Marshall Plan Report

PANNEXIN-1 KO MICE ARE UNRESPONSIVE TO TENOFOVIR INDUCED BONE LOSS

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Abstract

Human immunodeficiency virus (HIV) continues to be a worldwide public health challenge. Despite the advanced scientific knowledge of HIV and its mechanism, there is no cure for HIV-infected individuals. However, modern therapies such as highly active anti-retroviral therapy, have been established to treat HIV. One of the medications involved in this type of medication regimen is tenofovir, an anti-retroviral agent that prevents HIV reverse transcriptase. Clinical observations revealed increasing bone mineral abnormalities among human immunodeficiency virus (HIV)-infected patients strongly associated to tenofovir.

Tenofovir is an anti-retroviral agent commonly used to treat human immunodeficiency virus (HIV)-infected patients as part of the drug regimen known as highly active anti-retroviral therapy (HAART). As many as 15% of patients taking tenofovir develop osteopenia resulting in pathological fractures. Recent studies in our lab indicate that tenofovir-induced osteopenia is due to both the reduction of extracellular adenosine resulting from tenofovir-mediated blockade of Pannexin-1, a channel that transports ATP into the extracellular space. However, the effect of tenofovir on bone can be reversed by dipyridamole, an agent that blocks adenosine re-uptake and stimulates new bone formation. Thus, hypothesized that a blockade of pannexin-1 hemichannels by tenofovir phosphates could contribute to the antiretroviral effects of the drug by blocking HIV entry into the cell. To further confirm this hypothesis, I studied the effect of tenofovir and dipyridamole on osteoblasts (OB) and osteoclasts (OC) in Pannexin-1 knockout mice (PANX1KO) in vitro.

Since previous study has indicated that tenofovir treatment diminishes extracellular adenosine concentrations through suppression of ATP export via pannexin-1 channel, it was further investigated whether dipyridamole, an agent that increases local adenosine levels, potentially prevents tenofovir-induced bone loss.

As tenofovir blocks ATP release and decreases extracellular adenosine levels, we investigated whether tenofovir treatment affects bone homeostasis in mice. Here we report that tenofovir enhances osteoclast differentiation in an adenosine receptor

dependent fashion in vitro, which is reversible by treatment with dipyridamole. Moreover, tenofovir induces osteopenia in vivo and dipyridamole treatment prevents the development of osteopenia in mice

We hypothesized that tenofovir regulates bone resorption by diminishing endogenous adenosine levels and questioned whether dipyridamole may be a useful treatment to counteract the deleterious bone effects of tenofovir.

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List of Abbreviations

α-MEM	Alpha Modified Eagle Medium
A_1R	Adenosine 1 Receptor
$A_{2A}R$	Adenosine 2A Receptor
$A_{2B}R$	Adenosine 2B Receptor
A_3R	Adenosine 3 Receptor
AR	Adenosine Receptor
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
BMD	Bone Mineral Density
BMP-2	Bone Morphogenic Protein 2
BMU	Bone Multicellular Units
cAMP	Cyclic Adenosine Monophosphate
CBFA1	Core Binding Factor α1
cCM	Complete Culture Medium
CREB	Cyclic AMP Response Element Binding
CSF-1	Colony-Stimulating Factor 1
DMEM	Dulbecco's Modified Eagle Medium
DPBS	Dulbecco's Phosphate-Buffer Saline
DXA	Dual-Energy X-Ray Absorptiometry
ECM	Extracellular Matrix
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor

GCPR	G-Protein-Coupled Receptor
G_i	Inhibitory G Protein
G_s	Stimulatory G Protein
HAART	Highly Active Anti-Retroviral Therapy
HIV	Human Immunodeficiency Virus
HPLC	High-Pressure Liquid Chromatography
IGF	Insulin-Like Growth Factor
KO	Knock-Out
M-CSF	Macrophage Colony Stimulating Factor
NF-ĸB	Nuclear Factor-KappaB
OCIF	Osteoclastogenesis Inhibitory Factor
OPG	Osteoprotegerin
PANX1	Pannexin-1
PANX1KO	Pannexin-1 Knock Out
PKA	Protein Kinase A
P/S	Penicillin/Streptomycin
RANK	Receptor Activator of NF-ĸB
RANKL	Receptor Activator of NF-ĸB Ligand
TAF	Tenofovir Alafenamide
TDF	Tenofovir Disoproxil
TFV-DP	Tenofovir Diphosphate
TNF	Tumour Necrosis Factor
TRAP	Tartrate-Resistant Acid Phosphatase
WT	Wild Type

1 Introduction

Osteopenia is a degree of bone loss, measured by bone mineral density (BMD), a marker which expresses the strength of bone and the chances of breaking. This condition results in weaker bone prone to develop fragility fractures comparable to normal bone. Clinical observations have revealed increasing bone mineral abnormalities among human immunodeficiency virus (HIV)-infected patients resulting from several factors related to the host, the virus, and the antiretrovirals used, particularly in conjunction with tenofovir, a nucleoside analogue that inhibits HIV reverse transcriptase. As many as 15% of patients taking tenofovir develop osteopenia resulting in pathological fractures.

1.1 Bone Homeostasis

Bone is a living and growing tissue that constitute the vertebrate endoskeleton covering various functions in particular structure and support to the body, protection of internal organs, and facilitation of movement. Additionally, bone produces red and white blood cells, has a metabolic function with the storage of minerals, growth factors and fat, and acts as an endocrine organ [1]. Bone is composed of different types of cells and the extracellular matrix (ECM) which is mostly comprised of type I collagen fibres and numerous non-collagenous proteins [2]. Its structure and shape are constantly destructed and rebuild in a dynamic process between osteoblasts and osteoclasts, two cell types specialized to enable the maintenance of the bone integrity. This property of changing the architecture through removal of old bone and replacement by newly formed bone is called bone remodelling and is obligatory for the accomplishment of the following functions: i) removal of primary and infantile bone, and deposition of the mechanically competent secondary bone, ii) old bone renewing, iii) removal and substitution of ischemic or microfractured bone, and iv) regulation of calcium homeostasis [3, 4]. Bone remodelling strongly relies on the correct equilibrium between bone resorption by osteoclasts and bone formation by osteoblasts and occurs in the so called Bone Multicellular Units (BMUs) throughout

the skeleton and is classified in four phases: i) activation phase, ii) resorption phase, iii) formation phase, and iv) reverse phase (Figure 1).

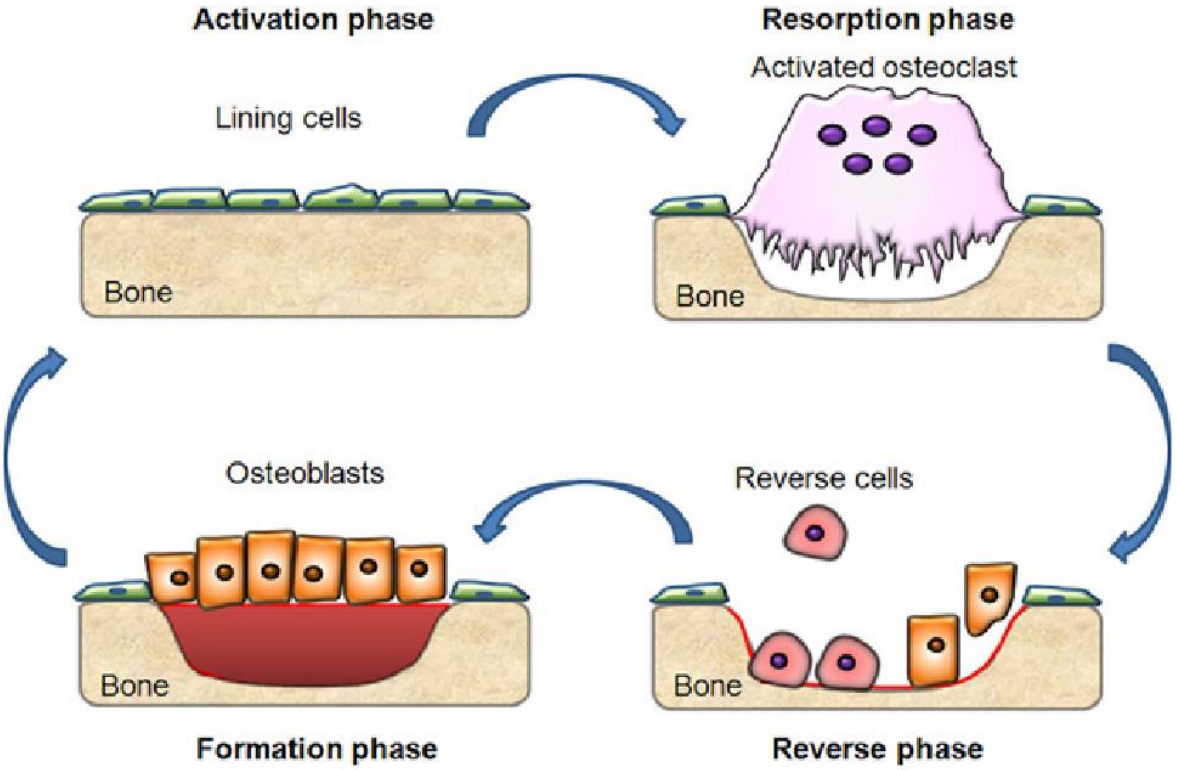

Figure 1. Schematic representation of the bone remodelling process
The bone remodelling has four phases: i) activation phase where the bone surface is exposed as the lining cells separate from underlying bone, osteoclasts precursor cells are attracted to the remodelling sites and fuse; ii) resorption phase where differentiation and activation of mature osteoclasts are conducted, approximately two weeks in duration; iii) reverse phase where bone resorption switch to bone formation, approximately four to five weeks in duration; iv) formation phase where mature osteoblast develop at the surface and release type 1 collagen-rich osteoid at the site and regulate osteoid mineralization, approximately four months in duration.

