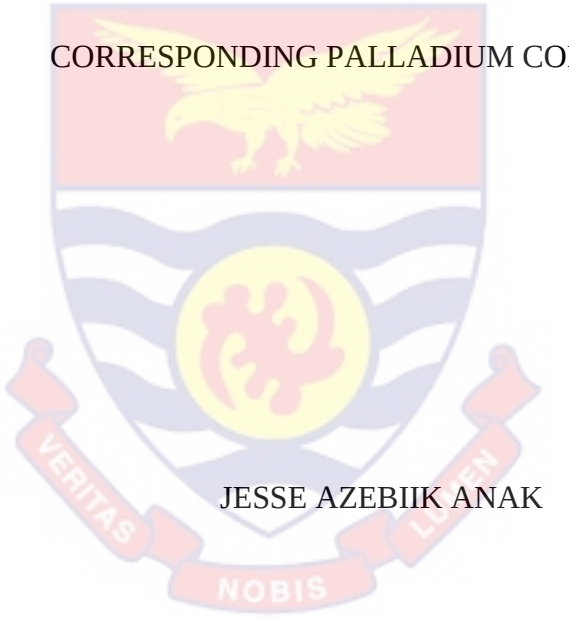
UNIVERSITY OF CAPE COAST

MODIFIED SULFONAMIDES AS ALTERNATIVE ANTIBACTERIALS: ACTIVITY OF NOVEL PYRAZOLE SULFONAMIDES AND THEIR CORRESPONDING PALLADIUM COMPLEXES



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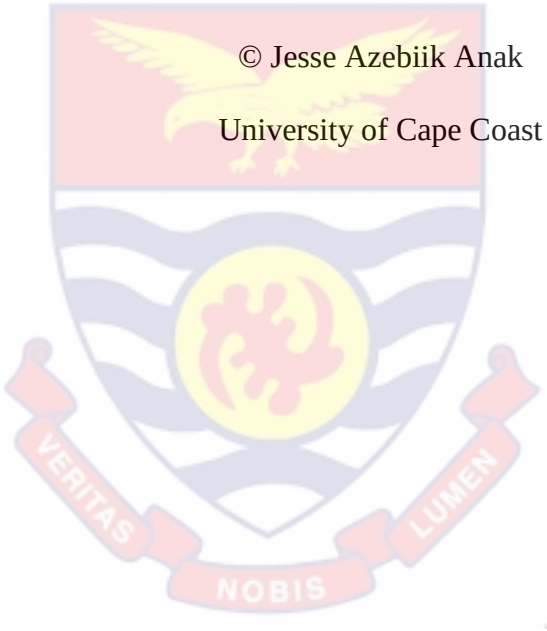

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BY

JESSE AZEBIIK ANAK

Dissertation submitted to the Department of Microbiology and Immunology of the School of Medical Sciences, College of Health and Allied Sciences, University of Cape Coast in partial fulfilment of the requirements for the award of Master of Philosophy degree in Infection and Immunity

NOVEMBER 2023

DECLARATION

Candidate's Declaration

I do hereby declare that this dissertation is the result of my own original research and that no part of it has been presented for another degree in this university or elsewhere.

Candidate's Signature: …… Date: …….

Name: Jesse Azebiik Anak

Supervisor's Declaration

I hereby declare that the preparation and presentation of the dissertation were supervised in accordance with the guidelines on supervision of dissertation laid down by the University of Cape Coast.

Principal Supervisor's Signature: …… Date ……

Name: Dr. Ewura Seidu Yahaya

Co-Supervisor's Signature: ……... Date ……

Name: Dr. Roland Osei Saahene

ABSTRACT

Antibiotic resistance has emerged as a global health emergency, necessitating the need for the development of novel drugs. This study evaluated antibacterial activity of pyrazolyl sulfonamide compounds and their palladium complexes. All compounds were coded. Broth microdilution method was utilized to determine minimum inhibitory and bactericidal concentrations (MIC and MBC). Whereas the compounds demonstrated antibacterial activity against *Staphylococcus aureus, Staphylococcus hominis, Staphylococcus haemolyticus, Staphylococcus epidermidis, and Corynebacterium striatum*, no activity was observed against *Staphylococcus cohnii* and *Enterococcus faecalis*. Compound CA11B, a derivative of N, N-tetramethyl sulfonamide, demonstrated the strongest antibacterial activity against *S. aureus, S. hominis,* and *S.haemolyticus* (MIC = 0.5 – 8 µg/mL). Although most of the test compounds were active against at least two organisms, the sulfamoyl-phenyl derivatives (CA45A/B), dichloro-palladium (CA50A/B/D), and dimethyl-phenyl sulfonamides (CA-032) derivatives were narrow spectrum in activity. When CA11B was combined with the positive control antibiotic (tetracycline), an antagonistic effect was observed in *S. haemolyticus and S. hominis.* Interestingly, all other compounds were synergistic with tetracycline when combined in the tested organism, except CA50B and CA45A, which were indifferent and antagonistic, respectively. The time-kill assays performed on synergistic combinations showed that CA45B, CA-O32, CA47, CO-3, CA48A at concentrations ≥ MIC, achieved 3 log10 CFU/mL colony reduction of *S. haemolyticus.* Similar trend was observed for CA50A, and CA50D in *S. hominis*. The time-kill kinetics results in this study showed the potent inhibitory and bactericidal activity of the tested compounds. In conclusion, the compounds showed promising potency against the different resistant strains tested. It is recommended that these compounds be further assessed for their toxicological effects and tested against a diverse array of bacteria to further ascertain their efficacy.

KEY WORDS

Antibacterial activity

Antimicrobial resistance

Palladium

Pyrazole

Sulphonamide derivatives

Synergistic activity

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DEDICATION

To my family, relatives, and friends.

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LIST OF ACRONYMS

Antimicrobial resistance	AMR
Corynebacterium striatum	*C. striatum*
Dihydropteroate synthase	DHPS
Enterococcus faecalis	*E. faecalis*
Para-aminobenzoic acid	PABA
Staphylococcus aureus	*S. aureus*
Staphylococcus cohnii	*S. cohnii*
Staphylococcus epidermidis	*S. epidermidis*
Staphylococcus haemolyticus	*S. haemolyticus*
Staphylococcus hominis	*S. hominis*

CHAPTER ONE

INTRODUCTION

The problem of antibiotic resistance is becoming an increasingly severe threat to public health. As a result, the development of innovative antimicrobial medications for treating bacterial infections has become very necessary. Sulfonamides are a class of antibiotics that have been used for a very long time to treat a wide variety of bacterial infections, including those of the urinary tract, the respiratory system, and the skin (Frieri, Kumar, & Boutin, 2017). The rise in antibiotic-resistant bacteria strains is making it harder to treat infections with common antibiotics like sulfonamides (Frieri et al., 2017). To combat the problem of antibiotic resistance, it has become necessary to research and create new antimicrobial agents. The modification of the chemical structure of existing antibiotics is one field being investigated as part of the pursuit of new antimicrobials. The study's primary objective was to assess the therapeutic potential of some novel structurally modified sulfonamide compounds against confirmed resistant bacterial pathogens.

Background of the Study

The transformative impact of antibiotics in the field of medicine within less than a century deserves careful consideration. Before the advent of the initial widely beneficial antibiotics in the mid-1930s, the average lifespan was considerably shorter, with infectious and associated disorders serving as the primary contributors to mortality. The introduction of antibiotics significantly reversed this trend (Surette & Wright, 2017). In a significant milestone, it can be observed that humans have reached a point in their history where the likelihood of mortality due to chronic diseases associated with advanced age,

such as cancer and cardiovascular ailments, has surpassed that of infectious diseases. Preventive strategies aimed at enhancing public health and facilitating vaccination accessibility have also been substantially influenced (Szydlowski & Luliak, 2020).

Nevertheless, antibiotics are the most productive approach for addressing acute infections. Antibiotics allow healthcare professionals to carry out procedures that were previously considered unfeasible without compromising infection control measures. Antibiotics have become integral in facilitating critical surgical procedures, such as cancer chemotherapy, organ transplantation, and other routine medical interventions. These anti-infective agents play a pivotal role in contemporary medical practise (Surette & Wright, 2017).

The growing epidemic of antibiotic resistance is emerging as a major threat to public health as a result of the spread of antibiotic-resistant traits among a wide variety of well-known pathogens, the shortage of new therapeutics discoveries, and the proliferation of new mechanisms of resistance (Adu-Oppong, Gasparrini, & Dantas, 2017; Liu, Wang, Walsh, Yi, Zhang, Spencer, Doi, Tian, Dong, & Huang, 2016). Sulfonamides are synthetic antimicrobial drugs extensively utilized in human and animal medicine (Kim, Thawng, Lee, Wellington, & Cha, 2019). The widespread utilization of sulfonamides on a global scale has resulted in environmental contamination. It poses a significant risk to public health due to the possible emergence and spread of antibiotic resistance (Kim et al., 2019).

Sulfonamides are organo-sulphur compounds with the $-SO_2NH_2$ and/or $-SO_2NH-$ group. These structures indicate the presence of the

sulfanilamide group and unique 6- or 5-membered heterocyclic rings. Sulfonamides (SNs) have limited biodegradability and can induce a range of adverse consequences, such as ailments affecting the gastrointestinal and respiratory systems (Sultan, 2015). According to Mathews et al., (2015), certain non-allergic symptoms might occur due to SN medication usage. These side effects include diarrhea, dizziness, nausea, vomiting, candidiasis, folate insufficiency, and migraines.

The administration of SN medications in high quantities has the potential to induce a potent allergic response, characterized by the occurrence of two particularly severe conditions known as Stevens-Johnson syndrome and toxic epidermal necrolysis (Shah, Moshirfar, & Hoopes, 2018). The prevalence of problems with medication related to sulfonamide intolerance is estimated to range from 3% to 8%, which is similar to the incidence observed for penicillin (Giles, Foushee, Lantz, & Gumina, 2019; Warrington, Silviu-Dan, & Wong, 2018). One crucial factor influencing allergic reactions is the substitution at the N4 arylamine group position, as seen in compounds like sulfamethoxazole, sulfasalazine, and sulfadiazine. This substitution generally increases the likelihood of an allergic response by promoting the formation of reactive metabolites, which can bind to proteins and trigger immune responses. Such substitutions are associated with heightened hypersensitivity, making compounds with an N4 arylamine group more likely to provoke allergic reactions (Dibbern Jr & Montanaro, 2008). According to Giles et al. (2019) and Khan, Knowles, and Shear (2019), it has been observed that non-arylamine-containing SN medicines generally do not elicit an allergic response, suggesting their potential for safe consumption. Depending on

whether or not they trigger an allergic reaction in the user, sulfonamides (SNs) are either (i) antibacterial (containing an aromatic amine) or (ii) nonantibacterial (lacking such an amine) (Igwe & Okoro, 2014; Yousef, Mansour, & Herbali, 2018; Zawodniak, Lochmatter, Beeler, & Pichler, 2010).

The medications derived from SN that have been developed thus far include sulfamethazine, sulfadiazine, sulfamethoxazole, sulfasalazine, sulfisoxazole, sulfamerazine, sulfadimethoxine, sulfafurazole, and sulphanilamide (Supuran, 2017). One of the SN compounds, known as sulphanilamide, was initially synthesized in 1906 but did not find application as an antibacterial drug until the late 1930s (Fernández-Villa, Aguilar, & Rojo, 2019). Sulfamethazine (SMZ) and sulfadiazine (SDZ) are aromatic amine-containing derivatives of the sulfonamides category of antibacterial medicines.

In veterinary medicine, the antibacterial agents SMZ and SDZ are frequently used to manage livestock infections, including those that affect the gastrointestinal and respiratory systems (Rama, Lucatello, Benetti, Galina, & Bajraktari, 2017). The utilization of SMZ in livestock diets or feed additives has been employed as a means to enhance the growth of livestock (Awaisheh, Khalifeh, Rahahleh, Ja'far, & Algroom, 2019; Chattopadhyay, 2014). In contrast, SDZ is predominantly employed to treat infection resulting from burn injuries (Dan et al., 2013). The combination of SDZ with the anti-malarial medication pyrimethamine has been utilized to treat toxoplasmosis in mammals (Eshghi et al., 2011). Multiple studies have been conducted on the environmental impact, antibacterial properties, and interactions with certain bio-macromolecules of SN, SMZ, and SDZ (Abdul Qadir, Ahmed, Aslam,

Waseem, & Shafiq, 2015; Bendjeddou, Abbaz, Ayari, Benahmed, Gouasmia, & Villemin, 2016; Biošić, Mitrevski, & Babić, 2017).

Sulfonamides represent a crucial category of antibiotic medications that exhibit a broad spectrum of efficacy, particularly against gram-positive bacteria and some Gram-negative organisms (White & Cooper, 2005). Several gram-negative bacteria, such as *Klebsiella sp*, *Salmonella sp*, *Escherichia coli*, and *Enterobacter sp*, are susceptible to sulfonamides. However, it has been observed that sulfonamides do not exhibit inhibitory effects against *Pseudomonas aeruginosa* and Serratia species, indicating the presence of bacterial resistance (Lavanya, 2017). Sulfonamides are effective in the treatment of various conditions, including tonsillitis, septicemia, meningococcal meningitis, bacillary dysentery, and multiple infections of the urinary tract (Wiedemann, Heisig, & Heisig, 2014). According to McFarland et al. (2016), sulfonamides have been found to exhibit inhibitory effects on some fungi, such as *Pneumocystis carinii*, as well as protozoa, including Toxoplasma and Coccidia. Several studies have documented the antibacterial properties of sulfonamide medicines, specifically sulfamethazine and sulfadiazine (Peng, Gao, Yang, Li, Lu, & Yang, 2020; Reddy, Rao, Chari, Kumar, Jyothy, & Himabindu, 2012; Tailor & Patel, 2015; Ueda, Tamura, Kawano, Shiono, Hobor, Wilson, & Hamachi, 2021). According to Isik and Özdemir-Kocak (2009), SN and its derivatives demonstrated significant antibacterial efficacy towards bacterial infections attributed to Nocardia, *Staphylococcus aureus*, and *Escherichia coli*. The SN drug group exhibited higher antibacterial properties when electron-withdrawing groups, like the nitro group, are introduced (Isik & Özdemir-Kocak, 2009; Tailor & Patel,

2015). Therefore, the present study aimed to examine the antibacterial properties of newly synthesized pyrazolyl sulfonamides and their related palladium complexes against selected Gram-positive bacteria isolated from Buruli ulcer patients.

Statement of Problem

The emergence of antibacterial resistance poses a significant danger to world health, food safety, and human growth. The misuse of antibiotics in clinical settings has rapidly led to the emergency and spread of multidrug-resistant bacteria in the past decades. This phenomenon has posed substantial challenges in medical treatment, resulting in increased mortality rates, increased therapy expenses, and prolonged illness recovery time (Bartley et al., 2019). The incorporation of heterocyclic moieties has garnered considerable interest and scrutiny in the advancement of pharmacologically active compounds (Khan, Alam, Verma, Akhtar, Akhter, & Shaquiquzzaman, 2016; Küçükgüzel & Şenkardeş, 2015). Pyrazole, a cyclic compound consisting of five carbon atoms arranged in an aromatic configuration, and its derivatives have attracted significant attention in drug development. These compounds have demonstrated diverse applications as antibacterial agents (Kaur, Kumar, & Gupta, 2015), antimicrobial agents (Malladi, Isloor, Peethambar, Ganesh, & Goud, 2012), anticonvulsants (Dawood, Eldebss, El-Zahabi, Yousef, & Metz, 2013; Kumar, Saini, Jain, & Jain, 2013), and anti-tubercular (Khunt, Khedkar, Chawda, Chauhan, Parikh, & Coutinho, 2012).

Sulfonamides can be regarded as structural analogues of para-aminobenzoic acid (PABA), the precursor involved in folic acid synthesis. The sulfonamide moiety holds considerable importance in medicinal chemistry due

to its manifestation of a wide range of biological activities, encompassing antibacterial and anti-diabetic effects, among others. According to a study conducted by Jain, Saravanan, and Singh (2013), sulfonamides are significant in the context of peptide hydrolysis as they operate as a robust permanent inhibitor of the enzyme cysteine proteases during anti-microbial actions, effectively mimicking the transition state. In recent times, there has been increasing use of these compounds as agents with antibacterial properties, as well as in the treatment of Alzheimer's disease (Kołaczek, Fusiarz, Ławecka, & Branowska, 2014). According to Sabounchei and Shahriary (2013), empirical investigations have demonstrated that the effectiveness of organic compounds and their metal complexes in inhibiting microbial growth is significantly influenced by the choice of central metal ion. Therefore, several metal complexes, including palladium and platinum, have demonstrated significant efficacy as antibiotic medicines. The labile nature of these complexes, in comparison to well-established chemicals like cisplatin and carboplatin, has been attributed (Budzisz, Krajewska, & Rózalski, 2004).

