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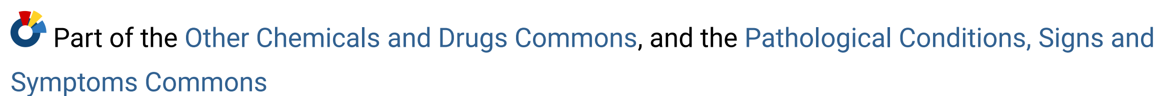
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BROMODOMAIN AND EXTRATERMINAL DOMAIN (BET) INHIBITOR RVX-208 AMELIORATES PERIODONTAL BONE LOSS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Physiology and Biophysics at Virginia Commonwealth University

By

Nicholas J. Clayton

Bachelor of Science in Sports Medicine, Pepperdine University, 2014

Director: Zhao Lin, BDS, MS, MMSc, PhD

Assistant Professor, Department of Periodontics

Virginia Commonwealth University

Richmond, Virginia

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ABBREVIATIONS

ABC: Alveolar bone crest

CEJ: Cementoenamel junction

CVD: Cardiovascular disease

DMEM: Dulbecco's modified Eagle medium

DMSO: Dimethyl sulfoxide

ELISA : Enzyme-linked immunosorbent assay

FBS: Fetal bovine serum

IL-1: Interleukin-1

IL-6: Interleukin-6

IL-18: Interleukin-18

IKK: IκB kinase

LPS: Lipopolysaccharide

MACE: Major adverse cardiac events

Micro-CT: Micro-computed tomography

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

PMA: Phorbol 12-myristate 13-acetate

PS: Penicillin and streptomycin

p-TEFb: Positive transcription elongation factor B

RANK: Receptor activator of nuclear factor kappa-B

RANKL: Receptor activator of nuclear factor kappa-B ligand

ROF: Roof of furcation

ROI: Region of interest

TGF-β: Transforming growth factor beta

TLR: Toll-like receptor

TNFα: Tumor necrosis factor alpha

VOI: Volume of interest

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Abstract

BROMODOMAIN AND EXTRATERMINAL DOMAIN (BET) INHIBITOR RVX-208 AMELIORATES PERIODONTAL BONE LOSS

By Nicholas J. Clayton, BSc

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Physiology and Biophysics at Virginia Commonwealth University

Virginia Commonwealth University, 2017

Major Director: Zhao Lin, BDS, MS, MMSc, PhD

Assistant Professor, Department of Periodontics

Periodontal disease affects 47% of Americans over 30 and is a growing global concern. Current treatments for periodontal disease focus on the mechanical elimination of periodontal biofilms. Very few treatments are available that target the rampant, unregulated host immune response that is ultimately responsible for tissue degradation. BET proteins have been shown to play critical roles in inflammatory gene regulation and are therefore potentially ideal therapeutic targets for treating periodontal disease. RVX-208 is a selective BET-inhibitor with a high affinity for Bromodomain 2 (BD2) as compared to BD1 in BET proteins. Our previous studies have shown that RVX-208 inhibits inflammatory cytokine production and suppresses osteoclast differentiation. Cell culture assays have provided proof of concept for RVX-208 and its feasibility as a treatment for periodontal disease. As such, our long term goal is to develop

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RVX-208 as a front-line treatment for periodontitis. The objectives of this study were to determine the ability of RVX-208 to reduce bone loss in a ligature-induced periodontitis model, and to further investigate the mechanisms through which RVX-208 mediates its anti-inflammatory and osteoclastogenesis-suppressive effects. The specific aims of this study were: 1) To further validate the *in vivo* effects of RVX-208 on a ligature-induced periodontitis model in rats, and 2) To determine the molecular mechanisms of RVX-208 on preventing alveolar bone loss in periodontal disease. To investigate, a ligature-induced periodontitis model was created in rodents. Those rodents were treated with increasing dosages of RVX-208 (0-2.5 mM) by subgingival injection every other day. After 2 weeks, the maxillae were harvested and analyzed via a micro-CT protocol that had been created and validated through statistical analyses. To study the ability of RVX-208 to suppress osteoclastogenesis, RAW264.7 cells were induced into osteoclasts by RANKL and then treated with RVX-208. To ensure RVX-208 was not species specific, THP-1 cells were challenged with either *E. coli*-LPS or *P. gingivalis* bacteria and then treated with RVX-208. Linear and volumetric micro-CT analysis showed that RVX-208 could significantly ameliorate bone loss in a ligature-induced periodontitis model. RVX-208 was shown to prevent osteoclast differentiation by suppressing the expression of genes closely associated with osteoclast differentiation and maturation. RVX-208 was found to not be species specific, as it was able to mediate its effects on a human cell line, and had consistent anti-inflammatory effects regardless of whole pathogen or LPS-induced inflammatory response. Therefore, RVX-208 is a promising therapeutic for treatment of periodontal diseases.

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CHAPTER 1: INTRODUCTION

1.1 SCOPE OF PERIODONTAL DISEASE

As one of the most common inflammatory diseases, periodontitis is systemic amidst global populations. It currently affects approximately 50% of Americans (1) and continues to increase steadily in prevalence worldwide (2). In addition to the well-documented inflammation and bone-loss attributed to periodontitis, periodontal disease in its more severe forms has also been found to increase the patient's risk of systemic diseases such as rheumatoid arthritis, atherosclerosis, adverse pregnancy outcomes, pneumonia, and even cancer (3-8). Partially due to its pervasiveness, periodontal disease has become a major burden on the healthcare systems in place around the globe, affecting 11% of the population and making it the 6th most prevalent health condition worldwide (9). Characterized by microbial dysbiosis and unregulated inflammation, severe periodontitis will ultimately result in destruction of the periodontal tissue and tooth loss. The wide range of symptoms, from swollen and/or sore gums, loose and/or drifting teeth, to halitosis and toothache, have a severe detrimental impact on the physical, social, and psychological aspects of a patient's quality of life.

1.2 PATHOGENESIS OF PERIODONTAL DISEASE

While a triad of oral anaerobic bacteria, including *P. gingivalis*, *Treponema denticola*, and *Tannerella forsythia*, are traditionally considered the primary causative agents for the disease

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itself (10), it is the host immune response to the microbiotic insults that ultimately leads to tissue damage (11). The dysbiosis of commensal oral microbiota and subsequent interaction with the host immune response persist through bouts of activity and quiescence, until the affected tooth or microbial biofilm is therapeutically removed and the inflammation subsides (12). Thus, the inflammatory cascade provides a vehicle through which the deleterious effects of periodontitis are administered. More specifically, a chronic and robust inflammatory response induces a constitutive overproduction of pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-a) and interleukin-6 (IL-6) (13). This overabundance of pro-inflammatory cytokines promotes the generation and release from the bone marrow of osteoclast precursors (14), which subsequently differentiate into osteoclasts, ultimately leading to irreversible tissue degradation and bone destruction. Additionally, formation of a periodontal pocket stemming from degradation of the gingival tissue creates an opportunity for unfavorable microbial colonization (15), furthering the oral dysbiosis and perpetuating the vicious cycle of bacterial infection common in periodontal diseases.

As the dysbiosis of periodontitis evolves, the overgrowth of microbial commensals drives the activation of specific pathogen recognition receptors (PRRs) including NOD1 and Toll-like receptors (TLRs). In addition to activating these recognition receptors, evidence suggests that bacterial pathogens are also able to induce extravagant epigenetic modifications, leading to the activation of pro-inflammatory pathways (16). The impact of specific epigenetic modifications, including DNA methylation and histone acetylation, on periodontal disease remains a topic of intense focus. It is well established that the acetylation of histones relaxes the chromatin and leads to enhanced transcription of many of the inflammatory genes commonly upregulated in

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periodontitis. While the specifics remain unclear, NF-kB signaling appears to play a key role in connecting histone modifications to disease progression (17). NF-kB activates the innate immune response and protects against infections (18). However, chronic NF-kB activation and overexpression cause the osteoclast differentiation and bone resorption characteristic of periodontitis pathogenesis (19). Thus, it is the disruption of equilibrium between the innate immune response and chronic activation of the NF-kB pathway that ultimately leads to overall tissue degradation in periodontal disease even after the pathogens have been removed. Martins et. al explored the ability of the bacteria known to cause periodontitis, *P. gingivalis*, to affect the NF-kB pathway through epigenetic modifications and found that the modifications were induced primarily through acetylation of histones and downregulation of DNA methyltransferase 1 (DNMT1) (17). Additionally, PRRs were found to induce histone modifications as well (17). These findings suggest that disruption of dysbiosis-mediated epithelial injury may inhibit PRRs and that targeting the disruption of histone acetylation may provide the foundation for treating epigenetically-relapsing periodontitis (17).

1.3 CURRENT TREATMENT STRATEGIES FOR PERIODONTAL DISEASE

Current treatment strategies for periodontitis focus on the eradication of oral pathogens at the site of infection through mechanical means, usually by surface debridement procedures with or without adjunctive therapies, including the use of antiseptics and/or antibiotics (20). While it is thought that the removal of subgingival plaque and elimination of the biofilm is sufficient to reduce the inflammatory response and remediate tissue destruction, recent meta-analyses suggest otherwise. Studies show that after initial mechanical debridement, the microbial load drops to 0.1% (21). However, the bacteria is able to recolonize within a week

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with less pathogenic composition (22), potentially leading to re-infection. Moreover, surface debridement does not account for the host immune response that is ultimately responsible for the inflammation, suggesting that mechanical means alone are insufficient despite remaining the treatment of choice for periodontal disease (23). In conjunction with mechanical methods of treatment, chlorhexidine is among the most common antiseptic treatments because of its broad-spectrum antimicrobial activity (24). Yet despite its efficacy and ease of use, many limitations have been reported, including tooth discoloration, oral mucosal erosion, parotid swelling, and bitter taste, all ultimately contributing to patient non-compliance (25). Alternatively, several classes of antibiotics have been suggested for the treatment of periodontal infections, including tetracyclines, macrolides, β-lactams, and nitroimidazoles (26, 27). Because systemically administered antibiotics can penetrate deep into periodontal tissue, they are able to reach oral pathogens that are not otherwise accessible via mechanical means or locally applied antibiotics (28). However, this treatment method requires a high patient compliance, has been associated with undesirable side effects, and can facilitate antibiotic resistance (29, 30). Additionally, concerns about the efficacy of the aforementioned antimicrobials in treatment of periodontitis have been raised within the last few years. Yamaguchi et. al. found that *P. gingivalis* cells residing in biofilms are less susceptible to antimicrobials such as chlorhexidine when compared with planktonic cells (31). More specifically, and perhaps more worrisome, biofilms can be up to 500 times less sensitive to antibiotics (32). Furthermore, several studies have examined the antibiotic susceptibility of subgingival microflora isolated from patients suffering from periodontitis and found that 74.2%

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of patients harbor pathogens resistant to at least one standard antibiotic (29), further validating concerns of an overall increase of antibiotic resistance.

1.4 BROMODOMAINS AS THERAPEUTIC TARGETS

When examining the epigenetic mechanisms through which inflammatory genes are expressed, it becomes clear that the post-translational modifications (PTMs) of histones are some of the most important determinants of chromatin structure and function. Among the many PTMs, histone acetylation (Fig. 1) is generally associated with enhanced DNA accessibility and transcriptional activation (33). Acetylation weakens the bond between histone and DNA by removing the positive charge on lysine residues and by introducing slight structural changes within the histone itself. As a result, the nucleosome loosens, and the DNA becomes more readily accessible. In addition to increasing DNA accessibility, acetylation of the histone also recruits transcription and chromatin-remodeling factors that lead to enhanced transcriptional activity (34). These factors are traditionally recruited via a bromodomain (BRD), an epigenetic reader domain that specifically recognizes ε–*N*-acetylated lysine residues (K_{ac}) (35). In humans, there are at least forty proteins that interact specifically with the bromodomain (36), including histone acetylases (HATs), helicases, scaffolding proteins and other co-factors that control gene transcription, among the most important of which is positive transcription elongation factor b (PTEFb). By inhibiting the BRD's interaction with these bromodomain proteins, the DNA would remain in a dormant state and the proteins for which that genomic sequence codes would be suppressed. Without BRDs, gene transcription is slowed or halted altogether. As such, the discovery of high-affinity small molecule BRD inhibitors has introduced new avenues through which to treat epigenetic symptoms in a wide range of diseases.

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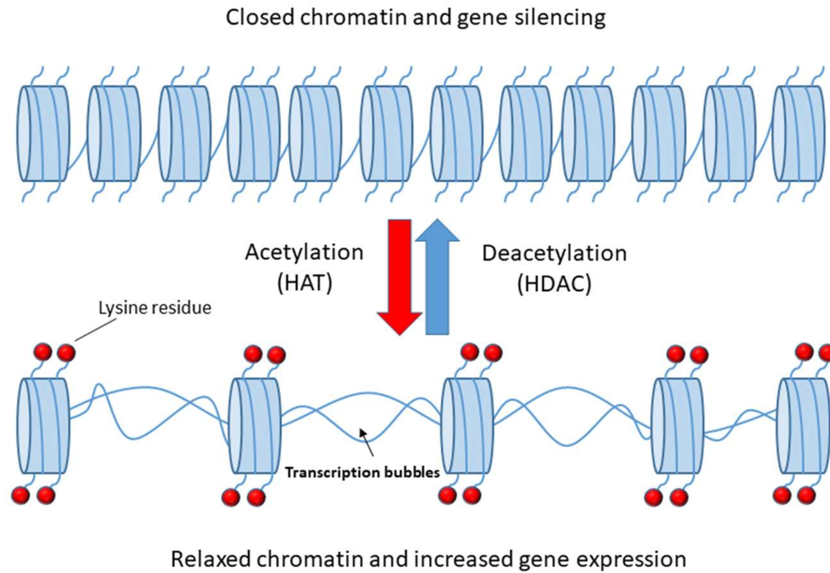

Figure 1: Histone acetylation and deacetylation regulated by HAT and HDAC enzymes alters chromatin structure. Acetylation of histones increases the accessibility of chromatin and allows DNA binding proteins to interact with exposed sites to activate the genes.

1.4.1 BET PROTEINS

The bromodomain and extra-terminal (BET) family is a distinct group of bromodomain proteins that includes BRD2, BRD3, BRD4, and BRDT, each of which are ubiquitously expressed in mammalian tissue except for BRDT, which is testis specific (37). Each BET isoform contains two consecutive bromodomains (Fig. 2) (BD1 and BD2) which share highly similar structures but differ in their functions regarding gene transcription (38-41). While the roles of BRD2 and BRD4 in cell cycle control (42) and transcription elongation (41) have been well established, their function in inflammatory responses *in vivo* remains poorly explored.

