Modulation of Tenofovir by Probenecid: Impact on Drug, Interleukin-1β, and Dopamine Concentration in the Prefrontal Cortex and Cerebellum.

By

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DECLARATION

I, **Simangele Shabalala** hereby declare that the dissertation entitled **"Modulation of Tenofovir by Probenecid: Impact on Drug, Interleukin-1β, and Dopamine Concentration in the Prefrontal Cortex and Cerebellum**" is the result of my own investigation and research, and it has not been submitted in part or in full for any other degree or to any other university. Where use of the work of others was made, it is duly acknowledged in the text.

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PLAGIARISM DECLARATION

School of Laboratory Medicine and Medical Sciences, College of Health Sciences

Master of Medical Science, 2023

1. I know that plagiarism is wrong. Plagiarism is to use another's work and pretend that it is one's own.
2. I have used the Vancouver Convention for citation and referencing. Each contribution to and quotation in this dissertation has been attributed, cited, and referenced.
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4. I have not allowed and will not allow anyone to copy my work to pass it off as his or her work.

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ACKNOWLEDGEMENTS

I am filled with immense gratitude and joy as I reach the culmination of my Masters studies journey. This dissertation would not have been possible without the unwavering support and guidance of numerous individuals whose contributions have been instrumental in shaping my academic and personal growth. Therefore, I extend my heartfelt appreciation to specific individuals and teams.

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	cerebellum and PFC at 1h,4h, and 6h time intervals.	

ABBREVIATIONS

Before use, abbreviations are defined when first used in the text throughout the dissertation.

STUDY OUTLINE

Chapter 1: consists of the introduction/literature review, aims, objectives and general methodology of the study. This chapter contains a brief background, current knowledge about the research theme and knowledge gaps. The aim of the present study is to fill the gaps in the literature, and the objectives depict how the aim of the study was answered. The general methodology of the study offers a preview regarding how the aims and objectives of the study were achieved.

Chapter 2 contains the first research study in a manuscript format that investigated the enhancement properties of probenecid on tenofovir disoproxil fumarate extent of brain penetration and concentration in the Prefrontal cortex and Cerebellum.

Chapter 3 presents the second research study within the manuscript format. This study examined the effects of administering tenofovir disoproxil fumarate and probenecid on the concentrations of interleukin-1β and dopamine in the prefrontal cortex of BALB/c mice.

Chapter 4, the last section of the study, gives a narrative of the discussion of the themes covered in the two studies. The discourse focuses on the linkages between the content covered by the studies in view of the broad study aim(s) and objectives. Appendices contain supplementary information that might be useful to the reviewers.

ABSTRACT

Tenofovir, a first-line antiretroviral therapy (ART) therapeutic agent is pivotal in the management of HIV. Its efficacy, tolerability, extended half-life, and inclusion in different fixed-dose forms make it attractive for treatment naive and experienced individuals. These benefits lead to successful viral suppression, enhanced patient quality of life, and improved HIV management. Nonetheless, due to limited central nervous system (CNS) penetration, tenofovir's positive impact is constrained in the CNS, potentially permitting ongoing HIV-related neurotoxicity. Studies have found that even at low concentration, tenofovir exhibits neurotoxic effects through increased inflammation and neurotransmitter dysregulation. Probenecid has shown potential as an adjuvant to improve therapeutic agent bioavailability in the CNS and exhibits neuroprotective properties. This makes it a candidate as a pharmacokinetic enhancer. Limited research exists on strategies to enhance tenofovir's passage through the blood-brain barrier (BBB) to have optimal brain concentration while cushioning its neurotoxicity. The effects of co-administering a single dose of tenofovir (5mg/kg) and probenecid (8.3mg/kg) in mice on therapeutic agent concentration in the prefrontal cortex (PFC) and the cerebellum after 1h, 4h, and 6h post-administration was investigated. Ninety-six BALB/c mice were divided into 3 groups: tenofovir, probenecid, and tenofovir with probenecid. Co-administration with probenecid increased tenofovir concentration in plasma, the PFC, and cerebellum, indicating improved CNS penetration. The impact of co-administering tenofovir and probenecid on IL-1β and dopamine concentration in the PFC was also evaluated. Tenofovir administration led to an increase in IL-1β concentration, indicating an inflammatory response but the co-administered probenecid did not prevent the tenofovir-induced inflammation. Neither tenofovir nor probenecid alone or combined affected dopamine concentration. In conclusion, the study findings demonstrate that co-administration of tenofovir with probenecid increases the former’s concentration in the plasma, PFC, and cerebellum. The failure by probenecid to prevent the tenofovir-induced inflammation could be attributed to the relatively short observation period employed in the study. This perhaps is an indication that assessing the impact of Probenecid on the tenofovir-induced inflammation might necessitate an extended observation period beyond that of the current study. The lack of impact on dopamine emphasises the need for further research intoTenofovir's influence on dopamine regulation in other brain regions. Incorporating other brain regions in investigating Tenofovir and

Probenecid's impact on dopamine regulation is essential due to the brain's intricate network and the diverse roles of dopamine in various regions of the CNS. While the prefrontal cortex is associated with cognitive functions and motivation, different brain regions contribute to reward processing, mood regulation, and motor control. Understanding how Tenofovir and Probenecid affect dopamine across these regions can provide a comprehensive picture of their influence on brain function, especially in conditions like HIV-associated neurocognitive disorders where dopamine dysregulation can have widespread effects beyond a single area.

Keywords: Tenofovir, Probenecid, Blood-Brain Barrier, Plasma, Prefrontal cortex, Cerebellum, Interleukin-1β, Dopamine

Chapter 1

1.1 Background

Despite the success of combination antiretroviral therapy (cART) in managing HIV-1 infection in the body, including the central nervous system (CNS), HIV-associated neurocognitive disorders (HAND) still prevail (1–3). HAND is a set of neurological disorders that result from the progressive deterioration of areas of the brain infected and affected by HIV (3). HAND can be categorised into three categories depending on the severity of the disorder asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorders (MND), and HIV-associated dementia(HAD) (4). Before the introduction of ART, HAD was highly prevalent in almost 15% ofthe infected population and this statistic has decreased significantly to 5% (1). The overall prevalence of HAND is approximated at 42.6%, while the prevalence of ANI is 23.5%,and MND is 13.3% (2,3). The progressive decline of HAD can be credited to managing the virus through antiretroviral therapy (ART) in the systemic and local environment. In the body, cART inhibits HIV-1 replication. This, in turn, allows the immune system to repair itself and prevent further damage. This is done by maintaining host defence, immune activation, ultimately limiting viral entry into the CNS (5). However, the direct suppression of CNS HIV-1 is the most critical in preventing the progression of ANI to severe forms of HAND and accelerated ageing. Two main reasons are responsible for the unsuccessful treatment of HIV in the brain and, ultimately, the treatment of HAND. These reasons are the limited penetration of ART across the blood -brain barrier (BBB) and ART neurotoxicity.

The brain is a protected environment by the selectively semi-permeable that results in poor CNS penetration by therapeutic antiretroviral (ARV) therapeutic agents (6) The optimal concentration of ARV drugs must reach the brain for the optimal management of HIV replication management and prevention of neurological injury and impairment. However, some therapeutic agents used as first-line treatment, for example, Tenofovir disoproxil fumarate (TDF), as they approach the BBB, they are effluxed by efflux protein transporters embedded in the BBB. The result is limited or no TDF passing into the brain and managing HIV replication in this system. TDF, a prodrug of tenofovir (9[9(R)-2-(Phosphonomethoxy) propyl] adenine, PMPA), is still the recommended first-line nucleos(t)ide reverse transcriptase inhibitors (NRTIs) in treating HIV. Studies have

shown that this therapeutic therapeutic agent has limited access to the CNS and that it (Tenofovir) accumulates at the choroid plexus at only approximately 5% of the plasma concentration, and in the brain, its concentrations are even lower(7,8). This small Tenofovir concentration has been shown to cause damage within the CNS. Numerous studies present evidence of impaired learning in the presence of Tenofovir (9). Tenofovir is said to induce neuroinflammation through oxidative stress (10,11). Studies looking at the influence of Tenofovir on neurotransmitters greatly lackevidence that shows mental illnesses associated with HIV and HIV medication (12–15). Neuro-HIV studies show a relationship between inflammation and depression (16–19). However, the role of Tenofovir is not fully elucidated. It should be mentioned that studies looking at the relationship between ART and other neurotransmitters need to be fully explored and consider societal impacts.

It is challenging to develop a definite mechanism related to neurological impairments without understanding the basic pharmacokinetics and pharmacodynamics related to ART in the brain, particularly concerning NRTIs in the CNS. This study sought to start the journey of fully understanding the mechanism of Tenofovir in the brain as an individual Therapeutic agent and ultimately in combination therapy, with the ultimate goal of crafting strategic and innovative ways to treat HIV in the brain and HAND.

1.2 Current state of HIV-1

In June 1989, HIV/AIDS was declared a chronic disease, marking a shift in the social definition of acute illness. However, HIV continues to be a significant global public health concern. At the end of 2021, the World Health Organisation (WHO) estimated that 38.4 million people live with HIV, while the virus claimed 650 000 lives in the same year (20). HIV is a retrovirus that attacks CD4 T lymphocytes, leading to their death resulting in severe immunodeficiency (21). The virus attaches glycoproteins to the host cell and then integrates its chromosomal material into the host cell allowing it to take over the cellular activity of the host cell by generating viral proteins and its genetic material (22). The host cell dies as the virus takes over, and other CD4 cells are infected.

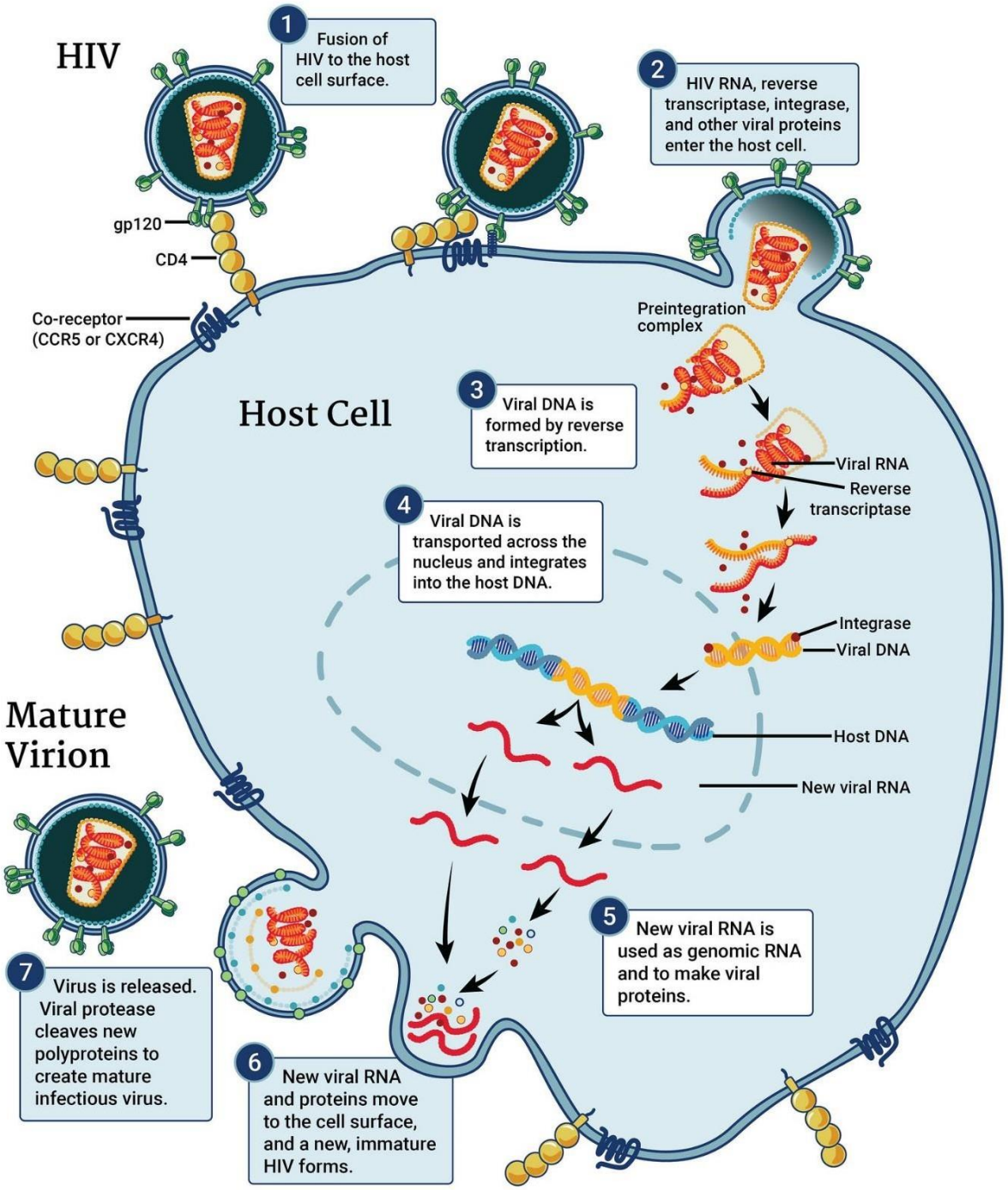

Fig. 1: HIV replication Cycle (23).

HIV has no cure but is managed by potent combination regimens that have seen infected individuals living long, healthy lives. These regimens are a combination of antiretroviral (ARV) therapeutic agents which are divided into seven classes. These classes include non-nucleotide reverse transcriptase inhibitors (NNRTIs); nucleoside reverse transcriptase inhibitors (NRTIs); protease inhibitors (PIs), fusion inhibitors; CCR5 antagonists, integrase strand transfer inhibitors (INSTIs), and post-attachment

inhibitors (2) . Barriers to successfully eradicating this disease exist; these include latent reservoirs. When the virus integrates into the host DNA, a subset of the HIV provirus remains transcriptionally silent. This means that the provirus does not produce the viralproteins nor the viral progeny unless it is reactivated, for example, by the interruptionof treatment, antiretroviral therapy (ART) (24). Because of this latency, some infectedcells can escape immune detection and elimination, and these cells make up viral reservoirs (25). Understanding the nature and persistence of viral reservoirs is essential for developing HIV eradication strategies. Hence, the following section dwellson the CNS as an HIV-1 reservoir.

