Review

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The Application of Prodrugs as a Tool to Enhance the Properties of Nucleoside Reverse Transcriptase Inhibitors

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Abstract: The effective treatment of human immunodeficiency virus (HIV), also known as acquired immunodeficiency syndrome (AIDS), has transformed a highly lethal disease into a chronic and manageable condition. However, better methods need to be developed for eliminating the virus from reservoirs, enhancing patient access and adherence to therapy, and improving treatment in the long term to reduce adverse effects. From the perspective of drug discovery, one promising strategy is the development of anti-HIV prodrugs. This approach aims to enhance the efficacy and safety of treatment, promoting the development of more appropriate and convenient systems for patients. In this review, we discussed the use of the prodrug approach for HIV antiviral agents and emphasized nucleoside reverse transcriptase inhibitors. We comprehensively described various strategies that are used to enhance factors such as water solubility, bioavailability, pharmacokinetic parameters, permeability across biological membranes, chemical stability, drug delivery to specific sites/organs, and tolerability. These strategies might help researchers conduct better studies in this field. We also reported successful examples from the primary therapeutic classes while discussing the advantages and limitations. In this review, we highlighted the key trends in the application of the prodrug approach for treating HIV/AIDS..

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1. Introduction

Considerable advancements in the reduction of mortality and morbidity related to Human Immunodeficiency Virus (HIV) were only possible after the development of the Highly Active Antiretroviral Therapy (HAART). However, social stigma, drug-related adverse effects, poor access to treatment, the persistence of a chronic inflammatory state and HIV reservoirs, the emergence of resistant strains, the lack of patient immune response, and the discovery of a safe and efficacious therapeutic approach are big problems that need to be addressed [1]. In 2021, the WHO estimated that 38.4 million people are living with HIV; only 75% of them have access to HAART [2].

More than 30 antiretroviral drugs distributed in different therapeutic classes have been approved by the Food and Drug Administration (FDA). These drugs can inhibit the main steps of the HIV viral cycle. The capsid inhibitor (i.e., lenacapavir) represents the most recent drug category to these treatment options [3]. New therapeutic strategies for treating HIV infections emerge in the horizon as perspectives, such as the allogenic transplant of stem cells from CCR5Δ32/Δ32 donors, 'kick and kill', 'block and lock', therapeutic vaccination, and gene editing (i.e., zinc finger nuclease and CRISPR-Cas-9) [1–3].

Combined therapy, in which at least 2–3 different antiretroviral drugs are administered, can be used to eliminate undetectable HIV-RNA in the plasma. This treatment strategy has greatly increased

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the life expectancy and quality of life of patients [3,4]. However, challenges, including the emergence of HIV-resistant strains, co-infections, and long-term adverse effects (e.g., poor tolerability, drug interactions, and toxicity), are factors that increase the chances of poor patient compliance. Safe and convenient regimens for non-occupational post-exposure prophylaxis (nPEP) and mainly for pre-exposure prophylaxis (PrEP) are key factors that influence patient adherence to treatment [3, 4].

The lack of treatment adherence is a complex phenomenon influenced by several factors, including intrinsic factors related to the patients (e.g., psychosocial, socioeconomic), medical conditions, and pharmacological therapy. A greater pill burden, complex posology schemes involving high intake frequency, the use of some class of therapeutic agents (e.g., protease inhibitors), adverse effects, and toxicity decrease adherence to HAART [5]. The lack of adherence to treatment and the interruption of therapy can lead to disease progression and viral rebound due to the transcription of viral genomes silenced in CD4+ T cell and macrophage reservoirs found in various anatomical sites, such as the central nervous system and lymphatic system [1, 6].

Among the strategies to overcome the limitations of antiviral drugs, prodrugs might be administered to modulate pharmacokinetic properties. This method can help maintain effective drug concentrations through a single dose/day regimen, reduce toxicity and adverse effects, and enhance the efficacy of drug delivery to target cells/organs [6–8]. Prodrugs are compounds with little or no pharmacological activity; however, chemical or enzymatic activation releases the active metabolite from prodrugs. Prodrugs comprise about 10% of all marketed drugs [8]. They are commonly used in drug design to improve water-solubility, increase lipophilicity, permeability, and oral absorption, promote parental and topical use, reduce toxicity and undesirable organoleptic properties, and decrease metabolic instability to accurately deliver the drug to the target site and prolong the duration of action [8–10].

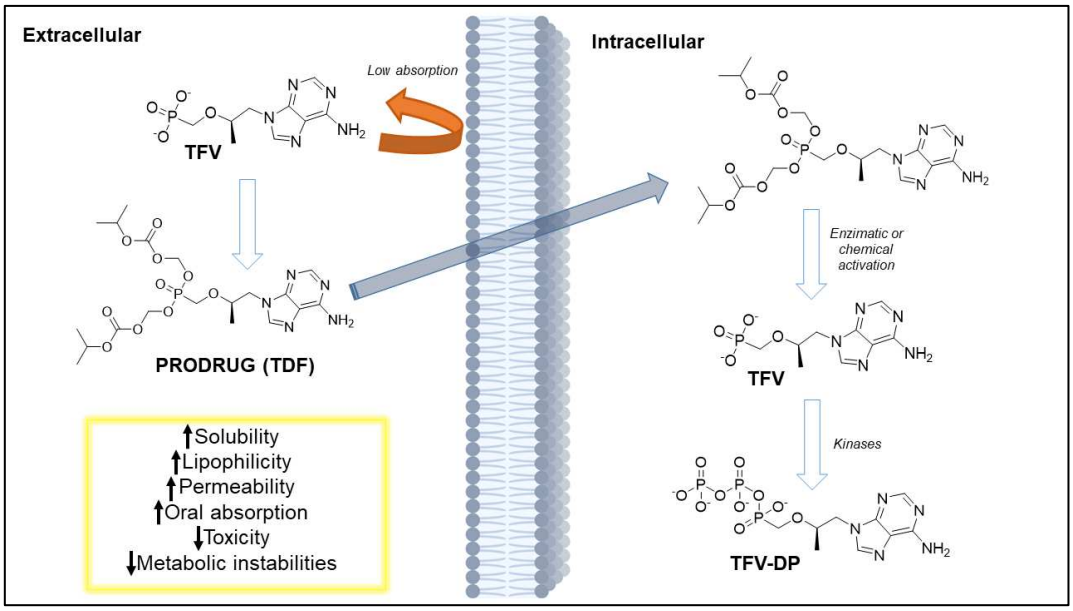

Figure 1. A diagrammatic representation of the prodrug approach to improve **TDF** properties. **TDF** can improve pharmacokinetic properties as it can cross the biological membrane.

This review covers recent advancements in the prodrug strategy linked to the creation of new HIV antiviral agents reported in the literature from 2010 to 2022. We emphasize key studies that have contributed to enhancing drug properties and delve into the most successful examples within various therapeutic classes.

2. Nucleoside Reverse Transcriptase Inhibitors – NRTIs

The synthesis of viral double-stranded DNA from viral RNA is performed by HIV reverse transcriptase. This asymmetric heterodimer enzyme has two subunits (p51 and p66) derived from a Gag-Pol polyprotein. It exhibits two enzymatic activities, including DNA polymerase and RNase H,

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which act synergistically to convert RNA into DNA. The DNA polymerase domain present in p66 is made of four distinct subdomains: fingers (residues 1–85; 118–155), palm (86–117; 156–236), thumb (237–318), and the connection (319–426). The nucleic acid is accommodated in the p66 thumb due to the contribution of αH and αI helices. The polymerase active site in the palm subunit of p66 exhibits three carboxylates (D110, D185, and D186) responsible for binding to the Mg^{2+} ion. These carboxylates (D185 and D186) are described as a highly conserved YXDD motif that is found in all retroviruses; in HIV RT, X represents methionine. Additional conserved sites include K65 and R72, which are involved in the interactions with the phosphates of deoxynucleoside triphosphate (dNTP), Y115, which interacts with the deoxyribose ring, and Q151, which interacts with the 3'-OH of dNTP [11].

Many FDA-approved drugs are inhibitors of HIV-1 reverse transcriptase, which belong to two different classes, including nucleoside RT inhibitors (NRTI) and non-nucleoside RT inhibitors (NNRTI). Examples of FDA-approved drugs include abacavir (ABC), didanosine (ddI), emtricitabine, lamivudine (3TC), stavudine (D4T), and tenofovir. All these drugs were developed through molecular modifications of natural nucleosides, and all of them involve the removal of the ribosyl 3'-hydroxyl group to induce chain termination blockade. Although this class is extremely important for controlling the replication of viruses, some issues in the common pharmacokinetic properties were found in NRTI, which encouraged the use of prodrugs to optimize these effects. Here, we discussed how the prodrug approach can improve the properties of NRTI-based compounds. We also presented examples of applications and perspectives.

2.1. Zidovudine prodrugs

Zidovudine (3'-azido-3'-deoxythymidine; AZT) is a nucleoside RT inhibitor (NRTI), which contains the azido group (N_3-R) at the third carbon of the dideoxyribose subunit. Although AZT was synthesized in 1964 by Horwitz et al., its effect as an anti-HIV drug was shown in 1986 by Yarchoan et al. It was the first HIV-1 drug approved; however, its use was discontinued because of serious concerns about bone marrow toxicity, neutropenia, macrocytic anemia, and granulocytopenia. Subsequently, safer and more effective therapeutic alternatives were introduced. Despite these limitations, this drug can be used as a prototype to investigate the use of the prodrug strategy. In this section, we presented some examples of zidovudine prodrugs.

The synthesis of AZT-glycerolipids prodrugs showed that this method can be used to develop liponucleosides. It can mimic the metabolism of natural triglycerides and can be directly transported to the lymphatic system without undergoing primary metabolism; thus, its bioavailability is high. In studies on chemical stability, the phosphodiester derivative (compound (**9**) (**Figure 2**) exhibited a half-life ($t_{1/2}$) of 20 h at pH 7.3, $t_{1/2}$ of > 20 h at pH 8.5, and $t_{1/2}$ of 8 h at pH 9.5. The maximum antiretroviral activity of compound (**9**) was 94.1% at 10 μg/mL, whereas the maximum antiretroviral activity of AZT was 96.9% at 1 μg/mL. Compound (**9**) also induced a decrease in the level of p24 antigen by 88.1% at 10 μg/mL. In toxicity studies, the compound showed cellular viability of 77.67% at 10 μg/mL [12].

A new dinucleoside phosphonate of AZT and β-L-2',3'-didesoxy-3'-tiacitidine (3TC) were synthesized to investigate the prodrug approach. The homodimer 3-azido-3-deoxythymidin-5-*O*-yl fluoromethylphosphonate (**2d**) (**Figure 2**) was found to be the most effective compound against HIV, with EC_{50} values of 0.015 μM in CEM-SS cells. However, it was five times more toxic than the parental drug (AZT), with a CC_{50} value of 29 μM, which indicated that it had a low selectivity index [SI (CC_{50}/EC_{50})] of 1,933. In this assay, AZT exhibited an EC_{50} value of 0.037 μM, CC_{50} value of 142 μM, and SI of 3,837. Studies on the stability and hydrolysis of the (**2d**) prodrug in human blood serum showed that it underwent fast hydrolysis and had a $t_{1/2}$ of 0.78 h, i.e., it was slightly less stable than the other compounds. A mixture of **AZT** and **AZT** monophosphonate products was found in the hydrolysis assay [13].

In a study, AZT phosphorodiamidate prodrugs were prepared and evaluated; prodrug (**21**) was a phosphodiamidate containing esterified L-alanine and a CH_2tBu radical (**Figure 2**). It exhibited the best antiviral activity as it could efficaciously deliver the monophosphate form of the parental AZT nucleoside into cells. This prodrug (**21**) also had a high cellular uptake due to its high lipophilicity.

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The results of cytotoxicity evaluation showed that prodrug (**21**) had CC_{50} values of 75 μM, which was higher than that of AZT. Its chemical and metabolic stability was evaluated using Trizma buffer (pH = 7.6) in the presence of carboxypeptidase Y, an enzyme that metabolizes the phosphorodiamidate subunit. The study was conducted using the NMR technique. The results showed that the phosphorodiamidate subunit was susceptible to hydrolysis by carboxypeptidase Y. Prodrug (**21**) exhibited anti-HIV activity with an EC_{50} value of 0.0083 μM (HIV-1) and 0.013 μM (HIV-2) [14].

Macromolecular azidothymidine polymeric prodrugs containing monomers with a self-immolative linker were synthesized and evaluated against HIV-1. The presence of disulfide contributed to the intracellular release of AZT. The self-immolative linker spontaneously decomposed after the cleavage of the trigger, releasing the parental drug (**Figure 2**). After synthesizing the monomer, this polymeric prodrug was prepared through the controlled radical polymerization technique (RAFT). The macromolecular prodrugs were obtained through the treatment of monomers with *N*-(2-hydroxypropyl)methacrylamide (HPMA). The synthesized prodrug exhibited an average molar mass ranging from 10 to 20 kDa. For them, it was observed constituting up to 20 mol% of the parental drug (AZT)."The prodrug also released AZT when it was exposed to 5 mM glutathione (GSH); the rate of release of AZT was high ($t_{1/2}$ < 30 min). HIV infectivity was determined after mammalian cells were incubated with the polymers for 24 h, and the level of the virus was quantified after 48 h. The macromolecular AZT polymeric prodrugs could decrease up to 80% of HIV-1 infectivity. To evaluate the effect of the self-immolative linker, the researchers also prepared and evaluated ester analogs. The thiol-responsive prodrugs were >10-fold more efficacious than the ester-based counterparts [15].