Palladium has garnered interest as a possible candidate in the area of drug discovery due to its comparable coordination chemical properties to platinum and certain advantages, such as higher solubility of its complexes in comparison to platinum (Eslami Moghadam, Divsalar, Abolhosseini Shahrnoy, & Saboury, 2016; Fanelli, Formica, Fusi, Giorgi, Micheloni, & Paoli, 2016; Fong, Lok, Chung, Fung, Chow, Wan, & Che, 2016; Jahromi, Divsalar, Saboury, Khaleghizadeh, Mansouri-Torshizi, & Kostova, 2016). In a study published by Medici et al. (2015), it was found that palladium (II) complexes exhibit notable cytotoxic effects against a range of cancer types,

such as head and neck squamous cancer, ovarian cancer, cancer of the breast, malignant melanoma, glioma, human colorectal cancer, osteogenic sarcoma, human chronic myelogenous leukemia, prostate cancer, cancer of the lungs, and human cervical epithelial cancers.

Nevertheless, the emergence of resistant strains of bacteria, viruses, and fungi to well-recognized biologically active substances has evolved into an urgent problem in clinical medicine. Due to these factors, there has been an increase in the production of novel chemical compounds to combat various diseases and infections. Various combinatory syntheses have been utilized to effectively produce highly active molecules, ensuring the suppression of resistant strains. The practise of incorporating numerous physiologically active moieties into a single pharmacophore is well-recognized as a means to enhance the bioactivity of the drug and its ability to combat microbial resistance (Khan, Alam, Verma, Akhtar, Akhter, & Shaquiquzzaman, 2016). The present study, which is part of a study at the Noguchi Memorial Institute for Medical Research titled: "Scaling up early detection and treatment of *Buruli ulcer* morbidity in the Asante Akim north district of Ghana." aims to examine the potential antibacterial properties of a collection of novel pyrazolyl sulfonamides and their associated palladium complexes against selected Gram-positive bacteria isolated from the Buruli ulcer patients. The compounds were synthesized using a condensation reaction involving suitable diketone and phenylhydrazine. The characterization of these compounds was carried out using several analytical techniques, including nuclear magnetic resonance (NMR), infrared spectroscopy (IR), mass spectrometry (MS), elemental analysis, and X-ray crystallography in specific instances.

Purpose of the Study

This study aimed to examine the antibacterial properties of newly synthesized pyrazolyl sulfonamides and their corresponding palladium complexes, intending to explore their potential as alternative antimicrobial agents that may be employed in the treatment of bacterial infections.

General Objective

The present study examines the potential antibacterial properties of a collection of newly synthesized pyrazolyl sulfonamides and their related palladium complexes against selected Gram-positive bacteria isolated from Buruli ulcer patients.

Specific Objectives

Specifically, the study sought to:

1. Determine the minimum inhibitory concentration (MIC) of the test compounds using the broth microdilution method.
2. Determine the minimum bactericidal concentration (MBC) of test compounds
3. Evaluate the synergistic effect of the test compounds.
4. Conduct time-kill kinetic test on successful synergistic combinations of the test compounds and antibiotics.

Research Questions

The following research questions guided the study:

1. What is the minimum inhibitory concentration of test compounds against clinically relevant selected bacteria using the broth microdilution test?

2. What is the minimum bactericidal concentration of the test compounds for each targeted microorganism?
3. Do the compounds synergize with other antibiotics against clinically relevant selected bacteria?
4. What is the rate and extent of bacterial killing over time for successful synergistic combinations identified in the time-kill kinetic test against clinically relevant selected bacteria?

Significance of the Study

The discovery of novel compounds that can potentially cure bacterial infections resistant to conventional antibiotics, such as sulfonamides, is a major reason why this study is of such significance. Upon demonstrating efficacy in inhibiting microbial growth, the newly synthesized compounds have the potential to serve as a novel category of antimicrobial drugs suitable for addressing a diverse spectrum of bacterial infections. The significance of synthesizing new derivatives of sulfonamides lies in their relevance to the escalating public health concern of antibiotic-resistant bacterial infections. The bacteria discussed herein are responsible for several diseases, including but not limited to urinary tract infections, respiratory tract infections, and skin infections. The treatment of these diseases has become progressively more difficult due to the rise of strains that are resistant to antibiotics. These infections provide significant challenges in terms of treatment and have the potential to result in serious consequences, such as sepsis and mortality. Identifying novel antimicrobial drugs capable of effectively targeting these bacteria has the potential to enhance the efficacy of treatment for various

bacterial illnesses, particularly those that pose challenges for conventional antibiotic therapies.

In addition, the necessity to discover novel antimicrobial agents arises from the need to mitigate the overreliance on conventional antibiotics, a practice that might engender the emergence and proliferation of antibiotic resistance. The study has the potential to contribute to the preservation of the efficacy of current antibiotics and the mitigation of the proliferation of antibiotic resistance by finding novel compounds suitable for treating bacterial illnesses.

Delimitation

This study specifically focused on evaluating the antimicrobial activity of novel pyrazole sulfonamides and their palladium complexes against selected Gram-positive bacteria (*Staphylococcus aureus, Staphylococcus hominis, Staphylococcus haemolyticus, Staphylococcus epidermidis*, and *Corynebacterium striatum*). It was designed as an in vitro study, utilizing broth microdilution to determine MIC and MBC, checkerboard synergy testing, and time-kill kinetics for selected synergistic combinations. The study did not include Gram-negative bacteria and fungi. Additionally, no in vivo experiments were conducted to assess pharmacokinetics, toxicity, or clinical efficacy, as the study was limited to preliminary antibacterial screening. Structural modifications of the sulfonamides were restricted to predefined derivatives, with no additional optimization or computational modeling. The results and conclusions drawn are, therefore, limited to the specific bacteria tested and the conditions under which the study was conducted.

Limitations

Several unavoidable constraints may have influenced the scope and outcomes of this study. First, the availability of bacterial strains was a limiting factor, as only a subset of Gram-positive pathogens could be tested, preventing a broader evaluation of antimicrobial activity. Second, the study was restricted to in vitro methods, and while these provide valuable preliminary insights, they do not fully replicate real-life physiological conditions, leaving uncertainty regarding the actual pharmacokinetics and toxicity of the compounds in living organisms. Third, resource constraints and time limitations prevented the inclusion of additional experimental approaches, such as mechanistic studies to determine how the compounds interact with bacterial targets or resistance profiling to assess the potential for bacterial adaptation. Fourth, while the study evaluated the antibacterial properties of predefined chemical structures, the synthesis process was constrained by available reagents and methods, meaning that potentially more effective modifications may not have been explored. Lastly, the study relied on standard laboratory conditions, and variations in environmental factors, bacterial strain mutations, or differences in clinical settings could influence the reproducibility of results in other contexts.

Definition of Terms

Antibacterial: Antibacterial refers to any substance that inhibits the growth or kills bacteria, preventing infections or bacterial proliferation (Butler et al, 2024). These agents can be classified as bacteriostatic (growth-inhibiting) or bactericidal (bacteria-killing) (Chen et al., 2024).

Sulfonamides: Sulfonamides are a class of synthetic antimicrobial agents that contain a sulfonamide (-SO_2NH_2) functional group and act by inhibiting bacterial folic acid synthesis, making them effective against a range of infections (Duan et al., 2022).

Activity: In microbiology, activity refers to the effectiveness of a compound in inhibiting or killing microbial pathogens, often measured using parameters like minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (Ovung & Bhattacharyya, 2021).

Pyrazole: Pyrazole is a five-membered heterocyclic compound containing two adjacent nitrogen atoms, widely used in pharmaceuticals for its bioactive properties, including antimicrobial and anti-inflammatory effects (Ebenezer et al., 2022).

Pyrazole Sulfonamides: Pyrazole sulfonamides are hybrid compounds that combine the pyrazole ring with sulfonamide functionality, enhancing antimicrobial activity through dual inhibition mechanisms (Chalkha et al., 2022).

Palladium: Palladium (Pd) is a transition metal widely used in medicinal chemistry due to its catalytic properties and ability to enhance bioactivity in metal-based drug complexes (Veisi et al., 2021).

Organization of Study

The present study is structured into five chapters sequentially numbered from one to five. The initial chapter provided a concise overview of the study's context and established the problem statement, research goal, objectives, and inquiries. Additionally, it offers valuable information regarding the research's importance and the study's structure. The second

chapter provides a comprehensive assessment of the pertinent literature on antimicrobial resistance and the antibacterial properties exhibited by sulfonamides, pyrazole, and palladium complexes, which have emerged as potential alternatives for combating microbial infections. The third chapter primarily provided information regarding the materials, reagents, samples, solutions preparations, and test organisms employed in the research. The data analysis and discussion of findings were expounded upon in chapter four. The concluding chapter five summarises the study's findings, concludes, and offers recommendations.

CHAPTER TWO

LITERATURE REVIEW

Introduction

The global concern surrounding antimicrobial resistance (AMR) has emerged due to its potential to compromise our ability to prevent and treat various diseases caused by bacteria, parasites, viruses, and fungi. Additionally, it increases the complications of surgical procedures and cancer chemotherapy. The emergence of antimicrobial resistance is a natural occurrence that occurs gradually, often due to genetic changes in bacteria following their exposure to antimicrobial drugs. The excessive use and inappropriate utilization of contemporary antibiotics have played a role in the emergence of microorganisms that exhibit resistance to the existing range of antimicrobial medications, leading to the current predicament of AMR.

Consequently, the efficacy of currently accessible pharmaceutical agents is compromised, leading to the persistence of infections within the host organism. This perpetuates an elevated threat to the well-being of patients and the dissemination of pathogens, thereby amplifying the financial burden associated with healthcare provision. Multidrug-resistant bacteria, including *Enterococcus faecium*, *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and various species of Enterobacteriaceae, pose a significant apprehension for the World Health Organisation (WHO) and health authorities worldwide (Bouley et al., 2016; Ng et al., 2013; Zhao et al., 2013). Methicillin-resistant *Staphylococcus aureus* (MRSA) is a prominent etiological agent responsible for a wide array of infections within healthcare settings and the community. This is primarily

attributed to its remarkable ability to develop resistance against various antibiotics and its capacity to produce toxins (Xia, Gao, Kokudo, Hasegawa, & Tang, 2013). It is, thus, apparent that an imperative requirement exists for the advancement of novel antimicrobial agents possessing enhanced mechanisms of action (Von Nussbaum, Brands, Hinzen, Weigand, & Häbich, 2006).

Amidst the growing AMR situation, prominent pharmaceutical enterprises have halted their endeavors in antibiotic drug discovery, thereby placing academia at the vanguard of unearthing novel classes of bioactive compounds, particularly targeting bacterial pathogens (Pawlowski, Johnson, & Wright, 2016; Talbot et al., 2019). The conventional methodology employed by medicinal chemists, which predominantly relies on organic compounds, is anticipated to yield a transient and constrained influence due to the propensity of pathogens to adapt and acquire resistance against novel therapeutics. In addition, only about 25 percent of the drugs currently undergoing clinical development possess entirely novel structural classes, as Frei has pointed out in recent research findings. On the other hand, the remaining 75% of pharmacological agents constitute essentially derivatives and modifications of antibiotics that have been approved in the past (Frei, 2020). Hence, the pressing demand for novel antimicrobial agents extends beyond the mere necessity for additional antibiotics, encompassing the imperative for entirely novel categories of chemical compounds to fulfil this objective of finding alternative therapeutics. Transition metal complexes present a promising avenue for exploration in this regard. Microorganisms exhibit diverse three-dimensional geometries, offering an extensive repertoire for manipulating

their coordination sphere to influence substitution kinetics, charge, lipophilicity, biological targets, and modes of action. These complex formations, nonetheless, continue to be regrettably disregarded by pharmaceutical corporations despite the undeniable utilization of numerous such compounds in medical facilities across the globe.

Brief History of Antimicrobials and Antimicrobial Resistance

The discovery of penicillin by Alexander Fleming in 1928 marked a significant medical milestone. This breakthrough led to the effective utilization of penicillin as the first antibiotic for treating and preventing bacterial infections, particularly among military people during World War II. However, before the introduction of penicillin in 1940, there were already documented reports on penicillin-resistant strains of Staphylococcus. In light of the initial appearance of penicillinases, the introduction of methicillin into the therapeutic armamentarium in 1959. Subsequently, in 1960, the scientific community documented the presence of a strain of Staphylococcus that displayed resistance toward methicillin (Sengupta, Chattopadhyay, & Grossart, 2013). Vancomycin, a potent glycopeptide antibiotic, was introduced in 1958 as a therapeutic agent targeting methicillin-resistant staphylococci. Several decades later, specifically in 1979, the emergence of coagulase-negative staphylococci exhibiting resistance to vancomycin was documented. Subsequently, ten years later, the manifestation of vancomycin resistance in enterococci was elucidated (Courvalin, 2006). Followed by the report of less-susceptible *S. aureus* in 1997 (vancomycin-intermediate *S. aureus*, VISA) strains in Japan (Levine, 2006). Another precedent is the introduction of tetracycline in 1950, followed by the report of tetracycline-resistant *Shigella*

strains in 1959. Moreover, the advent of levofloxacin in the realm of clinical application occurred in 1996, concomitantly with the emergence of levofloxacin-resistant *pneumococcus*, as documented within the annals of scientific literature (Sengupta et al. 2013).

During the period spanning from 1960 to 1980, the pharmaceutical industry exhibited a commendable capacity in generating novel antimicrobial agents. After the 1980s, the pace of unearthing novel antibiotic categories experienced a notable decline until a recent resurgence ignited a renewed interest (Parmar et al., 2018). A prominent global clinical practice issue is bacterial infections caused by multidrug-resistant or completely drug-resistant organisms due to rising antimicrobial resistance and a limited supply of novel antimicrobials.

Intrinsic, Acquired, and Adaptive Forms of Antimicrobial Resistance

The phenomenon of antibiotic resistance demonstrated by bacteria can manifest in various ways, namely intrinsic, acquired, or adaptive (Lee, 2019). The term "intrinsic resistance" refers to the resistance displayed by a bacterium due to its inherent features. Instances of intrinsic resistance can be observed in glycopeptide resistance displayed by Gram-negative bacteria. This resistance is attributed to the impenetrable nature of the outermost membrane layer within the Gram-negative bacteria's cell envelope.

Acquired resistance pertains to when a bacterium, which was previously sensitive to specific medications, changes its genetic makeup either through mutation or the acquisition of additional genetic material from an external source via horizontal gene transfer, resulting in the bacterium becoming resistant to those drugs. Three primary methods facilitate horizontal gene

transfer: transformation, transduction, and conjugation (Holmes et al., 2016; Munita, Arias, Unit, & Santiago, 2016).

Transformation: This phenomenon refers to a genetic recombination process when DNA fragments from a dead bacterium are introduced into a receiving bacterium, subsequently becoming integrated into its chromosome. A limited number of bacterial species possess the inherent ability to undergo spontaneous transformation.

Transduction: Transduction is a process of genetic exchange in bacteria where DNA is transferred from one bacterium to another via a bacteriophage (a virus that infects bacteria). This process allows for the incorporation of new genetic material into the recipient bacterium's genome.

Conjugation: This specific horizontal gene transfer route is widely considered the most crucial and significant. The mechanism involves the transfer of genetic material from a bacterial cell acting as a donor to another bacterial cell acting as a receiver, which is enhanced by the physical interaction between these microorganisms. The intercellular emergence of a conjugative pilus facilitates the horizontal transmission of a plasmid from the donor bacterial cell to the receiving bacterial cell. It is common for multiple resistance genes to coexist on a single plasmid, making it possible for multidrug resistance to be efficiently transferred via a single event of conjugation. Mobile genetic elements, such as transposons, integrons, and Insertion Sequence Common Region (ISCR) elements, can integrate many resistance genes onto a single plasmid. This makes it possible to create resistant strains of bacteria.

Adaptive resistance can be precisely characterized as the phenomenon wherein the efficacy of one or more antibiotics is diminished as a direct

consequence of exposure to a distinct environmental stimulus. Stress, growth state, pH, ion concentrations, nutritional circumstances, and even sub-inhibitory antibiotic concentrations are all examples of stimuli that may be involved. In contrast to intrinsic and acquired resistance, adaptive resistance demonstrates a temporary characteristic. Following the elimination of the provoking stimulus, the adaptive resistance mechanism, which facilitates bacteria's ability to promptly react to antibiotic exposure, typically reverts to its original condition (Fernández, Breidenstein, & Hancock, 2011; Lee, 2019; Motta, Cluzel, & Aldana, 2015; Salimiyan Rizi & Noghondar, 2018).

Adaptive resistance appears to arise from alterations in gene expression patterns in response to fluctuations in the surrounding environment. Instead of being instigated by genetic modifications, which typically yield permanent phenotypic alterations, adaptive resistance may arise from epigenetic modifications. In light of recent findings, it has been postulated that the process of DNA methylation mediated by the DNA adenine methyltransferase (DAM) methylase enzyme may underlie the manifestation of distinct gene expression patterns within a bacterial populace. This mechanism can confer heterogeneity and epigenetic inheritance of gene expression, thereby facilitating the emergence of adaptive resistance phenomena (Motta et al. 2015; Salimiyan-Rizi et al. 2018). Particularly, changes in the expression of the efflux pumps and porins are associated with the development of adaptive resistance (Motta et al. 2015).