1.3. Central Nervous System as an HIV-1 Reservoir

Infection of the brain by the HIV virus via a trojan horse-like mechanism where in the virus "hides" inside infected monocytes that cross the BBB.Once inside the brain, the virus is released from the infected monocytes and infects resident cells such as astrocytes and microglia (26). The HIV virus can also be "transported" into the CNS "hidden" in infected leukocytes that can cross the BBB using specific cell-tissue (endothelial cells) interaction (27). This regulated process also involves other molecules within and outside the brain among them chemokines, cytokines, cell adhesion molecules, tight junctions, and adherens junction proteins (28). Once the virus passes into the brain, the infected monocytes differentiate into perivascular macrophages (26). These macrophages harbour the virus and allow for its replication and infection. However, astrocytes although the "house" the virus, do not allow for HIV replication but the infected astrocytes do contribute to HIV-related brain damage through astrogliosis(29).

Studies have shown that astrocytes can be infected with HIV when co-cultivated with HIV-infected lymphocytes (30,31). While astrocytes are typically resistant to infection by cell-free viruses, the presence of HIV-infected lymphocytes significantly increases their susceptibility to disease through cell-to-cell transmission (32,33). This transmission is more efficient than a cell-free virus and occurs before HIV maturation is completed, with only immature viral particles entering astrocytes. The infection of astrocytes by HIV in this manner does not depend on the CD4 receptor cell but relies on the CXCR4 co-receptor (32,34). X4 or R5X4 viruses can infect astrocytes via cell-to-cell contact, while CCR5-tropic viruses are not detectable in cultured astrocytes (31).Some studies have reported the presence of X4-tropic or R5X4 dual-tropic viruses in the brain or cerebrospinal fluid of individuals with HIV-associated dementia (35–38).

Antiretroviral therapy can switch HIV tropism from R5 to X4 usage (39). Lymphocytes migrate into the brain, particularly in asymptomatic carriers, and HIV-infected and immune-activated macrophages produce IL-1β, which triggers the secretion of stromal derived factor (SDF)-1 from astrocytes (40). SDF-1 facilitates the migration of HIV-infected lymphocytes through the BBB, leading to contact with astrocytes and subsequent infection. *In vivo,* studies suggest that astrocytes may serve as an additional reservoir for HIV in the brain, with infected cells producing low levels or no virus (30). HIV infection of the CNS is characterised by increased leukocyte penetration, microglial activation, abnormal expression of inflammatory factors, neuronal dysregulation, neuronal loss, and disruption of the BBB (41).

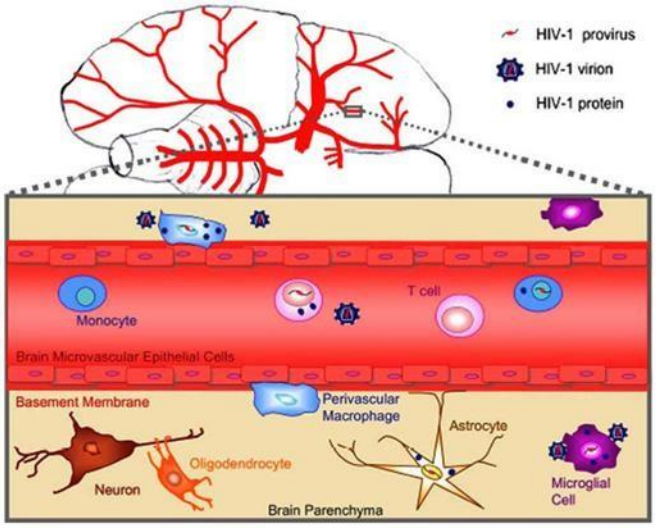

Fig. 2: Cellular HIV-1 reservoirs in the CNS (41)

Interestingly, individuals with HIV-infected comorbidities are as likely to develop HAND as those who were infected before the introduction of ARV medication (1,2). This can be attributed to various factors, including the limited ability of ARV therapeutic agents to penetrate the BBB, ongoing low-level replication of HIV-1 in the brain's reservoir, immune- inflammatory responses, the interaction between HIV infection and ageing, and potential toxicity of ARV medication in the brain (3). The limitations of ARV medication in penetrating the BBB and their potential toxicity to the brain, along with other contributing factors to the development of HAND, highlight the significance of understanding the CNS barriers' role in treatment delivery.

1.4. Antiretroviral Therapy: Access and Toxicity to the Central

Nervous System

The CNS comprises three barriers—the blood-brain barrier (BBB), the blood-CSF barrier, and the blood-arachnoid barrier—that separate the internal environment from the bloodstream (external environment) (42). These barriers regulate the exchange of substances, including therapeutic agent delivery between the blood and the brain parenchyma. The BBB, formed by the endothelial cells lining the cerebral capillaries, is almost impermeable compared to other barriers due to its non-fenestrated endothelial cells (43).The BBB serves to ensure efficient delivery of nutrients into the brain and the removal of waste products from the brain into the bloodstream and that maintenance and repair processes take place proficiently (3,4). Toxic compunds from the blood plasma are inhibited from entering the brain (44,45). The function of the barrier is attributed to a combination of three properties; the physical barriers held together by tight junctions (zonulae occluded) with extremely high electrical resistance (~1000Ωcm ^{2}). It is surrounded by a basal lamina excreted by the endothelial cells and end feet of astrocytes reduces paracellular permeability for hydrophilic molecules (42). In between the basal lamina and the astrocytes, the pericytes are embedded. This community of cells is called the neurovascular unit maintain the physical barrier of the BBB (46). Another function is transport regulation, composed of influx/efflux transporters and transcytosis that regulate the transcellular movement of molecules through the BBB. Lastly the BBB also performs a metabolic function wherein enzymes metabolise potentially harmful substances (47).

By protecting the internal environment of the brain, the BBB, in addition to blocking potentially harmful substances, also limits the penetration of therapeutics including ARV therapy from crossing into the brain which limits their efficacy in the brain (42, 48, 49). The makeup of the BBB limits the penetration of large hydrophilic molecules including tenofovir, ritonavir, and atazanavir (47). The disruption of the barrier to allow for increased penetration and ultimately increased concentration of antiretrovirals may have deleterious effects on the brain's internal system, such as neural cell damage and irreversible brain injury (43,48). Assessing the modification ofother BBB properties is thus essential.

The concentration needed to inhibit HIV replication in human brain tissue isunknown, as the tissue is only accessible at autopsy. Extracted brain tissue of HIV- infected individuals shows viral RNA and DNA in the tissues despite ARV treatment (3). It has

been demonstrated that macrophages derived from monocytes were highlyresponsive to combined ARV treatment (50). This raises questions about the responseof other brain cells to ARV treatment, the impact of individual ARV medication on the brain and which medication present the most neuronal toxicity. Answering these questions will provide data on which ARV medications are highly inclined to inhibit HIV replication withminimal toxicity.

Therapeutic agents use passive and facilitate diffusion to enter the brain (44). Successful distribution depends on the therapeutic agent's lipid solubility and molecular size (47,51). Antiretroviral compunds with a significant molecular weight >500 Da, due to their low diffusion rate, will likely have difficulty penetrating the BBB (51). The polarity of the therapeutic agent also plays a significant role in the diffusion rate. Polar therapeutic agents are hydrophilic, and these medicines cannot diffuse across the BBB, which is lipophilic by nature (47, 51). Protein binding is another therapeutic agent characteristic that plays a role in their ability to diffuse across the BBB. Protein transporters facilitate the entry and exit of xenobiotics and therapeutic therapeutic agents into and out of organs and the degradation and elimination of endogenous substances such as hormones and prostanoids (52). Thesetransporters have a high affinity for the specific therapeutic agent and allow the transporter to carry it into or out of the cells. For an ARV therapeutic agent to penetrate the BBB (or any other CNS barrier) and enter the internal system is dependent on whether the therapeutic agent is a substrate for any of the transporters present in the barrier and the level of expression.

The ATP-binding cassette (ABC) superfamily and, to a lesser extent, the solute carrier (SLC) superfamily are efflux transporters that mediate the removal of therapeutic agentout of the BBB (45,49). ABC transporters consist of a "cassette-like" domain responsible for catalysing ATP hydrolysis that then provide energy for the transportation of a molecule against the concentration gradient (42). Endogenous and exogenous substances are transported by this superfamily and are involved in xenobiotics' absorption, distribution, metabolism, and excretion (ADME) (42). These transporters play a role in transporting ARV therapeutic agents in cellular and anatomical reservoirs, as evidenced by their expression in the BBB, lymphocytes, $CD4^+$ T cells and microglia (49).

In addition to poor penetration, ARV therapeutic agents also carry the risk of

neurotoxicity and are associated with a range of disorders, such as peripheral neuropathy (16) and neuropsychiatric and neurocognitive deficits (53,54). One of the shortcomings of HAND etiology is the difficulty distinguishing adverse effects caused by ART and CNS injury resulting fromthe virus. This conundrum has been seen with HAND; as mentioned previously, the prevalence of these disorders has significantly decreased in the ART era. However, the rates of the mild and asymptomatic forms are increasing (2). It is suggested that either ART cannot adequately suppress the virus in the CNS and or that it contributes to the development of HAND (55).

A study by Robertson et al. to evaluate the direct effects of ARV therapeutic agents on neurons showed that no compound was highly toxic, but neural damage in the form of dendriticbeading and pruning was common (5). The study also noted that NNRTIs and PIs produced neuronal damage equal to and, to some extent, more significant than neuronal damage caused by NRTIs (56).

Therefore, understanding the pharmacokinetics and dynamics of antiretroviral therapy in the CNS is crucial to accurately quantify ART-induced toxicity and finding effective waysto manage the virus in the CNS. In particular, the poor penetration of certain ARV therapeutic agents like tenofovir, through the CNS BBB, combined with the risk of neurotoxicity, highlights the need for further investigation and evaluation of the properties of the therapeutics that influence CNS barrier penetration, such as molecular weight, lipid solubility, and protein binding.

1.5. Tenofovir Disoproxil Fumarate

The World Health Organisation (WHO) recommends that first-line ARV treatment include one non-nucleoside reverse transcriptase inhibitor (NNRTI), and two nucleos(t)ide reverse transcriptase inhibitors (NRTIs). According to the WHO 2013 report, efavirenz (EFV) is the preferred NNRTI in sub-Saharan Africa, and it is further recommended that one should either be tenofovir disoproxil fumarate (TDF) or zidovudine (ZDV) (57). As of 2019, the Minister of Health for South Africa introduced a new fixed-dose combination of ARV treatment that included tenofovir/lamivudine/dolutegravir (TLD) as a first-line treatment (4).

According to the CNS Penetration Effectiveness (CPE) ranking system, tenofovir has a score of 1, meaning it has a very low or diminished ability to penetrate the CNS barriers (6). Antiretroviral with CPE of 1 have low penetration effectiveness and those

with a score of 4 have a high capacity to penetrate the CNS barriers. Letendre and colleagues state that a low CPE score is directly related to an increased HIV viral load in the cerebrospinal fluid (CSF) (5). CNS barrier penetration depends on the physical and chemical makeup of the barrier as well as the therapeutic agents' physical and chemical properties chiefly molecular weight, protein binding, lipo-solubility, and degree of ionisation (51). Therapeutic agents with low molecular weight and high lipo-solubility have a better chance of diffusing across the BBB (7). Table 1 below shows the CPE scores of the various antiretrovirals.

Table 1. Antiretroviral treatment CNS Penetration-Effectiveness Scores

CNS Penetration-Effectiveness (CPE) Ranks (2010)

Table 1.	4	3	2	1
NRTIs	Zidovudine	Abacavir	Didanosine	Tenofovir
		Emtricitabine	Lamivudine	Zalcitabine
			Stavudine	
NNRTIs	Nevirapine	Delavirdine	Etravirine	
		Efavirenz		
PIs	Indinavir-r	Darunavir-r	Atazanavir-r	Nelfinavir
		Fosamprenavir-r	Atazanavir	Ritonavir
		Indinavir	Fosamprenavir	Saquinavir-r
		Lopinavir-r		Saquinavir
				Tipranavir-r
Fusion/Entry Inhibitors		Maraviroc		Enfuvirtide
Integrase Inhibitors		Raltegravir		

The central passage of entry for therapeutic therapeutic agents into the brain is through passive diffusion; a process whereby these agents move from the highly concentrated environment (blood plasma) to a low-concentrated environment (brain). Antiretroviral with a molecular weight >900 Da have a low diffusion rate are likely to have difficulty penetrating the BBB (47). The polarity of the therapeutic agent also plays a significant role in the diffusion rate; hydrophilic therapeutic agents cannot diffuse across the BBB due to its lipophilic nature (7,8) Antiretroviral therapeutic agents that do not bind to circulating plasma proteins such as albumin have an increased chance of crossing the BBB. Tenofovir-PMPA is a 99% unbound therapeutic agent with a molecular weightof 287.213g/mol (51); characteristics which would favour it to exhibit a high CPE score. However, it has poor penetration which is thought to be mediated by the two negative charges on the phosphoryl group and high polarity (47), thus resulting in low lipophilicity.

A study to understand the distribution of tenofovir in the CNS found that it was not

transported across the BBB but reported that it can cross the BCSFB (8). The researchers stated the difference between the physiological and functional characteristics of the BBB and BCSFB as the possible reason. It was further noted that the ability of tenofovir to accumulate in the choroid plexus (at approximately 5% of the plasma concentration) is due to transporters. Still, Anthonypillai and colleagues (7) suggested that transporters do not play a role in PMPA delivery through the BBB. This assertion still requires further investigation because substantial evidence shows that the transporters in the BBB interact and efflux ARV therapeutic agents into the plasma (49,58,59). It is essential to mention that a few studies demonstrate small concentrations of tenofovir in the brain (9,11,51) but don't mention its entry route. This project is, therefore, crucial inassessing quantitatively the role of transporters MRP4 in the transportation of tenofovir in the BBB.

Tenofovir fumarate (TDF) is a prodrug of tenofovir (9[9(R)-2-(Phosphonomethoxy) propyl] adenine, PMPA), an acyclic nucleotide analogue with potent *In vitro and In vivo* antiretroviral activity (8). Tenofovir PMPA has a low oral bioavailability due to its two negative charges (at physiologic pH) on the phosphoryl group. Thus, the prodrug was created. TDF is administered as a single 300mg tablet daily as treatment in HIV-naïve and -experienced patients because of its efficacy and tolerability (60). Figure 4below shows the chemical structure of tenofovir PMPA and its prodrug TDF.