Bolaamphiphilic are amphiphilic compounds that contain a hydrophobic spacer linked to two hydrophilic compounds. Such structures tend to form monolayers and are commonly used as carriers of drugs and genes. Asymmetric-bolaamphiphilic, which combines the molecular structure of the drugs zidovudine and didanosine as polar subunits and a phosphoryl-deoxycholyl (ZPDD) as a non-polar subunit, was synthesized and evaluated against HIV-1 (**Figure 2**). The prodrug ZPDD formed small spherical vesicles with an average size of 174 nm and high zeta potential (-31.3 mV), which caused repulsion between particles. The degradation of ZPDD was highly pH-dependent. It degraded at pH 2.0 (representing the stomach environment) in 0.5 h. However, it was stable in a neutral medium with a degradation half-life ($t_{1/2}$) of 61.3 h at pH 5.5 (intestine) and $t_{1/2}$ of >1,000 h at pH 7.4 (blood). Studies on the stability of ZPDD in the plasma of mice, dogs, monkeys, and humans showed that it was highly stable under most conditions; however, in mouse plasma, its $T_{1/2}$ was only 9 h. The anti-HIV effect of ZPDD is similar to that of AZT; the EC_{50} of ZPDD is 5 nM in infected MT4 cells. The half toxic concentration (TC_{50}) of ZPDD was found to be 42.22 μM (SI: 8444). Pharmacokinetic studies showed that the bioavailability of ZPDD was 30.8% and 90.5% after oral and intraperitoneal administration. The low oral bioavailability could be explained by certain chemical and enzymatic instability in this acidic environment. High levels of ZPDD were found in organs such as the lungs, liver, thymus, and lymph nodes. The degradation of ZPDD releases the parental drug AZT quickly and the drug didanosine (ddI) slowly. In mice, ZPDD administered by the oral route showed a $t_{1/2}$ of 15.7 min, C_{max} of 10 μg/mL, and area under the curve (AUC)0–120/μg.mL^{-1}/min of 575; when ZPDD was administered intraperitoneally, those values were 28.1 min, 41.4 μg/mL, and 1,690 μg.mL^{-1}/min, respectively. This prodrug can simultaneously deliver two different drugs to the target cells; thus, it can enhance the efficacy and concentration of those drugs in the target organs [16].

Nikavir (**5′-*H*-phosphonate**) is a prodrug of AZT that was approved in Russia in 1999 (**Figure 2**). The insertion of a phosphate group into the 5′ position of AZT reduced the cytotoxicity in comparison to AZT. This, in turn, decreased blood count shifts and improved its tolerance in patients during long-term treatments. Nikavir is four times less toxic than AZT, although it is one order of magnitude less efficacious. In vivo, nikavir is converted to AZT-5′-triphosphate, which inhibits viral replication. The decrease in cytotoxic effects is attributed to the pharmacokinetic parameters; the drug is slowly converted to AZT, which prevents high levels of the drug from accumulating in plasma [17, 18] Analogs of nikavir were prepared and evaluated to comprehend the SAR. These analogs showed

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low activity and low toxicity. The derivative **1c** (containing the Cl_3C group) **(Figure 2)** had the best profile with ID_{50} of 0.45 μM, CC_{50} of 1.34 μM, and a SI of 3,000 [19].

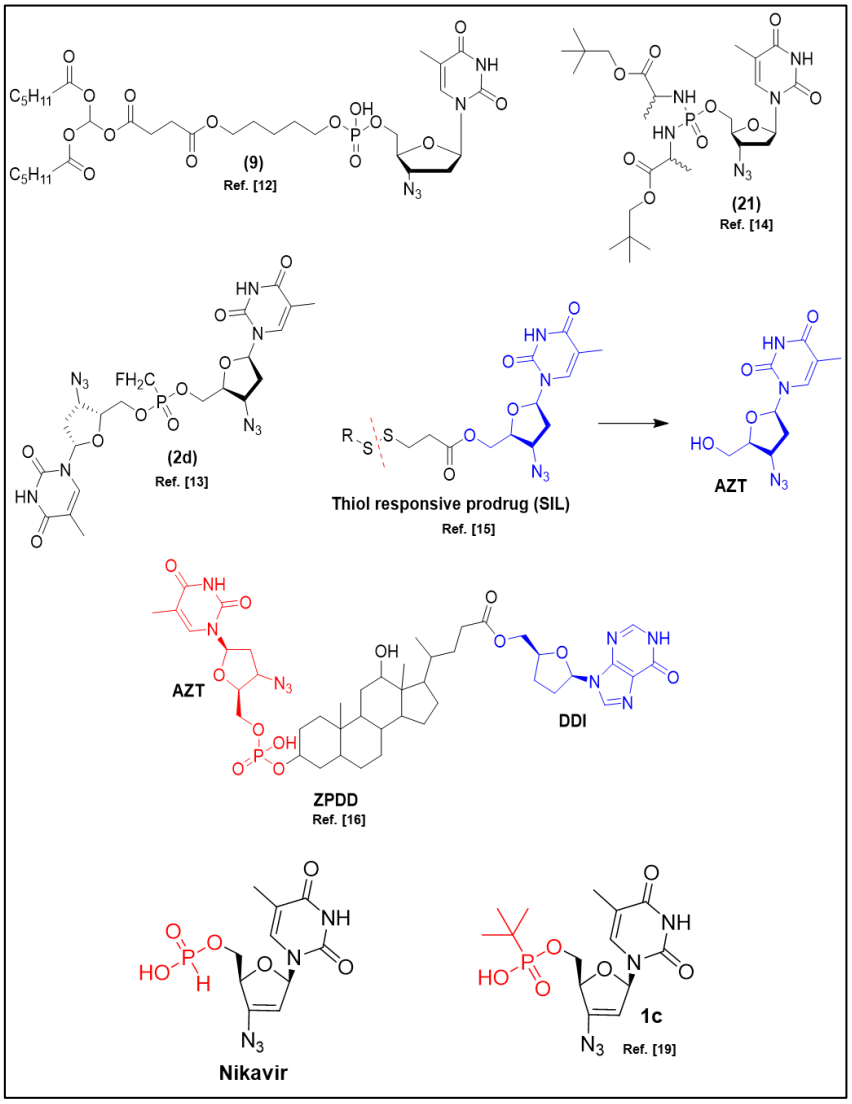

Figure 2. Zidovudine prodrugs.

NeuroAIDS, also known as HIV-associated neurocognitive disorders (HAND), has a complex and wide range of clinical symptoms characterized by cognitive and neurological issues commonly found in HIV-1-infected patients in the advanced stage. Administering neuroAIDS causes symptoms that include anxiety, depression, loss of attention, difficulty in memorizing, motor coordination, dementia, etc., which decrease the quality of life of patients [20, 21]. Cognitive impairment develops in up to 50% of HIV-positive patients, even after initiating HAART [22]. Chronic inflammation induced by HIV-1 can lead to blood-barrier damage, thus facilitating the entry of harmful substances into the central nervous system (CNS). The exact mechanism underlying neuroAIDS is not clear, but it is known that highly active antiretroviral therapy (HAART) can decrease the rate of progression of the infection and control inflammation.

Many studies have suggested the potential harmful effects of antiretroviral therapy on the brain [23]. This reinforces that a thorough assessment of the penetration of drugs into the CNS and the effects of these drugs is necessary. Some studies have proposed strategies to improve the delivery of antiretroviral drugs to the CNS [24].

Studies on rats showed that a single dose of **AZT** (50 mg/Kg) could achieve a C_{max} value in plasma of up to 55,976 ng/mL, whereas, for brain tissue, this concentration was 692 ng/mL [25]. To improve access to CNS, **AZT** and ursodeoxycholic acid (UDCA) were conjugated to circumvent the

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active efflux transport system (AET) of the central nervous system (CNS). This specific conjugation occurred at the 5'-ester of **AZT** with UDCA, which has antiapoptotic effects. The results of High-Performance Liquid Chromatography (HPLC) analysis revealed that the half-life ($t_{1/2}$) of the prodrug in rat blood was approximately 10 s, while in human plasma, it had a longer half-life of 7.53 h; thus, it exhibited controlled release of **AZT**. Upon administration, the prodrug (UDCA-**AZT**) was detected in the rat brain, where it underwent rapid hydrolysis and showed a $t_{1/2}$ of 7.24 min. In liver homogenates it had a $t_{1/2}$ of 2.70 min, suggesting that it might be released in this compartment. The permeation characteristics of the compound were investigated across physiological barriers using a human retinal pigment epithelium (HRPE) cell line. A polarized cell monolayer was formed with epithelial features. Compared to the active efflux of **AZT** from the CNS, the bidirectional permeation of 30 M **AZT** across this cell monolayer was governed by apparent permeability coefficients (PE) that were higher from the apical to the basolateral compartments (PE = 209 ±4 × 10^{-5} cm/min) than in the reverse direction (PE = 133 ±8 × 10^{-5} cm/min). In contrast, the permeability coefficients of 30 M UDCA-**AZT** were similar for inflow (PE = 39.1 10^{-5} cm/min) and efflux (PE = 31.3 x 10^{-5} cm/min), which suggested that the prodrug could accumulate in the CNS. Partial hydrolysis of the prodrug during its penetration through the cell membrane was associated with its relatively lower PE values. The molecular structure of UDCA might serve as a reference for designing drugs or prodrugs to evade active efflux transport systems, thus minimizing the chances of developing multidrug resistance. Neither UDCA nor UDCA-**AZT** showed interactions with the active efflux transport system [26].

To improve the effect of the prodrug UDCA-**AZT**, the same research group developed a delivery system using solid lipid nanoparticles for nasal administration. Medicines that can adhere to the olfactory mucosa can traverse into the cerebrospinal fluid (CSF) by diffusing across the mucosal layer. Additionally, the drug can enter the brain through the trigeminal or olfactory nerves that extend to the nasal cavity. These nanoparticles were spherical, and their diameters were around 7–16 μm. The researchers found that for the UDCA-**AZT** prodrug, the loading rate was 0.57% (w/w) when tristearin was used and 1.84% (w/w) when stearic acid was used. The main difference between these two methods is that tristearin can control the rate of release, whereas stearic acid can improve the dissolution rate of the system. Nasal administration is an effective strategy to increase permeation and brain uptake [27]. The solid lipid nanoparticles containing tristearin demonstrated a significant enhancement in prodrug stability with 75% retention after 30 min, whereas, in the presence of stearic acid, only 14% retention was recorded after 30 min. The nasal administration of stearic acid-based solid lipid nanoparticles facilitated prodrug uptake into the CSF, revealing a direct pathway from the nose to the CNS. The uptake of the prodrug improved after chitosan was included. By implementing this method, the uptake of the prodrug in CSF increased six-fold (up to 1.5 μg/mL within 150 min) after the post-nasal administration of 200 μg [28].

2.2. Tenofovir prodrugs

In 1993, some researchers reported different effects of enantiomers of acyclic nucleoside phosphonate – tenofovir (**TFV**) - against herpes virus and retrovirus. Although the drug has promising *in vitro* effects, at physiological pH 7.4, it is a negatively charged dianion due to the presence of a phosphate group. This property decreases membrane permeability and consequently, reduces oral bioavailability. Efforts to improve such limitations led to the synthesis of tenofovir disoproxil (**TFV-DP**), a tenofovir prodrug with higher oral bioavailability (~25%) than the parental drug tenofovir. The presence of food can increase the bioavailability by up to 35%. After oral administration, **TFV-DP** is hydrolyzed by plasma esterases and then phosphorated by cellular kinases to the active metabolite tenofovir diphosphate (**Figure 1**). TFV-DP is commonly synthesized as its fumarate salt and has favorable pharmacokinetic properties; it is generally administered at a dose of one tablet a day. The drug, used in combination, is effective in treating naïve patients. It is administered in most first-line treatments. However, its long-term administration is associated with kidney injury and a decrease in bone mineral density. Disturbances in the tenofovir secretory pathway (higher OAT-1 activity or lower MRP efflux transport activity) can increase the concentrations of TFV in the cell, which can cause depletion and dysfunction of mitochondrial DNA

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and proximal tubulopathy. This is a serious concern for the long-term treatment of HIV and hepatitis B, and strategies to reduce this inconvenient effect might be investigated using the prodrug approach [29].

Tenofovir alafenamide (**TAF**), approved by the FDA in 2016, is a prodrug of tenofovir that contains a phenoxy isopropylalanineamidate (alafenamide) subunit (**Figure 3**). In vitro studies have shown that **TAF** is about 10-fold more active than **TFV-DP** and up to 1,000-fold more active than parental **TFV**. **TAF** is a ProTide that efficiently delivers the nucleoside inside cells at high concentrations. This decreases the plasma levels of TFV and the adverse effects on the kidneys and bones. **TAF** can accumulate in some compartments, such as the lymphatic tissue and the liver, which is highly desirable for HIV and hepatitis B. Studies on the evaluation of the permeability and stability of **TAF** have shown that the ProTide uses the systemic circulation to effectively load target cells by saturating intestinal efflux transporters; this process is facilitated by its high water-solubility. In a study, **TAF** exhibited concentration-dependent permeability across the monolayers of Caco-2 cells, as well as oral bioavailability ranging from 1.7% at 2 mg/kg to 24.7% at 20 mg/kg. In intestinal and hepatic extraction in the portal vein of cannulated dogs, its half-life ranged from 0.12 h at 0.5 mg/kg (iv) to 0.34 h at 20 mg/kg (po). The systemic pharmacokinetic profile of **TAF** in dogs showed that after oral administration of the drug at 5 mg/kg, it was rapidly absorbed. The highest plasma concentration of the drug recorded was 1.58 μM 0.14 h after administration. **TAF** disappeared quickly (2 h), and this change was associated with an increase in the concentration of **TFV** in plasma. Exposure of **TAF** to plasma led to the rapid accumulation of **TFV-DP** in PBMC (18 μM after 1 h) [30].

In clinical trials, **TAF** was well-tolerated and presented potent antiviral activity. About 8 mg of TAF had antiviral effects similar to that of 300 mg of **TFV-DP**. Administering **TAF** increased intracellular levels of **TFV-DP** in peripheral blood mononuclear cells (PMBCs), which confirmed the observations of other in vitro studies [31–35].

A comparative study on tenofovir prodrugs, **TAF** and **TDF**, was conducted by Callebout et al. (2015). In that study, **TAF** showed better results than **TDF**, with higher antiviral activity against HIV-1 and HIV-2, the EC_{50} ranged from 0.10 to 12.0 nM (mean EC_{50} was 3.5 nM). For HIV-2 isolates, the mean EC_{50} was 1.8 nM for **TAF** and 6.4 nM for **AZT** (used as control group). [35]. A year prior, Markowitz et al. (2014) conducted other clinical studies that demonstrated comparable findings. The researchers also found mild adverse effects (headache, nausea, and flatulence) after administering **TAF** but not **TFV-DP**. TAF was also more effective in reducing the viral load (HIV-1 RNAm) and did not result in resistance mutations [32].