The presence of adaptive resistance could explain the inconsistencies noticed when evaluating the effectiveness of an antimicrobial agent in laboratory settings to that of real-life conditions. The presence of adaptive

resistance could also contribute to the limited success of antibiotic therapies in clinical settings. In addition, an upsurge in resistance that occurs as a result of exposure to an external stimulus might not completely reverse when the stimulus is removed, which would result in a gradual increase in the minimum inhibitory concentration (MIC) over time (Fernández et al. 2011). The ability of bacterial populations to proliferate in the face of sub-inhibitory concentrations of antibiotics through adaptive resistance has been hypothesized to eventually pave the way for the emergence of improved and long-lasting mechanisms of resistance (Fernández et al. 2011; Salimiyan Rizi et al. 2018).

Mechanisms of Antibiotic Resistance

The breakdown or modification of antibiotics and changes in antibiotic targets are common causes of antibiotic resistance. These target alterations can happen through several pathways, including target replacement, target site mutations, target site enzymatic alterations, target site protection, target overproduction, or target bypass. Resistance can also arise from reduced antibiotic accumulation, which can be attributed to diminished permeability and/or heightened efflux mechanisms. In an alternative perspective, antibiotic resistance may arise due to a widespread adaptation exhibited by bacterial cells on a global scale. The primary mechanisms of antibiotic resistance and its implications for clinical practise are covered in the paragraphs below.

Destruction of Antibiotic: Some bacteria exhibit antibiotic resistance by generating enzymes that neutralize or break down the antibiotic. In the context of sulfonamide antibiotics, this particular mechanism is relatively rare.

Sulfonamides function by obstructing the DHPS enzyme, which plays a role in folate production and is vital for bacterial proliferation.

Antibiotic Alteration: This resistance mechanism involves the addition, deletion, or transformation of functional groups within the antibiotic molecule, which reduces its potency. Concerning sulfonamides, this mechanism has not been extensively documented. Sulfonamides are structural analogues of para-aminobenzoic acid (PABA) and compete with PABA for the DHPS enzyme's active site, inhibiting the enzyme and obstructing folate synthesis. Any modification to the sulfonamide molecule that impacts its capacity to compete with PABA might lead to resistance (Venkatesan et al., 2023).

Modifications of Antibiotic-Activating Enzymes: This type of resistance occurs when alterations in the enzymes responsible for activating the antibiotic render it ineffective. Sulfonamide antibiotics do not necessitate activation by bacterial enzymes; rather, they directly inhibit DHPS. Consequently, this resistance mechanism does not apply to sulfonamides (Venkatesan et al., 2023).

Target Substitution or Bypass: In this resistance mechanism, bacteria develop an alternate metabolic pathway or enzyme that circumvents the antibiotic's target. With sulfonamides, certain bacteria can acquire an alternative DHPS enzyme that is not susceptible to sulfonamide inhibition. This enables bacteria to continue producing folate in the presence of sulfonamide antibiotics, thus conferring resistance (Venkatesan et al., 2022).

Alteration of Target Site (Through Mutation or Enzymatic Modification): Resistance can develop when the antibiotic's target site undergoes changes via genetic mutations or enzymatic modifications, which diminish the antibiotic's

affinity for the target. In the case of sulfonamide resistance, mutations in the gene encoding DHPS can result in structural changes in the enzyme, reducing the binding affinity for sulfonamides. Consequently, the enzyme can continue operating in the antibiotic's presence, allowing bacterial folate synthesis and growth.

Protection of Target Site: This mechanism entails the production of proteins that shield the target site from the antibiotic, preventing it from exerting its inhibitory effect. In the context of sulfonamide antibiotics, this mechanism has not been well-established. However, bacteria could evolve or obtain proteins that safeguard DHPS from the inhibitory action of sulfonamides, allowing the enzyme to function unhindered and conferring resistance.

Mutations in the DHPS enzyme: Mutations in the DHPS enzyme, particularly at key residues like 407, can lead to substantial resistance against sulfonamide antibiotics while preserving the enzyme's ability to bind PABA. This resistance arises because sulfonamides, which mimic PABA, normally inhibit DHPS by blocking folate synthesis, an essential pathway for bacterial growth. However, mutations at residue 407 alter the binding site to reduce sulfonamide affinity, allowing the enzyme to continue its function despite the presence of the antibiotic. Importantly, these mutations do not impede PABA binding, meaning that the enzyme retains its biological activity, enabling the bacterium to survive and proliferate even under antibiotic treatment.

Sulfonamides

Structure and Nomenclature

The characteristic configuration of a tertiary sulfonamide (SN) entails a central sulphur atom, which is connected to two oxygens through double

bonds. Additionally, this sulphur atom forms bonds with a nitrogen atom (occurring as a substituted amine) and an aniline group (as depicted in Figure 1a). It is worth noting that R1/R2 in this context can represent hydrogen, alkyl, aryl, or heteroaryl groups. The prototypical structure of a sulfonamide (SN) drug can be elucidated as an organic compound comprising aniline that has undergone derivatization with a sulfonamide moiety (Pareek, Rani, & Kishore, 2013). The discrepancy in the derivative arrangement of SN (Figure 1a) between SMZ and SDZ (Figures 1b and c) can be ascribed to an extra dimethyl group positioned at the 4th and 6th sites of the pyridine ring. The name the International Union of Pure and Applied Chemistry (IUPAC) assigned for SN is 4-amino benzenesulfonamide. In this particular instance, SMZ is identified as a derivative pharmaceutical compound referred to as 4-amino-N-(4, 6-dimethylpyrimidin-2-yl) benzene sulfonamide. In a similar vein, the derivative compound of SDZ is denoted as 4-amino-N-(pyrimidin-2-yl) benzene-1-sulfonamide (Robertson, Moodie, Holland, Jandér, & Göransson, 2020).

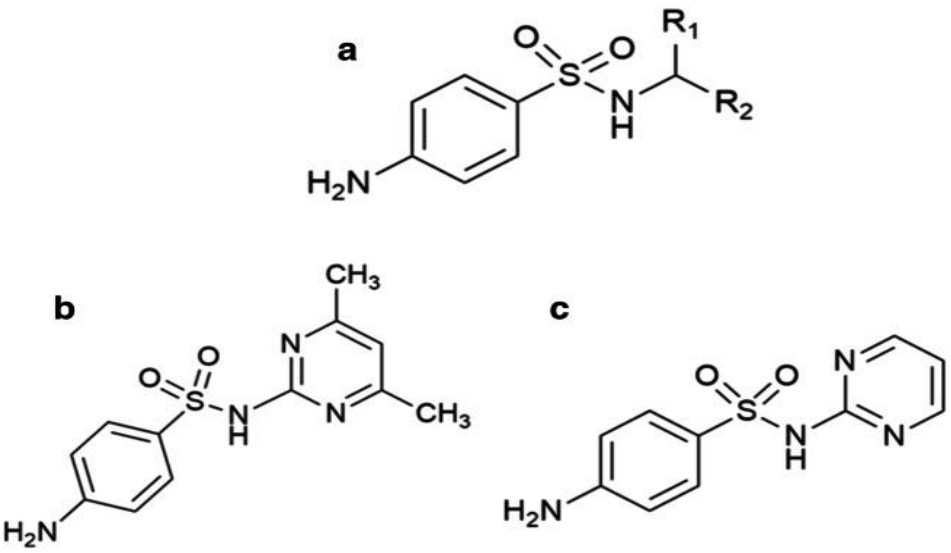


Figure 1*:* Chemical structures of sulfonamide antibiotics: **a**. generic tertiary sulfonamide; **b.** sulfamethazine; and **c.** sulfadiazine.

Sulfonamides pharmacodynamics

The sulfonamides, also called sulfa medicines, are a group of artificially produced bacteriostatic antibiotics that demonstrate a wide range of activity against various Gram-positive bacteria and a substantial proportion of Gram-negative germs. However, it is crucial to recognise that various strains within a certain species can demonstrate resistance. Because of their remarkable ability to function as antagonistic inhibitors of PABA, which is key in the folic acid synthesis cycle, sulfonamides can successfully block the multiplication of bacteria. The bacterial susceptibility remains consistent across the diverse spectrum of sulfonamides, and the development of resistance against a single sulfonamide implies resistance to the entire class. Most sulfonamides exhibit favourable oral bioavailability, allowing for efficient absorption into the systemic circulation.

Nevertheless, the parenteral route of administration poses considerable challenges due to the inherent alkalinity and tissue irritability exhibited by the soluble sulfonamide salts. The sulfonamides exhibit extensive distribution across various anatomical compartments. Significant concentrations are attained within the pleural, peritoneal, synovial, and ocular fluids. While it is true that these particular medications are no longer employed for the treatment of meningitis, it is worth noting that cerebrospinal fluid (CSF) levels tend to exhibit an elevation in the context of meningeal infections. The antibacterial efficacy is impeded by purulent exudate (Supuran, 2017; Tailor & Patel, 2015).

Mechanism of Action

Antibiotics, as chemotherapeutic agents, exert their pharmacological effects by selectively inhibiting bacterial growth. Sulfonamides, being competitive antagonists, function as structural analogues of PABA during the folic acid synthesis process. Folic acid is a crucial component required for subsequent DNA production in bacterial organisms (Zessel, Mohring, Hamscher, Kietzmann, & Stahl, 2014). The structural resemblance between SN and PABA facilitates the capacity of SN to impede and supplant PABA within the dihydropteroate synthetase enzyme (which plays a crucial role in folate synthesis), ultimately hindering the generation of dihydrofolate and tetrahydrofolate (as depicted in Figure 2). Consequently, this impedes bacterial DNA proliferation, cell division, and replication (Pareek et al. 2013). The utilisation of SN drugs, in conjunction with trimethoprim, serves the purpose of impeding the biosynthesis of tetrahydrofolate, thereby effectively halting the process of DNA replication. The drug's pharmacological properties elicit impediments in cellular mitosis, thereby conferring bacteriostatic attributes to the SN drugs as opposed to bactericidal ones (Böhni, 1976; Nemeth, Oesch, & Kuster, 2015).

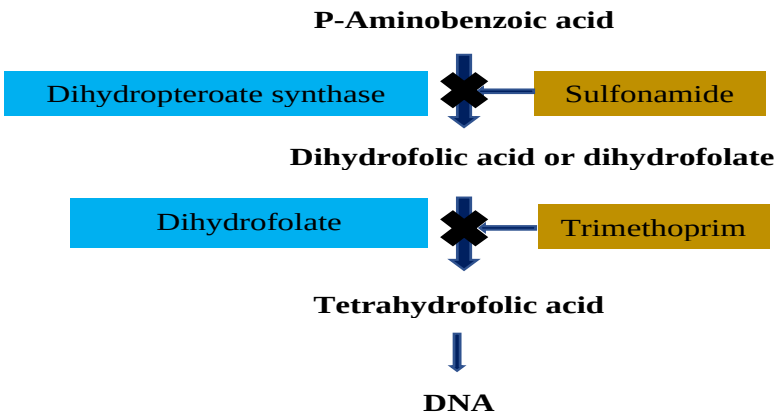


Figure 2*:* Synthesis of tetrahydrofolic acid and the mode of sulfonamide action
Source: (Lavanya, 2017; Pareek et al., 2013)

Folic acid, alternatively referred to as vitamin B9, is of paramount importance in facilitating cellular proliferation and maturation inside the human body. This essential nutrient is required for the complex procedures of DNA synthesis, repair, and methylation (Mahmood, 2014). In light of this, it is imperative to acknowledge the vital role of folic acid in female gestation, as it contributes significantly to promoting a thriving embryonic entity. Furthermore, it is noteworthy that folic acid also holds relevance for the male population, as it can potentially enhance sperm count and motility (Gao et al., 2016). Sulfa drugs do not disrupt animal cells because they do not interfere with folate production. In humans, folate is an essential nutrient, and folic acid works after being converted to tetrahydrofolic acid by the enzyme dihydrofolate reductase, which is believed to have lower activity in humans (Bailey & Ayling, 2009). Sulfonamides can cause disruptions in the synthesis of tetrahydrofolate, which can result in alterations in DNA due to an insufficient supply of methyl groups for methylation.

Antimicrobial Activity of Sulfonamides

Sulfonamides exhibit a wide range of efficacy and effectiveness against Gram-positive and select Gram-negative bacteria (White & Cooper, 2005). *Klebsiella, Salmonella, Escherichia coli*, and species of *Enterobacter* are examples of Gram-negative bacteria that are susceptible to sulfonamides. On the other hand, sulfonamides have no inhibitory activity against *Pseudomonas aeruginosa* and *Serratia species* (Lavanya, 2017). Sulfonamides find application in the therapeutic management of tonsillitis, septicaemia, meningococcal meningitis, bacillary dysentery, and various urinary tract infections (Wiedemann, Heisig, & Heisig, 2014). Sulfonamides exhibit

notable inhibitory activity against certain fungi, such as *Pneumocystis carinii*, and protozoa like *Toxoplasma* and *Coccidia* (McFarland et al., 2016). Drugs containing sulfonamide, sulfamethazine, and suladiazine have been shown to have antibacterial activity in some studies that have been published (Blanchard et al., 2016; Majewsky et al., 2014; Peng et al., 2020; Tailor et al., 2015; Ueda et al., 2020). The compound SN and its derivatives shown noteworthy antibacterial effectiveness in managing bacterial infections caused by *Nocardia sp., Staphylococcus aureus, and Escherichia coli* (Isik & Özdemir-Kocak, 2009). The observed augmentation in the antibacterial efficacy of the SN drug category was noted subsequent to the introduction of electron-withdrawing substituents, exemplified by the nitro group (Isik et al., 2009; Radha, KKl, & Thamaraichelvan, 2016; Tailor et al., 2015).

Bacteria Resistance to Sulfonamides

Sulfonamides, a class of synthetic antibacterial agents, exhibit a chemical composition characterised by a PABA structure. These compounds possess a sulfonamide group linked to an aromatic group, exerting a competitive inhibitory effect on the DHPS. DHPS, also known as dihydropteroate synthase, plays a crucial role in the folate synthesis, a vital process for producing bacterial DNA and RNA. This enzymatic activity relies on the utilisation of PABA as a substrate. However, the development of bacteria can be impeded by sulfonamides, which competitively inhibit DHPS, thereby hampering the synthesis of folate and subsequent bacterial growth (Mąka, Maćkiw, Ścieżyńska, Modzelewska, & Popowska, 2015; Sánchez-Osuna, Cortés, Barbé, & Erill, 2019; Xu et al., 2020). Consequently, these medications have a high level of effectiveness against a diverse range of

bacterial species. They can successfully suppress the growth of Gram-negative and Gram-positive bacteria that do not possess mechanisms that can defend against the inhibitory effects of DHPS (Nunes, Manaia, Kolvenbach, & Corvini, 2020).

Sulfonamides, renowned for their therapeutic efficacy, have been widely acknowledged as the pioneering pharmaceutical agents employed in veterinary medicine (Agyare, Boamah, Zumbi, & Osei, 2018; Lees, Pelligand, Giraud, & Toutain, 2021). The extensive utilisation of these agents has exerted significant selective pressures on bacterial populations, leading to the notable prevalence of sulfonamide resistance in predominantly Gram-negative bacterial strains obtained from both animal and human sources worldwide during the previous decade (Ben, Wang, Pan, & Qiang, 2017; Card et al., 2016; Liu, Klümper, Shi, Ye, & Li, 2019; Yuan, Ni, Liu, Zheng, & Gu, 2019).

Resistance to sulfonamides in bacteria is linked to sul genes that encode dihydropteroate synthase in a conformation that prevents the drug from exerting its inhibitory effects. It has been discovered that there are four distinct sul genes, more particularly *sul1*, *sul2*, *sul3*, and *sul4*, with each gene encoding a different resistance mechanism to sulfonamides (Mąka et al., 2015; Xu et al., 2020). The sul1 and sul2 genetic material have been previously discovered within the Enterobacteriaceae family, specifically in *Escherichia* and *Salmonella* strains (Xu et al., 2020). Perreten and Boerlin published their findings on the sul3 gene in 2003. This gene was found in *Escherichia coli* isolated from pigs in Switzerland (Perreten & Boerlin, 2003). In 2017, Razavi et al. successfully discovered the *sul4* gene, which confers clinical resistance within the Enterobacteriaceae family (Razavi et al., 2017). The transfer of Sul

genes among bacterial species can occur through various mechanisms, such as integrons, transposons, or plasmids. These genetic elements facilitate the horizontal gene transfer of sul genes, enabling their dissemination within microbial populations (Xu et al., 2020). As per the findings, it has been observed that the *sul3* gene exhibits detectability within *Salmonella spp*. strains originating from diverse sources and characterised by distinct serotypes, all harboured on many expansive plasmids (Guerra, Junker, & Helmuth, 2004). Nevertheless, the propagation of *sul1* and *sul2* genes among various bacterial species is frequently documented, surpassing the prevalence of the *sul3* gene (Mąka et al., 2015).

A systematic review published by Pavelquesi et al. revealed that the sulfonamide-resistance genes that garnered the highest number of research studies were as follows: sul1, which was the subject of 19 studies (82.6%); sul2, which was investigated in 13 studies (56.5%). Conversely, the sulfonamide-resistance genes that received comparatively less attention were sul3, examined in 7 studies (30.4%), and sul4, which was the focus of only 1 study (4.3%). The sul1 gene was detected in 18 out of 19 investigations that sought to identify its presence. The prevalence of this gene among bacterial species ranged from 0% to 89.7%, with an average occurrence of 45.6% in the isolates. The sul2 gene, a genetic element of interest, has been identified in 12 scientific investigations. Its occurrence within various bacterial species has exhibited a range of 0% to 97.8%, with an average prevalence of 44.5% among the isolated strains. The sul3 gene was detected in six independent investigations, wherein its occurrence within bacterial species exhibited a

range of 0% to 85.1% (with an average prevalence of 31.6% among the isolated strains) (Pavelquesi et al., 2021).