1.1.1 Osteoblasts

Osteoblasts are mononuclear cells derived from pluripotent mesenchymal stem cells (Figure 2). Under the influence of a specific suite of regulatory transcription factors they have the ability to differentiate into adipocytes, myocytes, chondrocytes, and osteoblasts [5]. Osteoblasts appear to be cuboid-shaped and contain a round nucleus situated in the centre of the cell [6]. They have a vital role in establishing and maintaining the skeletal structure as they are responsible for the mineralization of bone during bone formation and bone remodelling. In addition, osteoblasts

synthesize bone proteins including sialoprotein and osteocalcin [2]. In vitro, differentiation of osteoblasts require the presence of local and systemic factors including growth factors such as fibroblast growth factor (FGF) and insulin-like growth factor (IGF), ascorbic acid, beta-glycerophosphate, and dexamethasone. During the proliferation phase from precursor to mature osteoblast, these cells synthesize and secrete collagen type I [7], which make up the majority (95%) of organic matrix proteins in bone [8]. The complete development of the osteoblast cell lineage involves a complex cellular and molecular regulation by transcription factors including runt-related gene family such as core binding factor α1 (Cbfa1) and Runx2, members of runt-related gene family, as well as proto-oncogenes c-fos and c-myc [2, 9]. Ultimately, mature osteoblasts may undergo different fates and can either be subject to apoptosis, become inactive osteoblasts or bone lining cells or are trapped in the bone matrix giving rise to osteocytes (Figure 2) [10].

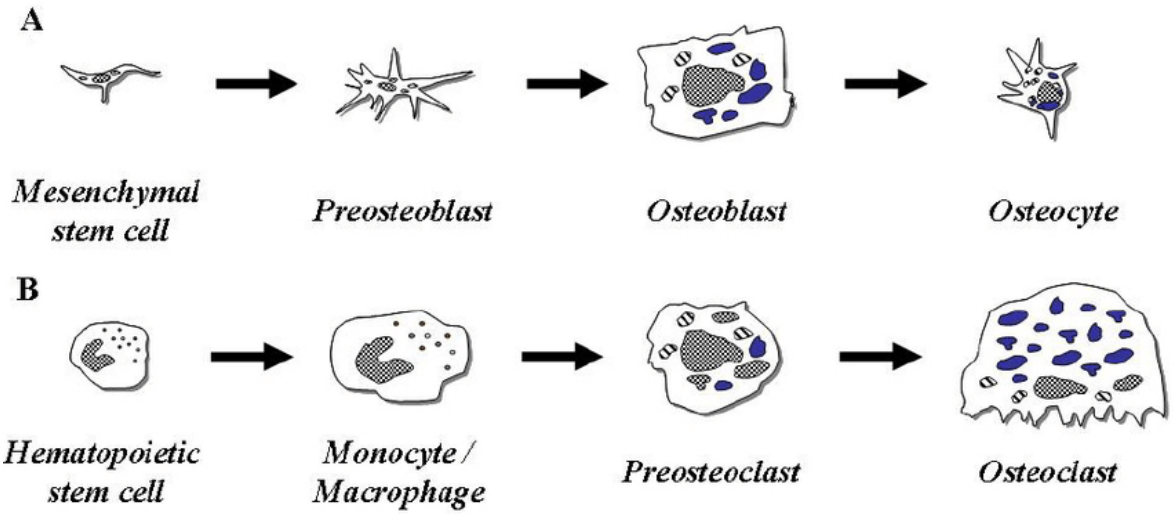

Figure 2. Osteoblast (A) and Osteoclast (B) differentiation
Pre-osteoblasts are derived from mesenchymal stem cells in the bone marrow. The differentiation to mature osteoblasts last until they have achieved their location and phenotype. By mineralization of osteoid through osteoblasts, these cells become osteocytes. On the contrary, osteoclasts differentiate from hematopoietic stem cells of the monocyte/macrophage lineage in the bone marrow.

1.1.2 Osteoclasts

Osteoclasts are differentiated myeloid cells derived from hematopoietic monocytic precursor cells (Figure 2) [11, 12]. The unique adaption of these cells are responsible for resorbing mineralized bone material on the bone surface. Their distinct morphological and phenotypic characteristics, are routinely used measures for identification including multinuclearity, high expression of tartrate-resistant acid phosphatase (TRAP), cathepsin K and the calcitonin receptor [13]. In order for osteoclasts precursor cells to survive, expand, and differentiate into mature osteoclasts in vitro, stimulation by macrophage colony stimulating factor (MCS-F); also known as colony-stimulating factor 1 (CSF-1) and a complex network of regulatory factors that includes systemic hormones, locally produced cytokines and cell-cell and cell-matrix interactions is required [14, 15]. Receptor activator of NF-ĸB ligand (RANKL) belongs to the Tumour Necrosis Factor (TNF) superfamily of extracellular cytokine and plays a crucial part in the formation and activation of osteoclasts [16-18]. Upon binding of RANKL to its receptor, RANK, stimulation of numerous intracellular pathways such as nuclear factor-kappaB (NF-ĸB), c-Fos, PLCγ, and nuclear factor of activated T-cells c1 (NFATc1) are activated [19-21]. Osteoclastogenesis is tightly regulated by osteoblasts by expressing RANKL as a membrane surface molecule and secreting osteoclast stimulus in form of several cytokines such as IL-1beta, IL-6, PTH related Peptide (PTHrP) and TNF-alpha. RANKL/RANK signaling is dependent on the cell-cell contact between osteoblasts and the osteoclast precursors. Osteoprotegerin (OPG) also known as osteoclastogenesis inhibitory factor (OCIF) is a soluble decoy receptor for RANKL. It negatively mediates osteoclastogenesis; thus, inhibits osteoclast differentiation [22, 23].

1.2 Adenosine

Adenosine are organic compounds known as purine nucleoside. The purine nucleoside, made up of the base adenine and the sugar D-ribose, is not only ubiquitously available in the human body as a component of numerous biochemical structures, but also fulfils many regulatory tasks in the fundamental physiological processes of a somatic cell. It serves as a metabolite of adenosine triphosphate (ATP), present

in cells and extracellular fluids. As a result of rapid metabolism it is only has a short half-life. It is generated both intra- and extracellularly from the catabolism of adenine nucleotides in response to ischemia and stress such as inflammation and hypoxia.

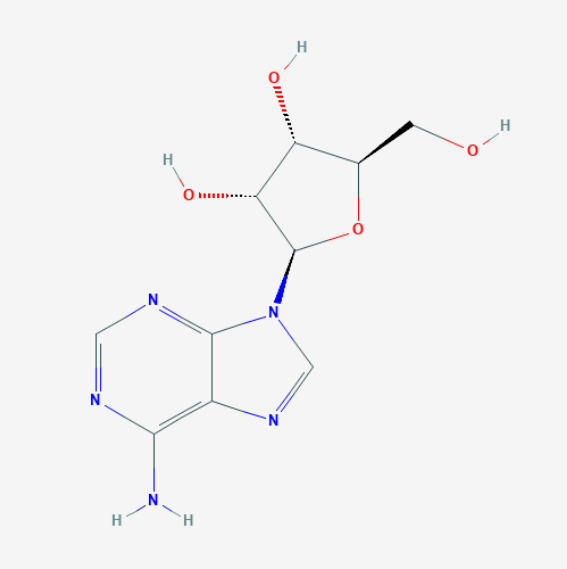

Figure 3. Two-Dimensional (2D) Structure of Adenosine
A 2D depiction of an adenosine comprised of an adenine molecule in blue and a D-ribose in red.

In the extracellular space it modulates various important physiological and pharmacological functions via interaction with a family of 4 related 7-transmembrane spanning G-protein-coupled receptors (GCPRs) – referred to as adenosine receptors (ARs) A_1, A_{2A}, A_{2B}, and A_3. Each AR is subject to a selective pharmacologic profile and posses unique functions when activated upon binding of its endogenous ligand adenosine. Each of the four AR are encoded by different genes and the expression of the various ARs differ among tissues as well as cell types. Classification is based on their differential influence on the adenylyl cyclase activity to regulate cyclic AMP (cAMP) [24, 25]. Both A_1 and A_3 receptors have suppressing functions through G_i-linked proteins and inhibit cAMP production resulting in diminished protein kinase A (PKA) activity and phosphorylation of the cyclic AMP response element binding protein (CREB). In contrast the A_{2A} and A_{2B} receptors stimulate the adenylyl cyclase via G_s and G_{olf} signaling and increase cAMP levels leading to activation of phospholipase C pathway, EPAC and PKA.

Adenosine plays a vital role in many biochemical pathways and contribute to various functions in the body such as in the cardiovascular and central nervous system. However, with the advanced knowledge on AR and better understanding of agonists and antagonists, there is growing evidence that these receptors are potential therapeutic targets.

1.2.1 Adenosine and Bone Metabolism

As early as in the years 1989 research within the field of purinergic signalling was first proposed where study of regulation of bone metabolism by extracellular nucleotides was looked into [26]. These studies were recently reviewed [27, 28] but the knowledge on the role of adenosine and its influence on bone metabolism was limited. Due to its short half-life, adenine nucleotides are degraded even though they are present at high concentration levels in the cell [29]. On the contrary, the adenosine concentration increase under pathological conditions for instance, when a tissue or organ is experiencing stress or inflammation [30]. Effectively, adenosine and its receptors are involved in the (patho)physiologic regulation of different pathways in various tissues and organs (e.g. vasodilation airway tone).

