# Methylene blue analogues: In vitro antimicrobial activity and in silico pharmacophore modelling


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Dissertation accepted in fulfilment of the requirements for the degree Master of Sciences in Pharmaceutical Chemistry at the North-West University

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# PREFACE

This MSc dissertation is submitted in article format. The article (chapter 3) has been accepted for publication by the European Journal of Pharmaceutical Sciences and is available online. The materials and methods, results and discussion of additional work done by the student that was not included in the article is presented in chapter 4.

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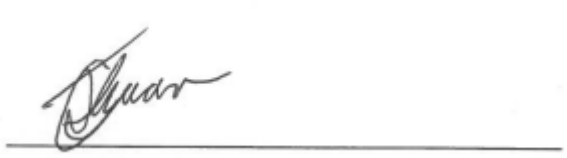
## DECLARATION

This dissertation is submitted in fulfilment of the requirements for the degree of Master of Science in Pharmaceutical Chemistry, at the North-West University, Potchefstroom campus.

I the undersigned, Louis Thesnaar, hereby declare that the dissertation with the title: “Methylene blue analogues: *In vitro* antimicrobial activity and *in silico* pharmacophore modelling” is my own work and has not been submitted at any other University either whole or in part.



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Signed at Potchefstroom on the 14th day of October 2020.

# LETTER OF PERMISSION

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To whom it may concern.

CO-AUTHORSHIP OF RESEARCH ARTICLE

The undersigned are co-authors of the research article listed and hereby give permission to Mr. Louis Thesnaar to submit this article as part of the degree Magister Scientiae in Pharmaceutical Chemistry at the North-West University (NWU) Potchefstroom campus:

"Methylene blue analogues: In vitro antimicrobial activity and in silico pharmacophore modelling"

Sincerely,

Dr Theunis Cloete

Prof. Anél Petzer

Prof. Jacques Petzer

Dr. Jaco Bezuidenhout

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# ABSTRACT

Antibiotics have drastically reduced the morbidity and mortality associated with fatal infections and have made previously impossible procedures, *e.g*. heart surgery, possible by allowing infections to be prevented or treated. Unfortunately, the misuse of antibiotics has resulted in the development of numerous antimicrobial resistant bacterial strains. It is estimated that by the year 2050, deaths caused by antimicrobial resistant infections will surpass deaths caused by cancer. Lipopeptides and oxazolidinones are the only newly developed antibiotic classes that have been registered in the past two decades, both are indicated for the treatment of Gram-positive bacteria. The rapid spread of antimicrobial resistance as well as the lack of new antibiotics underlines the need for novel antimicrobial agents.

Methylene blue was developed in 1876 as a dye in the textile industry and has since found numerous applications in different fields, *e.g*. biology, chemistry and medicine. Methylene blue has also been used as a lead compound in the development of quinolines (antimalarial agents) and phenothiazines (neuroleptic agents). Methylene blue is known to have antimicrobial properties although few studies have determined its minimum inhibitory concentration, which is the gold standard used to measure antimicrobial activity. Methylene blue's precise antimicrobial mode of action is still unclear and to our knowledge no pharmacophore model has been created to investigate its mode of action.

Computer-aided drug design is used in various aspects of drug discovery. When the drug target is unknown, ligand based drug design can be used to create a pharmacophore model by using the activity of known compounds. The pharmacophore model can be used to identify important chemical features that are necessary for activity and can also be used to predict the activity of other compounds. However, to obtain reliable results the pharmacophore model has to be properly validated.

The aim of this study was to firstly screen methylene blue, methylene green, neutral red, new methylene blue, azure B, dimethyl methylene blue, methylene violet, cresyl violet, acriflavine, nile blue and ethylthioninium chloride for activity against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Klebsiella pneumonia*, *Salmonella enterica* and *Candida albicans*. Thereafter, the minimum inhibitory concentration (MIC) as well as the minimum bactericidal concentration (MBC) of the analogues with activity was determined against the relevant pathogens. The MIC data was used to develop and validate a common feature pharmacophore model. The pharmacophore model was validated with three statistical metrics, *i.e*. the enrichment factor (EF), hit rate (HR) and the area under the curve of a receiver operator characteristic (ROC-

AUC), which determined the pharmacophore model's ability to differentiate between active/decoy compounds. The validated pharmacophore model was used to map similar antibacterial compounds in an attempt to elucidate methylene blue's antimicrobial mode of action.