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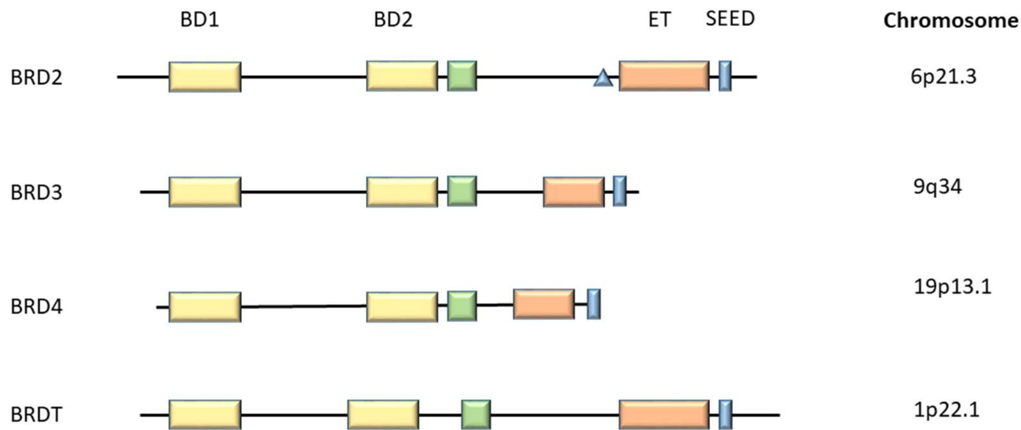

Figure 2: Motif alignment of double bromodomain-containing BETs. The dual tandem BDs are always positioned at the amino terminus. The recruitment of transcription factors takes place either through ET domains or through SEED domains rich in Ser, Glu, and Asp.

1.4.2 THE ROLE OF BET PROTEINS IN THE INFLAMMATORY RESPONSE

BRD4 has recently emerged as a key transcription regulator of NF-kB-dependent inflammatory gene expression due in part to its ability to activate CDK9 of P-TEFb (Fig. 3), ultimately facilitating the RNAPII-dependent transcription elongation (42-44). NF-kB is an inducible transcription factor that is widely considered to be the master regulator of innate and immune responses. NF-kB is a heterodimer composed of ReIA and p50 and remains sequestered in the cytoplasm by its association with IkBα (45). Acetylation of the ReIA subunit at lysine-310 regulates the transcriptional activation of NF-kB target genes (46). When stimulated, IkBα is phosphorylated and degraded, facilitating the nuclear translocation of NF-kB and the activation of NF-kB target genes (45, 47) (Fig. 4). Importantly, one of NF-kB's target genes is its inhibitor, IkBα, creating a negative-feedback regulation that prevents sustained NF-kB activation and prolonged inflammatory response (45). In addition to this negative feedback loop, the NF-kB pathway is subjected to many intricate layers of regulation, including various epigenetic modifications. The eukaryotic translation initiation factor eIF4E has been shown to

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be the node of the translational control of immune response via the mTOR signaling pathway or the MAPK-Mnk1-Mnk2-eIF4E pathway (48). Bao et. al showed that in response to LPS stimulation, deletion of *BRD4* in macrophages led to the sustained expression of *Mknk2* and the enhanced activation if eIF4E, which stimulates the translation of IkBα mRNA, resulting in decreased NF-kB-dependent inflammatory gene expression and compromised innate immune response (49). Additionally, BRD4 has been shown to bind to acetylated lysine-310, suggesting that BRD4 helps to maintain constitutively active NF-kB by binding to acetylated RelA (43, 46). These findings provide clear evidence that BRD4 has an essential role in the innate immune response and justify the protein as a reasonable target to influence the NF-kB inflammatory response. Furthermore, a recent genetic disruption model in mice suggested that BET protein function is required for inflammatory cytokine gene expression (37), prompting further study of their relevance in the host response while leaving the exact mechanisms an enigma. Because the deleterious effects of periodontitis stem from the host-response inflammatory cascade, elucidating the roles of the other BETs in inflammation may prove beneficial to mitigating the symptoms of the disease. Namely, BET bromodomain inhibition has been shown to suppress LPS-induced expression of pro-inflammatory cytokines in murine bone marrow-derived macrophages (37).

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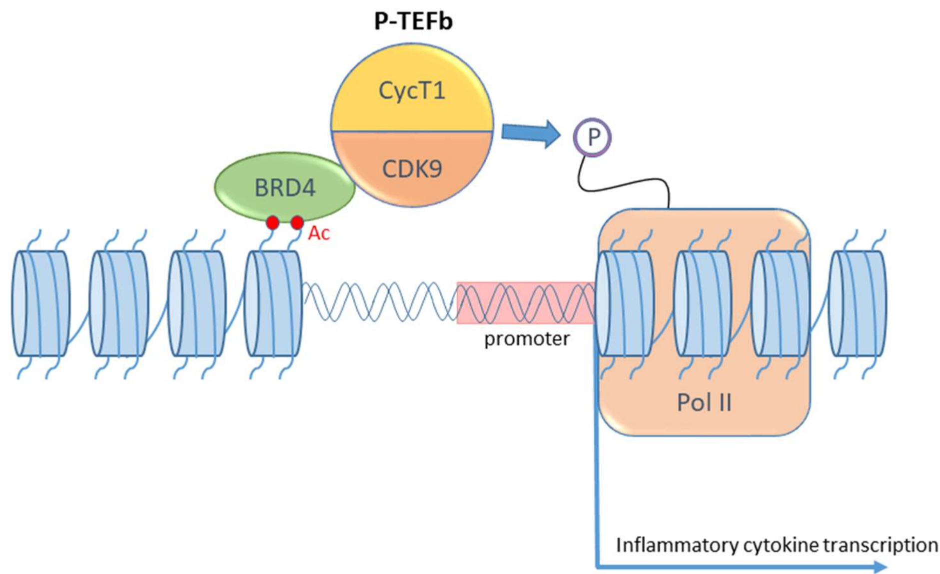

Figure 3: The binding of BRD4 to acetylated histones recruits and activates cyclin-dependent kinase 9 (CDK9) of the positive elongation factor b complex (P-TEFb) to phosphorylate RNA polymerase II (Pol II), leading to increased transcription of inflammatory cytokines.

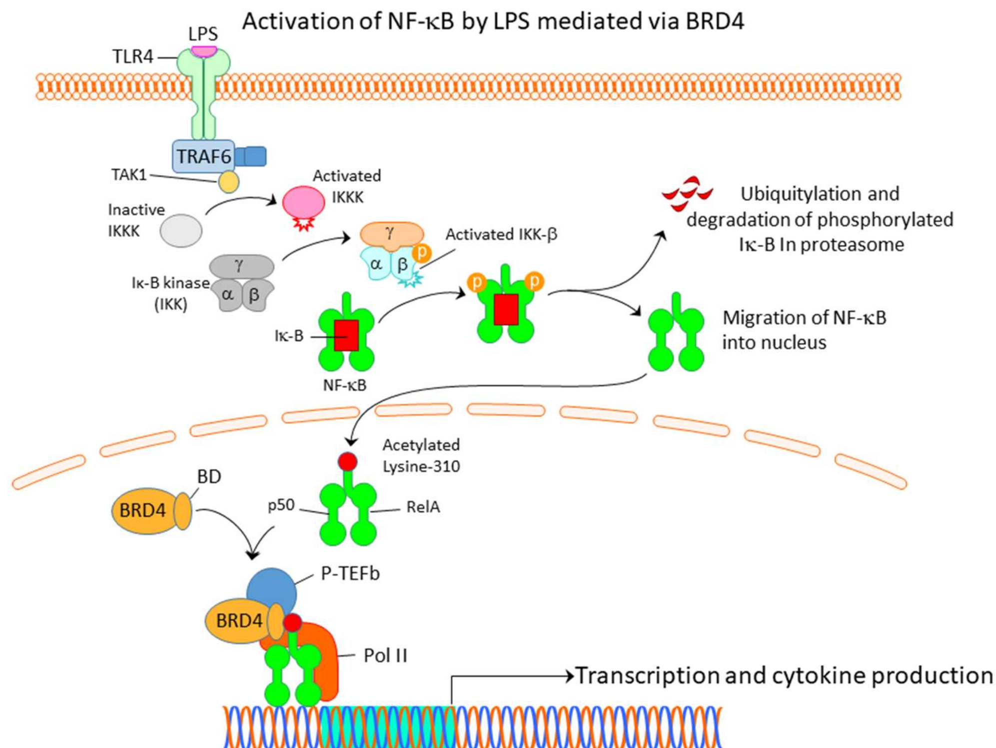

Figure 4: The stimulation of Toll-like receptor 4 by LPS leads to the degradation of Iκ-B and NF-κB. BRD4 then binds to acetylated Lysine-310 on the ReiA subunit via its two BDs and recruits p-TEFb, phosphorylating RNA Polymerase II and promoting the transcriptional activation of a subset of NF-κB target genes, including inflammatory cytokines.

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1.5 SUPPRESSION OF INFLAMMATION BY BET-PROTEIN INHIBITORS

In the past decade, several small molecules have been developed to block the interactions between the BRDs of BET proteins and acetylated lysine residues in histones (Fig. 5). So far, many small molecule inhibitors targeting the BET family are being developed for clinical use, with most being non-selective inhibitors that display equivalent activity in both bromodomains BD1 and BD2. In 2010, Nicodeme et al. successfully synthesized a compound (IBET762) that mimicked acetylated histones and disrupted chromatin complexes responsible for the expression of key inflammatory genes in activated macrophages, while also conferring protection against LPS-induced endotoxic shock and bacteria-induced sepsis (50, 51). Almost simultaneously, Filippakopoulos et al. described a cell-permeable small molecule (JQ1) that binds competitively to bromodomains and displaces the BRD4 fusion oncoprotein from chromatin (52). Both of these landmark findings established proof-of-concept for targeting protein-protein interactions of epigenetic "readers," and ushered in a new era of epigenetic drug discovery. Like all BET-inhibitors, JQ1 and IBET762 interact with the BRD pocket in a manner competitive with acetylated peptide binding, resulting in the displacement of BET proteins from acetylated chromatin in cells exposed to these inhibitors (34). Systemic treatment of JQ1 prevented bone loss in inflammatory arthritis (53) and estrogen-related osteoporosis (53, 54). For periodontal disease in particular, intraperitoneal injection of JQ1 in a murine periodontitis model inhibited inflammatory cytokine expression and prevented periodontal alveolar bone loss (55). Mechanistically, JQ1 inhibits TLR2/4 expression and NF-kB phosphorylation and nuclear translocation in macrophages and suppresses the gene expression of the master osteoclast regulator NFATC1. While JQ1 displays a high affinity for BRD4, it also

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demonstrates an affinity for other BRDs, opening the door for potential undesirable off-target effects. On the other hand, IBET762 displayed very similar selectivity profiles and binding affinities to JQ1, but also demonstrated the added bonus of suppressing several important inflammatory genes *in vivo* (50). Taken together, the discovery and characterization of JQ1 established the plausibility of optimizing small-molecule inhibitors of epigenetic targets, leading to advances in the treatment of cardiovascular disease, obesity-associated type 2 diabetes, and cancer (56-61).

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Table 1: Summaries of the current BET-inhibitors in the treatment of inflammatory diseases

Inhibitor	Target	Potency	Scaffold	*in-vitro* Results	*in-vivo* Results	Affiliation	Reference
JQ1	BET	K_D = 50-190 nM (BRD2-4, BRDT)	Triazolodiazepine	Inhibits IL-6, IL-8, and TNFa cytokine secretion and expression of matrix metalloproteinases	Reduced synovial inflammation and joint destruction in mice with collage-induced arthritis, and has shown promising results in a mouse model of psoriasis.	Dana-Farber Cancer Institute	Filippakopoulos et al. (41)
I-BET151	BET	K_D = 20-100 nM (BRD2-4)	Dimethylisoxadole	Suppresses expression of key LPS-inducible cytokines and chemokines, including IL-6 and IL-8.	Prevents or attenuates death in mice induced with endotoxic shock, and protected mice against death by sepsis.	GSK	Mirguet et al. (48)
I-BET762	BET	K_D = 50-61 nM (BRD2-4)	Triazolodiazepine	Suppresses expression of IL1B and IL-6, as well as chemokines CXCL9 and CCL12.	Suppressed inflammation in a mouse model of severe sepsis, which prevented or delayed death from endotoxic shock. Mice showed reduced serum levels of pro-inflammatory cytokines.	GSK	Nicodeme et al. (39)
CBP30	CBP/p 300	K_D = 26 nM (CBP)	Dimethylisoxadole	Inhibits IL-17A production in primary human cells and Th17 responses from patients with AS and PSA.	N/A	SGC	Hay et al. (94, 95)
CPI-203	BET	IC_{50} = 37 nM (BRD4)	Triazolodiazepine	Suppresses Th17 cell responses in explanted CF tissue and inhibited IL-17-driven chemokine production in HBEC.	Decreased inflammation in an acute lung infection mouse model.	N/A	Chen et al. (96, 97)
PFI-1	BET	IC_{50} = 220 nM (BRD4(1))	Dihydroquinazolin-one	Inhibits release of IL-6 and IL-8, but despite its tight interaction with BRD4, it displays a weak reduction in pro-inflammatory cytokines.	N/A	Pfizer	Fish et al. (98)
RVX-208	BET (2nd BRD)	K_D = 140 nM (BRD4(2))	Quinazolone	Stimulates ApoA1 transcription leading to a dose-dependant increase in ApoA1 levels.	In African green monkeys, daily treatment increased serum ApoA1 and HDL-C levels. Showed usefulness in treating a mouse model of atherosclerosis and hypercholesterolemia, inhibited production of proinflammatory cytokines, and reduced the formation of aortic lesions.	Resverlogix	Bailey et al. (71)
RVX-297	BET	IC_{50} = 20-80 nM (BRD2-4(1))	Quinazolone	Suppresses pro-inflammatory gene expression, including IL-6, IL-1B, and TNFa.	Reduced both cytokine production and gene expression in endotoxemic mice. Additionally, countered disease pathology in rodent models of polyarthritis and prevented disease progression of a murine model of human multiple sclerosis.	Zenith Epigenetics	Kharenko et al. (99, 77)
MS-402	BET	K_D = 77 nM (BRD4 (1))	Cyclopentanone	Inhibits Th-17 cell differentiation.	Ameliorates adaptive T-cell transfer-induced colitis in mice.	N/A	Cheung et al. (67)

Abbreviations: GSK, GlaxoSmithKline; SGC, Structural Genomics Consortium, AS, ankylosing spondylitis; PSA, psoriasis and psoriatic arthritis

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Among the many pan-BET inhibitors that demonstrate therapeutic potential, I-BET151 was found to be one of the more potent and selective. I-BET151 preferentially inhibits BRD4 (K_d = 50 nM), and has also been suggested as having a role in the control of the inflammatory response (62). Because BRD4 associates with acetylated NF-kB (37, 63), it was theorized that I-BET151 could selectively modulate the expression of pro-inflammatory genes associated with the NF-kB cascade. Interestingly, I-BET151 was found to preferentially reduce IL-6 production induced by LPS in RAW267.4 cells, while having no effect on the activation of NF-kB (albeit at low concentrations). Furthermore, it was found that I-BET151 does not affect TNFα, IL-1β, or IL-10, suggesting that bromodomain proteins inhibited by I-BET151 are selectively involved in the control of the production of IL-6 (62). Similarly to JQ1, systemic treatment of I-BET151 also prevented bone loss in inflammatory arthritis (53) and estrogen-related osteoporosis (53, 54). I-BET151 also showed promise in the treatment of multiple sclerosis, as administration of I-BET151 in the mouse experimental autoimmune encephalomyelitis (EAE) delayed onset of the disease and was well tolerated over a prolonged treatment period of nearly 40 days (62). However, the dosage of I-BET151 was unable to prevent progression of the disease, suggesting that other pathogenic mechanisms in the experimental model of multiple sclerosis were not affected by I-BET151, and highlighting the need for further study on the protein-protein interactions of BRD4 and I-BET151.