Mediero et al have found that the A_{2A} receptor ($A_{2A}R$) has shown significant importance in bone metabolism. Upon stimulation of $A_{2A}R$, inhibition of osteoclast formation and its function were attained. Moreover, it was proven that a complete deletion of $A_{2A}R$ leads to osteopenia in mice, thereby reducing the bone density which is corresponding to an increase in the number of TRAP-positive osteoclasts [31]. In further studies, Mediero et al have reporter another significance of $A_{2A}R$ activation in osteoclasts. This of high importance in surgeries involving hip and knee replacements, where a number of individuals suffer from severe pain and degenerative diseases of joints. Based on the studies, it was demonstrated in an in vivo model of wear particle-induced osteolysis that $A_{2A}R$ had the ability to decrease porosity and loss of the bone by reducing the number of osteoclasts as well as diminish joint inflammation which is usually caused by implant degradation. These findings suggest enhancement of implant survival which could potentially delay or even eliminate the need for revision arthroplasty surgery [32]. Lastly, Mediero et al have studied upon activation of $A_{2A}R$ by the compound that blocks ENT1 transporter,

dipyridamole, increases endogenous adenosine levels and thereby stimulates the formation of new bone as well as bone morphogenic protein 2 (BMP-2).

It has been established that adenosine receptor became targets of several drugs such as dipyridamole disease-modifying anti-rheumatic drug methotrexate. Further findings point out the importance of adenosine and its receptors in bone metabolism and have opened new areas in investigating the apprehension of endogenous regulation of bone metabolism.

1.3 Pannexin

Pannexins are a class of membrane channel proteins with three human pannexin isoforms Pannexin-1 (PANX1), Pannexin-2 (PANX2) and Pannexin-3 (PANX3). Its structural similarities are comparable to connexin channels, however they do not share related sequence homology. Both pannexins and connexins possess four transmembrane domains with two extracellular loops, an intracellular loop with cytoplasmic amino and carboxyl termini [33, 34]. The primary function of pannexins is to form transmembrane channels allowing the passage and exchange of small molecules between the cytosol of the cell and the extracellular membrane which include for instance ions and ATP. PANX1 is ubiquitously expressed in many human tissues (e.g. heart, brain, skeletal muscle, ovary, lung, etc.) while PANX2 is abundantly localized in the central nervous system and PANX3 in connective tissues and skin [35, 36].

1.3.1 Structure of Pannexin

Its name derives from the Greek pan = all/everywhere and the Latin nexus = connection/binding and indicates its wide distribution pattern (see below) and the channel function. Pannexins occur in both vertebrates and invertebrates and thus face the connexins, which occur only in the former [37]. Similar to the connexins, they have four transmembrane domains (TMD) connected by two extra- (between TMD1 and TMD2) and one intracellular (between TMD2 and TMD3) loop (Figure 4).
Furthermore, they each contain an intracellular carboxy- and amino-terminal end [38]. One of the differences between the two protein families is that pannexins, like the innexins of the invertebrates, have only two instead of three preserved cysteine residues at each

extracellular loop [36]. These cysteine radicals are involved in the regulation of the channel function [39, 40].

Additionally, there is an N-glycosylation (connection of a sugar residue to an asparagine residue), which is located at the first (PANX2 and PANX3) or the second (PANX1) extracellular loop [41, 42]. This probably prevents sterically from docking neighbouring pannexins to each other [43] and is important for the positioning of the protein in the cell and at the cell membrane [34].

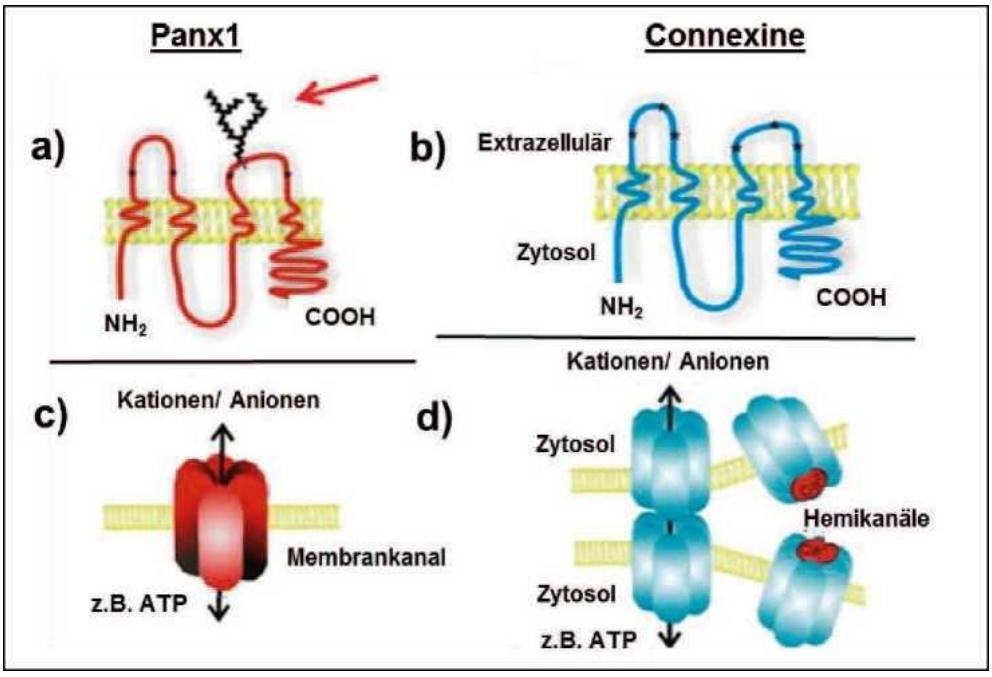

Figure 4. Possible secondary structure of PANX1 (a) with two extracellular cysteine residues (*) and glycosylation (red arrow) compared to that of the Connexine (b)
PANX1 acts primarily as a c) membrane channel, while Connexine as d) hemichannels by docking to each other form gaps between the cytosol of adjacent cells (Modified according to [43]).

Pannexin-1 is a protein that is 426 amino acid-long with a cytoplasmic N- and C-termini. This protein contains two extracellular loops and four transmembrane domains. Six subunits of PANX1 proteins form a hexameric channel termed a pannexon. As mentioned above PANX1 and connexins have no shared homology however some parts of the pore structure are similar between the two types of proteins. A main difference between the pore of a connexin and the pore of PANX1 is that the N-terminus is part of the connexin pore and the C-terminus is in the pore of PANX1.

1.3.2 Function of Pannexin

Exchange of small molecules (approximately 1kD) between the intra- and extracellular matrix belongs to the main function of pannexins. This includes passing of ions (e.g. calcium ions), secondary messenger substances (e.g. ATP), neurotransmitters, and amino acids. Interactions between pannexins and purinergic receptors are linked to the export of ATP.

Numerous functions have been attributed to PANX1 because of its electrophysiological properties and broad expression pattern. Particularly, the permeability for the extracellularly nucleotide ATP, which is of fundamental significance as a signal molecule in the context of various signal transduction pathways [44].

1.4 Tenofovir

Tenofovir is an anti-retroviral agent from the group of reverse transcriptase inhibitors and it is also called 9-(R)-[45]adenine or PMPA. In 2001 it was approved by the FDA to treat HIV infection based on a drug regimen known as highly active anti-retroviral therapy (HAART). Recently Tenofovir has also been approved for use in treating chronic hepatitis B infection [46]. The effects are based on the inhibition of the enzyme reverse transcriptase, an enzyme that converts the viral RNA into DNA and is important in virus replication. Tenofovir is available in medicinal products as a prodrug in tenofovir disoproxil (TDF) or tenofovir alafenamide (TAF). Both phosphonamidate prodrugs have tenofovir diphosphate (TFV-DP) as their intracellular active metabolite which is responsible to inhibit HIV-1 reverse transcriptase.

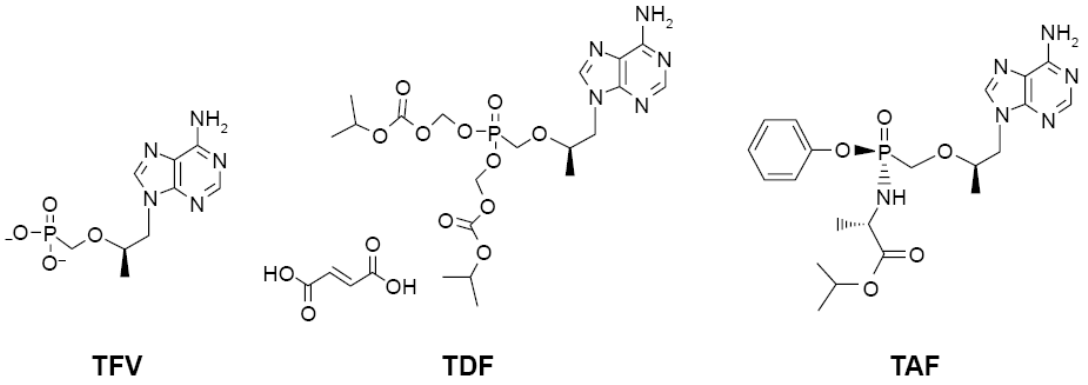

Figure 5. Chemical Structure of Tenofovir, Tenofovir Disoproxil Fumarate and Tenofovir Alafenamide
Tenofovir is a nucleoside-based drug present in the prodrugs Tenofovir disoproxil fumarate and tenofovir alafenamide. Prodrugs are drugs that are pharmacologically inactive and are only converted into their actual active form through a conversion step in the body.
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HIV infection is predominantly recognized for its immunologic effects as it weakens the immune system by incapacitating important immune cells, the so-called T helper cells (also called T4 helper cells, as CD4 cells or CD4+ lymphocytes) and monocytes/macrophages. The virus penetrates the cells and causes them to produce more HI-viruses. The T-helper cells have, among other things, the function of controlling other cells of the immune system in the defense against pathogens. These important defense functions are destroyed by the HIV infection inducing severe lymphopenia and immunodeficiency. Furthermore, HIV infection disturbs other organs and tissues as for example the heart, liver and kidney, as well as accelerates aging which include fragility, sarcopenia and osteoporosis. These alterations within the body are likely associated with the virus, antiretroviral therapies, or chronic inflammation [47].

