Sodium Butyrate and Calcium Phosphate Influence on Transformed Human Mast Cells

by

Nishita Hiresha Verma

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Department of Chemical and Materials Engineering

University of Alberta

Abstract:

Mast cells (MCs) are crucial components of the innate immune system, playing a significant role in immune responses to allergens and pathogens. These sentinel cells are primarily located at interfaces between the external environment and the body and can be categorized into connective tissue MCs and mucosal MCs. MRGPRX2 is a G protein-coupled receptor that plays a key role in MC activation, particularly in response to certain drugs, peptides, and endogenous compounds. Recent studies have shown that MRGPRX2 can mediate pseudo-allergic reactions and contribute to MC-related diseases. Sodium Butyrate (NaBu) is a histone deacetylase inhibitor that exhibits anti-inflammatory properties by altering gene expression and promoting regulatory T-cell differentiation. Calcium phosphate ($Ca_3(PO_4)_2$) has been shown to be vital to calcium signaling and immune response modulation. It is thought that MRGPRX2 receptors are modulated in the presence of these compounds because of their known roles in immune regulation. We hypothesize that the introduction of these two compounds will modulate MRGPRX2-mediated activation in HMC-1.2 cells. The HMC-1.2 cell line was used since these cells have MRGPRX2 receptors. This hypothesis was tested by introducing HMC-1.2 cells to these compounds in a dose-dependent manner and characterizing cell viability, proliferation, and degranulation. It was found that NaBu did not affect MRGPRX2-mediated degranulation or cell viability but inhibited cell proliferation, suggesting selective modulation of immune responses without compromising essential MC functions. Moreover, $Ca_3(PO_4)_2$ did not significantly impact MRGPRX2-mediated degranulation but did exhibit a dose-dependent inhibition of cell proliferation, suggesting an influence on cellular growth without MRGPRX2 response. Our results did not confirm our hypothesis, as they did not lead to MRGPRX2-mediated responses in HMC-1.2 cells. That said, these findings did show an inhibition of cell proliferation that may be applicable to managing inflammatory and allergic diseases. Further research is recommended to explore the precise pathways and conditions under which these

compounds can modulate immune cell function, particularly focusing on different immune cell types and receptor-specific pathways.

Keywords: Mast Cells, MRGPRX2 Activation, Sodium Butyrate, Calcium Phosphate, Immune Response, Modulation

Preface:

This thesis is an original work by Nishita Hiresha Verma. I was responsible for the data collection, data analysis, and manuscript writing.

Part of Chapter 2 of this thesis has been submitted to be published as “Butyrate increases glycosaminoglycan synthesis and storage in human mast cells,” *Cells* 2024*, 13;* Syed Benazir Alam, Zhimin Yan, Nishita Hiresha Verma, Larry D. Unsworth, and Marianna Kulka.

A part of the research in Chapter 2 of this thesis has also been shared at the following scientific conferences:

- Oral and poster presentation: "Identification and *In-Vitro* Evaluation of Peptide Inhibitors of Mast Cell Receptor MRGPRX2," Deep Tech in Health + Medical Innovation, October 16-17, 2023, Edmonton, Alberta.
- Poster presentation: "Sodium butyrate modulates human mast cell activation through MRGPRX2," ImmuNet Research Day, June 23, 2023, Edmonton, Alberta.

Dr. Larry D. Unsworth and Dr. Marianna Kulka were the supervisory authors and were involved with concept formation and manuscript composition.

Dedication:

This thesis is dedicated to the two pillars of my life: my mother, whose love and sacrifices have been the foundation of my strength, and my brother, whose guidance and support have continually inspired and motivated me. Their unwavering belief in me has shaped this journey in ways I will always cherish.

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List of Abbreviations

AC: Adenylate cyclase

ACP: Amorphous Calcium Phosphate

BCP: Biphasic Calcium Phosphate

β-hex: β-hexosaminidase

Ca^{2+}: Calcium

$Ca_3(PO_4)_2$: Calcium Phosphate

cAMP: Cyclic Adenosine Monophosphate

CKD: Chronic Kidney Disease

CKD-MBD: CKD-related mineral and bone disorders

CRAC: Calcium release-activated calcium

CTMCs: Connective Tissue Mast Cells

DAG: Diacylglycerol

DNA: Deoxyribonucleic Acid

DRG: Dorsal Root Ganglia

ERK: Extracellular Signal-Regulated Kinase

FBS: Fetal Bovine Serum

FcεRI: High-affinity Fc-epsilon receptor-I

FGF23: Fibroblast Growth Factor 23

GPCR: G Protein-Coupled Receptors

GPCRs: G Protein-Coupled Receptors

HDP: Host Defense Peptide

HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

HDAC: Histone Deacetylase

HMC-1.2: Human Mast Cell line 1.2

HuMC: Human Mast Cells

IBD: Inflammatory Bowel Disease

IBS: Irritable Bowel Syndrome

IgE: Immunoglobulin E

IL-33: Interleukin 33

IMDM: Iscove's Modified Dulbecco's Medium

IP3: Inositol 1,4,5-trisphosphate

JNK: c-Jun N-terminal kinases

KIT: A receptor tyrosine kinase

LAD2: Laboratory of Allergic Diseases 2 cell line

LAT-B: Latrunculin B

MAPK: Mitogen-Activated Protein Kinase

MC: Mast Cell

MRGPRX1-X4: MAS-related G protein-coupled receptors 1-4

MRGPRX2: MAS-related G protein-coupled receptor X2

NaBu: Sodium Butyrate

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

PAMPs: Pathogen-Associated Molecular Patterns

PACAP: Pituitary Adenylate Cyclase-Activating Polypeptide

PBS: Phosphate Buffered Saline

PI3K: Phosphoinositide 3-kinase

PIP2: Phosphatidylinositol 4,5-bisphosphate

p-NAG: Poly-N-acetylglucosamine

ROS: Reactive Oxygen Species

SCF: Stem Cell Factor

SOCE: Store-operated calcium entry

ST2: A receptor for IL-33

STIM1: Stromal Interaction Molecule 1

TLRs: Toll-Like Receptors

TNF: Tumor Necrosis Factor

Tregs: Regulatory T cells

VC: Vascular Calcification

Chapter 1: Introduction

1.1 Background

1.1.1 Role and Importance of Mast Cells in Immunity

Mast Cells (MCs) are innate immune cells that play a crucial role in regulating immune responses to allergies and infections. MCs are densely packed with secretory granules containing pre-formed mediators such as histamine, TNF, heparin, chondroitin sulfates, and MC-specific proteases (Albert-Bayo et al., 2019; T. C. Moon et al., 2014). Upon activation, MCs undergo degranulation, releasing the contents of granules through membrane fusion and producing pro-inflammatory leukotrienes, prostaglandins, chemokines, and cytokines (Genta et al., 2024; Urb & Sheppard, 2012). This cascade of events underscores the role of MCs in promoting host resistance to allergies and infections. Additionally, MC activation leads to the rapid release of granules and the production of inflammatory mediators, including cytokines and lipid mediators such as interleukins, which are essential for maintaining allergic inflammation (Atiakshin et al., 2022). Consequently, MCs can recognize a variety of danger signals from pathogens, tissues, and other immune cells, thereby regulating immune responses according to the stimuli they encounter (Genta et al., 2024; Suárez Vázquez et al., 2024). MCs are primarily located at junction points between the body and the external environment, such as at antigen entrance sites in the gastrointestinal tract, skin, and respiratory epithelium (Rönnberg et al., 2012; Theoharides et al., 2000).

Based on recent research, Human MC heterogeneity is better understood as a spectrum of phenotypes that vary in their protease content and tissue distribution (Tauber et al., 2023; West & Bulfone-Paus, 2022). In

humans, mast cells are primarily classified based on their protease content into three main subtypes: MC_T (containing only tryptase), MC_TC (containing both tryptase and chymase), and MC_C (containing only chymase, which is relatively rare) (West & Bulfone-Paus, 2022). While MC_T are predominant in the mucosa of the lung and small intestine, and MC_TC are more prevalent in the skin, synovium, and intestinal submucosa, most human tissues contain a mixed population of MC subtypes with varying ratios. Recent single-cell RNA sequencing studies have revealed even greater heterogeneity in human mast cells. A comprehensive study identified six distinct mast cell clusters/states (MC1-6) distributed across 12 organs, each with unique transcriptomic signatures (Tauber et al., 2023, 2024). For example, MC1 are preferentially enriched in the skin and lungs, MC2, MC3, and MC4 in the skin and bladder, MC5 in the lymph node and vasculature, and MC6 in the trachea and lungs (Tauber et al., 2024). This heterogeneity extends beyond protease content to include differences in cytokine production, receptor expression, and functional responses (T. C. Moon et al., 2010; Tauber et al., 2023). The specific tissue microenvironment plays a crucial role in shaping mast cell phenotypes, allowing for plasticity and adaptation to local conditions. Factors such as embryonic origin, tissue-specific cues during maturation, and even sex can influence mast cell characteristics (Tauber et al., 2023; West & Bulfone-Paus, 2022).

1.1.2 Key Mast Cell Receptors

MC receptors are summarized in Table 1.1. Stem Cell Factor (SCF) is essential for the survival, proliferation, and differentiation of MCs. SCF binds to KIT, activating downstream signaling pathways crucial for MC development, and mutations in KIT can lead to disorders such as systemic mastocytosis (Griesenauer & Paczesny, 2017; Sandig & Bulfone-Paus, 2012). High-Affinity Immunoglobulin E (IgE) Receptor (FcεRI) plays a fundamental role in allergic responses through binding to IgE antibodies and

triggering MC degranulation, releasing histamines and other mediators that contribute to allergic symptoms such as anaphylaxis and urticaria (Y. Li et al., 2022; Roy et al., 2021). The IL-33 receptor (ST2) is involved in amplifying inflammatory responses; IL-33 binds to ST2, promoting the release of pro-inflammatory cytokines and chemokines, crucial in chronic inflammatory diseases like asthma and rheumatoid arthritis, without inducing MC degranulation (Callahan et al., 2020; Dziadowiec et al., 2024). Toll-Like Receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs) and modulate the innate immune response by driving cytokine and chemokine production (Choi et al., 2023; Tsai et al., 2022). G Protein-Coupled Receptors (GPCRs), including adenosine receptors, prostaglandin E2 receptors, complement receptors C3a/C5a, and notably, the MAS-related G protein-coupled receptor X2 (MRGPRX2), mediate various physiological responses such as modulation of vascular tone, pain perception, and immune cell recruitment (Molfetta et al., 2005; Zhou et al., 2023). MRGPRX2 is particularly important for its role in pseudo-allergic reactions, as it can induce MC degranulation in response to various polybasic compounds (Krystel-Whittemore et al., 2016; Zhou et al., 2023). Degranulation is the rapid release of histamine and other vasoactive substances from MCs upon activation and is crucial to the acute-phase allergic reaction and can cause immediate hypersensitivity reactions ranging from mild itching to life-threatening anaphylaxis (Takematsu et al., 2022). However, not all receptors induce both degranulation and cytokine production; for instance, IL-33 and TLRs primarily stimulate cytokine production rather than degranulation, indicating that different receptors tailor MC responses to various stimuli, thereby modulating different aspects of the immune response (Agier et al., 2018; Pavlyuchenkova et al., 2024).

Table 1.1 Key mast cell receptors and their functions.

S. No.	Receptor	Function		References
1	SCF/KIT	Survival, proliferation, differentiation	Crucial for MC development; mutations lead to systemic mastocytosis	(Roy et al., 2021; Sandig & Bulfone-Paus, 2012)
2	FcεRI	Binds IgE, triggers degranulation	Fundamental in allergic responses, releases histamines	(Griesenauer & Paczesny, 2017; Y. Nagata & Suzuki, 2022; Ogasawara & Noguchi, 2021)
3	IL-33/ST2	Amplifies inflammatory responses	Promotes release of pro-inflammatory cytokines, crucial in chronic inflammatory diseases	(Saluja et al., 2015; Tsai et al., 2022)
4	TLRs	Recognize PAMPs	Lead to cytokine and chemokine production, vital in innate immune response	(Choi et al., 2023; Griesenauer & Paczesny, 2017)
5	GPCRs (e.g., MRGPRX2)	Modulate various physiological responses	Induce degranulation in pseudo-allergic reactions, immune cell recruitment	(Roy et al., 2021; Supajatura et al., 2002)

Immunoglobulin E (IgE)-dependent and IgE-independent pathways are the two routes for MC activation. In IgE-dependent MC activation, the antigen binds to the variable region of the antibody, and the Fc portion of IgE binds to the high-affinity IgE receptor (FcεRI) on the surface of mast cells. This binding leads to the cross-linking of the IgE-bound FcεRI receptors, triggering MC activation and degranulation (Vitte et al., 2022; Yang et al., 2023). Although IgE-dependent signaling is the main pathway for MC activation, IgE-independent MC activation is induced by the ability of MCs to alter their responses to many other internal and external signals (Finkelman et al., 2016; Pałgan, 2023). This suggests that the various regulatory receptors act as regulators of these responses. One such family of regulatory receptors is G protein-coupled receptors (GPCRs) (L. Cheng et al., 2023; Suno, 2024). All GPCRs share the same seven membrane-spanning α-helical segments, which are separated into segments by alternate internal and extracellular loop regions. They convert extracellular signals into biological responses *via* G proteins and β-arrestins (L. Cheng et al., 2023; Suno, 2024). GPCR is activated as a result, which causes the release of cyclic adenosine 3,5-monophosphate (cAMP), the mobilization of calcium, or the phosphorylation of extracellular regulated protein kinases 1/2 (pERK1/2) (L. Cheng et al., 2023; Suno, 2024). The majority of cellular responses to hormones and neurotransmitters are mediated by GPCRs, the largest family of membrane proteins (L. Cheng et al., 2023; Suno, 2024). When ligands or agonists bind to a GPCR, they activate the associated trimeric GTP-binding protein (G protein). When activated, G proteins contain an α-subunit that binds guanine nucleotide and hydrolyzes GTP to GDP, as well as β and γ subunit complex. As shown in Figure 1.1, the effector adenylate cyclase (AC) is activated by the activation of the Gαs-coupled receptors, which allows cytosolic ATP to be converted to cyclic AMP (cAMP). On the other hand, AC is inactivated when Gαi-coupled receptors are activated. Phospholipase C cleaves membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol (1,4,5) trisphosphate (IP3) and

diacylglycerol when the Gαq subfamily is activated (DAG). Ca2+ is released when IP3 interacts with IP3 receptors that are present in the endoplasmic reticulum membrane. Researchers use each of these GPCR activation-related small molecules as a target to investigate GPCR function and to develop assays for the identification of molecules that can influence GPCR activity (L. Cheng et al., 2023; Suno, 2024).