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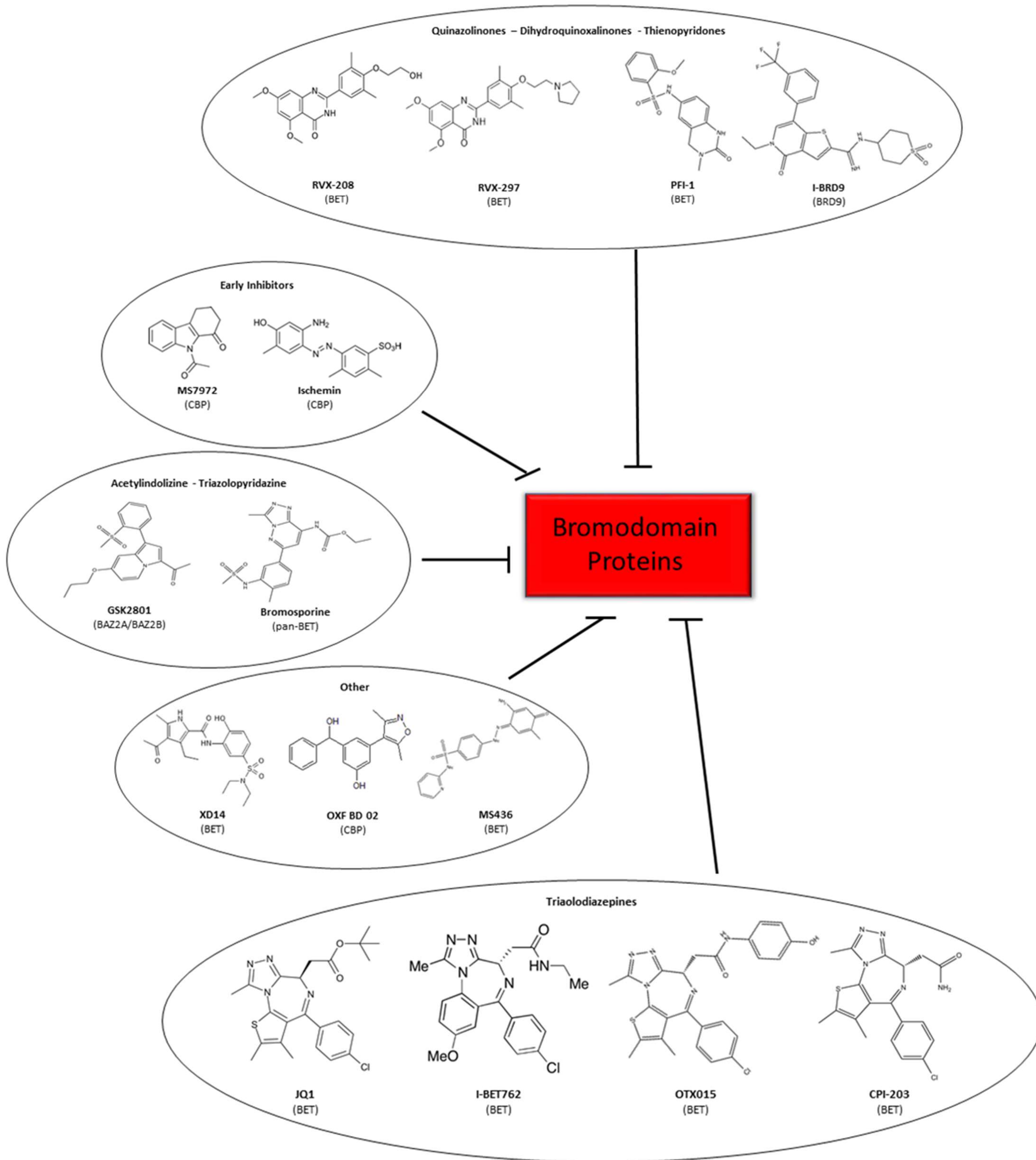

Figure 5: Bromodomain (BRD) inhibitors. Chemical structures of representative BRD inhibitors, arranged by chemical class or scaffold. In parentheses are reported the target BRDs (adapted from (34)).

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1.5.1 PROBLEMS WITH PAN-BET INHIBITORS

While early clinical use of the aforementioned pan-BET inhibitors has shown some efficacy for treatment of hematological cancers and solid tumors (64-68), the significant toxicities of these compounds limit most clinical translations. For example, mice treated with JQ1 at efficacious exposures demonstrated dose-dependent decreases in both B- and T-lymphocytes with a concomitant decrease in peripheral white blood cells (69). At higher doses, JQ1 caused significant body weight loss that forced the researchers to euthanize their subjects early (69). JQ1 is also responsible for significant and unsatisfactory side-effects, including a reactivation of HIV in patients with latent infections (70), uncontrollable increases in blood insulin (71), and temporary male infertility due to inhibition of the testis-specific variant BRDT (72). Young mice treated with the brain-permeable pan-BET inhibitor I-BET858 showed pharmacological suppression of BET proteins resulting in neuronal gene suppression followed by an autism-like syndrome (73). Another pan-BET inhibitor currently in Phase I clinical trials for the treatment of hematological malignancies, OTX015, displayed a wide array of undesirable side-effects including thrombocytopenia, diarrhea, fatigue, nausea, dysgeusia, and hyperglycemia (74). Given the far-reaching effects of BET proteins on gene transcription, the severe systemic toxicity observed in these cases is not surprising given the rampant and unselective blocking of BET-protein function by pan-BET inhibitors. Thus, the need for BET inhibitors that can selectively target individual BET proteins is imperative for a safe and efficacious transition of these inhibitors into the clinic.

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1.5.2 THE CASE FOR SELECTIVE BET-INHIBITORS

In order to minimize the known side effects of BET protein inhibition, clinical use of BET proteins must be geared toward inhibitors that are selective among specific BET family members and coupled with tissue-specific drug targeting (75). In order to accomplish this, researchers focused on elucidating the distinctive functions of the two bromodomains (BD1 and BD2) in BET proteins, which facilitate their interactions with different lysine-acetylated histones and/or transcriptional proteins. Most efforts to develop selective inhibitors have been focused on BRD4 due to the discovery of a gene rearrangement that links BRD4 to aggressive carcinoma (76). The first bromodomain (BD1) of BRD4 anchors the molecule and its associated proteins to the target gene promoter and enhancer sites in chromatin through binding to histone proteins, while the second bromodomain (BD2) is involved in the recruitment of non-histone proteins (such as the pTEFb complex) to target genes (40). Conversely, in BRD3, it is the BD1 that binds the non-histone protein hematopoietic transcription factor GATA1 (77). In oligodendrocytes, only the inhibition of BD1 function promoted differentiation, while the reverse process was observed when the function of just BD2 or both BDs were blocked (39). Similarly, the blocking of only BD1 by the small molecule MS-402 selectively suppressed Th17 cell differentiation while leaving CD4+ cells unaffected (78). These findings all suggest that BD1 and BD2 have distinctive roles, despite their highly-conserved structures.

In addition to establishing the distinct functions of the two BDs, several studies have explored the roles of individual BET protein family members in regards to the regulation of cytokine gene transcription. In primary epithelial cells, a knockdown of BRD2 with siBRD2 had no effect on the secretion of IL-6, an effect that would otherwise have been masked by a pan-

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BET inhibitor (79). Further substantiating this role for BRD2, a knockdown of BRD2 inhibited LPS-induced secretion of pro-inflammatory cytokines from mouse bone marrow-derived macrophages (75). While BRD4 is still present, BRD2 is the only BET protein that becomes enriched at the TNF-α promoter upon LPS stimulation (37). Finally, a substantial cache of evidence (37, 72, 80) has been established in recent years that reinforces the definitive role BRD2 plays in regulating the responses of immune cells crucial to the inflammatory cascade. In light of the ubiquitous nature and diverse functions of BET proteins, these observations suggest that establishing the exclusive effects of individual BET proteins on the regulation of cytokine networks is imperative to advancing treatment options. Based on the evidence, selective BRD2 inhibitors, designed in the context of anti-cancer treatments, would be advantageous to alleviating inflammatory diseases. Furthermore, the ability to discriminate between BD1 and BD2 pharmacologically presents an opportunity to achieve more selective transcriptional effects, potentially reducing the disastrous side effects of the known pan-BET inhibitors.

1.6 RVX-208 AS A SELECTIVE BET-INHIBITOR

RVX-208, a derivative of the plant polyphenol resveratrol and belonging to the quinazoline family (Table 1.), was first identified in 2010 as a pharmaceutical candidate for cardiovascular disease due to its ability to increase apolipoprotein A-I (ApoA-1) gene expression (81, 82). Pharmacokinetics and oral bioavailability studied in a number of species indicated good oral absorption. Additionally, RVX-208 has demonstrated a low systemic clearance rate (5 mL/min/kg), a moderate volume of distribution (0.81/kg) at steady state, a relatively short half-life (1.5 hours) and an oral bioavailability of 44% in cynomolgus monkeys (83). In recently concluded Phase 2b trials, RVX-208 significantly increased apoA-I levels, HDL-C levels, large HDL

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particle levels, and decreased levels of high-sensitivity C-reactive proteins (hsCRP), all in a dose-dependent manner (84). Moreover, a post-hoc analysis demonstrated a 55% risk reduction in the major adverse cardiac events (MACE) in patients receiving RVX-208, which was even more pronounced in patients with diabetes (up to 77%) (85). In another phase II study, short durations of RVX-208 treatment were shown to reduce overall glucose absorption and endogenous glucose production (85). In addition to its beneficial effects on blood lipid profile, the MACE-reducing actions of RVX-208 are largely due to its novel anti-inflammatory actions, suggesting a role in the anti-inflammatory cascade. To verify this, Jahagirdar et al. demonstrated a significant reduction in circulating adhesion molecules and pro-inflammatory cytokine interferon gamma inducible protein 10 (IP-10) when apo-E deficient mice were orally treated with RVX-208 in conjunction with a low-fat diet for 14 weeks (86), while simultaneously decreasing the level of C-C motif chemokine ligand-18 (CCL18) and interleukin-18 (IL-18). These results show that the anti-atherogenic activity of RVX-208 occurs via a combination of lipid-profile changes and anti-inflammatory activities. A phase III clinical trial that will examine the effect of RVX-208 on MACE in high-risk coronary artery disease patients and further elucidate its cardioprotective mechanisms is currently underway and recruiting patients.

1.7 THE SELECTIVITY OF RVX-208

Recently, it was found that RVX-208 preferentially binds to BD2 of BRD2 and BRD3 proteins (86), making it a more selective BET inhibitor than the previously detailed pan-BET inhibitors. RVX-208 competitively binds to the BET bromodomains and causes dissociation of the BET proteins from the chromatin, resulting in altered transcription. This variability in

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transcription results in an elevation of apoA-I and HDL, along with a suppression of inflammatory pathways, especially apparent in decreased levels of IL-6 (86).

In the early years of RVX-208, the selectivity for BD2 remained poorly explored, and the molecular basis of the selective inhibition for BD2 over BD1 was enigmatic. Because the active pockets of BD1 and BD2 are so highly conserved (their sequence similarity approaches 95%), developing selective inhibitors remains a significant challenge. In mid-2017, with the help of extensive classical molecular dynamics simulations and hybrid density function theory/molecular mechanics (DFT/MM) simulations, Cheng et al. revealed that the selective inhibitory effect towards BD2 is achieved by the distinctive structural dynamics of the ZA-loop ("in/out" conformations) in BD1 and BD2, which originate from the existence of residue Asp144 in BD1 which is replaced by His433 in BD2 (87). This small alteration in amino acid residues confers a more stable inherent H-bond, accounting for the high inhibitory activity of RVX-208 in BD2 (87). Furthermore, additional molecular dynamics simulations have shown that Val435 and His433 are the distinguishing residues of BD2 from BD1, with Leu383 and Asn429 being the most key residues for BD2 binding to RVX-208 (88). With one step closer to fully understanding the differences between the two domains and the selectivity mechanism for preferential inhibition of BRD2-BD2, the development of clinically translatable BET-inhibitors seems close at hand.

When examined collectively, the anti-inflammatory effects of RVX-208 denote a high therapeutic potential for the treatment of periodontal disease. Its ability to suppress the host immune response in conjunction with its excellent safety profile observed in the completed and ongoing clinical trials suggest next steps are ready to be made in the development of RVX-208

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as a treatment option. In order to do so, the mechanisms of action of RVX-208 need to be better understood to avoid any undesirable off-target effects. Additionally, *in vivo* studies are still needed to verify the effects seen *in vitro*.

1.8 SUMMARY

Periodontitis is caused by a disruption in the oral microbial biome in conjunction with severe inflammation, often resulting in degradation of periodontal tissue. The high prevalence of periodontitis, debilitating impacts on the quality of life of patients, limitation of current therapies, and the omnipresent global threat of antimicrobial resistance coupled with the current data concerning biofilm-mediated resistance in dental practice (89), all underscore the importance of implementing innovative treatment interventions to address the disease. Efficacious, safe, and economically-viable therapies to control the host immune response are an unmet need for current periodontal disease treatment. Mechanical debridement does nothing to address the hyper-inflammatory state of the periodontal tissue while leaving much to be desired in terms of preventing re-infection, and a widespread use of broad-spectrum antibiotics may lead to drug resistance issues in the near future. Subsequently, epigenetic modifiers have emerged as promising therapeutic targets for several inflammatory diseases, including cancer. Because the disease pathogenesis is dictated by the host immune response, targeting the overly exuberant inflammatory response is critical for controlling periodontal disease, especially in susceptible individuals who exhibit severe periodontal inflammation but often minimal plaque accumulation. Amelioration of tissue inflammation not only halts the tissue degradation, but also facilitates the restoration of bacterial homeostasis in periodontal pockets (90). Thus, a need arises for host-response modulation therapies. Because of RVX-208's

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success in clinical trials, favorable safety profile, and documented effects on both reducing the inflammatory response and suppressing osteoclast differentiation, we believe there exists a potential therapeutic application of the drug in the treatment of periodontal disease.

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CHAPTER 2: SPECIFIC AIMS

Excessive periodontal inflammation results in the degradation of gingival tissue and surrounding bone, which ultimately results in tooth loss. Many small molecule BET-inhibitors have been developed that either directly or indirectly target the host immune response ultimately responsible for periodontal disease progression, but very few show as much promise as RVX-208. The overall objective of this study is to elucidate the therapeutic potentials of RVX-208 as treatment for periodontal disease, specifically examining the molecule's ability to reduce bone loss and inflammation. The main hypothesis was that RVX-208 could inhibit alveolar bone loss in a ligature-induced experimental model of periodontitis by inhibiting the host inflammatory response and by suppressing osteoclast formation.

2.1 Specific Aims

The following aims were developed to test our main hypothesis:

Specific Aim 1: To further validate the *in vivo* effects of RVX-208 on a ligature-induced periodontitis model in rats.

Sub-aim 1: To validate the micro-CT protocol for analyzing linear and volumetric data from the periodontitis model in rats. In order to quantitatively analyze any specimens from the experimental model, a standardized and validated protocol was needed that ensured reliable results.