While treatment of HIV infection with tenofovir shows great success, it has also been clinically reported to cause unwanted side effects within the patients undergoing HAART. Among those adverse events, extended exposure to tenofovir has implicated bone loss with diminishing degrees of bone mineral density consequently, putting patients at great risk for bone injuries including osteoporosis, osteopenia, and bone fractures [45, 48]. A specific mechanism for tenofovir-related bone

reduction has not yet been established, however potential hypothesis included the alteration of gene expression of osteoclasts (indicating an increase of bone resorption), osteoblasts (implying a decrease of bone formation), or changes within both cell types simultaneously. Further findings suggest an indirect effect of tenofovir on bone through the renal system. Exposure to tenofovir has proven dysfunction of the renal system, mainly in the proximal renal tubules. This condition is called Fanconi's syndrome and in its most severe form resulted in bone demineralization (osteomalacia) caused by bicarbonate and phosphate wasting.

In a separated in vivo study led by Feig and colleagues two different murine models of adenosine and $A_{2A}R$-mediated fibrosis were used to prove the hypothesis that tenofovir diminishes fibrotic scarring by interfering adenosine passage. Results substantiated that blockage of ATP release through pannexin-1 channel diminished extracellular adenosine levels and subsequently resulted in prevention of hepatic and dermal fibrosis when treated with tenofovir. A model was established to characterize the action of tenofovir within the adenosine axis and ultimately raised understanding of the antifibrotic effect [49].

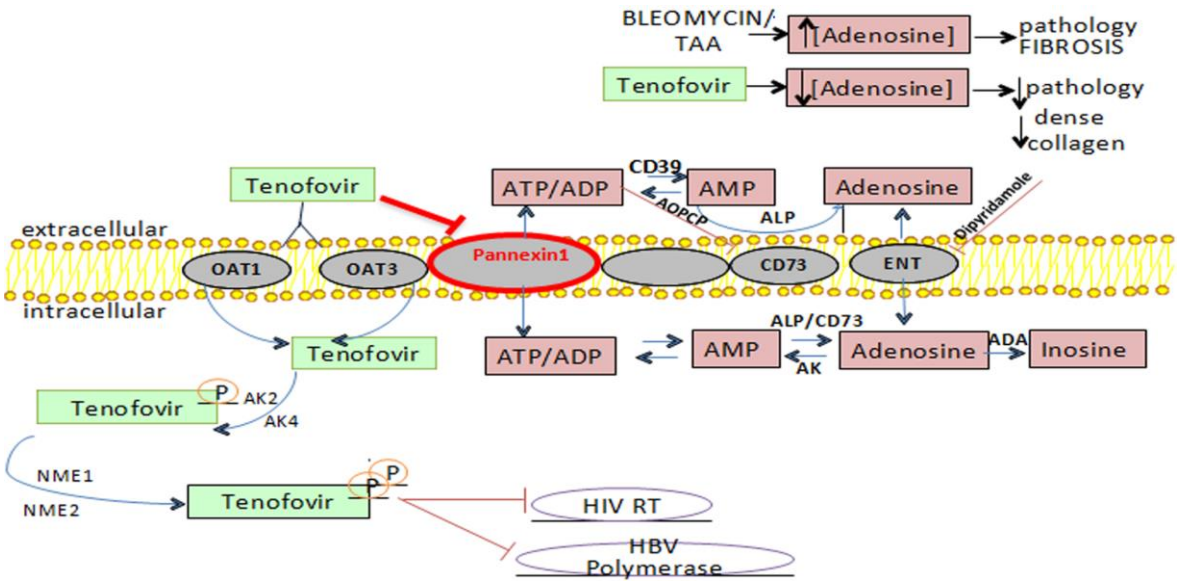

Figure 6. Scheme of tenofovir activity in the framework of the adenosine axis
Tenofovir enters the intracellular space through organic anion transporter 1 (OAT1) and organic anion transporter 3 (OAT3), crucial transporters contributing to tenofovir release. Within cells, the enzymes adenylate kinase (AK2 and AK4) and nucleotide diphosphate kinases (NME1 and NME2) phosphorylate tenofovir. Tenofovir controls further HIV

proliferation by inhibiting the mechanism of HIV-1 reverse transcriptase. This is achieved by competing tenofovir diphosphate against the nucleotide deoxyadenosine 5'-triphosphate for integration into newly synthesized viral DNA. Once incorporated, it results in cessation of DNA elongation and further DNA-synthesis is halted.
The purine nucleoside adenosine is found intra- as well as extracellularly and is generated by ATP. Intracellularly formed adenosine is either re-phosphorylated to ATP by the enzyme adenosine kinase (AK) or deaminated from adenosine to inosine through adenosine deaminase (ADA). On the contrary, extracellular adenosine is the outcome of a cascade mechanism involving ectonucleotidases (CD39 and CD73) and alkaline phosphatase (ALP).

The model demonstrates that the pro-drug tenofovir suppresses ATP export mediated by pannexin-1 which result into reduced adenosine concentrations in the extracellular space. These diminished adenosine levels lead to decreased $A_{2A}R$-mediated fibrosis. In contrast, bleomycin, a drug commonly used in chemotherapy, increases adenosine levels in the extracellular space which lead to higher risks of developing fibrosis disorders. The study recommended potential chances of tenofovir as a treatment of fibrosis diseases.

1.5 Objective of the Project

The presentation of altered adenosine levels impacted by tenofovir exposure, raised attention to the question whether this specific process also contributes to the toxic side effects experienced in tenofovir therapy. Primarily, HIV-infected individuals who undergo HAART with tenofovir have revealed growing appreciation in bone alteration most notably in the drop of BMD and the rise of bone fractures. Although it was not evidently confirmed that tenofovir is in fact the root cause for bone loss in HIV-infected people, clinical findings strongly suggest that tenofovir treatment is a substantial risk factor.

The main goal of this project was to determine the impact of tenofovir in bone homeostasis utilizing two different mice models. Furthermore, it was investigated whether dipyridamole may potentially be an effective counteract treatment to the adverse bone effects of tenofovir.

2 Material and Methods

2.1 Reagents

Dulbecco's Modified Eagle Medium (DMEM). Alpha Modified Eagle Medium (α-MEM), Fetal Bovine Serum (FBS), Penicillin/Streptomycin (P/S), Antibiotic-Antimycotic (Anti-Anti), Dulbecco's Phosphate-Buffer Saline (DPBS), were from Thermo Fisher Life Sciences (New York, NY, USA). Murine Macrophage Colony-Stimulating Factors (M-CSF) and Receptor Activator of NFkB Ligand (RANKL) were from R&D (Minneapolis, MN, USA). Dipyridamole, Sodium Acetate, Glacial Acetic Acid, Naphtol AS-MX Phosphate Disodium Salt, Fast Red Violet LB, Sodium Tartrate, Alizarin Red, Dexamethasone, β-glycerophosphate and L-Ascorbic Acid were from Sigma-Aldrich (St. Louis, MO, USA). Collagens I and II were from Santa Cruz Biotechnology (Dallas, TX, USA). ATP Determination Kit (A22066) was from Molecular Probes. Tenofovir was from Sequoia Research Products (Carbosynth Limited, Berkshire, UK)

2.2 Osteoclast Differentiation from Bone Marrow Cells

Bone marrow cells (BMCs) were isolated from 6- to 8-week-old female C57BL/6, also called wild type (WT), and pannexin-1 knock-out (PANX1KO) mice (n = 6 each). First, a single incision trough the skin, starting at the top of the sternum and ending a few millimetres above the genitals, was done with a sterile number 10 scalpel. Another incision was made at the hips deep enough to reach the femur. The femur and tibia were removed and thoroughly cleaned with the scalpel by scraping off all the muscles. Remaining adherent soft tissue with sterile alcohol wipes. In a 6-well plate, three femurs and three tibiae were places into each well containing DPBS and 1% Anti-anti. The 6-well plate was incubated at room temperature (RT) for one hour. After incubation time each well containing femurs and tibia were washed with DPBS + 1% Anti-Anti. The bone ends, known as epiphyses, were cut off and the bone marrow cavity was flushed out with the complete culture medium (cCM) containing DMEM, 10% FBS and 1% Anti-Anti from one end of the bone using

a sterile 21-gauge needle. The bone marrow was carefully collected in a new 6-well plate containing cCM and incubated at 37°C for at least 24 hours to obtain single-cell suspension. On the following day, nonadherent cells were collected and each transferred into one 50 mL falcon tube. The cells were centrifuged at 10,000x for 10 minutes and the supernatant poured off. Into each falcon tube new cCM was added and the cells were resuspended. The cells were seeded onto a 48-well plate, at a density of 200,000 cells per well containing cCM and 30 ng/mL M-CSF. The 48-well plate was incubated at 37°C for 2 days (at least 48 hours). On day 3, which is day 0 (zero) of differentiation, 30 ng/mL RANKL was added into the culture in the together with tenofovir (10 µM, 1µM, 0.1 µM, 0.01 µM) either alone or in the presence of dipyridamole (10 µM). Within the cultures the medium and reagents were changed to new every third day. On day 7 of differentiation, the cells were stained for tartrate-resistant acid phosphatase (TRAP) in order to determine the efficacy of tenofovir on osteoclast formation.

