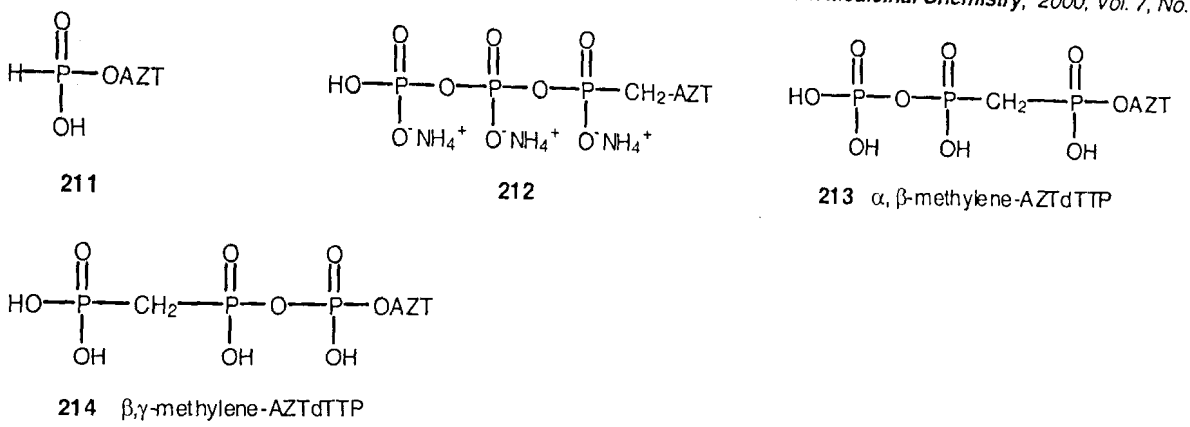


Fig. (28). Hydrogen and methylene phosphonate derivatives of AZT (**211-214**).

where it was shown that the H-phosphonate of AZT undergoes facile conversion to AZT-MP. AZT was not detected in the extract of the AZT-5'-H-phosphonate-treated cells. This observation rules out the possibility of prior conversion of AZT-5'-H-phosphonate to AZT followed by conversion of the latter to AZT-5'-MP [208]. Additional studies are required to elucidate the metabolic pathway(s) involved in this conversion [208, 213]. However, the observation that the AZT H-phosphonate showed no anti-HIV activity in a TK^- cell line [207] suggests that thymidine kinase is necessary for AZT H-phosphonate to exert its antiviral effect [213].

3'-Azido-3',5'-dideoxythymdine-5'-methylphosphonic acid diphosphate (**212**) (Fig. **28**) [271] is an inactive anti-HIV-1 agent in MT4 infected cells. Substitution of a methylene group for the 5'-oxygen atom of AZT-TP reduced the rate constant for RT-catalyzed phophodiester bond formation by a factor of at least 1800, which would explain the inactivity of **212** as an anti-HIV-1 agent. Balzarini and co-workers prepared the 5'-α,β-methylene (**213**) and 5'-β,γ-methylene (**214**) triphosphate analogs of AZT (Fig. **28**) that were reported to be > 400-fold, and 10-40 fold, respectively, less protective than AZT against the cytopathic effect of HIV-1 using ATH8 cells [272].

Future Directions

Extensive efforts to synthesize lipophilic prodrugs to AZT have not yet provided an anti-HIV prodrug agent with a clear-cut therapeutic advantage for clinical use. Further research to identify prodrugs with distinct advantages, relative to AZT and other nucleoside RT inhibitors, is warranted. The various prodrug design concepts described in this review provide an introduction to this area of drug research. Promising data from these prodrugs suggests that more selective prodrugs can be designed by optimization of drug delivery using other 5'-*O*-ester prodrugs. It is concluded that the ester prodrug approach could serve as a useful method to improve the *in vivo* clinical properties of AZT.

In comparison to AZT, several prodrugs exhibited similar anti-HIV potency and superior properties such as increased plasma half-life, lipophilicity and cellular uptake, and decreased toxicity. Data from *in vitro* and *in vivo* evaluation of ester prodrugs have shown improved site delivery and/or pharmacokinetic parameters for the parent nucleoside.

A therapeutic approach based on the prodrug concept could provide a better drug to reduce the body HIV viral particle titre and/or prevent HIV infections from moving to more advanced stage of AIDS. When prodrugs are designed, it is essential that they will be appropriate for clinical use in terms of production, stability, metabolism, toxicology and side effects.

The utility of 5'-*O*-ester prodrugs as therapeutic agents will be enhanced by a clearer understanding of the mechanisms pertaining to their selective uptake (including lymphatic transport), bioconversion, and cellular incorporation. As well, an appreciation for the contribution of the prodrug ester moiety to the ultimate anti-HIV effect, in addition to its AZT effect, must be developed.

Acknowledgment

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