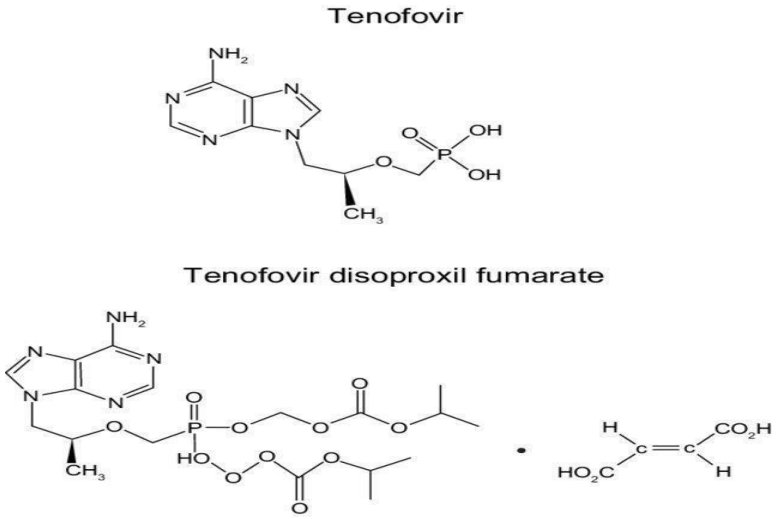

Figure. 4: Structures-of-tenofovir-and-tenofovir-disoproxil-fumarate-TDF

The mechanism of conversion from TDF to tenofovir PMPA within the human bodyis that once absorbed into the cells, cellular enzymes enzymes convenrt it to tenofovir diphosphate an active metabolite that inhibits HIV reverse transcription and terminates

the chain of the viral cDNA (9,10).

To reach the infected brain cells, antiretroviral treatment need to pass through the BBB. The tight junctions and efflux transporters at the endothelial cells in the BBB prevent therapeutic agents from entering through the BBB by paracellular diffusion and transcellularpathway. Tenofovir is a substrate for enzyme transporters organic anion transporters(OAT) 1 and 3, and MRP 2 and 4, found in the BBB and the BCSFB (42,61). Studies on the interaction of tenofovir and its transporters at the BBB are limited. However, substantial studies have been done concerning tenofovir-transporter relationship in the renal system (9,11,53,62–65). Even at low concentrations, tenofovir in the brain induces toxicity, as evidenced by numerous studies (11–13). Another study found that tenofovir affects peripheral nerve function and cause CNS inflammation and mitochondrial biogenesis in an *in vivo* setting (10). Given the neuroprotective potential of probenecid (14–16) and its established role in enhancing therapeutic agent bioavailability, exploring its co-administration with tenofovir may hold promise in overcoming BBB limitations and mitigating neurotoxicity in HIV treatment within the CNS.

1.6 Probenecid

Probenecid, also known as benemid, was first synthesised in 1949 and initially studied to decrease the renal clearance of penicillin (67). It was similar to other therapeutic agents developed during World War II, which increased penicillin and para-aminosalicylic acidserum levels but had limited clinical applications due to side effects or high oral doses (67). Probenecid was found to be effective in enhancing the retention of antibiotics and was used as adjunct therapy with penicillin. It was also discovered that probenecidenhanced the renal excretion of urate, leading to its clinical use in treating gout (68,69). In the 1960s, researchers found a new service for probenecid in studying the brain's serotonin synthesis rate by blocking acid metabolites from exiting the central nervous system (67,70, 71). This led to the "Probenecid Test" for studying depression and neurological diseases (67).

Over the past two decades, probenecid has mainly been employed in research with isolated myocytes to study calcium transients (67, 72). This is because it inhibits anion transport and prevents the leakage fura-2 from cells to the extracellular fluid. In 2007,

probenecid was discovered to act as an agonist of transient receptor potential vanilloid 2 (TRPV2), a critical cation channel in the respiratory tract, central nervous system, and immune system (17). Recently, it has also been located in the heart (73).

Probenecid has found utility as a transporter inhibitor, enhancing the concentration and effectiveness of various medications like antiretroviral therapy, chemotherapy, and antibiotics (67,70). Its inhibitory effect on transporters has been observed in the restraint of cAMP or cGMP release in erythrocytes and the release of ATP in glial cells (74,75). An investigation conducted by Clemente and colleagues revealed that when MRP4 inhibitors and NSAIDs were present, zidovudine, emtricitabine, and tenofovir increased concentration due to reduced efflux rates by the transporter, ultimately amplifying their impact on infected lymphocytes (76). The poor penetration of tenofovir across the BBB shares a similarpredicament with bumetanide (77). Bumetanide is a diuretic for epilepsy and difficult-to-treat seizures (77–79). This therapeutic agent inhibits neuronal Na–K–Cl cotransporter (NKCC1), which is implicated in the pathophysiology of this disease. However, like tenofovir, bumetanide has poor brain penetration by passive and transcellular diffusion(18). The poor BBB penetration by tenofovir is due to its high polarity (carries two negative charges), low lipid solubility increased ionisation and plasma protein binding of bumetamedine comrpmises its penetration (39,77). Both tenofovir and bumetanide are substrates for MRP4 and OAT3 (77,80,81). Töllner et al. demonstrated that systemic administration of probenecid inhibits MRP4 and OAT3, and the inhibition of these transporters increased the concentration of bumetanide in the brain.

Many factors, among them, infection and toxins trigger neuroinflammation. Inflammation involves inflammasomes, protein complexes that regulate the inflammatory response in cells (82). In the CNS, inflammasomes consist of nucleotide-binding oligomerisation domain, leucine-rich repeat-containing family proteins (NLRP), ASC adaptor protein, and caspase-1 enzyme (83). These inflammasomes can be activated by toxins, misfolded proteins, reactive oxygen species, and elevated extracellular ATP and K^+ concentrations (83, 84). Once activated, inflammasomes release proinflammatory cytokines Interleukin (IL)-1β and IL-18, leading to pyroptosis, a form of proinflammatory cell death (85). Pannexin 1 (Panx1) hemichannels play a crucial role in inflammasome activation by mediating ATP release, which acts as a damage-associated molecular pattern (DAMP) during tissue damage (68). The increased activity of Panx1 hemichannels promotes ATP release, affecting neuron-

astrocyte communication and contributing to neurotoxicity (76). Interestingly, research indicates that dopamine, besides its well-known role in cognition, motivation, and movement, may also act as an immunomodulatory agent (86). Previous studies have shown dopamine's ability to alter the production of inflammatory factors (18,86). Despite the advances in cART, overt dopamine neurodegeneration and behavioural symptoms of dopaminergic dysfunction remain in HIV-infected patients, albeit in subtler forms (19). Moreover, deficits in attention and motivation seen in HIV+ individuals are thought to reflect specific dopaminergic dysfunction (12,87). Post-mortem analyses of dopaminergic gene and protein expression have revealed post-synaptic changes associated with neuroinflammation and neurocognitive impairment (19).

Inhibition of Panx1 hemichannels with probenecid has been shown to prevent inflammasome activation, reduce reactive oxygen species (ROS) production, and improve astrocyte survival in cultured astrocytes under oxygen-glucose deprivation conditions (85,88). Furthermore, probenecid has demonstrated a protective effect by blocking Panx1 hemichannels in brain lesions after a subarachnoid haemorrhage in mice, leading to reduced expression of inflammasome-related proteins and purinergic receptors and decreased levels of ROS and cytokines (89).

Given the knowledge of probenecid's role in enhancing medication concentration and mitigating inflammation in the brain, this study sought to investigate whether probenecidcan increase the engagement of tenofovir, an antiretroviral agent, and assess its potential to alleviate inflammation induced by tenofovir and dopamine dysregulation.

1.7. Justification of Study

While ART has profoundly impacted HIV management in the CNS and clinical outcomes, neurological impairment still occurs in patients who adhere to treatment. In some patients, it may be essential to consider antiretroviral medication entry and its effects within the CNS (5). To find ways to successfully treat HIV in the CNS with minimal adverse effects from anti-HIV medication, it is essential to understand the pharmacokinetics and pharmacodynamics of ARV therapeutic agents, individually and in combination, in the system. Studies on TDF, particularly in the brain, are scanty. This study aimed to contribute to the body of knowledge regarding the effects of co-administering tenofovir with probenecid in the treatment of HIV and HAND in the CNS.

1.8 Aim(s)

The study investigated the effect of co-administering tenofovir with probenecid on the concentration of Tenofovir in the prefrontal cortex (PFC) and cerebellum of BALB/c mice. Additionally, the study explored the potential effects of Tenofovir on inflammation and neurotransmitter levels in the PFC.

1.9 Objectives

- Determine if co-administration of tenofovir and probenecid leads to altered concentrations of tenofovir in the prefrontal cortex and cerebellum compared to individual administration.
- Quantify the extent of brain penetration of tenofovir in the presence andabsence of Probenecid.
- Measure the concentrations of dopamine and interleukin-1 beta (IL-1β) in the PFC to assess the potential influence of tenofovir on neurotransmitter levels and inflammation, respectively.
- Conduct correlation analyses to explore any associations between tenofovir concentrations in the PFC and the levels of IL-1β and dopamine.

1.10 Potential Benefits

The potential benefit of this study lies in exploring the use of probenecid as a pharmacokinetic enhancer to improve the CNS penetration of tenofovir, a therapeutic agent with low brain concentrations and neurotoxic potential. By investigating the co-administration of tenofovir and probenecid, the study evaluated the potential enhancement of tenofovir delivery to the brain targeting to improve improve HIV treatment in the central nervous system. Potentially, the study could contribute to a strategy that optimizes tenofovir bioavailability in the CNS but at the same time mitigating its neurotoxic effects thus overall contributing treatment efficacy and safety in HIV patients.

1.11 Methodology Overview

Following approval of all animal experimentation by the Animal Research Ethics Committee of the University of KwaZulu-Natal (AREC/00003133/2021). Male BALB/c mice aged 8-12 weeks were obtained and kept in the university/s Biomedical Research Unit under standard laboratory conditions. The mice were maintained under normal laboratory conditions of constant temperature (22 ± 2 °C), CO_2 content (< 5,000 P.M.), relative humidity (55 ± 5%) and illumination (12 H light/dark cycle, lights on at 07H00). The noise level was maintained at less than 65 decibels. The mice, which were *ad libitum* to water and feed, were randomly assigned into four groups (n=24): TDF treated (5mg/kg), probenecid treated (8.3mg/kg), and TDF + probenecid treated. The

mice were acclimatised to the experimental environment for a week. Each day of the experimental period was dedicated to the focused administration of the designated therapeutic agent to the respective groups. Mice within each group received a single oral dose, with the prescribed dosage of 0.15mg/day TDF and/or 0.24 mg/day of probenecid. For the TDF + probenecid group, TDF was administered first, followed by the administration of probenecid after a 30-minute interval. This sequential dosing strategy was implemented to mimic the protocol established by Anthonypillai et al. The sequential dosing strategy incorporated a 30-minute waiting period, referencing the timing observed in a study conducted by Tollner et al (18,19).It is noteworthy that all animals received their designated drug simultaneously, emphasizing uniformity in the timing of drug administration across groups. This methodical approach aimed to minimize variability and enhance the reliability of the experimental result. The oral administration route was selected for its clinical relevance, mirroring the way patients commonly ingest the prescribed therapeutic agents. This choice aligns with real-world scenarios, enhancing the translational potential of the study to practical applications in patient care (2,20) . After administering the designated drug to all animals, I initiated the sacrifice of six mice at the specified time points of 0, 1, 4, and 6 hours. The plasma, prefrontal cortex and cerebellum were collected for analysis. HPLC was used to measure the concentration of tenofovir in the presence and absence of probenecid, and the extent of brain penetration by each therapeutic agent was calculated using the partition coefficient in the collected tissues. Prefrontal cortex IL-1β and dopamine concentrations were measured using ELISA. Statistical comparisons were carried out using appropriate tests in GraphPad Prism version 8 (San Diego, California, USA), to analyze the data and establish statistical significance. The results were presented as mean ± SEM. For study 1, a two-way ANOVA followed by Bonferroni *post hoc* test was employed, while for study 2, a one-way ANOVA followed by Tukey's multiple comparison test and a two-way ANOVA followed by Bonferroni *post hoc* test was conducted. Additionally, a Pearson correlation analysis was performed to assess the linear relationship. Statistical significance was set at $p < 0.05$ in both studies.

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155. ACID (DOPAC) IN RAT ADRENAL GLAND Akio KAWAGUCHI *, Masami NIWA and Masayori OZAKI ** of Pharmacology of Medicine , Japan Accepted January Abstract-Although norepinephrine and epinephrine are the predominant catecholamines (CAs) in peripheral tissues . 1983;617:611–7.

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Chapter 2

Prologue

Manuscript 1

Tenofovir exhibits restricted permeability across the BBB because of its physiochemical characteristics. This leads to limited access to infected macrophages in the brain. As tenofovir treats HIV infection, poor brain permeability may contributeto developing HAND. Probenecid has been recognised for its capacity to augment tenofovir concentration in plasma. However, the specific mechanism for this enhancement still needs to be better understood. We sought to assess whether probenecid can elevate tenofovir concentration in the brain, particularly in the prefrontal cortex and cerebellum.

Investigating the Pharmacokinetic Enhancement of Tenofovir Concentration by Probenecid in the Prefrontal Cortex and Cerebellum.

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Investigating the Pharmacokinetic Enhancement of Tenofovir Concentration by Probenecid in the Prefrontal Cortex and Cerebellum of BALB/c Mice.

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Abstract

Tenofovir's poor blood-brain barrier penetration limits its ability to clear HIV from brain reservoirs. probenecid, a pharmacokinetic enhancer, increases tenofovir concentration in plasma and the renal system. This study sought to find out if probenecid has similar effects on tenofovir concentration in the brain as its effects in plasma and renal system. Ninety-six maleBALB/c mice were divided into three equal groups: Tenofovir disoproxil fumarate (TDF) treated, probenecid treated, and TDF + probenecid treated. The therapeutic compunds administered orally, and six mice per group were sacrificed after 1h, 4h, and 6h post-treatment to collect blood, prefrontal cortex (PFC) and the cerebellar tissue. The co-administration of tenofovir with probenecid resulted in increased tenofovir concentration with peak concentration at the 6-hour time point. Tenofovir concentration was highest in the cerebellum than in the PFC. Therefore, we showed that probenecid enhanced tenofovir's ability to pass through the blood-brain barrier suggesting that probenecid is a suitable pharmacokinetic enhancer for tenofovir in the brain.

Keywords: Tenofovir, Probenecid, Blood-Brain Barrier, Plasma, Cerebellum, Prefrontal cortex.