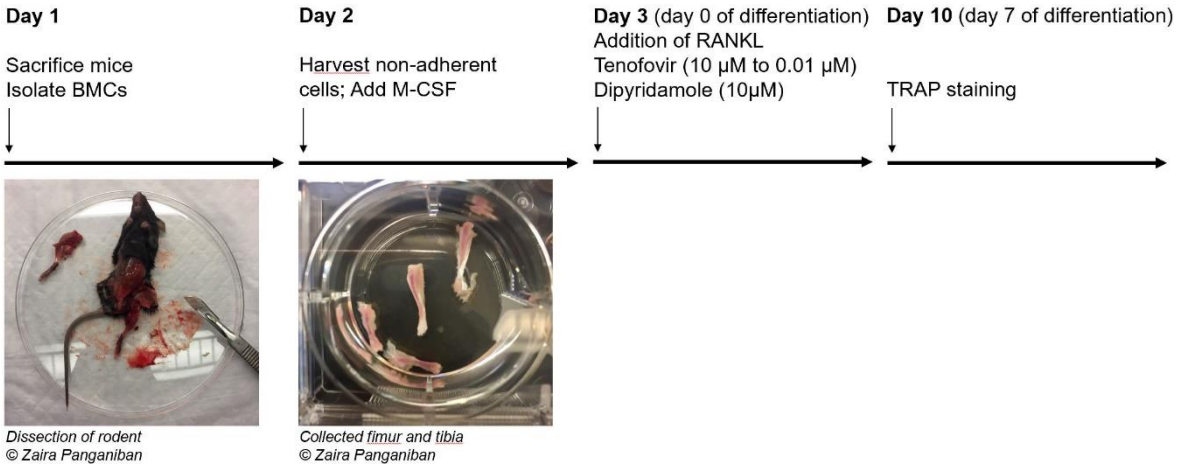

Figure 7. Timetable of osteoclast differentiation
It displays timeframe from sacrificing the mice all the way to TRAP staining the formed osteoclasts.

2.3 Tartrate-Resistant Acid Phosphatase Staining

For the TRAP staining, the TRAP buffer, which is composed of 0.1M Acetate Buffer, 0.3M Sodium Tartrate, 10 mg/mL Naphtol AS-MX Phosphate, Triton X-100, 0.3 mg/mL Fast Red Violet LB Stain and distilled water, was prepared and warmed in a 37°C water bath. First, the medium in the culture was removed and the cells were

washed with prewarmed PBS. The PBS needed to be prewarmed so that it can create an approximate temperature environment of 37°C. Subsequently, the cells were fixed with 50 µL of 10% formalin for 10 minutes and washed again with prewarmed PBS twice. Afterwards, the cells were treated with the prewarmed TRAP staining solution and incubated at 37°C for 10 minutes. The TRAP buffer was removed and the treated cells were washed with PBS twice to remove all the excess staining solution. Under a light microscope the differentiated osteoclasts were observed and counted. Evaluation of the mature osteoclasts was determined by the number of nuclei per cell. Mature osteoclasts were considered TRAP-positive multinucleated cells with ≥ 3 nuclei/cell.

2.4 Osteoblast Differentiation from Bone Marrow Cells

Bone marrow cells (BMCs) were isolated from 6- to 8-week-old female C57BL/6, also called wild type (WT), and pannexin-1 knock-out (PANX1KO) mice (*n* = 6 each). First, a single incision trough the skin, starting at the top of the sternum and ending a few millimetres above the genitals, was done with a sterile number 10 scalpel. Another incision was made at the hips deep enough to reach the femur. The femur and tibia were removed and thoroughly cleaned with the scalpel by scraping off all the muscles. Remaining adherent soft tissue with sterile alcohol wipes. In a 6-well plate, three femurs and three tibiae were places into each well containing DPBS and 1% Anti-anti. The 6-well plate was incubated at room temperature (RT) for one hour. After incubation time each well containing femurs and tibia were washed with DPBS + 1% Anti-Anti. Once the bone marrow cavity was flushed out, the diaphyses were put back into the corresponding wells and incubated over night at 37°C with cCM. On the following day, the medium was removed from the wells and the diaphyses cut into little pieces of approximately 1-2 mm² using a sterile scissor. The bone pieces were washed three times with the solution composed of DPBS and 1% Anti-anti and after that incubated for two hours in 4 mL collagenase II solution at 37°C. Every 30 minutes the well plate was shaken vigorously by hand in order to remove all remaining soft tissue and adherent cells. Once the incubation time was over the well plate was shaken one more time and the bone pieces were rinsed three times with cCM, shaking the solution containing the pieces for a few

seconds during every wash step. The bone pieces were incubated with 4 mL of cCM at 37°C. The culture medium was replaced three time per week. After each medium replacement, it was made sure that the bone fragments were evenly placed over the well to achieve an even distribution of osteoblasts. Adult mouse bone cells started to migrate from the bone chips after 3-5 days. On average the osteoblasts growing from the bone fragments were ready for use after 11-15 days. For the PANX1KO mice osteogenic medium was used (α-MEM containing 1μM dexamethasone, 50 μg/mL ascorbic acid, 10mM β-glycerophosphate).

2.5 Determination of ATP Release into the Supernatant

Once the osteoblasts, which were cultured in a 6-well plate, finally achieved confluency ATP levels were able to be quantified. Before applying the Molecular Probes® ATP Determination Kit (A22066), first each well containing mature osteoblast cells was washed with sterile 1x PBS for three times. In each well 500 µL of 1x PBS was added and the plate incubated at 37°C for one hour. After the hour, each sample was collected and put into a sterile microcentrifuge tube. All samples were centrifuged at 5000x RPM at 4°C to pellet any cells that may have pulled off the plate during sample collection and transfer of the cell supernatant to sterile microcentrifuge tube and immediately kept on ice until further steps. Regular media was added into the culture and places into the 37°C incubator until further experimental use.

Release of ATP to the extracellular space was measured in WT and PANX1KO osteoblast cell lines on the basis of the bioluminescence assay. With the ATP Determination Kit quantification of ATP with recombinant firefly luciferase and its substrate D-luciferin was accomplished. For this 1 mL of1x Reaction Buffer was prepared which composed of 50 µL of 20x Reaction Buffer and 950 µL of deionized water. This amount of volume was sufficient to prepare 1 mL of 10 mM D-luciferin stock solution which is comprised of 1 mL of 1x Reaction Buffer and one vial of D-luciferin. It was important to protect the 10 mM D-luciferin stock solution from light until usage because the solution is reasonably stable and efficient for several weeks if stored at ≤ -20°C. Then a 100 mM DTT stock solution (adding 1.62 mL of deionized water to the bottle with 25 mg of DTT) was made. With this aliquots of 100 µL each

was prepared. Lastly low-concentration ATP standard solution, ranging from 1 nM to 1 µM, was prepared by diluting the 5 mM ATP solution in deionized water. All components of the reaction were combined in a 15 mL falcon tube to make 10 mL of a standard reaction solution, in this recommended order: 8.9 mL deionized water, 0.5 mL 20x Reaction Buffer, 0.1 mL 0.1 M DTT, 0.5 mL of 10 mM D-luciferin and 2.5 µL of firefly luciferase 5 mg/mL stock solution. To mix the solution the tube was gently inverted and protected from light. For the reaction 20 µL of the sample was put to a 96-well plate and 180 µL of the standard reaction solution was added into each well ending up with a total volume of 200 µL. A standard curve had to be determined as well, therefore 200 µL of only standard reaction solution was put into the well. All assays were performed in triplicates. The luminescence was read at 560 nm immediately.

2.6 Quantification of Adenosine Levels by High-Pressure Liquid Chromatography (HPLC)

For the quantification of adenosine levels in the osteoblast culture a similar initial procedure as for the determination of ATP concentration was obtained. Here each well containing mature osteoblast cells was washed with sterile DMEM media which did not contain any serum for three times. In each well 500 µL of DMEM media was added and the plate incubated at 37°C for one hour. After the hour, each sample was collected and put into a sterile microcentrifuge tube. All samples were centrifuged at 5000x RPM at 4°C to pellet any cells that may have pulled off the plate during sample collection and 400 µL of the cell supernatant was transfer into a new sterile microcentrifuge tube. Into the tube 400 µL of trichloroacetic acid (10% vol/vol) was added to denature the proteins in the supernatants. The tubes were vortexed and kept on ice for 10-30 minutes. Then 800 µL of freon-octylamine solution (consisted of 2.25 mL Tri-n-Octylamine and 7.75 mL Trichloro-trifluoro-enthane) was added and vigorously vortexed for about 15 seconds. The tubes were spun for 2-4 minutes at 14,000x RPM at 4°C. The top aqueous layer was taken from the tube and transferred to a new sterile microcentrifuge tube and stored at -80°C before analysis.

