Title Page

Title:

Cell and tissue specific metabolism of nucleoside and nucleotide drugs: case studies and implications for precision medicine

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Running Title Page

Running title:

Tissue specific metabolism of nucleoside/nucleotide drugs

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Number of text pages: 18

Number of figures: 1

Number of tables: 0

Number of references: 140

Number of words in abstract: 236

Number of words in introduction: 368

Number of words in minireview body (excluding introduction and conclusion): 4,698

Number of words in minireview conclusion: 570
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<td>CMV</td>
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Abstract

Many clinically used antiviral drugs are nucleoside or nucleotide analogue drugs, which have a unique mechanism of action that requires intracellular phosphorylation. This dependence on intracellular activation presents novel challenges for the discovery and development of nucleoside/nucleotide analogue drugs. Contrary to many small molecule drug development programs that rely on plasma pharmacokinetics and systemic exposures, the precise mechanisms that result in efficacious intracellular nucleoside triphosphate concentrations must be understood in the process of nucleoside/nucleotide drug development. The importance is highlighted here, using the following as case studies: the herpes treatment acyclovir, the cytomegalovirus therapy ganciclovir, and human immunodeficiency virus (HIV) treatments based on tenofovir, which are also in use for HIV prophylaxis. For each drug, the specificity of metabolism that results in its activation in different cells or tissues is discussed, and the implications explored. Acyclovir’s dependence on a viral enzyme for activation provides selective pressure for resistance mutations. Ganciclovir is also dependent on a viral enzyme for activation, and suicide gene therapy capitalizes on that for a novel oncology treatment. The tissue of most relevance for tenofovir activation depends on its use as treatment or as prophylaxis, and the pharmacogenomics and drug-drug interactions in those tissues must be considered. Finally, differential metabolism of different tenofovir prodrugs and its effects on toxicity risk are explored. Taken together, these examples highlight the importance of understanding tissue specific metabolism for optimal use of nucleoside/nucleotide drugs in the clinic.
Significance Statement

Nucleoside and nucleotide analogue drugs are cornerstones in current antiviral therapy and prevention efforts that require intracellular phosphorylation for activity. Understanding their cell and tissue specific metabolism enables their rational, precision use for maximum efficacy.
Introduction

Nucleoside and nucleotide analogue drugs play a key role in human health, as they are the cornerstone of many antiviral and anticancer treatments (Jordheim et al., 2013). These small molecule drugs tend to interact with enzymes involved in the processes of DNA or RNA replication, a crucial part of the viral or cancerous cell life cycle. Recent efforts to repurpose nucleoside analogues as antibacterial drugs highlight their versatility and importance (Thomson and Lamont, 2019). In order to be recognized by target enzymes, nucleoside/nucleotide drugs must be phosphorylated and mimic the nucleotide triphosphates that are the natural substrates. Phosphorylation needs to occur intracellularly, as triphosphate molecules are too polar to cross cell membranes, let alone the tissues that are barriers to oral drug absorption. Typically the first phosphorylation step that forms a monophosphate analogue is rate limiting (Zenchenko et al., 2021). One development strategy that has shown some success for certain nucleoside analogs in overcoming this limitation is the use of nucleoside phosphoramidate prodrugs (Slusarczyk et al., 2018).

Due to this dependence on intracellular phosphorylation for activity, it is now understood that the development of nucleoside and nucleotide analogue drugs cannot rely solely on information gleaned from plasma pharmacokinetics. In order to enable their most optimal use, the intracellular processes that activate these drugs must be understood (Varga et al., 2016). Furthermore, the processes required for activation must occur in the cells containing the target and it is expected that enzymatic activities may vary across cell and tissue types. In the current review, I highlight examples of cell or tissue specific metabolism of antiviral nucleoside/nucleotide drugs and explore the implications of this specificity. There are examples of antiviral selectivity gained through activation only in viral infected cells and efforts to...
combine such selectivity with gene therapy to repurpose an antiviral into an oncology treatment. Tissue specific drug activation results in unique considerations regarding pharmacogenomics and drug-drug interactions within mucosal tissues. Different prodrug moieties applied on the same drug may also be differentially metabolized by different cells--this can alter the toxicity profile and clinical relevance of the drug. Chemical structures of drugs discussed in detail, acyclovir, ganciclovir, and tenofovir, are shown in Figure 1, along with their relevant prodrugs.