A subcategory of GPCRs that triggers MC degranulation is the mas-related G protein-coupled receptor (Mrgpr). One member of the Mrgpr family is mas-related GPCR-X2 (MRGPRX2), which is highly expressed in human skin and cord blood MCs (Saluja et al., 2015). Instead of directly interacting with G-proteins, basic secretagogues, such as c48/80, activate connective tissue MCs *via* MRGPRX2 (Krystel-Whittemore et al., 2016).

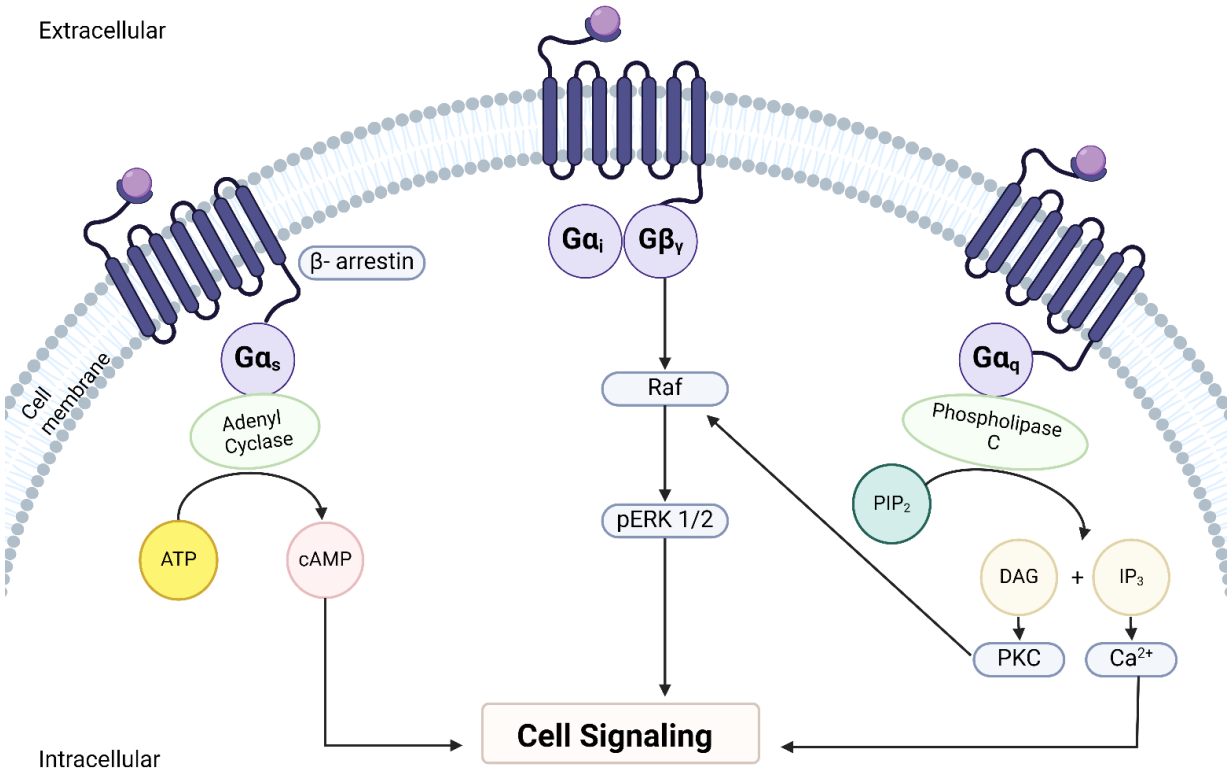

Figure 1.1 GPCR activation of cell signaling. (Created with BioRender.com)

1.1.3 MRGPRX2 and its Significance

MRGPR is a large family of seven transmembrane receptors expressed in MCs and sensory neurons of the dorsal root ganglia (DRG) that have previously been studied in the context of itch, pain, and inflammation (Ando & Kitaura, 2021; Gour & Dong, 2024). Humans have four MRGPRX families (MRGPRX1-X4) and Mrgpr D-H (Baran et al., 2023; Mayr et al., 2002).

Table 1.2 MRGPR in various cell types.

S. No.	Cell Type	MRGPR (Human ortholog)	Activation Mode	References
1	Mast Cells	MRGPRX2	Degranulation	(McNeil et al., 2015)
2	Basophils	MRGPRX2	Degranulation	(Gour & Dong, 2024)
3	Eosinophils	MRGPRX2	Degranulation	(Gour & Dong, 2024)
4	Dorsal Root Ganglion Cells		Signal transmission for itch/pain	(Baran et al., 2023)
			Signal transmission for itch/pain	(Mendoza et al., 2021; Xu et al., 2022)
		MRGPRX1	Signal transmission for itch/pain	(Tseng et al., 2019)
			Regulates Mrgpr D's trafficking	(Green, 2021)
		MRGPRX2	Signal transmission for itch/pain	(Ogasawara & Noguchi, 2021)
5	Keratinocytes	MRGPRX3 MRGPRX4	Wound healing through activation of keratinocytes	(Kiatsurayanon et al., 2016)

The basic peptides that MRGPRX2 particularly recognizes and employs to initiate the degranulation of human connective tissue mast cells (CTMCs) include mastoparan, somatostatin, c48/80, and pituitary

adenylate cyclase-activating polypeptide (PACAP) (Kumar et al., 2021; Ogasawara & Noguchi, 2021). MRGPRX2 may be used to identify danger signals from cytotoxicity since the enzymatic breakdown products of intracellular proteins, such as chaperonin-10, released after cytotoxicity or cell death also activate MCs as MRGPRX2 ligands (Ogasawara & Noguchi, 2021). In addition to MCs, MRGPRX2 is also expressed in basophils and eosinophils, mediating degranulation (Babina, 2020; Toscano et al., 2023). According to one study, basophils and eosinophils strongly expressed MRGPRX2, whereas neutrophils did not (Babina, 2020). Basophils and MCs function similarly in many ways, including the production of highly specific IgE receptors and the release of histamine in response to stimulation (Shah et al., 2021). In the case of eosinophils, they are recognized to contribute to a number of inflammatory disorders through cross-talk with MCs. One research study investigated the expression of MRGPRX2, as well as its agonists, cortistatin, and major basic protein (MBP), in the lesional skin of patients where eosinophils are present (Wedi et al., 2020). Blood eosinophils express MRGPRX2, although less so than MCs, according to a study (Wedi et al., 2020).

Some studies indicate that MRGPRX2 is also expressed in dorsal root ganglion (DRG) neurons. Although MrgprA3+, MrgprC11+, and MrgprD+ are involved in the itch expression, MrgprA3+ and MrgprD+ were observed to be specifically expressed in DRG sensory neurons (Babina, 2020). A study indicates that MrgprA3+ may have dual modalities that transmit both pain and itch to the spinal cord (Babina, 2020). MrgprD+ resides deep in the epidermis and can be considered a somatosensory marker (Babina, 2020). The rodent MrgprC subfamily shares similarities with human MRGPRX1, and both are specifically expressed in small-diameter DRG neurons(Babina, 2020). Meanwhile, in the rodent subfamily of MrgprC, only MrgprC11+ was found to be expressed. MrgprE is also expressed in DRG neurons, but very little information is known regarding this receptor. It is known to have a role in regulating MrgprD trafficking

(Babina, 2020). One study suggests that MRGX3 and MRGX4 facilitate the AG-30/5C molecule to activate human keratinocytes, which can be used for wound healing. AG-30/5C is a modified form of AG-30 that activates cytokine and chemokine production (Babina, 2020). Since the AG-30 molecule is very unstable and is easily degraded by proteases, AG-30/5C has been used in the study. AG-30/5C enhanced keratinocyte migration and proliferation by inducing the release of cytokines and chemokines *via* the MRGX3 and MRGX4 receptors (Babina, 2020).

1.1.4 Role of MRGPRX2 in Mast Cell Activation

The MAS-related G protein-coupled receptor X2 (MRGPRX2) plays a pivotal role in MC activation through IgE-independent pathways, as illustrated in Figure 1.2 (Ogasawara & Noguchi, 2021; Yang et al., 2023). This receptor's activation mechanism is crucial for several physiological and pathological processes. In host defense, MRGPRX2-mediated MC activation enhances the innate immune response by promoting the elimination of bacterial infections and facilitating wound healing. This underscores the receptor's importance in maintaining host defense against pathogens (Callahan et al., 2020; Lu et al., 2021). Additionally, MRGPRX2 is involved in neurogenic inflammation, contributing to conditions such as postoperative pain, chronic itch, and type 2 inflammation. The activation of MRGPRX2 by neuropeptides leads to the release of inflammatory mediators that exacerbate these conditions, highlighting the receptor's role in neuro-immune interactions (Dziadowiec et al., 2024; Roy et al., 2021; Thapaliya et al., 2021).

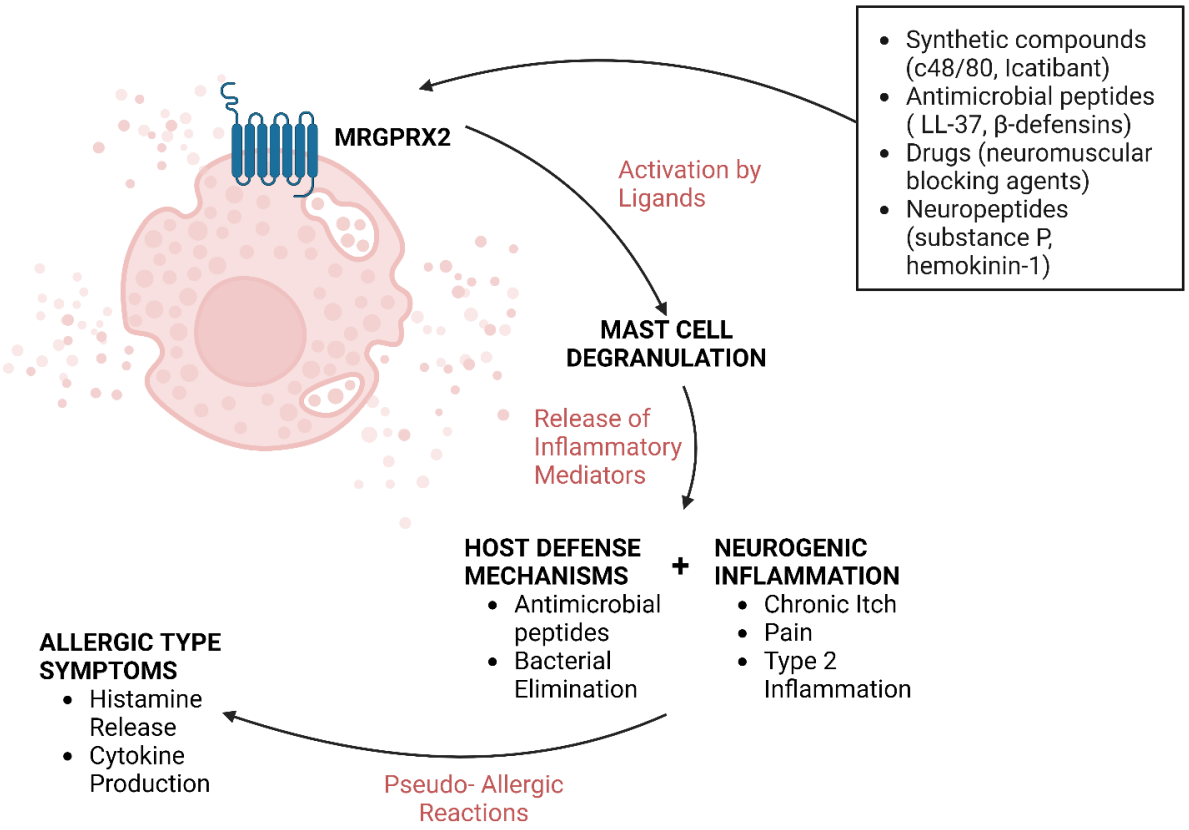

***Figure 1.2 Role of MRGPRX2 in MC Activation.* (*Created with BioRender.com*)**

1.1.5 Activating MCs using Compound 48/80 and A23187

MC activation using Compound 48/80 (c48/80) involves several biochemical pathways leading to cell degranulation *via* the MRGPRX2 receptor and the subsequent release of mediators. There are several pathways activated upon introducing c48/80 to MCs, including phospholipase C (PLC) and mitogen-activated protein kinase (MAPK). PLC activation splits phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG). The release of IP3 facilitates the liberation of calcium ions from internal stores, thereby activating downstream signalling cascades vital for further

cellular responses. This process is particularly crucial for degranulation, where the elevated intracellular calcium levels lead to the fusion of granules with the plasma membrane and the subsequent release of their contents. The increase in calcium concentration specifically triggers the exocytosis of granules, a key step in degranulation, making this outcome more significant compared to other cellular responses.(Porebski et al., 2018; F. Zhang et al., 2022). MAPK pathway involves the stimulation of key kinases, including extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 MAPK. The engagement of the MAPK pathway is essential for regulating gene expression and orchestrating the degranulation (Dziadowiec et al., 2024; F. Zhang et al., 2022).

MC activation using A23187, a calcium ionophore, hinges predominantly on the influx of extracellular calcium ions, which leads to cell degranulation and the release of mediators. Unlike receptor-mediated pathways, A23187 directly causes the intracellular calcium levels to rise. This surge in calcium ions triggers various signaling pathways, notably activating PLC, which subsequently results in the production of IP3 and DAG. The elevation in calcium levels not only activates PLC but also prompts the further release of calcium from internal stores, thereby amplifying the signaling required for effective MC degranulation (Lee et al., 2017; Subramanian et al., 2016).