Most analogues only had activity against *S. aureus*, *S. epidermidis* and *K. pneumonia*. Dimethyl methylene blue, new methylene blue and acriflavine proved to be the most active compounds against *S. aureus* [1 µg/ml (dimethyl methylene blue); 4 µg/ml (new methylene blue); 8 µg/ml (acriflavine)], *S. epidermidis* [1 µg/ml (dimethyl methylene blue); 4 µg/ml (new methylene blue); 2 µg/ml (acriflavine)] and *K. pneumoniae* [8 µg/ml (dimethyl methylene blue); 0.5 µg/ml (new methylene blue); 2 µg/ml (acriflavine)]. During method development, both the standard- and a less common bactericidal test were unable to deliver reliable MBC results. Comparing these two methods to one another it was found that acriflavine is bactericidal against *S. aureus*, and both methylene blue and azure B are bactericidal against *K. pneumoniae*. A common feature pharmacophore model was created (rank score: 26.664, max. fit value: 4), which was able to accurately identify active methylene blue analogous out of the test set ($EF^{2\%}$: 51, $HR^{2\%}$: 100%, ROC-AUC: 1.00 ± 0.00). Six phenothiazine derivatives with known antibacterial activity had high fit values when mapped with the validated pharmacophore model, *i.e*. >3.4.

In conclusion, this study found that dimethyl methylene blue, new methylene blue and acriflavine had potent antibacterial activity against *S. aureus*, *S. epidermidis* and *K. pneumonia*. A common feature pharmacophore model was developed and validated and was able to identify methylene blue analogous with known *in vitro* antibacterial activity out of a database containing active/decoy compounds. The common feature pharmacophore model highlighted the importance of two hydrophobic features, an aromatic feature and a hydrogen bond acceptor. The validated pharmacophore model also correctly mapped six phenothiazine derivatives with known antibacterial activity suggesting that they may share a common antibacterial mechanism of action.

For future studies the MIC data will allow other researchers to compare the activity of methylene blue analogues across different laboratories. The validated pharmacophore model can also be used to identify other compounds with similar activity as well as shed light on a possible antimicrobial mode of action.

**Keywords:**

Discovery Studio; Methylene blue; Antimicrobial activity; Common feature pharmacophore modelling; Minimum inhibitory concentration.

# OPSOMMING

Antibiotika het die morbiditeit en mortaliteit wat verband hou met dodelike infeksies drasties verminder en voorheen onmoontlike prosedures, bv. hartchirurgie, moontlik gemaak deur infeksies te voorkom of te behandel. Ongelukkig het die misbruik van antibiotikums gelei tot die ontwikkeling van talle antimikrobiese weerstandige bakterie stamme. Daar word beraam dat sterftes weens antimikrobiese weerstandige infeksies teen 2050 sterftes as gevolg van kanker sal oortref. Lipopeptiede en oksasolidinone is die enigste nuut ontwikkelde antibiotikum klasse wat die afgelope twee dekades geregistreer is. Albei is aangedui vir die behandeling van Gram-positiewe bakterieë. Die vinnige verspreiding van antimikrobiese weerstand sowel as die gebrek aan nuwe antibiotikums, beklemtoon die behoefte aan nuwe antimikrobiese middels.

Metileenblou is in 1876 ontwikkel as ’n kleurstof in die tekstielbedryf en het sedertdien talle toepassings in verskillende velde gevind, bv. biologie, chemie en medisyne. Metileenblou is ook gebruik as 'n leidraadverbinding in die ontwikkeling van kinoliene (antimalaria) en fenotiasiene (neuroleptika). Dit is bekend dat metileenblou antimikrobiese eienskappe het, hoewel min studies die minimum inhiberende konsentrasie daarvan al bepaal het, wat die goue standaard is om antimikrobiese aktiwiteit te meet. Metileenblou se presiese antimikrobiese werkingsmeganisme is nog onduidelik en na ons wete is geen farmakofoormodel geskep om die werkingsmeganisme daarvan te ondersoek nie.