1. Introduction

Tenofovir is a nucleotide analogue of adenosine 5'-monophosphate; in this form, it is a dianion at physiologic pH (90) resulting in its low oral bioavailability and poor permeability within the system (90–92). To improve the delivery and pharmacokinetics of tenofovir, a prodrug tenofovir disoproxil fumarate(TDF) was created (93,94). The prodrug improves the delivery and pharmacokinetics of tenofovir by, amongst others, enhancing oral absorption and prolonging its half-life in plasma (90,95). TDF is an essential therapeutic agent used in the treatment of HIV, specifically as a component of highly active antiretroviral therapy (96, 97). Its therapeutic significance lies in its potent antiviral properties and favourable resistance profile, making it a cornerstone in managing HIV infection (90, 96, 98). However, despite its efficacy, TDF faces a limitation in crossing the blood-brain barrier (BBB) (51,99–101).As a result, its brain concentration is significantly lower than in other organs (100,102).This has several implications, including reduced efficacy against HIV in the central nervous system (CNS), leading to continued HIV-related injury (45,103,104). These implications contribute to neurological complications including cognitive impairment, motor dysfunction and behavioural changes (3,105–107). Nonetheless, TDF can traverse the blood-cerebrospinal fluid barrier, providing some therapeutic benefits within the CNS (7, 8, 108).

Pharmacokinetic enhancers such as Probenecid can improve the properties of therapeutic agents including TDF by improving their entry into the brain (70, 76). Probenecid can enhance TDF concentration by increasing its half-life (70, 97, 109) largely by inhibiting its excretion, prolonging its elimination half-life that contribute to slower clearance from the body (81,109,110). Probenecid also functions as a potent inhibitor of organic anion transporters (OATs) and multidrug resistance proteins (MRPs) located at the luminal and abluminal membranes of the intestine, liver, kidney, lungs, placenta, testis, and BBB (42). These transporters play a crucial role in therapeutic compund transport byfacilitating the movement of substances into and out of cells (111–113). Studies have shown a relationship between tenofovir concentration increase and inhibition ofthese transporters by probenecid in the renal system (26, 27). A Study has also shown that the concentration of tenofovir increases in plasma in the presence of probenecid (28). While previous studies have investigated nanoparticle-based approaches to enhance tenofovir penetration across the BBB, the high cost of nanoparticle therapeutics in low-income countries necessitates the exploration

of more affordable alternatives (115–118). Investigating the potential of pharmacokinetic enhancers, specifically in the prefrontal cortex (PFC) and cerebellum, becomes crucial in this context. The PFC is a pivotal brain region renowned for its involvement in intricate cognitive processes. This brain region plays a crucial role in higher brain functions, including complex thinking and decision-making. The notable impact of HIV/AIDS on the PFC, where severe cortical thinning is observed, is evident, with this effect extending not only to the PFC itself but also to related areas. (21). This unique pattern of neurodegeneration in AIDS patients, distinct from other forms of dementia, exerts a profound influence on cognitive function. Additionally, Gruenewald et al further elaborates the impact of HVI on the PFC by revealing a connection between reduced heme oxygenase-1 (HO-1) protein expression in the PFC of individuals with HIV and conditions like HIV-related neurocognitive impairment and encephalitis (22). This reduction in HO-1 is intimately linked to the level of HIV in the brain, interferon-stimulated gene expression, and macrophage activation, emphasizing the intricate interplay between HIV infection and the PFC. Notably, the research also detected HO-1 protein reduction in the neostriatum, suggesting regional variations in the brain's response to HIV infection, as supported by Gelman et al.'s findings of increased interferon-stimulated genes and endothelia-associated genes in the neostriatum of individuals with HIV (23), indicating broader host antioxidant and neuroinflammatory responses extending beyond the PFC.

Traditionally recognized for its part in motor coordination and balance, the cerebellum also plays a role in attention, language, and specific memory functions (24–26). Research by Wächter et al further accentuates the cerebellum's relevance by demonstrating its involvement in HAND (27). Clinical features of HAND encompass cognitive deficits in attention, memory, and executive function, along with cerebellum-associated motor symptoms such as gait disturbances and limb weakness. Moreover, the study highlights unexplained cerebellar atrophy in HIV patients, signifying that HIV-induced cerebellar neurodegeneration precedes high viral load and inflammation during AIDS progression.

Probenecid has shown promise in increasing tenofovir concentrations in the renal system and plasma, but its role in facilitating tenofovir penetration acrossthe BBB remains inadequately explored. This study investigated the potential impact of co-administering TDF with probenecidon tenofovir's ability to penetrate the BBB and subsequently enhance its concentration in the PFC and cerebellum.

2. Materials and Methods

2.1 Reagents

Tenofovir disoproxil fumarate (TDF) and probenecid were procured from Aspen Pharmacare in Port Elizabeth, South Africa. An internal standard, Adefovir disoproxil, was obtained from Sigma-Aldrich in JHB, South Africa, and acetonitrile was sourced from Merck in JHB, South Africa, and used in the study.

2.2 Experimental Animals

Ninety-six BALB/c male mice aged 8-12 weeks, bred and housed in the Biomedical Resource Unit (BRU) of the University of KwaZulu Natal, were used in the study. The mice were acclimatized to the experimental environment prior to study commencement. The mice were maintained under standard laboratory conditions of constant temperature (22 ± 2 °C), CO^2 content (< 5,000 P.M.), relative humidity (55 ± 5%) andillumination (12 H light/dark cycle, lights on at 07H00). The noise level was less than 65 decibels. All animal experimentation was approved by the Animal Research Ethics Committee of the University of KwaZulu-Natal (AREC/00003133/2021). The mice which were given *ad libitum* access to standard rat chow and water, were randomly placed in three groups (n=24 per group) viz: TDF treated, probenecid treated, and TDF + probenecid treated.

2.3 Experimental Protocol

After a week of acclimatization, the mice were randomly allocated into three groups (n=24): the tenofovir-only, the probenecid-only, and the tenofovir + probenecid. All animals were treated simultaneously. The chosen method of oral administration aligns with the way these medications (tenofovir and probenecid) are typically taken by patients, which is a crucial factor in ensuring relevance to human clinical scenarios (2,28). The administration procedure involved a single oral dose of 5mg/kg of TDF and 8.3mg/kg of Probenecid. These dose calculations for TDF and Probenecid were determined as per Zulu et al. (11), based on the human daily dose of TDF (300mg/60kg human = 5mg/kg; 29g mouse = 0.15mg/day)

In the Tenofovir + Probenecid treated group, TDF (0.150 mg/day) was administered initially, followed by Probenecid (0.24 mg/day) 30 minutes later. TDF was dissolved in

5 ml of distilled water, while Probenecid was dissolved in 10 ml of saline with dilute NaOH (77,119). For each time point (1 hr, 4 hrs, and 6 hrs), six mice from each group were sacrificed. At each time point, blood samples were obtained via cardiac puncture. Following blood collection, the mice were decapitated using a guillotine, and the brain was dissected to collect the prefrontal cortex and cerebellum for further analysis.

2.4 Tissue Harvesting

Blood was collected via cardiac puncture, transferred into a heparin tube, and centrifuged at 10000 × g for 10 min (Eppendorf centrifuge 5403, Germany). Plasma was collected and stored at -80°C in a bio ultra-freezer until further analysis. The brain removal procedures followed established protocols (29,30). In summary, the mice were decapitated with a guillotine, and their skulls were meticulously removed using fine scissors, surgical blades, and forceps. Precise dissection techniques were employed to access the brain without causing any damage. Following decapitation, the whole brain was removed immediately and placed in a frozen 0.9% saline slush. Subsequently, the PFC and cerebellum were isolated and weighed in pre-cooled Eppendorf tubes. Afterwards, they were snap-frozen in liquid nitrogen and stored at -80°C in a bio ultra-freezer until further analysis. An illustration of the microdissection process is provided in Appendix 1 and 2.

2.5 Biochemical Analysis

Brain samples. The tissues were prepared as described by Ntshangase et al. (120). Brain tissue collected was briefly weighed and transferred into a tissue culture dish containing 10ml PBS. Cells were dissociated by mechanical trituration and enzyme digestion (0.25% trypsin diluted in PBS for 20 minutes at room temperature). The cells were then neutralised with cell culture medium (serum added), and the suspension was filtered through a 40µm cell strainer to eliminate clumps and debris. The sample was centrifuged at 500 x g for 4-5 minutes at 2-8°C, and the supernatant was discarded. The cells were washed with a cell staining buffer and centrifuged at 400 x g for 5 minutes at 4°C, and the supernatant was discarded (this procedure was repeated). The pellet was resuspended in a cell staining buffer, and a cell count and viability analysis were performed.

High-performance liquid chromatography (HPLC) Instrumentation: The liquid chromatography experiments were conducted on an Agilent 1200 series® liquid chromatography system equipped with a G1379B Micro Vacuum Degasser, a G1312B SL binary pump, a G1367C high-performance autosampler (Hip-ALS SL), a G1330B FC/ALS Therm, a G1316B TCC SL and a G1315C DAD SL (all from Agilent Technologies, Inc., CA, USA). Chromatographic separation was performed on a Zorbax SB C18 column (4.6 × 150 mm, 5 µm, Agilent) with a guard-column cartridge (Agilent, SB C18). All data were analysed using the Agilent Chemstation software (Agilent Technologies, Inc., CA, USA).

Chromatographic conditions: The mobile phase was composed of (A) water containing 0.05% formic acid (v/v) and 0.05% acetonitrile (v/v) and (B) methanol containing 0.1% formic acid (v/v) and 0.05% acetonitrile (v/v). Before application, water and methanol were filtered using a Millipore glass filter system with a nylon membrane (0.2 µm) and degassed for 10–12 min. Analytical separations were conducted at 35°C. The column elute was monitored by DAD at 295 nm, preceded byan RRLC in-line filter, 4.6 mm, 0.2 µm. The gradient elution was applied at a flow rateof 1.0 ml /min, starting at 45% A and 55% B from 0 min linearly programmed to 5% Aand 95% B in 6 min; then the gradient was changed to 45% A and 55% B and maintained for 2 min. The total running time was 8 min.

Standards and calibration curves. Different stock solutions of TDF and internal standard (IS), adefovir disoproxil (ADV) were prepared by dissolving 10mg of each substance in 10Ml of methanol, and the solutions were stored at refrigerated temperature (0-4°C). A series of TDF working standards solutions and an IS solution will be prepared by appropriate dilutions of their stock solutions with acetonitrile: deionised water (1:1, v/v). Calibration standards were prepared by spiking working standard solutions into 100 µL of blank mice plasma or different brain tissue homogenates of untreated mice to yield tenofovir concentrations of 10-1500 ng/Ml.

Plasma sample preparation. 5µL of the internal standard was added to 100 µL ofthe plasma homogenate sample. The mixture was vortexed briefly, and 895µL of methanol was added to extract the target analyte while protein precipitation is induced. The combination was vortexed again for 30 sec and centrifuged at 1000 xg for 10 min at 4°C to remove the precipitate. The supernatant was filtered througha super-select HLB (30mg/ ml) solid-phase extraction (SPE) cartridge. The selection of the SPE cartridge

will be based on analyte percentage recovery after filtration. The extract was collected into autosampler vials and briefly vortexed, and then 5µL was injected into the HPLC system.

Brain samples. Each brain tissue area of interest (prefrontal cortex, cerebellum) was weighed and homogenised in three volumes of ultrapure water (3 ml /g tissue). The same extraction procedure used for the blood plasma sample was followed for each brain tissue homogenate sample.

Calculation of tenofovir/probenecid concentration and extent of tenofovir/probenecid penetration. Each sample collected was ran through HPLC, and from the chromatogram produced, the peak of the compound of interest (Tenofovir and Probenecid) was recorded. Using the calibration curve equation, each sample's concentration was calculated. The extentof tenofovir brain penetration was calculated to quantify the amount of tenofovir/probenecid that reached the brain by describing the partition coefficient $K_{b,brain}$:

$$K_{b,brain} = \frac{C_{tot,brain}}{C_{tot,plasma}}$$

3. Statistical Analysis

The data was analysed using GraphPad Prism version 8 (San Diego, California, USA). All results are presented as the mean + standard error of mean (SEM). Two-way ANOVA followed by Bonferroni*post hoc* test was used to analyse the data, and $p < 0.05$ was considered significant.

4. Results

4.1 Plasma, Cerebellum and PFC tenofovir/probenecid Concentrations

When administered alone or in combination, the probenecid concentration was analysed in plasma, cerebellum, and PFC at 1,4, and 6-hour intervals (Figure 1). A change in probenecid concentration was observed in the PFC and cerebellum during the experimental period [$F(5,90) = 9.284$, $p<0.0001$].

When probenecid was administered alone, a higher concentration was present in the cerebellum than in plasma at the 1h time point $^{\theta}$(Plasma Probenecid vs Cerebellum Probenecid at 1h, $p<0.05$ (Figure 1). When probenecid was co-administered with tenofovir, a higher probenecid concentration in the PFC was present at 6h than in plasma $^{\pi}$(Plasma co-administered Probenecid at 6h vs PFC co-administered Probenecid at 6h, $p<0.05$ (Figure 1)).

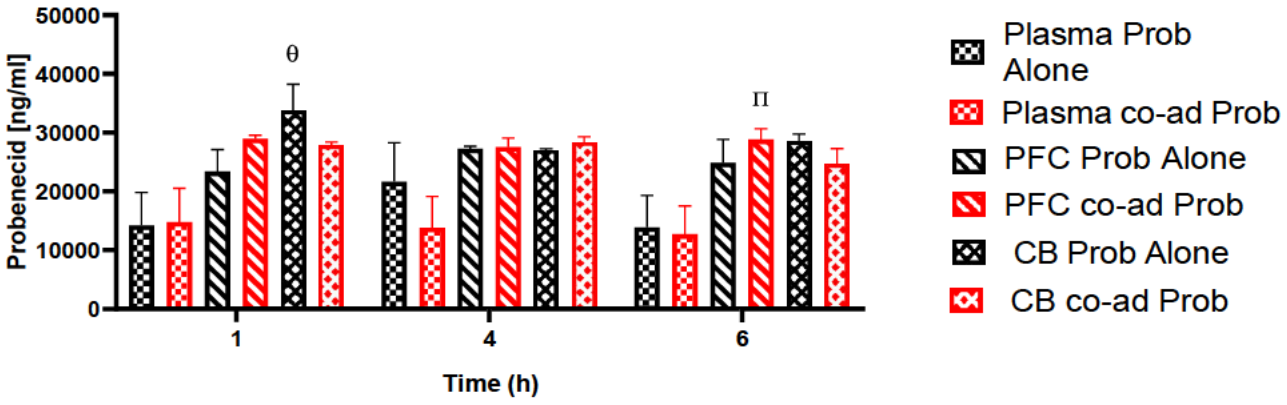

Figure 1: Concentration of probenecid in the plasma, cerebellum, and PFC at 1h, 4h, and 6h time intervals. $^{\Theta}p<0.05$ denotes comparison with plasma Probenecid alone at 1h, $^{\pi}p<0.05$ denotes comparison with plasma Probenecid co-administered at 6h. (Prob=Probenecid, CB=cerebellum, co-ad=co-administration)

The concentration of tenofovir, when administered alone or co-administered, was analysed in plasma, cerebellum, and PFC at 1, 4-and 6-hour intervals (Figure 2). co-administration resulted in increased tenofovir concentration [$F(5,77)=16.42$, $p<0.0001$].