**Acyclovir: a safe and well-tolerated herpes simplex virus and varicella zoster virus treatment that is activated in virus infected cells**

Herpesviruses are a family of large DNA viruses consisting of a linear double stranded DNA core, an icosahedral protein capsid, an unstructured protein layer known as the tegument, and an outer envelope. This outer envelope is composed of lipids obtained from the host cell and contains viral glycoproteins which play a role in cell entry (Zarrouk et al., 2017). After primary infection, herpes simplex viruses (HSV-1 and HSV-2) establish latent populations in the host and may sporadically reactivate, resulting in orofacial and genital lesions which can be accompanied with symptoms such as pain, burning sensations, fever, and headache (Whitley and Roizman, 2001). Patients with clinical symptoms are more likely to shed viral particles, though shedding is known to occur in asymptomatic patients as well. In immunocompromised or neonatal patients, herpesviruses can cause much more severe disease, and there is significant risk of morbidity and mortality resulting from viral reactivation in post-transplant patients (Ivana et al., 2022; Jenkins et al., 2003). Given the prevalence of HSV, affecting 60-95% of human adults, herpesvirus infections represent a significant and ongoing disease impacting human health (Fatahzadeh and Schwartz, 2007).
Acyclovir has been in use for the management of HSV infections since the early 1980s (Whitley and Baines, 2018). Acyclovir is an analogue of guanosine, with a 2-hydroxyethoxyxymethyl acyclic side chain in place of the cyclic sugar (Majewska and Mlynarczyk-Bonikowska, 2022). The lack of a 3’ hydroxy group results in DNA chain termination when the active moiety, acyclovir triphosphate, is incorporated in the process of viral DNA synthesis. Valacyclovir is a L-valine ester prodrug of acyclovir that is taken up via dipeptide transporters in the gut and rapidly converted by intestinal and hepatic esterases to release acyclovir into the systemic circulation (MacDougall and Guglielmo, 2004). Oral administration of valacyclovir significantly improves on acyclovir’s oral exposure, increasing bioavailability from 15-30% to 54% (Bras et al., 2001; Majewska and Mlynarczyk-Bonikowska, 2022; Soul-Lawton et al., 1995). This improved exposure of valacyclovir allows for less frequent dosing and greater efficacy, while maintaining acyclovir’s safety and tolerability (Beutner et al., 1995; Chen et al., 2017). However valacyclovir has not been evaluated in comprehensive clinical trials for all of the indications for which acyclovir is standard of care, and valacyclovir is not approved for use in immunocompromised patients (Vigil and Chemaly, 2010). Despite the oral bioavailability advantages of valacyclovir, intravenous acyclovir continues to be used in pediatric and neonatal populations due to their increased risk of severe disease (Abdalla et al., 2020; Harris and Holmes, 2017; Poole and Kimberlin, 2018). Intravenous acyclovir is also administered for adult patients with symptoms of serious HSV disease such as hepatitis, meningitis, or encephalitis (Johnston, 2022). The intravenous administration is advantageous for rapidly increasing systemic acyclovir concentrations, and after severe symptoms have subsided, patients transition to oral suppressive therapy. Acyclovir and its prodrug valacyclovir remain the first line antivirals for treatment and suppression of lesions occurring from herpes simplex virus
infections (Majewska and Mlynarczyk-Bonikowska, 2022). They are also effective in managing varicella-zoster virus (VZV) infections and reactivation (Boeckh et al., 2006; Cunha and Baron, 2017).

The active moiety resulting from administration of both valacyclovir and acyclovir that interacts with the herpesvirus DNA polymerase is acyclovir triphosphate. Acyclovir triphosphate competes with deoxyguanosine triphosphate for incorporation into the newly synthesizing DNA chain by viral DNA polymerase (Furman et al., 1984). This triphosphate needs to be formed intracellularly once acyclovir enters a cell, through three phosphorylation steps. The first step is known to be carried out by herpes virus specific thymidine kinase UL23 and the remaining two steps by human guanylate kinase and human phosphoglycerate kinase (Deville-Bonne et al., 2010; Elion, 1983; Fyfe et al., 1978). HSV infected cells produce 40-100 times more intracellular acyclovir triphosphate than uninfected cells (Elion, 1982). Acyclovir triphosphate is also a 10-30 fold more potent inhibitor of HSV DNA polymerase compared to cellular DNA polymerase. Combined, the two above factors result in up to a 3000- fold more potent inhibition of HSV-1 multiplication versus inhibition of uninfected host cell growth (Elion et al., 1977). This reliance on a viral enzyme for activation of acyclovir results in an exquisite specificity for virus infected cells, thereby improving selectivity and decreasing risk of drug related adverse events.

As expected, the dependence of acyclovir on viral thymidine kinase results in resistant strains of virus with mutations in thymidine kinase. Exposure of VZV-infected human fibroblasts to acyclovir resulted in selection for VZV strains that had decreased thymidine kinase function or altered DNA polymerase susceptibilities (Biron et al., 1982). Mutations in the thymidine kinase gene appear to be more prevalent clinically—in one study, 43 strains of HSV-1 or HSV-2 with acyclovir resistance were isolated, and 36 of those strains had mutations in the thymidine kinase
gene (Sauerbrei et al., 2011). It is estimated that the molecular basis behind 95% of acyclovir resistant HSV isolates is deficient production of thymidine kinase or alteration of thymidine kinase specificity (Burrel et al., 2010).