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Sub-aim 2: To determine the effects of RVX-208 on alveolar bone loss in an animal periodontitis model. *The hypothesis of this experiment was that RVX-208 would reduce bone loss in the periodontitis model in a dose-dependent manner*. The maxillae of experiment subjects were harvested, and subsequently analyzed via the aforementioned micro-CT protocol. Both linear and volumetric bone measurements were taken.

Sub-aim 3: To perform a histological analysis of samples from the animal periodontitis model. *The hypothesis of this experiment was that the presence of inflammatory cell infiltrate and osteoclast activity would be significantly reduced in tissue treated with RVX-208.* Samples from the harvested maxillae were demineralized and placed on slides. The slides were then stained with H&E to investigate the morphology and to qualitatively show the extent of the inflammatory response.

Specific Aim 2: To determine the molecular mechanisms of RVX-208 on preventing alveolar bone loss in periodontal disease.

Sub-aim 1: To determine the extent to which RVX-208 inhibits osteoclast gene expression. *The hypothesis of this experiment was that RVX-208 would significantly suppress the expression of the major genes associated with osteoclast differentiation and maturation.* RAW264.7 cells were induced to osteoclasts via treatment with RANKL, then treated with RVX-208 for 24 hours. RT-PCR was performed on the harvested RNA.

Sub-aim 2: To determine the extent to which RVX-208 suppresses the inflammatory cytokine production when challenged by *P. gingivalis* bacteria. *The hypothesis of this experiment was that RVX-208 would significantly suppress inflammatory cytokine*

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production in RAW264.7. Recent findings suggest that *P. gingivalis* bacteria elicits a specific response different than that of *P. gingivalis*-LPS alone. RAW264.7 were challenged with *P. gingivalis* bacteria and treated with RVX-208 for 24 hours.

Sub-aim 3: To determine through which bromodomain protein of the BET-protein family the effects of RVX-208 are mediated.

The hypothesis of this study is that RVX-208 mediates its anti-inflammatory effects by primarily inhibiting the actions of BRD2. Initially, we needed to determine that the effects of RVX-208 were not species specific, as our cell-culture assays almost exclusively utilized a murine cell model. In order to be clinically applicable, we need to show that RVX-208 is equally as effective in human cells. Secondly, once a viable human cell line is determined, a human with a knockdown of the BRD2 protein would provide insight as to whether or not the effects of RVX-208 can still be seen without the presence of BRD2.

2.1 SIGNIFICANCE OF THE STUDY

The purpose of these studies is to provide insight into the effects of RVX-208 on inflammatory diseases outside the realm of CVD, while also furthering the development of the molecule as a therapeutic drug for the treatment of periodontal disease. Because the current therapies available for the treatment of periodontitis focus on the removal of bacterial pathogens, they do little to address the host immune response and often leave the periodontal tissue in a hyper-inflammatory state. Thus, efficacious, safe, and economical therapies to directly control the inflammatory response are an unmet need for current periodontal disease treatment. Recently, epigenetic modifiers have emerged as promising therapeutic targets for

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several inflammatory diseases. In our preliminary studies, we found that one such epigenetic modifier currently in phase III clinical trials, RVX-208, is a potent inhibitor for the inflammatory response induced by the keystone pathogen of periodontitis, *P. gingivalis*. Because it not only suppresses inflammatory cytokine production but also inhibits osteoclast differentiation, RVX-208's regulation of both upstream and downstream events that lead to periodontal tissue destruction paves the way for a new class of therapeutics for periodontal diseases. To further our understanding of RVX-208, the safety and efficacy of RVX-208 was tested via a rodent experimental periodontitis model in conjunction with cell culture assays and histological analysis. Additionally, we examined the mechanisms of action of RVX-208 through the generation of a BET-protein knockout cell line by CRISPR/Cas9. In the long run, our study may lead to an innovative solution to target the host immune response in periodontal patients, with the potential to improve the life quality of millions of periodontitis patients and reduce the economic burdens associated with the disease.

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CHAPTER 3: METHODS

3.1 SPECIFIC AIM 1: TO FURTHER VALIDATE THE *IN VIVO* EFFECTS OF RVX-208 ON A LIGATURE-INDUCED PERIODONTITIS MODEL IN RATS

Studies in Aim 1 seek to expand on data our lab obtained throughout a ligature-induced periodontitis model study in rats by developing and standardizing the protocol for quantitative micro-CT analyses and performing histological assessments of our samples. With our ultimate goal being the clinical application of RVX-208 in the treatment of periodontal diseases, and having established reliable proof-of-concept results in cell culture assays, the next step in progression towards that goal relies on *in vivo* studies. Not only were dosage ranges narrowed and refined, but we also standardized the quantification of our data assessment and reinforced our preliminary *in vitro* findings. The hypothesis is that RVX-208 will significantly ameliorate bone loss in an experimental model of periodontitis.

3.1.1 AIM 1.1: TO VALIDATE THE MICROCT PROTOCOL FOR ANALYZING LINEAR-AND VOLUMETRIC MEAUREMENTS IN THE PERIODONTITIS MODEL IN RATS

A reproducible and standardized micro-CT protocol is critical for the purposes of our research. We wanted to examine both linear and volumetric bone data, and establish a standardized and reliable protocol that produced repeatable results. The samples examined were maxillae harvested from the rat subjects from a ligature-induced periodontitis model that

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were first placed in 10% formalin for 2 days, then transferred to 70% ethanol for preservation before and after scanning. A maxilla sample was initially scanned using a micro-CT machine (Bruker SkyScan 1173 High Energy Micro-CT). An image was reconstructed using NRecon Reconstruction software (Bruker) which was then opened in DATAVIEWER (Bruker) in order to visualize the three separate planes of the sample. The planes were then standardized to ensure that every sample would have identical alignment when performing subsequent analysis. For linear measurements, the standardized sagittal plane was isolated. The cementoenamel junction (CEJ) midpoint between maxillary molars M1 and M2 was located using line tools within the software. From that midpoint, a perpendicular line was drawn to the alveolar bone crest (ABC), and the measurement of that line represented your linear bone loss measurement.

For volumetric measurements, a region of interest (ROI) was established in the standardized transverse plane. The top boundary was just above the roof of furcation (ROF), the bottom boundary included all roots of M2, the right boundary was just outside the most distal roots of M1, and the left boundary was just outside the most mesial roots of M3. That ROI was then saved and uploaded to CTAn Visualization software (Bruker). In order to keep the measurements standardized, we found that the bone volume results were most reproducible when the volume of interest (VOI) included approximately 1 mm below the ROF. The VOI boundaries were set with the top of the selection as the ROF and the bottom of the selection 80 layers or 1 mm below the ROF, depending on initial scan settings. A detailed ROI was then drawn by hand beginning with the lowest layer, so that the midpoints of the roots represent the connecting boundaries of your ROI. The process of drawing the ROI was repeated approximately every 5 layers. The frequency at which the ROI was redrawn will vary; the

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deeper roots will not have dramatic movement layer-to-layer, while the layers closer to the ROF will vary greatly and require more frequent redrawing of the ROI. Once the ROI is completed, the image was thresholded and a 3D analysis was performed within the software. The analysis provided tissue volume (TV), bone volume (BV), and a ratio of the two (BV/TV) that represented the percentage of total bone. All data was stored on an 8 terabyte (TB) hard drive (Backup Plus Hub, Seagate).

3.1.2 AIM 1.2: TO DETERMINE THE EFFECTS OF RVX-208 ON ALVEOLAR BONE LOSS IN AN ANIMAL PERIODONTITIS MODEL

Validating the model: A preliminary experiment was conducted in order to confirm that a ligature-induced periodontitis model would indeed produce bone loss that could imitate a periodontal disease state in male Sprague-Dawley rats. Briefly, a 3-0 silk ligature was placed around the maxillary left second molar. The accumulation of biofilm and physical irritation ultimately resulted in periodontal tissue destruction, which was quantified by our validated micro-CT protocol.

Male Sprague-Dawley rats (approximately 250-300 g, aged 9-10 weeks) were anesthetized with isoflurane. Rats undergoing suture placement were administered buprenorphine for pain management. 3-0 silk sutures were placed into the gingival sulci around the left first and second maxillary molars. Sutures were displaced apically, or replaced when necessary, into the gingival sulci weekly to ensure a subgingival position. The sutures were left in place for two weeks to induce experimental periodontitis. A total of 18 rats, with 3 rats per group, were randomized into the following groups:

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1) Ligature ligation only

2) Vehicle treatment (1% DMSO)

3) 2.5 mM RVX-208 (10% DMSO)

4) 0.5 mM RVX-208 (2% DMSO)

5) 0.1 mM RVX-208 (0.4% DMSO)

6) 0.02 mM RVX-208 (0.08% DMSO)

The drug was administered to the animal locally by gingival injection, once every other day, starting from the day of ligature ligation. There were two injection sites of 2µL, for a total volume of 4µL per animal. After 2 weeks, the animals were euthanized by CO_2 and the maxillae were harvested. The maxillae samples were placed in 10% formalin for 2 days, and then transferred to 70% ethanol before being analyzed for bone loss via the aforementioned micro-CT protocol.

3.1.3 AIM 1.3: TO PERFORM A HISTOLOGICAL ANALYSIS OF MAXILLAE SAMPLES FROM THE ANIMAL PERIODONTITIS MODEL

After micro-CT analysis, maxillae samples were decalcified in a 14% EDTA solution that was pH balanced to 7.2-7.4 with 5N NaOH. The samples were placed in the solution with a stir bar to ensure constant movement, and placed in a cold room for approximately 14 days. Once soft, the samples were rinsed four times with H_2O and placed in 30% EtOH for 30 minutes to begin the dehydration process. The samples were then placed in 50% EtOH for another 30 minutes. Finally, the samples were placed in 70% EtOH until ready for sectioning. With help from the CMMC (Cancer Mouse Models Core, Massey Cancer Center, Richmond, VA), the

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samples were embedded in paraffin blocks and sectioned in the sagittal plane from the buccal side so that the roots of M1 and M2 were visible. The sections were placed on slides and stained with Hematoxylin and Eosin (H&E). The H&E stain helped visualize tissue morphology and allowed for qualitative analysis of the samples.

3.2 SPECIFIC AIM 2: TO DETERMINE THE MOLECULAR MECHNISMS OF RVX-208 ON PREVENTING ALVEOLAR BONE LOSS IN PERIODONTAL DISEASE

Studies in Aim 2 seek to examine the genetic and epigenetic effects of RVX-208 on both murine and human cell lines, specifically in regards to its abilities to suppress inflammatory cytokine production and inhibit osteoclast differentiation. The reduction of the host immune response and inhibition of bone loss could ultimately halt periodontal disease progression. The hypothesis is that RVX-208 would suppress the inflammatory response induced by the keystone pathogen of periodontal disease, while also inhibiting osteoclast differentiation by suppressing osteoclast-associated gene expression.

3.2.1 AIM 2.1: TO DETERMINE THE EXTENT TO WHICH RVX-208 INHIBITS OSTEOCLAST GENE EXPRESSION

RAW264.7 cells were seeded in a 48-well plate with low glucose DMEM + 10% heat-inactivated fetal bovine serum (HI-FBS) + 1% penicillin and streptomycin (PS) at a density of 5000 cells/well. Cells were incubated at 37° C for 24 hours until confluent and adherent. On Day 1, the medium was changed, and cells were treated with 100 ng/mL RANKL and either 0, 5, or 10 μM RVX-208. The cells were placed back in the incubator and left undisturbed. On Day 3,

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the medium was changed and the cells were retreated. Cell supernatant was harvested on Day 5 and centrifuged (4° C, 10 minutes, 16.0 RCF). 350 µL of Lysis Buffer RLY (Bioline) was added to each well after the supernatant was collected. RNA was collected and purified per the ISOLATE II RNA Mini Kit (Bioline). The concentrations of the RNA were obtained using a NanoDrop One (Thermo Scientific). cDNA was then generated from the sample RNA using SensiFAST cDNA Synthesis Kit (Bioline). Finally, Real Time-PCR was conducted on the cDNA samples using SensiFAST Probe Lo-ROX Kit (Bioline). The 8 primers used during RT-PCR were those thought to be associated with osteoclast differentiation and included *nfatc-1, c-fos, Oscar, ctsk, trap, clcn7, ostm,* and *traf6*. QuantStudio 3 (AppliedBiosystems) was the machine utilized, and the data was analyzed in an Excel Spreadsheet.

3.2.2 AIM 2.2: TO DETERMINE THE EXTENT TO WHICH RVX-208 SUPPRESSES THE INFLAMMATORY CYTOKINE PRODUCTION WHEN CHALLENGED BY *P. gingivalis* BACTERIA

RAW264.7 cells were seeded in a 48-well plate with low glucose DMEM + 10% HI-FBS + 1% PS at a density of 50,000 cells/well. Cells were incubated at 37° C for 24 hours until confluent and adherent. On Day 1, the medium was changed and the cells were treated with either 0, 5, 10, or 30 µM RVX-208 and challenged *P. gingivalis* bacteria at an MOI of 500. The supernatant was harvested at 12 hours and centrifuged (4° C, 10 minutes, 16.0 RCF). Murine IL-6 ELISA was performed on the supernatant, and the results were normalized to DNA content.

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3.2.3 AIM 2.3: TO DETERMINE THROUGH WHICH BROMODOMAIN PROTEIN OF THE BET-PROTEIN FAMILY THE EFFECTS OF RVX-208 ARE MEDIATED

We first ensured that the effects of RVX-208 were not specific to only murine cells and that they could elicit a response in human cells as well. THP-1 cells were seeded in a 48-well plate with 1640 RPMI + 10% FBS + 1% PS at a density of 100,000 cells/well. PMA (10 ng/mL) was added to ensure cell adherence. Cells were incubated at 37° C for 24 hours until confluent and adherent. On Day 1, the medium was changed and the cells were treated with either 0, 10, 30, or 90 µM RVX-208 and challenged with *E. coli*-LPS (500 ng/mL) in one trial and with heat-killed *P. gingivalis* bacteria (MOI 500) in another. The supernatant was harvested at 12 hours and centrifuged (4° C, 10 minutes, 16.0 RCF). Human IL-6 ELISA was performed on the supernatant, and the results were normalized to DNA content.

Once THP-1 cells were determined to be viable candidates for investigating the effects of RVX-208, a knockdown cell line for BRD2 was created. Initially, 2 separate gRNAs were created using the known sequence of BRD2, and one additional gRNA was purchased (GenScript). The forward and reverse gRNA oligonucleotides were then annealed by adding 1 µL of the forward and 1 µL of the reverse oligonucleotide along with 8 µL of dH_2O and thermocycled (GenAmp PCR System 9700, Applied Biosystems) at 37° C for 30 minutes, 95° C for 5 minutes, and then ramped down to room temperature (25° C) at 5° C/minute. To digest the vector plasmid, 2 µL Tango Buffer, 1 µL BsmBI enzyme (Thermo Fisher), 1 µg lentiCRISPRv2 (Addgene) and dH_2O were combined for a total volume of 20 µL and placed in a warm water bath (37° C) for 1 hour. Finally, the annealed oligonucleotides and digested vector were ligated by adding 2.5 µL T4 DNA Ligase Buffer, 1 µL of the annealed oligonucleotides, and 1.5 µL T4

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DNA Ligase directly to the digested vector solution. For the control, 1 µL of dH_2O was substituted for the oligonucleotides. The resulting solution was placed into a warm water bath for 1 hour.