In plasma, there was a co-administration effect on tenofovir concentration at 6h $^{\sigma}$(co-administered tenofovir at 1h vs co-administered tenofovir at 6h $p<0.05$, (Figure 2).

In the PFC, there was a co-administration effect on tenofovir concentration at the 1 h time point *(tenofovir alone at 1h vs co-administered tenofovir at 1h, $p<0.05$, (Figure2). At the 6h time point, the concentration of tenofovir was notably higher $^{\alpha}$(tenofovir alone at 6h vs co-administered tenofovir at 6h, $p<0.05$, Figure 2).

In the cerebellum, tenofovir concentration was highest at 6h following co-administration $^{\omega}$(tenofovir alone at 6h vs co-administered tenofovir at 6h, $p<0.05$, Figure 2), $^{\#}$(co-administered tenofovir at 1h vs co-administered tenofovir at 6h, $p<0.05$, Figure 2), and $^{\beta}$(co-administered tenofovir at 4h vs co-administered tenofovir at 6h, $p<0.05$, Figure 2).

Additionally, tenofovir concentration following co-administration was higher in the cerebellum than in the PFC at 6h $^{\varepsilon}$(PFC co-administered tenofovir at 6h vs cerebellum co-administered tenofovir at 6h,$p<0.05$).

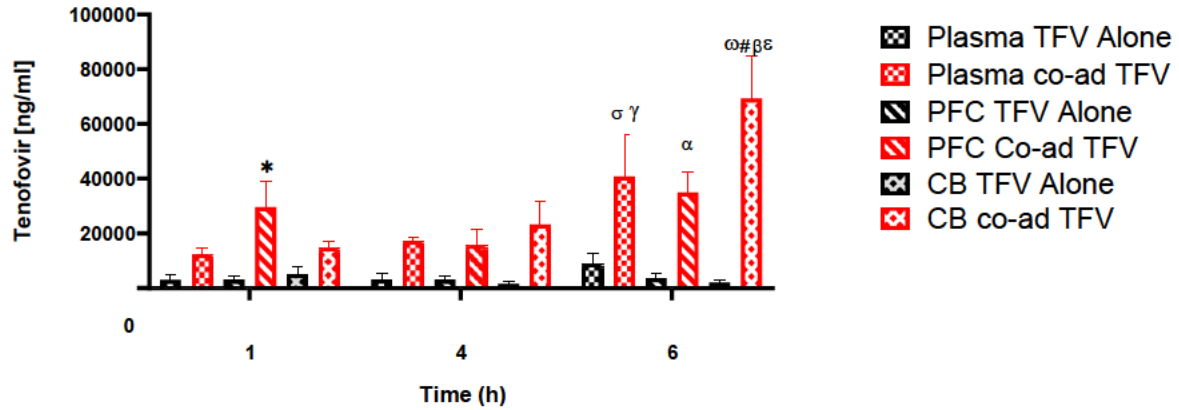

Figure 2: Concentration of tenofovir in the plasma, cerebellum, and PFC at 1h, 4h, and 6h time intervals. *$p<0.05$ denotes comparison with PFC tenofovir Alone at 1h, $^{\sigma}p<0.05$ denotes comparison with plasma Tenofovir co-administered at 1h, $^{\gamma}p<0.05$ denotes comparison with plasma tenofovir co-administration at 4h, $^{\alpha}p<0.05$ denotes comparison with PFC tenofovir Alone at 6h, $^{\omega}p<0.05$ denotes comparison with cerebellum

tenofovir alone at 6h, $^{\#}$p<0.05 denotes comparison with cerebellum co-administered Tenofovir at 1h, $^{\beta}$p<0.05 denotes comparison with cerebellum co-administered tenofovir at 4h, and $^{\ell}$p<0.05 denotes comparison with PFC co-administered Tenofovir at 6h.(TFV=tenofovir)

4.2 The extent of probenecid and tenofovir penetration in the brain

The extent of brain penetration by each compund (tenofovir and probenecid) when administered alone or in combination was compared in the cerebellum and PFC at 1h, 4h, and 6h time intervals (Table 1). We did not observe any significant change in drug penetration.

Table 1: Extent of brain penetration by tenofovir and probenecid (administered individually and in combination) in the cerebellum and PFC at 1h,4h, and 6h time intervals.

	Probenecid Alone			**Tenofovir Alone**			**Co-administered Tenofovir+Probenecid**					
							Probenecid			TENOFOVIR		
	1 hr	4 hr	6 hr	1hr	4 hr	6 hr	1 hr	4 hr	6 hr	1 hr	4 hr	6 hr
cerebellum	1,27	0,86	1,10	0,44	0,12	0,11	1,01	1,10	1,05	1,21	1,36	2,23
PFC	0,88	0,87	0,96	0.27	0,25	0,21	1,05	1,07	1,23	2.39	0,91	1,13

5. Discussion

Tenofovir disoproxil fumarate, the prodrug tenofovir, is an effective and safe therapeutic agent for HIV treatment (121,122), but it has limited ability to cross the BBB (5, 8,102). In this study, we postulated that since co-administration of TDF with a pharmacokinetic enhancer increases TDF concentration in certain tissues and organs (109,123), co-administration will increase tenofovir BBB penetration. The primary objective of this study was to assess the potential of probenecid as a pharmacokinetic enhancer for tenofovir, with a specific focus on its impact on tenofovir penetration across the BBB and its concentration in the cerebellum and PFC. Our findings indicate that probenecid positively influences the pharmacokinetic properties of tenofovir. These results suggest that probenecid holds promise as a pharmacokinetic enhancer for tenofovir.

Although no change was observed in the extent of probenecid penetration in the cerebellum and PFC when the therapeutic agent was administered alone, an increase (1h) in probenecid concentration was observed in the cerebellum following co-administration with tenofovir. This effect was observed at the 6h time point in the PFC. This may assist in understanding the therapeutic agent's behaviour in these brain regions. It can be postulated that the lack of change in the extent of Probenecid penetration in the cerebellum and PFC when administered individually compared to when co- administered with TDF may be due to the therapeutic agent's inherent properties and its interaction with the BBB (69, 70, 124). Probenecid has similar permeability characteristics in both scenarios, indicating that TDF does not affect its ability to cross the BBB.

Our results demonstrate a noticeable decrease in the extent of brain penetration and concentration of tenofovir in the cerebellum and PFC over the experimental period. This outcome can be attributed to two main factors. Firstly, the decrease in the therapeutc agent's half-life reduces therapeutic agent penetration (109,123). Secondly, tenofovir relies on active transporters, specifically organic anion transporters (OAT) 1 and 3, as well as multidrug resistance proteins (MRP) 4 and 5, which are found on the BBB (51,125). This therapeutic agent has high polarity and relatively low lipid solubility, making it a substrate for these efflux transporters (95). Thus, the transporters actively pump tenofovir out of the brain and back into the bloodstream, limiting its accumulation in the brain over time.

Our results align with previous studies that showed tenofovir concentration decreases over time (51, 120), but these studies did not include the extent of therapeutic agent penetration. However, they did calculate the brain/plasma ratio that showed that at a maximum concentration of 54.5ng/g (31), Tenofovir's brain/plasma ratio was 0,6% compared to elvitegravir's maximum concentration of 976.5ng/g at 3.2%. Taken together, we can deduce that tenofovir has a low ability to cross the BBB when administered individually, and the level of BBB penetration by tenofovir influences the concentration of the therapeutic agent inside the brain.

When TDF was co-administered with probenecid, there was an increase in tenofovir penetration. The highest concentration was at 6 hrs. when tenofovir was co-administered; its concentration was higher in the cerebellum than in the PFC. Consequently, probenecid prolongs the presence of tenofovir in the bloodstream, leading to an extended circulation time (98,109). As a result, the brain concentration of tenofovir is increased. Moreover, at the BBB, Probenecid competes with and inhibits the active transporters responsible for the efflux of Tenofovir from thebrain (51,70,102). This inhibition prevents the transporters from actively pumping Tenofovir out of the brain.

Our results in the cerebellum contrast with Anthonypillai et al., who did not find a change in Tenofovir concentration following co-administration (23). However, this study had a shorter timeline, measuring concentrations up to 30 minutes. It should also be noted that while we administered the therapeutic agent in its prodrug form, in Anthonypillaiet al., it was issued in its molecular structure, tenofovir. The state in which tenofovir is administered (prodrug or drug) is essential. Numerous studies have shown that TDFhas excellent permeability and cellular accumulation than tenofovir, resulting in considerably higher concentrations of TDF in the body (127–130). It is noted that TDF in prodrug form can diffuse passively across the plasma membrane (129). However, what is emphasised in these studies is the work of protein transporters (129,130). Interaction between TDF and transporters depends on the tissue, as it has been shown that the transporters of choice in crossing the BBB are different from those used by the same therapeutic agents in organs such as the placenta and female genital tract cells (8,51,129,130). The expression of transporters can also influence the route of

administration. For example, the intraperitoneal bioavailability of Tenofovir dramatically exceeds that of oral administration (131). However, it has been shown that bioavailability is higher in dogs than in mice and monkeys following oral administration (131,132). Cundy et al suggested that this was due to the presence of active transporters in the intestine of dogs (131). With this information, we opted for oral administration of TDF as it is done in human beings (133,134)

We showed that tenofovir brain penetration is increased following co-administration with probenecid. This resulted in increased therapeutic agent concentration in the cerebellum and PFC, in contrast to the decreasing concentration observed when tenofovir was administered alone. This suggests that probenecid enhances the ability of tenofovir to cross the BBB and reach these regions. These findings are significant because they indicate that probenecid may improve the delivery of tenofovir to the CNS, potentially enhancing its therapeutic effects in the brain. The concentration of tenofovir in the cerebellum and PFC also increased over time when co-administered, in contrast to the decreasing concentration observed when tenofovir was administered alone. These findings suggest that probenecid also helps maintain higher tenofovir concentration over an extended period in these brain regions. This prolonged exposure to tenofovir may have clinical implications, such as improved efficacy in treating HIV in the CNS and HIV-associated neurological diseases. While probenecid is known to enhance the systemic exposure of certain therapeutic agents, including tenofovir, its impact on brain penetration and concentration of tenofovir has not been extensively studied. The comparison of tenofovir penetration and concentration in two specific brain regions, the cerebellum and PFC, provides valuable insight into the therapeutic agent’s distribution in different brain areas. This information is crucial as other brain regions may exhibit varying responses to therapeutic agent therapies.

Lastly, monitoring the extent of therapeutic agent brain penetration and concentration at multiple time points was essential to the study. It allowed for a better understanding of how thetherapeutic agent and enhancer interact over time, providing insight into their pharmacokinetic profile and potential clinical implications.

6. Conclusion

In conclusion, probenecid positively influences the pharmacokinetic properties of tenofovir, as evidenced by increased therapeutic agent brain penetration and concentration over the experimental period. These results suggest that probenecid holds promise as a pharmacokinetic enhancer for tenofovir, potentially improving its therapeutic efficacyin the central nervous system. However, further research is needed to understand the specific mechanisms underlying the concentration changes observed in this study, mainly focusing on the role of transporters on the BBB and the implications of increased Tenofovir concentration for HIV-related injuries and management.Monitoring therapeutic agent brain penetration and concentration over time provides valuable insight into the pharmacokinetic profile of Tenofovir and its potential clinical applications.

Declaration of interest:

None

Author contributions:

Miss Shabalala conceptualized the research idea, designed the methodology, literature review, and conducted experiments. She collected and analysed data, interpreted results, and wrote the dissertation. Prof. Mabandla and Dr. Luvuno guided the research direction and provided invaluable feedback on refining the research question. They offered insights into data analysis techniques and experimental design, reviewed drafts, provided constructive criticism, and aided in structuring the dissertation. They also provided guidance during the data analysis phase and contributed to result discussions. Furthermore, they reviewed the dissertation, offering expertise-based feedback, and acted as mentors, supporting the overall research process.

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Ethical approval:

Ethical approval was obtained from the Animal Research Ethics Committee (AREC/00003133/2021) of the University of KwaZulu Natal

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155. ACID (DOPAC) IN RAT ADRENAL GLAND Akio KAWAGUCHI *, Masami NIWA and Masayori OZAKI ** of Pharmacology of Medicine , Japan Accepted January Abstract-Although norepinephrine and epinephrine are the predominant catecholamines (CAs) in peripheral tissues . 1983;617:611–7.

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Chapter 3

Prologue

Manuscript 2

In the first study, the co-administration of tenofovir with probenecid resulted in an increase in tenofovir concentration in the brain. These results hold promise for effectively managingHIV and related brain injuries. However, Tenofovir possesses neurotoxic properties, even at low concentration. Therefore, the increased tenofovir concentration may exacerbate its neurotoxic effects. To address this concern, the focus transitions to identifying an enhancer that increases Tenofovir concentration and mitigates its associated neurotoxicityTherefore, the subsequent investigation aims to assess whether Probenecid can effectively regulate the alterations in IL-1β, and whether dopamine concentration can be influenced by TenofovirBy studying these parameters, we can gain valuable insight into the potential neuroprotective properties of Probenecid following co-administration with Tenofovir.

"Evaluating anti-inflammatory and neuroprotective effects of probenecid against the neurotoxic effects of tenofovir disoproxil fumarate: effects on Interleukin-1 Beta and Dopamine concentration in the prefrontal cortex of BALB/c mice."

The current manuscript will be submitted for publication in the journal of **Frontiers in Neuroscience.** The manuscript has been prepared in the style used in papers published in **Frontiers in Neuroscience.**

Evaluating anti-inflammatory and neuroprotective effects of Probenecid against the neurotoxic effects of tenofovir disoproxil fumarate: effects on Interleukin-1 beta and dopamine concentration in the prefrontal cortex of BALB/c mice.

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Abstract

Human Immunodeficiency Virus (HIV) infection and the use of antiretroviral therapy contribute to neurotoxicity through inflammation and neurotransmitter dysregulation. The present study aimed to investigate the effects of co-administering tenofovir with probenecid on interleukin-1β (IL-1β) and dopamine concentration in the prefrontal cortex (PFC). BALB/c mice were assigned to three treatment groups viz. tenofovir only, probenecid only, and tenofovir + probenecid. These groups were given a single dose of their designated therapeutic agent orally. The PFC was collected at 0-, 1-, 4-, and 6 hours following Therapeutic agent administration. We showed that Tenofovir administration increased IL-1β concentration at all intervals post-administration, while Probenecid did not affect IL-1β concentration. When Tenofovir and Probenecid were co-administered, the IL-1β concentration followed the same trend as when Tenofovir alone was given. This suggests that the anti-inflammatory effects of Probenecid might require additional time to manifest fully. The order of administration and the timing of Probenecid after Tenofovir could potentially influence the observed outcomes. Both Tenofovir and Probenecid did not affect dopamine concentration. Furthermore, correlation analysis demonstrated no association between inflammation and dopamine. Prior research has emphasised dopamine disruption due to oxidative stress and inflammation in different brain regions, motivating the need for expanded study encompassing multiplebrain areas. In conclusion, Probenecid's limited impact on IL-1β concentration suggests that its anti-inflammatory properties may take more than 6h to take full effect. The absence of direct influence on dopamine in the experimental period may suggest that neurotoxicity should be investigated in areas of the mesocortical pathwaycloser to the ventral tegmental area where it is synthesised.