Zhu et al., (2013) observed that within the cohort of 91 isolates exhibiting resistance to sulfonamide, a staggering 97.8% (n = 89) possessed, at minimum, one of the genes under investigation (*sul1*, *sul2*, or *sul3*). The *sul2* gene exhibited the highest prevalence (97.8%, n = 89) in comparison to the *sul1* and *sul3* genes (both at 50.5%, n = 46) (Zhu et al., 2017). As per the findings of Maka et al. (2015), the propagation of *sul1* and *sul2* genes among various bacterial species has been observed to occur with greater frequency than that of the *sul3* gene. Xu et al. (2020) also documented the co-occurrence of *sul1* and *sul2* genes at comparable frequencies within sulfonamide resistant Gram-negative isolates. Furthermore, it is worth noting that surveys of environmental bacteria consistently report the existence of *sul* genes, with *sul2* being the most prevalent, closely followed by sul1. However, it is important to mention that *sul3* remains relatively scarce in these surveys (Machado, Coque, Cantón, Sousa, & Peixe, 2013).

The sul genes are frequently identified within plasmids and strongly associated with the prevalent and firmly established sulfonamide resistance reported in Gram-negative bacteria (Xu et al., 2020). The presence of the *sul1* gene is frequently detected in class 1 integrons and frequently found in close proximity to other genes that confer resistance. On the other hand, the *sul2* gene is primarily associated with small multicopy plasmids or large transmissible plasmids that provide resistance to several antimicrobial drugs (Jiang et al., 2019; Sánchez-Osuna et al., 2019). The presence of the sul3 gene was identified in conjugative plasmids found in *Escherichia coli*. In contrast,

the *sul4* gene was discovered by a comprehensive examination of class 1 integron genes in sediment samples from Indian rivers (Sánchez-Osuna et al., 2019).

Per Perreten and Boerlin's findings, it is noteworthy that sul1 and sul2 genes present in *Escherichia coli* exhibit a DNA identity of 57%. Furthermore, the sul3 gene demonstrates an overall amino acid identity of 50.4% to the sul2 gene found in the plasmid of *Salmonella enterica subsp. enterica.* Additionally, sul3 displays an amino acid identity of 40.9% to the sul1 gene from the plasmid of *Escherichia coli*. Based on the analysis of amino acid homology and phenotype, it was determined that sul3 exhibits characteristics indicative of a novel sulfonamide-resistant dihydropteroate synthase (DHPS). As per the findings of Razavi et al. (2017), the sul4 gene exhibited a sequence similarity of 31-33% with established mobile genes associated with sulfonamide resistance, namely sul1, sul2, and sul3. The terminology "sul4" was suggested based on its capacity to confer resistance against sulfonamides, its mobile nature evidenced by its integration into integrons, and its similarity to previously identified genes associated with sulfonamide resistance. The structural prediction of sul1, sul2, sul3, and sul4 reveals robust overall homologies. The genetic composition encompasses the loci harbouring binding regions for 7,8-dihydropterin pyrophosphate, PABA, and sulfonamide. Once the DHPP compound has successfully established its binding within the molecular structure, the sulfonamide moiety subsequently engages in binding interactions close to the protein's surface. Hence, the binding of sulfonamide is influenced by alterations in the vicinity of the surface of the DHPS (Razavi et al., 2017).

The genetic elements *sul1*, *sul2*, *sul3*, and *sul4* can proceed through horizontal gene transfer processes such as conjugation or transformation. This allows resistance determinants to be horizontally disseminated among bacterial populations, regardless of the species affiliation (Jiang et al., 2019; Xu et al., 2020). Several investigations concerning sulfonamide-resistant isolates, in which none of the sul genes have been identified, have been documented in scientific literature. However, no additional plasmid-borne sulfonamide resistance gene has been documented (Ma et al., 2017).

Mechanism of Resistance to the Sulfonamides

The acquisition of mutations in the folP gene is the first step in the process of developing resistance to sulfonamides. The folP gene encodes DHPS, an enzyme essential for folate synthesis in bacteria, which is crucial for DNA synthesis and cell growth. Mutations in the folP gene, particularly those altering the DHPS binding site, can lead to resistance against sulfonamide antibiotics, which act by inhibiting this enzyme. Such mutations typically reduce the affinity of DHPS for sulfonamides while preserving its ability to bind PABA, allowing folate synthesis to continue even in the presence of the antibiotic, thereby enabling bacterial survival and resistance. The second step is the horizontal transfer of exogenous genes that encode for DHPS variants that demonstrate high sequence divergence and, as a result, confer insensitivity to sulfa drugs. Both of these mechanisms can lead to the development of sulfonamide resistance in bacteria. The molecular underpinnings of resistance, made possible by mutations occurring in the folP gene, have been substantially explained by detailed structural investigations. It has been determined which specific residues within loops 1 and 2 of DHPS are responsible for giving

resistance, and these alterations have been mapped out. In various bacterial species, this binding region, which is responsible for the interaction between pABA and sulfonamide, is surrounded by loops 1 and 2 (Griffith et al., 2018; Yun et al., 2012). These genetic variations result in an elevation of the Michaelis-Menten constant values specifically for sulfonamides, while the impact on Michaelis-Menten constant for pABA is comparatively less pronounced. Consequently, these mutations bestow upon the DHPS enzymes the ability to differentiate between substrates, a capability absent in the wild-type enzymes (Griffith et al., 2018).

The presence of plasmid-encoded sulfonamide resistance genes has been extensively documented in clinical isolates of Gram-negative pathogenic species, such as *Escherichia coli, Acinetobacter baumannii,* and *Klebsiella pneumoniae* (Carvalho et al., 2021; Liu et al., 2020). Transferable sulfonamide resistance was initially observed during the 1950s and 1960s, wherein *Shigella* and *Escherichia coli* demonstrated interchanging resistance in both in vitro and in vivo experimental settings (Kasuya, 1964). Nevertheless, the elucidation and characterization of this transmissible resistance phenomenon did not occur until 1975 and subsequent years.

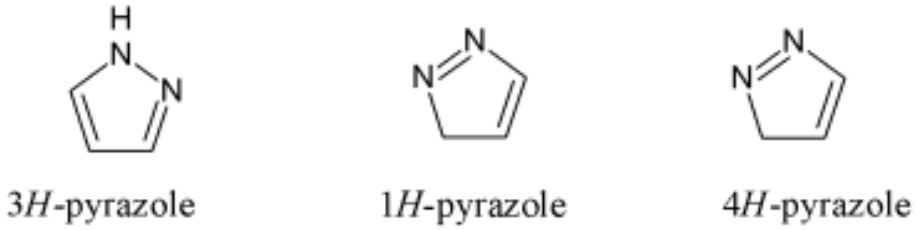
Pyrazole

Structure and Antimicrobial Activity

Heterocycles represent a fundamental and distinctive group of compounds within the field of chemistry. They constitute more than half of all identified organic compounds and exhibit diverse physical, chemical, and biological characteristics. Heterocycles encompass a wide range of reactivity and stability, making them highly versatile in various scientific applications

(Ansari, Ali, & Asif, 2017). Moreover, the synthetic utility of these compounds as intermediates in organic synthesis, their application as protective groups, chiral auxiliaries, organo-catalysts, and metallic compounds in asymmetric catalysis for medicinal drugs have made them subjects of significant interest. Within the heterocyclic compounds exists a diverse and extensive category consisting of five-membered rings that incorporate nitrogen atoms. This group exhibits various biological activities, showcasing its significant impact and potential in various biological contexts (Alam et al., 2016; Ferreira et al., 2010; Gregory et al., 1989). The constituents of this group, including pyrazole, imidazole, oxazole, triazole, tetrazole, oxadiazole, thiazole, and isoxazole, hold significant significance as antibacterial and antifungal agents (Ferreira et al., 2010; Gregory et al., 1989; Ramtohul et al., 2010). The structure depicted in Figure 3 represents the pyrazole ring, a heterocyclic compound consisting of a five-membered ring with two nitrogen atoms close. This moiety is commonly present in numerous compounds and has many uses.

Moreover, it is widely recognised that both naturally occurring pyrazoles and their synthesised derivatives exhibit a wide range of biological features. In recent years, pyrazole derivatives have been used in the development of FDA-approved and commercially available medications, including those protected by patents. This suggests the significance of incorporating these chemical groups in creating novel bioactive compounds.



3*H*-pyrazole 1*H*-pyrazole 4*H*-pyrazole

Figure 3: General chemical structures of Pyrazole

Pyrazole derivatives are a class of organic compounds that have attracted significant attention for their diverse range of pharmacological activities, including antimicrobial properties (Kumar, Ram, Sewwandi, Honda, & Chaminda, 2020). In recent years, a growing body of research has been conducted on the antimicrobial activity of pyrazole derivatives and their potential applications in medicine (Alagarsamy, Sulthana, Chitra, Solomon, & Saravanan, 2022). The increasing prevalence of multidrug-resistant pathogens has heightened the demand for novel antimicrobial agents, and pyrazole derivatives have shown promise in this regard (Alagarsamy et al., 2022; Patel et al., 2012). A notable characteristic of these derivatives is their ability to act against various pathogens, including bacteria, fungi, and parasites (Kumar et al., 2020). This broad-spectrum activity has made pyrazole derivatives a promising area of research in the ongoing battle against antimicrobial resistance.

The pyrazole-based sulfonamides are an important subclass of pyrazole derivatives with promising antimicrobial activities. Sulfonamides are widely used in medical practice as antibacterial agents, and their combination with pyrazole moieties has been shown to enhance their antimicrobial efficacy (Patel et al., 2012). A study found that pyrazole-based sulfonamide derivatives exhibited significant antibacterial activity against both Gram-positive and

Gram-negative bacterial strains, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant Enterococcus faecalis (VRE) (Sriram, Jun, Satheesh, & Coates, 2017). These derivatives were also found to have a favorable selectivity index, which indicates a higher toxicity to bacterial cells than to mammalian cells, thus suggesting potential applicability in human medicine (Sriram et al., 2017). In addition, the ability of pyrazole-based sulfonamides to act synergistically with existing antibiotics may offer a strategy for overcoming resistance in some cases (Patel et al., 2018).

Pyrazole derivatives have also been studied for their potential antimicrobial effects against fungus and other microbes. El-Sharkawy and colleagues synthesized and tested several pyrazole derivatives for antifungal efficacy against Candida species (El-Sharkawy, Brough, & Freeman, 2020). The findings showed that several of the compounds that were examined possessed powerful antifungal activity, with MICs comparable to those of the antifungal medicines used as reference; fluconazole and amphotericin B. The findings indicated that, these pyrazole derivatives have the potential to act as lead molecules in the research and development of novel antifungal drugs (El-Sharkawy et al., 2020). In addition to their activity against fungi, some pyrazole derivatives have also shown promising activity against parasites, such as *Plasmodium falciparum*, the causative agent of malaria (Kumar et al., 2020).

The mechanism of action for the antimicrobial activity of pyrazole derivatives, including sulfonamides, has been extensively studied. It has been proposed that pyrazole-based sulfonamides act by inhibiting the enzyme DHPS, which is essential for synthesising folic acid in microorganisms (Patel

et al., 2018). This inhibition results in a deficiency of folic acid, which is necessary for the production of nucleic acids and ultimately leads to the death of the microorganism (Sriram et al., 2017). Some pyrazole derivatives have also been shown to interfere with other essential cellular processes, such as cell wall synthesis.

Pyrazolyl Sulfonamides Derivatives

The concurrent presence of pyrazole and 1,2,4-triazole moieties exhibited significant and robust biological activity (Reddy et al., 2015; Reddy et al., 2016; Vijesh, Isloor, Shetty, Sundershan, & Fun, 2013). In light of this knowledge, Mustafa et al. synthesised 1,2,4-triazole-appended pyrazoles and investigated these compounds' carbonic anhydrase (CA) inhibitory activity (Mustafa et al., 2019). Most of the tested compounds displayed CA-inhibiting activities at micromolar to nanomolar concentrations. In particular, the 2,4-dichlorophenyl analogue had the greatest inhibitory effect, with KI (non-competitive inhibition) values of 55.4 and 24.4 nM, respectively, when tested against Human Carbonic Anhydrase (hCA) II and IX. The inhibitory activity of 4-nitrophenyl derivative 2 was seen to be marginally reduced for hCA II (KI = 67.3 nM) and further decreased for hCA IX (KI = 120.7 nM). A comparable level of hCA II activity (KI = 84.6 nM) was seen for the furan analogue, whereas it demonstrated a highly effective inhibitory activity against hCA IX (KI = 24.4 nM). The Structure Activity Relationship (SAR) analysis of the assessed compounds demonstrates that incorporating the carboxylic acid functional group led to enhanced activity compared to the ester functional group. Including dichlorophenyl, nitrophenyl, or furan moieties resulted in an augmentation of inhibitory action against hCA II and hCA IX.

Alkylamide linkers were strategically incorporated between pyrazole and benzenesulfonamide moieties to create modified sulfonamide derivatives (Angeli et al., 2021). The majority of the compounds tested effectively suppressed the activity of hCA II, which is one of the several isoforms of CA. Among the compounds examined within the pyrazole series, it was observed that the tricyclic pyrazole analogue exhibited the highest degree of potency in terms of inhibiting hCA II and hCA IX. The KI values for hCA II and hCA IX were determined to be 3.3 nM and 6.1 nM. Although it exhibited low hCA I activity and moderate hCA XII inhibitory activity (with a KI value of 80.5 nM). In addition, it is noteworthy that a particular isoform had comparable inhibitory effects on hCA II and hCA IX, as evidenced by their respective KI values of 16.2 and 16.1 nM.

In contrast, the isoform that exhibited the highest hCA II inhibitory action was observed when a chloro group was introduced on the phenyl ring and N-ethylation of the amide linker occurred. Most of the assessed compounds demonstrated a level of hCA XII activity ranging from moderate to poor. The results suggest that the combination of 5,6-dimethoxy-2,3-dihydro-1H-indene with a pyrazole ring resulted in a good outcome in terms of its ability to inhibit hCA IX. Regarding inhibitory action against hCA II, compounds containing N-ethylamide had relatively superior activity. The inclusion of 5-chloro-2-hydroxyphenyl proved to be an advantageous component. Furthermore, incorporating the 2-hydroxy-5-methylphenyl group led to the highest observed hCA IX activity when comparing various pyrazole amide derivatives.

Yamali et al., (2021) conducted a study to examine the carbonic anhydrase (CA) inhibitory activity of sulfonamides incorporating thiophene and pyrazole-benzenesulfonamide compounds. This investigation was motivated by the selective inhibitory activity of thiophene-bearing sulfonamides against hCA XII, as well as the utilisation of hydrazine coupler as a bioisosteric group of urea in efficient CA inhibitors (Yamali et al., 2021). In contrast to acetazolamide (AZA), most of the assessed compounds exhibited limited inhibitory activity against hCA I and hCA II. Furthermore, the pyrazole-benzenesulfonamide demonstrated significant efficacy (with a KI value of 16.8 nM) against hCA IX. The 3-hydroxy-4-methoxyphenyl analogue exhibited high hCA IX activity (KI = 8.1 nM) and showed favourable selectivity (1009) compared to hCA I. Incorporating the thiophene ring resulted in the most potent hCA IX activity (KI = 7.0 nM) and the highest selectivity of 1232. Both drugs exhibited hCA IX activity that was roughly three times greater than AZA. The study's results suggest that introducing electron-withdrawing groups often resulted in enhanced catalytic activity, as indicated by SAR analysis. Furthermore, the augmentation of substituted phenyl groups with heterocycles such as furan and thiophene increased hCA IX activity and selectivity compared to hCA I.

In addition, the same research team led by Yamali synthesised new pyrazolo-benzenesulfonamides incorporating thiophene moieties and subsequently assessed their inhibitory action against carbonic anhydrase (Yamali et al., 2021). Compared to AZA, pyrazolo-benzenesulfonamides demonstrated varying inhibitory capabilities against carbonic anhydrase (CA), ranging from promising to poor. Regarding the inhibition of hCA I, it was

observed that all the synthesised compounds exhibited a moderate level of activity. Among the compounds assessed, the pyrazolo-benzenesulfonamides exhibited the most potent inhibitory effect against hCA IX, with a KI value of 29.3 nM. Pyrazolo-benzenesulfonamides exhibited comparable hCA II action to AZA, but their inhibitory effect against hCA XII was reduced by a factor of two. Substituting the ethylbenzene group with a methylbenzene group led to a nearly equivalent level of hCA XII activity, causing a drop in hCA II activity. The corresponding KI values for hCA XII and hCA II were 9.7 and 54.3 nM, respectively. The analysis of structure-activity relationships (SAR) indicates that the presence of methyl and ethyl substitutions and their specific locations exerts a more significant influence on the inhibition of human carbonic anhydrase (hCA). In the instances of hCA II and hCA XII, introducing replacement at the 2-position resulted in enhanced activity. Replacing the methyl group at the 4-position led to an increase in hCA I activity. The inhibitory activity of halogen-substituted derivatives was not found to be substantial. Nevertheless, notable selectivity indices were observed.