Mutations in both the conserved and nonconserved regions of HSV-1 thymidine kinase are associated with acyclovir resistance (Sergerie and Boivin, 2006). The thymidine kinase deficient strains may have reduced pathogenicity, however those with altered specificity may retain pathogenicity and neurovirulence (Darby et al., 1981). Work is ongoing to characterize the virulence and resistance phenotypes of specific mutations in acyclovir resistant strains (Bleymehl et al., 2011; Frobert et al., 2005; Schubert et al., 2014). This will enable the use of genotypic testing of acyclovir resistant isolates to guide rational clinical practice (Piret and Boivin, 2011; 2016).

Ganciclovir: a cytotoxic nucleoside drug activated in virus infected cells with implications for cytomegalovirus and oncology treatment

Cytomegalovirus (CMV) is a β-herpesvirus with worldwide prevalence estimated to range from 40%-100% of various human populations. Rates tends to be lower in Western Europe and the United States, whereas CMV prevalence is as high as 100% among adults in Africa, South America, and Asia (de Melo Silva et al., 2020; Jerry Teng et al., 2021). Similarly to HSV, the CMV virion consists of a double stranded DNA core, an icosahedral protein capsid, an unstructured integument, and an outer lipid bilayer envelope. Subsequent to primary infection, lifelong latency of CMV is established in cells of the myeloid lineage in a homeostatic balance with the immune system (Al Mana et al., 2019). In healthy adults, CMV infection is typically asymptomatic or manifests as mild symptoms including fatigue, fever, and sore throat. However
in neonates and immunocompromised adults, such as post-transplant patients or those with acquired immunodeficiency syndrome (AIDS), CMV infection is associated with more significant adverse effects (Drew, 1988; Lancini et al., 2014). Congenital CMV infections, wherein the virus is transmitted \textit{in utero} from mother to fetus, are a known cause of birth defects and developmental disabilities (Cannon et al., 2010; Hyde et al., 2010). CMV infection is a complication of both allogeneic hematopoietic stem cell and solid organ transplants which can cause retinitis, pneumonitis, hepatitis, colitis, and myriad other symptoms (Jerry Teng et al., 2021; Lumbreras et al., 2014). Active CMV disease in post-transplant patients leads to a poor prognosis and higher chance of morbidity and mortality (Azevedo et al., 2015).

First line therapy for CMV treatment and prophylaxis is intravenous ganciclovir or oral valganciclovir. Ganciclovir is an acyclic guanosine analogue that differs from acyclovir by having a hydroxymethyl group which places -OH at the 3’ position of the putative “sugar”. Despite this, ganciclovir is 33-fold more potent against CMV than acyclovir (Fletcher and Balfour, 1989). The oral bioavailability of ganciclovir is poor, with absolute bioavailability estimated to be 2.6%-8.9% in CMV or HIV infected patients. (Anderson et al., 1995; Spector et al., 1995). The exposure of ganciclovir in humans increases when taken with food, as shown in a crossover study that observed a 21.6% increase in AUC$_{0-8h}$ \((p < 0.001)\) from co-administration of ganciclovir with a high fat meal versus after an overnight fast--however this is still low bioavailability (Lavelle et al., 1996). Valganciclovir is a valyl ester prodrug which is rapidly metabolized by intestinal and hepatic esterases to release ganciclovir into the systemic circulation. Oral valganciclovir has been shown to increase systemic bioavailability of ganciclovir up to 60% (Stockmann et al., 2015; Wiltshire et al., 2005).
Whether valganciclovir or ganciclovir is the administered drug, ganciclovir triphosphate is the species that inhibits CMV DNA polymerase. The first phosphorylation step of ganciclovir is carried out by CMV kinase UL97, helping to accumulate ganciclovir triphosphate specifically in CMV-infected cells (Freitas et al., 1985; Matthews and Boehme, 1988). Subsequent phosphorylation steps can be performed by cellular guanylate kinase and phosphoglycerate kinase. Similarly to acyclovir, this reliance on a kinase only present in virus infected cells for initiation of ganciclovir phosphorylation confers favorable selectivity. As expected, this reliance results in resistant CMV strains that alter their ability to phosphorylate ganciclovir (UL97) rather than their DNA polymerase (UL54) (Drew, 1991; Stanat et al., 1991). Ganciclovir resistant CMV via DNA polymerase mutation also occurs, and these strains have the added benefit of cross-resistance to second line drugs foscarnet and cidofovir as well (Erice, 1999; Smith et al., 1997). However the majority of ganciclovir resistant clinical isolates tend to have mutations in the UL97 phosphotransferase gene (Chou, 2008; Chou et al., 1995). Ganciclovir is also phosphorylated by HSV thymidine kinase, however acyclovir is the preferred therapy for HSV infection due to its greater tolerability and decreased risk (Smee et al., 1985).