Keywords: Tenofovir, Probenecid, IL-1β, dopamine, cognitive function, inflammation

1. Introduction

Before the advent of combination anti-retroviral treatment, HIV infection in the CNS resulted in significant neuropathology, including microglial activation, multinucleated giant cell formation, reactive astrogliosis, inflammation, myelin loss, and regional atrophy, particularly in the prefrontal cortex (87,135,136). While combination anti-retroviral treatment has greatly reduced and altered the severity of these effects, more subtle forms of neuropathology persist in most infected individuals (137). Despite viral replication being suppressed in the current era of HIV treatment, HIV-associated neurocognitive disorders (HAND) continue to affect approximately 50% of infected individuals (3). These disorders manifest as cognitive impairment, including attention deficit, working memory problems, executive function difficulties, processing speed deficits, behavioral changes, and motor coordination issues (10,11). These are accompanied by antiretroviral therapy (ART)-induced neuroinflammation and disruptions in neurotransmission, even in virally suppressed individuals suggesting neurotoxic effects of the treatment (12–16). To better understand the underlying mechanisms contributing to HAND, the present study focuses on the prefrontal cortex (PFC) based on compelling evidence from previous studies. In a study investigating the alterations in synapse structure in the frontal neocortex associated with HIV encephalitis and HAND, revealed dysregulation of synaptic preproenkephalin and dopamine receptor D2L in the frontal neocortex of HIV-infected individuals. Indicating potential implications for neuropsychological outcomes (23). Using a mouse model, Cirino et al investigated the effects of HIV-1 Tat on neurons in specific brain regions. They found excitatory effects in prefrontal cortical neurons (32). These findings support our choice to investigate the PFC in the context of providing a foundation for exploring ART-induced neuroinflammation and neurotransmitter dysregulation.

Among antiretroviral treatments, nucleoside reverse transcriptase inhibitors (NRTIs) like Tenofovir disoproxil fumarate (TDF) induce mitochondrial dysfunction (9,10,16). *In Vitro* studies have demonstrated that NRTIs inhibit mitochondrial DNA synthesis by blocking polymerase gamma, leading to inadequate energy production and malfunctioning mitochondria (11,138). Additionally, NRTIs disrupt other mitochondrial functions, causing oxidative damage, enzyme inhibition, electron transport chain uncoupling, and impaired ATP synthesis, ultimately

triggering cellular apoptosis (10, 56). ART-induced stress in mitochondria activates inflammasomes like nucleotide-binding oligomerisation domain (NOD)-like receptor protein 3 (NLRP3), increasing cytokines such as the pro-inflammatory Interleukin-1β (IL-1β) (139).

It has been shown that innate immune activation and the release of inflammatory cytokines have a preferential impact on reward pathways and brain dopamine levels, leading to reduced motivation and slowing motor function (18,19,140). Dopamine in the prefrontal cortex is primarily synthesized in the ventral tegmental area, located in the midbrain. It reaches the prefrontal cortex through the mesocortical pathway, playing a crucial role in the regulation of behaviour,

motivation, and cognitive function (141,142). Evidence supports the link between inflammation and brain dopamine from neurochemical and behavioural studies conducted in rodents administered with acute or sub-chronic interferon (IFN)-α doses (18). Other studies demonstrated that IFN-α releases the cytokines IL-1, IL-6,and tumour necrosis factor. These studies reported varied effects on dopamine and dopamine metabolites, accompanied by depressive behaviours and changes in locomotor activity (19,143,144). Recent data points to dopamine playing a crucial role as a regulator of inflammation (140,145). Therefore, the interplay between inflammation and dopamine in HAND becomes apparent. Tenofovir has shown significant benefits in HIV management and is recommended for pre- or post-exposure prophylaxis (10, 98, 123). However, mitigating potential neurotoxicity has been unsuccessful.

Notably, Probenecid's role as a pannexin (Panx)1 hemichannel blocker has been highlighted for reducing neuroinflammation, a significant factor in various CNS alterations (146–148). Under inflammatory conditions, Panx1 is upregulated in microglia and astrocytes, suggesting a role in inflammatory responses to injury or infection (68,69). These studies show that Probenecid blocks Panx1 hemichannel currents, thereby reducing neuroinflammation, a secondary factor in various brain pathologies.

Probenecid also reduces the excretion of acidic metabolites of dopamine and serotonin, prompting investigations in the psychiatric field (85,149). Pioneering studies have demonstrated that Probenecid blocks the transport of neurotransmitter metabolites, including homovanillic acid for dopamine, vanillyl-mandelic acid for catecholamines, and 5-hydroxy indoleacetic acid for serotonin, limiting their subsequent excretion in urine (12,19,87,149). These findings have led to its use as a clinical test for depression (18,150).

Studies have shown that Tenofovir has been highly beneficial in managing and controlling HIV, and it is still the most recommended NRTI in HIV pre-exposure or post-exposure prophylaxis (109). Therefore, exploring ways to allow Tenofovir to exert its total activity while mitigating related injury is essential. Thus, the present study aimed to elucidate the effects of co-administering Tenofovir with Probenecid

on IL-1β and dopamine concentrations in the PFC of mice at different time intervals.

2. Materials and Methods

2.1 Chemicals

Tenofovir disoproxil fumarate (TDF) and probenecid were purchased from Aspen Pharmacare in Port Elizabeth, South Africa. An internal standard, Adefovir disoproxil, was obtained from Sigma-Aldrich in JHB, South Africa, and acetonitrile was sourced from Merck in JHB, South Africa, and used in the study.

2.2 Animals

Ninety-six BALB/c male mice aged 8-12 weeks, bred and housed in the Biomedical Resource Unit (BRU) of the University of KwaZulu Natal, were used in the study. The mice were maintained under standard laboratory conditions of constant temperature (22 ± 2 °C), CO_2 content (< 5,000 P.M.), relative humidity (55 ± 5%) andillumination (12 H light/dark cycle, lights on at 07H00). The noise level was less than 65 decibels. All animal experimentation was approved by the Animal Research EthicsCommittee of the University of KwaZulu-Natal (AREC/00003133/2021). Mice weregiven access to standard rat chow and water *ad libitum*. Mice were randomly placed in three groups (n=24): TDF treated, Probenecid treated, and TDF + Probenecid treated. The mice were acclimatised to the experimental environment for a week.

2.3 Experimental protocol

The mice were grouped into three groups (n=24): Tenofovir only, Probenecid only, and Tenofovir+ Probenecid. Mice were administered a single oral dose of 5mg/kg of TDF or 8.3mg/kg of Probenecid. The dosage was calculated as previously described by Zulu et a (11). Briefly, the dose calculation for human TDF daily dose: 300 mg/60 kg human=5 mg/kg; 29g mouse=0.15mg/ day. Treatment included 0.150 mg/kg for the tenofovir-only,0.24 mg/kg for the probenecid, and the in the tenofovir + probenecid treated, TDF (0.150 mg/day) was administered first, and Probenecid (0.24 mg/day) was administered 30min later. TDF was dissolved in distilled water (5 ml), and Probenecid was dissolved in 10 ml saline (dilute NaOH) (77,151). After treatment, six mice in each group were decapitated using a guillotine at three different time points: 1

hour, 4 hours, and 6 hours. Subsequently, the brain was dissected to collect both the prefrontal cortex (PFC) and the cerebellum.

2.4 Tissue harvesting

The brain removal procedures adhered to established protocols (10,11). In brief, mice were decapitated with a guillotine, and their skulls were meticulously dissected using fine scissors, surgical blades, and forceps to access the brain without causing any damage. The entire brain was swiftly extracted after decapitation and placed in a frozen 0.9% saline slush. Subsequently, the prefrontal cortex (PFC) and cerebellum were isolated, weighed, and placed in pre-cooled Eppendorf tubes. They were then rapidly frozen in liquid nitrogen and stored at -80°C in a bio ultra-freezer for subsequent analysis. An illustration of the microdissection process is provided in Appendix 1 and 2.

2.5 Biochemical analysis

ELISA

Before the ELISA technique, the brain samples were thawed, and they were homogenised in PBS (0.01M, pH 7.4) in a ratio of 1:9 (tissue weight (g): PBS (mL) volume) with an ultrasonic cell disruptor (sonicator). The homogenate was centrifuged for 10 min at 5000 xg at 2°C to get the supernatant. The ELISA was conducted according to the manufacturer's (Elabsciences) instructions to quantify the levels of IL-1 beta and dopamine in the supernatant.

3. Statistical analysis

The data was analysed using GraphPad Prism version 8 (San Diego, California, USA). All results are presented as the mean ± SEM. The data was analysed using a one-way ANOVA followed by Tukey's multiple comparison test and a two-way ANOVA followed by Bonferroni post hoc test. A Pearson correlation analysis was also conducted to assess the linear relationship. A $p < 0.05$ was considered significant.

4. Results

4.1 IL-1β concentration

The analysis of variance revealed a significant effect of the tenofovir alone treatment condition on IL-1β (F (1.315,3.944) =68.17, p=0.0011), indicating that there was a statisticallysignificant difference within the group at different time intervals (Figure 1A). There wasa Tenofovir (alone) effect on IL-1β concentration at the 1 h interval *(0h vs 1 h, p< 0.0001, Figure 1A). There was a subsequent decrease at the 4 and 6h time points #(1hvs 4h, 1h vs 6h, p< 0.0003, Figure 1A). The PFC IL-1 β concentration did not return tobaseline *(0h vs 4h and 0h vs 6h, p< 0.0003, Figure 1A).

A tenofovir + probenecid treatment effect was present on IL-1β concentration (F (1.048,3.145) =6.69, p=0.0031). A co-administration effect was present at 1h *(0h vs 1h, p<0.0001, Figure 1C). There was a subsequent decrease at the 4 and 6 hr marks #(1h vs 4h,p< 0.0003 and 1h vs 6h, p<0.0001, Figure 1C). concentration was greater than at baseline, *(0h vs 4h, p<0.0004, 0h vs 6h, p<0.0018, Figure 1C).

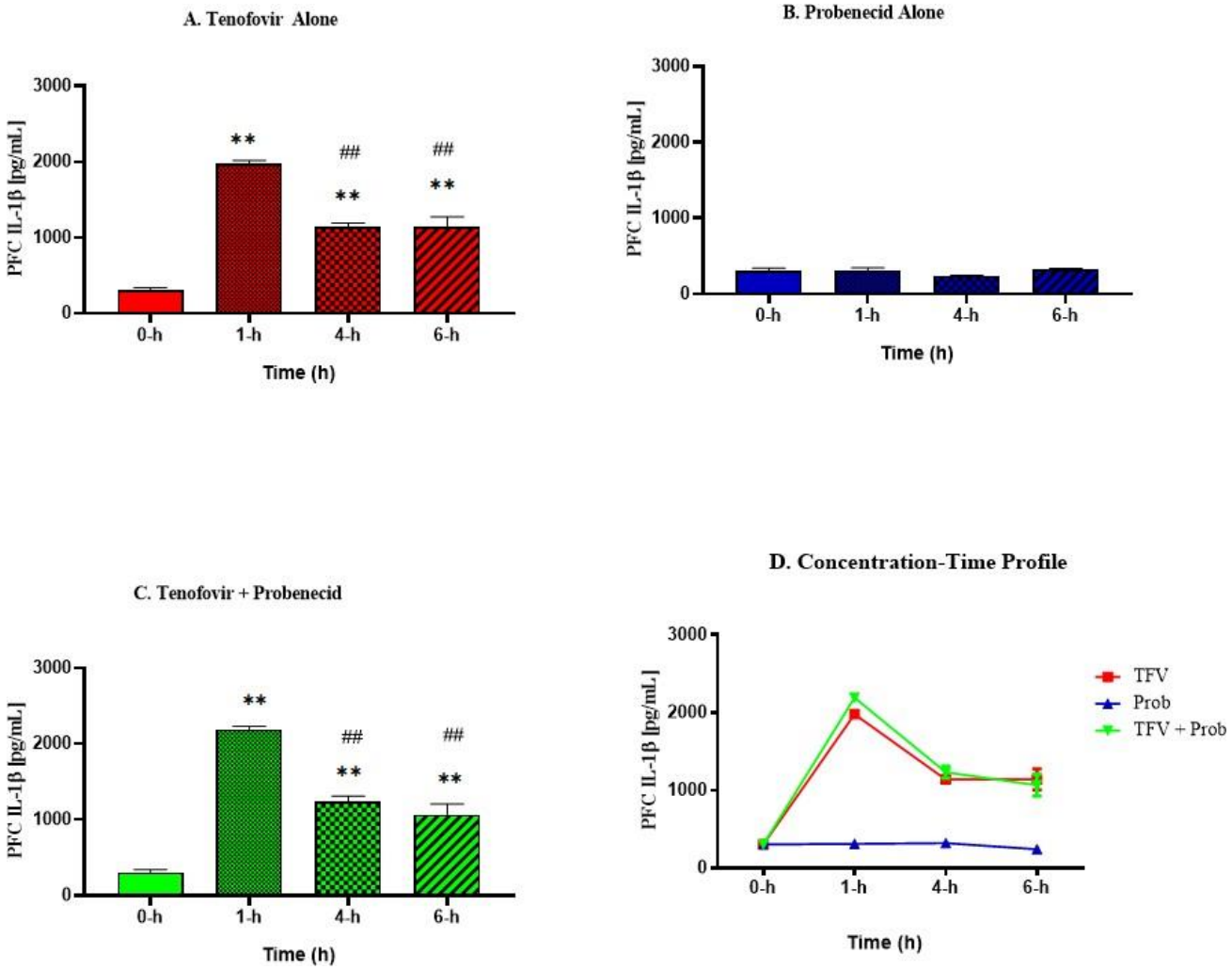

Fig.1 PFC IL-1β concentration of the tenofovir, probenecid, and tenofovir + probenecid groups (n=3) in 0-h, 1-h, 4-h, and 6-h time intervals. Values are presented as mean ± SEM. *p<0.05 denotes comparison with 0-h; #p<0.05 denotes comparison with 1-h.