The adenosine concentration of the supernatants was determined by reverse-phase HPLC. Before running the reverse-phase HPLC two buffers, Buffer A and Buffer B were prepared. Buffer A contained 100 mL ammonium persulfate stock and 900 mL ultrapure water. The pH was adjusted with two drops of ammonium hydroxide to attain a pH of 5.5. Buffer B comprised of 600 mL of buffer A and 400 mL MetOH. The buffer was sonicated for 5-10 minutes. Before the samples were ran some buffer were ran to check the pressure. For the samples preparation, adenosine and inosine standards (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}) from the stock solution (10^{-3}) as well as 2-chloro-adenosine 10^{-4} from the stock solution, were prepared. For the standard (1.5 mL) in the vial 150 µL of 2-chloro-adenosine 10^{-4}, 150 µL of adenosine or inosine (one of the concentrations 10^{-4} to 10^{-7}) and 1200 µL of diethyl pyrocarbonate water. The same was done for the biological samples with 150 µL of 2-chloro-adenosine and 1350 µL of sample. Adenosine was identified by retention time and the characteristic UV absorbance spectrum, and the concentration was calculated by comparison to standards. In some experiments, the adenosine peak was digested by treatment with ADA (0.15 IU/ml, 30 min at 37°C) to confirm that the peak identified contained only adenosine. All samples were run in triplicates, and results were expressed as adenosine concentration (arbitrary unit).

2.7 *In-Vivo* Tenofovir Treatment of WT and PANX1KO Mice

In vivo procedures were conducted by the post-doc. However, the study is directly correlating to the in vitro study and therefore included in this research.

Male C57BL/6 mice, also referred to as WT, were divided into four groups, where each group received daily treatments with either saline 0.9%, tenofovir 75 mg/kg, dipyridamole 25 mg/kg or a combination of tenofovir 75 mg/kg and dipyridamole 25 mg/kg (n = 8 each). Saline 0.9% was considered the control group. The treatments were administered via injection. The mice were weighed weekly. Water and food were given ad libitum until euthanization. After a duration of 4 weeks the mice were sacrificed by euthanizing them in a CO_2 chamber. The same procedure was performed with PANX1KO mice.

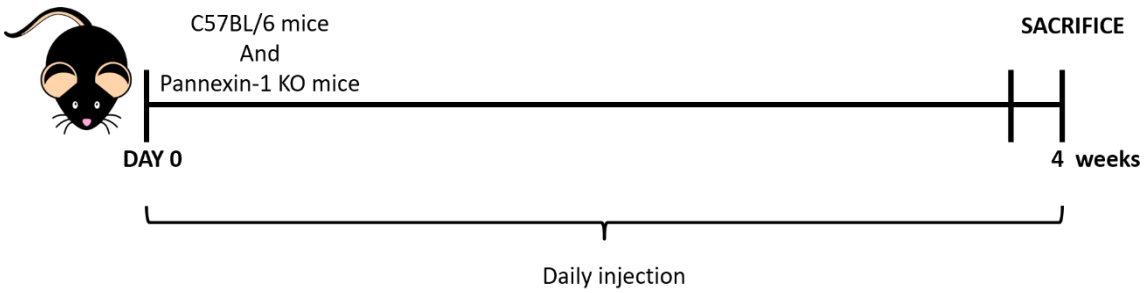

Figure 8. In vivo Study in C57BL/6 and Pannexin-1 KO Mice
It illustrates injection treatment in mice starting from day 0 until sacrifice. The daily injection lasted 4 weeks.

2.8 Measurement of Bone Mineral Density

BMD (g/cm2) of whole skeletons by PIXImus bone densitometer (Lunar, Madison, WI, USA) was assessed. Before each scanning period the instrument was calibrated, with the help of a phantom with known BMD, according to the manufacturer's guidelines. Mice were anesthetized by ketamin/xylacin anesthesia and placed in the prone position on the specimen tray for scanning of the entire skeleton.

2.9 Statistical Analysis

Statistical significance for differences between groups was determined by use of one-way ANOVA and Bonferroni post hoc test or Student's t test, as appropriate. All statistics were calculated using GraphPad software (GraphPad, San Diego, CA, USA).

3 Results

Tenofovir promoted WT bone marrow-derived osteoclast differentiation while the addition of dipyridamole suppressed osteoclast differentiation. On the contrary, osteoclasts differentiation derived from PANX1KO bone marrow were also inhibited by dipyridamole, but a reverse effect with tenofovir was not observed. Moreover, WT mature osteoblasts obtained from the long bones showed significant reduction of extracellular ATP and adenosine when treated with tenofovir. In vivo studies expressed a decline of BMD in WT mice upon treatment with tenofovir alone. Treatment combined with dipyridamole in addition to tenofovir reversed the effect in the animals. PANX1KO mice did not demonstrate the same influence of tenofovir as in the WT mice and indicated no significant change in BMD upon treatment.

3.1 Tenofovir stimulates Osteoclast differentiation

To confirm whether tenofovir directly influences osteoclast differentiation, murine primary BMCs were isolated and mature osteoclasts were differentiated in the presence of tenofovir alone or in combination with dipyridamole. Mature osteoclast were defined by the number of nuclei/cell. A cell which contained ≥ 3 nuclei was considered a positive TRAP-stained mature osteoclast.

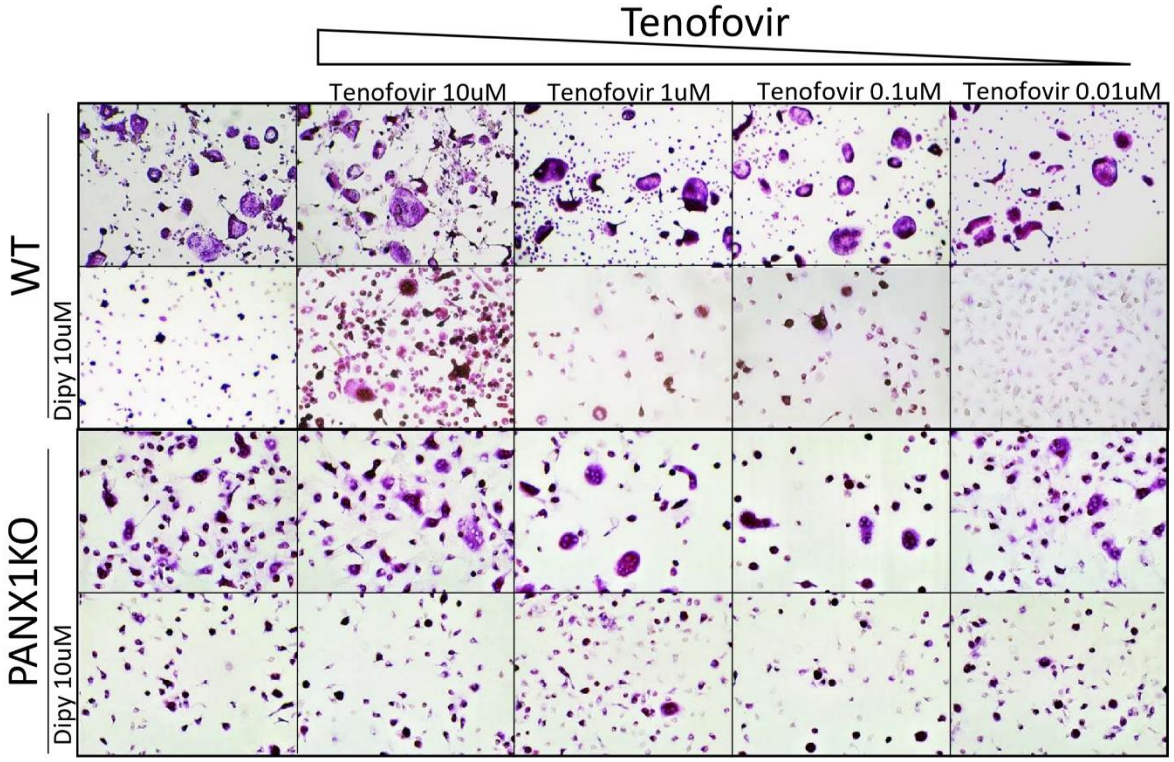

Figure 9. Positive TRAP-Stain of Murine-Derived Osteoclasts in WT and PANX1KO Mice Tenofovir increased osteoclast differentiation derived from WT bone marrow. A reversed effect is given when the cells were cultured for 7 days with 10 µM dipyridamole in addition to tenofovir (concentration ranging from 10 µM to 0.01 µM). Murine-derived osteoclasts were fixed and stained for tartrate-resistant acid phosphatase (TRAP)-positive cells. Cells with three or more nuclei were counted as mature osteoclasts. The results are expressed as the means of five different assays carried out in duplicate.

WT bone marrow-derived osteoclast cultures treated with tenofovir alone demonstrated an enhanced formation of osteoclasts in a dose-dependent manner. The decrease of tenofovir concentration in the cells correlated to a decreasing number of mature osteoclasts. Cells treated with 10µM tenofovir showed the greatest number of mature osteoclast differentiation, while cells who received treatment with tenofovir concentration 0.01µM showcased the least number of mature osteoclast. In the WT bone marrow cell cultures, combination treatment with 10µM of dipyridamole completely revoked the effect of tenofovir and triggered suppression, resulting in a decreased amount of mature osteoclasts. Cells treated with 10µM dipyridamole in addition to the lowest tenofovir concentration of 0.01µM resulted in the greatest inhibition of osteoclast formation. Contrary, in the absence of pannexin-1 channel (PANX1KO bone marrow-derived cells) a drop of mature osteoclasts

differentiation is visible in both tenofovir-only-treatment and combination-treatment with tenofovir and dipyridamole compared to WT cell cultures. In the PANX1KO cell cultures, the number of mature osteoclasts remained constant in both types of treatments. PANX1KO bone marrow-derived cells that received the combination treatment with tenofovir and dipyridamole diminished the formation of mature osteoclasts even more when compared to the PANX1KO cell cultures treated by tenofovir alone.