Clinical use of ganciclovir is associated with multiple adverse events including neutropenia and myelosuppression (Kimberlin, 2002; Winston et al., 1988). Testing in preclinical animal models indicate that ganciclovir may be mutagenic, teratogenic, and carcinogenic, and additionally result in reproductive toxicity (FDA, 2017; Kimberlin, 2002). It is recommended to determine creatinine clearance and adjust the ganciclovir or valganciclovir dosage based on renal function to help avoid adverse effects while maintaining efficacy and decreasing the risk of resistance (Lumbreras et al., 2014). Therapeutic drug monitoring in the
course of ganciclovir treatment helps to guide treatment, however more investigation to clarify the therapeutic window is necessary (Martson et al., 2022; Martson et al., 2019).

Efforts are underway to evaluate ganciclovir as part of a suicide gene therapy approach to treat tumors (Duzgunes, 2019). This approach relies on the introduction of the HSV thymidine kinase gene into cancer cells, followed by treatment with ganciclovir (Beck et al., 1995). HSV thymidine kinase performs the first phosphorylation step of ganciclovir, thus allowing ganciclovir triphosphate to form selectively inside the HSV thymidine kinase expressing cancer cells (Smee et al., 1985). An additional advantage to this approach is that ganciclovir’s toxicity is associated with rapidly dividing cells, a hallmark of cancerous cells (Matthews and Boehme, 1988).

Early clinical trials used retroviral vectors to introduce the HSV thymidine kinase gene, however tumor progression continued due to limited propagation of the vector from the original injection site (Harsh et al., 2000). Increasing the bystander effect, wherein HSV thymidine kinase expressing cells may deliver ganciclovir triphosphate to neighboring HSV thymidine kinase non-expressing cells via gap junctions, may help overcome this limitation (van Dillen et al., 2002). More recently, a phase II trial using replication-deficient adenovirus vectors with ganciclovir treatment resulted in improved survival for high-grade glioma patients (Ji et al., 2016). HSV thymidine kinase gene transduction may be more well tolerated and efficacious with adenoviral rather than retroviral vectors (Rangel-Sosa et al., 2017). Gene delivery vectors under the control of a tumor-specific promoter such as human telomerase offer more opportunity for enhancing the efficacy of this approach (Tian et al., 2013). There is also evidence that the HSV thymidine kinase protein acts as a superantigen, stimulating the adaptive immune system and supplementing ganciclovir tumor cell killing with an anti-tumor immune response (Rangel-Sosa et al., 2017).
et al., 2017). Overall, this is an intriguing strategy, combining the cell specific metabolism of an antiviral nucleoside drug with its toxicity towards rapidly dividing cells to result in an innovative oncology treatment.

Tenofovir: dissimilarities in phosphorylation in different tissue compartments informs the fine tuning of HIV treatment and prophylaxis

Acquired immunodeficiency syndrome (AIDS), caused by the human immunodeficiency virus (HIV), continues to have a significant impact on human health. Due to lack of robust medical infrastructure, training, and resources, the HIV epidemic is more pronounced in low and middle income countries (Cohn et al., 2020a; Kabir et al., 2020). The hallmark of HIV infection is the decrease of CD4+ T cells, which leads to immunodeficiency and increases a patient’s susceptibility to opportunistic infections and comorbidities (Chang et al., 2013). After a patient begins highly active antiretroviral therapy, active viral replication is inhibited but the HIV genome persists in latent reservoirs that may reactivate (Cohn et al., 2020b). To date, only three patients have been cured of HIV infection, via stem cell transplantations that were intended to treat additional disorders in these patients. —The remaining population infected with HIV have persistent viral reservoirs and must remain on chronic antiretroviral therapy (Hsu et al., 2022).

The HIV genome consists of two single stranded RNA molecules, contained within the viral capsid along with its reverse transcriptase enzyme. HIV reverse transcriptase is responsible for transcribing viral single strand RNA into its complementary DNA and next into double stranded proviral DNA, which then integrates into the host cell genome and establishes infection (Deeks et al., 2015). Antiretroviral treatments for HIV are classified into seven categories based on their mechanism of action: fusion inhibitors, CCR5 antagonists, nucleoside reverse
transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, integrase inhibitors, post-attachment inhibitors, and protease inhibitors (NIH, 2022; Shin et al., 2021).