For antiviral drugs, the use of ProTide technology exhibits promising application to allow the delivery of monophosphates and monophosphonate nucleoside intracellularly, which improves the exposure and oral bioavailability of drugs and decreases collateral effects on non-target tissues. ProTide can be prepared using three distinct routes. First, the coupling reaction of the diarylphopshite with a nucleoside followed by oxidation of the amino group; second, the treatment of phosphorochloridate with the desirable nucleoside; third, the treatment of arylphosphate of a certain nucleoside with an amino acid through a coupling reaction [36]. By masking the monophosphate group, which is negatively charged at a physiological pH of 7.4, the poor cellular uptake of nucleosides can be decreased. The activation of ProTide inside the cell involves the cleavage of the ester by esterases. Two main esterases are involved in cleaving esters and include carboxylesterase 1 (CES1) and cathepsin A (**Figure 3**). The nature of the ester group can influence the lipophilicity, along with the steric and electronic parameters, leading to the release of carboxy metabolites at different rates. In the second step, the negative charge of the amino acid facilitates a nucleophilic attack on the phosphonate or phosphate that contains a partial positive charge at this electron-deficient site. This results in the elimination of the aryl-leaving group and subsequent cyclization. This step leads to the formation of an unstable five-membered ring that opens after hydrolysis and forms a phosphoramide intermediate. In the last step, a phosphoramide-type enzyme once again hydrolyzes the phosphorus-nitrogen (P-N) bond, producing the triphosphorylated nucleoside. This nucleoside is then released by cellular kinases, enabling its effectiveness ediagainst virus-encoded DNA or RNA polymerases (**Figure 3**) [37, 38].

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The tyrosine-based prodrug (*ProTide* strategy) of **TAF**, in which the phenol group of the traditional compound was replaced by a tyrosine derivative, was identified as a ketone-containing compound (**3v**), which showed high antiviral potency against HIV-1. Prodrug (**3v**) (**Figure 3**) (slow-eluting epimer) showed an EC_{50} value of 6 pM against HIV-1 and cytotoxicity with CC_{50} of 9.2 μM. The SI of this compound was 1.500.000, which was about a 300-fold increase in SI compared to **TAF** (EC_{50} of 0.0064 μM; cytotoxicity with CC_{50} of 35 μM). For human hepatocytes, this ProTide showed subnanomolar EC_{50} values for hepatitis B virus (HBV) with a 26-fold higher SI than that of TAF. In a metabolomics study in which lymphoid cells (CCRF-CEM) were used, **3v** showed higher cellular uptake. The intracellular concentration of the prodrug was 102.36 pmol/10^6 cells, with a higher concentration of **TDF** (148.09 pmol/10^6 cells) than the parent drug **TAF**. Thus, the ProTide **3v** showed higher antiviral activity. These findings highlighted that **3v** is an effective pro-pharmaceutical agent that can release a sufficient amount of **TDF** [34].

Other researchers modified the ProTide technology to form stable nanocrystals. Phenylalanine and alanine amino esters containing long-chain fatty acids were synthesized to investigate the ProTide approach. Both prodrugs, i.e., M1TFV and M2TFV (**Figure 3**), showed higher lipophilicity than tenofovir. Both compounds also showed higher intracellular drug uptake and retention, mainly in the important compartments for HIV, such as the liver, spleen, lymph nodes, gut, lymphocytes, and rectal and vaginal tissues. In vivo experiments using Sprague Dawley rats showed that after intramuscular injection of both prodrugs, a sustainable level of tenofovir-diphosphate was maintained at four times the effective dose during two months of the study. These findings were promising, including for antiviral pre-exposure prophylaxis (PrEP), considering that **TDF** is the choice for PrEP therapy in many countries [39].

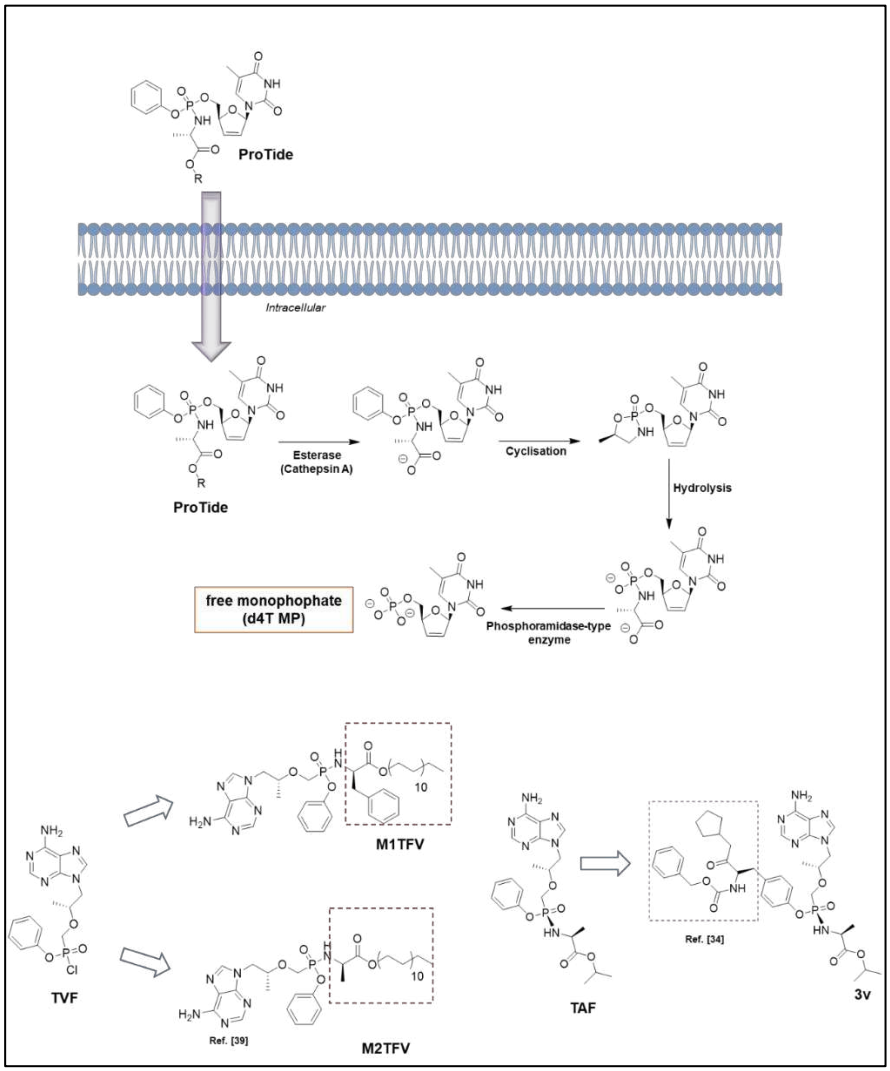

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Figure 3. The proposed mode of action of ProTides.

Prodrugs containing drug-lipid conjugates can effectively enhance lipophilicity, enable sustained release of the parental drug, elevate concentrations in specific body compartments (organs, tissues, and cells) through targeted drug delivery, and offer the potential to alter the route of administration. These prodrugs are being extensively investigated in the field of drug discovery [40]. Drug-lipid conjugation can be performed using different lipophilic substances, such as fatty acids (e.g., squalene, stearic acid, oleic acid, palmitic acid, linoleic acid, lauric acid, and octadecanoic acid), steroids (cholesterol, ursodeoxycholic acid, and lithocholic acid), glycerides, phospholipids, among others. The choice of the lipid-acid chain depends on its physicochemical properties, stability, and additional biological effects of the designed prodrug. Additionally, distinct chemical bonds might be assessed to design conjugates consisting of esters, amides, hydrazone, disulfide, carbamates, carbonates, and phosphodiesters. The selection of this chemical bond is determined by considerations of reactivity, stability, selective delivery, drug release rates, and modulation of pharmacokinetic properties [40]. Among the bonds, esters and amides are the most studied. Amide bonds are more stable and exhibit slower rates of hydrolysis compared to ester bonds. Other examples include the hydrazone bond, in which the sensitivity to acidic environments, such as that found in lysosomes, can lead to the hydrolysis and release of the drug and the lipid [40].

The lymphatic system is an important site for HIV viral replication. Some studies have found a correlation between the concentration of antiviral drugs in lymphoid tissues and suppression of HIV, as well as viral persistence [41, 42].

Many researchers have used lipid conjugates and the prodrug approach to deliver drugs to the HIV reservoir in the gut lymphatics using dendrimers, nanoparticles, nanosuspensions, micelles, conjugated polymers, etc. [43]. During lipid metabolism, lipophilic drugs can be organized and packaged into intestinal lipoproteins as chylomicrons and secreted into the mesenteric lymph (**Figure 4**). These drugs can enter the systemic circulation through the thoracic lymphatic duct (**Figure 4**). Several studies have shown that highly lipophilic compounds with suitable physicochemical characteristics (log D7.4 > 5, Log P > 5, large particles, solubility >50 mg/mL) can be effectively transported by the lymphatic system through the intestinal tract when administered orally with a high-fat diet (**Figure 4**). Antiretrovirals, for example, when absorbed by the intestinal lymphatic vessels, are involved (talvez trocar por "wrap") in lipid vesicles in the enterocyte and transported by lymph to the lymph nodes (**Figure 4**). Thus, they are distributed according to the lymph flow pattern (**Figure 4**). Lipophilic antiretrovirals are more likely to perfuse through the middle of the lymph node, where cell density is high and fluid flow is low [44].

This type of transport can avoid a first-pass effect. The reach of the lymphatic system is a result of triglycerides being re-esterified after their hydrolysis in the small intestine by pancreatic lipases. This results in the formation of metabolites (2-monoglycerides and fatty acids) that are absorbed by enterocytes and re-esterified into triglycerides, which are organized into chylomicrons and secreted into the mesenteric lymph nodes. Lymph nodes are tissues where viral RNA and DNA are repeatedly verified in non-human primates, humanized mice, and humans undergoing antiretroviral therapy. Thus, lymph nodes are important in the pathology of HIV, as they contain large populations of CD4+ T cells and other immune cells organized in specific regions that facilitate the immune response. HIV spreads to regional lymph nodes within 3–6 days due to its large target population of CD4+ T cells and constant movement of cells by elevated endothelial venules (HEVs) and lymphatic vessels. Systemic spread of HIV occurs within 6–25 days. This reservoir can be maintained by two mechanisms during antiretroviral therapy, including low levels of viral replication and clonal expansion of infected latent CD4+ T cells. The incomplete inhibition of viral replication in lymph nodes might be due to the low concentration of antiretroviral agents in this tissue compared to their concentration in plasma [45, 46]

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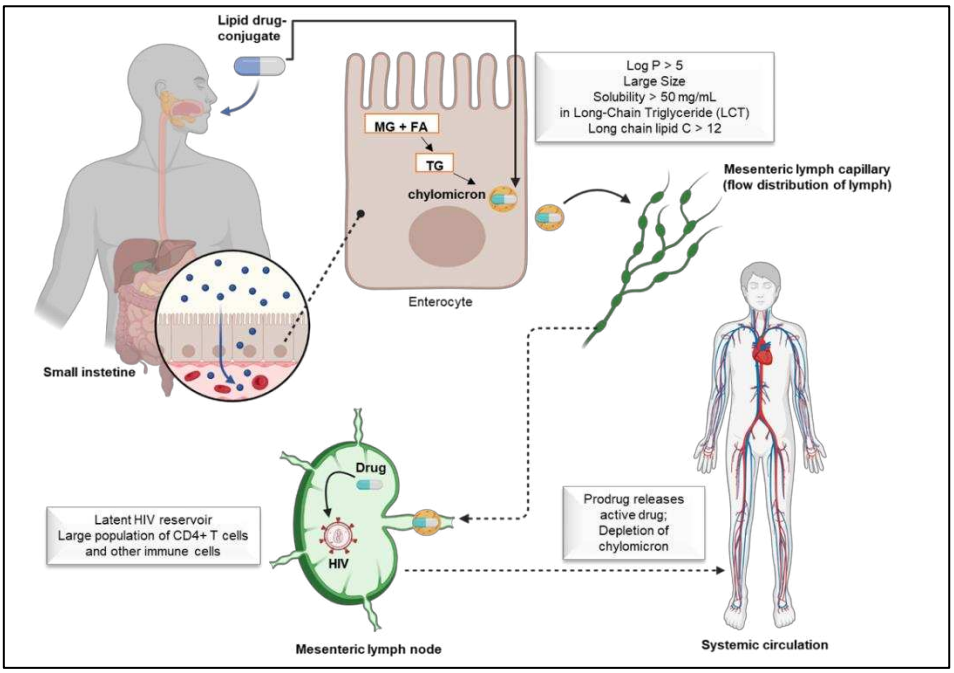

Figure 4. Lipophilic drugs during mechanism until reach the systemic circulation.

TFV lipid-conjugates were specifically designed and synthesized to mimic lysophosphatidylcholine, aiming to target the natural lipid pathway and attain elevated intracellular concentrations of the active antiviral agent. This approach enhances the effectiveness against both wild-type and mutant HIV strains. **TDF**-exalidex (**CMX157**) (**Figure 5**), {3-(hexadecyloxy)propyl hydrogen [(*R*)-1-(6-amino-9*H*-purine 9-yl)propan-2-yloxy]methylphosphonate. Hexadecyloxypropyl TFV [HDP-TFV], was found to be active against all major HIV-1 subtypes in PBMCs, with EC_{50} values ranging from 0.20 to 7.2 nM for the 27 different viruses evaluated. CMX-157 is a lysoglycerophospholipid conjugate prodrug designed to increase intracellular concentrations of TFV and decrease the circulating levels and systemic presence of TFV. CMX-157 has low bone and renal toxicity that is often reported following the long-term administration of TFV. In vitro studies have also shown that this lipid prodrug is 97-fold more active against HBV and up to 267-fold more active against HIV-1 [47, 48]. This prodrug mimics the uptake pathways of lysolecithin in the gut. CMX-157 can be cleaved by phospholipase C to release the parental drug TFV; however, the long-chain fatty acid is metabolized by CYP450 omega-hydrolylases in the liver. These undesired reactions hindered access to HIV-1 infected cells, and the resulting metabolite may have potential organ toxicity risks [47, 49]. Pharmacokinetic studies revealed substantial hepatic extraction, implying that this approach may find more promising applications in the treatment of hepatitis rather than HIV infection [50].

These results suggested that the cellular uptake of **CMX157** was high, which resulted in higher intracellular levels of the active antiviral anabolic agent, **TFV-diphosphate (TFV-PP)**. No cytotoxic effects were observed up to the high-tested concentration of 1.000 nM. It also showed activity against HIV-2 in PBMCs (mean EC_{50} ±SD = 3.5 ±1.5) and against six HIV-1 isolates in MDMs (mean EC_{50} ±SD = 2.5 ±1.7 nM) **[51]**. Human PBMCs were used to evaluate the relative levels of **TFV-PP** produced by **CMX157** and **TFV**. Human PBMCs exposed to 1.0 μM **CMX157** for 24 h produced approximately 34 times more **TFV-PP** than that produced after exposure to 1 μM **TFV**. The lipid-TFV conjugate is not a substrate for human organic anion transporters (hOATs 1 to 3). After absorption, it is activated in human PBMCs, which increases its efficacy and decreases the toxicity imparted by **TFV**. No antagonistic interactions were recorded between **CMX157** and any FDA-approved antiretroviral drug, such as NRTI. Thus, the prodrug could deliver high levels of the active metabolite and reduce toxicity [51].