While both c48/80 and A23187 share a common pathway through PLC activation, their mechanisms of action markedly differ. C48/80 relies on receptor binding for PLC pathway activation, whereas A23187 bypasses this step, directly elevating intracellular calcium levels to trigger the pathway. These differences potentially influence the intensity and duration of MC degranulation and the profile of mediator release. Additionally, while c48/80 also triggers the MAPK pathway, influencing gene expression and mediator release, A23187 does not directly initiate this pathway, which may result in distinct outcomes in terms of inflammatory response and mediator profile (Kim & Choi, 2010; Lee et al., 2017)

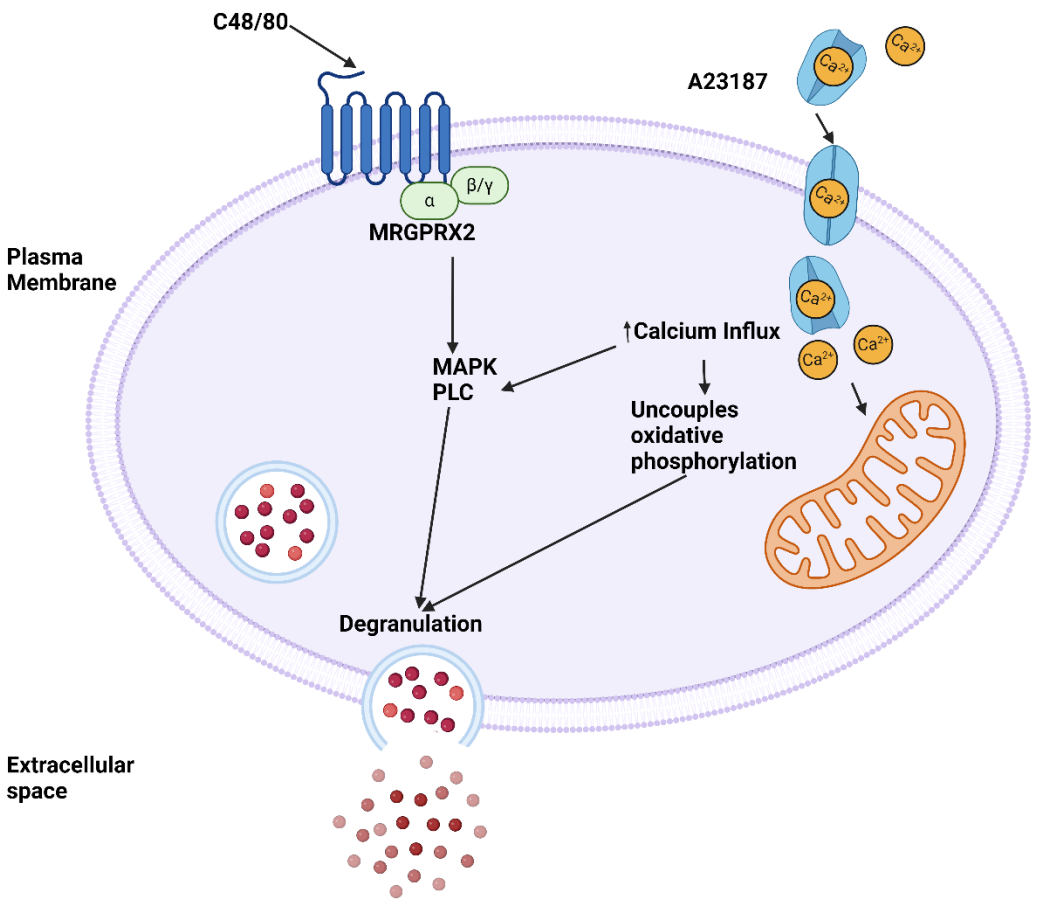

Figure 1.3 Role of C48/80 and A23187 in MC activation. (Created with BioRender.com)

1.1.6 Applications of Inhibition of MRGPRX2 in Mast Cell Disorders

Targeting Mas-related G-protein coupled receptor X2 (MRGPRX2) for inhibition presents a ground-breaking strategy for addressing MC-related disorders, including chronic urticaria and asthma. MCs are crucial to the immune system, particularly in allergic reactions and inflammatory processes. When MRGPRX2 is activated, it triggers MC degranulation, releasing various inflammatory mediators such as histamines, cytokines, and proteases. This degranulation contributes to the symptoms seen in conditions

like chronic urticaria, characterized by persistent hives, and asthma, marked by airway inflammation and constriction (Dziadowiec et al., 2024; Roy et al., 2021).

By focusing on MRGPRX2, researchers aim to disrupt this specific activation pathway. MRGPRX2 is implicated in MC degranulation, which plays a crucial role in the pathogenesis of asthma and urticaria (Puxeddu et al., 2024). In asthma, MRGPRX2 activation leads to airway inflammation and hyperresponsiveness, while in urticaria, it triggers histamine release, causing hives and itching (Wollam et al., 2023; Yang et al., 2023). Understanding the molecular mechanisms and signaling pathways through which MRGPRX2 operates allows for the development of targeted inhibitors (Guo et al., 2023). These inhibitors can effectively block the receptor's activity, thereby preventing MC degranulation and the ensuing inflammatory response. (Lerner et al., 2024; Ogasawara & Noguchi, 2021; Wong et al., 2024; C. Yao et al., 2023).

One of the significant advantages of this approach is the specificity of the inhibition. By selectively targeting MRGPRX2, it is possible to mitigate the adverse effects associated with MC degranulation without impairing other essential functions of MCs. This specificity is crucial because MCs are involved in various physiological processes, including defense against pathogens and wound healing. Therefore, a targeted inhibitor would ideally reduce pathological inflammation while preserving the beneficial roles of MCs (Guo et al., 2023; Kumar et al., 2023).

This novel therapeutic approach holds promise not only for improving the management of chronic urticaria and asthma but also for enhancing the quality of life for patients suffering from these chronic conditions. As research progresses, the development of MRGPRX2 inhibitors could lead to more effective

and tailored treatments, minimizing side effects and providing a new avenue for managing MC-related disorders (Callahan et al., 2020; Mackay et al., 2021).

1.1.7 Overview of NaBu and Its Biological Implication

NaBu, a potent histone deacetylase (HDAC) inhibitor, plays a crucial role in modifying chromatin structure and regulating gene expression. By inhibiting HDACs, histone acetylation is promoted, resulting in a more open chromatin structure that enhances gene expression (Ozkan et al., 2023; D. Wang et al., 2023). Additionally, NaBu has notable anti-inflammatory properties, reducing inflammatory cytokine production in MCs (Jiang et al., 2023; MacDonald et al., 2023). It impacts various cellular mechanisms, including gene regulation through histone acetylation, and influences key signaling pathways, such as MAPK and NF-κB, essential for cell survival, proliferation, and immune responses (Y. Cheng et al., 2021; Messier et al., 2022). It also plays a role in epigenetic modifications like DNA methylation and microRNA expression, which further influence gene expression and cellular phenotypes (De Lazari et al., 2023; Sarkar et al., 2023).

There is a need to investigate the effects of NaBu on the MRGPRX2 receptor, particularly in the context of MC degranulation, due to its ability to regulate immune responses and its anti-inflammatory properties, which suggest it could influence MC activity crucial in allergic reactions and inflammation (Zong et al., 2023). The involvement of NaBu in pathways like MAPK and NF-κB, linked to MC activation and degranulation, highlights the potential for NaBu to affect MC behaviour through the MRGPRX2 receptor (Sivamaruthi et al., 2023). Nabu has been shown to regulate immune responses and have anti-inflammatory properties, but the specific mechanism has yet to be fully defined. It has been shown that MRGPRX2-induced MC activation and NaBu activities share commonalities, such as the activation of

MAPK and NF-κB pathways. Because of this, it is hypothesized that NaBu may activate these pathways by interacting with the MC receptor MRGPRX2. The involvement of NaBu in pathways like MAPK and NF-κB, linked to MC activation and degranulation, highlights the potential for NaBu to affect MC behaviour through the MRGPRX2 receptor.

1.1.8 Biological Role of Calcium Phosphate

The immune system relies on calcium signaling for MC functions crucial for allergic reactions and defense against pathogens, including degranulation, cytokine production, and eicosanoid generation. MCs rely on calcium influx through calcium release-activated calcium (CRAC) channels to trigger these immune responses (J. Li et al., 2024; Z. Sun et al., 2021). Recent studies have shown that $Ca_3(PO_4)_2$ nanoparticles can act as effective adjuvants, enhancing both innate and adaptive immune responses by stimulating MCs to produce cytokines and other mediators (Humbert et al., 2019; Z. Sun et al., 2021). This suggests a potential link between calcium signaling and the activation of MRGPRX2, a receptor known to be involved in MC activation and degranulation. NaBu, which has been shown to regulate immune responses and possess anti-inflammatory properties, shares commonalities with MRGPRX2-induced MC activation pathways, such as MAPK and NF-κB. Thus, it is hypothesized that NaBu may influence these pathways through interactions with the MRGPRX2 receptor, potentially affecting MC behavior and contributing to allergic reactions and inflammation (Zong et al., 2023; Sivamaruthi et al., 2023). Existing research supports this hypothesis, indicating that further investigation into the effects of NaBu on the MRGPRX2 receptor is warranted.

1.1.9 Transformed Human Mast cells: HMC 1.2 cell line

The HMC-1.2 cell line is a subline of the original HMC-1 cell line, which was derived from a patient with mast cell leukemia. HMC-1 cells are notable for their metachromatic granules containing histamine and tryptase, and they exhibit many characteristics of tissue mast cells. The HMC-1.2 subline specifically harbors two mutations in the KIT gene: V560G and D816V. These mutations result in constitutive phosphorylation of KIT, which is crucial for the cells' growth and survival in the absence of stem cell factor (SCF) (Arock et al., 2018; Sundström et al., 2003).

HMC-1.2 cells rapidly proliferate, have a stable phenotype, and are a model for studying the pathophysiology of mast cell-related diseases and the effects of various therapeutic agents. The presence of the KIT D816V mutation in HMC-1.2 cells makes them particularly relevant for studying systemic mastocytosis and other conditions driven by KIT mutations (Arock et al., 2018). The Mas-related G-protein-coupled receptor X2 (MRGPRX2) is prominently expressed in mast cells and is involved in degranulation upon binding with various ligands. However, the expression and functionality of MRGPRX2 in HMC-1.2 cells have been subjects of debate.

Recent studies have demonstrated that HMC-1.2 cells do express MRGPRX2 at lower levels compared to other human mast cell lines like LAD2 and HuMC. Functional assays have shown that HMC-1.2 cells can undergo degranulation in response to MRGPRX2-specific stimuli, such as c48/80 (Arock et al., 2018; Hermans et al., 2021).

1.2 Hypothesis

Sodium butyrate and calcium phosphate are hypothesized to modulate MRGPRX2-mediated activation in HMC 1.2 cells, influencing mast cell proliferation, viability and degranulation.

1.3 Research Objectives

This thesis focuses on the influence of biomolecules on MRGPRX2-mediated activation of transformed human MCs (HMC 1.2). The objectives of this thesis are as follows:

- To examine the impact of Sodium Butyrate on MRGPRX2-mediated and calcium signaling-mediated proliferation, viability, and degranulation in transformed human mast cells.
- To evaluate the effect of Calcium Phosphate on MRGPRX2-mediated proliferation, viability, and degranulation in transformed human mast cells.

1.4 Organization of Thesis

This thesis presents an investigation into the effects of sodium butyrate and calcium phosphate on MRGPRX2-mediated activation of transformed human mast cells (HMC 1.2). Organized into four chapters: Chapter 1 is an introduction to the research topic, background information, and the significance of the study; Chapter 2 is focused on the effect of Sodium Butyrate on MRGPRX2-mediated and calcium signaling-mediated degranulation in HMC 1.2 cells; Chapter 3, is where the effect of Calcium Phosphate on MRGPRX2-mediated degranulation in HMC 1.2 cells is given; Chapter 4, is where the research findings are summarized and future work outlined.

Chapter 2: Sodium Butyrate Inhibits Cell Proliferation and Viability at High Concentrations in Transformed Human Mast Cells Without Impacting MRGPRX2-Induced Degranulation

2.1 Introduction

2.1.1 Chemical Properties of NaBu

NaBu ($C_4H_7NaO_2$) is highly soluble in water and has a molecular weight of 110.09 g/mol. This high solubility facilitates swift cellular absorption and subsequent utilization, making it an effective compound for various biological applications. NaBu is widely recognized for influencing the function of various immune cells, including macrophages, dendritic cells, and T cells. For instance, NaBu can inhibit the secretion of pro-inflammatory cytokines such as TNF-α and IL-6 from macrophages and dendritic cells, thereby exerting anti-inflammatory effects (Kushwaha et al., 2022; Nicese et al., 2023). Additionally, NaBu promotes the differentiation of regulatory T cells (Tregs), which play a crucial role in maintaining immune homeostasis and preventing chronic inflammation (Mayorga-Ramos et al., 2022). NaBu also supports gut health by maintaining intestinal barrier integrity and reducing inflammation, which is crucial for preventing metabolic disorders.

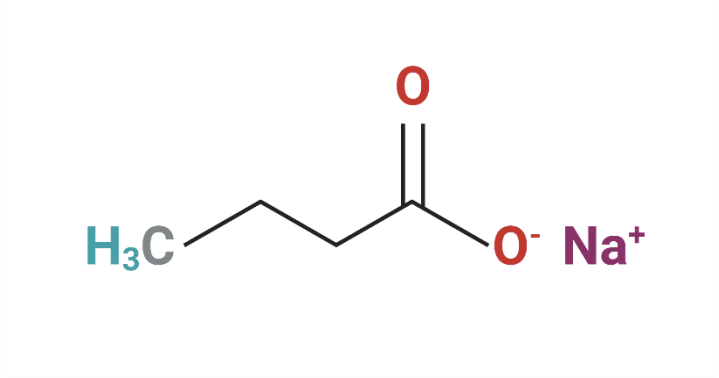

Figure 2.1 Structure of NaBu.

2.1.2 Biological Functions of NaBu

HDACs are enzymes that typically remove acetyl groups from histone proteins, leading to chromatin condensation and suppression of gene activity. As a potent HDACi, NaBu plays a pivotal role in the modulation of chromatin structure and gene expression. The inhibition of these enzymes by NaBu results in enhanced histone acetylation, promoting a more open chromatin conformation that supports increased gene expression (Folkerts, Redegeld, et al., 2020).

Moreover, NaBu exhibits notable anti-inflammatory properties. It regulates immune responses by curtailing the production of inflammatory cytokines and modulating the activity of various immune cells. For instance, NaBu has been shown to inhibit the secretion of pro-inflammatory cytokines such as TNF-α and IL-6 from macrophages and dendritic cells, thereby exerting anti-inflammatory effects (MacDonald et al., 2023; K. Nagata et al., 2024). Additionally, NaBu promotes the differentiation of regulatory T cells (Tregs), which play a crucial role in maintaining immune homeostasis and preventing chronic inflammation (Uranga et al., 2020).

2.1.3 NaBu in Mast Cells

NaBu has been shown to significantly impact mast cell (MC) function, particularly in the context of degranulation and cytokine production. MCs are critical effector cells in allergic reactions and other immune responses, releasing various inflammatory mediators upon activation. NaBu inhibits both IgE- and non-IgE-mediated MC degranulation, reducing the release of histamine and other pro-inflammatory cytokines (Kaag & Lorentz, 2023). This inhibition is achieved through the suppression of HDACs, leading to changes in gene expression that reduce MC activation.