Tenofovir (TFV) is a nucleotide based reverse transcriptase inhibitor that is instrumental in pre-exposure prophylaxis (PrEP) efforts and post-exposure therapy (De Clercq, 2021). As an acyclic nucleoside phosphonate analogue of adenosine 5’monophosphate, TFV skips over the first, typically rate limiting, phosphorylation step towards activation (Zenchenko et al., 2021). Only two phosphorylation steps are required, and tenofovir diphosphate (TFV-DP) is the active species mimicking a nucleoside triphosphate which inhibits HIV reverse transcriptase (Cherrington et al., 1995; Robbins et al., 1998). Lack of a 3’ hydroxy group results in chain termination. The intracellular half life of TFV-DP is reported to be 12 hours to greater than 60 hours, allowing for once-daily administration to maintain efficacy (Kearney et al., 2004; Robbins et al., 1998). Tenofovir itself has poor bioavailability and is administered in one of two prodrug forms: tenofovir disoproxil fumarate (TDF) or tenofovir alafenamide (TAF).

In 2012, once daily oral Truvada became the first therapy to be approved by the US FDA for use in HIV PrEP (CDC, 2012). Truvada contains 300 mg TDF and 200 mg emtricitabine (FTC), another nucleoside based reverse transcriptase inhibitor. The efficacy of Truvada for preventing establishment of HIV infection is supported by multiple clinical trials evaluating oral TDF alone (Baeten et al., 2012; Choopanya et al., 2013), or co-formulated with FTC (Baeten et al., 2012; Grant et al., 2010; Thigpen et al., 2012) against a placebo. In 2019 Descovy, also a once daily pill containing 25 mg TAF and 200 mg FTC, was approved for HIV PrEP, though not in individuals at risk from receptive vaginal sex (FDA, 2019). Oral TAF/FTC was shown to be non-inferior to TDF/FTC in preventing HIV transmission, and improved on bone mineral density and renal safety endpoints (Mayer et al., 2020). Efforts are ongoing to evaluate topical
formulations and vaginal rings for PrEP, highlighting the importance of understanding antiretroviral drug metabolism and disposition specifically at the mucosal sites of infection (Obiero et al., 2021; Palanee-Phillips et al., 2022).

Significant investigation has gone into elucidating the enzymes responsible for TFV phosphorylation. Initially adenylate kinase was shown to perform the first phosphorylation step, and nucleoside diphosphate kinase was shown to perform the second phosphorylation (Robbins et al., 1995; Robbins et al., 1998). Both are ubiquitously expressed and highly active throughout all stages of the cell cycle. It was later shown that TFV metabolites are weak substrates for human nucleoside diphosphate kinase due to limited intermolecular interactions, and creatine kinase is more efficient at TFV phosphorylation (Koch et al., 2009). An additional study confirmed that recombinant creatine kinase efficiently phosphorylates tenofovir monophosphate, and that pyruvate kinase also demonstrates activity (Varga et al., 2013).

The dissimilarities in phosphorylation of TFV within different tissue compartments was recently studied with a side by side comparison of PBMCs (peripheral blood mononuclear cells), colorectal biopsies, and vaginal biopsies. It was determined that in all three tissue types, adenylate kinase 2 performs the first phosphorylation step of TFV to produce tenofovir monophosphate (Lade et al., 2015). However in PBMCs and vaginal tissues, pyruvate kinases perform the second phosphorylation step, whereas in colorectal tissues this phosphorylation is catalyzed by creatine kinase, muscle (Lade et al., 2015). Furthermore, genetic variants of the kinases that phosphorylate TFV have been identified in clinical samples (Figueroa et al., 2018a; Lade et al., 2015). These genetic variants have the potential to decrease phosphorylation efficiency. Six adenylate kinase 2 variants were predicted to result in decreased enzyme function, and four of them showed ≥ 30% decreased activity towards TFV in vitro (Figueroa et al., 2018b).
Notably, purified adenylate kinase 2 was able to perform both phosphorylation steps of TFV, though its exact contribution towards phosphorylation of TFV monophosphate in the presence of additional kinases remains to be determined (Figueroa et al., 2018b). Four naturally occurring variants of creatine kinase, muscle were also shown to produce < 5% of TFV-DP compared to wild type enzyme (Mosher et al., 2021). The work thus far has shown the effects of kinase variants on TFV phosphorylation in vitro, and verification of these findings in relevant preclinical animal models or in human clinical trials would be very interesting. It is important to understand this compartment specific phosphorylation and the pharmacogenetics of the kinases identified in order to guide the use of TFV in HIV treatment and prevention (Hamlin et al., 2019).