To avoid the metabolic issues related to CMX-157, ω-functionalized lysoglycerophospholipid prodrugs derived from **TDF**-exalidex were synthetized and evaluated to improve hepatic stability,

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decrease **TFV** organ toxicity, and reduce CYP-mediated ω-hydroxylation. Optimizing the length of the chain was expected to improve hepatic stability and increase the half-life. From the series prepared, two compounds (**21** and **23**) (**Figure 5**) showed the best pharmacokinetic profiles. The saturated CF_3 derivate (**21**) exhibited an IC_{50} value of 0.049 μM for HIV-1 and a CC_{50} value >100 μM using human embryonic kidney (HEK293T) cells. For this prodrug (**21**), the therapeutic index (CC_{50}/HIV IC_{50}) was found to be greater than 2,040. Prodrug (**21**) exhibited metabolic stability using mouse liver microsome experiments with a half-life ($T_{1/2}$) greater than 120 min. The values for the inhibition of CYP 2D6 and 3A4 were greater than 20 μM [52]. The TMS acetylenyl prodrug **(23)** showed an IC_{50} value of 0.069 μM and a CC_{50} value >100 μM (HEK293T cells), with a therapeutic index greater than 1,450. The $T_{1/2}$ was higher than 120 min, and the IC_{50} values for CYP 2D6 and 3A4 were 21.7 μM and >20 μM, respectively. Prodrug (**23**) presented a higher metabolic stability than **TDF**-exalidex, which in the same experiments showed an IC_{50} value of 0.018 μM for HIV-1, a CC_{50} value of 97.6 ±2.68 (therapeutic index of 5,420), and $T_{1/2}$ of 42 min. Preliminary SAR revealed that chain lengths of 18 and 20 atoms were optimal for antiviral activity. Analogs containing ether or thioether linkers had equal metabolic stability but higher antiviral activity than methylene linkers. The ω-terminal functionalized groups, such as acetylenyl, TMS acetylenyl, CF_3, and CF_3 acetylenyl showed higher metabolic stability in HLM compared to prodrugs with unfunctionalized terminals, probably due to a decrease in the metabolism by ω-oxidases. The antiviral activity of the substituents TMS acetylenyl, CF_3, and CF_3 acetylenyl derivatives was similar to that of **TDF**-exalidex, whereas undecorated acetylenyl congeners showed significantly lesser antiviral activity. Such differences occurred due to the variations in cellular permeability and metabolism. The mouse plasma pharmacokinetic profile showed AUC0−24 h (h·ng/mL) of 301, 1,280, and 550 for the compounds **TDF**-exalidex, prodrug (**21**), and prodrug (**23**), respectively. Such effects suggested a decrease in the metabolism catalyzed by ω-oxidases [52].

The same research group examined the effect of modifying the length of the carbon chain by performing bioisosteric replacement of the carbon-carbon bond to a disulfide bond. Lipid phosphomonoester disulfide-linked prodrugs do not rely solely on reduction for prodrug cleavage; instead, enzymatic activation is required to release **TFV** into the target cell. The researchers suggest that these prodrugs likely depend on enzymatic hydrolysis to facilitate S-S bond reduction. The researchers prepared two different series. In the first study, they found that the prodrug (**2B**) ((*R*)-4-(Hexadecyldisulfanyl)butyl (((1-(6-Amino-9*H*-purin-9-yl)- propan-2-yl)oxy)methyl)phosphonate) (**Figure 5**) exhibited anti-HIV effects with an EC_{50} value lesser than 0.0005 μM, a CC_{50} value greater than 50 μM, and a therapeutic index greater than 100,000. Prodrug (**2B**) was more efficacious than TFV (EC_{50} = 0.320 μM, CC_{50} > 100 μM, and TI > 300). Prodrug (**2B**) was activated by 5-exotet cyclization to yield non-electrophilic tetrahydrothiophene after enzymatic hydrolysis, in which phospholipase C and/or sphingomyelinase may have participated. The researchers hypothesized that its high antiviral activity was due to the presence of a "twisted" S-S bond in this prodrug. The bond involved a 90° dihedral angle that increased membrane fluidity to accelerate the translocation of the conjugate from the outer side of the plasma membrane to the inner side, where it was enzymatically cleaved to release **TFV** into the cytosol. However, this disulfide conjugate showed low aqueous solubility, and the mutagenic potential of thiirane formed by the decomposition of *S*-acyl-2-thioethyl (SATE) and dithioethanol (DTE) encouraged further investigation of ways to mitigate this deleterious effect [50].

In the second series, lipid phosphomonoester disulfide-linked prodrugs were synthesized that did not require enzymatic activation to release TFV into the target cell. Lipid conjugates (**12c**), (**12d**) and (**12e**) (**Figure 5**) had alkyl chains of 14 to 18 carbon atoms and showed maximal antiviral activity against HIV, with EC_{50} values in PBMCs of 0.00050 μM, 0.00065 μM, and 0.00060 μM, respectively. Compounds (**12c**) and (**12d**) displayed robust stability in human plasma with half-lives of >24 h, probably due to the formation of micelles that protected the fragile S-S bond from the action of reductases circulating in human serum. Stability assays using carbonate/bicarbonate buffer (pH 9), PBS (pH 7.4), and DMEM (a nucleophilic medium) showed that (**12c**) and (**12d**) exhibited similar stabilities with $t_{1/2}$ > 2 h. After optimization, the lipid conjugates significantly improved the potency

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of the original nucleoside by more than 600-fold, showing that the compounds had adequate stability [53].

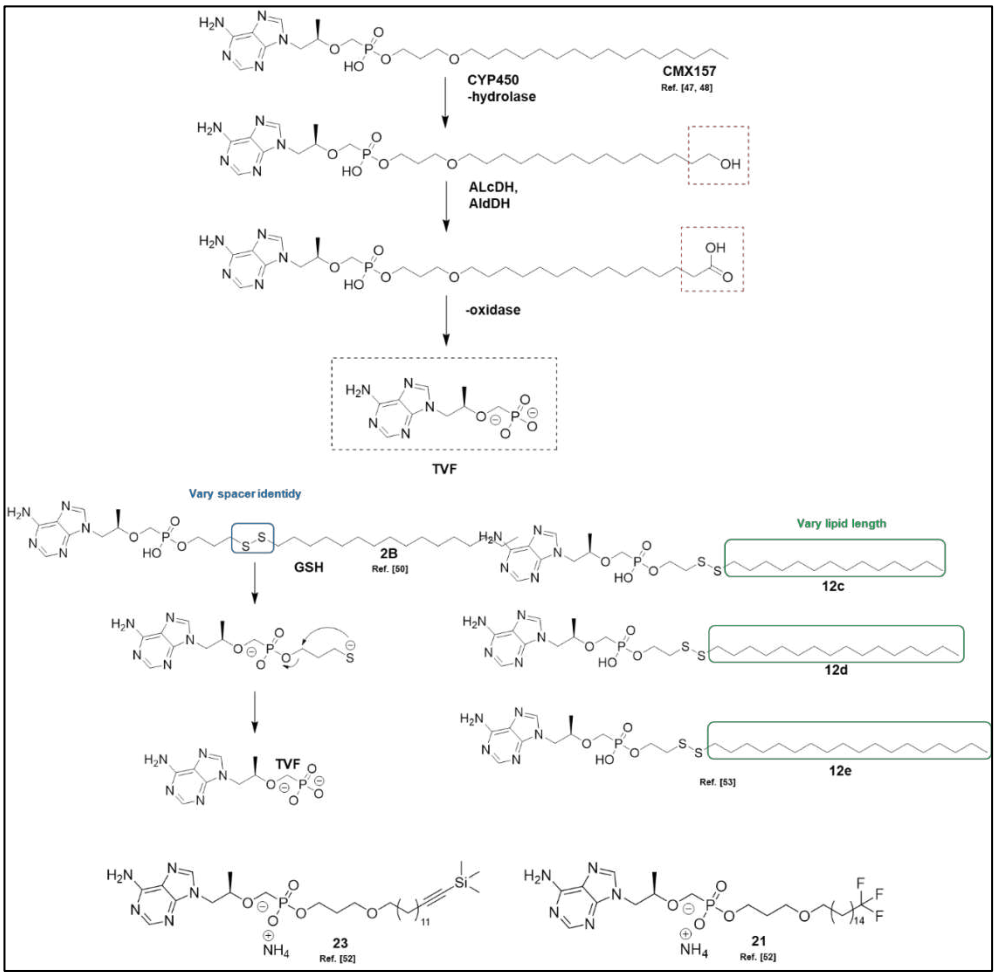

Figure 5. TDF prodrugs.

2.3. Lamivudine prodrugs

Lamivudine, also known as 2′,3′-dideoxy-3′-thiacytidine (**3TC**), was designed by the bioisosteric replacement of the 3′-methylene hydroxide group with a sulfur atom in the ribose scaffold. The drug is efficaciously absorbed after oral administration, and its bioavailability is 86%. Nevertheless, the prodrug approach must address challenges such as peripheral neuropathy and gastrointestinal issues (e.g., nausea, diarrhea, and vomiting) to ensure the sustained, long-term use of **3TC**.

Phosphonate-based derivatives of **3TC** were synthesized and their anti-HIV activity was assessed using infected MT-4 cells. The IC_{50} value for the most active prodrug (i.e., compound (**1**)) (**Figure 6**) was 3.4 μM, which was considerably higher than that of **3TC** (IC_{50} = 0.44 μM). However, compound (**1**) exhibited lower toxicity (CC_{50} > 3410 μM) than **3TC** (EC_{50} = 43.6 μM) and had a selective index (SI) one order of magnitude greater than that of **3TC**. The stability of these prodrug compounds was evaluated across a pH range of 5.5–8.5; the half-life was found to be slightly over 24 h. In the whole blood of dogs, compound (**1**) showed a half-life of 5 h. The pharmacokinetic profiles of compound (**1**) were similar when the compound was administered orally to dogs and intragastrically to rabbits. Following administration, the t_{max} values increased 2–3 times, C_{max} was nearly halved, and the AUC was reduced by 1.5–2 times compared to **3TC** administered alone [54].

The lipophilicity attributed to the length of the carbon chain at the 5′-*O*-position was modulated for lamivudine derivatives to improve their capacity to cross the cellular membrane through passive diffusion, and they were further studied to design new prodrugs. Thus, a series of **3TC** carbonate prodrugs were prepared using the coupling agent *N*,*N*-carbonyldiimidazole, and certain alcohols.

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The effectiveness of these prodrugs was evaluated against HIV-1 infected cells. Compared to **3TC** with a log P value of –0.91, the carbonate prodrugs were more lipophilic and showed log P values ranging from –0.81 to 1.72. Some derivatives had IC_{50} values higher than that of **3TC**; for example, the prodrug **3TC**-penta (compound **8**) (**Figure 6**) showed an IC_{50} of 0.065 μM and SI greater than 19,700. *In vitro* assessments in PBMCs highlighted the relationship between the increase in cytotoxicity and the elongation of the carbon chain of the alcohol moiety, suggesting that a chain containing five carbon atoms is ideal for such effects [55].

After lamivudine was used against HIV, its efficacy against the hepatitis B virus (HBV) was assessed. Studies have shown that 3TC is effective in reducing HBV replication. To improve the lipophilicity of the drug, researchers designed **3TC** prodrugs (compounds **I** and **II**) in which stearate was used as the carrier. This approach increased the log P value from –0.95 (**3TC**) to 1.82 (prodrug). Then, the researchers developed a particulate delivery system to improve the uptake of the prodrug using stearic acid-g-chitosan oligosaccharide (CSO-SA) micelles. After the stearic-3TC prodrug was incorporated into micelles (average size: 460 nm), the zeta potential increased (29.7 mV). This modification increased its uptake by HepG2.2.15, decreased its cytotoxicity, and exhibited a linear kinetic of hydrolysis to release **3TC**; these changes helped in reducing the viral infection [56].

A liver-specific prodrug (**3TCSD**) (**Figure 6**) derived from the antiviral compound lamivudine (**3TC**) was prepared through the coupling of **3TC** to dextran (~25 kDa) using succinate as the spacer. The synthesized **3TCSD** had >99% purity and a substitution degree of 6.5 mg of **3TC** per 100 mg of the conjugate. Dextrans are promising systems/carriers that might be further investigated in the field of drug discovery, as plasma kinetics and distribution of dextran carriers in tissues depend on the molecular weight (Mw) of the polymer. For example, dextrans with a MW of 20–70 kDa showed high specificity for the liver, probably because of their size, which permitted their passage through the larger pores of the liver sinusoids [57]. The prodrug showed high chemical stability under different pH conditions (4.4 and 7.4) and in the rat blood, releasing **3TC** at slow rates. Plasma concentration-time curves after i.v. administration was also examined, and the results showed that for **3TC**, C_{max} was 4.94 $\mu g.mL^{-1}$ and $t_{1/2}$ was 37.9 min, whereas, for the prodrug, C_{max} was 52.5 $\mu g.mL^{-1}$ and $t_{1/2}$ was 125 min, respectively. The linkage of **3TC** to dextran considerably decreased drug clearance and distribution volume by factors of 40 and 7, respectively. The prodrug level was 50 times higher than the level of the parental drug (**3TC**) in the liver. The prodrug concentration was also high in the kidney, but it was almost absent in the lungs, heart, brain, and spleen [58].

Drug-releasing systems, such as hydrogels, nanogels, and polymer conjugates, are effective ways to prolong the half-life and reduce the cytotoxic effects of nucleosides. In certain scenarios, the kinetics of hydrolysis are contingent upon the chemical nature and stability of the bond between the drug and the system, which plays a pivotal role in regulating drug delivery. Polymers containing hydroxyl and sulfonated side chains linked to **3TC** through a disulfide self-immolative linker were synthesized, characterized, and evaluated against HIV-1 infected cells (HeLa-derived TZM-bl cells). Polymer sulfonation is important to avoid HIV-1 entry; about 50% of sulfonated monomers were found to inhibit reverse transcriptase. Sulfonate monomers also exhibit activity against DNA−DNA polymerase, which further enhances its antiviral effects. These mechanisms together considerably improve the antiviral effects. Thus, incorporating such mechanisms while developing prodrugs can greatly enhance their half-life and decrease their cytotoxicity [59].