To transform the plasmids into Stbl3 bacteria, a vial of chemical competent Stbl3 cells was thawed on ice and 5 µL of plasmid DNA was carefully pipetted onto the top layer of bacteria and then incubated on ice for 30 minutes. The vials were then heat-shocked in a hot water bath (42° C) for 45 seconds, and then placed back on ice for 2 minutes. 250 µL of pre-warmed (37° C) S.O.C. Medium (New England Bio Labs) was added to each vial and the vials were shaken horizontally at 37° C for 1 hour at 225 in a shaking incubator. The vials were then removed from the incubator and 100 µL of solution from each transformation were spread on pre-warmed agar plates selective for ampicillin resistance. The agar plates were then incubated overnight at 37° C.

To isolate the plasmid DNA from the bacteria, 2 colonies from each plate were selected and inoculated in 4 mL of LB Broth (Fisher Scientific) and 4 µl ampicillin overnight. The resulting solution was then centrifuged (12,000 RCF, 4° C, 15 minutes) and the plasmid DNA was extracted and purified following the protocol for the PureLink Quick Plasmid DNA Miniprep Kit (Invitrogen). The resulting DNA was then sequenced (DNA Core, Nucleic Acids Research Facilities, Virginia Commonwealth University) and compared to the initial gRNA sequences to ensure successful transformation. The verified sequences were then transformed into Stbl3 bacteria, re-plated, and then inoculated overnight to amplify the desired DNA. The 4 mL of inoculated bacteria was then placed into 100 mL of LB Broth and allowed to inoculate overnight.

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The 100 mL of inoculated verified bacteria was then centrifuged (12,000 RCF, 4° C, 15 minutes), and the amplified plasmid DNA was extracted and purified following the protocol for the HiSpeed Plasmid Midi Kit (Qiagen). The resulting DNA was then sequenced and again compared to the initial gRNA sequences to ensure successful transformation.

To infect THP-1 cells with the verified plasmid DNA, HEK 293FT cells were first seeded in a 10-cm dish at a density of 5 x 10^6 cells in complete DMEM medium. On Day 1, old medium was removed and the 293FT cells were transfected with the target plasmid. 1 mL opti-MEM (Gibco Laboratories), 12 µg target plasmid, 6 µg pMD2G (Addgene), and 6 µg psPAX (Addgene) were combined in a 1.5 mL Eppendorf tube, while 1 mL opti-MEM and 60 µL Lipofectamine 2000 were combined in a separate tube. Both tubes sat at room temperature for 20 minutes before they were combined and added dropwise to the 10-cm dish with fresh medium. The 293FT cells were incubated at 37 C undisturbed for 48 hours. On Day 2, THP-1 cells were seeded at 5 x 10^5 cells/well in a 6-well plate with 1640 RPMI + 10% FBS + 1% PS to ensure ~50% confluency by Day 3. On Day 3, the THP-1 cells were infected with the viral supernatant. The supernatant was collected from the 293FT cells and centrifuged (1,800 RCF, 4° C, 15 minutes). The centrifuged supernatant was filtered through a 0.45 µm syringe filter into a 15 mL tube. The supernatant was then added to the wells at a ratio of 1.1 µL/1000 cells, along with polybrene (8 µg/mL) and mixed by pipetting up and down. The cells were then mixed via pipette every 2-3 hours, and allowed to culture overnight in a 37° C incubator. After culturing for 24 hours, the viral supernatant was removed and replaced with fresh medium. After 48 hours, the medium was replaced with medium selective for puromycin resistance (1 µg/mL). The puromycin-selective medium was changed every 3-5 days.

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To confirm BRD2 knockdown in the THP-1 cell line, a Western Blot was performed. First, 1 mL of Pierce RIPA Buffer (Thermo Scientific) and 10 µL of Halt Protease Inhibitor Cocktail (Thermo Scientific) were combined on ice. The THP-1 cells were centrifuged (180 RCF, 4° C, 7 minutes), the old medium was removed, and the lysis buffer was added to the pellet in an approximate ratio of 1:3. The lysis solution was then sonicated at 30% amplitude in 3 second pulses 8-10 times, and centrigued (13,000 RCF, 4° C, 10 minutes). The resulting protein was assayed using a macro BCA protein assay (Pierce) to determine protein concentration.

For the Western Blot, a Mini-PROTEAN TGX Gel (BioRad) was loaded with 10 µg of the isolated protein and run at 120 mV for 45 minutes. The gel was then transferred to an Immun-Blot PVDF Membrane (BioRad) at 300 mA for 90 minutes on ice. The resulting membrane was then blocked by placing in 5% milk and rocked for 1 hour. To attach the primary antibody, the membrane was placed in a plastic sleeve and filled with 10 mL 5% milk and 2 µL Rabbit Anti-BRD2 antibody (Bethyl Laboratories) (1:5,000 dilution). The sleeve was then sealed and placed on a rocking table in a cold room (4° C) overnight. The membrane was removed and washed in 0.1% PBST 3 times for 5 minutes each. To attach the secondary antibody, the membrane was placed in a plastic sleeve and filled with 10 mL 5% milk and 1 µL Goat Anti-Rabbit IgG (SBI System Biosciences) (1:10,000 dilution). The sleeve was sealed and rocked at room temperature for 2 hours. The membrane was then washed in 0.1% PBST as above, and allowed to soak in 2 mL SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). The prepared membrane was then imaged using blue BioExcell 5x7 Autoradiographic Film (World Wide Medical Products Inc.). All imaging was performed in a dark room.

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3.3 STATISTICAL ANALYSIS

ELISA and bone volume data were calculated in Excel spreadsheet. The results were input into GraphPad Prism 7 software to create bar graphs and histograms. Statistical analysis was done in this software using a parametric one way ANOVA ($p<0.05$) and Tukey's multiple comparisons test. P-values were indicated using asterisks as follows: * (p,0.05), ** ($p<0.01$), *** ($p<0.001$), **** ($p<0.0001$).

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CHAPTER 4: RESULTS

4.1 <u>SPECIFIC AIM 1:</u> TO FURTHER VALIDATE THE *IN VIVO* EFFECTS OF RVX-208 ON A LIGATURE-INDUCED PERIODONTITIS MODEL IN RATS

After establishing and validating a reliable and repeatable micro-CT protocol, samples from the ligature-induced periodontitis model were analyzed using the micro-CT protocol and other statistical regressions.

4.1.1 AIM 1.1: TO VALIDATE THE MICRO-CT PROTOCOL FOR ANALYZING LINEAR AND VOLUMETRIC DATA FROM THE PERIODONTITIS MODEL IN RATS

To ensure accurate quantitative measurements of samples from the ligature-induced periodontitis animal model, the protocol we created to measure the bone volume of the samples needed to be stream lined and verified by multiple trials. Previously, our group performed a ligature-induced periodontitis model and ligatures were ligated around the left maxillary M2 of three rats. The alveolar bone loss was compared to the right maxillary molars of the same animals, as well as the left maxillary molars of three rats without ligature ligation. Statistical analyses (Fig. 6) of volumetric analysis data showed that the right side of the experimental animal was highly similar to control group. Additionally, there was no significant difference between different analyses of the same sample using the volumetric protocol when

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repeated by two separate researchers. These findings suggest that our protocol is repeatable and reliable.

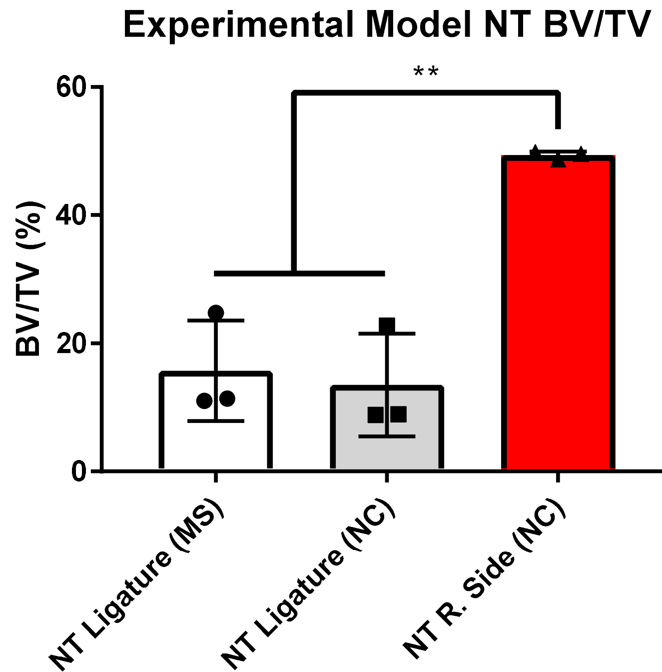

Figure 6: Bone volume of maxillae samples from the experimental model – the left side of the maxilla that received the ligature had significantly less bone volume than the right side of the maxilla of the same animal. Two separate researchers NC (Nicholas Clayton) and MS (Mingxu Sun) verified the measurements of the left side no treatment group following the developed protocol and a similar trend was observed in their analyses.

4.1.2 AIM 1.2: TO DETERMINE THE EFFECTS OF RVX-208 ON ALVEOLAR BONE LOSS IN AN ANIMAL PERIODONTITIS MODEL

To begin *in vivo* testing, we first had to verify that the experimental periodontitis model could reliably induce bone loss similar to what you would expect to find in patients suffering from periodontal disease. In our first experimental trial, ligature placement induced significant alveolar bone loss in the animals both in linear (Fig. 8) (ligature: $p<0.001$) and volumetric measurements (Fig. 10) (ligature to control: $p<0.0001$, ligature to R.side: $p<0.001$). Once the

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experimental model was verified, the model was replicated and rats were injected with varying concentrations of RVX-208 (0-2.5 mM) for two weeks. The left maxillae were harvested, and the samples were analyzed for linear (Fig. 7) and volumetric (Fig. 9) bone loss using the validated MicroCT protocol. Linear bone loss was significantly reduced both between M1 and M2 as well as between M2 and M3 for medium to high doses (0.5-2.5 mM, $p<0.01$) (Fig. 11). A dose-dependent response was observed in volumetric measurements, as BV/TV was positively correlated with RVX-208 dosages, although it did not reach statistical significance due to the small sample size (n=3) (Fig. 12). The decrease in linear and volumetric bone loss indicates that RVX-208 can ameliorate bone loss in patients suffering from periodontal disease.

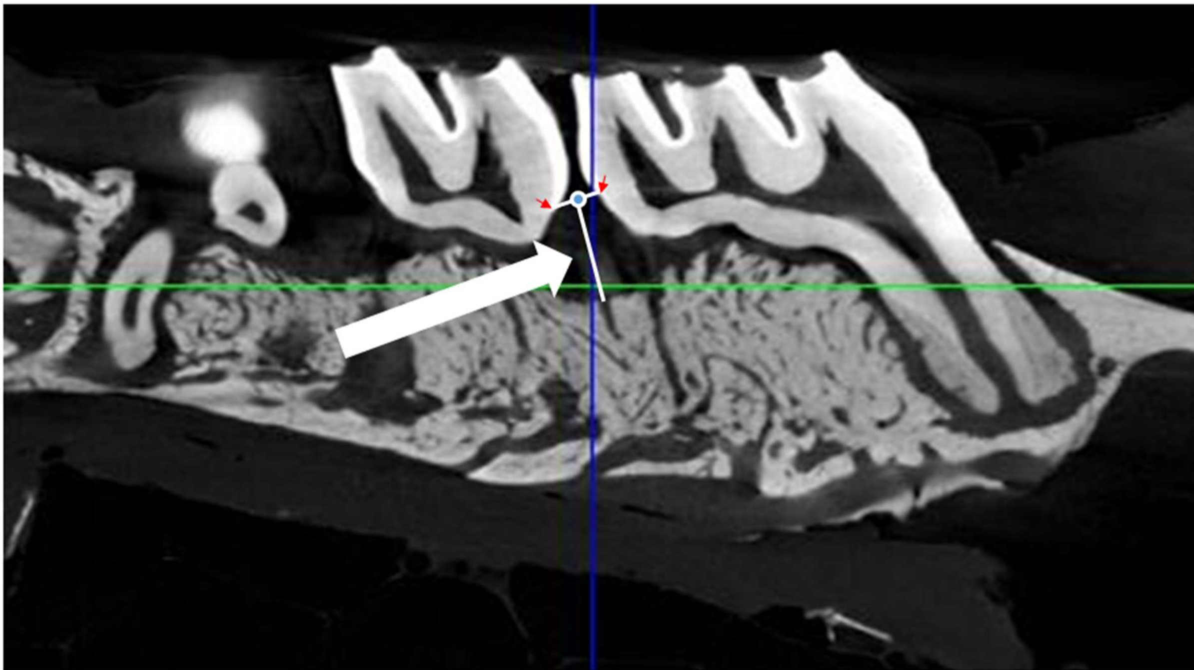

Figure 7: Linear measurement of alveolar bone crest (ABC) and cementoenamel junction (CEJ) – micro-CT scans were analyzed in the sagittal plane to find the midpoint (blue dot) between the CEJs (red arrows) of M1 and M2. The distance measured was from the midpoint to the ABC, denoted by the white arrow.