The prevention of HIV infection goes hand-in-hand with prevention of unwanted pregnancy. As such, understanding the effects that contraceptives may have on TFV phosphorylation is also an important area of study. In a macaque study, it was shown that depot medroxyprogesterone acetate (DMPA) use resulted in increased rectal concentrations of TFV-DP after an oral dose of TAF (Daly et al., 2021). There were no differences in PBMC TFV-DP concentrations, and vaginal tissue levels were undetectable in both groups. The CONRAD A10-114 study showed that vaginal TFV-DP levels increase approximately 3-fold upon DMPA use, and did not observe a statistical difference in vaginal TFV-DP levels due to oral contraceptive pill use (Thurman et al., 2019). A separate study examining women receiving TDF/lamivudine pills demonstrated 76% greater TFV-DP concentrations in cervical tissues among those using DMPA versus women using nonhormonal contraception (Nicol et al., 2020). The mechanism for the increase in vaginal TFV-DP concentrations when individuals are also using DMPA is yet unclear. Hypotheses include hormonal effects on inflammatory pathways or perhaps direct interaction of contraceptives with kinases or transporters known to activate and transport TFV.
(Hu et al., 2022). The increase due to DMPA use would be expected to maintain protection against HIV infection, but that may not be true for all contraceptives or other hormone treatments. For example, transgender women taking oestrogens as part of gender-affirming hormone treatment appeared to have 27% lower plasma exposure of TFV compared to cisgender men receiving the same oral TDF/FTC regimen (Shieh et al., 2019). The lower plasma exposure of TFV likely results in decreased intracellular TFV-DP levels which hint at decreased efficacy; however this has yet to be confirmed in this population. A full understanding of the potential interactions between hormonal therapies and TFV based regimens, and how they may affect success of each, would be valuable and warrants further investigation.

Development work is ongoing towards vaginal administrations of TFV that are co-formulated with contraceptives, allowing individuals dual protection in a single product (Palanee-Phillips et al., 2022). A phase 1 trial evaluating an intravaginal ring that delivered 10 mg TFV/day alone or with levonorgestrel at 20 ug/day showed that the rings were well tolerated and delivered the two drugs into vaginal tissue at levels that would be expected to be protective against HIV infection and effective for contraception (Thurman et al., 2018). The vaginal TFV-DP levels did not differ between the TFV only and TFV co-formulated with levonorgestrel rings in this study. As TFV products continue to be used for the prevention of HIV infection, the potential drug-drug interactions that may arise must be elucidated. A greater understanding of the tissue specific activation of TFV, especially in the mucosal tissues of infection, is an integral piece of that effort.

**Tenofovir prodrugs: lymphoid specific metabolism decreases toxicity risk and hepatic specific metabolism increases clinical benefit against hepatitis B**
It is worth examining the differences between TDF and TAF metabolism that result in intracellular TFV delivery and subsequent phosphorylation. TFV itself has poor oral bioavailability and was initially administered as daily infusions in clinical trials. This poor oral bioavailability is due to the two negative charges on the phosphonyl group, which are capped with two isopropyloxymethylcarbonyl groups to form prodrug TDF (Robbins et al., 1998). For the majority of TDF delivered orally to a patient, the prodrug moieties of TDF are cleaved by esterases in the gastrointestinal tract, hepatocytes, and plasma, delivering TFV into the systemic circulation to be uptaken by cells. When PBMCs are incubated with TDF, the intracellular TFV-DP levels are greater than 1,000-fold higher than PBMCs incubated with TFV (Robbins et al., 1998). The anti-HIV activity of TDF is also 90-fold greater than that of TFV in MT-2 cells, and 36-fold greater in PBMCs. When administered in preclinical species, TDF improves the oral bioavailability of TFV from 2% to 20% in mice and 17.1% to 30.1% in dogs (Kearney et al., 2004). Clinical use of TDF results in oral bioavailability in humans of 25% when fasted, and 39% when fed (Barditch-Crovo et al., 2001). Drug elimination after oral TDF administration is through intracellular dephosphorylation of TFV-DP to yield TFV, which is then released into plasma and undergoes glomerular filtration and renal tubule secretion into urine (Fung et al., 2002; Gallant and Deresinski, 2003; Ray et al., 2016). This intracellular dephosphorylation is proposed to be carried out by 5’-nucleotidase enzymes; however the activity of nucleotidases on TFV has not been fully elucidated. TFV treatment has been shown to affect nucleotidase activity in epithelial cells, immune cells, and fibroblasts from the human female reproductive tract, and similar differences were noted in blood derived macrophages after TFV treatment (Biswas et al., 2013; Biswas et al., 2014). These effects imply an interaction between TFV (or TFV metabolites)
and 5'-nucleotidases, and further work is warranted to clarify the potential role of nucleotidases in TFV elimination, especially in different compartments.