Drug delivery to specific compartments, such as the liver (for treating hepatitis), is a promising approach to enhance the effectiveness of treatment. This strategy ensures higher concentrations of the active antiviral agent in the liver with minimum exposure of other tissues to the drug. HepDirect prodrugs were developed to achieve these aims. In this approach, the chemical structure of the antiviral drug is modified to develop a compound that remains inactive until it reaches its target, i.e., the liver. In the liver, the prodrug undergoes a specific biochemical transformation triggered by liver enzymes, mainly CYP450 isoforms. This transformation converts the prodrug into its active form, which leads to the release of the antiviral agent at the precise site of infection. Studies have shown that this technique has higher antiviral activity and a better safety profile than the traditional systemic administration of antiviral agents [60, 61].

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The **3TC** prodrugs containing 5'-O-carbonates were synthesized to perform pre-formulation studies. The water solubility of all prodrugs ranged from 0.24 to 17 mg/mL, whereas, for **3TC** the solubility was 65.9 mg/mL at 25 °C. The increase in the carbon chain length of the substituent led to a reduction in aqueous solubility. Most of the 5'O-carbonate prodrugs showed favorable chemical stability, indicating that their structures affected the hydrolysis rate. At pH 7.4, the half-life for all prodrugs ranged from 22.85 h to 378.77 h, whereas, for enzymatic hydrolysis, the half-life ranged from 5.44 min to 35.20 min. The log P values for prodrugs were found to vary between -0.81 and 1.72 [62]. The intestinal permeability of two of these prodrugs (**3TC**-Etha and **3TC**-Buta) (**Figure 6**) was evaluated, and the results showed an increase of two-fold and 10-fold compared to the intestinal permeability of **3TC**. However, **3TC**-Etha was a substrate of P-glycoprotein, and 3-TC-Buta underwent enzymatic hydrolysis during permeation [63]. Even small chemical modifications, using homology series, for example, can greatly affect the physicochemical properties and the kinetics of hydrolysis. Thus, chemical modification is an important parameter to evaluate the design of new prodrugs [62].

The efficacy of 3TC therapy for HBV is not very high, and the virus shows considerable drug resistance. This is probably because of the poor bioconversion of **3TC** to the triphosphate form that is necessary to inhibit HBV DNA polymerase. As the HepDirect prodrug is resistant to esterase cleavage, it first needs to undergo oxidative cleavage by CYP450 (CYP3A4) to be converted into nucleoside monophosphate, and then, into the active nucleoside triphosphate. Some researchers found that the **3TC** HepDirect prodrug (compound (**6**)) can increase the concentration of **3TC**-triphosphate by up to 35-fold in rat hepatocytes. This prodrug also showed a sustainable release in rat hepatocytes compared to **3TC**. After i.v. administration in rats, the concentration of the prodrug in the liver was 29.4 nmol.g/h, and that of **3TC** was 2.6 nmol.g/h. The concentration of **3TC** after prodrug metabolism in plasma was <12.8 μM, indicating that its concentration in the liver was > 320-fold higher than the concentration of **3TC** [64].

Co-drugs, also known as mutual prodrugs, have two or more drugs/active compounds covalently bound through a spacer link or a direct link. The primary goal of co-drug development is to achieve synergistic effects by combining compounds that target different aspects of a disease or its underlying mechanisms. This approach can improve therapeutic outcomes, decrease drug resistance, and overcome the limitations associated with single-drug therapy. Co-drugs can be especially effective in treating complex diseases, where multiple pathways contribute to the pathogenesis, or where single-drug treatments have limited efficacy. However, finding the balance between the chemical properties of the individual drugs is a major challenge in the development of co-drugs. Thus, the stability, solubility, and pharmacokinetics of both active compounds should be compatible [65, 66].

Mutual prodrugs of **3TC** and **TDF** were synthesized to obtain a heterodinucleotide (**3TCpPMPA**) (**Figure 6**). The ability of this prodrug to protect against '*de novo*' HIV-1 infection was evaluated. Cells were incubated with parental drugs, the prodrug, and the combination (**3TC** + tenofovir) for 18 h before HIV-1 infection, and then, viral production was evaluated for 35 days. Although the combination decreased viral production by ~40.7%, the prodrug was more active, leading to only 8.5% viral production in 35 days. The activation of 3TCpPMPA was evaluated using murine plasma, which contains enzymes capable of cleaving the phosphate bridge of the prodrug. The results showed that **3TCpPMPA** was stable in murine plasma ($t_{1/2}$ = 14 h), and its half-life was higher than that of **3TC**. The prodrug was stable in erythrocytes. Experiments in which prodrug-loaded erythrocytes were added to macrophages showed that the treatment had a protective effect against viral infection. However, pharmacokinetic investigations in mice showed that **3TCpPMPA** was quickly removed from the bloodstream ($t_{1/2}$ = 15 min), and it did not have any benefits compared to the administration of individual drugs [67].

Another co-drug, involving **3TC** and ursolic acid, was synthesized and evaluated. Ursolic acid has hepatoprotective effects. Some researchers hypothesized that the co-drug might be effective against infectious diseases such as hepatitis. The chemical stability was studied at pH 7.4, using plasma and in the presence of lipase. The researchers found that **LMX** (**Figure 6**) was rapidly

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hydrolyzed in the presence of lipase, exhibiting a half-life of 1.4 h, which was shorter than its half-life in phosphate buffer (pH 7.4) (half-life of 11.2 h) and in buffered human plasma (half-life of 5.4 h). Studies on the chemical stability at pH 3–6 showed that the co-drug was highly stable, and its half-life was greater than 40 h. Hepatoprotective effects were determined using the acute liver injury model induced chemically by carbon tetrachloride (CCl_4) and acetaminophen. Animals treated orally with 200 μmol/Kg of the co-drug showed effects similar to that found for the reference hepatoptotective compound ursolic acid. Histopathologic studies showed a decrease in inflammation and hepatocellular necrosis for the co-drug group. Anti-HBV effects were investigated in vivo using the DHBV-infected duckling model. Animals treated with the co-drug at 150 and 250 mg/kg/d showed lower levels of DHBV-DNA, as determined by PCR. These findings suggested that the 3TC-ursolic acid co-drug should be investigated for HBV infections [68].

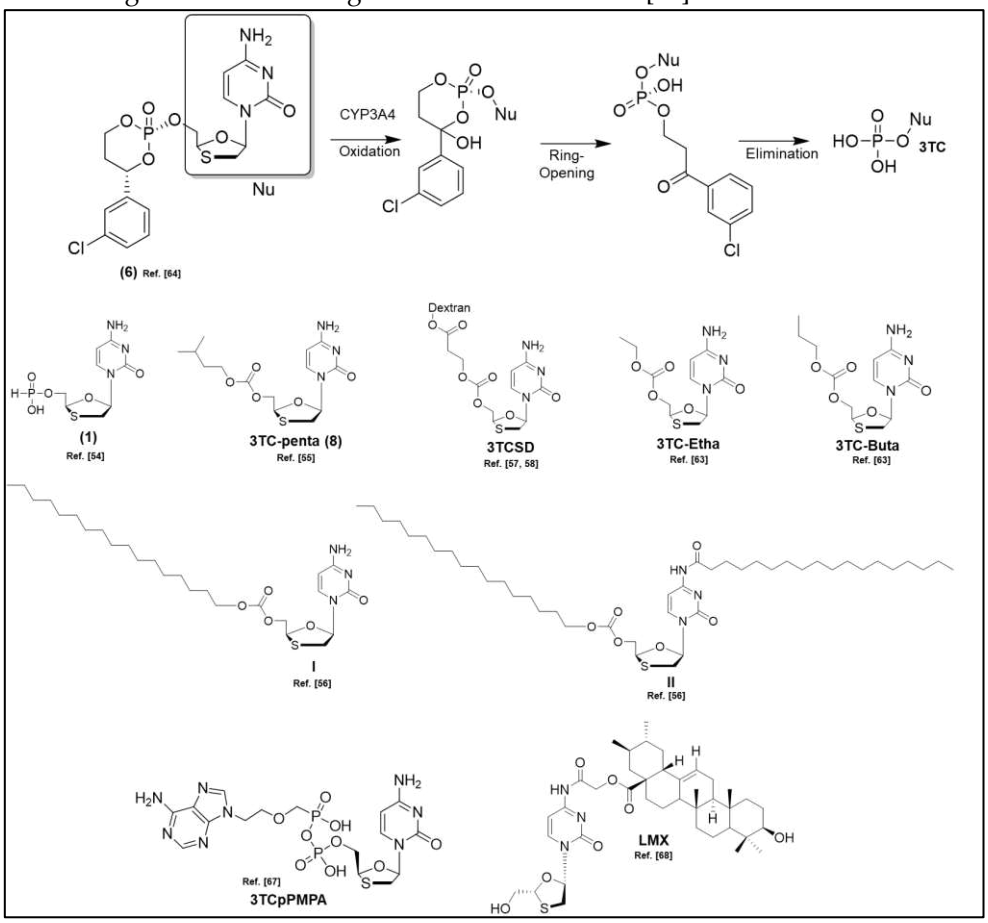

Figure 6. Lamivudine prodrug.

2.4. Stavudine (d4T) prodrugs

Stavudine (2′,3′-didehydro-2′,3'-dideoxythymidine, **d4T**) is a pyrimidine nucleoside, which differs from thymidine in that the 3′-hydroxyl group is replaced by a hydrogen atom and a double bond is present in the ribose ring between the 2′ and 3′ positions. The bioavailability is estimated to be around 85% after oral administration; however, the drug has a short half-life (~ 1.5 h). In contrast, the triphosphate metabolite has a half-life of 3.5 h [69].

Myristoylated prodrugs derived from d4T (stavudine) were designed and synthesized by a research group. Among these prodrugs, the bi-functional compound (**4**) (**Figure 7**), known as 2′,3′-didehydro-2′,3′-dideoxy-5′-O-(11-thioethylundecanoyl) thymidine, containing a C10 alkyl chain, exhibited the most potent antiviral activity against HIV-1. In MT-4 cells, it showed a very low EC_{50} of 0.03 μM and minimal cytotoxicity. Its CC_{50} exceeded 16.85 μM in MT-4 cells. These properties were more favorable than those found for stavudine. This compound could inhibit HIV replication at two different stages: reverse transcription and post-transcriptional processing of various proteins. In

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contrast, stavudine displayed an EC_{50} of 0.15 μM and some level of cytotoxicity, indicated by a CC_{50} of 41.60 μM, in MT-4 cells [70].

A research group synthesized a series of glycosidic-nucleoside prodrugs. Among them, compound (**10b**) (**d4T**-glucose-phosphodiesters) (**Figure 7**) was prepared by condensing glycosyl phosphoramidite 7 with the d4T nucleoside using an activating agent. This prodrug showed high anti-HIV-1 activity with an EC_{50} of 0.34 μM in PBM cells. This improved potency might be attributed to the presence of free nucleosides within the compound. Compound (**10b**) showed negligible cytotoxicity in PBM cells (>100 μM) and Vero cells (>100 μM), and it had a modest IC_{50} value of 76.8 μM in CEM cells. This indicated that the conjugation performed did not fully stop the activity of the original d4T nucleoside, which exhibited an anti-HIV-1 activity with an EC_{50} value of 0.073 μM Additionally, all prodrugs, including (**10b**), exhibited higher solubility in water (average solubility of 16.7 mg/mL) than the original d4T compound (solubility of 5.4 mg/mL). Thus, prodrug (**10b**) could enhance the oral bioavailability of nucleosides and improve their absorption in the intestine [71].

Mononucleotide prodrugs, also known as pronucleotides, derivatized from **d4T**, 2′,3'-dideoxythymidine (**ddT**), and 2′,3′-dideoxyadenosine (ddA), were synthesized and evaluated by some researchers. For developing these prodrugs, two o-pivaloyl-2-oxyethyl substituents were integrated into a phosphorodithiolate structure, promoting biolabile phosphate protection. Among these compounds, the derivative of ddA (compound (**3**)) (**Figure 7**) was found to be the most potent antiviral agent against HIV. Its EC_{50} value was 0.025 μM in CEM-SS cells, 0.27 μM in MT-4 cells, 0.02 μM in PBMC, and 0.00023 μM in MDM cells. This compound also had low cytotoxicity, with CC_{50} values greater than 10 μM in CEM-SS cells. In comparison, the original drug 2′,3'-dideoxyadenosine (ddA), exhibited less potent antiviral effects against HIV. Its EC_{50} values were 0.49 μM in CEM-SS cells, 7.8 μM in MT-4 cells, 0.24 μM in PBMC, and 0.12 μM in MDM cells. Its CC_{50} values were greater than 100 μM in CEM-SS cells, MT-4 cells, PBMC, and MDM cells. Tests to evaluate the stability of the compound in non-enzymatic environments, such as phosphate buffer and RPMI, showed that the synthesized prodrug underwent minimal hydrolysis. The corresponding metabolites formed slowly, with half-lives of several days ($t_{1/2}$ of 2.9 days in phosphate buffer at pH 7.4; $t_{1/2}$ of 2.2 days in RPMI at pH 7.45). The prodrug was also converted more rapidly into its diester metabolite, indicating accelerated conversion that coincided with the intended release of mononucleotides in cells [72].

Some researchers synthesized a series of α- and β-carboxylated phospholipid prodrugs derived from dideoxy nucleosides (**d4A**, **d4T**, and **ddC**) and evaluated them against HIV-1 infected cells. The phospholipid scaffold increased lipophilicity and, thus, cellular penetration, while the α-carboxylated subunit contributed to the phosphodiester bond cleavage. The α-hydroxy D4T compound (**18**) (**Figure 7**) showed an IC_{50} value of 0.4 μM against both HIV-1 and HIV-2 in infected MT-4 cells, but it was inactive in CEM cells. In contrast, the β-hydroxy D4T compound (**19**) (**Figure 7**) was about 30-fold less active than (**18**), and both prodrugs were less active than stavudine (d4T). In the MT-4 cell model, the prodrug phosphodiester **d4A-20**, characterized by the presence of an α-hydroxy stearic acid d4A conjugate, showed an IC_{50} value of 0.19 μM and a CC_{50} value of 97.8 μM. In comparison, the reference compound, dideoxy nucleoside **d4A**, showed an IC_{50} value greater than 277.5 μM and a CC_{50} value of 277.5 μM. As these prodrugs showed high inhibitory ability, **d4A**-20 might undergo more facile enzymatic degradation in cells, which explains its superior effect [73].