Antimicrobial properties of palladium complexes

Palladium complexes have garnered increasing attention in recent years due to their diverse applications, including catalysis, materials science, and medicinal chemistry (Sorinezami et al., 2019). Among the various biological activities exhibited by palladium complexes, their antimicrobial properties have been a subject of significant research interest (Khan, Stapleton, Summers, Rice, & Willcox, 2020). The development of novel antimicrobial agents is crucial in mitigating multidrug resistance among pathogens, and palladium complexes have shown potential in this area (Nobre,

Cavalheiro, & Duarte, 2018). These complexes possess a unique combination of electronic and steric properties, which can be fine-tuned through carefully selecting compounds, thereby modulating their biological activities (López-Gil et al., 2020).

Various studies have investigated palladium complexes' antibacterial activity against Gram-positive and Gram-negative bacteria (Mansouri-Torshizi et al., 2019; Khan et al., 2020). For example, a study by Mansouri-Torshizi et al. (2019) evaluated the antibacterial activity of palladium (II) complexes containing bidentate Schiff base compounds against a range of bacterial strains, including *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. The results demonstrated that these complexes exhibited substantial antibacterial activity, with minimum inhibitory concentrations (MICs) comparable to those of standard antibiotics such as gentamicin and ciprofloxacin. The research indicated that the compounds' nature and the palladium complexes' coordination geometry played a significant role in determining their antibacterial potency (Mansouri-Torshizi et al., 2019).

In addition to their antibacterial properties, some palladium complexes have demonstrated antifungal activity (López-Torres et al., 2015; Khan et al., 2020). A study by López-Torres et al. (2015) investigated the antifungal activity of various palladium (II) complexes against Candida species, including fluconazole-resistant strains. The study found that the tested palladium complexes exhibited potent antifungal activity, with MIC values within the same range as those of the reference antifungal drugs, such as amphotericin B and caspofungin. Moreover, the antifungal activity of the complexes was found to be influenced by the nature of the compounds, with

certain ligand types, such as dithiocarbamates, showing enhanced activity compared to others (López-Torres et al., 2015).

The antimicrobial mechanisms of action of palladium complexes have been the subject of extensive research (Khan et al., 2020; Mansouri-Torshizi et al., 2019). Some studies have suggested that the interaction between the complexes and key cellular biomolecules, such as DNA and proteins, may contribute to their antimicrobial properties (Khan et al., 2020). For instance, DNA-binding studies have demonstrated that certain palladium complexes can intercalate into the DNA double helix, inhibiting DNA replication and transcription (Mansouri-Torshizi et al., 2019). Additionally, some palladium complexes have been found to inhibit bacterial enzymes involved in cell wall synthesis, such as penicillin-binding proteins, or disrupt the integrity of the cell membrane, leading to cell death (Nobre et al., 2018). These diverse modes of action may contribute to the broad-spectrum antimicrobial activities observed for some palladium complexes.

Chapter Summary

The literature review highlights the escalating global challenge of AMR and its implications for healthcare, particularly due to the limited efficacy of existing antibiotics. The emergence of multidrug-resistant bacteria and the associated resistance mechanisms, including intrinsic, acquired, and adaptive forms, underscore the critical need for innovative antimicrobial agents. The chapter underscores the potential of sulfonamides, pyrazole derivatives, and palladium complexes as promising candidates for addressing this challenge, given their unique structural attributes and mechanisms of action. It further identifies gaps in current research, particularly in the limited

exploration of novel compounds with improved efficacy against resistant pathogens. This review establishes a compelling case for investigating new pyrazole-based sulfonamides and their palladium complexes, aiming to contribute to the development of alternative therapeutics to combat AMR.

CHAPTER THREE

RESEARCH METHODS

Introduction

In this chapter, the research methods employed in the study have been described. The chapter also offers an in-depth overview of the research paradigm and design underpinning this study. Furthermore, it details the various tests performed on the compounds, the sample organisms used, and the methodologies adopted. All compounds used were synthesized by synthetic chemists in the chemistry department of the University of Ghana. They have undergone a comprehensive characterization process to ascertain their identity, purity, and intrinsic properties.

Ethical Consideration

This thesis was part of study at the Bacteriology Department at the Noguchi Memorial Institute for Medical Research (NMIMR) titled: "Scaling up early detection and treatment of *Buruli ulcer* morbidity in the Asante Akim north district of Ghana." Before the commencement of the study, clearance was sought from the NMIMR Institutional Review Board (Appendix A1). Also, the study was approved by the ethics committee of the Korle Bu Teaching Hospital (KBTH-IRB/000106/2018) before sample collection from patients.

Research Approach and Design

A research approach involves the general direction, model and analytical procedures which operationalises the research paradigm. Creswell identified three main types of study approach. These are qualitative, quantitative and mixed approach (Creswell, 2013). A research approach and

its suitability depend on the research problem, research paradigm, and the data gathering procedure (Anlo, 2012; Denscombe, 2017). The choice of a quantitative approach was informed by the nature of our research questions, the type of data collected, and the statistical methods used to analyse the data.

Experimental design refers to the systematic approach of doing research in a manner that is objective and controlled, aiming to maximise precision and enable the derivation of precise findings of a certain hypothesis statement (Gravlee, 2022). The research design for this study is experimental. Experimental research design is characterized by a high level of control over the variables and conditions under which the study is conducted and the ability to determine cause-and-effect relationships.

In this study, the independent variables are the different conditions under which the novel compounds were synthesized and tested. These conditions could include factors like the concentration of the compounds, the type of bacteria used in the tests, and the temperature and duration of the tests, among others. The dependent variables are the outcomes of the tests, such as the MIC, MBC, results of the synergy tests, and the time-kill tests.

The researchers have control over the independent variables and observe how changes in these variables affect the dependent variables. This allowed them to establish causal relationships and predict how the compounds will behave under different conditions. The experimental design is further characterized by rigorous testing and validation methods to ensure the reliability and validity of the findings. The researchers conduct multiple tests using analytical techniques such as NMR, IR, mass spectrometry, and X-ray crystallography to validate the synthesized compounds. The experimental

research design aligns well with the positivist paradigm and the deductive research approach adopted in this study, as it allows for objective measurement and data analysis and the testing of hypotheses.

Test Sample

A total of eighteen novel pyrazolyl sulfonamides and their corresponding palladium complexes were synthesised at the chemistry department of the University of Ghana. The synthesis process was carried out via a condensation reaction involving a suitable diketone and phenylhydrazine, followed by a carefully monitored reaction process. The careful orchestration of this process was aimed at ensuring the production of the desired compounds with the required molecular structure and properties. After the synthesis, each compound was characterized using several analytical methods, as this was a crucial step in confirming their identity and purity. The characterization process incorporated nuclear magnetic resonance (NMR) spectroscopy, infrared (IR) spectroscopy, and Mass spectrometry. These techniques allowed for detailed structural elucidation, identification of functional groups, and determination of molecular mass, respectively. Furthermore, elemental analysis was carried out to determine the elemental composition of each compound. In selected cases, X-ray crystallography was used to determine the three-dimensional atomic and molecular structure of the compounds, thus providing a profound understanding of the spatial orientation of atoms and the chemical bonds that hold the atoms together. An initial phase of this project including the detailed synthesis of the compounds was published (see Amoah et al., 2021).

Preparation of Stock Solution

All synthesized test samples were scheduled for thorough antimicrobial assessments. A standard stock solution of each test compound, at a concentration of 2.0 mg/mL, was prepared in 1% Dimethylsulfoxide (DMSO). These solutions were stored at -20 degrees Celsius in an air-tight container to prevent degradation or contamination. Appropriate dilutions of the stock solutions (ranging from 64 µg/mL through 0.0625 µg/mL) were used in microbial experiments. As a necessary measure, currently recommended antibiotics, the specific choice of which depends on the microbial organism being studied, were included in each experiment as positive controls. This provided a basis of comparison, allowing us to gauge the relative efficacy of the novel pyrazolyl sulfonamides and their corresponding palladium complexes against currently used antimicrobial agents.

Test Organisms

The study used clinical isolates of Gram-positive organisms, including various Staphylococcus species (*Staphylococcus aureus, Staphylococcus haemolyticus, Staphylococcus epidermedes, Staphylococcus cohnii, Staphylococcus hominis), Corynebacterium striatum, and Enterococcus faecalis*. These organisms were selected for their medical relevance, as they are known to cause a range of infections, from common skin conditions to severe systemic diseases. To maintain these organisms, nutrient agar (Oxoid ltd, UK) was used for culture, while Mueller-Hinton Broth (Oxoid ltd, UK) was utilized as growth media. This standard and widely-used medium provides compatibility with a diverse array of organisms, facilitating the study of growth dynamics and susceptibility testing.

Kirby-Bauer Method for AST

Bacteria strains and Quality Control strain *Staphylococcus aureus* ATCC 25923 were cultured on a Nutrient Agar plate and incubated overnight for 16-18 hrs. Muller Hinton Agar (MHA) plates were dried in the incubator before preparing inoculum for AST. A standard inoculum of bacteria (0.5Mcf) was prepared in sterile saline water from a pure culture on appropriate isolation medium. Swab was immersed in the suspension (inoculum) and excess liquid was removed by swirling the swab against the sides of glass tube before spreading on a dried MHA. The entire agar plate was rubbed, rotating the dish to ensure even distribution. Antibiotics were inserted in a dispenser, and this was placed on the surface of the MHA. Petri dishes were incubated at 37℃ in the incubator for 16-18hrs and turned upside down. The plates were examined by measuring the zone of inhibition using a ruler. The inhibition diameter was compared against the CLSI guideline to determine the resistance (R), Immediate (I) and sensitive (S) of the strains to the antibiotics. The results of the AST are presented in Table 1.

Minimum Inhibitory Concentration

Broth Microdilution

The study adopted the procedure for broth microdilution outlined by (CLSI, 2020; Needs et al., 2022) with modifications. Clinical isolates were cultured on MHA and incubated at 36 – 37 ^{o} C for 24 hours. Pure cultures were adjusted to 0.5 McFarland equivalent corresponding to approximately 1–2 × 10^{8} CFU/mL. The culture was diluted 1:150 in Mueller Hinton Broth (OXOID CM0337) to give 1 x 10^{6} CFU/ mL suspension. Optical density (O.D) was measured at 600 nm using the Tecan Spark (Tecan life sciences,

Switzerland) and compared to the standard (O.D at 600 nm = 0.08 – 0.1). concentrations ranging from 64 µg/mL through 0.0625 µg/mL were considered in this study. In each 96-well plate, each column contained one antimicrobial agent and each row contained a different concentration of the antimicrobial agent. All experiments were run in triplicates.

Inoculation of 96-well Plate

Working concentrations of each test compound (0.0625 µg/mL – 64 µg/mL) was prepared by doing a two-fold serial dilution using 100µL of MHB in 96-well plates. Each test well was then inoculated with 100 µL of the microbial inoculum prepared in MHB medium and adjusted to 0.5 McFarland standard resulting in an in-well test sample concentration of 0.5 µg/mL – 64 µg/mL. Positive control wells were prepared similarly, using standard antibiotics at concentration of 0.0625 to 16 µg/mL. Growth control wells had only MHB media (100 µL) and inoculum (100 µL), whilst sterile control wells had only MHB media (200 µL).

To determine the purity, a sample of 0.01 mL was taken from the growth control well shortly after inoculation. After that, the sample was diluted in 10 mL of saline, which resulted in a dilution of one part in 1:1000 dilution. After the mixture was thoroughly combined, a 0.1 mL amount was taken out and spread out evenly across the MHA plate. After an incubation period of 16-18 hours, the presence of around 50 colonies indicated an inoculum density of 5×10^5 colony-forming units per millilitre (CFU/mL). After positioning the plates in an incubator shaker, the plates were incubated at a temperature of 37 degrees Celsius for a period ranging from 16 to 18 hours. The Tecan Spark V3.2 multimode microplate reader was utilized to

obtain the absorbance value at a wavelength of 600 nm. Simply put, the MIC is the lowest concentration of an antimicrobial drug that significantly slows the observable growth of a microorganism.

Minimum Bactericidal Concentration

The minimum bactericidal concentration of all active compounds was assessed using the broth microdilution method (Flamm, Farrell, Rhomberg, Scangarella-Oman, & Sader, 2017). A compound will be considered to exhibit bactericidal activity against a particular organism if the MBC/MIC ration is less or equal (≤) 4. After the MIC test, 10 µL sample was taken from the MIC well and above and cultured on a fresh MHA plate containing no antimicrobial. The plates were kept in an incubator at 36–37 degrees Celsius for 16–18 hours. After a period of incubation, the plates were checked for the presence of bacterial growth. If a compound's MBC/MIC ratio is less than or equal to 4, it is regarded to have bactericidal properties. Generally speaking, bacteriostatic properties are attributed to the molecule if the ratio is greater than 4 (Mogana, Adhikari, Tzar, Ramliza, & Wiart, 2020).

Synergistic Test

Synergistic activity was determined using the Checkerboard synergy testing method. Compounds were tested alone and in combination with tetracycline (CAYMAN, 1.800.364.9897) against each organism. The interpretation of the fractional inhibitory concentration (FIC) was applied as follows: synergy, ≤ 0.5; indifference, > 0.5 to ≤ 4.0; antagonism, > 4.0 (Flamm et al., 2017).

Checkerboard Method

The checkerboard method is a commonly used assay to determine the synergistic, additive, indifferent, or antagonistic interactions between two antimicrobial agents. This method allows for a range of concentrations for each drug to be tested in combination. The study adopted the checkerboard procedure outlined by (Bellio, Fagnani, Nazzicone, & Celenza, 2021). In a 96-well plate, 100 µL of MHB was dispensed into the wells using a multichannel pipette. A 4-fold (256 µg/mL) of the highest concentration (64 µg/mL) of the compound to be tested was prepared by dispensing 25.6 µL of the stock solution of the test compound and diluting to 200 µL using broth. With the multichannel pipette set at 100 µL, serial dilutions were performed and the last 100 µL from the last well was discarded. A 2-fold (128 µg/mL) of the highest concentration of tetracycline to be tested was also prepared. This was achieved by dispensing 12.8 µg/mL of the antibiotic and diluting with broth to 200 µL. The antibiotic was diluted in each well using a multichannel pipette set at 100 µL. In the 96-well plate, well A1 to G1 was designated to only the test compound and H2 to H8 was designated to only the antibiotic. H1 was a used as a growth control well (only MHB and bacterial inoculum). Using a multichannel pipette, 100 µL of approximately 1.6 $x10^6$ of the bacteria suspension was added to each well resulting in 200 µL with a final bacteria density of 5 x 10^5. A total of 77 combinations were tested and fractional Inhibitory Concentration index (FICI) calculated as follows:

$$FICI = \frac{MIC_{drug\ test\ compound\ in\ combination}}{MIC_{drug\ test\ compound\ alone}} + \frac{MIC_{drug\ antibiotic\ in\ combination}}{MIC_{drug\ antibiotic\ alone}}$$

The FICI is interpreted as follows:

- FICI ≤ 0.5 indicates synergy
- 0.5 < FICI ≤ 1 indicates additivity (or indifference)
- 1 < FICI ≤ 4 indicates no interaction
- FICI > 4 indicates antagonism

Time-kill Kinetics

Time-kill kinetics is a powerful method for evaluating the bactericidal activity of antimicrobial agents. Time-kill kinetics is used to observe the effects of antimicrobial agents on bacterial growth over time. In this study, bacterial growth curve analysis was performed using a range of concentrations of the antimicrobial agents in accordance with (Appiah, Boakye, & Agyare, 2017). Specifically, we used concentrations 0.5×, 1×, 2×, and 4×, the Minimum Inhibitory Concentration (MIC) against each organism. These concentrations were chosen to thoroughly understand the antimicrobial activity at both sub-inhibitory and supra-inhibitory levels. Sample from the bacterial populations were sampled at five specific time points: at the beginning of the experiment (time zero, T_0), 2 hours (T_2), 4 hours (T_4), 8 hours (T_8), and 24 hours later (T_{24}). These time points were selected to observe both the initial response of the bacteria to the antimicrobial agents and the longer-term effects over 24 hours. Each time-kill curve was determined in triplicates. This replication was to ensure the results' accuracy and assess the variability of the antimicrobial effects. In interpreting our results, bactericidal activity was defined as a decrease of at least 3 logarithmic units (≥3 -log reduction) in the bacterial counts, when expressed in log10 Colony Forming Units (CFU) per millilitre (Appiah et al., 2017).

Data Collection Procedures

Experiments were conducted at the Bacteriology Department of the Noguchi Memorial Institute for Medical Research and the School of Medical Sciences laboratories (UCC). Three independent experiments were conducted for each test sample to ensure uniformity of findings. Clinical isolates, test procedures including MIC, MBC, synergy testing, and time-kill kinetics were performed at the Noguchi Memorial Institute for Medical Research. Stock solutions of test compounds were prepared at the School of Medical Sciences laboratories and transported to Noguchi.

Data Management

Each data represented an average of three independent analysis. Data was securely saved on a desktop computer, external drive, and google drive after each analysis to prevent loss.