Recent studies have highlighted the role of NaBu in modulating MC activity through the Mas-related G protein-coupled receptor X2 (MRGPRX2) (Dziadowiec et al., 2024; Ogasawara & Noguchi, 2021). MRGPRX2 is a receptor that mediates non-IgE-dependent MC degranulation in response to various stimuli, including neuropeptides and basic molecules (Callahan et al., 2020; Ogasawara & Noguchi, 2021). Understanding the role of NaBu in MRGPRX2-mediated MC degranulation is crucial because it offers insights into potential therapeutic strategies for conditions characterized by excessive MC activation, such as chronic urticaria and other allergic diseases.

2.2 Methods

2.2.1 HMC 1.2 Cell Culture and Maintenance

HMC-1.2 cells, generously provided by John Butterfield from the University of Pennsylvania, were cultured in Iscove's Modified Dulbecco's Medium (IMDM) from Life Technologies, New York, United States. The medium was supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

The cells were passaged 2-3 times a week and maintained at a density of 1 x 10^5 to 1 x 10^6 cells/mL at 37 °C.

2.2.2 NaBu Treatment

HMC 1.2 cells were cultured at a concentration of 5 x 10^5 cells in 5 mL of IMDM media. The cells were treated with NaBu (Sigma-Aldrich, Oakville, ON, Canada) at varying concentrations (0, 0.1, 1, and 10 mM) by preparing a NaBu stock solution in PBS. This culture was maintained for five days.

2.2.3 Cell Proliferation and Viability Assessed Using the Trypan Blue Exclusion Method

HMC-1.2 cells were seeded at 1 × 10^5 cells/mL density and incubated with 0, 0.1 and 1 mM NaBu for 1 to 5 days. Cell counts were performed each day post-treatment using a hemocytometer with trypan blue dye to stain dead cells. Cell proliferation was calculated using the Equation 1:

***Equation 1**: Cells/mL= (Number of live cells) x (Dilution factor) x 10^4*

Cell viability was measured using the following Equation 2:

***Equation 2**: % Cell Viability = [Number of live cells / (Number of live cells + Number of dead cells)] x 100*

2.2.4 XTT Assay

HMC-1.2 cells were seeded at 1 × 10^5 cells/mL density and incubated with 0, 1 and 10 mM NaBu for 1, 2 and 3 days. At each time point (1, 2 and 3 days), 100 µL of cells were collected and added to 50 µL of XTT reagent (Roche, Laval, QC, Canada) in a 96-well plate and incubated for 4 hours at 37 °C with 5%

CO_2. Absorbance at 405 nm was measured using a VarioSkan Lux plate reader (ThermoFisher), and the results were plotted using Microsoft Excel software.

2.2.5 β-hexosaminidase Assay

HMC-1.2 cells were treated with NaBu at concentrations of 0.1, 1, and 10 mM for 1, 2, and 3 days to examine its effect on degranulation. Following treatment, cells were washed and resuspended in HEPES buffer (containing HEPES, NaCl, KCl, $Na_2HPO_4 \cdot 7H_2O$, Glucose, $CaCl_2 \cdot 2H_2O$, and $MgSO_4 \cdot 7H_2O$). They were then stimulated with C48/80 (100 µg/mL) or A23187 (100mM) for 30 minutes at 37 °C. After stimulation, cells were centrifuged at 200 x g for 5 minutes to separate them from the supernatant. The β-hexosaminidase activity in both the supernatant and the cell pellet was assessed by incubating the samples with Poly-N-acetylglucosamine (p-NAG) for 1.5 hours, followed by the addition of Glycine buffer (pH 10.7). Absorbance was measured at 405 nm and 570 nm using a Varioskan plate reader (ThermoFisher Scientific, Mississauga, ON, Canada) at 37 °C.

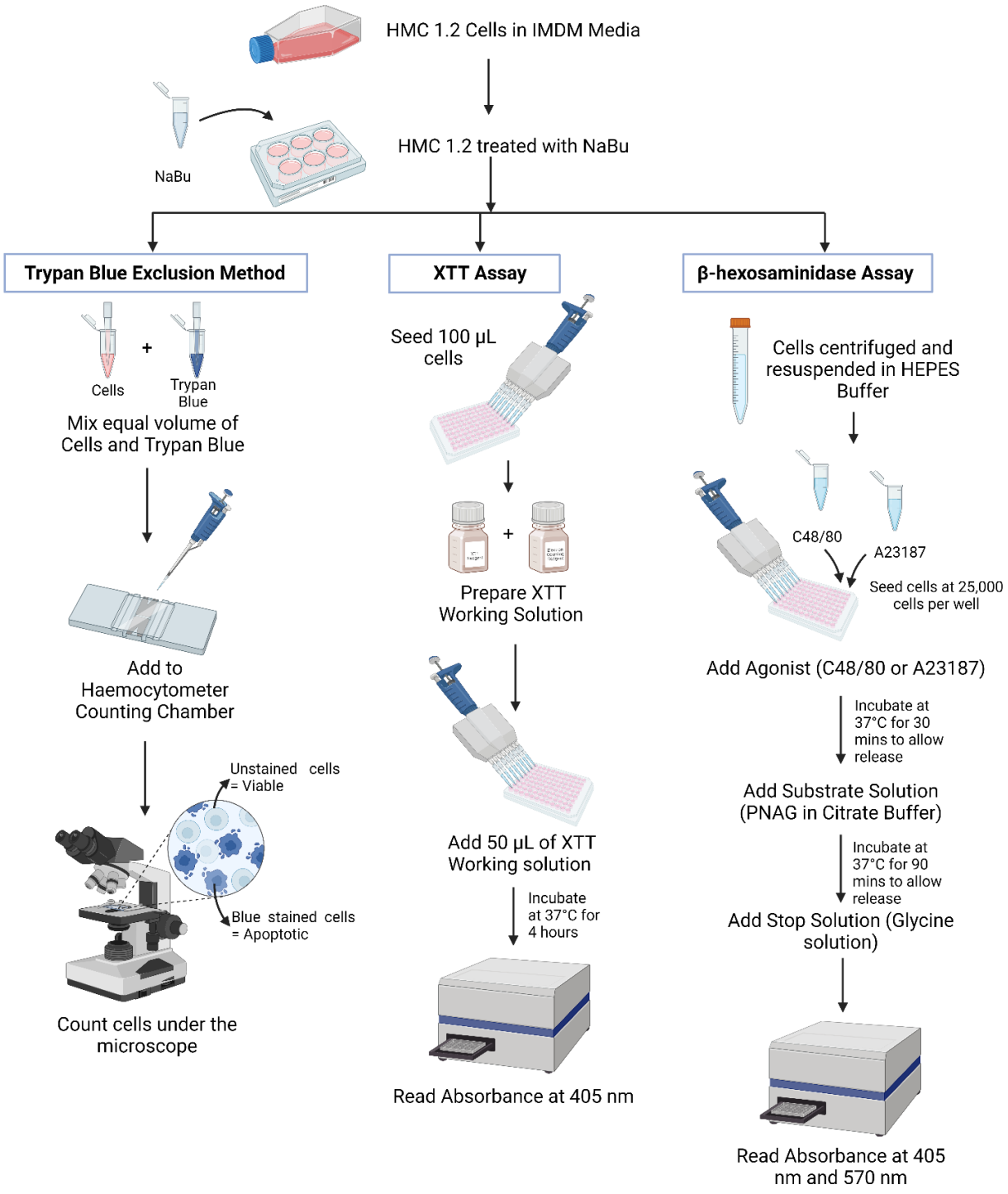

Figure 2.2 Schematic of all methods for NaBu effects on MCs. (Created with BioRender.com)

2.3 Results

2.3.1 Cell Proliferation and Viability Post-NaBu Treatment

NaBu inhibited cell proliferation in a concentration-dependent manner (Figure 2.3), where cell proliferation was reduced from 0.1 mM (Figure C, appendix) to 1 mM NaBu in HMC-1.2 cell culture. Specifically, HMC-1.2 cells treated with 1 mM NaBu showed a marked decrease in proliferation over the 5-day exposure period when compared to untreated cells (Figure 2.3A). Cell viability was not significantly affected and remained over 80% at all concentrations of NaBu, indicating that NaBu inhibits cell proliferation without inducing cytotoxicity (Figure 2.3B).

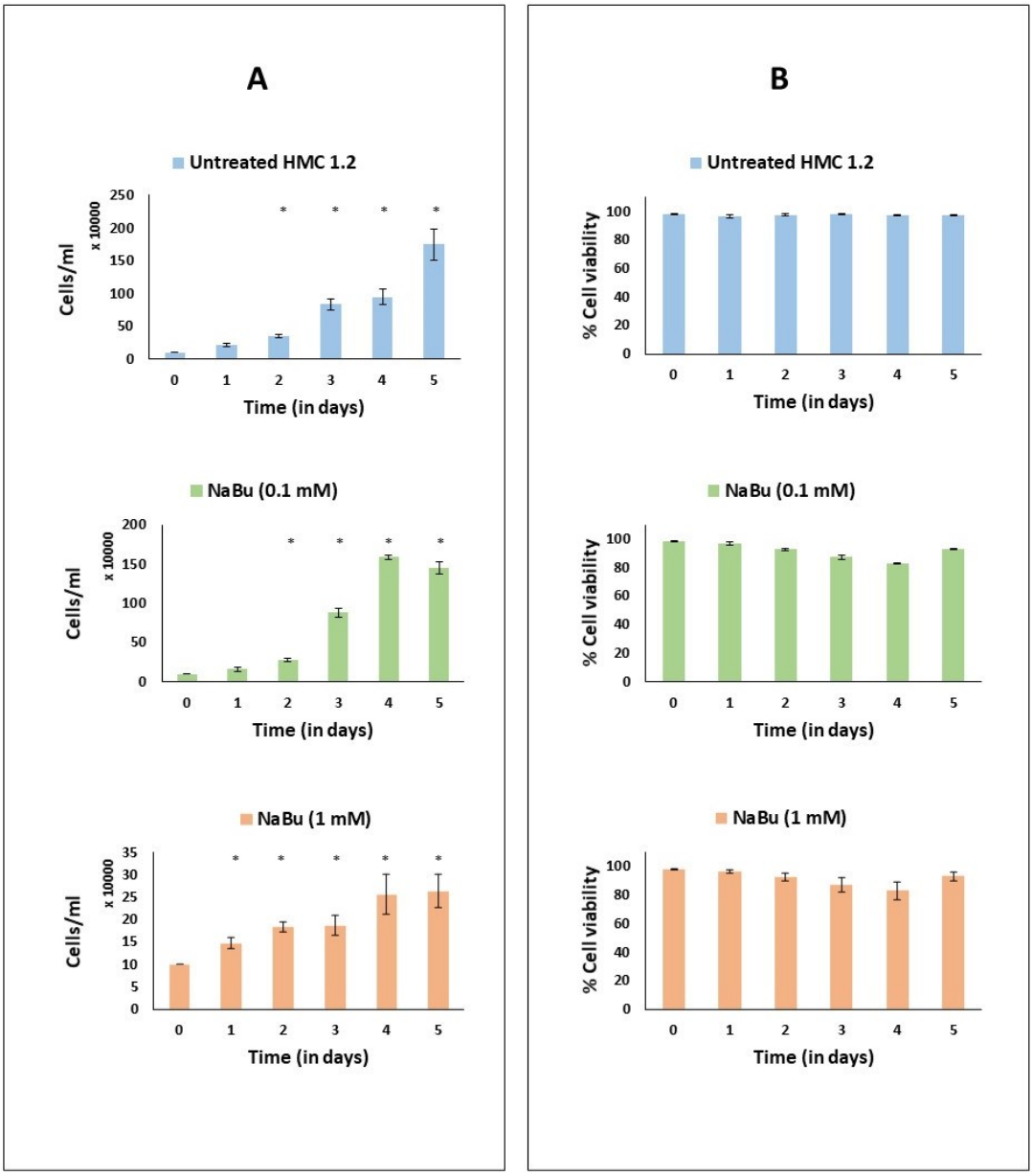

Figure 2.3 NaBu Inhibits HMC-1.2 Cell proliferation without affecting cell viability. *HMC-1.2 cells were cultured with NaBu (0, 0.1 and 1 mM) for a 5-day exposure time, and cell proliferation (A) and cell viability (B) were measured (n = 7 for days 1 to 3 and n=3 for days 4 and 5). The student's t-test was performed to determine the statistical significance (*p < 0.01) relative to the 0 (inactivated) samples.*

2.3.2 Metabolic Activity Effect Post-NaBu Treatment

The metabolic activity of HMC 1.2 cells treated with 10 mM NaBu shows a marked reduction over a three-day period compared to untreated (0 mM NaBu) control cells (Figure 2.4). On day 1, the metabolic activity of the treated cells decreased by 20%, demonstrating an initial impact of NaBu on cellular metabolism. This reduction in metabolic activity became more pronounced by day 2, with a 67% decrease relative to the control observed, indicating a progressive impairment of cellular functions. By day 3, the metabolic activity of the NaBu-treated HMC 1.2 cells was reduced by 87%, highlighting a significant ($P < 0.01$) and sustained inhibitory effect. These results clearly indicate that NaBu exerts a time-dependent reduction in the metabolic activity of HMC-1.2 cells, emphasizing its potent impact on cellular metabolic processes over prolonged exposure. It is important to note that NaBu at a concentration of 10 mM was toxic to the cells, especially after 3 days, contributing significantly to the observed decrease in metabolic activity. The significant decrease in metabolic activity over the three-day period underscores the potential of NaBu to modulate cellular metabolism significantly ($P < 0.01$).