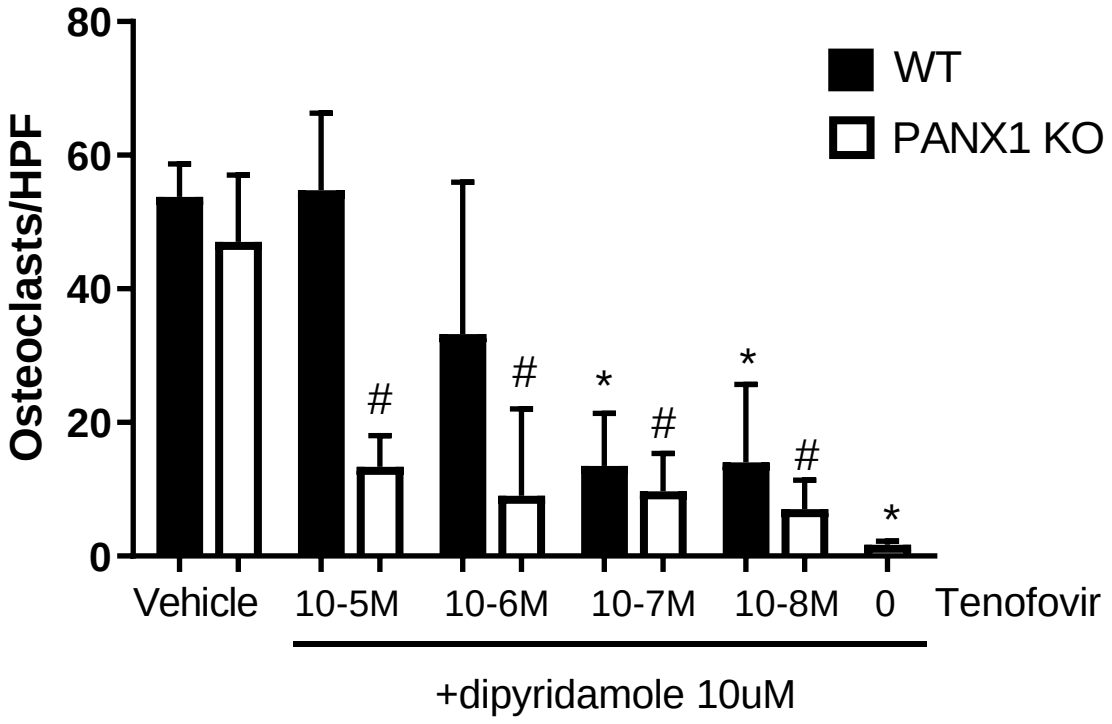

Figure 10. Tenofovir diminishes osteoclast formation in PANX1KO mice
Bar Graph displays the quantitative analysis of positively formed osteoclasts/high power field (HPF). Y-axis presents the number of formed osteoclasts in % against the concentration levels (in MOL) of tenofovir and dipyridamole showcased in the x-axis. Results are presented as mean±SEM. *p<0.05 WT, #p<0.05 PANX1KO compared to vehicle.

The statistical depiction of the formation of mature osteoclasts revealed enhancing osteoclast differentiation in WT bone marrow-derived osteoclast cultures with the combination treatment of tenofovir and dipyridamole. In contrast, PANX1KO cell cultures, that received identical combination treatment, showed significantly less formation of mature osteoclasts. Both cell cultures obtained the same dipyridamole concentration of 10 µM. Administration of tenofovir at 10^{-5} M concentration on WT

bone marrow-derived cells resulted in nearly 60% osteoclast formation whereas, bone marrow-derived cells from PANX1KO mice only formed slightly 10% osteoclasts. A decline of osteoclast differentiation for both WT and PANX1KO was visible in a dose-dependent manner. The lowest percentage of mature osteoclast formation was identified at the lowest tenofovir concentration of 10^{-8} M.

3.2 Tenofovir inhibits ATP export via Pannexin-1 Channel

Previous research studies reported that increase of adenosine in the extracellular matrix inhibits osteoclast formation, stimulates osteoblast formation and therefore controls bone turnover. ATP which is released to the extracellular space is converted to adenosine through the ecto-enzymes CD39 and CD73. In order to further understand the direct contribution of tenofovir on bone alteration, the influence of tenofovir on bone forming cells, osteoblasts, was investigated. Extracellular ATP levels of both WT and PANX1KO osteoblasts was measured. Here fore, osteoblasts, obtained from bone fragments, were cultured for 15 days and subsequently treated overnight with either 1µM of tenofovir, 1µM of dipyridamole alone, or in combination (1µM tenofovir + 1 µM dipyridamole). The control group did not receive any treatment.

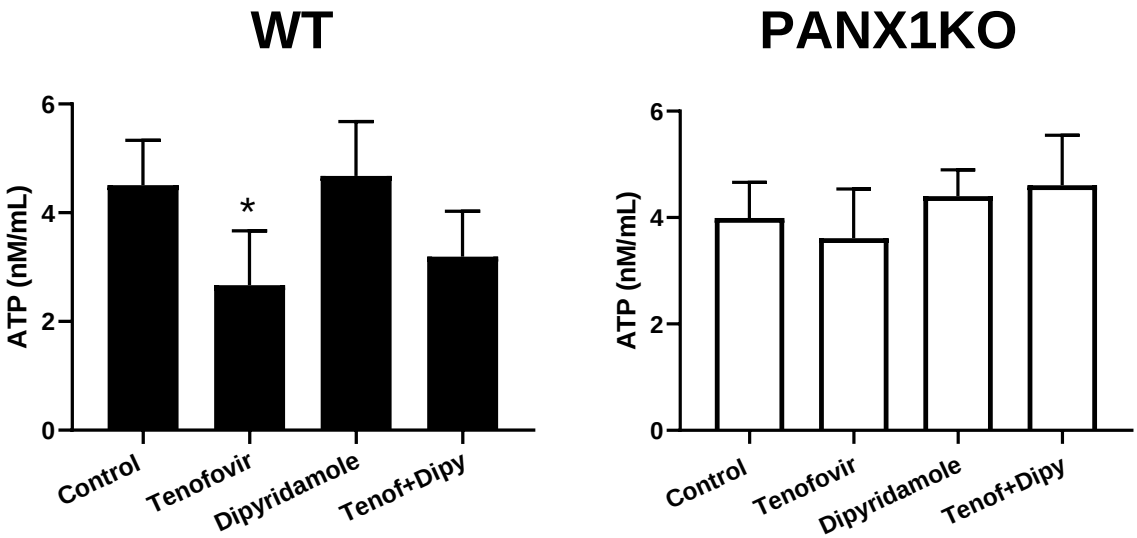

Figure 11. Tenofovir inhibits ATP export via Pannexin-1 Channel
In vitro ATP determination assay was conducted on osteoblasts for both WT and PANX1KO based on the manufacturer's protocol. Tenofovir dose-response effects are given. Results are presented as mean±SEM. *p<0.05 WT compared to control.

Overnight incubation of WT osteoblasts treated with 1 µM tenofovir alone expressed significant decrease of ATP concentration, resulting in almost half of the amount of ATP levels compared to the control group. In contrast, treatment with only 1 µM dipyridamole did not result in a reduction of ATP concentration and a similar level to the control group was attained. Exposure to the combination treatment of 1µM tenofovir and 1 µM dipyridamole revealed diminishing levels of ATP when compared to the control.

Within the PANX1KO osteoblast culture, no significant changes in ATP levels were observed. The control group as well as all three treatment groups (1 µM tenofovir, 1 µM dipyridamole, or 1 µM tenofovir + 1 µM dipyridamole) obtained an ATP concentration of around 4 nM/mL.

3.3 Tenofovir diminishes Extracellular Adenosine Levels

The suppression of ATP release through pannexin-1 channels causes adenosine concentrations within the extracellular space to decline. For this project, extracellular adenosine concentrations were statistically identified. Both WT and PANX1KO osteoblast cultures received treatments with identical principles described in the ATP determination assay. Evaluation of extracellular adenosine levels in the supernatant were measured via HPLC, as described in the methods section.

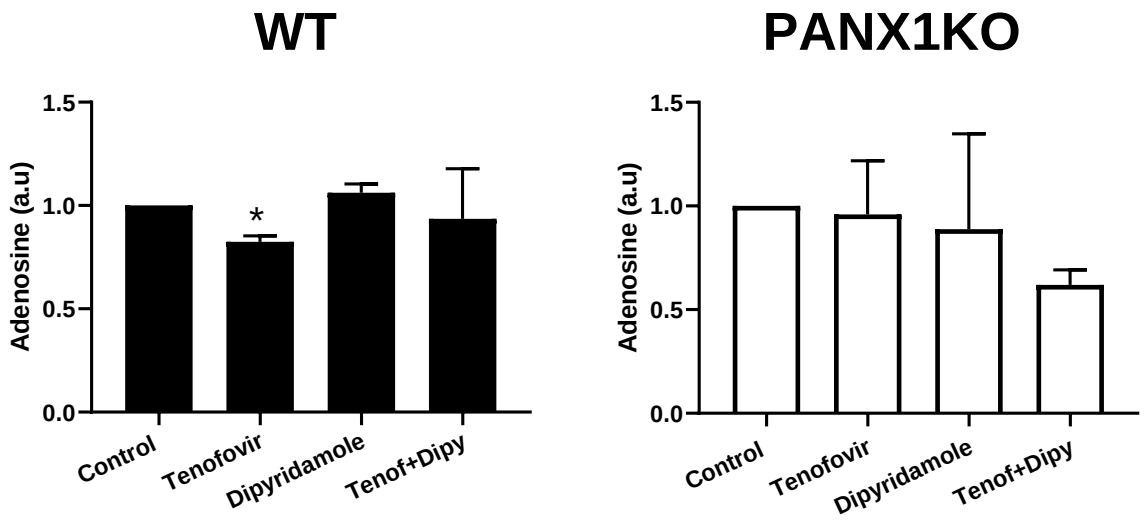

Figure 12. Tenofovir modulates Extracellular Adenosine Levels
Quantification of adenosine concentration in osteoblasts is ascertained via high-pressure liquid chromatography (HPLC). The experimental procedure was performed on both WT and PANX1KO. Results are presented as mean±SEM. *p<0.05 WT compared to control.