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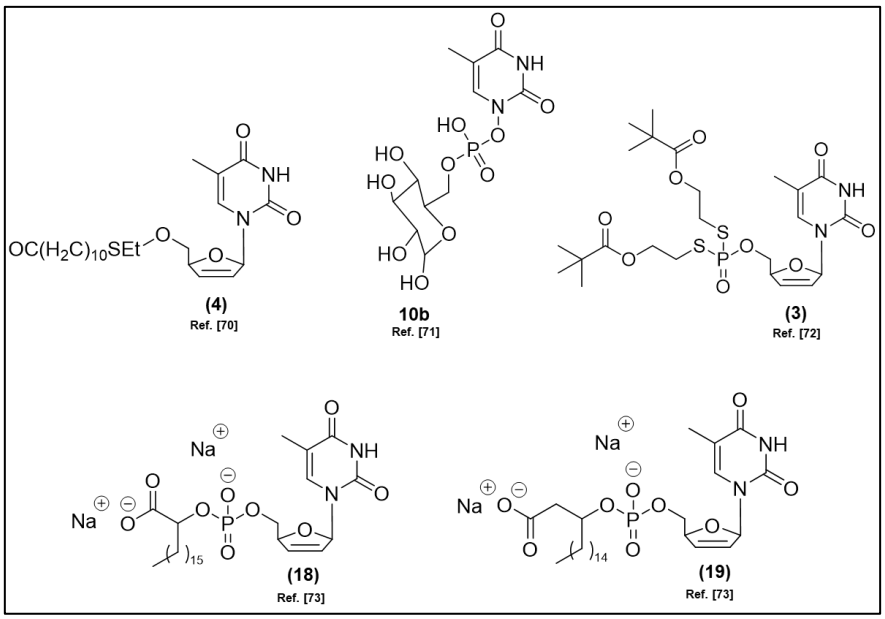

Figure 7. Stavudine prodrugs.

A prodrug delivering nucleoside diphosphates and triphosphates directly inside cells can overcome inconveniences, such as fast metabolism or side effects, compared to the parental nucleoside [74]. Thus, methodologies to avoid such problems can help in evading enzymatic processes, such as nucleoside deamination or the dephosphorylation of nucleoside monophosphates. Researchers have developed some approaches to solve this issue by using cycloSal-technology [75] Di*PP*ro- and TriPPPro – approaches [74, 76, 77].

CycloSal prodrugs have a cyclic salicylate moiety integrated into the prodrug structure. It undergoes cleavage through chemically induced processes (**Figure 8**). This cyclic group serves as a carrier and can be enzymatically cleaved in vivo to release the active drug in its therapeutic form, which allows fine-tuning of release kinetics and targeting. The cyclic structure provides several benefits, such as high stability during circulation, protection against metabolic degradation, targeted delivery to specific tissues or cells, and the control of drug release that reduces the side effects [74].

Symmetric and non-symmetric nucleoside diphosphate prodrugs (known as Di*PP*ro) can be used to perform high levels of selective delivery. Di*PP*ro exhibits HIV-1 antiviral activity, even in mutant thymidine-deficient (CEM/TK-) cells [78]. An advantage of asymmetric Di*PP*ro is that the levels of nucleoside monophosphate are also detected along with the levels of nucleoside diphosphate in the cellular extract. The level of nucleoside monophosphate was found to be correlated with the stability of the prodrug. This stability depended on the length of the alkyl residues denoted as "R", which were attached via an ester linkage to the 4-hydroxybenzyl group. Nucleoside monophosphate is formed due to a competitive reaction, where water or hydroxide ions interact with the phosphorus atom of the β-phosphate group, leading to the cleavage of the phosphate anhydride bond and the release of nucleoside monophosphate [79].

Non-symmetric DiPPro-Nucleoside Diphosphate (NDP) prodrugs were designed using active anti-HIV nucleoside analogs of **d4T** and **AZT**. Two different masking units were attached to the β-phosphate group of the respective nucleoside diphosphate. The mechanism of delivery of these prodrugs was confirmed through hydrolysis studies conducted using phosphate buffer, RPMI culture medium, CEM/0 cell extracts, and pig liver esterase. Experiments on the enzymatic hydrolysis of prodrugs, using cell extracts and pig liver esterase, led to the formation of nucleoside diphosphates. Additionally, PBS (phosphate-buffered saline) facilitated the production of nucleoside diphosphates and nucleoside monophosphates. Among the various compounds investigated, the DiPPro-d4TDPs compounds (**7**) and (**8**) (**Figure 8**) were prominent due to their unique composition. These compounds featured distinct aliphatic ((R2 = CnH2n+1) and aromatic (R_2 = X-Ph) acyl moieties

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as lipophilic masks. Along with short aliphatic acyl moieties (R_1 = CH_3, C_4H_9) in the acyloxybenzyl-masking group, these non-symmetric DiPP compounds exhibited high activity, often similar to or even higher than the activity of the original nucleosides in wild-type CEM cell cultures. Specifically, the D4TDP derivatives (**7**) and (**8**), containing aliphatic ester functions in the acyloxybenzyl units, showed comparable activity against HIV-1 and HIV-2. This was observed within the same concentration range as that for the original **d4T** compound in wild-type CEM cell cultures. Compound (**8c**) was found to be the most potent, with an EC_{50} value of 0.10 μM for HIV-1 and 0.28 μM for HIV-2. In contrast, D4T showed an EC_{50} value of 0.52 μM for HIV-1 and 2.23 μM for HIV-2 in CEM cell cultures. The observed antiviral efficacy was also recorded in the lipophilic compounds DiPPro (**7b**) and (**8b**) (**Figure 8**), as well as compound (**8c**) (**Figure 8**), in thymidine (TK-) deficient mutant CEM cells infected with HIV-2. The antiviral activity was correlated with the lipophilicity of these compounds. This relationship occurred due to the introduction of acyl portions of the DiPPro prodrugs. These findings highlighted the efficient cellular uptake and intracellular delivery of a phosphorylated form of **d4T** (**d4T** diphosphate) [79].

Some researchers synthesized and evaluated DiPPro nucleotides, specifically a bis(benzyloxybenzyl)nucleoside diphosphate prodrug. Among these, the prodrug DiPPro-d4TDP, featuring a hydrogen donor at position 4 of the benzoyl segment (referred to as **4c**) (**Figure 8**), showed higher activity than the original compound against both HIV-1 and HIV-2. In CEM/0 cells, its EC_{50} value was 0.40 μM for HIV-1 and 0.30 μM for HIV-2. The prodrug showed an EC_{50} value of 0.85 μM against HIV-2 in CEM/TK- cells. In comparison, the unmodified compound **d4T** showed an EC_{50} of 0.86 μM for HIV-1 and 2.3 μM for HIV-2 in CEM/0 cells, as well as an EC_{50} of 173 μM for HIV-2 in CEM/TK- cells. Although the cytotoxicity of the prodrug was slightly high, with a CC_{50} of 36 μM in CEM/0 cells, the CC_{50} value of the original compound was greater than 250 μM. The chemical stability of the prodrug was assessed using PBS at pH 7.3 and CEM/0 cell extracts. It showed high stability, with a half-life ($t_{1/2}$) of 82 h at pH 7.3 and a $t_{1/2}$ of 7 h in CEM/0 cell extracts. An evaluation of the hydrolysis process and the role of enzymes indicated that the prodrug had a low retention time (tR) of 9.41 min in CEM/0 cell extracts, suggesting lower lipophilicity. Trace amounts of d4TMP were detected in this prodrug. These findings might be attributed to the dephosphorylation of d4TDP by phosphatases present in the cell extracts or the cleavage of the anhydride-phosphate bond after the hydrolysis of the benzoyl ester [80].

Schulz, Balzarini, and Meier (2014) synthetized and evaluated lipophilic **d4T** diphosphate prodrugs using the Di*PPro* approach. The convergent synthesis was performed with the protection of the β-phosphate group of nucleoside diphosphates as bis(acyloxybenzyl)phosphate esters. The **(4f)** ((*N*[nBu]4)-Bis-(4-decanoyloxybenzyl)-d4TDP) compound (**Figure 8**) exhibited an EC_{50} value of 0.080 μM for HIV-1 and 0.32 μM for HIV-2 in CEM cells, 0.11 μM for HIV-2 in CEM/TK^-, and a CC_{50} value of 62 μM. The parent drug **d4T** showed an EC_{50} value of 0.86 μM for HIV-1 and 2.3 μM for HIV-2 in CEM cells, 173 μM for HIV-2 in CEM/TK^-, and a CC_{50} value >250 μM. Compared to the parent nucleoside **d4T**, this antiviral activity was 1,570 times greater in TK-deficient CEM cells; the prodrug was more active in infected wild-type CEM cells. To assess the effects of the counterion, **C9-DiPProd4TDP 4** was saltified with ammonium and tetra-n-butylammonium. Cytotoxicity and antiviral activity remained unaffected. In studies on stability conducted in aqueous 25 mM phosphate buffer (PBS; pH 7.3), the $T_{1/2}$ for the initial hydrolysis process was 63 h with the formation of **d4TDP**, and $T_{1/2}$ of the second step was 280 h, with the formation of **d4TMP**. These findings showed that by making the prodrugs more lipophilic than the compounds synthesized earlier, the Di*PPro* method was improved [74].

Some researchers performed molecular modification of the analogs of **d4TTP** (the triphosphate form of **d4T**) involving lipophilic γ-alkyl groups and acyloxybenzyl prodrugs, to deliver *γ*-alkyl-d4TTP into cells. After synthesis, the effectiveness of those prodrugs was assessed against HIV-1 and HIV-2 infected cells. The γ-alkyl-nucleoside triphosphates were highly stable under enzymatic dephosphorylation treatment in cell extracts. They remained stable for a minimum of 30 h. These modified compounds exhibited high antiviral efficacy against both HIV-1 and HIV-2 in CD4+ T-lymphocyte CEM cell cultures (CEM/0). Additionally, they were effective against HIV-2 in cell

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cultures deficient in thymidine kinase (CEM/TK-cells), which indicated that they could pass through the cell membrane. Among these compounds, γ-C18-alkyl-d4TTP (**7z**) (**Figure 8)** was the most potent in CEM/0, showing an EC_{50} value of 0.11 μM against HIV-1 and HIV-2. Its activity against HIV-2 in CEM/TK-cells was especially high, with an EC_{50} value of 0.05 μM, representing a 2,700-fold increase in potency compared to **d4T**. These compounds were found to exclusively function as substrates for HIV-RT in primer extension assays, and they were not recognized by DNA-polymerases α, β, or γ. Thus, these prodrugs with high selectivity toward viral polymerase are promising agents for efficaciously delivering nucleoside triphosphates [81].

Due to the negative charge, nucleoside triphosphate (NTP) demands optimization using a prodrug approach. NTPs containing reversible modifications due to the inclusion of the γ-phosphate were synthesized and evaluated by some researchers. These modifications included two lipophilic masking units at the γ-phosphate and d4T. These modifications were aimed at augmenting the limited phosphorylation found in nucleoside reverse transcriptase inhibitors. A compound (**3j**) (**Figure 8**); c-Bis-(4-octadecanoyloxybenzyl)-d4TTP (R = $C_{17}H_{35}$)), characterized by two identical 4-alkanoyloxybenzyl groups, showed higher antiviral efficacy against HIV compared to the original **d4T** compound. This increase was attributed to its lipophilicity, facilitating cellular permeation. In mesenchymal stem cells (MSC) cells, its EC_{50} value was 0.17 μM for HIV-1 and 0.31 μM for HIV-2. In MSC/TK- cells, it showed an EC_{50} value of 0.28 μM for HIV-2 and a CC_{50} value of 29 μM in MSC cells. In contrast, the original d4T compound exhibited an EC_{50} value of 0.33 μM against HIV-1 in CEM cells, 0.89 μM against HIV-2 in CEM cells, and 150 μM against HIV-2 in CEM/TK- cells. It also showed a CC_{50} value of 79 μM in CEM cells. The evaluation of the hydrolysis of the prodrug in PBS at pH 7.3 showed that its half-life for the first removal of one masking unit ($t_{1/2}$ (1)) to generate intermediate (**4**) was 50 h. For the second hydrolysis step ($t_{1/2}$ (2)) leading to the formation of triphosphate (**19**), its half-life was 583 h. Enzymatic hydrolysis using cell extracts (CEM cells) showed a $t_{1/2}$ (1) of 13 h. These findings highlighted the occurrence of intracellular enzymatic hydrolysis, directly leading to the intracellular formation of phosphorylated d4T metabolites, specifically **d4TTP** in this case [82].

The TriPPPro approach is an extremely promising strategy in the field of nucleotide prodrugs, as this method has a unique ability to penetrate cell membranes without relying on cellular kinases for activation. In the nucleoside triphosphate delivery system, the terminal γ-phosphate group is concealed using a pair of lipophilic acyloxybenzyl (AB) groups that are susceptible to enzymatic cleavage [82]. The high permeability of such prodrugs leads to their accumulation in thymidine kinase-deficient CEM/TK(-) cells [77].

Prodrugs of d4T nucleoside triphosphate (NTP) analogs were obtained by investigating the TriPPPro approach. For this, the researchers incorporated distinct biodegradable masking units and an alkoxycarbonyloxybenzyl (ACB) moiety (that had a carbonate and an elongated lipophilic aliphatic chain), along with an acyloxybenzyl (AB) moiety as an ester, into the γ-phosphate group of the compound. Using this method, compounds (**8**) (γ-ACB;AB-d4TTP) and (**13**) (γ-(ACB;ACB)-d4TTP) were synthesized. Among these compounds, prodrug (**8by**) (R_1: C_2H_5; R_2: $C_{16}H_{33}$) (**Figure 8**) exhibited high antiviral activity against HIV-1 and HIV-2 in wild-type CEM/0 cell cultures. It was effective, considering that it had an EC_{50} of 0.027 μM for HIV-1 and an EC_{50} of 0.0048 μM for HIV-2. In comparison, the reference compound d4T showed EC_{50} values of 0.43 μM against HIV-1 and 0.31 μM against HIV-2. Prodrug (**8by**) showed higher activity against HIV-2 (EC_{50}: 0.11 μM) in mutant CEM/TK- cells due to its high lipophilicity. In contrast, the reference d4T showed a substantially higher EC_{50} value (31.05 μM) against HIV-2 under the same conditions. The chemical stability of these compounds was assessed in PBS at pH 7.3. Prodrug **8by** (C2-AB; C16-ACB) showed high stability with a half-life ($t_{1/2}$) of 83 h, in contrast, the reference d4TTP had a $t_{1/2}$ greater than 500 h. When the TriPPPro composite (**8by**) was hydrolyzed, the researchers found a specific cleavage of the biodegradable moiety, which resulted in the formation of an intermediate known as γ-(C16-ACB)-d4TTP or (**20y**) (**Figure 8**). This intermediate (**20y**) initially accumulated and underwent subsequent cleavage to finally yield d4TTP. In hydrolysis assays involving exposure to human CD4+ T-lymphocyte cell extracts, prodrug (**8by**) (with a $t_{1/2}$ of 1.9 h) displayed efficient cleavage, which led to the production of the intermediate γ-(C16-ACB)-d4TTP (**20y**). These findings collectively elucidated

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the asymmetric TriPPPro concept, where the γ-phosphate of the NTP was modified in a bioreversible manner. This mechanism facilitated the targeted delivery of d4TTP with high selectivity through an enzyme-activated pathway. Using this strategy, the intracellular phosphorylation steps can be avoided [83].