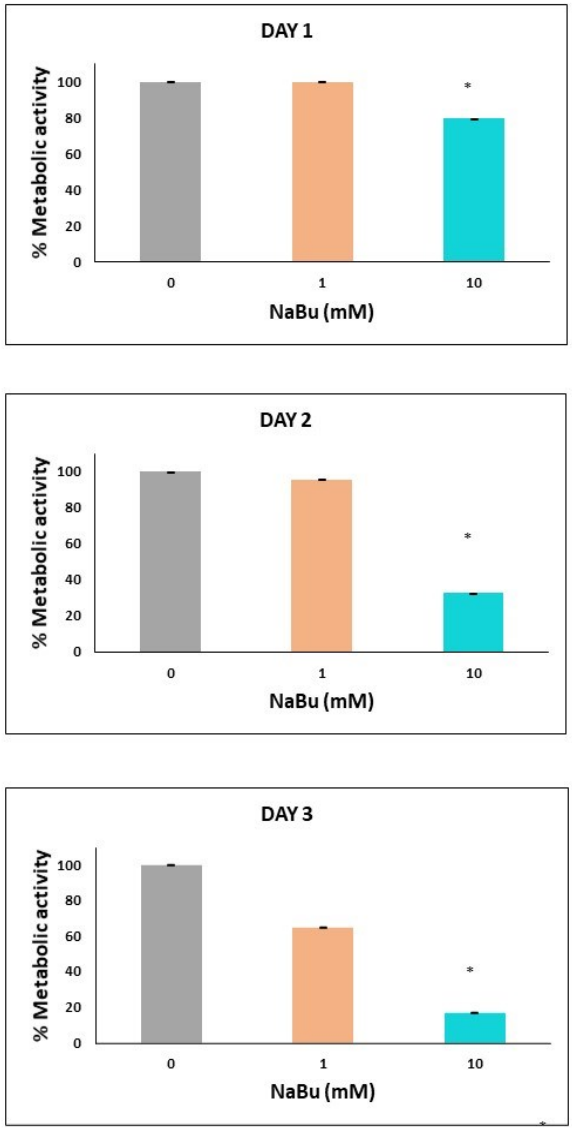

Figure 2.4 NaBu Significantly Reduces Metabolic Activity in HMC 1.2 Cells Over 3 Days. *HMC 1.2 cells were treated with 10 mM NaBu, and their metabolic activity was measured over a three-day period (n=7 for each day). Statistical significance was determined using the student's t-test (*$p < 0.01$) relative to the untreated samples.*

2.3.3 NaBu-induced Mast Cells Degranulation

NaBu did not affect the degranulation of HMC-1.2 cells through MRGPRX2 activation. This conclusion is based on experiments where cells treated with NaBu and subsequently stimulated with either C48/80 (as shown in Figure 2.5) or A23187 (as shown in Figure 2.6) exhibited no significant changes in the release of β-hexosaminidase (degranulation marker). In the provided graphs, "0 (Inactivated)" refers to untreated HMC 1.2 cells that were not subjected to activation by C48/80 (Figure 2.5) or A23187 (Figure 2.6), serving as baseline controls. The "0" condition represents untreated cells that were activated by either C48/80 or A23187, illustrating the typical response to these stimuli. For the conditions labeled "0.1" and "1", HMC 1.2 cells were treated with 0.1 mM and 1 mM concentrations of NaBu, respectively, after being activated by C48/80 or A23187. Despite these treatments, there were no significant differences observed in the levels of β-hexosaminidase release compared to the untreated, activated controls.

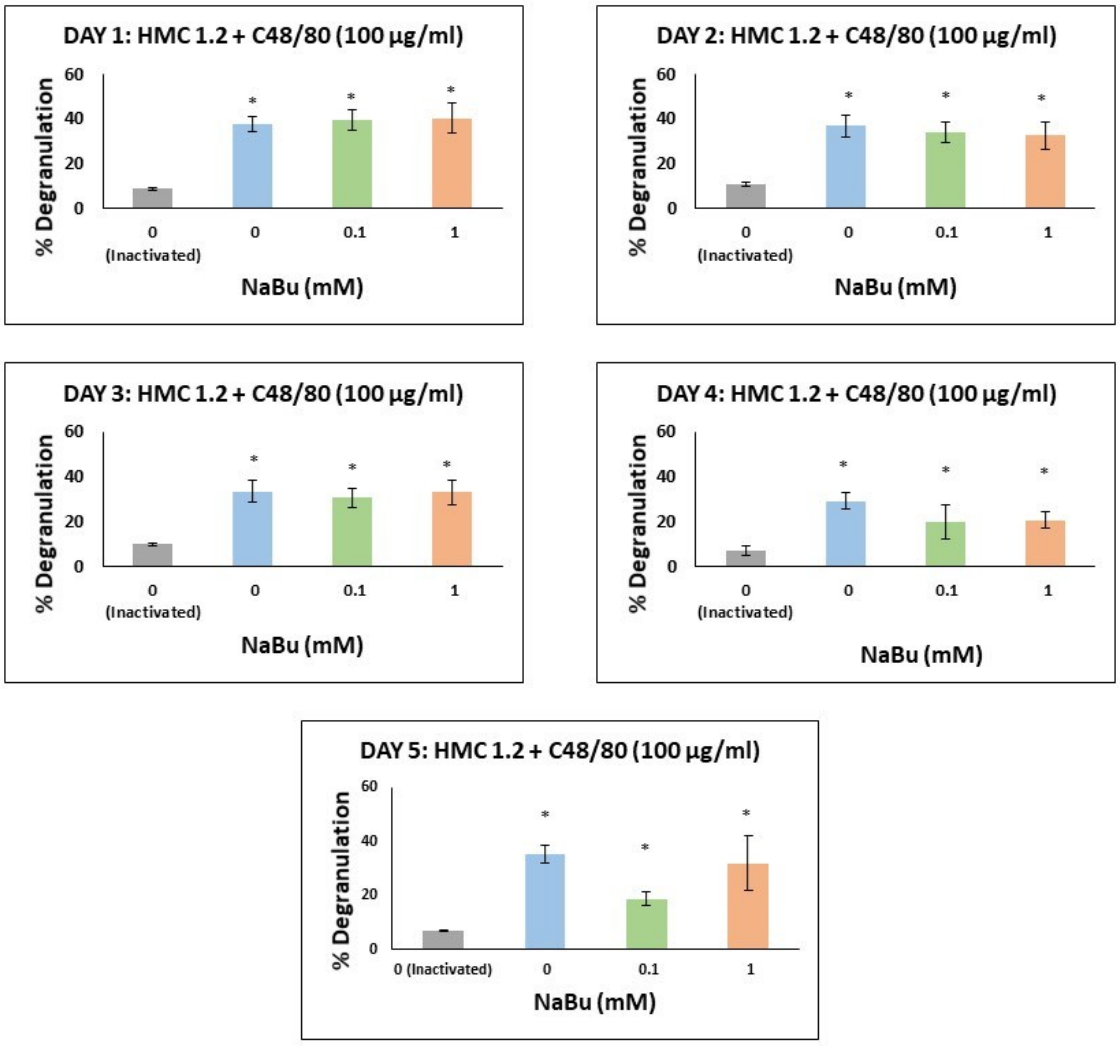

Figure 2.5 NaBu has no significant effect on HMC-1.2 Cell Degranulation through C48/80. *HMC-1.2 cells were exposed to different concentrations of NaBu (0, 0.1, and 1 mM) for 5 days and were activated with C48/80 (100 µg/mL). β-hexosaminidase release was measured to evaluate degranulation (n = 7 for days 1 to 3 and n=3 for days 4 and 5). The student's t-test was performed to determine the statistical significance (*p < 0.01) relative to the 0 (inactivated) samples.*

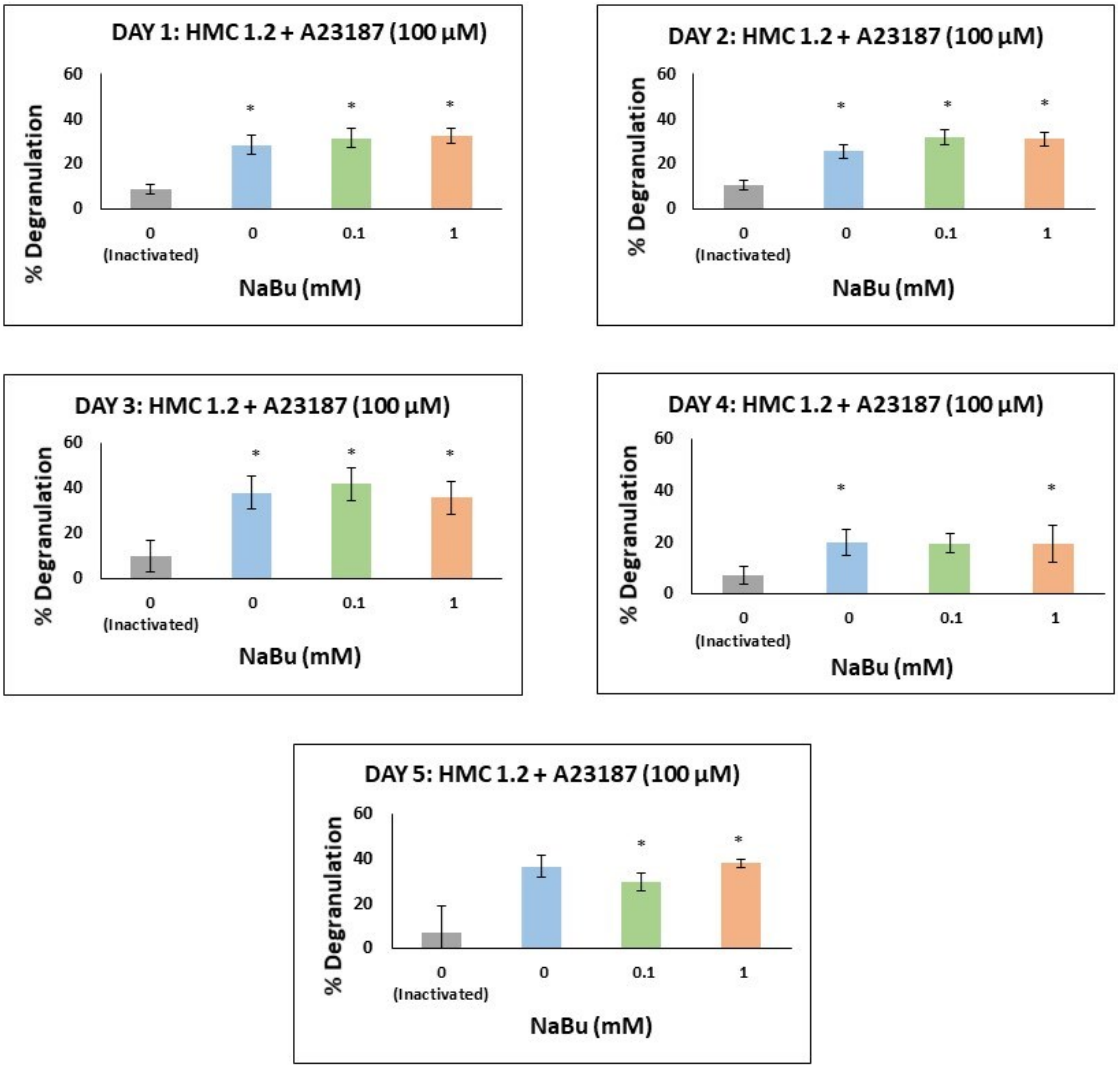

Figure 2.6 NaBu has no significant effect on HMC-1.2 Cell Degranulation through A23187. *HMC-1.2 cells were exposed to different concentrations of NaBu (0, 0.1, and 1 mM) for 5 days and were activated with A23187 (100 mM). β-hexosaminidase release was measured to evaluate degranulation (n = 7 for days 1 to 3 and n=3 for days 4 and 5). The student's t-test was performed to determine the statistical significance (*p < 0.01) relative to the 0 (inactivated) samples.*

2.4 Discussion

2.4.1 NaBu Inhibits Proliferation but not Viability at lower Concentrations

NaBu's ability to inhibit cell proliferation without significantly affecting cell viability at 0.1 and 1 mM concentrations (Figure 2.3) highlights its potential as a selective therapeutic agent. The observed concentration-dependent inhibition aligns with previous studies where NaBu has demonstrated anti-proliferative effects in various cancer cell lines (Davie, 2003; Milani et al., 2022). The mechanism primarily involves the induction of cell cycle arrest through the upregulation of cyclin-dependent kinase inhibitors and the downregulation of cyclins and cyclin-dependent kinases (CDKs) (Archer et al., 2017). These findings suggest that NaBu can modulate cell growth by altering the expression of genes involved in cell cycle regulation.

2.4.2 High Concentration of NaBu reduces Metabolic Activity of HMC 1.2

Metabolic activity assays indicated that 1mM NaBu-treated cells maintained normal metabolic functions similar to the Untreated cells, as evidenced by consistent XTT assay results over a 3-day time period (Figure 2.4). However, it is observed that 10 mM NaBu-treated cells have lower metabolic activity over 1 day period and show a decrease in cell viability by days 2 and 3.

2.4.3 NaBu and Cell Viability

The retention of cell viability despite the inhibition of proliferation suggests that NaBu does not induce cytotoxic effects at the concentrations tested. This characteristic is particularly advantageous for

therapeutic applications, as it implies that NaBu can suppress the growth of unwanted proliferative cells, such as in cancer, without damaging healthy cells. Previous studies have shown that NaBu can induce differentiation and apoptosis in cancer cells while sparing normal cells, thus supporting its role in selective cancer therapy (Tang et al., 2017).

2.4.4 NaBu and MRGPRX2-Induced Degranulation

One of the critical findings of this study is that NaBu does not affect MRGPRX2 (C48/80) or calcium ionophore (A23187) induced degranulation of HMC-1.2 cells (Figure 2.5 and Figure 2.6). The concentrations of C48/80 (100 µg/mL) and A23187 (100 mM) were chosen based on the data in Figures A and B in the Appendices section, where the most degranulation was observed at these concentrations. The lack of effect on MRGPRX2-induced degranulation suggests that NaBu can be used therapeutically without compromising essential immune functions mediated by MCs. This finding is consistent with other studies that have shown selective modulation of immune responses by NaBu (Babina et al., 2022).

2.5 Conclusion

The results of this study suggest that NaBu does not modulate MRGPRX2-mediated activation in HMC 1.2 cells, indicating that the expected degranulation and inflammatory responses were not influenced by this compound. One possible explanation for these findings is that the mechanism by which NaBu exerts its anti-inflammatory effects might not involve direct interaction with MRGPRX2 or its downstream signaling pathways. Instead, NaBu might predominantly act on other receptors or through systemic metabolic pathways that were not the focus of this study (Hermans et al., 2021). Additionally, the cellular context and specific receptor environment in HMC 1.2 cells might limit the efficacy of NaBu, as different

cell types and receptors can exhibit varying sensitivity to HDAC inhibition and short-chain fatty acids (M. Li et al., 2018; Zou et al., 2021). Other than this, NaBu did inhibit cell proliferation without affecting viability, which supports existing studies (Qiu et al., 2017). These results highlight the necessity for further investigation to delineate the precise pathways and conditions under which NaBu can modulate immune cell function and receptor-mediated responses (Kushwaha et al., 2022; Siddiqui & Cresci, 2021; Xi et al., 2021).