Overnight incubation of WT osteoblasts with 1µM tenofovir alone showed diminishing levels of extracellular adenosine. Treatment with only 1µM dipyridamole did not result in reduction of extracellular adenosine when compared to the control group. The exposure to 1µM tenofovir and 1µM dipyridamole together displayed a smaller drop of adenosine levels. By contrast, osteoblast obtained from PANX1KO bone fragments kept a more constant value of adenosine concentration within the control group, tenofovir only treatment and dipyridamole only exposure. Nonetheless, combination treatment with tenofovir and dipyridamole gave rise to a statistically outlying result.

3.4 Tenofovir reduces Bone Mineral Density *In-Vivo*

BMD also defined as bone mass, is the amount of bone mineral, an inorganic component, within the bone tissue. Poor bone density is highly associated with great risk of bone fractures and potential development of osteoporosis.

The in vivo study was performed by the post-doc to determine the bone mineral density of WT as well as PANX1KO. Here C57BL/6 (WT) and PANX1KO male mice were split into four groups: saline 0.9%, tenofovir 75 mg/kg/day, dipyridamole 25 mg/kg/day, combination tenofovir 75 mg/kg/day and dipyridamole 25 mg/kg/day. Each group, consisting of 8 mice, were injected daily with either saline, tenofovir, dipyridamole, or a combination of tenofovir and dipyridamole. In this experimental procedure, the control group was given saline 0.9%. Treatment was held for 4 weeks and the mice were sacrificed and scanned with a dual-energy x-ray absorptiometry (DXA).

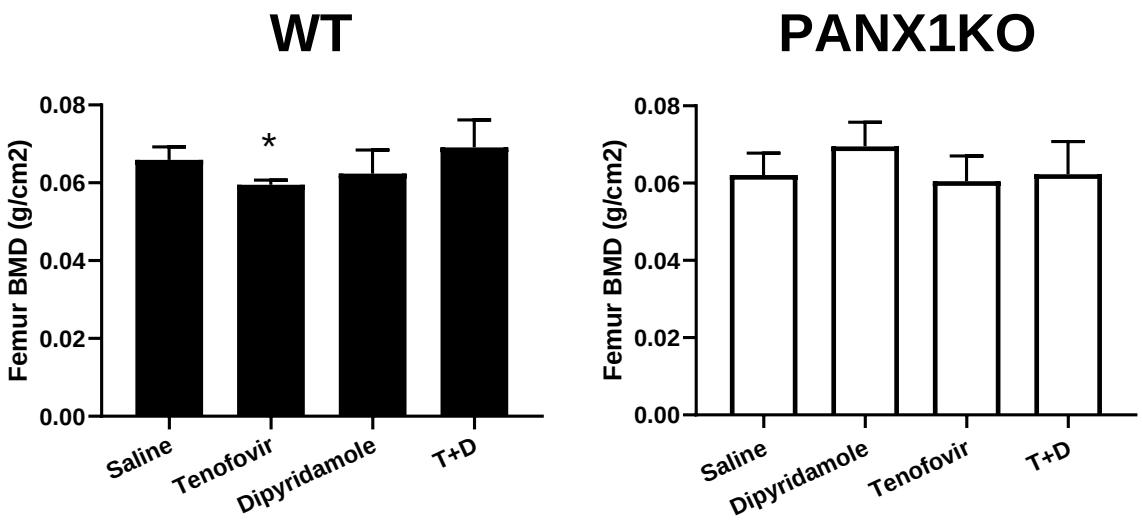

Figure 13. Tenofovir decreases Femur BMD
Femur BMD is quantified via DXA scanning and given in g/cm^2. The experimental procedure was conducted in vivo on C57BL/6 (WT) and PANX1KO male mice. Results are presented as mean±SEM. *n* = 8 each, *p<0.05 WT compared to saline (control).

As previously described, tenofovir promotes osteoclast differentiation; therefore it was assumed that tenofovir might lead to bone loss and subsequently cause osteopenia through greater bone resorption and lower bone remodeling. DXA scanning of the femur revealed that 4-week treatment with tenofovir 75 mg/kg/day alone induced a drop of 10% BMD in WT. It was observed that a reversed effect was attained in the presence of dipyridamole. Combination treatment with tenofovir and dipyridamole stimulated growth of bone minerals in bone tissue and increased the BMD. In comparison, the BMD in PANX1KO remained constant despite treatment of tenofovir. Additionally, cotreatment with dipyridamole presented a stable value in BMD as well.

4 Discussion

Clinical findings have reported great success rate of antiviral therapies in HIV-infected patients. Nonetheless, in course of infection and its treatment, various alteration within the body are visible. Little is known what causes these pathological changes, but it is presumed that they are triggered by the host, the virus, or the antiviral therapies administered. One of the conditions observed among people living with HIV is the increasing abnormality in bone mineralization. Tenofovir, an antiretroviral prodrug which is applied in the treatment regimen called highly active anti-retroviral therapy (HAART), is often associated with the bone disorders osteopenia and fragility fractures.

Previous studies proved that adenosine, which is generated extracellularly by catabolism of adenine nucleotides, is an crucial player in osteoclastgenesis. Activation of $A_{2A}R$ by adenosine regulates osteoclast formation and function. Reduction of extracellular adenosine production or inhibition of $A_{2A}R$ activities, increased osteoclast formation was reported. Furthermore, complete deletion of $A_{2A}R$ led to osteopenia in mice in vivo [31]. In a different study approach which investigated the effect of tenofovir in fibrosis indicated that tenofovir blocks fibrosis by diminishing extracellular adenosine levels in two murine models [49]. Its mechanism demonstrated that cells absorb tenofovir and transform it to tenofovir polyphosphates. These polyphosphates are responsible for the antiviral effects of the substance. An interference of the activity of ecto5'nucleotidase was not observed despite tenofovir being an analogue of AMP.

In this research project, the characteristics of tenofovir on murine derived bone cells were analyzed. Additionally, the counteracting effect of dipyridamole, an agent that increases local adenosine concentration, was tested. The obtained results propose that 1) tenofovir's influence on bone loss is independent from HIV-infection; 2) tenofovir promotes formation of bone degrading cells; 3) tenofovir reduces extracellular adenosine by blocking pannexin-1 mediated cellular ATP release. These findings were all reversed by dipyridamole.

In the presence of pannexin-1, tenofovir promoted osteoclast formation and function. The effect could be counteracted by the addition of dipyridamole. Contrary within the PANX1KO mice no impact of tenofovir was observed on the osteoclast differentiation and function. This outcome suggest the hypothesis that tenofovir phosphates block pannexin-1 channels and support the antiretroviral effect of the drug by hindering access of HIV into the cell. Furthermore in vitro data indicate that tenofovir reduced osteoblast differentiation and altered osteoblast function in a dose-dependent manner. A novel process between adenosine A2AR and β-catenin recently demonstrated the effect of bone homeostasis impairment by tenofovir. Although the in vivo study discovered no decline in osteoblast numbers, an alteration of osteoblast function likely explain the inhibition of mineralization and suppression of bone formation [50].

In the absence of pannexin-1, tenofovir prevents release of ATP which ultimately led to diminishing levels of extracellular adenosine in vitro. Adenosine and its receptors play important roles in osteoclast homeostasis and considered key mediators. According to findings, blockage or deletion of A1AR leads to enhancement of bone density and prevention of ovariectomy-induced bone loss in mice [51, 52]. In addition, activation of A2AR suppresses osteoclast formation and function while enhancing the percentage amount of immature osteoclast precursors [31]. Taking these facts into consideration, osteoclast differentiation likely increases due to the reduced concentration of adenosine resulted by tenofovir-mediated inhibition of ATP export. This occurrence might contribute to the development of osteopenia in patients treated with tenofovir.

5 Summary

In conclusion, bone homeostasis is directly affected by the antiretroviral agent tenofovir and can be reversed by treatment with dipyridamole.

Osteoclasts were successfully differentiated from bone marrow cells and treated with tenofovir or dipyridamole. The results indicate increase of osteoclast formation in the presence of pannexin-1 upon receiving treatment with tenofovir alone. This effect was counteracted with dipyridamole treatment. Upon knock-out of pannexin-1, osteoclast differentiation was also suppressed by dipyridamole, however the effect was not reversed by tenofovir treatment.

Moreover, ATP and adenosine concentrations were successfully quantified and revealed lower levels in WT mice upon treatment with tenofovir. PANX1KO mice did not result in decrease ATP or adenosine levels in the cell supernatants.

Lastly, in vivo studies imply reduction of BMD through tenofovir treatment in WT mice. Treatment together with dipyridamole reversed this effect and did not result in BMD decline. In PANX1KO mice, treatment with tenofovir did not impact the BMD.

These discoveries support the hypothesis that the commonly used drug tenofovir influences bone homeostasis by enhancing bone resorption and repressing bone formation. More studies are necessary to be able to confirm this hypothesis, nevertheless it raises awareness to utilize agents like dipyridamole that increase local adenosine concentrations as potential adjuvants to prevent bone loss within the highly active anti-retroviral therapy with tenofovir.

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