While synthesizing γ-non-symmetrical nucleoside analog triphosphates through the TriPPPro method, two distinct AB-masks were incorporated, which were attached to the γ-phosphate group. The evaluation was conducted in CD4+ T-lymphocyte CEM cell cultures. The compounds, denoted as γ-(AB, ab)-**d4T**, showed high efficacy against HIV-2 and comparable or slightly higher performance against HIV-1 compared to the parent nucleosides **d4T** or **d4TTP**. Among these compounds, the effect of compound (**3ce**) was prominent. This compound is a γ-(AB-iso-C_4H_9,ab-$C_{14}H_{29}$)-**d4TTP** ammonium salt. It exhibited high potency, with an EC_{50} value of 0.17 μM against HIV-1 in CEM cells and the same EC_{50} value against HIV-2. The CC_{50} value was 24 μM. The compound (**3ce**) (**Figure 8**) exhibited a 128-fold increase in activity compared to the parent compound d4T. However, the newly synthesized compounds discussed in this study had slightly lower potency than the Tri*PP*Pro compounds containing two alkylacyl AB-masks. Assessment of the chemical stability of compound **3ce** (characterized by a branched iso-butyl group) in an aqueous solution of 25 mM phosphate buffer (PB) at pH 7.3 showed a half-life ($t_{1/2}$) of 64 h. Additionally, the principal hydrolysis products identified included the original drugs (**d4TTP** and **d4TDP**) and small quantities of **d4TMP** (with concentrations less than 4%) [84].

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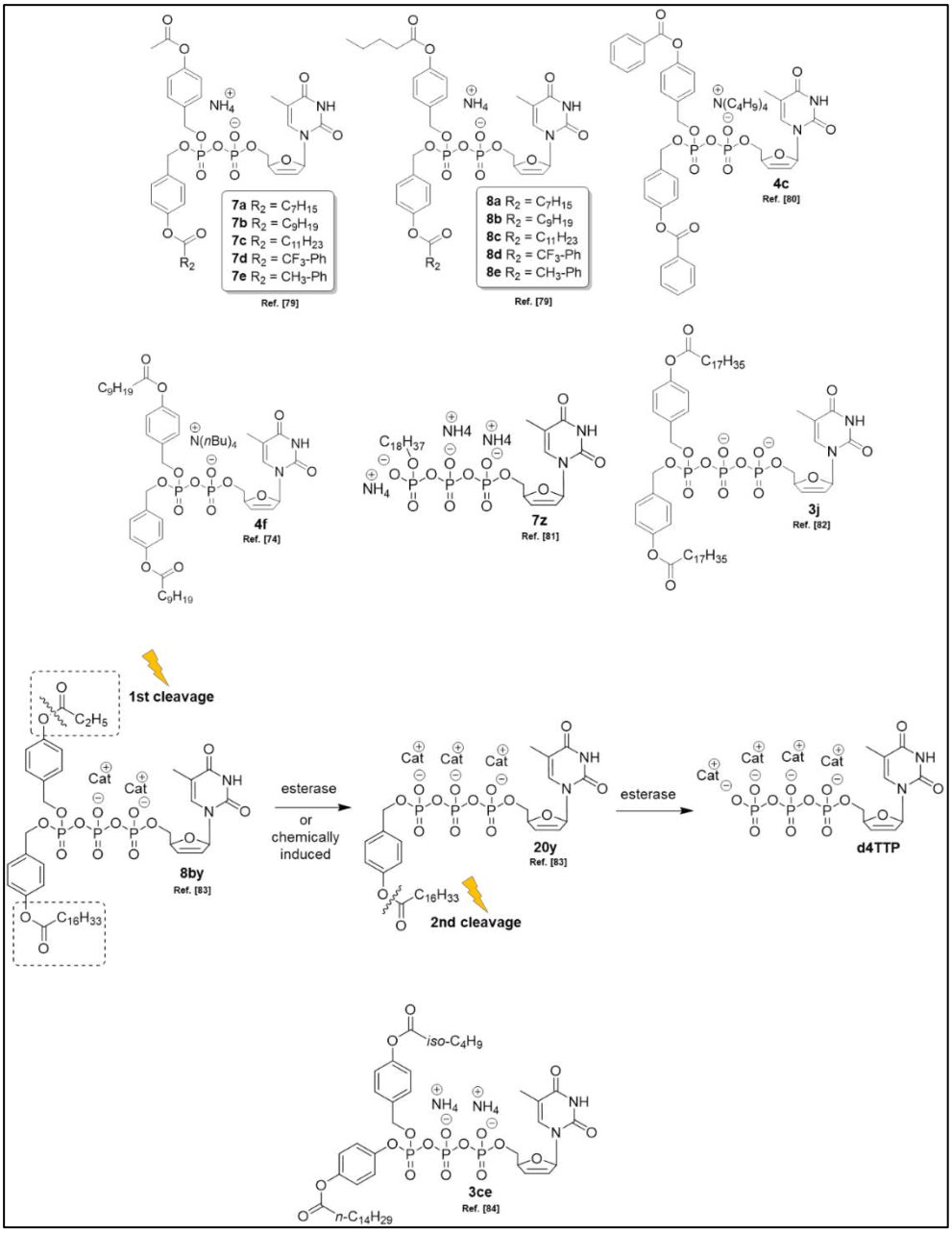

Figure 8. Stavudine prodrugs.

2.5. Emtricitabine prodrugs

Emtricitabine (**FTC**) is a nucleoside transcriptase reverse inhibitor administered orally once a day; the (-) enantiomer is more active than its antipode. It is used in combination with antiviral drugs for HIV treatment, as well as in pre-exposure prophylaxis (PrEP). The drug has a relatively long half-life, and its main adverse effects include headache, nausea, diarrhea, and fatigue. In some cases, it can cause changes in kidney function and bone density. Lactic acidosis and hepatomegaly with steatosis are rare events [85].

Extended-release injectable (ERI) formulations have been investigated for the treatment of several diseases, including AIDS, as they can address challenges linked to medication non-adherence. For HIV treatment, the approval of ERI containing rilpivirine and cabotegravir provided a formulation that allowed therapeutic plasma concentrations of drug for up to 20 weeks [86, 87]

Three **FTC** prodrugs were synthesized (**PD1 – PD3**) (**Figure 9**) and incorporated into injectable aqueous semi-solid prodrug nanoparticles (SSPNs). The pharmacokinetics of SSPNs containing

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prodrugs were evaluated over 28 days through intramuscular injection in Wistar rats, New Zealand white rabbits, and Balb/C mice. Two formulations were selected to evaluate their potential to prevent HIV infection in NSG-cmah−/− humanized mice, based on the study of pre-formulations. After injection, the peak concentration (Cmax) was reached by 12 h in rats, 48 h in rabbits, and 24 h in mice. Plasma concentrations fell below the detectable threshold of 2 ng/mL by day 21 in rats and rabbits and by day 28 in mice. Even 28 days after infection, HIV RNA remained undetectable in the plasma, spleen, lung, and liver samples [87].

Targeting the primary reservoir of HIV lymphoid tissues and infected cells is a huge challenge that needs to be solved to eliminate the virus and achieve a functional cure in the next few years [88]. Class A scavenger receptors (SR-A), which are expressed in myeloid type cells (i.e., monocytes, macrophages, and dendritic cells), might be used to selectively deliver the drug to those cells, which can decrease undesirable systemic effects [89]. Endothelial scavenger receptor-A (SR-A) facilitates the transcytosis of modified LDL in the lymphatic system. This provides an opportunity for targeted delivery via SR-A to the lymphatic system [89].

The **FTC** prodrug, known as **PLS-FTC** (**Figure 9**), was designed through esterification by conjugating it with poly(L-lysine succinylated) (PLS) to increase its distribution in the lymphatic system. In vitro evaluations of PLS-**FTC** showed a release half-life of 15 h in human plasma and 29 h in plasma with inhibited esterase. After incubation for 24 h in peripheral blood mononuclear cells (PBMCs), the drug released from PLS-FTC was converted into the active metabolite **FTC** triphosphate. The ratio of active metabolite to precursor (FTC-TP/**FTC**) was 1.3 compared to the initial ratio of 0.4, which indicated that the conversion of the prodrug was more efficient. In pharmacokinetic assessments conducted in rats, PLS-**FTC** concentrations were 7–19 times higher than **FTC** concentrations in lymphatic tissues. This finding emphasized the enhanced distribution potential of PLS-**FTC** in lymphatic tissues, highlighting that this macromolecular platform might be effective [90].

FTC-based ester conjugates were prepared and evaluated against cell-free and cell-associated HIV-1 viruses. This myristoylated **FTC** conjugate, referred to as compound (**5**) (**Figure 9**), exhibited high lipophilicity with a log p-value of 5.96, whereas for **FTC**, the calculated value was –1.29, which limited its permeability and its effective internalization by cells. At the highest tested concentrations, prodrug (**5**) showed negligible cytotoxicity (CC_{50} > 200 μM). This myristoylated conjugate showed high anti-HIV activity. Against cell-free viruses, it exhibited an EC_{50} of 0.07–0.1 μM, which was greater than the EC_{50} of **FTC** alone by approximately 10–19 times (EC_{50} = 0.7–1.9 μM). Compound (**5**) showed an EC_{50} of 3.7 μM against cell-associated viruses and outperformed **FTC** by approximately 24-fold (EC_{50} = 88.6 μM). When evaluated against clinical HIV isolates, prodrug (**5**) demonstrated potent anti-HIV activity against clade B and C clinical isolates, with IC_{90} values of 6.6 and 10.9 nM, respectively. These values were considerably higher than those of **FTC** (IC_{90} = 32.4 and 161.9 nM), exhibiting a 5-fold to 15-fold increase in potency. Additionally, when tested against drug-resistant viral mutations, specifically those resistant to non-nucleoside reverse transcriptase inhibitors (B-NNRTI) and those exhibiting **TDF**-NRTI resistance (B-K65R), prodrug (**5**) exhibited IC_{90} values of 15.7 nM and 16.1 nM, respectively. This presented a substantial decrease in potency compared to FTC (IC_{90} = 103 and 567 nM) with reductions of 6.6-fold and 35.2-fold. Cellular uptake studies in which human T-lymphoblastoid cells (CCRF-CEM, ATCC No.CCL-119) were exposed to prodrug 5 (50 μM) for 1–24 h at 37 °C confirmed through HPLC analysis that prodrug (**5**) was internalized within 1 h of incubation (retention time: 18.2 min). However, at the 12th hour, intracellular levels of prodrug (**5**) decreased, suggesting hydrolysis to **FTC** catalyzed by intracellular esterases. The HPLC profiles also revealed peaks at 1.7–2.9 min, indicating that intracellular hydrolysis occurred and potentially phosphorylated products were present. Prodrug (**5**) disappeared from cell extracts after 24 h, with a distinct peak indicating the presence of metabolic products at 1.7–2.9 min [91].

NTP prodrugs can considerably enhance cellular uptake by infected cells. The antiviral activity profiles against HIV-1 and HIV-2 displayed substantial variations in potency, depending on the lipophilicity of the NTP prodrugs. Studies using prodrugs of emtricitabine (FTC), denoted as 8dy, were conducted on cultures of infected wild-type CD4+ CEM T-cells and thymidine kinase-deficient

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CD4+ T-cells (CEM/TK−). Specifically, the NTP prodrug (**8dy**) (**Figure 9**) (with R1: C_4H_9 and y-(C4-AB; C16-ACB)), showed high antiviral effectiveness against HIV-2 CEM/TK−. This effectiveness was indicated by a lower EC_{50} of (**8dy**) (EC_{50} = 0.029 μM) compared to that of its parent nucleoside FTC (EC_{50} = 0.046 μM). For infected wild-type CD4+ CEM T-cells, prodrug (**8dy**) exhibited a robust HIV-1 EC_{50} of 0.0043 μM and an HIV-2 EC_{50} of 0.0087 μM. The prodrug outperformed the reference compound FTC with EC_{50} values of 0.010 μM for HIV-1 and 0.016 μM for HIV-2. When the stability of prodrug (**8dy**) in cell extracts was assessed via hydrolysis, it showed a considerably rapid formation of nucleoside analog triphosphates in human T-lymphocyte CD4+ CEM cell extracts. Their half-lives ranged from 1.1 h to 5 h, independent of the specific attached nucleoside. This finding suggested that an enzymatic cleavage occurred. Compound (**8dy**) underwent rapid hydrolysis, generating the corresponding NTPs in concentrations similar to that recorded in other stability assays (CEM/0 $t_{1/2}$ 2.78 h). This confirmed the release of NTPs in biological media, such as T-lymphocyte extracts. These findings indicated that **FTC** prodrugs can be further modified to improve the effects of nucleoside analogs into potent biologically active metabolites [83].

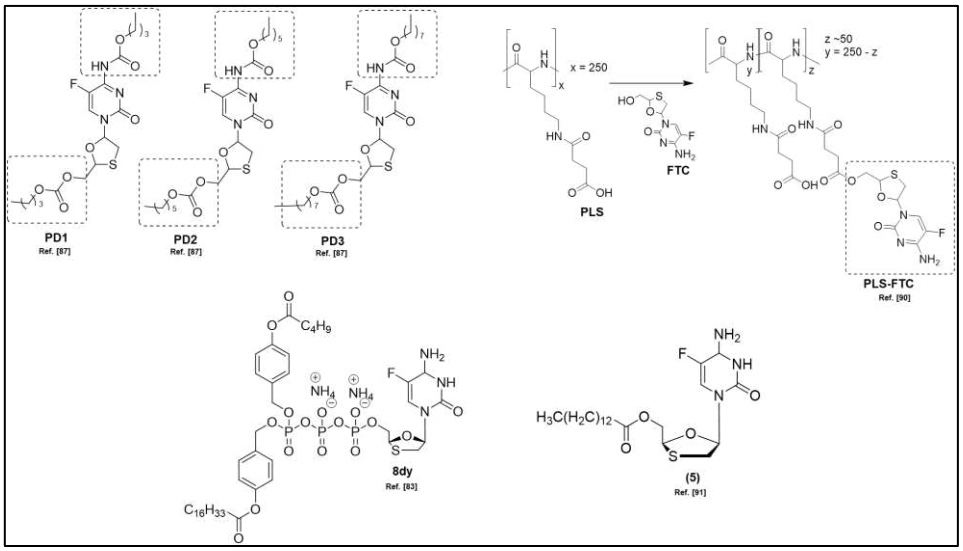

Figure 9. Emtricitabine prodrugs.