4.2 Dopamine Concentration

Figure 2 below displays the measurement of dopamine concentration in the Tenofovir, Probenecid, and Tenofovir + Probenecid groups at 1-hour, 4-hour, and 6-hour time intervals. There was no significant change in dopamine concentration.

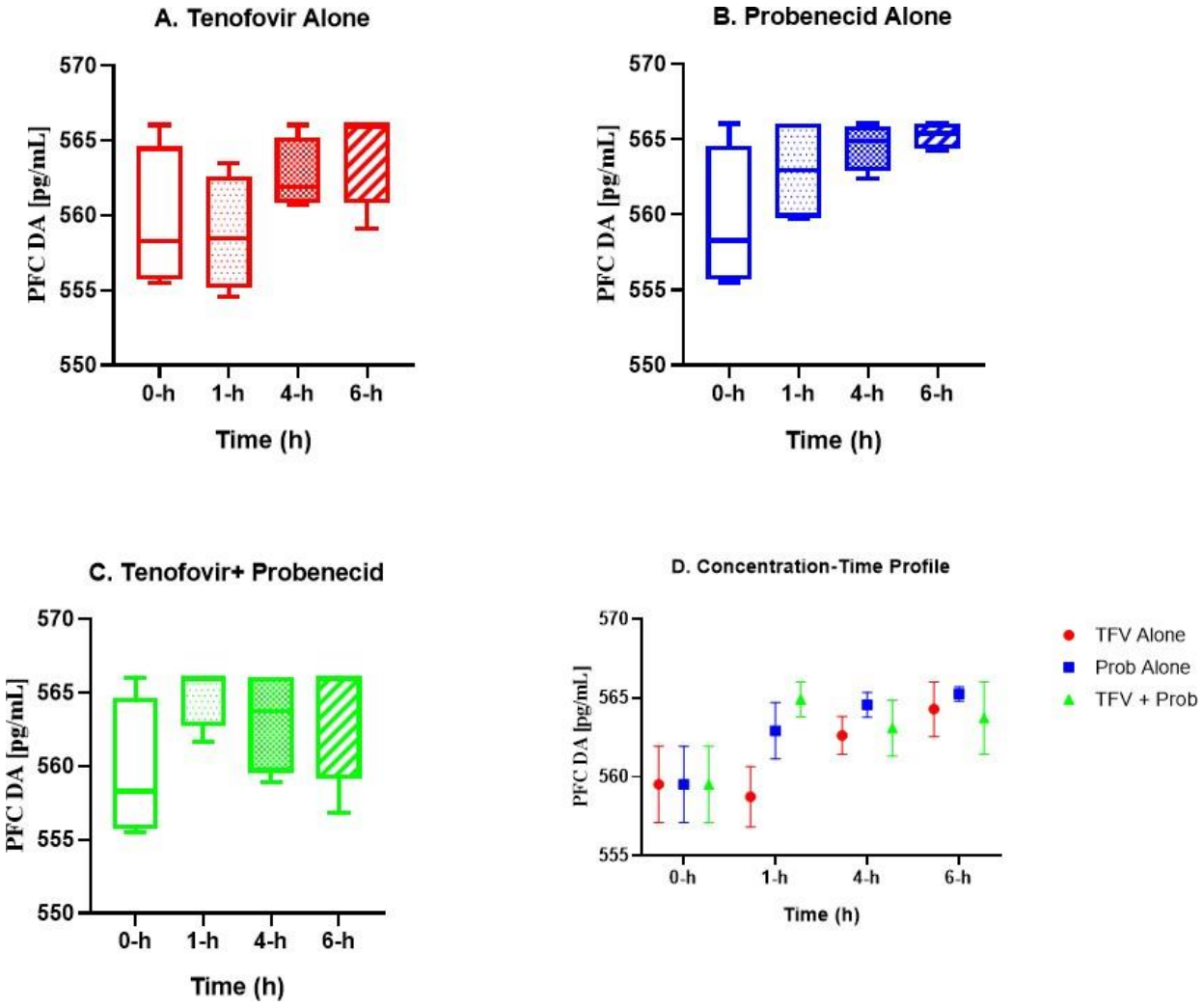

Figure 2: PFC dopamine concentration in the tenofovir, probenecid, and tenofovir + probenecid groups (n=3) at 0-h, 1-h, 4-h, and 6-h time intervals.

4.3 IL-1β and Dopamine Concentration Correlation.

A Pearson correlation coefficient was computed to assess the linear relationship between time and the concentration of IL-1β and dopamine (Figure 3). No changes were observed.

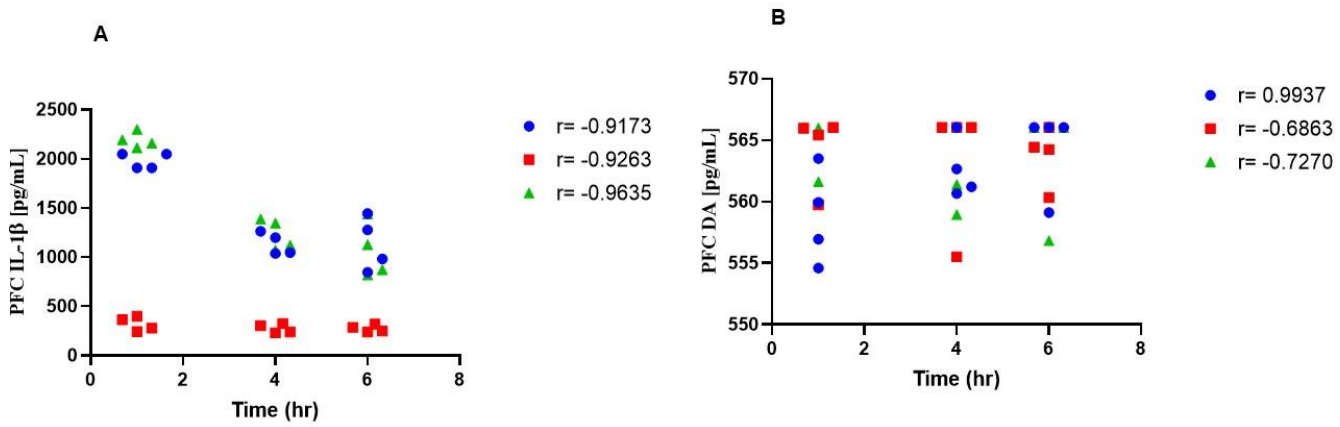

Figure 3: Correlation analysis of tenofovir and probenecid PFC IL-1β concentration (A), and PFC dopamine (B) concentration with 0-h, 1-h, 4-h, and 6-h time intervals in the Tenofovir group, probenecid group, and tenofovir + probenecid group (n=3).

5. Discussion

Antiretroviral therapy-associated neurotoxicity contributes to the exacerbation of HAND in HIV-infected individuals (55, 56). Therapeutic strategies are needed to target neurotransmitter dysregulation and the neuroinflammation induced by these therapeutic agents. Studies show that low CNS tenofovir concentration (5% of plasma concentration) can lead to neurotoxicity through mitochondrial dysfunction (9,16). The stress induced by tenofovir on mitochondria activates inflammasomes, such as NLRP3, resulting in increased inflammatory cytokine release, such as IL-1β (152). This inflammation has been found to impact brain dopamine concentration, thus affecting the reward pathway, causing reduced motivation and impaired motor function (153).

In this study, we aimed to assess whether probenecid could attenuate the inflammatory response and the dopamine dysregulation induced by tenofovir treatment. To achieve this, we examined the effects of combined therapy with tenofovir and probenecid on IL-1β concentration and investigated the impact on dopamine concentration in the PFC. Both tenofovir alone and the tenofovir co- administered with probenecid groups showed an initial peak in IL-1β concentration followed by a gradual decline until the end of the experiment. Notably, neither tenofovir nor probenecid, alone or in combination, affected dopamine concentration.

Neuroinflammation is evidenced by elevated levels of pro-inflammatory cytokines such as IL-1 β and tumour necrosis factor-α (TNF-α) (144,145). In the 1st hr following single administration of tenofovir, there was a substantial increase in IL-1 β concentration. This was followed by a gradual decrease that stabilised at levels higher than the baseline concentration. These findings are consistent with studies showing that tenofovir induces brain inflammation (11,16). In the co-administration group, the IL-1β concentration displayed a similar trend to tenofovir alone, indicating that the presence of probenecid did not seem to counteract the tenofovir-induced neuroinflammation. Our results suggest that the neuroinflammatory response is swift,as evidenced by the peak increase in the 1st hr. At the same time, the anti-inflammatory effects of probenecid might require more time to manifest fully. Moreover, it's plausible that probenecid's impact on tenofovir-induced IL-1B response wasn't observable within the confines of the experimental timeline, owing to the subsequent administration of probenecid post-tenofovir. This theory is reinforced by Wonnenberg et al., where the anti-inflammatory properties of probenecid were scrutinised (154). In

this study, probenecid was initially administered orally for two consecutive days, followed by *P. aeruginosa* infection two hours after the last probenecid dose. The outcomes of this research indicate that Probenecid effectively mitigates inflammation and curbs the production of inflammatory mediators like IL-1B and TNF-α. Building upon this understanding, studies with a longer observation period (109,154) have described probenecid's capacity to diminish inflammation and suppress the production of inflammatory mediators. This supports the concept that the full scope of probenecid's anti-inflammatory effects might become more pronounced in experiments exceeding the 6-hour timeframe. Hence, it would be prudent to embark on subsequent studies of longer duration to comprehensively assess probenecid's anti-inflammatory attributes and its potential to alleviate Tenofovir-induced neuroinflammation. Considering that people with HIV often take these therapeutic agents as chronic medication, conducting long-term studies would provide valuable insight into probenecid's long-term impact and potential benefit in mitigating the inflammation associated with tenofovir administration.

In the probenecid group, our results show that the therapeutic agent does not invoke IL-1β release.This was also demonstrated in our correlation analysis. Our results agree with literature that showed that probenecid does not affect IL-1β concentration (68,70,85).

In contrast to the observed changes in IL-1β concentration, there were no discernible fluctuations in PFC dopamine concentration. This finding implies that tenofovir, probenecid, and their combined administration did not directly impact the concentration of dopamine in the PFC throughout the experimental period. Interestingly, previous long-term studies have shown that probenecid can increase dopamine concentration (149,155). Despite the evident presence of inflammation in the context of tenofovir exposure, our correlation analysis has shown an absence of a connection between inflammation and dopamine. This finding diverges from research that has found a link between inflammation and dopamine (18,140,145). Research has established a link between inflammation and dopamine, mainly through changes in dopaminergic gene and protein expression, as Gelman et al. demonstrated (87,136). They found that alterations in the brain's dopamine system, including decreased expression of the dopamine receptor, DRD2L, are associated with increased dopamine activity and neuroinflammation, leading to neurocognitive impairment. This

relationship between inflammation and dopamine has also been

extensively studied in the context of HAND (88,144,156). However, it is crucial to note that these prior investigations did not encompass tenofovir-induced inflammation. To the best of our knowledge studies assessing the influence of tenofovir on dopamine concentration are lacking. Nonetheless, existing research has investigated the impact of oxidative stress and inflammation in disrupting dopamine function across various brain regions, including the substantia nigra, ventral tegmental area, and hypothalamus (17,86). Given that tenofovir triggers oxidative stress and causes neuroinflammation (10,11,118), we propose extending our experimental scope to encompass other brain regions. Furthermore, we anticipate that such research might also illuminate the role of probenecid as a pharmacokinetic enhancer.

In summary, our study showed that tenofovir rapidly triggered brain inflammation, while the presence of probenecid didn't seem to counteract this effect. Although inflammation from tenofovir was evident, we didn't find a direct connection between inflammation and dopamine levels in the prefrontal cortex. It must be noted that the source of dopamine in the prefrontal cortex is the ventral tegmental area that is located in the midbrain (17). This may imply that effects on dopamine concentration may take longer than 6 h to manifest. To get a clearer picture, further research could investigate a broader range of brain regions, helping us understand how tenofovir, inflammation, and dopamine are linked and how probenecid might play a role.

6. Conclusion

A single dose of tenofovir triggered acute inflammation in the prefrontal cortex that was not attenuated by probenecid. Additionally, we did not find a link between inflammation and dopamine concentration in our experimental time interval. Further investigation covering a broader range of brain regions over an extended experimental duration could elicit a better understanding of how this interaction occurs. It is suggested that this relationship be further investigated in a chronic state, similar to the constant exposure to treatment in HIV positive patients.

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7. References

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Chapter 4: Synopsis

HIV-associated neurocognitive disorders (HAND) can occur in people living with HIV. Despite advances in HIV treatment, the virus can still affect the brain in certain individuals. HAND can lead to impairment in memory, attention, thinking, and movement difficulties, thus impacting the quality of life. The virus can directly or indirectly cause neuroinflammation, leading to these cognitive issues. HIV can also disrupt function of neurotransmitters that are essential for communication in the brain. This neurotransmitter imbalance can further contribute to mental and behavioural problems in people living with HIV.

An example of brain areas affected by HAND are the prefrontal cortex (PFC) which plays a significant role in controlling higher brain functions suchas complex thinking and decision-making. In HIV-infected individuals, the immune cells in the PFC can be affected, leading to chronic inflammation that disrupts brain connections.

The cerebellum is primarily known for its role in coordinating movement and balance. It is also involved in attention, language use, and certain kinds of memory. In HIV-infected individuals, viral damage can impair motor coordination and cerebellar-related cognitive function. Additionally, HIV can damage the cerebellum's white matter,leading to difficulties in movement and thinking.

The current treatment regimen for HIV has not completely eradicated the effects of the virus on the brain. In this project, we focused on finding better ways to improve the treatment of HAND. Even with advancements in antiretroviral therapy, while some antiretroviral compounds have shown promise in managing HIV, they still have difficulty reaching the brain.

One such compound is tenofovir, which is effective against HIV but faces difficultyto pass through the blood-brain barrier. tenofovir, even at low concentration, can induce neurotoxicity by triggering inflammation and neurotransmitter dysregulation. To

address this issue, we explored the use of probenecid, a therapeutic agent that has long been utilised for various clinical purposes, from treating gout to acting as a coadjutant for antibiotic agents. Besides its diverse attributes, probenecid's capacity to maintain elevated concentrations of multiple metabolites and substances in the CNS and its minimal adverse effects has established it as a valuable pharmacological asset in clinical and basic science research. probenecid is thought to mitigate inflammation byinterfering with the activation of certain immune responses, potentially by reducing cytokine production. Additionally, it's suggested that probenecid may regulate dopamine by influencing the reuptake of this neurotransmitter, thus modulating its availability in brain synapses.