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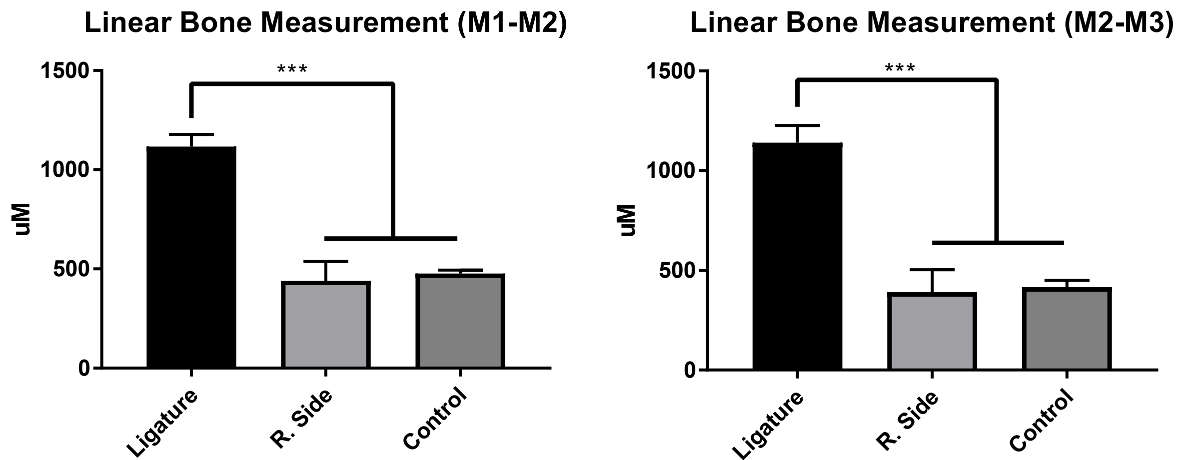

Figure 8: Linear bone measurement of ligature model validation experiment – Measurements were made between the cementoenamal junction and alveolar bone crest. The linear measurements of the ligature samples were significantly larger than both the right side of the same animal and the control group, denoting greater bone loss (*: p<0.001).**

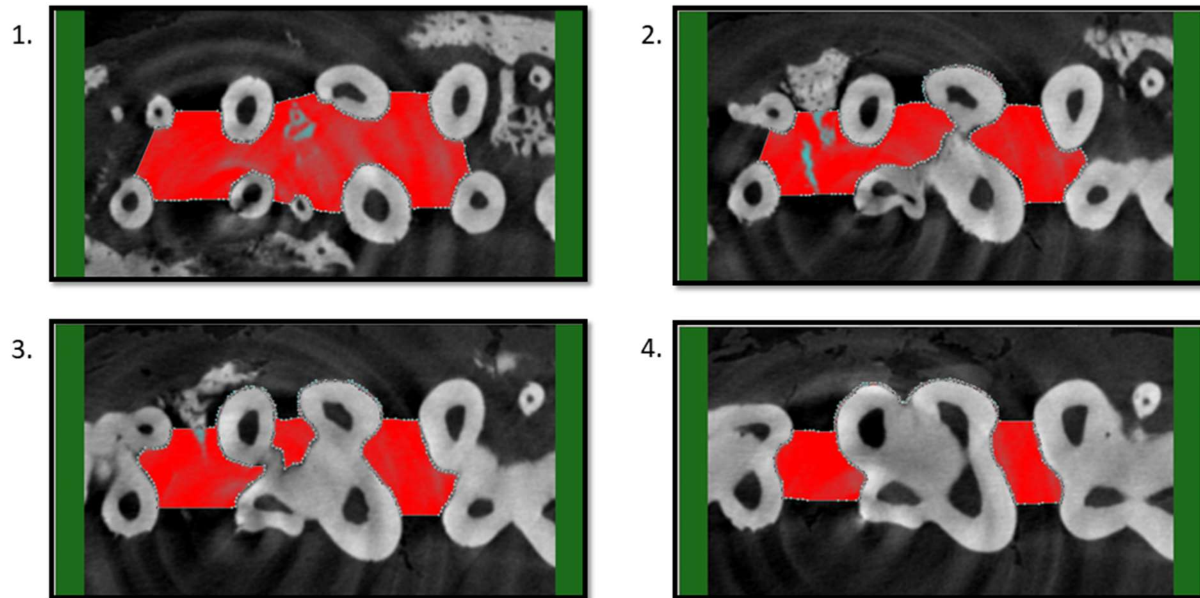

Figure 9: Volumetric analysis – micro-CT scans were isolated for a region of interest in the transversal plane with the maxillary second molar M2 as the center, the most mesial root of M3 as the left boundary, and the most distal root of M1 as the right boundary. 1.) A volume of interest (VOI, shown in red) was established beginning 80 layers or 1 mm below the roof of furcation, and included non-tooth tissue encompassed by the midpoints of each root. 2-3.) The VOI was redrawn every 4-5 layers to account for root migration 4.) The roof of furcation represents the top-most boundary of the VOI.

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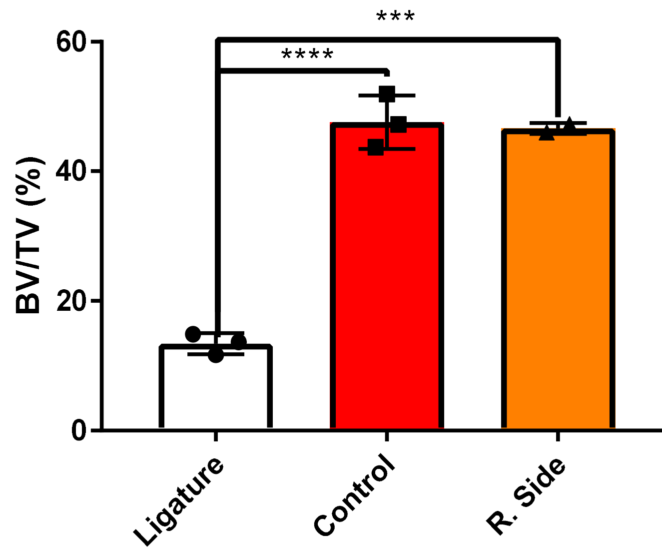

Figure 10: Bone volume of maxillae samples from the ligature-induced experimental periodontitis model – the ligature group showed a significant loss of bone volume when compared to the right side of the same animal and the control group (*: p<0.001, ****: p<0.0001). There was no difference between the right side of the experimental animal and the control group.**

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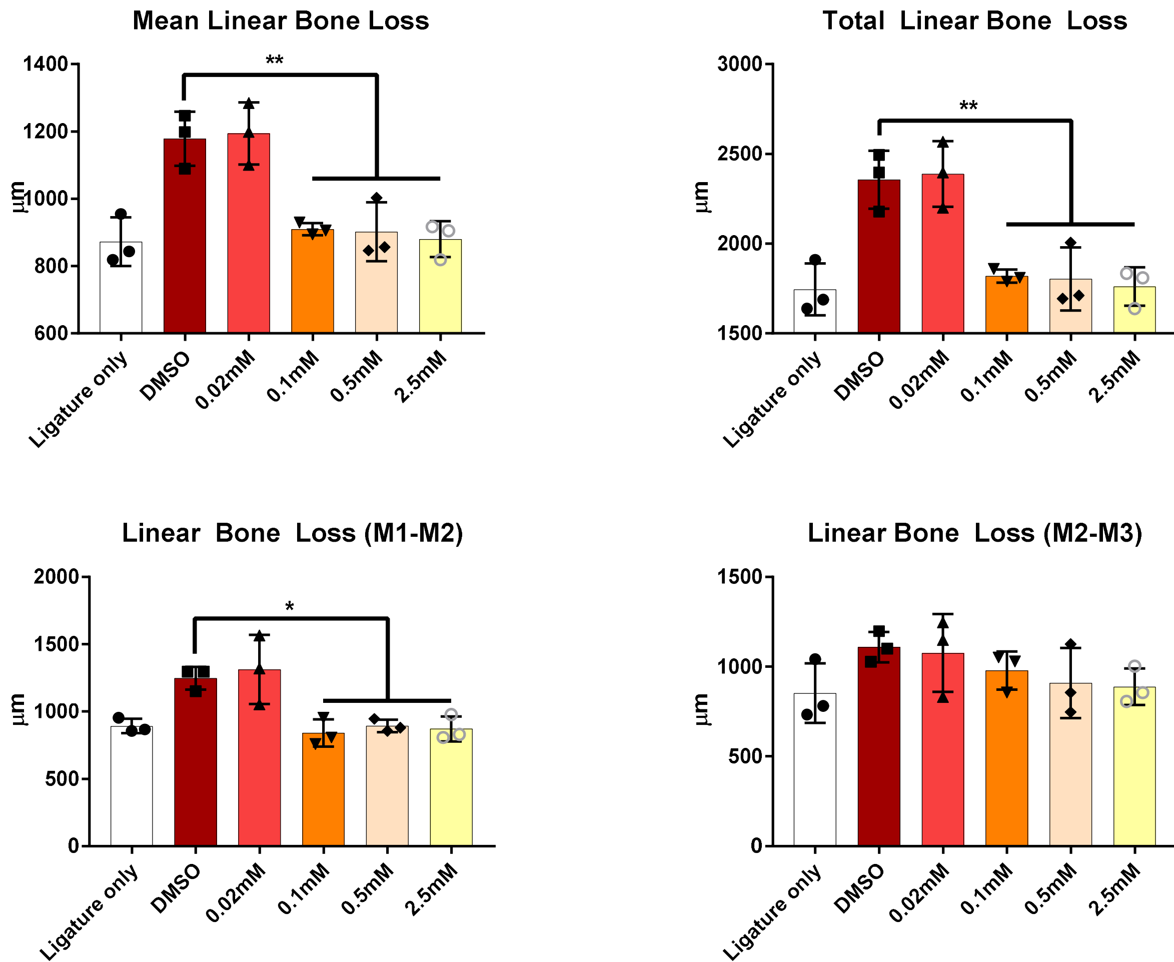

Figure 11: Linear measurements of samples from the ligature-induced periodontitis model – Measurements were made between the cementoenamel junction and the alveolar bone crest. Mean linear bone loss was calculated by averaging the linear measurements of M1-M2 and M2-M3. Total linear bone loss was calculated by adding the total measurements of M1-M2 and M2-M3. There was significantly less bone loss when RVX-208 was administered at medium to high doses (0.1 mM-2.5 mM: $p<0.01$).

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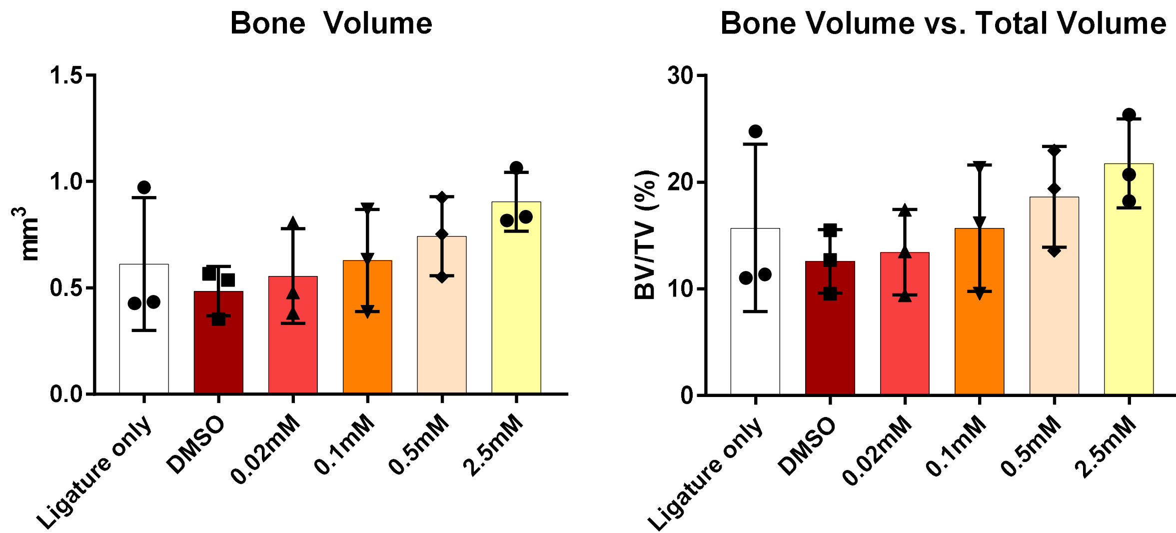

Figure 12: Bone volume measurements of samples from the ligature-induced periodontitis model. The slight upward trend indicates a positive correlation between an increased dose of RVX-208 and an increase in retained bone volume.

4.1.3 AIM 1.3: TO PERFORM A HISTOLOGICAL ANALYSIS OF MAXILLAE SAMPLES FROM THE ANIMAL PERIODONTITIS MODEL

Aside from quantitative analyses of the ligature-induced periodontitis model, histological analysis can provide insight as to what effects RVX-208 may have on a tissue morphology. Harvested maxillae samples were decalcified in EDTA, embedded in paraffin, and sectioned serially. The sections were then stained with H&E to highlight the morphology of the periodontal tissue between M1 and M2. As shown in Fig. 13, the distance between the alveolar bone crest and cementoenamel junction is dramatically decreased in the higher doses of RVX-208, while also appearing to have a slight effect on decreasing bone volume.

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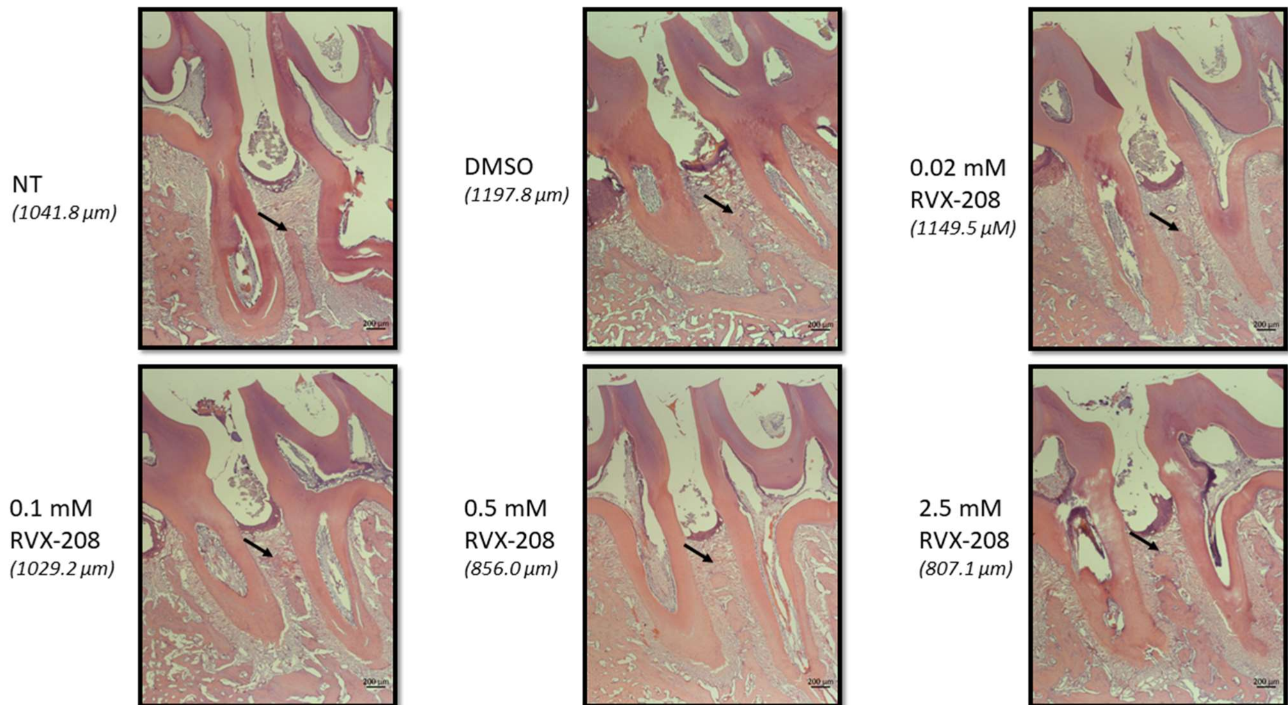

Figure 13: H&E stained histology sections from a ligature-induced periodontitis model. The distance between the CEJ and the ABC is in italics. The ABC is marked by the black arrow. Qualitatively, the most profound difference can be seen between the DMSO group and the higher dosages of RVX-208 (0.5 mM and 2.5 mM).

4.2 SPECIFIC AIM 2: TO DETERMINE THE MOLECULAR MECHNISMS OF RVX-208 ON PREVENTING ALVEOLAR BONE LOSS IN PERIODONTAL DISEASE

Cell culture assays were performed to determine the mechanisms through which RVX-208 suppresses osteoclast differentiation, its ability to suppress the inflammatory response initiated by whole pathogens, and to determine if its effects were species-specific. Finally, a knock down human cell line was generated and confirmed for future studies

4.2.1 AIM 2.1: TO DETERMINE THE EXTENT TO WHICH RVX-208 INHIBITS OSTEOCLAST GENE EXPRESSION

RAW264.7 cells were induced into osteoclasts by RANKL and then treated with RVX-208 (0-10 µM). cDNA was generated from the extracted RNA and RT-PCR was run to determine

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RVX-208's ability to suppress the expression of genes associated with osteoclast differentiation. When RVX-208 was added, the expression of genes most closely associated with osteoclast differentiation were significantly suppressed(*trap*: $p<0.0001$, *oscar*: $p<0.0001$, *ctsk*: $p<0.0001$, *nfatc-1* 5 µM RVX-208: $p<0.001$, and *clcn7* 5 µM RVX-208: $p<0.01$, 10 µM RVX-208: $p<0.001$) (Fig. 14) at both doses (5 and 10 µM). *c-fos, traf-6,* and *ostm-1* were unaffected by RVX-208.