Data Analysis

The data obtained from the experimental procedures was subjected to statistical analysis using GraphPad Prism software, version 9.5.1. The study utilized a one-way Analysis of Variance (ANOVA) approach, followed by Tukey's post hoc test. The ANOVA is a statistical technique that can be utilized to determine whether or not there are differences in the means of three or more independent groups that can be considered statistically significant. These groups represent the different experimental conditions under which the antimicrobial agents were tested (varying concentrations). Following the ANOVA, Turkey’s post hoc test was employed. This test is specifically designed to compare several treatments with a single control. This test helped determined which specific groups differ from the control group (standard

antibiotic), providing further insights into the effectiveness of the antimicrobial agents under different conditions.

Quality Assurance

All equipment was calibrated before use to uphold the quality and reliability of the research findings. Standard operating procedures were strictly adhered to. Furthermore, to enhance the robustness of the data and account for any potential variability, all experiments were repeated three times. This replication ensured that the findings were not a product of random error or outlier data points, but were representative of consistent observations.

Chapter Summary

This study employed an experimental in vitro design to evaluate the antimicrobial activity of novel pyrazole sulfonamides and their palladium complexes against selected Gram-positive bacteria. The broth microdilution method was used to determine the MIC and MBC of the test compounds. The checkerboard synergy assay was conducted to assess potential interactions between the compounds and tetracycline, with the FICI used to classify the interactions as synergistic, additive, indifferent, or antagonistic. Time-kill kinetics were performed for selected synergistic combinations to evaluate bactericidal activity over time. Data were analyzed using descriptive statistics and ANOVA and results were presented as mean values. All experiments were conducted under controlled laboratory conditions to ensure reproducibility and reliability of the findings.

CHAPTER FOUR

RESULTS AND DISCUSSION

Introduction

This chapter provides an in-depth presentation of the findings obtained from the study. This work aimed to investigate the antibacterial properties of novel pyrazole sulfonamides and the palladium complexes that correspond to them. The compounds were synthesized using a condensation reaction involving diketone and phenylhydrazine. Broth microdilution was employed to obtain the MIC and MBC values of the compounds. Synergistic testing was conducted to determine whether or not the chemicals were more effective when combined with tetracycline. To determine the killing effect (bacteriostatic and bactericidal) of the drugs, time-kill kinetics assay was employed to successful synergistic combinations. GraphPad Prism, version 9.5.1, was utilized throughout every stage of the data analysis process.

AST Results

The AST confirmed high resistance among the Gram-positive isolates. All were resistant to oxacillin, indicating methicillin resistance. Isolates of *S. haemolyticus, S. epidermidis, S. hominis,* and *C. striatum* were resistant to erythromycin and chloramphenicol, while *S. cohnii* and *E. faecalis* showed multi-drug resistance. Also, *S. aureus* and *S. haemolyticus* had intermediate susceptibility to vancomycin and gentamicin, while *S. epidermidis* and *S. hominis* were fully resistant. Tetracycline was the most effective antibiotic, with all isolates susceptible except *S. haemolyticus*, which showed intermediate resistance. These findings confirm antibiotic resistance, justifying the need to test the novel pyrazole sulfonamides (Table 1).

Table 1: AST results of antibiotic to the selected Gram-positive isolates

Bacterial Isolate	ID	Oxacillin	Vancomycin	Erythromycin	Chloramphenicol	Gentamycin	Amikacin	Tetracycline
S. Aureus	045W 10/11/23 MAC	R	16 I	23 S	11 R	16 I	28 S	30 S
S. Haemolyticus	042W 9/11/23 MAN	R	18 S	10 R	12 R	12 R	15 I	28 S
S. Epidermidis	046W 16/11/23 MAC	R	20 S	11 R	10 R	10 R	9 R	29 S
S. Cohnii	013W 20/2/23 C2 MAN	R		6 R				
S. Hominis	045B 10/11/23 MAC	R	10 R	6 R	10 R	10 R	22 S	28 S
C. Striatum	042W 14/11/23 MAN	R	21 S	9 R	10 R		6 R	30 S
E. Faecalis	032W 28/7/23 MAC	R		15 I			6 R	20 S

Test Compounds

A total of 14 novel derivatives of pyrazole-sulfonamides were synthesised, with modifications in structure at the R site of the sulfonamide (involving substituted cyclohexyl/phenylethylamine compounds) and at the R1 site with the addition of a methyl group on the pyrazole ring as shown in Figure 1. The compounds were synthesised using a condensation procedure involving the suitable diketone and phenylhydrazine. Subsequently, they were subjected to several characterization techniques, including nuclear magnetic resonance (NMR), infrared (IR) spectroscopy, mass spectrometry, elemental analysis, and, in some instances, X-ray crystallography. This study involved the synthesis of new small compounds based on N-substituted pyrazol-5-carboxylate and pyrazolopyrimidine scaffolds. The compounds and their corresponding IUPAC names are provided in Appendix A2.

Antimicrobial Activity of Compounds

This study tested fourteen newly synthesized pyrazole sulfonamides against seven (7) Gram-positive organisms, including *S. aureus, S. hominis, S. epidermidis, S. haemolyticus, S. cohnii, C. striatum, and E. faecalis.*

A preliminary examination of the compounds' potential antibacterial effects was carried out via broth microdilution. The antibacterial activity of the compounds is compared in Table 2 against a variety of Gram-positive bacterial species, as was previously described. Compounds CA47 and CA11B demonstrated activity against the tested Gram-positive organisms. These two compounds exhibited activity against three out of the seven Gram-positive species that were tested up to the maximum concentration that was tested, which was 64 µg/mL. CA47 was active against *S. aureus, S. epidermidis, and*

S. haemolyticus, CA11B demonstrated activity against *S. aureus, S. haemolyticus, and S. hominis.* The most susceptible organism was *S. haemolyticus*, with half of the tested compounds having activity against it up to the maximum tested concentration. On the other hand, *S. cohnii* and *E. faecalis* were resistant to all the sulfonamide derivatives tested up to 64 µg/mL.

Table 2: Activity of compounds against selected Gram-positive bacterial species

Compound ID	*S. aureus*	*S. epidermidis*	*S. Haemolyticus*	*S. cohnii*	*S. hominis*	*E. faecalis*	*C. striatum*
CA45A	-	-	+	-	-	-	-
CA45B	-	-	+	-	-	-	-
CA45C	-	-	-	-	-	-	-
CA45E	-	-	-	-	-	-	-
CA47	+	+	+	-	-	-	-
CA48A	+	-	+	-	-	-	-
CA50A	-	-	-	-	+	-	-
CA50B	-	-	-	-	+	-	-
CA50C	-	-	-	-	-	-	-
CA50D	-	-	-	-	+	-	-
CA-032	-	-	+	-	-	-	-
CA11B	+	-	+	-	+	-	-
CO-2	-	-	-	-	-	-	-
CO-3	-	-	+	-	-	-	+

+ = Activity; - = No Activity
Source: Field Survey (2023)

In the CA50 series, three compounds; CA50A, CA50B, and CA50D were exclusively active against *S. hominis*, showing no activity against any other bacterial species tested. These compounds share similar chemical structure, which could account for this observation. The compounds CA45A, CA45B, and CA-032 were solely active against *S. Haemolyticus*, with no activity noted against other bacteria. Compound CO-3 demonstrated activity against *S. Haemolyticus* and *C. striatum* and was inactive against all other bacterial species. The rest of the compounds, CA45C, CA45E, CA50C, and

CO-2, showed no activity against any bacterial species when tested up to 64 µg/mL.

MIC and MBC of Test Compounds

The MIC is the lowest concentration of an antimicrobial agent that inhibits the visible growth of a microorganism after 24 h incubation. Therefore, reliable antimicrobial susceptibility data is crucial because it helps clinicians determine which drug to prescribe for a particular microbial infection. Unlike MIC, which only looks at growth inhibition, MBC is the concentration at which the bacteria are actually killed. The MIC and MBC values ranged from 0.0625 ug/mL to 64 ug/mL (Table 2). In the case of *S. Haemolyticus*, compounds CA45A, CA48A, CA-032, CA11B, and CO-3 show varying degrees of efficacy, with MIC and MBC values ranging from 2 to 8 µg/ml. Notably, CA47 demonstrated strong inhibitory and bactericidal effects with the lowest MIC and MBC values of 1 µg/ml, while CA11B and CA45B appeared less effective with MIC and MBC of 8 and 16 µg/ml respectively. For *S. hominis*, the compounds in CA50 series (Appendix A2. [**l, n**, and **o**]) and CA11B were the only compounds that demonstrated positive results, with the CA50 series showing MIC and MBC values ranging from 8 to 32 µg/ml. CA11B also showed potential with MIC and MBC values at 4 µg/ml. Compounds CA47 and CA48A recorded MIC values of 64 µg/ml and 0.5 µg/ml against *S. aureus*. For *C. striatum*, only CO-3 showed potential, with MIC value of 64 µg/ml. For Tetracycline the MIC and MBC values ranged from 0.25 to 2 µg/ml for MIC and 0.5 to 2 µg/ml for MBC in the tested organisms.

Table 3: Minimum inhibitory and bactericidal concentrations (ug/ml) of test compounds against selected Gram-positive bacterial species

Compound ID	*S. Haemolyticus*		*S. hominis*		*S. aureus*		*S. epidermidis*		*C. striatum*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
CA45A	2	4	-	-	-	-	-	-	-	-
CA11B	8	32	4	16	0.5	2	-	-	-	-
CO-2	-	-	-	-	-	-	-	-	-	-
CO-3	2	8	-	-	-	-	-	-	64	-
CA-O32	2	4	-	-	-	-	-	-	-	-
CA45B	16	32	-	-	-	-	-	-	-	-
CA45C	-	-	-	-	-	-	-	-	-	-
CA45E	-	-	-	-	-	-	-	-	-	-
CA12A	-	-	-	-	-	-	-	-	-	-
CA47	1	2	-	-	64	-	64	-	-	-
CA48A	2	4	-	-	64	-	-	-	-	-
CA50A	-	-	32	64	-	-	-	-	-	-
CA50B	-	-	16	32	-	-	-	-	-	-
CA50C	-	-	-	-	-	-	-	-	-	-
CA50D	-	-	8	16	-	-	-	-	-	-
PC	0.25	0.5	0.25	1	2	4	0.25	0.5	1	2

Note: - indicates inactivity at the highest tested concentration; PC: positive control. *S. cohnii* and *E. faecalis* were excluded since the compounds showed no activity against them.
Source: Field Survey (2023)

The inhibitory activity of compound CA11B in *S. aureus*, compared with the positive control is as shown in Figure 4. The compound's activity generally increases from a lower concentration of 0.25 µg/mL, and peaked at 16 µg/mL. Comparatively, the IC_{50} of CA11B in *S. aureus* (2.55 µg/mL) was slightly higher than that of the positive control (2.02 µg/mL).

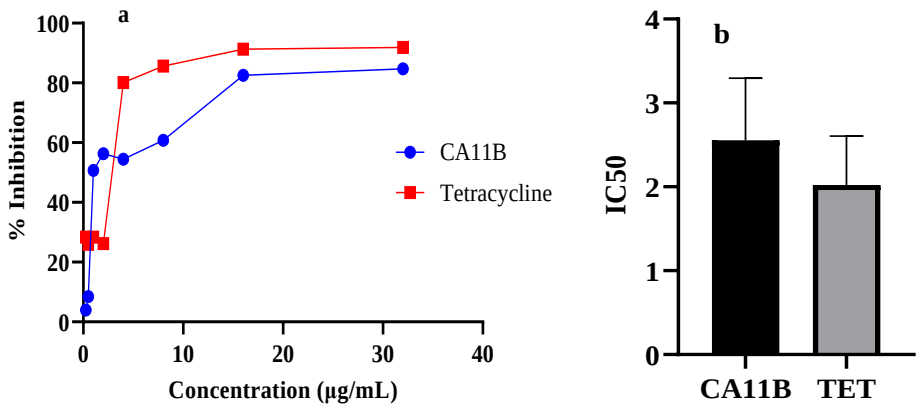


Figure 4: Percent inhibitory activity of CA11B in S. aureus
TET = Tetracycline
Source: Field Survey (2023)

The percent inhibitory activity of compounds CA50A, CA50B, CA50D, CA11B against *S. hominis* was similarly assessed and presented on Figure 5. The most active compound against *S. hominis* was CA11B, with an IC_{50} value of 2.4 µg/mL. On the other hand, CA50A demonstrated the weakest activity against this organism, with a recorded IC_{50} value of 10.88 µg/mL. However, the positive control was much more active against *S. hominis* (IC_{50} = 0.80) when compared with the compounds.

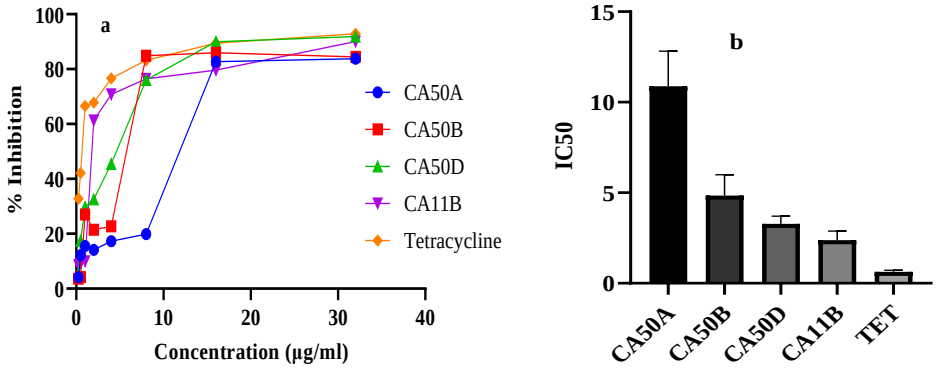


Figure 5: Inhibitory activity of compounds in *S. hominis*
Source: Field Survey (2023)

In *S. haemolyticus* (Figure 6), the lowest IC_{50} value (0.92 µg/mL) was recorded for CA48A, whilst CA45A recorded the highest value in *S. haemolyticus* (10.28 µg/mL). Although CA48A was the most active in *S. haemolyticus*, its IC_{50} was slightly higher than that of the positive control (IC_{50} = 0.8 µg/mL).

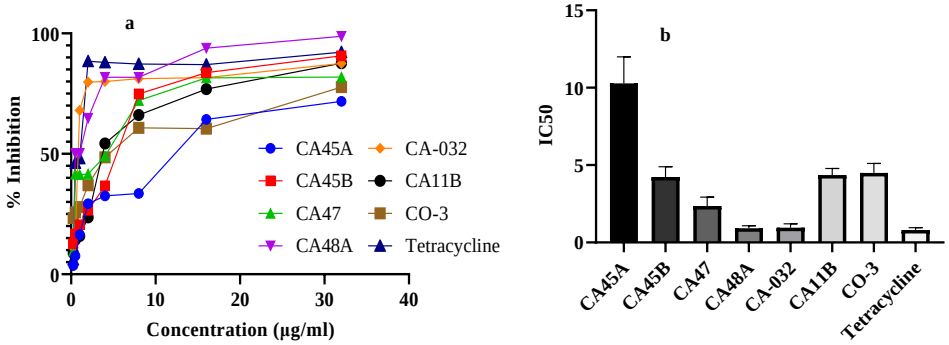


Figure 6: Inhibitory activity of compounds against *S. haemolyticus* growth
Source: Field Survey (2023)

Synergistic Assessment of Test Compounds

The broth microdilution checkerboard method was used to assess the test compounds' synergistic activity when combined with tetracycline (Table

3). Compounds with MIC values 64 µg/ml and above were excluded in this test. The results from the checkerboard assay reveal diverse interaction patterns between the test compounds and tetracycline across the bacteria strains tested. For the combination of CA11B with tetracycline, no interaction was observed against *S. haemolyticus* and *S. hominis*, indicated by FICIs of 2.0078 and 0.2656, respectively. However, against *S. aureus*, the interactional mode was indifferent, with a FICI of 0.6250. Similarly, the combination of CA45A with tetracycline exhibits no interaction against *S. haemolyticus*, reflected by a FICI of 1.0312. Contrastingly, several combinations demonstrate synergistic mode of interactions, particularly against *S. haemolyticus*. The combinations of CA45B, CA-032, CA47, CO-3, and CA48A with tetracycline all exhibit synergy, evidenced by FICIs of 0.5039, 0.2813, 0.3125, 0.2813, and 0.2813, respectively. In the case of *S. hominis*, the combinations of CA50A and CA50D with tetracycline also display synergistic mode of interactions, with FICIs of 0.2519 and 0.2578, respectively. However, the combination of CA50B with tetracycline showed no interaction towards *S. hominis*, as indicated by a FICI of 1.0078.