Chapter 3: Calcium Phosphate ($Ca_3(PO_4)_2$) Inhibits Cell Proliferation Without Affecting Viability and Has No Significant Effect on MRGPRX2-Induced Degranulation in Transformed Human Mast Cells

3.1 Introduction

3.1.1 Chemical Structure of $Ca_3(PO_4)_2$

Calcium phosphate ($Ca_3(PO_4)_2$) is a calcium salt of phosphoric acid and exists in various forms, including tricalcium phosphate and hydroxyapatite (Mishchenko et al., 2023; Yasar et al., 2021). The structure of $Ca_3(PO_4)_2$ can also include hydroxide (OH^-) and carbonate (CO_3^{2-}) ions in some forms, such as hydroxyapatite and amorphous calcium phosphate (ACP) (Mishchenko et al., 2023; Yasar et al., 2021). In MC, $Ca_3(PO_4)_2$ plays a crucial role in the regulation of calcium mobilization, which is essential for various cellular functions, including degranulation and cytokine production. The mobilization of calcium ions in MCs is tightly regulated by store-operated calcium entry (SOCE) mechanisms, involving the interaction of stromal interaction molecule 1 (STIM1) and Orai1 channels, which are critical for maintaining calcium homeostasis and facilitating MC activation in response to stimuli (Holowka et al., 2012; Rivera et al., 2008). Recent studies have highlighted the importance of calcium signaling in MC functions, emphasizing the role of $Ca_3(PO_4)_2$ in these processes (Hong et al., 2022; Liu et al., 2023).

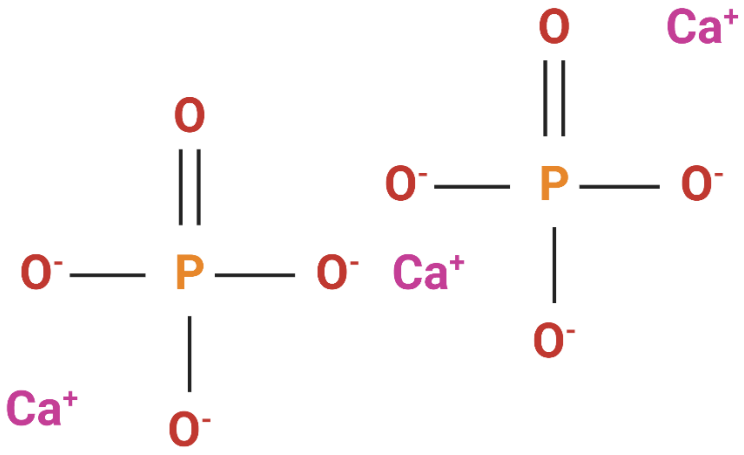

Figure 3.1 Structure of $Ca_3(PO_4)_2$.

3.1.2 The Multifaceted Role of $Ca_3(PO_4)_2$ in Mast Cell Function and Disease

$Ca_3(PO_4)_2$ plays a significant role in the regulation of Mast cell (MC) functions, particularly in the context of inflammatory diseases such as uremia and psoriasis. In uremia, elevated calcium levels have been linked to increased itching intensity, suggesting a connection between $Ca_3(PO_4)_2$ metabolism and MC activity, although skin MC numbers did not significantly differ between uremic patients and controls (Knapp et al., 2023). In psoriasis, MCs contribute to the pathophysiology by releasing pro-inflammatory cytokines like IL-17A, which are crucial for the recruitment of other immune cells and the perpetuation of inflammation. Calcium signaling is essential for MC degranulation and cytokine release, highlighting the importance of $Ca_3(PO_4)_2$ in these processes (R. M. Wang et al., 2023). Additionally, $Ca_3(PO_4)_2$ role extends to bone diseases, where it influences osteogenesis and immune responses. For instance, nano-scaled biphasic calcium phosphate (BCP) materials have shown promise in modulating osteogenesis and

attenuating inflammation induced by lipopolysaccharide (LPS) in macrophages, suggesting potential clinical applications for bone tissue repair (Su et al., 2023). Furthermore, $Ca_3(PO_4)_2$ cements are actively resorbed by macrophages and osteoclasts, which regulate bone regeneration during normal homeostasis and fracture healing (Lukina et al., 2023). In chronic kidney disease (CKD), vascular calcification (VC) is a common complication, and it has been established that VC is not merely a passive process of $Ca_3(PO_4)_2$ deposition but an actively regulated and cell-mediated process, sharing similarities with bone formation (Ding et al., 2023). Recent studies have also elucidated that phosphate regulates fibroblast growth factor 23 (FGF23) in bone, affecting phosphate homeostasis and contributing to CKD-related mineral and bone disorders (CKD-MBD) (Mace & Lewin, 2023). The interplay between $Ca_3(PO_4)_2$ and MCs, particularly in inflammatory and bone-related conditions, underscores the potential for targeted therapies that modulate calcium homeostasis to treat these diseases.

3.2 Methods

3.2.1 Cell lines used (HMC 1.2) and their Maintenance

HMC-1.2 cells were maintained the same as described in Section 2.2.1 of Chapter 2.

3.2.2 $Ca_3(PO_4)_2$ Treatment

HMC 1.2 cells were grown at a density of 5 x 10^5 cells in 5 mL of IMDM medium. They were exposed to different concentrations of $Ca_3(PO_4)_2$ (0, 0.1, 1, 5, and 10 mM) prepared from a $Ca_3(PO_4)_2$ stock solution in PBS. The culture was incubated for 24 hours.

3.2.3 Cell Proliferation and Viability Assessed Using the Trypan Blue

Exclusion Method

HMC-1.2 cells were seeded at 1×10^5 cells/mL density and incubated with 0, 0.1, 5 and 10 mM $Ca_3(PO_4)_2$ for 24 hours. Cell counts were performed post-treatment using a hemocytometer with trypan blue dye to stain dead cells. The same formula was used for Cell Proliferation and Cell viability calculations, as indicated in Section 2.2.3 of Chapter 2.

3.2.4 β-hexosaminidase Assay

HMC-1.2 cells were treated with $Ca_3(PO_4)_2$ at concentrations of 0.1, 1, 5, and 10 mM for 24 hours to examine its effect on degranulation by activating them by C48/80 (). The same procedure was followed as mentioned in section 2.2.5 of Chapter 2.

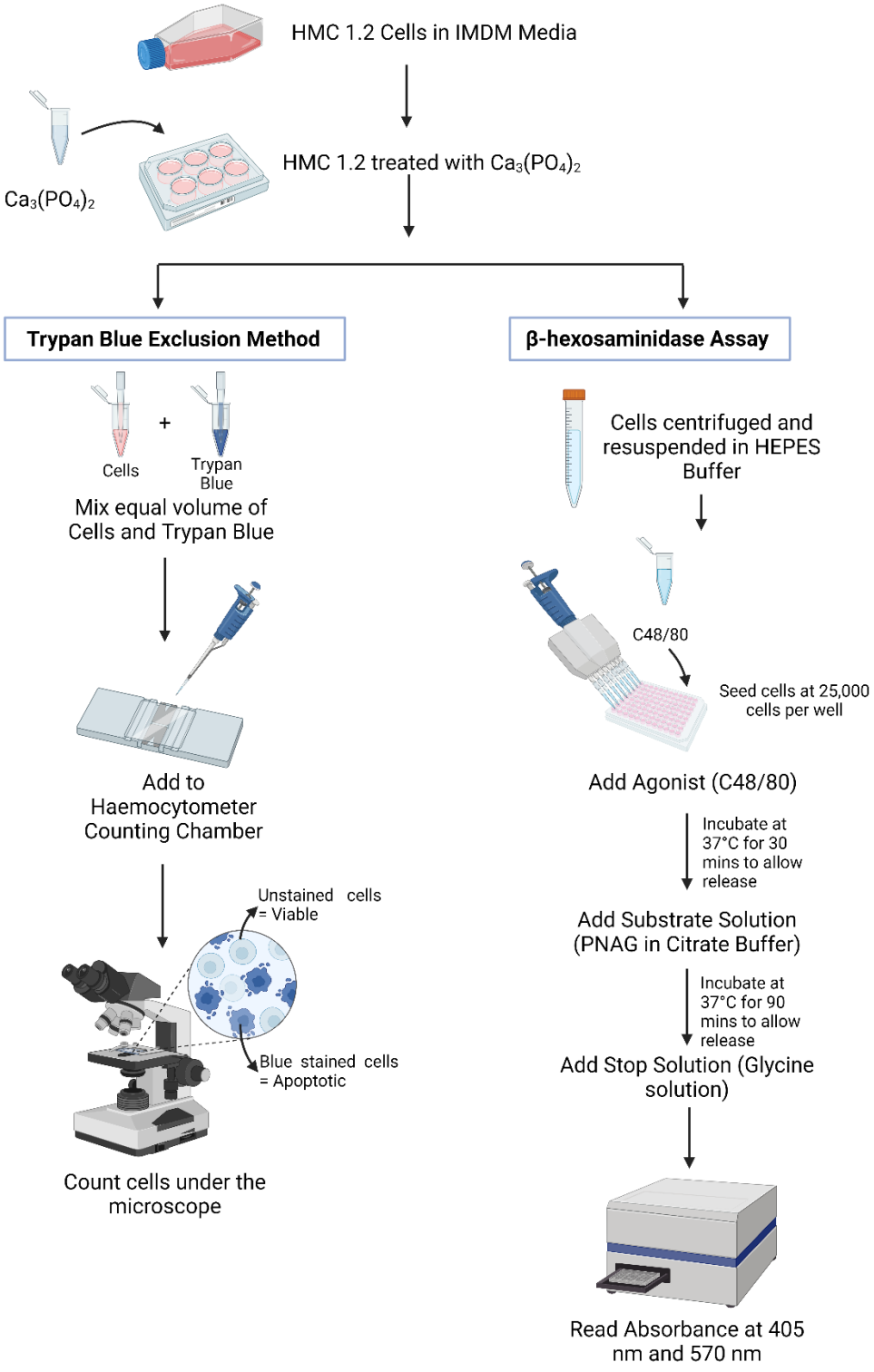

Figure 3.2: Schematic of all methods for $Ca_3(PO_4)_2$ effects on MCs. (Created with BioRender.com)

3.3 Results

3.3.1 Cell Proliferation Post- $Ca_3(PO_4)_2$ Treatment

HMC 1.2 cells treated with $Ca_3(PO_4)_2$ exhibited a dose-dependent decrease in cell proliferation over a 24-hour period, compared to untreated (0 mM) control cells (Figure 3.3). Specifically, at concentrations of 0.1 and 1 mM $Ca_3(PO_4)_2$, cell growth was reduced by 20%. This inhibitory effect on cell proliferation became more pronounced at higher concentrations, with a 25% reduction observed at 5 mM and a significant 44% decrease at 10 mM $Ca_3(PO_4)_2$ in comparison with the untreated control. These results indicate that higher concentrations of $Ca_3(PO_4)_2$ impede cell proliferation, suggesting a concentration-dependent cytostatic effect on HMC 1.2 cells. The findings underscore the potential impact of $Ca_3(PO_4)_2$ on cell growth regulation, highlighting its role in modulating cellular proliferation at varying concentrations.

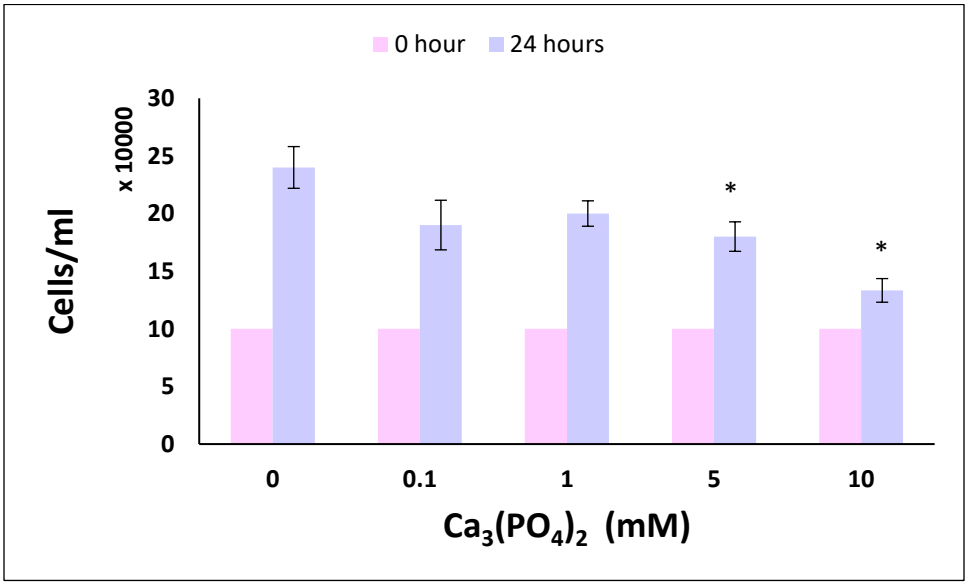

Figure 3.3 The effect of $Ca_3(PO_4)_2$ treatment on cell proliferation over 24 hours. *Cells were treated with $Ca_3(PO_4)_2$ at a concentration of 0,0.1,1,5 and 10 mM, and viability was assessed at 24 hours post-treatment. The students' t-test was performed to determine the statistical significance (*p < 0.05, **p < 0.01, ***p < 0.001) relative to the 0 (Inactivated) samples.*

3.3.2 Cell Viability Post- $Ca_3(PO_4)_2$ Treatment

HMC 1.2 viability after being treated with varying concentrations of $Ca_3(PO_4)_2$ exhibited a slight reduction with increasing concentration; however, this decrease was not significant, with cell viability consistently remaining over 90%. This high level of viability indicates that the cells maintained their viability throughout the treatment period, even at higher concentrations of $Ca_3(PO_4)_2$.

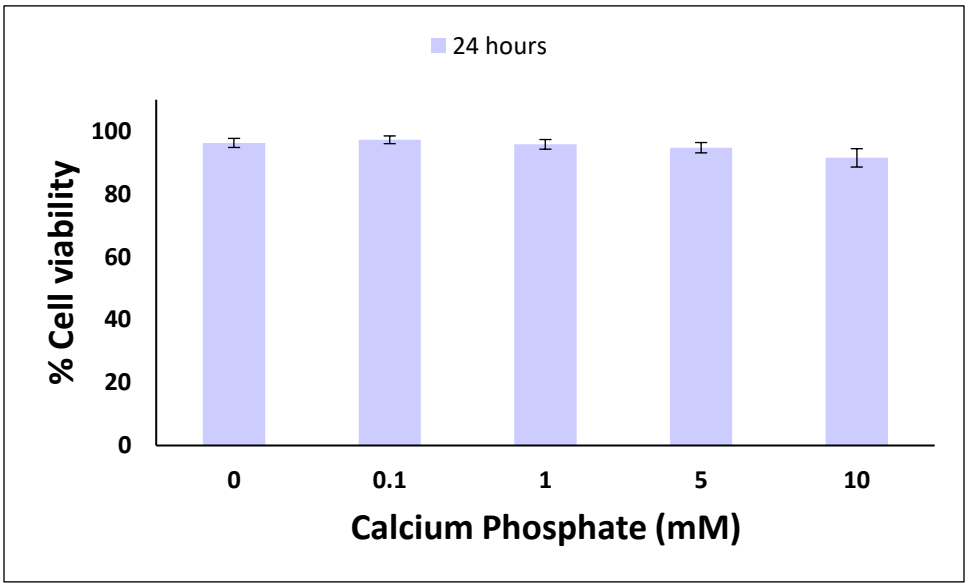

Figure 3.4 The effect of $Ca_3(PO_4)_2$ treatment on viability over 24 hours. Cells were treated with $Ca_3(PO_4)_2$ at a concentration of 0, 0.1, 1, 5 and 10 mM, and viability was assessed at 24 hours post-treatment.