Clinical administration of TAF results in lower plasma exposure of TFV and higher intracellular TFV concentrations compared to TDF administration, maintaining efficacy while decreasing systemic drug exposure (Ray et al., 2016). In one phase I/II study, a daily dose of 40 mg oral TAF demonstrated plasma TFV $C_{\text{max}}$ of 13 ng/mL (0.45 uM) and AUC$_{0-t}$ of 383 ng*h/mL (1.33 uM*h) with intracellular PBMC TFV concentration of 8.2 uM. In the same study, patients receiving daily 300 mg oral TDF displayed plasma TFV $C_{\text{max}}$ of 207 ng/mL (0.72 uM), AUC$_{0-t}$ of 1810 ng*h/mL (6.3 uM*h), and intracellular PBMC TFV concentration of 0.9 uM (Markowitz et al., 2014). Concordantly, the mean decrease in HIV RNA from baseline to 14 days of drug administration was also more pronounced for the TAF cohort, -1.57 log$_{10}$copies/mL versus -0.94 log$_{10}$copies/mL for the TDF cohort ($P = 0.025$).

The increased efficacy resulting from lower doses of TAF vs TDF is due to the unique metabolism of TAF by Cathepsin A and resultant enrichment in lymphoid tissues (Birkus et al., 2007; Lee et al., 2005). Cathepsins are proteases localized to the intracellular lysosome, and cathepsin A in particular is known to be highly expressed in lymphoid cells such as antigen presenting cells and platelets (Tan et al., 2013; Yadati et al., 2020). TAF is also stable in human plasma with a half-life of 90 min, in contrast to TDF and its human plasma half-life of 0.41 min (Lee et al., 2005). The intracellular species observed in vitro after TAF is administered to PBMCs constitute TFV, intermediate TFV alanine, and TFV mono- and diphosphate (Eisenberg et al., 2001). Thus TAF is the species which enters the target cells and undergoes cathepsin A cleavage to efficiently deliver intracellular TFV, allowing intracellular phosphorylation to pharmacologically active TFV-DP (Birkus et al., 2008). In contrast, oral TDF is primarily
cleaved in the process of intestinal absorption and relies on systemic TFV with its uncapped negatively charged phosphonyl to enter the target cells.

Clinical use of TDF has generally been well tolerated, however TDF regimens are associated with renal adverse events and bone abnormalities (Nelson et al., 2007). The renal events linked to TDF therapy include acute kidney injury, proximal tubular dysfunction, and chronic kidney disease (Hughes, 2021). Concomitant risk factors for renal events linked to TDF therapy have been identified, and it is recommended that clinicians monitor such patients closely. Decrease in bone mineral density is consistently observed upon initiation of antiretroviral treatment, and this reduction seemed to be greater when treatment naïve patients began TFV regimens (Powderly, 2012). Demineralization tends to stabilize after one year of antiretroviral treatment, though the long-term effects remain unclear.

Data on TFV renal excretion from TDF and TAF studies suggest a link between plasma TFV exposure and adverse renal events. This is supported by observations that patients taking TDF and ritonavir boosted protease inhibitor regimen had a greater decline in estimated glomerular filtration rate than those taking TDF and a non-nucleoside reverse transcriptase inhibitor (Gallant and Moore, 2009). It was previously shown that protease inhibitors are able to inhibit the intestinal efflux transporters to which TFV is susceptible (Tong et al., 2007). This explains the observed increase in plasma TFV exposure when some protease inhibitors are co-administered with TDF, and links plasma TFV exposure to renal adverse events (Cihlar et al., 2007). TAF administration, which results in decreased systemic exposure of TFV, has a more favorable renal and bone safety profile than TDF administration. Multiple meta-analyses of efficacy and safety markers in TDF vs TAF trials have been carried out, encompassing data from over 6000 or 9000 patients. These surveys show that TAF regimens maintain similar virologic
efficacy to TDF regimens while improving renal and bone safety outcomes (Gupta et al., 2019; Tao et al., 2020).

In addition to providing improved efficacy against HIV and decreased risk of toxicities compared to TDF, the differential metabolism of TAF also has implications for hepatitis B patients. Hepatitis B infection continues to have significant impacts on global public health despite the availability of a safe and effective vaccine to prevent establishment of infection (Trepo et al., 2014). There is no current cure for hepatitis B infection; antiviral therapy is focused on limiting viral replication and hepatic inflammation which may lead to cirrhosis and hepatocellular carcinoma (Trepo et al., 2014). TDF has been shown to be efficacious for treatment of chronic hepatitis B infection, effectively blocking viral infection in liver cells (Jenh et al., 2009). This activity is due to active metabolite TFV-DP and its potent competitive inhibition of hepatitis B polymerase (Delaney et al., 2006). TAF administration to dogs results in nearly 2-fold higher TFV-DP levels in liver compared to TDF administered at the same TFV equivalent (Murakami et al., 2015). This is due to the high passive permeability of TAF, active uptake of TAF into hepatocytes via organic anion-transporting polypeptides 1B1 and 1B3, and cleavage by carboxylesterase 1, which is highly expressed in hepatocytes (Murakami et al., 2015). Multiple clinical studies have shown that TAF is equally effective to TDF in managing chronic hepatitis B infection, and has the additional advantage of decreasing the risk of renal and bone toxicities (Agarwal et al., 2015; Byun et al., 2022; Lim et al., 2022; Ma et al., 2021). TAF was approved by the FDA for use as an HBV treatment in 2016, demonstrating the clinical benefit from understanding differential metabolism of nucleotide drugs (Vermes, 2016).