Table 4: Synergistic activity of test compounds in combination with tetracycline

Bacterial Strain	Compound ID	MIC alone (µg/mL)		MIC in combination (µg/mL)		FICI	Interaction pattern
		S	T	S	T		
S. haemolyticus	CA45A	2	0.25	0.0625	0.0625	0.2813	Synergistic
	CA11B	8	0.25	0.0625	0.5	2.0078	Indifferent
	CO-3	2	0.25	0.0625	0.0625	0.2813	Synergistic
	CA-O32	2	0.25	0.0625	1	4.0313	Antagonistic
	CA45B	16	0.25	0.0625	0.125	0.5039	Indifferent
	CA47	1	0.25	0.0625	0.0625	0.3125	Synergistic
	CA48A	2	0.25	0.0625	0.0625	0.2813	Synergistic
S. aureus	CA11B	0.5	2	0.0625	4	2.1250	Indifferent
S. hominis	CA11B	4	0.25	0.0625	2	8.0156	Antagonistic
	CA50A	32	0.25	0.0625	0.0625	0.2520	Synergistic
	CA50B	16	0.25	0.125	2	8.0078	Antagonistic
	CA50D	8	0.25	0.0625	0.0625	0.2578	Synergistic

FICI - fractional inhibitory concentration index; MIC - minimum inhibitory concentration; S - sulfonamide compound; T – tetracycline.
Source: Field Survey (2023)

Time-kill Kinetics of Synergised Compounds

Time-kill kinetic assays were conducted for all synergistic combinations to assess the compounds antibacterial activities over time in accordance with (Appiah et al., 2017). The assay time was limited to 24 h. A growth control was included as a negative control. In most cases, exposure to high antimicrobial concentrations resulted in a lower asymptote of the test compounds and higher concentrations had a slightly additional effect on the growth rate (Figures 7, 8). All bacteria inoculum density was adjusted to 5 x 10^5 CFU/mL for all experiments. Five dilutions (0.5x, 1x, 2x, 4x MIC) were plotted.

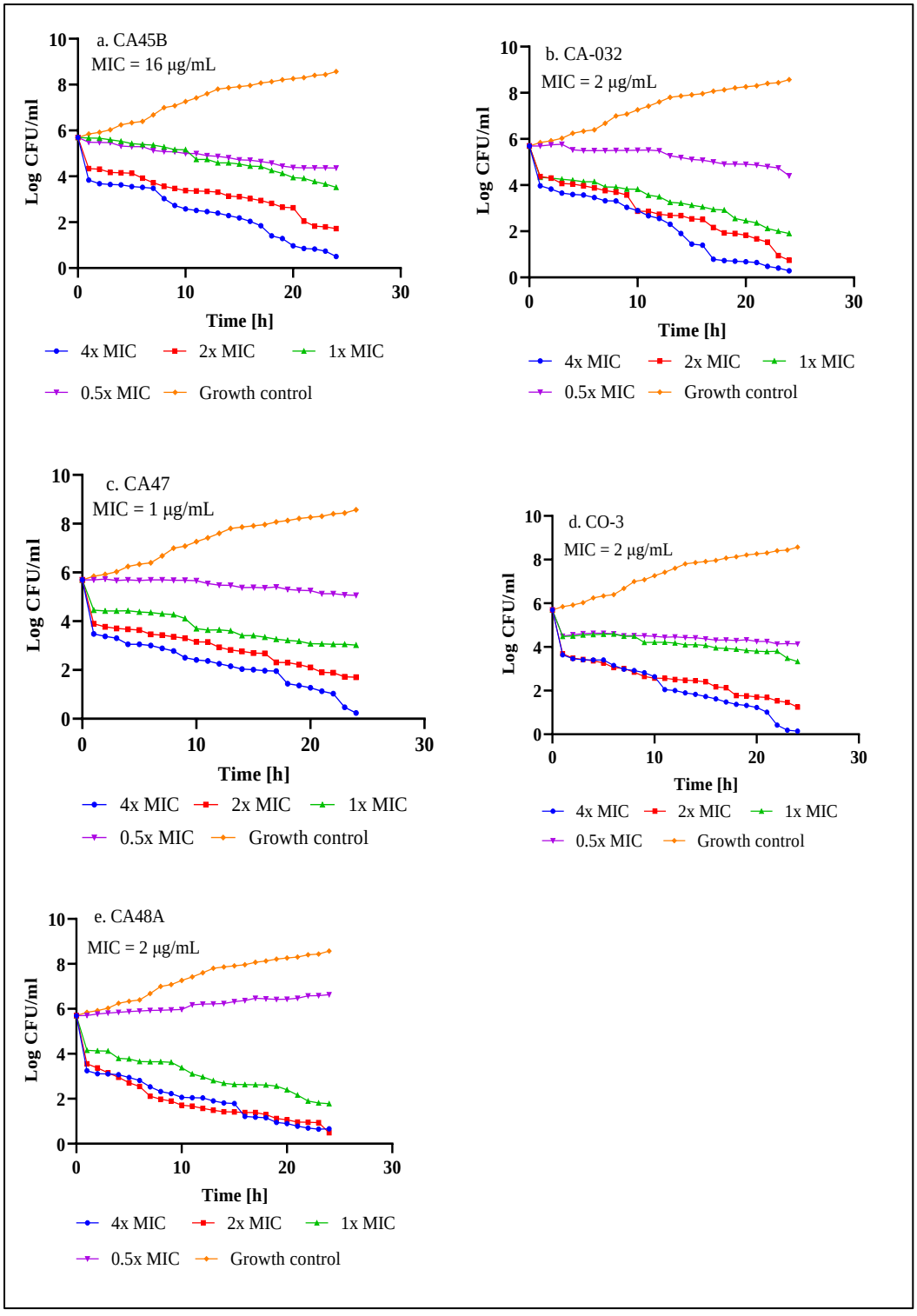


Figure 7: Time-kill curves of Synergised compounds against *S. haemolyticus*.
Source: Field Survey (2023)

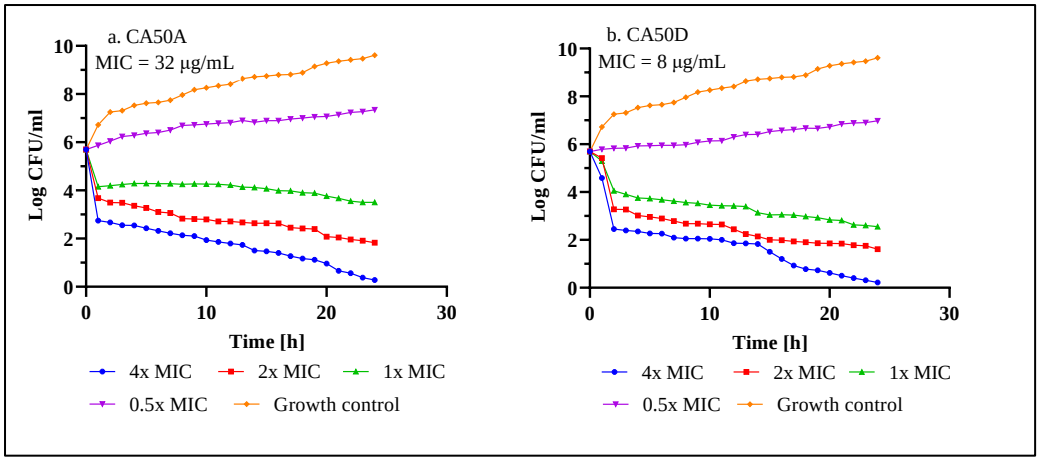


Figure 8: Time-kill curves of Synergised compounds against *S. hominis*
Source: Field Survey (2023)

Most notably and in all cases, concentrations of 0.5x MIC of the test compounds resulted in no reduction in bacteria growth rate as the bacteria grew to a level similar to the growth control. As shown in Figures 7 and 8 for *S haemolyticus* and *S. hominis* respectively, concentrations of 2x and 4x MIC all exhibited killing effect against *S. haemolyticus* and *S. hominis* reducing the initial Log CFU/mL by greater than 3 Logs. Concentrations equal the MIC also exhibited bacteriostatic effects, as the log CFU/mL over time remained roughly the same as the starting Log CFU/mL concentration.

Discussion

The sulfonamides have long been used clinically to manage diverse microbial infections. Like many antibiotics, the menace of antimicrobial resistance has limited the efficacy of the sulfonamide antibiotics. Various approaches are being explored in an attempt to overcome the resistance. This study determined the efficacy of novel synthesised sulfonamide derivatives against a selected clinically important Grams positive organisms.

The antibacterial activity of the pyrazolyl sulfonamide compounds (a – k, see Appendix A2) and their corresponding palladium complexes (l – o, see

Appendix A2) was evaluated against seven Gram-positive bacteria, namely S. *aureus, S. hominis, S. haemolyticus, S. epidermidis, S. cohnii, Enterococcus faecalis, and C. striatum*. The microorganisms described above are examples of what are known as human pathogenic bacteria. These bacteria are notorious for their propensity to cause various diseases in humans, including infections of the urinary system, the intestinal tract, and the respiratory tract, amongst others. In addition, organisms can evolve specialized resistance mechanisms, such as the deactivation of antimicrobial drugs and the change of microbial targets within the host organism. This can make it more difficult for antimicrobial drugs to be effective. These mechanisms reduce the efficacy of pharmaceuticals and antibacterial drugs (Chellat, Raguž, & Riedl, 2016), and the release of antimicrobial compounds from the bacterial cell for the bacteria to continue living inside the host (Walsh, 2000). There is an increasing need for the development of novel formulations to effectively address and overcome resistance.

The study assessed the minimum inhibition concentration (MIC) values throughout a range of concentrations, specifically 0.0625 µg/mL, then doubling to 64 µg/mL. The compounds demonstrated varying degrees of antibacterial activity, from weak to strong, as indicated in Table 2. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values for all the compounds tested were seen to be within the ranges of 0.5 µg/mL to 64 µg/mL. Nevertheless, as seen in none of the compounds exhibited any discernible activity against the Gram-positive bacteria strains *S. cohnii* and *E. faecalis*, as seen in Table 1. Additionally,

trends comparable to the activity observed for the compounds could be made for their palladium complexes.

A major significant observation was that including palladium in the compounds resulted in enhanced activity for certain compounds. For instance, CO-3 and CA-032 exhibited little activity against *S. hominis*. However, their palladium adducts demonstrated significant activity, comparable to that of the standard medication, as indicated in Table 2. In earlier research, Solmaz et al. were able to successfully prepare platinum (II) complexes of N, N-Di-(R)-N'-(4-chlorobenzoyl) thiourea. The antibacterial activity of these complexes were evaluated against a variety of microorganisms, including *S. aureus, S. pneumonia, E. coli, P. aeruginosa, A. baumanni* (Solmaz et al., 2018). The compounds had MIC value of 3.90 g/mL, indicating they were highly effective against *Streptococcus pneumoniae, Pseudomonas aeruginosa, and Acinetobacter baumannii*. In addition, they exhibited modest effectiveness against *E. coli, S. aureus, and Candida albicans*, with MIC value of 15.62 g/mL. Using a bidentate ligand, (E)-3-(4-(dimethylamino)phenyl)-1-(pyridin-2-yl)prop-2-en-1-one, Gaber et al. studied the production of chalcone complexes of palladium(II) and platinum(II) in a more recent work (Gaber, El-Ghamry, & Mansour, 2018). The platinum (II) complex had low IC_{50} values, but almost no antibacterial potency (MIC value of 30 mg/mL) against *Candida albicans, Aeromonas flavus*, *Escherichia coli*, or *Staphylococcus aureus*.

In treating and managing bacterial infections, primarily those caused by mycobacteria belonging to the Mycobacterium tuberculosis complex, the use of metal-complexed sulfonamides has been shown to offer substantial potential as a practical method. Previous research has shown this to be the case

(Agertt et al., 2013) as well as against pathogens belonging to the group of non-tuberculous mycobacteria (Agertt et al., 2013; Siqueira et al., 2018). Furthermore, various investigations have documented the antibacterial properties of these complexes with Gram-negative pathogens, specifically *E. coli* (Rigon Mizdal et al., 2017) and *P. aeruginosa* (Mizdal et al., 2018), and Gram-positive microorganisms, such as MRSA (Mizdal et al. 2018). Chohan and colleagues also investigated the sulfonamide complexes with Co, Cu, Ni, and Zn. They reported on the antifungal properties of these compounds against *Candida albicans, Aspergillus niger, Microsporum canis, and Trichophyton mentagrophytes*, highlighting the significance of investigating metal-coordinated sulfonamides as potential antimicrobial agents (Chohan, Sumrra, Youssoufi, & Hadda, 2010).

Furthermore, the MIC values obtained for the salts employed in synthesising the compounds investigated in this study confirm that the observed enhancement in biological activity can be attributed to the formation of metal complexes rather than the inherent properties of the salts. The current synthesized pyrazole compounds showed significant antibacterial efficacy against various Gram-positive bacteria. Several pyrazoles that have a trichloromethyl (CCl3) group have exhibited notable antibacterial activity. Aggarwal et al. synthesized fifteen compounds, which were subsequently subjected to in vitro screening to evaluate their antibacterial efficacy against multiple bacterial strains. The compounds exhibited strong efficacy against *Pseudomonas aeruginosa*, as shown by a minimum inhibitory concentration (MIC) of 8.822 µg/mL. The concentrations of ampicillin and chloramphenicol were determined to be 1.396 µg/mL and 0.969 µg/mL, respectively (Aggarwal

et al., 2014). Bhavanarushi et al. synthesized a collection of bispyrazoles including a pair of trifluoromethyl (CF_3) moieties. The antibacterial activity of all produced compounds was assessed in vitro against *Pseudomonas aeruginosa, Xanthomonas protophormiae, Bacillus licheniformis, and Staphylococcus aureus*. The MIC values for most of the compounds ranged from 25 to 50 mg/mL, in comparison to the control group treated with ciprofloxacin (12 µg/mL) (Bhavanarushi, Kanakaiah, Bharath, Gangagnirao, & Vatsala Rani, 2014).

The results obtained in the present study align with prior research, as evidenced by the range of minimum inhibitory concentration (MIC) values observed, which spanned from 0.5 µg/mL to 32 µg/mL. In the words of Efenberger-Szmechtyk and colleagues, the modification in the structure of sulfonamides led to many modes of action, including the inhibition of protein and ATP synthesis, resulting in alterations of metabolic processes and the inhibition of DNA synthesis (Efenberger-Szmechtyk, Nowak, & Czyzowska, 2021) which has the potential to result in cellular instability.

Additionally, it was shown that many antimicrobial drugs tested exhibited equivalent MIC and MBC values. This observation suggests that these compounds, when present at such concentrations, demonstrate a suppressive impact on the growth of bacteria, leading to increased mortality rates. Utilizing in-vitro techniques to assess the synergy and additive effects of drugs contributes to the advancement of understanding in combination therapy, enhancing the efficacy of bacterial clearance. The interpretation of the results was based on the fractional inhibitory concentration index (FICI) method, which determined if the combination of antibiotics exhibited

synergistic, additive, or antagonistic effects. From literature, FICI index of 0.5 or lower was classified to have a synergistic effect, whereas a FICI index of 4 or above was regarded to have an antagonistic effect. Nevertheless, multiple research studies have yielded disparate FICI indices on additive and indifference effects (Lorian, 2005). In their research, the combination of colistin with meropenem demonstrated a synergistic effect of 65% and an additive impact of 35% (Kaelin, 2005; Keith, Borisy, & Stockwell, 2005; Sharom, Bellows, & Tyers, 2004). Based on the FIC results obtained in this study, CA-032 emerged as the most effective compound, inhibiting 94% of S. haemolyticus growth. CA45B and CA47 trailed closely behind it, with 93% and 90% inhibition, respectively. Medications that demonstrate synergy are always regarded to be superior to medications that do not show synergy. This is because synergistic combinations of two or more drugs may prevent toxicity and other side effects that are connected to high doses of single treatments. This is because synergy can be produced in various ways, such as by neutralizing biological compensation, sparing doses on each chemical, or exploiting context-specific multitarget processes.

The strong bacteriostatic capacity was a result of antibacterial agents' ability to circumvent bacterial resistance development (Sun, Ansari, Battini, Bheemanaboina, & Zhou, 2019; Wang, Battini, Bheemanaboina, Zhang, & Zhou, 2019). Therefore, time-kill assays were performed on synergised compounds for the respective bacteria strains. Five compounds (CA45B, CA-032, CA47, CO-3, CA48A) exhibited synergism against *S. haemolyticus* while two compounds (CA50A, CA50D) demonstrated significant synergistic mode of interaction against *S. hominis*. As depicted in Figure 4, all the test

compounds exhibited strong bacteriostatic activity and the inhibiting effect of CA47 toward *S. Haemolyticus* was stronger than CA45B, CA-032, CO-3 and CA48A. About 3 log10 CFU/mL colony reduction of *S. Haemolyticus* was achieved by CA47 at 2x and 4 × MIC (1 μg/mL) for 1.5 h, which was more potent than CA45B, CA-032, CO-3 and CA48A. These results demonstrated that tert-butyl substituent on CA47 pyrazole group possessed excellent inhibiting capacity against *S. Haemolyticus*, which was conducive to shortening the treatment time. It was also observed that, with time up to 24 h, concentrations 2x and 4x MIC values maintained steady decrease in viable cell count. This suggests that the compounds can maintain appreciable amount of their molecules in the cell membrane to maintain the antimicrobial effect for this period. CA50A with MIC of 32 μg/mL exhibited a bactericidal effect within the first hour by reducing the starting bacteria load of 5 x 10^5 CFU/mL by 3 $\log_{10}$ CFU/mL after inoculation. It maintained steady decrease in bacteria load for the 24 h. This indicates that CA50A is more potent than CA50D (MIC = 8 μg/mL), exhibiting a bactericidal effect after 2 h. This potency was enhanced by substituting the hydrogens of the amide group with -CH3 groups. In the presence of the compounds evaluated, the death curve showed a decrease in the survival of *S. haemolyticus* and *S. hominis* in the first 2 h of treatment.