3.3.3 Responsiveness of Mast Cells to Degranulation by $Ca_3(PO_4)_2$ Effect

Experiments where HMC-1.2 cells were treated with NaBu and subsequently stimulated with either C48/80 (as shown in Figure 3.5) confirmed that Ca3(PO4)2 does not significantly affect degranulation through MRGPRX2 activation. These experiments showed no significant changes in the release of β-hexosaminidase, an enzyme commonly used as a marker for degranulation. In the provided graphs, "0 (Inactivated)" refers to untreated HMC-1.2 cells that were not subjected to activation by C48/80 (Figure 2.5) or A23187 (Figure 2.6), serving as baseline controls. The "0" condition represents untreated cells that were activated by either C48/80 or A23187, illustrating the typical response to these stimuli. For the conditions labelled 0.1, 1, 5, and 10, HMC-1.2 cells were treated with $Ca_3(PO_4)_2$ (mM) with being activated by C48/80 or A23187. Despite these treatments, no significant differences were observed in the levels of β-hexosaminidase release compared to the untreated, activated controls.

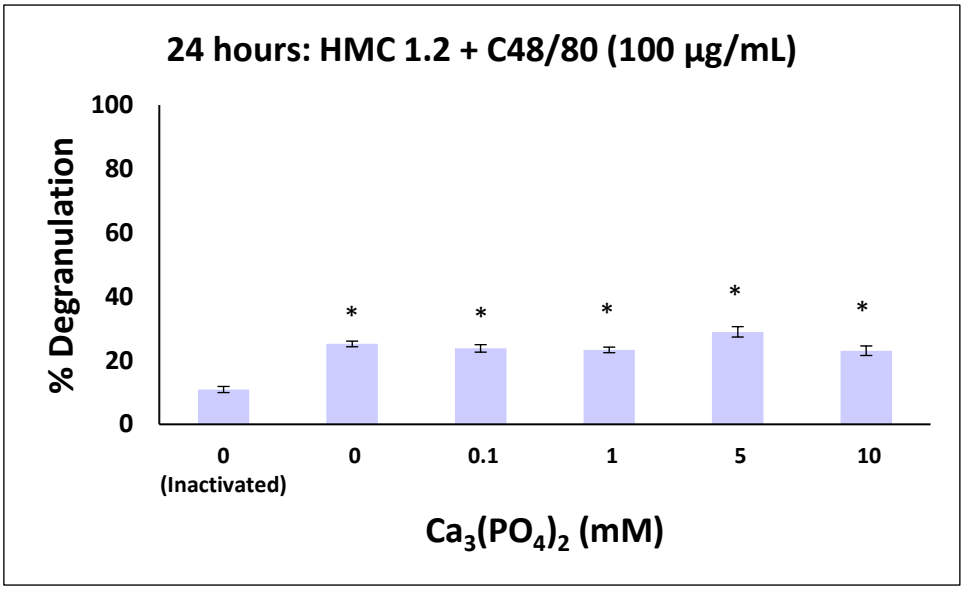

Figure 3.5: $Ca_3(PO_4)_2$ has no significant effect on HMC-1.2 Cell Degranulation through MRGPRX2 (C48/80). HMC-1.2 cells were exposed to different concentrations of $Ca_3(PO_4)_2$ (0, 0.1, 1, 5, and 10 mM)

for 24 hours and were activated with C48/80 (100 µg/mL). β-hexosaminidase release was measured to evaluate degranulation (n = 3). The students' t-test was performed to determine the statistical significance (*$p < 0.01$).

3.4 Discussion

3.4.1 $Ca_3(PO_4)_2$ Inhibits HMC 1.2 Cell Proliferation in a Dose-Dependent Manner without affecting viability.

Recent studies have shown that $Ca_3(PO_4)_2$ can influence cell proliferation, differentiation, and metabolic activity in various cell types. Calcium ions, a key component of $Ca_3(PO_4)_2$, play a crucial role in cellular signaling pathways essential for regulating cell growth and function (DerMardirossian, 2024). Calcium signaling is involved in gene expression, enzyme activity, and cell cycle progression, which could explain the observed modulation of cell proliferation by $Ca_3(PO_4)_2$ (Feng et al., 2023). Research found that higher concentrations of $Ca_3(PO_4)_2$ reduced proliferation in osteoblast-like cells, similar to the dose-dependent decrease observed in HMC 1.2 cells (Z. Wang et al., 2023).

Another study showed that $Ca_3(PO_4)_2$ nanoparticles could induce cell cycle arrest and apoptosis in human mesenchymal stem cells (hMSCs), supporting the notion that $Ca_3(PO_4)_2$ has cytostatic effects that are not cell-type specific (Jin et al., 2023). These findings indicate that $Ca_3(PO_4)_2$ can modulate cell proliferation in a dose-dependent manner, with potential applications as a cytostatic agent in various biomedical fields. However, it is important to note that the effects of $Ca_3(PO_4)_2$ on cell behavior can vary depending on factors such as particle size, surface properties, and the specific cell type being studied (Gou et al., 2024).

3.4.2 $Ca_3(PO_4)_2$ and MRGPRX2-Induced Degranulation

The findings from these experiments suggest that $Ca_3(PO_4)_2$ does not play a significant role in MRGPRX2-mediated degranulation. The lack of significant effect by $Ca_3(PO_4)_2$ on MRGPRX2-induced degranulation observed in this study could be due to several factors. One possibility is that the intracellular signaling pathways activated by MRGPRX2 do not require additional extracellular calcium influx provided by $Ca_3(PO_4)_2$. This hypothesis is supported by studies indicating that MRGPRX2 activation primarily triggers G protein-dependent pathways leading to degranulation, which might not be heavily reliant on extracellular calcium sources (Lerner et al., 2024; Schulman et al., 2023). Overall, these findings contribute to a nuanced understanding of the factors influencing mast cell degranulation *via* MRGPRX2.

3.5 Conclusion

The hypothesis that $Ca_3(PO_4)_2$ would affect MRGPRX2-mediated activation of HMC 1.2 cells, thereby influencing their degranulation and inflammatory responses, was based on previous research indicating that $Ca_3(PO_4)_2$ has been shown to modulate immune responses and influence cell signaling pathways (Brun et al., 2023; Diarimalala et al., 2023). Calcium ions, a key component of $Ca_3(PO_4)_2$, are known to play a crucial role in cellular signaling, including in the activation and function of immune cells (van Dijk et al., 2023). Furthermore, $Ca_3(PO_4)_2$ has been reported to induce the activation of inflammasomes and influence cytokine production (J. Yao et al., 2024), suggesting potential interactions with MC receptors like MRGPRX2. Given these roles, it was hypothesized that $Ca_3(PO_4)_2$ could modulate MRGPRX2-mediated pathways, affecting MC degranulation and inflammatory responses. However, the results demonstrated that $Ca_3(PO_4)_2$ did not significantly affect the activation or degranulation of HMC 1.2 cells

via MRGPRX2, indicating that its effects might be limited or non-specific to this particular receptor and cell type.

Chapter 4: Summary and Future Research Directions

4.1 Key Findings

4.1.1 NaBu and Mast Cell Modulation: Insights and Future Directions

Sodium Butyrate (NaBu) is well-documented for its role in inhibiting HDACs, leading to increased acetylation of histones, which in turn influences gene expression profiles toward an anti-inflammatory state (Chriett et al., 2019; Davie, 2003). However, the specific interaction between NaBu and MRGPRX2 has not been extensively studied. NaBu did not affect MRGPRX2-mediated activation in HMC 1.2 cells, suggesting a complex interplay of factors governing its efficacy. Further research is required to elucidate these interactions, particularly focusing on different immune cell types and receptor-specific pathways, to fully understand the potential therapeutic applications of NaBu in inflammatory diseases.

4.1.2 $Ca_3(PO_4)_2$ and Mast Cell Modulation: Insights and Future Directions

$Ca_3(PO_4)_2$ does not induce MRGPRX2-mediated activation of HMC 1.2 cells. One possible explanation for these results is that the pathways $Ca_3(PO_4)_2$ affects might not involve direct modulation of MRGPRX2 or its downstream signaling. Instead, $Ca_3(PO_4)_2$ could be acting through other cellular mechanisms or receptors that were not the focus of this study (Amantini & Morelli, 2023). Additionally, the specific cellular context and receptor environment in HMC 1.2 cells might limit the ability of $Ca_3(PO_4)_2$ to influence MRGPRX2-mediated responses. The intricate signaling networks and cellular heterogeneity within different immune cell types could result in varying sensitivity to $Ca_3(PO_4)_2$ (Su et al., 2023). We also observed a decrease in and one possible explanation is that $Ca_3(PO_4)_2$ might influence cellular mechanisms that indirectly affect cell growth and division rather than directly modulating MRGPRX2-mediated pathways. $Ca_3(PO_4)_2$ could be interacting with other cellular receptors or signaling pathways that regulate cell proliferation. Additionally, the specific cellular context of HMC-1.2 cells might inherently limit the ability of $Ca_3(PO_4)_2$ to impact proliferation through MRGPRX2. The complexity and heterogeneity of signaling networks within these immune cells could lead to varying responses to $Ca_3(PO_4)_2$, potentially causing the observed decrease in proliferation. Further research is necessary to elucidate the precise mechanisms and pathways involved. These results highlight the complexity of calcium signaling and its diverse effects on immune cells, suggesting that further research is needed to fully understand the context-dependent interactions and mechanisms involved.

4.1.3 HMC 1.2 as a Model for MRGPRX2-Mediated Degranulation

The HMC-1.2 cell line, while valuable for studying MRGPRX2-mediated degranulation, has several limitations that must be considered. One significant limitation is its poor degranulation capacity compared to other MC lines, such as LAD2 and HuMC. This reduced capacity can hinder the detection of subtle

degranulation effects, necessitating the use of techniques like pre-incubation with Latrunculin B (Lat-B) to enhance degranulation responses (Hermans et al., 2021). Additionally, the high constitutive activity of the KIT receptor in HMC-1.2 cells may negatively influence MRGPRX2-mediated activation, complicating the interpretation of results related to MRGPRX2 functionality (Folkerts, Gaudenzio, et al., 2020; Hermans et al., 2021).

Another limitation is the variability in degranulation kinetics and the higher concentrations of agonists required to induce degranulation in HMC-1.2 cells compared to other cell lines. For instance, C48/80-induced degranulation in HMC-1.2 cells requires significantly higher concentrations and longer stimulation times than in LAD2 cells, which can affect the reproducibility and reliability of experimental outcomes (Hermans et al., 2021). Furthermore, the presence of both V560G and D816V mutations in the KIT gene of HMC-1.2 cells introduces additional complexity, as these mutations confer different growth and signaling characteristics that may not be representative of primary human MCs (Arock et al., 2018; Bandara et al., 2023; Sundström et al., 2003).

Studies have also highlighted the challenge of using HMC-1.2 cells for cytokine production studies, as MRGPRX2 activation in these cells primarily induces degranulation with minimal cytokine release, limiting their utility in comprehensive MC activation studies (Hermans et al., 2021; Z. Wang et al., 2022). Moreover, the high division rate of HMC-1.2 cells, while advantageous for large-scale studies, may lead to genetic drift and phenotypic changes over extended culture periods, potentially affecting experimental consistency (Bandara et al., 2023; Hermans et al., 2021).

To address these limitations, researchers have employed various strategies, such as using higher cell numbers to improve statistical power and employing more sensitive detection methods like single-cell

RNA-Seq or CyTOF to capture subtle degranulation effects (Folkerts, Gaudenzio, et al., 2020). Additionally, the development of new MC lines, such as ROSA KIT D816V, which exhibit more consistent degranulation responses and better represent primary human MC biology, offers alternative models for studying MC function and drug responses (Saleh et al., 2014).

4.2 Implications and Applications

4.2.1 Implications and Significance of the Findings

The findings from the investigation of NaBu and $Ca_3(PO_4)_2$ on MC modulation reveal critical insights into the nuanced roles of these compounds in immune cell function. The most significant effect observed was a decrease in cell proliferation for both compounds, which warrants further exploration. There are several possible mechanisms through which NaBu and $Ca_3(PO_4)_2$ could be exerting this effect. NaBu is known to inhibit histone deacetylases (HDACs), which leads to the expression of anti-inflammatory genes and subsequent reduction in cell proliferation (Anshory et al., 2023). This pathway could involve the inhibition of specific HDAC isoforms that regulate cell cycle progression and apoptosis (Zaiatz-Bittencourt et al., 2023). For instance, HDAC inhibition can lead to the upregulation of cyclin-dependent kinase inhibitors such as p21, causing cell cycle arrest (MacDonald et al., 2023).

Conversely, $Ca_3(PO_4)_2$'s effects on cell proliferation might not involve direct modulation of MRGPRX2-mediated responses. Instead, $Ca_3(PO_4)_2$ could be affecting other signaling pathways or cellular processes

(Su et al., 2023). For example, calcium ions released from $Ca_3(PO_4)_2$ could interact with calcium-dependent signaling pathways that regulate cell proliferation and apoptosis, such as the Ca2+/calmodulin-dependent protein kinase (CaMK) pathway or the calcineurin/NFAT pathway (Wu et al., 2024). The lack of modulation by $Ca_3(PO_4)_2$ on MRGPRX2-mediated responses suggests that its immune modulatory effects might be mediated through these alternative pathways rather than through direct interaction with MRGPRX2. Understanding these mechanisms could provide deeper insights into how these compounds affect immune cell proliferation and function (Hu et al., 2023).