Our research aimed to investigate the synergistic effect of co-administering tenofovir and probenecid, focusing on enhancing tenofovir concentration in the brain while mitigating the associated neurotoxicity. This study explored the modulation of tenofovir facilitated by probenecid and its impact on biochemical factors within the prefrontal cortex and cerebellum.

We found that concomitant administration of tenofovir and probenecid elevated tenofovir concentration in the PFC and cerebellum. This observation emphasises the potential of probenecid to influence the pharmacokinetics of tenofovir, potentially leading to altered compund concentrations in the brain.

In a parallel investigation, the second aspect of modulation emerged. tenofovir administration in isolation correlated with a substantial elevation in IL-1β concentration across various post-drug administration intervals. Intriguingly, probenecid demonstrated no discernible impact on IL-1β levels when administered alone. However, the co-administration of tenofovir and probenecid showcased a corresponding trend to that of the tenofovir administration alone. This suggests a delayed manifestation of probenecid's anti-inflammatory effects, potentially requiring an extended duration to materialise fully. Notably, neither tenofovir nor probenecid exhibited an influence on dopamine concentration, highlighting their lack of impact on this neurotransmitter in the brain region investigated. This outcome presents a divergence from prior research, warranting an expanded focus. Given the acknowledged understanding of tenofovir-induced oxidative stress and inflammation in the brain, we propose broadening the experimental scope to encompass related

brain regions. This expansion could offer a better comprehension of the intricate interplay between therapeutic agent modulation, neuroinflammation, and neurotransmitter dynamics across distinct cerebral areas.

This study contributes to understanding interactions between tenofovir and probenecid, their implications on neuroinflammation, and their potential consequences for dopamine physiology in the brain. The findings illuminate the complex nature of therapeutic agent interactions and their impact on neurochemistry, paving the path for further exploration into therapeutic interventions and their potential effect on neurological well-being.

Recommendations

Despite advancements in antiretroviral therapy, cognitive impairment persists in specific individuals with HIV. This emphasises the need for a better understanding of the mechanisms underlying tenofovir transport into the brain. Further investigation is recommended to elucidate the interplay between tenofovir and neurotransmitters. This may require the integration of behavioural studies to assess cognitive outcomes comprehensively. By explaining these intricate relationships, we aim to advance therapeutic strategies for HAND and enhance the overall well-being of individuals living with HIV.

Chapter 5: Conclusion

These studies contribute insight into the potential enhancement and the challenges associated with co-administering tenofovir and probenecid in the context of HIV treatment. The first study highlighted the potential of probenecid as a pharmacokinetic enhancer for tenofovir in the brain. However, this enhancement does not extend to mitigating tenofovir-induced neurotoxicity in an acute setting. The relationship between tenofovir, probenecid, and blood brain barrier penetration was investigated, with probenecid positively influencing the delivery of tenofovir in the prefrontal cortex and cerebellum.

The impact of tenofovir and probenecid co-administration on neuroinflammation and

dopamine concentration showed that a time-dependent nature of the anti-inflammatory effects of probenecid and a swift neuroinflammatory response induced by Tenofovir.

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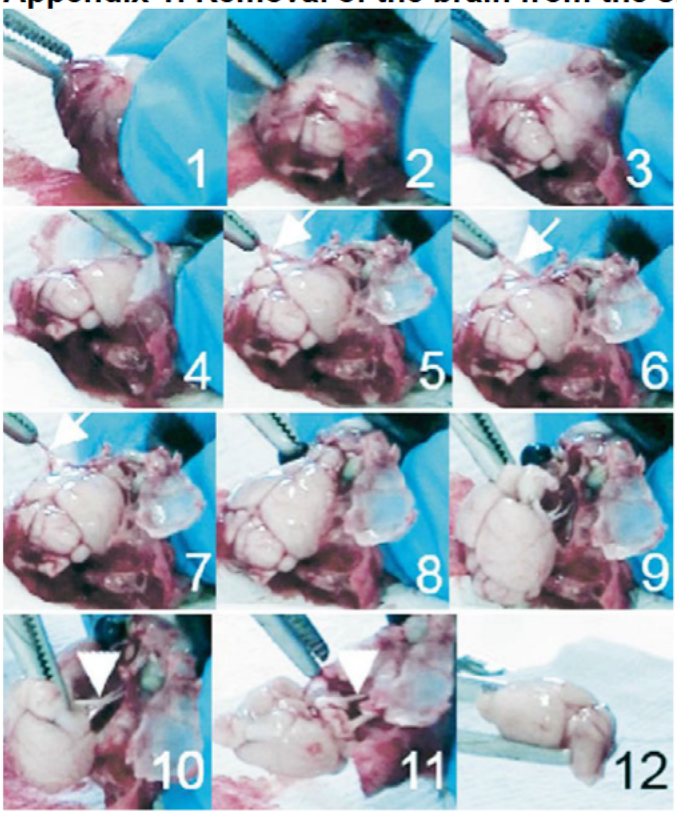
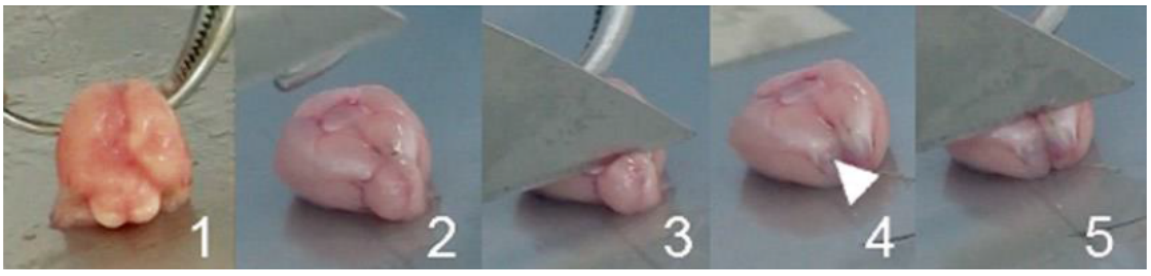
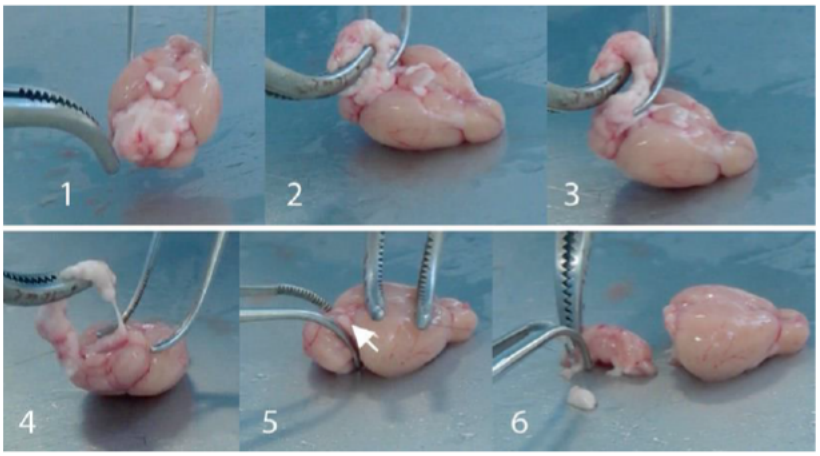
APPENDICES

Appendix 1: Removal of the brain from the skull.(30)



Appendix 2 : Dissection of the PFC & cerebellum (30)





Appendix 3: ethical clearance letters

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

29 November 2021

Ms Simangele Ntombifikile Euphemia Shabalala (221072879))
School of Laboratory Medicine & Medical Sciences
Westville Campus

Dear Ms Shabalala,

Protocol reference number: AREC/00003133/2021
Project title: Investigating the ability of Tenofovir to cross the blood-brain barrier during MRP4 inhibition.

Full Approval – Research Application

With regard to your revised application received on 16 November 2021, the Animal Research Ethics Committee has accepted the documents submitted and FULL APPROVAL for the protocol has been granted.

Please note: There must be adherence to national and institutional COVID-19 regulations and guidelines at all times. Researchers will be personally responsible and liable for non-adherence to national regulations. If in doubt, please contact the Research Ethics Chair and/or the University Dean of Research for advice.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 28 November 2022.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

[redacted]

Dr Sanil D Singh, BVSc, MS, PhD
Chair: Animal Research Ethics Committee

/kr

cc Supervisor: Dr Thabisile Mpofana and Dr Khethelo Richman Xulu
cc BRU Manager: Dr Jaca

Animal Research Ethics Committee (AREC)
Ms Karen Reinertsen (Administrator)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 8850 Facsimile: +27 (0) 31 260 4609 Email: animalethics@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

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Appendix 4: summary of guidelines to authors- Elsevier: Neuropharmacology

Article structure

Subdivision - numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its separate line.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient details to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarised and indicated by a reference. If quoting directly from a previously published way, use quotation marks and cite the source. Any modifications to existing methods should also be described.

Theory/calculation

A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.

Results

Results should be described clearly, concisely, and logically, with sub-headings as appropriate to guide the reader. For describing statistical results, please provide details of the statistical test used and full statistical reporting. Full statistical reporting should include the statistical value, the degrees of freedom, and the exact p-value. For example, an ANOVA would be reported as $F(1,13) = 15.484$; $p = 0.0017$, and a t-test as $t(39) = 3.83$, $p = 0.0004$.Individual data points should be shown wherever possible. This requirement may be relaxed for full concentration/dose-response curves or time-series data, but individual data points should be shown elsewhere, for example, in bar charts. Authors should provide the full, untruncated images of any gels or blots included in the figures as a supplemental figure in DOC, PDF or PPT format, not as a compressed file.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The study's main conclusions may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly, for tables and figures: Table A.1; Fig. A.1, etc.

Appendix 3: summary of guidelines to authors-Frontiers in Neuroscience

Author Guidelines

Title

The title should be concise, omitting implicit terms and, where possible, be a statement of the main result or conclusion presented in the manuscript. Abbreviations should be avoided within the title.

Witty or creative titles are welcome only if relevant and within measure. Consider if a title meant to be thought-provoking might be misinterpreted as offensive or alarming. In extreme cases, the editorial office may veto a title and propose an alternative. Authors should avoid:

- titles that are mere questions without giving the answer
- unambitious titles, for example, starting with 'Towards,' 'A description of,' 'A characterisation of', or 'Preliminary study on.'
- vague titles, for example, starting with 'Role of', 'Link between', or 'Effect of' that do not specify the role, link, or effect.

- including terms that are out of place, for example, the taxonomic affiliation apart from species name.

For Corrigenda, General Commentaries, and Editorials, the title of your manuscript should have the following format:

- 'Corrigendum: Title of Original Article'
- General Commentaries: 'Commentary: Title of Original Article' 'Response: Commentary: Title of Original Article.'
- 'Editorial: Title of Research Topic'

The running title should be a maximum of five words in length.

Authors and affiliations

All names are listed together and separated by commas. Provide exact and correct author names, which will be indexed in official archives. Affiliations should be keyed to the author's name with superscript numbers and be listed as follows:

- Laboratory, Institute, Department, Organization, City, State abbreviation (only for United States, Canada, and Australia), and Country (without detailed address information such as city zip codes or street names).

Example: Max Maximus1 1 Department of Excellence, International University of Science, New York, NY, United States.

Correspondence

The corresponding author(s) should be marked with an asterisk in the author list. Provide the exact contact email address of the corresponding author(s) in a separate section. Example: Max Maximus* maximus@iuscience.edu If any authors wish to include a change of address, list the present address(es) below the correspondence details using a unique superscript symbol keyed to the author(s) in the author list.

Abstract

As a primary goal, the abstract should make the work's general significance and conceptual advance accessible to a broad readership. The abstract should be no longer than a single paragraph and should be structured, for example, according to the IMRAD format. For the specific structure of the abstract, authors should follow the

requirements of the article type or journal to which they're submitting. Minimise using abbreviations, and do not cite references, figures, or tables. For clinical trial articles, please include the unique identifier and the URL of the publicly accessible website on which the trial is registered.

Keywords

All article types require a minimum of five and a maximum of eight keywords.

Text

The document should be single-spaced and contain page and line numbers to facilitate the review process. The manuscript should be written using either Word or LaTeX. See above for templates.

Nomenclature

The use of abbreviations should be kept to a minimum. Non-standard abbreviations should be avoided unless they appear at least four times and must be defined upon first use in the main text. Consider also giving a list of non-standard abbreviations at the end, immediately before the acknowledgements.

Equations should be inserted in editable format from the equation editor.

Italicise gene symbols and use the approved gene nomenclature where it is available. Please refer to the HUGO Gene Nomenclature Committee (HGNC) for human genes. New characters for human genes should be submitted to the HGNC here. Common alternative gene aliases may also be reported but should not be used alone in place of the HGNC symbol. Nomenclature committees for other species are listed here. Protein products are not italicised.

We encourage the use of Standard International Units in all manuscripts.

Chemical compounds and biomolecules should be referred to using systematic nomenclature, preferably using the International Union of Pure and Applied Chemistry (IUPAC) recommendations.

Astronomical objects should be referred to using the nomenclature from the International Astronomical Union (IAU) provided here.

Life Science Identifiers (LSIDs) for ZOOBANK registered names or nomenclatural acts should be listed in the manuscript before the keywords. An LSID is represented as a uniform resource name (URN) with the following format: urn:lsid:<Authority>:<Namespace>:<ObjectID>[:<Version>]

For more information on LSIDs, please see the 'Code' section of our policies.

Sections

Headings and subheorganisedganize the manuscript. The section headings should be those appropriate for your field and the research itself. You may insert up to 5 heading levels into your manuscript (i.e., 3.2.2.1.2 Heading Title).

For Original Research articles, organising your manuscript in the following sections or their equivalents for your field is recommended.

Introduction Succinct, with no subheadings.

Materials and methods This section may be divided by subheadings and should contain sufficient detail so that all procedures can be repeated when read in conjunction with cited references. An ethics approval statement should be included in this section for experiments reporting results on animal or human subject research (for further information, see the 'Bioethics' section of our policies.)

Results This section may be divided by subheadings. Footnotes should not be used and must be transferred to the main text.

Discussion This section may be divided by subheadings. Discussions should cover the key findings of the study: discuss any prior research related to the subject to place the novelty of the discovery in the appropriate context, discuss the potential shortcomings and limitations on their interpretations, discuss their integration into the current understanding of the problem and how this advances the current views, speculate on the future direction of the research, and freely postulate theories that could be tested in the future.

For further information, please check the descriptions defined in the journal's 'Article types' page in every journal page's 'For authors' menu.

Acknowledgements

This short text acknowledges the contributions of specific colleagues, institutions, or agencies that aided the authors' efforts. Should the manuscript's content have previously appeared online, such as in a thesis or preprint, this should be mentioned here, in addition to listing the source within the reference list.