In exploring structure-activity relationships among the synthesized compounds, a pivotal observation was the pronounced influence of the sulfonamide substituents on antibacterial activity. While sharing a core structure, the compounds CA45A and CA45B exhibit differences in activity against *S. haemolyticus*, with MIC values of 2 μg/ml and 16 μg/ml,

respectively. This divergence in activity could be attributed to the alteration in the alkyl chain from tert-butyl to propyl group, which potentially modulates lipophilicity, membrane permeability, and interaction with bacterial cellular targets. Similarly, the variation in the sulfonamide substituent between CA47 and CA48A, from tert-butyl to propyl, results in a significant disparity in activity against *S. aureus*, with MIC values of 1 µg/ml and 64 µg/ml, respectively. This underscores the role of the sulfonamide group in determining the interaction with bacterial cellular machinery and modulating antibacterial efficacy. Previous studies have documented the pivotal role of alkyl chain variations in modulating pharmacological activity (Biošić, Mitrevski, & Babić, 2017). The alteration in activity due to the change from tert-butyl (branched chain) to propyl (straight chain) could increase the lipophilicity of the compounds which is consistent with literature indicating that lipophilicity and membrane permeability are crucial determinants of drug-receptor interactions (Frei, 2020).

A noteworthy aspect of the modification is the introduction of palladium complexes CA50A, CA50B, and CA50D, which demonstrate the potential of metal incorporation in enhancing antibacterial activity. These complexes exhibit selective and varied activity against *S. hominis*, with CA50D showcasing superior potency with MIC of 8 µg/ml and 92% inhibition, as opposed to 16 µg/ml (90% inhibition) and 32 µg/ml (83% inhibition) for CA50B and CA50A, respectively. The variation in activity among these complexes highlights the critical role of the nature of the ligand attached to the palladium centre in determining interaction with bacterial proteins and obstructing essential biological processes. These findings align

with various studies investigating the antibacterial activity of palladium complexes against both Gram-positive and Gram-negative bacteria (Khan et al., 2020; Sorinezami et al., 2019). For example, a study by Mansouri-Torshizi et al. (2019) evaluated the antibacterial activity of palladium (II) complexes containing bidentate Schiff base compounds against a range of bacterial strains, including *Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa*.

The results demonstrated that these complexes exhibited substantial antibacterial activity, with minimum inhibitory concentrations (MICs) comparable to those of standard antibiotics such as gentamicin and ciprofloxacin. The study indicated that the compounds' nature and the palladium complexes' coordination geometry played a significant role in determining their antibacterial potency (Mansouri-Torshizi et al., 2019). This finding illuminates the potential of metal complexes as customizable antimicrobial agents, with the ability to tune their activity through ligand modifications. The compounds CA11B and CO-3 offer insights into the impact of nitrogen substituents on the spectrum of antibacterial activity. CA11B, characterized by tetramethyl substituents, exhibits a broad spectrum of activity, including a potent MIC of 0.5 µg/ml against *S. aureus*. In contrast, CO-3, with dimethyl substituents, demonstrates selectivity towards *C. striatum*, with MIC of 64 µg/ml. This divergence in activity profiles emphasizes the significance of nitrogen substituents in modulating interactions with bacterial enzymes and cellular structures, thereby influencing the selectivity and potency of the compounds against different bacterial strains.

Chapter Summary

The study evaluated the antimicrobial activity of fourteen novel pyrazole sulfonamides synthesized by a condensation reaction of the appropriate diketone and phenylhydrazine. Variations in alkyl chains and the introduction of palladium complexes showcased diverse activities against specific bacterial strains, noting the potential of such modifications in enhancing lipophilicity, membrane permeability, and interaction with bacterial targets. The compounds demonstrated good antibacterial activity with MIC and MBC values ranging from 0.5 µg/mL to 64 µg/mL. The checkerboard assay revealed that most compounds exhibited synergism with tetracycline, especially against *S. haemolyticus* and *S. hominis*. Time-kill assays further revealed the compounds' sustained antimicrobial effects and synergism, notably with CA47 exhibiting significant inhibitory capacity against *S. haemolyticus*. The findings align with existing literature, highlighting the promising nature of these compounds against a spectrum of Gram-positive bacteria and the potential of structural alterations in inhibiting essential biological processes, thereby addressing challenges associated with bacterial resistance and biofilm formation.

CHAPTER FIVE

SUMMARY, CONCLUSION, AND RECOMMENDATIONS

Introduction

This study evaluated the antibacterial acitivty of fourteen novel pyrazole sulfonamides and their corresponding palladium complexes. The compounds were synthesised using a condensation reaction of the appropriate diketone and phenylhydrazine. This chapter presents a detailed summary of the study findings in a rundown. It provides conclusion and recommendations based on the study's findings. Finally, the chapter closes with suggestions for future research.

Summary

The sharp decline in the availability of newer antibiotics, the worldwide growth in AMR, and the current COVID-19 pandemic are all indications that action plans for the improved treatment of infectious microorganisms are urgently required. Preserving the effectiveness of antibiotics and investing in research and development of new antibiotics could positively contribute to global initiatives and programs already in place to combat antimicrobial resistance. Even while this can be a step in the right direction toward combating the silent pandemic, it is time for a paradigm shift in AMR research to focus on innovations in antibiotic discovery. The development of AMR is practically every countermeasure, and as a result, the world's options for treating infections are becoming increasingly restricted. To reduce AMR, complex and customized multi-sectoral strategies are required (Dutescu & Hillier, 2021). In light of this, fourteen pyrazole sulfonamides and their corresponding palladium complexes were synthesized and categorised by

nuclear magnetic resonance (NMR) spectroscopy, infrared (IR) spectroscopy, mass spectrometry, and x-ray crystallography in selected cases. The biological activity of the compounds was evaluated using the broth microdilution method at the Noguchi Memorial Institute for Medical Research. MIC and MBC were determined using the broth microdilution method. Bacteria suspension of density 5 x 10^5 CFU/mL inoculated in a 96-well plate with serial dilutions of the test compounds with concentrations ranging from 0.0625 to 64 µg/mL. After 24h incubation, absorbance was read using the TECAN SPARKCONTROL. The MIC was determined by identifying the well with the lowest concentration of the antimicrobial that shows no increase in absorbance, implying no bacterial growth. The plates were re-incubated for an additional 24 h and MBC was determined by identifying well with the lowest antimicrobial concentration that shows no absorbance increase, implying no bacterial death.

Synergistic activity was determined using the Checkerboard synergy testing method. Compounds were tested alone and in combination with tetracycline against each organism. The interpretation of the efficacy of the combinations was done using the fractional inhibitory concentration index. Time-kill kinetics were conducted on synergised combinations to confirm the checkerboard assay results. A compound was considered bactericidal if the compound was able to reduce the starting CFU/mL by 3 Log_{10}.

Key Findings

Structural-activity Relationship

In evaluating the structure-activity relationships among the synthesized compounds, the study unveiled the significant impact of sulfonamide

substituents on antibacterial activity. Notably, compounds CA45A, CA45B, CA47, and CA48A exhibited significant activities and MIC values against *S. haemolyticus* and *S. aureus*, attributed to alterations in alkyl chains on the amide group. Furthermore, incorporating palladium complexes, specifically CA50A, CA50B, and CA50D, manifested a promising avenue for enhancing antibacterial efficacy. Among these, CA50D stood out, displaying superior potency and a narrow spectrum of activity against *S. hominis*, underscoring the pivotal role of the nature of the ligand in bacterial protein interaction. Moreover, the compounds CA11B and CO-3 provided valuable insights, revealing the significant influence of the nitrogen substituents on the spectrum of antibacterial activity, with CA11B demonstrating broad-spectrum efficacy and CO-3 showing selectivity towards *C. striatum*. These findings collectively emphasize the significant interactions and modifications that contribute to the antibacterial potency of the compounds.

Biological Activity of Compounds

In this study, pyrazolyl sulfonamide compounds and their palladium complexes were evaluated for their antimicrobial activity against seven Gram-positive bacteria, including strains known for causing various infections and developing resistance strategies. The broth microdilution method was used to determine growth inhibitions and minimum inhibitory concentration (MIC) values, revealing that the compounds exhibited a range of antibacterial activities, with MIC and MBC values falling within 0.5 µg/mL to 64 µg/mL, compared to the control antibiotic tetracycline (0.25 µg/mL to 2 µg/mL). Interestingly, all compounds showed no activity against *S. cohnii* and *E. faecalis.* A significant finding was that the improved activities of some

compounds, such as CA50D and CA50A, demonstrated activity comparable to the standard drug upon the addition of palladium.

The study also investigated the synergy and additive effects of the drugs, revealing that five combinations showed synergism against *S. haemolyticus*, and two compounds demonstrated significant synergistic interaction against *S. hominis*. CA-032 was the most efficacious, inhibiting of *S. haemolyticus* growth, followed by CA45B and CA47. The time-kill assays performed on synergised compounds showed that all compounds achieved about 3 log10 CFU/mL colony reduction of *S. haemolyticus*, demonstrating their strong inhibitory and bactericidal activity. The compounds maintained a steady decrease in viable cell count for up to 24 hours, suggesting their sustained antimicrobial effect.

Conclusion

In an era where antimicrobial resistance poses a significant threat to global health, the quest for innovative solutions has never been more critical. The growing prevalence of multi-drug-resistant microbial infections underscores the urgency of discovering novel antimicrobial agents, emphasizing the importance of drug modifications as a pivotal approach to unveil new therapeutic possibilities. By exploring the antibacterial potential of pyrazolyl sulfonamide compounds and their palladium complexes, this study holds considerable significance in this ongoing battle against antimicrobial resistance. The findings illustrate the promising efficacy of these synthesized compounds, especially with the incorporation of palladium and in synergistic combinations, offering a glimpse of their potential role in addressing the challenges of bacterial resistance and infections.

Recognizing the implications of this study in the field of drug discovery, it becomes evident that there is a pressing need for continued research in this domain. The success of the compounds tested in this study serves as a stepping stone, encouraging further exploration and optimization to develop effective and reliable antibacterial agents. In conclusion, this research contributes valuable knowledge to the scientific community. It emphasizes the ongoing need for dedicated efforts in drug discovery to combat the ever-evolving threat of antimicrobial resistance.

Recommendations

Based on the findings of the study, the study made the following recommendations:

Assessment of toxicology and safety profile: The compounds demonstrated good antimicrobial activity against a range of bacteria strains, however, it is essential to conduct comprehensive toxicology studies on the promising compounds identified in the study. Evaluating the safety profile, including potential side effects, cytotoxicity, and impact on non-target cells, will ensure the compounds are not only effective but also safe for human use

In-depth study of mechanism of action: Given the differences in modes of action observed, conducting an in-depth study into the mechanisms by which these compounds exert their antibacterial effects is crucial. Understanding the interaction with bacterial enzymes, cellular structures, and the inhibition of essentially biological processes will provide insights into optimizing the compounds and predicting their behaviour against different bacterial strains. Investigating the compounds' pharmacokinetics and pharmacodynamics will also contribute to understanding their behaviour within the body, aiding in

dosage determination and route of administration. This step is crucial in developing any new antimicrobial agent to prevent unforeseen adverse effects and ensure patient safety.

Testing against an array of bacterial strains: The compounds exhibited varied activities against different Gram-positive bacteria. Expanding the testing to include a diverse range of Gram-positive and Gram-negative bacteria is advisable. This will help in understanding the spectrum of activity of these compounds and their potential role in treating different bacterial infections.

Suggestions for Future Studies

Given the observed variations in antibacterial activity among the synthesized compounds, it is recommended that further research be conducted to optimize the structures of these compounds. This includes exploring different substituents and metal complexes, enhancing lipophilicity, membrane permeability, and interaction with bacterial cellular targets to improve efficacy against a broader spectrum of bacteria. Future studies should consider modifying other broad-spectrum antibiotics such as the beta-lactamase inhibitors to find alternative treatment options to fight resistant bacteria infections.

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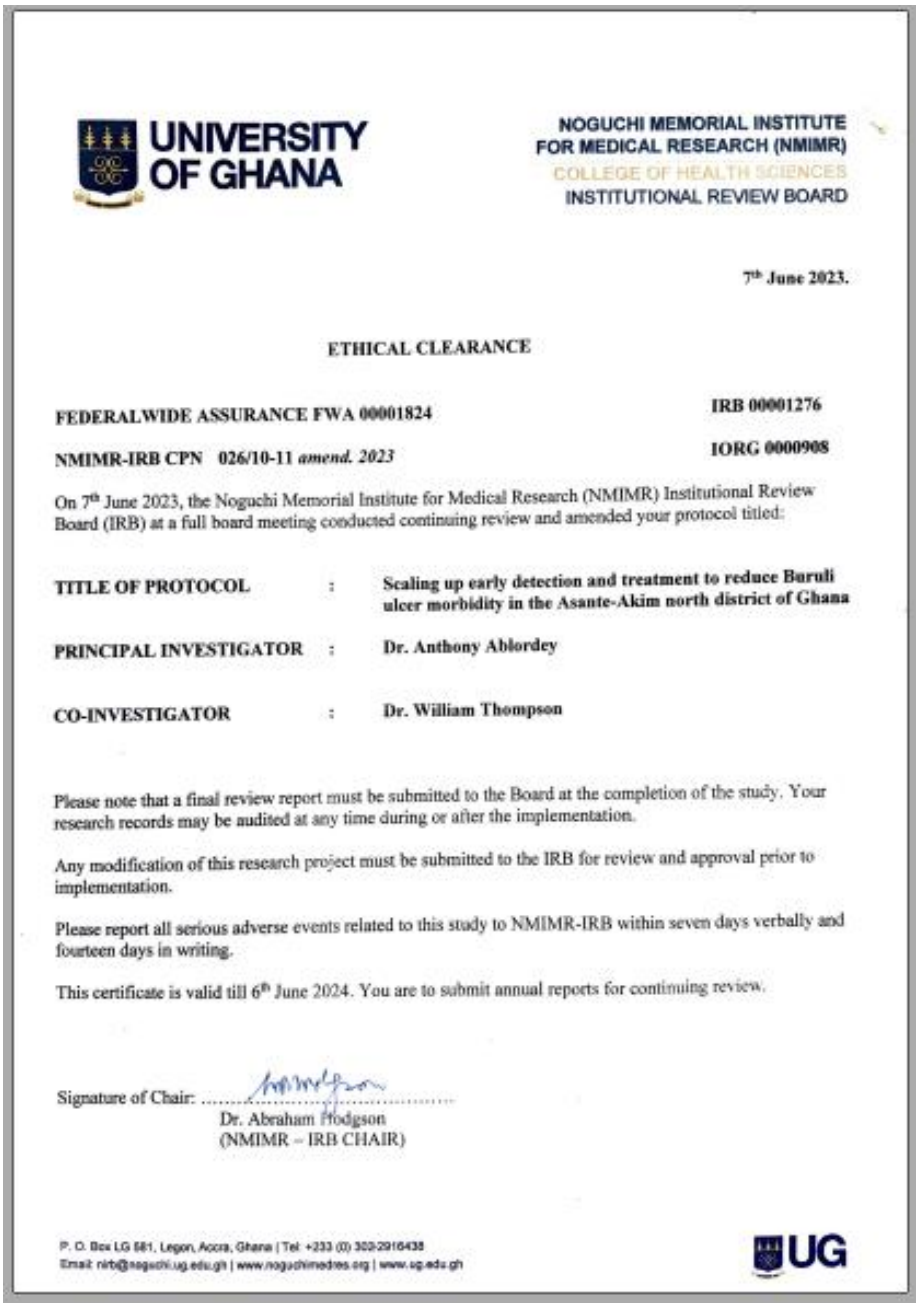
APPENDIX

Appendix A1: Ethical Clearance



UNIVERSITY OF GHANA

NOGUCHI MEMORIAL INSTITUTE
FOR MEDICAL RESEARCH (NMIMR)
COLLEGE OF HEALTH SCIENCES
INSTITUTIONAL REVIEW BOARD

7th June 2023.

ETHICAL CLEARANCE

FEDERALWIDE ASSURANCE FWA 00001824 IRB 00001276

NMIMR-IRB CPN 026/10-11 *amend. 2023* IORG 0000908

On 7th June 2023, the Noguchi Memorial Institute for Medical Research (NMIMR) Institutional Review Board (IRB) at a full board meeting conducted continuing review and amended your protocol titled:

TITLE OF PROTOCOL	:	Scaling up early detection and treatment to reduce Buruli ulcer morbidity in the Asante-Akim north district of Ghana
PRINCIPAL INVESTIGATOR	:	Dr. Anthony Ablordey
CO-INVESTIGATOR	:	Dr. William Thompson

Please note that a final review report must be submitted to the Board at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to the IRB for review and approval prior to implementation.

Please report all serious adverse events related to this study to NMIMR-IRB within seven days verbally and fourteen days in writing.

This certificate is valid till 6th June 2024. You are to submit annual reports for continuing review.

Signature of Chair: .....
Dr. Abraham Hodgson
(NMIMR – IRB CHAIR)

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