2.6. Abacavir (ABC) prodrugs

The presence of HIV in critical locations, like the central nervous system, poses a significant challenge to antiretroviral therapy, as penetrating the blood-brain barrier is difficult. This issue contributes to the maintenance of the HIV-1 reservoir. P-glycoprotein (P-gp), a member of the ATP-binding cassette (**ABC**) family of multidrug resistance transporters, is a major contributor to this limitation. P-gp is highly expressed in brain capillaries, which further hinders access to antiretroviral drugs. Strategies involving P-gp inhibition might be effective in eradicating the HIV reservoir. One such strategy involves **ABC**, a P-gp substrate. **ABC** dimers were synthesized and evaluated by inhibiting P-gp efflux at the blood-brain barrier and reverting to monomeric therapeutic forms under reducing conditions. **ABC** dimers were evaluated in vitro using a P-gp overexpressing T lymphoblastoid cell line (12D7-MDR) via flow cytometry assay to determine their inhibitory effect on P-gp-mediated transport of fluorescent substrates calcein-AM and 4-Fluoro-7-nitro-2,1,3-benzoxadiazole-abacavir (NBD-**ABC**). All dimers strongly inhibited P-gp efflux for the fluorescent substrates. Among these, **ABCS2Me4** (**Figure 10**), characterized by the linkage of two monomers via an ester containing a disulfide and featuring four additional methyl units adjacent to the ester carbonyl in the tethers (represented as Me4), showed the highest activity. Its IC_{50} values were 0.6 μM for Calcein-AM, 0.7 μM for NBD-ABC, and 65 nM for [^{125}I] IAAP competition. **ABCS2Me4** exhibited an extended half-time of over 100 h in human plasma and 17.2 h in dithiothreitol (DDT). This significant improvement in stability, which was approximately four-fold greater than **ABC**, indicated the reversion rate of abacavir prodrug dimers. **ABCS2Me4** was inactive in an in vitro reverse

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transcriptase (RT) assay, suggesting that its cellular antiviral activity was linked to the reversion of the prodrug dimer to the RT-active monomeric parental drug **ABC**. Thus, the researchers inferred that the conversion of **ABC** from the dimer form to the therapeutic form in the reducing environment of the cell was responsible for its antiviral activity. This mechanism can be used to modify the release rate of the abacavir monomer from the prodrug [92].

Some researchers synthesized and evaluated lipophilic NTP-**ABC** (triphosphate prodrugs of abacavir), along with their 1′,2′-cis-disubstituted analogs, in T-lymphocyte cell cultures (CEM cells) infected with either HIV-1 or HIV-2. Compared to their respective parent compounds, prodrug (**21**) (**NTP-ABCTP**, ammonium salt) (**Figure 10**) showed 4.5 times higher activity against HIV-1. Prodrug (**21**) showed EC_{50} values of 1.3 μM for HIV-1 and 1.4 μM for HIV-2 in CEM/0 cells. It also showed an EC_{50} of 2.1 μM for HIV-2 in CEM TK-cells and a CC_{50} of 60 μM in CEM T-cell cultures. In comparison, the parent drug monophosphate **ABC** showed EC_{50} values of 5.9 μM for HIV-1 and 5.2 μM for HIV-2 in CEM cells. It also exhibited an EC_{50} of 7.0 μM for HIV-2 in CEM TK-cells and a CC_{50} value of 135 μM in CEM T-cell cultures. No activity of 1′,2'-cis substituted nucleoside was detected. The provided triphosphates were not eliminated, and the compounds could not be rephosphorylated from their monophosphate or diphosphate forms. Even if triphosphates are produced, HIV reverse transcriptase may not use them as substrates. This aspect might be further investigated by conducting primer-extension tests using reverse transcriptase and separated triphosphates [93].

ABC-S2-NFV1 (**Figure 10**), a linked heterodimeric compound consisting of **NRTI ABC** and the protease inhibitor nelfinavir, was synthesized as a prodrug to address the therapeutic evasion of P-glycoprotein (P-gp) through a Trojan horse strategy involving a combination of dual mode of action. This mutual prodrug could strongly inhibit HIV P-gp efflux, even in human brain endothelial cells. The linkage between ABC and nelfinavir was established via an ester linkage with a disulfide-containing ether. In the cellular reducing environment, the central disulfide is reduced, generating thiols that rearrange to release the monomeric drugs. **ABC-S2-NFV1** showed high anti-HIV activity, as determined by the p24 protein levels in 12D7 cell lines infected with HIV-1LAI. At concentrations of 0.08, 0.31, and 1.25 μM, ABC-S2-NFV1 decreased the content of p24 (< 100 pg/mL) to levels lesser than that recorded in the control (> 500 pg/mL and < 1,000 pg/mL) and similar to that recorded after treatment with a 1:1 mixture of ABC and Nelfinavir (< 100 pg/mL of p24). ABC-S2-NFV1 also showed a dose-dependent potent inhibition of P-gp efflux in assays involving fluorescent substrates calcein-AM and NBD-ABC. This was found in 12D7-MDR cells (CD4+ T lymphocytes with P-gp overexpression) and hCMEC/D3 cells (immortalized human brain capillary endothelial cells expressing P-gp). The IC_{50} values for **ABC-S2-NFV1** were submicromolar, ranging from 0.41 to 0.77 μM for different substrates and cell types. In comparison, nelfinavir had higher IC_{50} values (ranging from 1.7 to 9.1 μM), indicating that its inhibitory effect was less potent. The results of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assays showed that **ABC-S2-NFV1** maintained cell viability (>95%) without inducing toxicity at its maximum tested concentration (20 μM). In experiments that simulated a reducing environment, where disulfide bonds within the heterodimers yielded thiols that rearranged into monomeric drugs (**ABC** and nelfinavir), the regeneration process was fast (full reduction in 1 h). The half-life ($t_{1/2}$) of ABC regeneration from heterodimer ABC-S2-NFV1 was 31.8 h with dithiothreitol (DTT) and 29.4 h with glutathione (GSH). Nelfinavir regeneration had a $t_{1/2}$ of 1.2 h with DTT and 10.3 h with GSH, releasing faster than ABC, due to the phenolic moiety's better leaving group ability in nelfinavir within ABC-S2-NFV1 [94].

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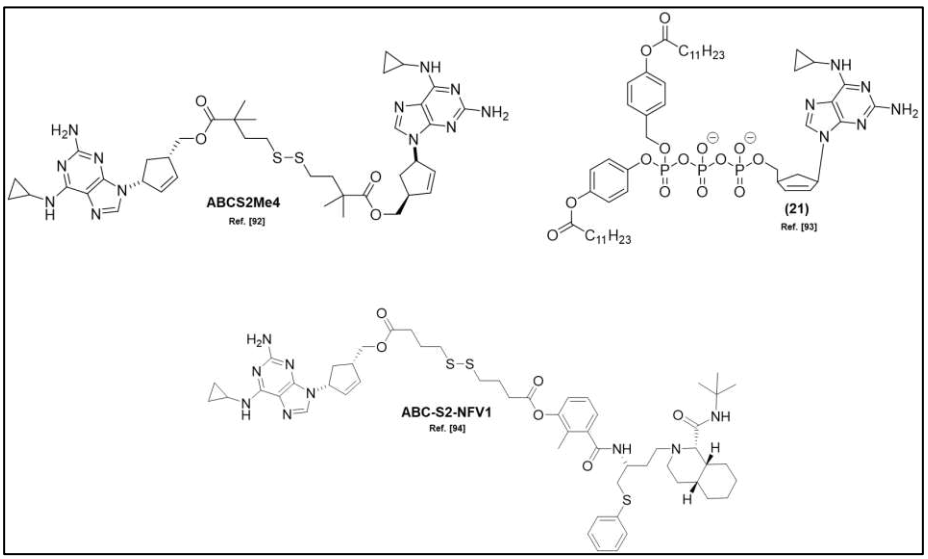

Figure 10. Abacavir prodrugs.

2.7. Others

Membrane-associated drugs can profoundly influence the pharmacokinetic, safety, and efficacy attributes of medicinal agents. This approach is particularly valuable in drug discovery, especially for compounds facing pharmacokinetic challenges. Many membrane transporters can perturb the internal movement of bioactive substances and alter their absorption and biodistribution; these transporters include members of the ATP-binding cassette (**ABC**) and solute carrier (SLC) transporter superfamilies. For example, certain SLC transporters like OAT1 (SCL22A6) act as selective substrates for compounds such as adefovir, ciclofovir, zidovudine, lamivudine, and tenofovir. These substrates are present in organs like the placenta and the proximal tubule of the kidneys. They affect drug disposition and elimination, and thus, play a role in drug-drug interactions [95, 96]

One of the best examples of drug design exploring the use of membrane transporters to increase bioavailability is the antiviral valacyclovir. This drug is commonly prescribed for treating genital herpes (herpes simplex virus type 2), cold sores (herpes simplex virus type 1) in adults, and shingles (herpes zoster). It can reduce the severity of herpes outbreaks in individuals with a weak immune system, such as those living with HIV/AIDS [97]. Valacyclovir exhibits 3–5 greater oral bioavailability (~ 55%) than its parental drug acyclovir, which is attributed to its recognition and transport by PepT1 [98-100].

Enhancing the ability of antiretroviral drugs to penetrate the CNS can decrease the severity of HIV-1 infection in the brain and mitigate the cognitive impairments linked to it [101, 102]. One example of this mechanism is the transporter called Breast cancer resistance protein (BCRP), which is expressed in human brain microvessel endothelial cells and mouse brain capillaries. For this transporter, Abcg2-/- knockout mice exhibited elevated levels of abacavir in the brain, suggesting the in vivo participation of Bcrp in transporting antiretroviral drugs across the blood-brain barrier (BBB) [103]. Another example includes the transporter MRP1 involved in the entry of emtricitabine inside lymphocytes. Mrp2-/- deficient rats showed a considerable decrease in the hepatobiliary elimination of **TDF** [104, 105].

Investigating membrane transporters for designing drugs is a difficult task for HIV therapy, as many factors can influence the expression of transporters responsible for the influx and efflux of drugs [106] Efflux transporters, such as P-gp and BCRP, which are highly expressed in the apical surface of enterocytes can be influenced by protease inhibitors (e.g., ritonavir and darunavir). An increase in the level of **TDF** in the bloodstream was also associated with the inhibition of P-glycoprotein (P-gp) due to concurrently administered protease inhibitors, such as atazanavir, lopinavir, ritonavir, and darunavir [107].

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Intestinal permeability can also be altered by food intake, inflammatory status, and the presence of viral proteins (e.g., Tat, gp120). High levels of pro-inflammatory cytokines and oxidative stress can also regulate **ABC** transporters [108, 109] These findings showed that **ABC** drug transporters might be modulated by inflammation and oxidative stress linked to HIV-1, potentially leading to changes in the distribution of antiretroviral drugs in different organs [96].

Utilizing drug delivery systems can improve drug transportation while preventing the concurrent inhibition of multiple transporters, a situation that might otherwise lead to harm by enabling the permeation of toxins. Pharmacologically modifying specific transporters by simultaneously administering HAART and transporter-targeted inhibitors is an effective strategy for elevating levels of anti-HIV drugs in tissues [101]. Miller et al. (1997) reported that micelles of pluronic copolymers can inhibit P-gp, and thus, can influence drug transport [110]. Incorporating antiviral drugs (e.g., **AZT** and **3TC**) with pluronic copolymers into human monocyte-derived macrophages infected with HIV-1 resulted in a considerable reduction in the percentage of HIV-1-infected monocyte-derived macrophages (8–22% of the control). The antiviral impact of this modification exceeded that of the antiretroviral drugs, reaching 38% of the control [111].

The function, distribution, and transport potency need to be further studied to design new therapeutic antiviral drugs. New prodrugs need to be designed to enhance the passage of drugs through blood-testicular, blood-mucosa, blood-cerebrospinal fluid, and blood-brain barriers.

3. Conclusions and Oulook

Although HIV treatment has progressed considerably, several limitations and challenges still need to be addressed, including therapy non-adherence, long-term toxicity, improvements in drug efficacy, and elimination of the viral reservoir. The prodrug approach is a promising tool to address these main drawbacks, particularly for overcoming the problems related to NRTIs. An example of the successful utilization of the prodrug approach is **TDF** alafenamide, which received FDA approval in 2016.

In this review, we focused on NRTIs and presented several illustrative examples demonstrating the promising application of prodrugs to enhance solubility, bioavailability, pharmacokinetics, and drug delivery. Challenges related to targeting specific tissues/organs, such as the central nervous and lymphatic systems, have been effectively addressed through this approach. We highlighted several perspectives for designing prodrugs to treat neuroAIDS, where inadequate NRTI levels in the CNS are a common challenge. We also discussed approaches to reduce HIV reservoirs by investigating ways to deliver drugs to the lymphatic system. The combination of the prodrug approach with suitable formulations is an effective strategy to enhance the efficacy and safety of treatment, and it might considerably reduce the duration of treatment. Understanding the function and distribution of membrane transporters might help deliver and accumulate drugs in desired compartments. In this review article, we only highlighted promising strategies that should be pursued to advance therapy in the future.

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Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, L.F.R., J.R.L., A.F.B., J.L.B.P., C.B.S. and J.L.D.S.; methodology, L.F.R., J.R.L., A.F.B., J.L.B.P., C.B.S. and J.L.D.S.; software, L.F.R., J.R.L., A.F.B., C.B.S. and J.L.D.S; validation, L.F.R., J.R.L., A.F.B., J.L.B.P., C.B.S. and J.L.D.S.; formal analysis, L.F.R., J.R.L., A.F.B., C.B.S. and J.L.D.S.; investigation, L.F.R., J.R.L., A.F.B., J.L.B.P., C.B.S. and J.L.D.S.; resources, C.B.S and J.L.D.S.; data curation, L.F.R., J.R.L., A.F.B., C.B.S. and J.L.D.S.; writing—original draft preparation, L.F.R., J.R.L., A.F.B., J.L.B.P., C.B.S. and J.L.D.S; writing—review and editing, L.F.R., J.R.L., A.F.B., J.L.B.P., C.B.S. and J.L.D.S; visualization, L.F.R., J.R.L., A.F.B., J.L.B.P., C.B.S. and J.L.D.S; supervision, C.B.S. and J.L.D.S; project administration, L.F.R., J.R.L., A.F.B., C.B.S. and J.L.D.S; funding acquisition, C.B.S., J.L.B.P. and J.L.D.S. All authors have read and agreed to the published version of the manuscript."

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