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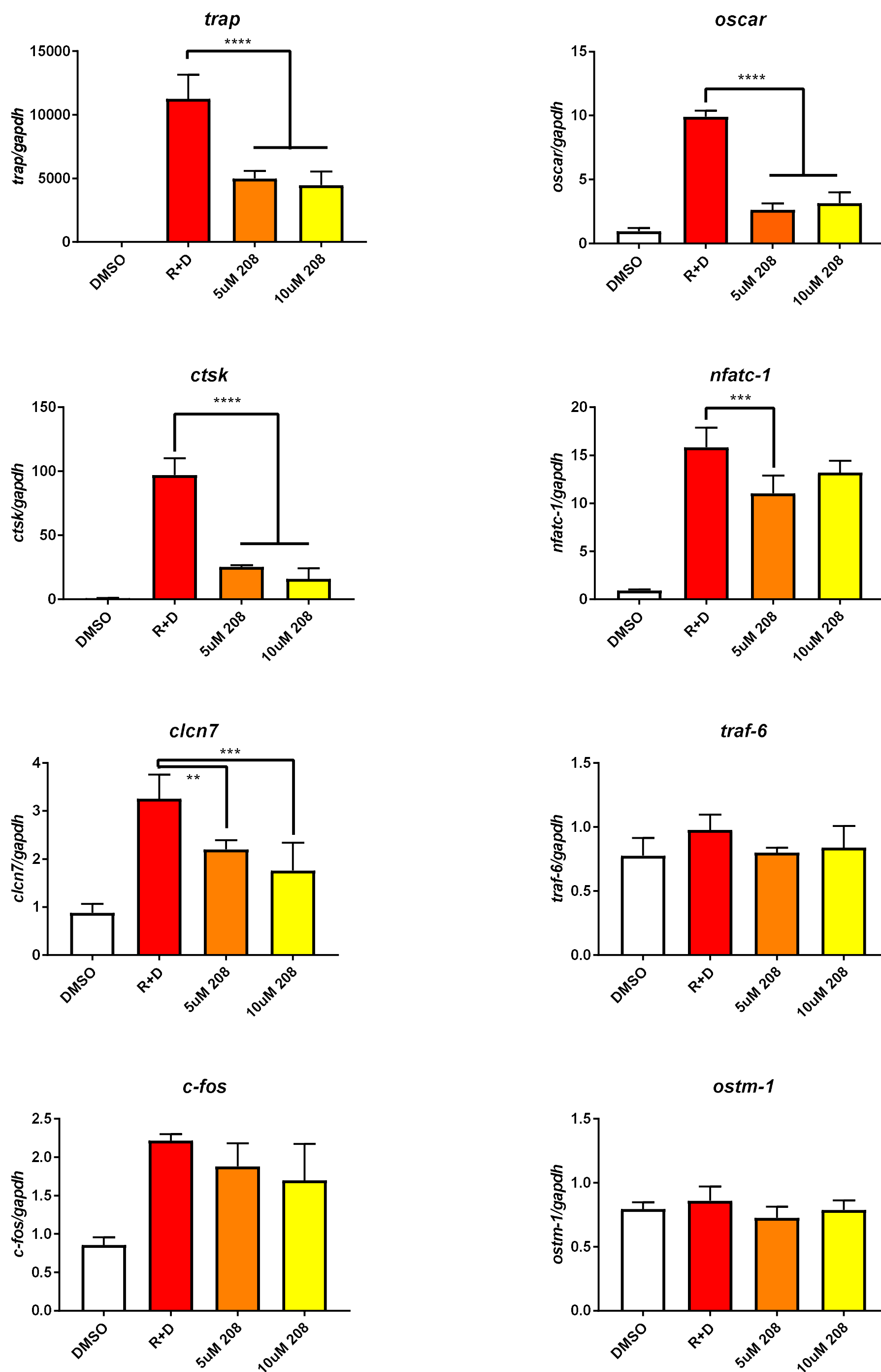

Figure 14: RT-PCR of relative gene expression of murine osteoclasts treated with RVX-208 – RAW264.7 cells were seeded at a density of 5000 cells/well in a 48-well plate. Cells were treated with RANKL to induce differentiation into osteoclasts and then treated with varying doses of RVX-208. After 7 days, the cells were harvested and cDNA was synthesized from harvested RNA. RT-PCR was run to examine a panel of genes associated with osteoclast differentiation. The expression of *trap, oscar, ctsk, nfatc-1,* and *clcn7* genes were all significantly suppressed (: p<0.01, ***: p<0.001, ****: p<0.0001) when treated with RVX-208. The expression of *traf-6, c-fos,* and *ostm-1* genes were not affected significantly.**

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4.2.2 AIM 2.2: TO DETERMINE THE EXTENT TO WHICH RVX-208 SUPPRESSES THE INFLAMMATORY CYTOKINE PRODUCTION WHEN CHALLENGED BY *P. GINGIVALIS* BACTERIA

Recent studies have suggested that the mechanism through which *P. gingivalis* bacteria activates the host immune response does not rely solely on its LPS, as other virulence factors may also be involved including bacterial DNA. Because our preliminary studies utilized LPS exclusively, we needed to verify that RVX-208 could also mediate the potentially varied immune response activation mechanisms utilized by *P. gingivalis* bacteria in order to justify clinical applicability. Murine macrophages were challenged with killed *P. gingivalis* bacteria and treated with increasing concentrations of RVX-208 (0, 5, 10, and 30 μM) for 12 hours, and IL-6 ELISA was performed to determine the effect on inflammatory response through cytokine production. As shown in Fig. 15, RVX-208 was able to significantly reduce the IL-6 production of cells challenged with *P. gingivalis* bacteria (*: $p<0.5$, ****: $p<0.0001$). These findings are in concordance with our findings for the effects of RVX-208 on *P. gingivalis*-LPS, and suggest RVX-208 can suppress the inflammatory response regardless of the initiating factor.

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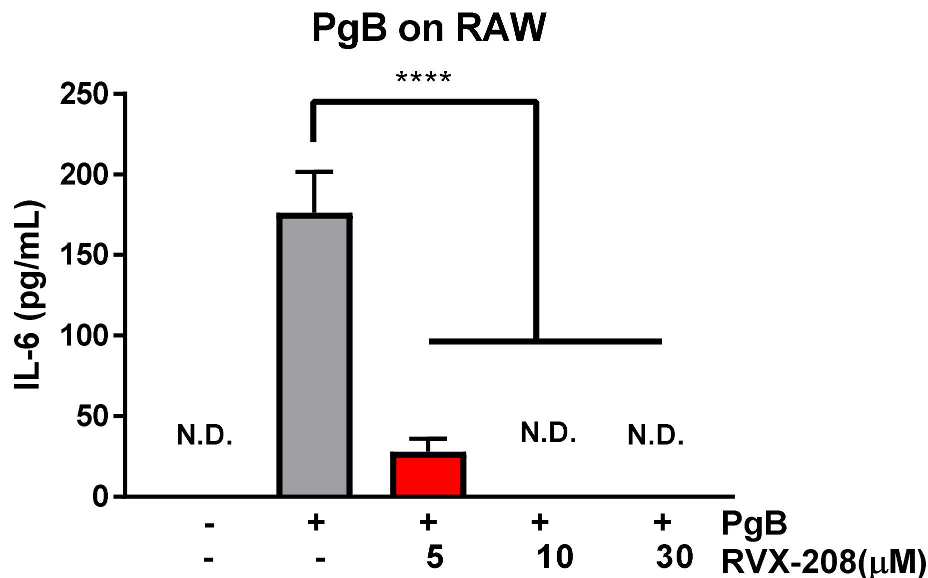

Figure 15: ELISA IL-6 Results – RAW264.7 cells were seeded at a density of 50,000 cells/well in a 48-well plate. The cells were challenged with *P. gingivalis* bacteria at an MOI of 500 and then treated with varying concentrations of RVX-208 for 12 hours. The assay shows a significant suppression of cytokine expression (**: $p<0.0001$). N.D. = non-detectable: the IL-6 kit utilized was not able to detect the lowest concentrations of IL-6 produced in our trials.**

4.2.3 AIM 2.3: TO DETERMINE THROUGH WHICH BROMODOMAIN PROTEIN OF THE BET-PROTEIN FAMILY THE EFFECTS OF RVX-208 ARE MEDIATED

Before investigating the roles of specific bromodomain proteins, we needed to verify that the effects of RVX-208 were not species specific. Human clinical trials are essential to the development of RVX-208 as a treatment option for periodontal disease, thus, the effects of RVX-208 need to be established in a human cell line. To test this, THP-1 cells were challenged with *P. gingivalis* bacteria and then treated with RVX-208 for 12 hours, and IL-6 ELISA was performed to determine the effect on inflammatory response through cytokine production. As a positive control, THP-1 cells were challenged with *E. coli*-LPS and treated with RVX-208 as before. As shown in Fig. 16, not only were *E. coli*-LPS and *P. gingivalis* bacteria able to stimulate the immune response of THP-1 cells, but RVX-208 was able to combat that immune response by

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significantly suppressing the production of inflammatory cytokines (****: p<0.0001). These findings confirm that the effects of RVX-208 are not species specific in regards to eliciting a response in both human and mouse cell lines, and that THP-1 cells are a viable cell line to examine the effects of specific bromodomain proteins.

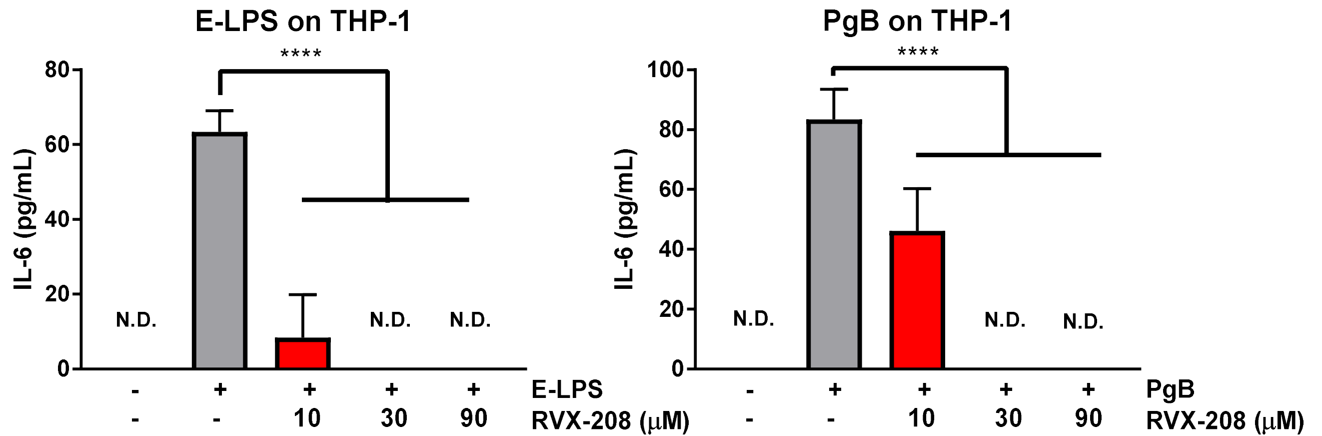

Figure 16: ELISA IL-6 Results – THP-1 cells were seeded at a density of 100,000 cells/well in a 48-well plate. The cells were challenged with *E. coli*-LPS (*left*) or *P. gingivalis* bacteria (*right*) and then treated with increasing concentrations of RVX-208. Both trials demonstrate a significant suppression of IL-6 production when treated with RVX-208 (**: p<0.0001). N.D. = non-detectable: the IL-6 kit utilized was not able to detect the lowest concentrations of IL-6 produced in our trials.**

Once a viable human cell line was established, the first step in isolating the effects of each bromodomain protein is to generate a cell line in which the protein of interest is knocked down via a CRISPR/Cas9 system. Since it has been shown that RVX-208 has a higher binding affinity to the BD2 domain of BRD2 when compared to other BRD proteins, we asked the question if the effects of RVX-208 on periodontal inflammation was directly associated with BRD2. In order to investigate this, we designed two separate guide RNAs representing different target regions of the genomic indels of BRD2, while a third guide RNA was purchased. Fig. 17 shows the sequence of BRD2 and where the gRNA were designed to produce their respective indels. Those gRNAs were ligated into a plasmid vector, and that plasmid was transfected into

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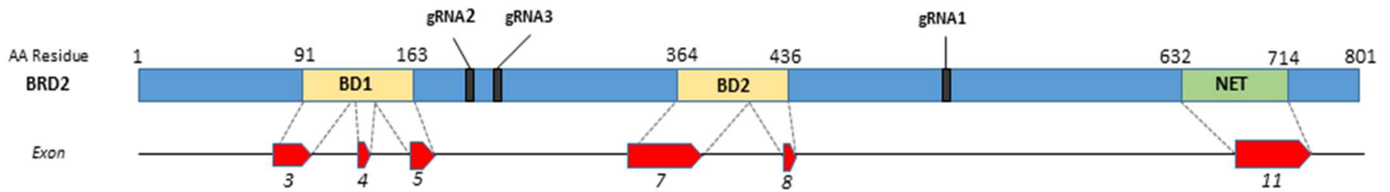
chemically-competent *Stbl3* bacteria to amplify the plasmid DNA. After a miniprep, the isolated plasmid DNA was sent to be sequenced to confirm the presence of our gRNA. The validated sequences were re-transfected into the bacteria, and a midiprep was performed to further amplify the plasmid DNA. The verified plasmid DNA was then transduced into HEK293FT cells, which produced a viral supernatant containing our vectors. The viral supernatant was used to infect THP-1 cells, which were then selected with puromycin. Those cells were grown in medium until stable, and a Western Blot was performed to examine the extent of BRD2 knockdown. Western Blot results (Fig. 18) demonstrate a significant knockdown effect observed in all three gRNAs, the most dramatic of which can be seen in gRNA2-2. These findings demonstrate a successful knockdown of BRD2 protein function, and can be used in later experiments to investigate the role of BRD2 in mediating the anti-inflammatory effects of



RVX-208 in periodontal disease.

Figure 17: Sequence layout of BRD2 and indel locations of designed guide RNAs (gRNA) – each gRNA was designed directly preceding a PAM sequence to ensure proper CRISPR function. gRNA 1 was purchased, while gRNAs 2 and 3 were designed using the described genomic sequence of BRD2.