These insights are significant as they expand the understanding of how these compounds can influence immune cell behaviour, potentially leading to new therapeutic strategies for managing inflammatory and allergic conditions. The ability of NaBu to alter gene expression and promote regulatory T-cell differentiation can be particularly beneficial in autoimmune diseases, where immune tolerance is disrupted (Humbert et al., 2019; Zou et al., 2021).

4.2.2 Implications for MRGPRX2 Mediated Responses

The modulation of MCs by NaBu and $Ca_3(PO_4)_2$ has direct implications for the treatment of inflammatory and allergic diseases. MCs play a crucial role in these conditions by releasing histamine and other inflammatory mediators. NaBu's ability to suppress pro-inflammatory cytokine production and promote anti-inflammatory pathways offers a promising approach to mitigating allergic responses and chronic inflammation (Occhiuto et al., 2020; Sarkar et al., 2023).

Furthermore, understanding the limitations of $Ca_3(PO_4)_2$ in modulating MRGPRX2-mediated responses helps refine the approach to developing treatments for allergic diseases. Identifying the precise pathways

and receptors influenced by $Ca_3(PO_4)_2$ can lead to the development of more targeted therapies that leverage its immune-enhancing properties without unintended effects on MC activation (Che et al., 2023; MacDonald et al., 2023).

4.2.3 Broader Implications in Immunity and Diseases

The broader implications of these findings have significant potential across numerous areas of immunity and disease management. By deepening our understanding of how compounds such as NaBu and $Ca_3(PO_4)_2$ influence immune cell functions, researchers can develop innovative therapeutic strategies. These strategies aim to enhance immune tolerance, thereby preventing the immune system from overreacting to harmless substances or the body's own cells, which is a key factor in autoimmune diseases and allergies (MacDonald et al., 2023; Salimi et al., 2017). One of the promising aspects of this research is the potential to reduce chronic inflammation, a common underlying factor in many diseases. Chronic inflammation is linked to a variety of health conditions, including cardiovascular diseases, diabetes, and neurodegenerative disorders (W. Zhang et al., 2023). By leveraging the anti-inflammatory properties of NaBu and $Ca_3(PO_4)_2$, new treatments could be designed to alleviate persistent inflammation, improving patient outcomes and quality of life (Moyse et al., 2022; Ozkan et al., 2023).

Moreover, these findings can lead to better management of allergic diseases, such as asthma and allergic rhinitis, where the immune system's exaggerated response to allergens causes significant health problems. By modulating the activity of immune cells, these compounds could help reduce the severity and frequency of allergic reactions (Henein et al., 2022; Khalifehzadeh & Arami, 2020). The ability to fine-tune the immune response also holds promise for cancer therapy. Inflammation often plays a role in tumor progression, and by reducing inflammation, these compounds might help slow down or even halt the

growth of certain cancers (Tsalamandris et al., 2019; Yuksel et al., 2022). Additionally, enhancing immune tolerance can be crucial in preventing the body from rejecting treatments or transplants, thereby broadening the scope of therapeutic applications (Sarkar et al., 2023; Zhao et al., 2021).

In summary, the implications of understanding how NaBu and $Ca_3(PO_4)_2$ modulate immune responses are vast. They pave the way for developing targeted therapies that can enhance immune tolerance, reduce chronic inflammation, and offer better management of inflammatory, allergic, and possibly even cancerous conditions. This research not only contributes to the scientific knowledge base but also opens up new avenues for improving patient care and treatment outcomes in a variety of diseases (Sorriento & Iaccarino, 2019; M. Wang et al., 2024).

4.3 Future Directions

Future research should prioritize the detailed study of NaBu and $Ca_3(PO_4)_2$ to fully elucidate their therapeutic potential and mechanisms of action. The findings that $Ca_3(PO_4)_2$ does not modulate MRGPRX2-mediated activation in HMC 1.2 cells suggest the need to explore alternative pathways and cellular mechanisms through which $Ca_3(PO_4)_2$ might exert its effects. This could include investigating its impact on specific receptors in mast cells, such as the high-affinity IgE receptor (FcεRI) and the Mas-related G-protein coupled receptor member B2 (MRGPRB2). Expanding research to other MC lines and primary human MCs, as well as other immune cells such as macrophages, dendritic cells, and T lymphocytes, will help clarify the broader immunomodulatory effects of $Ca_3(PO_4)_2$ (J. Li et al., 2024; Minaychev et al., 2024; Su et al., 2023).

Further investigations should also delve into the molecular effects of NaBu across various immune cells to understand its therapeutic potential comprehensively. NaBu has shown promise in modulating immune responses through the inhibition of histone deacetylases (HDACs), leading to altered gene expression and anti-inflammatory effects (Bridgeman et al., 2021; H.-R. Moon & Yun, 2023; Ozkan et al., 2023). Detailed studies should examine how NaBu influences the behavior, activation, and cytokine production of different immune cells in both physiological and pathological conditions. Understanding these interactions will be crucial for optimizing NaBu's use in treating inflammatory and autoimmune diseases (Che et al., 2023; Hua et al., 2024; J. Sun et al., 2024). Long-term studies on the safety and efficacy of NaBu are essential, particularly for chronic treatments. These studies should assess the therapeutic benefits and potential adverse effects or toxicity associated with prolonged use (Bawish et al., 2023; Cleophas et al., 2019). Investigating the synergistic effects of NaBu with other anti-inflammatory or immunomodulatory agents could provide valuable insights into optimizing treatment protocols. This includes exploring combination therapies to enhance efficacy or reduce side effects, which could significantly improve patient outcomes (Shuwen et al., 2023).

Advanced techniques such as single-cell RNA sequencing and proteomics should be employed to provide deeper insights into the specific pathways affected by NaBu and $Ca_3(PO_4)_2$. Single-cell RNA sequencing can reveal gene expression heterogeneity within cell populations and identify specific subsets of cells responding to treatment (McNulty et al., 2023). Proteomics can complement transcriptomic data by identifying changes in protein expression and modifications, highlighting key signaling molecules and pathways modulated by these compounds (Zheng et al., 2023). Integrating these advanced techniques will enable researchers to map out the molecular landscape influenced by NaBu and $Ca_3(PO_4)_2$, leading to the

identification of biomarkers for efficacy and safety and guiding the development of targeted therapeutic strategies (Gutierrez-Martinez et al., 2024).

In summary, a multi-faceted research approach involving detailed cellular studies, long-term safety and efficacy assessments, and advanced molecular techniques is essential to fully elucidate the therapeutic potential of NaBu and $Ca_3(PO_4)_2$. This comprehensive investigation will pave the way for developing more effective and safer treatments for various inflammatory and immune-mediated diseases.

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Appendices

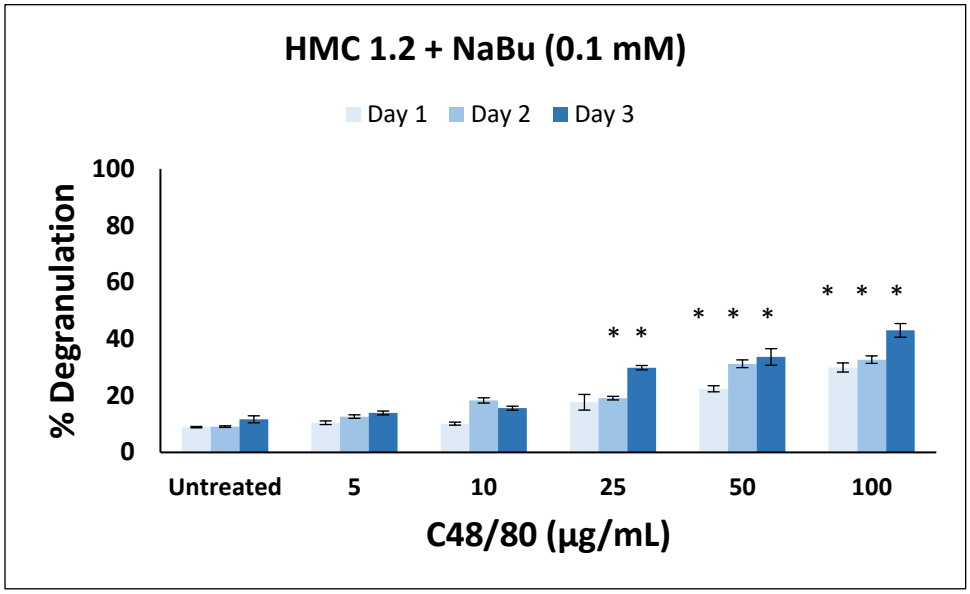

Figure A: C48/80 shows the highest degranulation at 100 µg/mL concentration: *HMC-1.2 cells were exposed to NaBu (0.1 mM) for 3 days and were activated with different concentrations of C48/80 (5, 10, 25, 50 and 100 µg/mL). β-hexosaminidase release was measured to evaluate degranulation (n = 4). The student's t-test was performed to determine the statistical significance (*p < 0.01) relative to the untreated samples.*

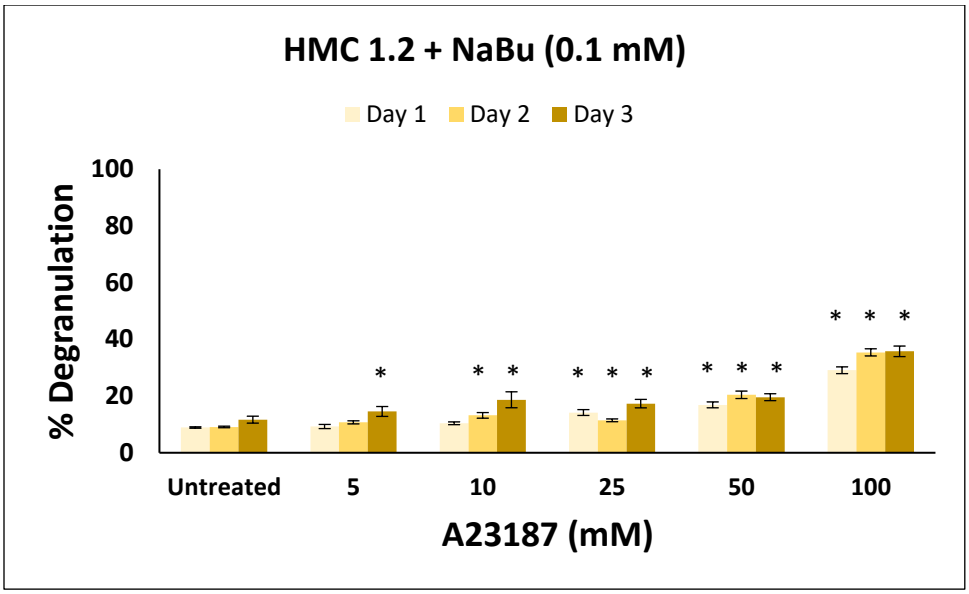

Figure B: A23187 shows the highest degranulation at 100 µg/mL concentration: *HMC-1.2 cells were exposed to NaBu (0.1 mM) for 3 days and were activated with different concentrations of A23187 (5, 10, 25, 50 and 100 mM). β-hexosaminidase release was measured to evaluate degranulation (n = 4). The student's t-test was performed to determine the statistical significance (*p < 0.01) relative to the untreated samples.*

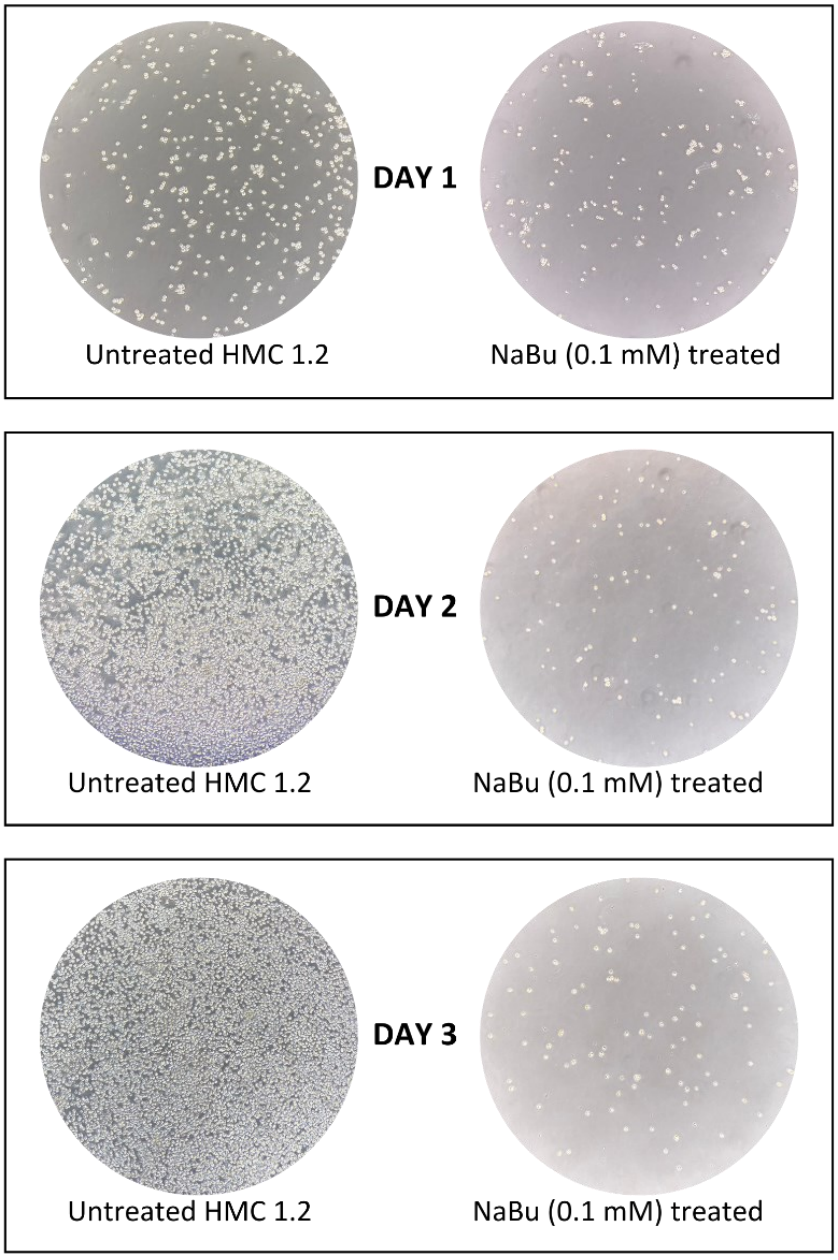

Figure C: Comparison of Untreated and NaBu-Treated Cells Over 3 Days. *This figure illustrates the morphological differences between untreated cells and those treated with 0.1 mM NaBu over a three-day period. Images of both untreated and NaBu-treated cells were captured daily using a microscope to monitor changes.*