Conclusion & future perspectives
In summary, above we see that understanding the cell/tissue specific metabolism of nucleoside/nucleotide drugs provides unique perspective of their effects and guides their most optimal use. Acyclovir is activated solely in HSV infected cells and is therefore highly selective. However, this results in acyclovir resistant variants tending to have mutations in the enzyme responsible for acyclovir activation, rather than the enzyme that activated acyclovir targets. Ganciclovir is similarly activated solely in viral infected cells. Suicide gene therapy attempts to use ganciclovir as an anticancer therapy by introducing ganciclovir’s activation enzyme into cancer cells, thereby redirecting ganciclovir’s selectivity of activation. The use of tenofovir for the prevention of HIV infection highlights the importance of high tenofovir diphosphate concentrations in the mucosal tissues of infection. This contrasts with the use of tenofovir for HIV treatment, where focus lay on activation in white blood cells. Thus, it is particularly interesting that tenofovir metabolism differs between vaginal tissues, colorectal tissues, and PBMCs, and that genetic variants of the enzymes may have varying activities. Drug-drug interactions between tenofovir and contraceptives in the vaginal mucosa are also emerging. Further explorations can clarify the clinical impacts of these genetic variants and drug-drug interactions, and how they may affect the ability of tenofovir-based therapies to prevent HIV infection. Lastly the differential prodrug metabolism of tenofovir alafenamide versus tenofovir disoproxil fumarate decreases toxicity risk and dosage required, while maintaining antiviral efficacy against both HIV and HBV.

Respiratory tissues are an emerging tissue of interest for antiviral development, especially considering the ongoing coronavirus disease 2019 (COVID-19) pandemic. Zoonotic respiratory viruses have caused many of the global pandemics in recent years, and are expected to continue doing so (Gray and Abdelgadir, 2021; Gray et al., 2021). Additional respiratory viruses
including influenza virus and respiratory syncytial virus also continue to negatively impact human health. Antiviral drugs that can specifically target lung tissue would be ideal therapies for use. Remdesivir, the first FDA-approved antiviral for treatment of COVID-19, is a phosphoramidate adenosine analogue (Cihlar and Mackman, 2022). Recently, some of the enzymes involved in activating remdesivir in lung cells were determined to be carboxylesterase 1, cathepsin A, and histidine triad nucleotide binding protein 1 (Li et al., 2021). Carboxylesterase 1 and cathepsin A hydrolyze remdesivir to its alanine intermediate MetX, which undergoes further hydrolysis by histidine triad nucleotide binding protein 1 to produce the monophosphate metabolite intracellularly. These enzymes are expressed and active in lung tissues, and work in concert to produce the monophosphate form in the lung cells relevant for SARS-CoV-2 infection and replication. The enzymes responsible for phosphorylating the monophosphate to produce the active triphosphate have yet to be determined. Continued exploration into lung specific metabolism of nucleoside/nucleotide drugs is crucial to inform current therapies and guide the development of novel antiviral drugs (Mackman, 2022).

Much of the research into nucleoside and nucleotide analogue metabolism has focused on phosphorylation and prodrug metabolism. This is understandable, as ensuring efficacy while minimizing undesired adverse events is the primary goal in any drug discovery journey. However triphosphates do not necessarily remain triphosphates—nucleotidases are endogenous enzymes that function to dephosphorylate nucleotides (Varga et al., 2016). There remains much to be elucidated with regards to dephosphorylation mechanisms, their kinetics, and their specificity. A comprehensive understanding of the mechanisms that produce and degrade active triphosphorylated metabolites, and the specific cell/tissues in which they occur, enables the optimal precision use of nucleoside/nucleotide analogue drugs in the clinic.
Authorship Contributions

Wrote or contributed to the writing of the manuscript: To, E.
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Footnotes

Funding: This work received no external funding
Conflicts of interest: To, E. is an employee of Gilead Sciences, Inc. and may own company stock.

Figure Legends

Figure 1. Chemical structures of acyclovir together with its prodrug valacyclovir and active metabolite acyclovir triphosphate, ganciclovir together with its prodrug ganciclovir and active
metabolite ganciclovir triphosphate, and tenofovir together with its prodrugs tenofovir disoproxil fumarate and tenfovir alafenamide and active metabolite tenofovir diphosphate. Prodrug moieties are highlighted in red and phosphates that require intracellular phosphorylation are highlighted in blue.
Figure 1