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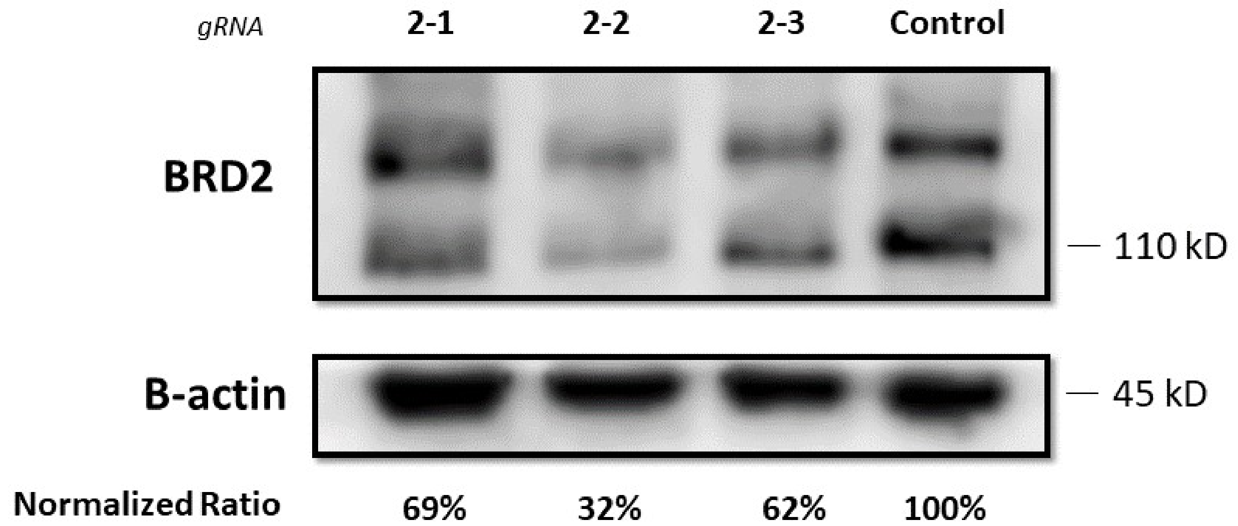

Figure 18: Western blot panel of BRD2 knockdown in THP-1 cells – 10 µg of cell lysate was loaded onto a gel and run to examine the efficacy of plasmid transfection into THP-1 cells. The weaker signals of the constructed gRNAs compared to the control indicates some degree of knockdown in our THP-1 cell lines. The relative normalized ratio was calculated by normalizing the experimental bands to β-actin, which was then normalized to the control group Guide RNA (gRNA) sequence locations are detailed in Fig. 17.

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CHAPTER 5: DISCUSSION

The findings presented in this work showcase the ability of the selective bromodomain and extraterminal domain (BET) protein inhibitor, RVX-208, to ameliorate bone loss in a ligature-induced experimental periodontitis model in rodents, while also detailing optimized methods to quantify the results of the model. RVX-208 was able to significantly suppress the expression of genes directly associated with osteoclast differentiation and maturation in murine RAW264.7 cells, and was shown to dampen the inflammatory response initiated by both *E. coli*-LPS and *P. gingivalis* bacteria in human THP-1 cells. Finally, this study also details the successful generation of a BRD2-knockdown THP-1 cell line that can be utilized to investigate the roles that the specific bromodomain protein BRD2 plays in the mediation of the effects of RVX-208.

In Aim 1, we began by validating the micro-CT protocol we had devised given the objectives of our study. We performed a small scale animal study in which we placed ligatures on one group of rodents, and left the others as a control. After analyses by two different researchers (MS and NC), we found that not only did our ligature model significantly induce bone loss, but that our micro-CT protocol for quantifying the data was reliable and reproducible based on the high consistency between separate analyses. Once the model was established and verified, our *in vivo* periodontitis experiment demonstrated RVX-208's ability to significantly reduce the bone loss seen in our control groups. Linear measurements showed

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that the amount of bone loss decreased as the dose of RVX-208 increased (up to 2.5 mM), while the volumetric measurements suggested a similar relationship without being statistically significant. It is our belief that had the sample size been greater than 3 rodents per group, the volumetric data would have shown a significant correlation between a decrease in bone loss and an increase in the dosage of RVX-208, based on the obvious trend in Fig. 5. Our study provided valuable preliminary data enabling us to perform powerful analysis for our future experiments. Interestingly, we saw more pronounced bone loss on average in the periodontal space between M1 and M2 when compared with the area between M2 and M3. To further verify our findings, histological sections were created from the maxillae samples harvested from the periodontitis model. Qualitatively, the slides stained with H&E demonstrated a clear morphological difference in the periodontal tissue between samples that had been treated with higher doses of RVX-208 and samples that had not been treated with RVX-208. In the future, those histological slides can be used to quantify the effects of RVX-208 *in vivo* through the analysis of inflammatory cell infiltrate in conjunction with a TRAP stain to examine osteoclast activity.

In Aim 2, we explored some of the mechanisms through which RVX-208 is able to mediate the effects seen in Aim 1. We showed that RVX-208 is able to suppress or halt bone loss altogether through the suppression of genes closely associated with the differentiation and maturation of osteoclasts. The genes *trap, oscar, ctsk, nfatc-1,* and *clcn7* were all shown suppressed to a highly significant degree. The suppression of *oscar*, a gene that codes for the osteoclast-associated Ig-like receptor, severely impedes an osteoclast-precursor's ability to differentiate into an osteoclast, ultimately reducing the overall presence of mature osteoclasts.

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Additionally, the suppression of *ctsk*, which codes for cathepsin K, a protease involved in bone remodeling and resorption, and *clcn7*, which codes for an important H+/Cl- exchange transporter, all retard the cell's ability to mature into a fully functional osteoclast, decreasing the effectiveness of the few precursors that are able to differentiate. Finally, RVX-208 had no effect on the expression of *traf6*, the gene that codes for TNF-associated receptors as well as some Toll-like receptors, demonstrating that RVX-208 does not interfere with the upstream cascade of the NF-κβ pathway, but rather suppresses the inflammatory cytokine gene expression directly through its inhibition of BRD proteins.

Recent evidence has suggested that whole pathogens may induce immune responses varying from those induced by lipopolysaccharides (), so we wanted to ensure that the effects of RVX-208 were universal, regardless of the initiating mechanism. We demonstrated that RVX-208 could significantly reduce the inflammatory response initiated by *P. gingivalis* bacteria, which was in agreement with our previous findings on the suppressive effects of RVX-208 on cells challenged with *P. gingivalis*-LPS. This has direct implications in the clinical applicability of RVX-208 as a therapeutic drug for periodontal disease, as we've shown that RVX-208 consistently suppresses the inflammatory cascade regardless of the mediator, and is especially effective against the clinical keystone pathogen of periodontal disease, *P. gingivalis*.

We also demonstrated that the effects of RVX-208 are not species specific. THP-1 cells, a human monocytic cell line derived from an acute monocytic leukemia patient, are commonly utilized in studies investigating oral pathogenesis. We demonstrated that RVX-208 could suppress the inflammatory response initiated by both *E. coli*-LPS and *P. gingivalis* bacteria in THP-1 cells. By establishing a viable human cell line that responds positively to the anti-

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inflammatory effects of RVX-208, further studies on RVX-208 can be directly applied to humans. Additionally, this allowed us to begin the generation of a knockdown cell line to examine the effects of RVX-208 on specific members of the BET protein family. Of the three BET proteins, RVX-208 has shown to be highly selective towards BD2 of BRD2 (). Cheng, et al. examined the mechanics of a novel orally active BETi with selectivity for BD2, RVX-297, and found that RVX-297 exhibited a higher inhibitory activity to BD2 as compared to RVX-208. Because RVX-297 has been shown to reduce inflammatory cytokines, reduce inflammatory gene expression, counter the pathology of inflammatory diseases like polyarthritis, and even prevent disease development in models of human multiple sclerosis in rats (77), the elucidation of the molecular mechanisms responsible for the selectivity of selective BET inhibitors, like RVX-208 and RVX-297, is a key step in advancing BET-inhibitor design for anti-inflammatory and autoimmune therapeutics. As such, we designed a CRISPR/Cas9 system to knock down the BRD2 protein in THP-1 cells, and verified the knock down via Western Blot. The Western Blot confirmed a successful knock down of BRD2 for at least one of our designed gRNAs. This cell line can be used in the future to investigate whether RVX-208 can still suppress the inflammatory response without the presence of BRD2.

While RVX-208 is the first and only BET inhibitor to reach Phase III clinical trials, a few lingering issues regarding the widespread use of BET inhibitors in a clinical setting still remain. Studies have indicated that BET protein BDs function cooperatively (35). How BD1 is impacted, either directly or indirectly, through BD2-selective inhibition requires further investigation. Additionally, because BET inhibitors simultaneously downregulate multiple inflammatory processes in contrast to targeting single entities in inflammatory pathways per traditional

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treatment methods, adverse effects on systemic suppression of inflammatory cascades could be potentially detrimental, highlighting the need for tissue-specific targeting. One problem that still persists is the method through which RVX-208 can be applied orally. Due to its short half-life, a method needs to be developed in which the lifespan of the drug is sustained while also delivering an efficacious dose. Additionally, suppression of the host immune response by RVX-208 raises concerns about overall immune susceptibility. While methods for measuring the systemic immunity remain inexact, one method for determining the ability of RVX-208 to impair the body's ability to fight off infection could be through utilization of a salivary swab and then subsequent next-gen DNA sequencing for bacterial composition done before and after treatment with RVX-208. If RVX-208 had a negative effect on the body's immune system, then that pathogen oral biofilm would theoretically be different than from that of a healthy immune system. While more studies are still needed to evaluate the efficacy of RVX-208 as a treatment for periodontitis, past results on selective BET inhibitors in conjunction with the findings detailed in this study for its direct application are highly promising.

In future studies, the effects of RVX-208 should be examined *in vivo* in larger animals, and with a larger sample size. The significance of our volumetric data was lacking due to our small sample size, yet a dose dependent effect was noticed. Using the BRD2 knock down cell line generated in this study, the interplay between BRD2 and RVX-208 should be elucidated. Additionally, knock down cell lines should be generated for the remaining two BRD proteins, BRD3 and BRD4. Finally, a mode of application needs to be investigated, as subgingival

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injections twice a day are not feasible for human patients.

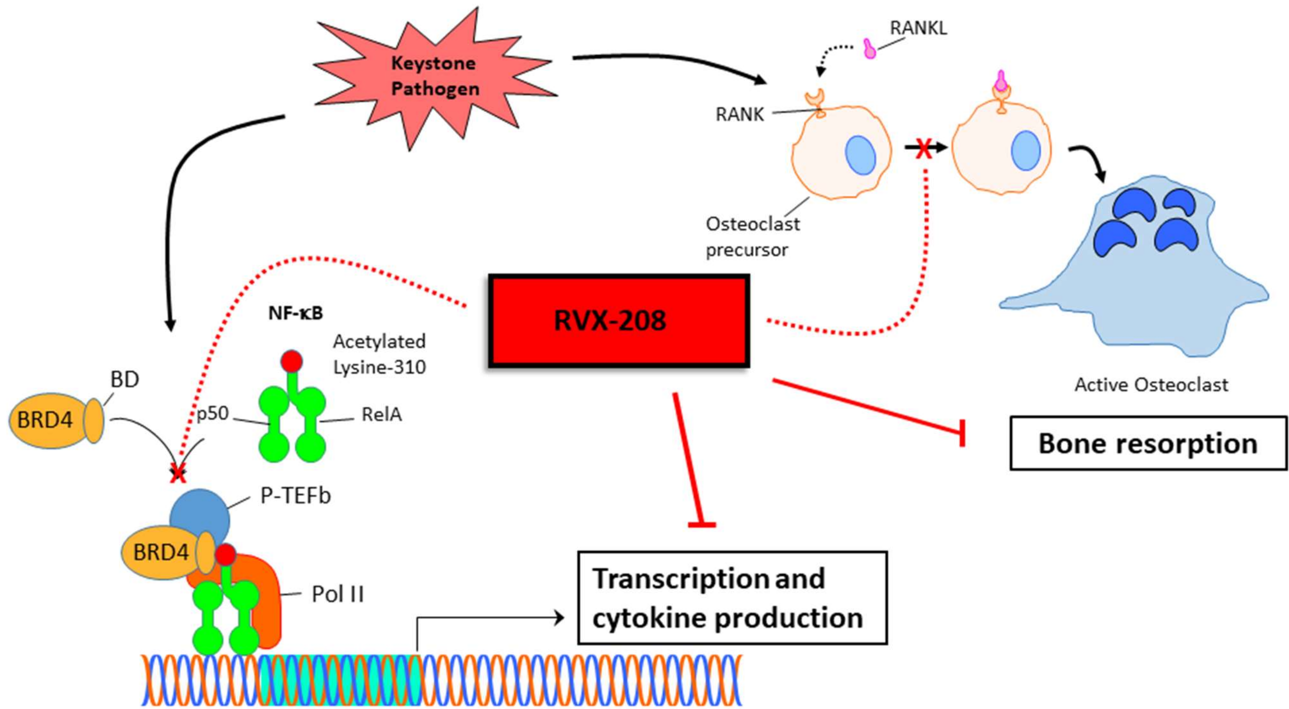

Figure 19: Summary of the effects of RVX-208 in the host immune response initiated by a keystone pathogen of Periodontitis. By suppressing the genes associated with osteoclastogenesis and by preventing the recruitment of transcription factors, RVX-208 can suppress the inflammatory response and inhibit bone resorption.

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CHAPTER 6: CONCLUSION

Periodontal disease therapies currently focus on mechanical debridement, which does little to combat the over-exuberant host immune response that is ultimately responsible for periodontal tissue degradation. With these therapies, bacterial reinfection is common, and antibiotic resistance is a growing concern. The development of host modulation therapies are an unmet need in the treatment of periodontal disease. One such host response modulator, BET-inhibitor RVX-208, may provide an alternative method through which to control the host immune response, suppress inflammation, and decrease the bone loss that is characteristic of periodontal disease.

Our study sought to observe the effects of RVX-208 on reducing inflammation and ameliorating bone loss in a ligature-induced model of periodontitis. As the exact mechanism of RVX-208 is relatively unknown, we also sought to examine the mechanisms through which RVX-208 mediates its effects. RVX-208 significantly decreased bone loss in a ligature induced periodontitis model. Qualitatively, the linear distance between the ABC and the CEJ was reduced as the dosage of RVX-208 increased. We showed that RVX-208 can stifle bone loss through suppression of genes closely associated with the differentiation and maturation of osteoclasts such as *trap, oscar, ctsk,* and *clcn7*. Finally, we demonstrated that the effects of RVX-208 are not species specific, and can successfully combat the host immune responses initiated by both *P. gingivalis*-LPS and whole pathogen bacteria in humans. Larger sample sizes

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should be utilized in future *in vivo* studies, while also testing alternative drug delivery techniques. Overall, we can conclude that treatment with RVX-208 can ameliorate bone loss in a ligature-induced periodontitis model through directly suppressing the expression of osteoclast genes, while also suppressing the inflammatory response initiated by multiple pathogens. Thus, RVX-208 remains at the forefront of promising host-modulation therapies for combating periodontal disease.

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VITA

Nicholas Clayton was born in Columbus, Ohio on February 7, 1992. He graduated from Heritage High School in Leesburg, VA in 2010, and matriculated from Pepperdine University in Malibu, CA in 2014 with a B. Sc. in Sports Medicine. Nicholas began the Premedical Graduate Health Sciences Certificate Program at Virginia Commonwealth University in 2016. Through this program, he started research for his Masters in Physiology and Biophysics degree, with an anticipated matriculation date of April 2018.

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