Rekenaar-gebaseerde geneesmiddelontwerp word in verskillende aspekte van medisyne ontdekking gebruik. Wanneer die geneesmiddelteiken onbekend is, kan ligand-gebaseerde geneesmiddelontwerp gebruik word om 'n farmakofoormodel te skep deur die aktiwiteit van bekende verbindings te gebruik. Die farmakofoormodel kan gebruik word om belangrike chemiese kenmerke wat nodig is vir aktiwiteit te identifiseer, en kan ook gebruik word om die aktiwiteit van ander verbindings te voorspel. Om betroubare resultate te verkry, moet die farmakofoormodel egter goed gevalideer word.

Die doel van hierdie studie was om eerstens metileenblou, metileengroen, neutraal rooi, nuwe metileenblou, asuur B, dimetielmetileenblou, metileenviolet, kresielviolet, akriflavien, nylblou en etielthioniniumchloried te ondersoek vir aktiwiteit teen *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella enterica* en *Candida albicans*. Daarna is die minimum inhiberende konsentrasie (MIK) sowel as die minimum bakteriedodende konsentrasie (MBK) van die analoë met aktiwiteit bepaal teen die betrokke patogene. Die MIK data is gebruik om 'n algemene kenmerk farmakofoormodel te ontwikkel en te valideer. Die farmakofoormodel is gevalideer met drie statistiese maatstawwe, naamlik die verrykingsfaktor

(VF), trefkoers (TK) en die oppervlakte onder die kurwe van 'n ontvanger operateur-eienskap (OOE-AOK), wat die vermoë van die farmakofoormodel om te onderskei tussen aktiewe en onaktiewe verbindings bepaal. Die gevalideerde farmakofoormodel is gebruik om soortgelyke antibakteriese verbindings te pas in 'n poging om metileenblou se antimikrobiese werkingsmeganisme te verduidelik.

Die meeste analoë het slegs aktiwiteit teen *S. aureus*, *S. epidermidis* en *K. pneumoniae* gehad. Dimetielmetileenblou, nuwe metileenblou en akriflavien was die mees aktiewe verbindings teen *S. aureus* [1 µg/ml (dimetiel metileenblou); 4 µg/ml (nuwe metileenblou); 8 µg/ml (akriflavien)], *S. epidermidis* [1 µg/ml (dimetiel metileenblou); 4 µg/ml (nuwe metileenblou); 2 µg/ml (akriflavien)] en *K. pneumoniae* [8 µg/ml (dimetiel metileenblou); 0.5 µg/ml (nuwe metileenblou); 2 µg/ml (akriflavien)]. Tydens metodeontwikkeling, kon beide die standaard- asook 'n minder algemene bakteriedodende toets nie betroubare MBK resultate lewer nie. Deur hierdie twee metodes met mekaar te vergelyk is gevind dat akriflavien bakteriedodend is teen *S. aureus*, en beide metileenblou en asuur B bakteriedodend is teen *K. pneumoniae*. 'n Algemene farmakofoormodel is geskep (rangtelling: 26.664, maksimum paswaarde: 4), wat aktiewe metileenblou analoë akkuraat kon identifiseer vanuit die toets stel ($VF^{2\%}$: 51, $TK^{2\%}$: 100%, ROE-AOK: 1.00 ± 0,00). Ses fenotiasien derivate met bekende antibakteriese aktiwiteit het hoë passingswaardes gehad toe dit met die gevalideerde farmakofoormodel geëvalueer was, d.w.s. > 3.4.

Ter afsluiting, hierdie studie het bevind dat dimetielmetileenblou, nuwe metileenblou en akriflavien kragtige antibakteriese aktiwiteit het teen *S. aureus*, *S. epidermidis* en *K. pneumoniae*. 'n Algemene kenmerk farmakofoormodel is ontwikkel en kon metileenblou analoë met bekende *in vitro* antibakteriese aktiwiteit identifiseer uit 'n databasis wat aktiewe en onaktiewe verbindings bevat het. Die algemene kenmerk farmakofoormodel het die belangrikheid van twee hidrofobiese kenmerke, 'n aromatiese kenmerk en 'n waterstofbindingontvanger, beklemtoon. Die gevalideerde farmakofoormodel het ook ses fenotiasien derivate met bekende antibakteriese aktiwiteit korrek gepas, wat daarop dui dat hulle moontlik 'n gemeenskaplike antibakteriese werkingsmeganisme kan hê.

**Sleutelwoorde:**

Discovery Studio; Metileenblou; Antimikrobiese aktiwiteit; Algemene kenmerk farmakofoor modellering; Minimum inhiberende konsentrasie.

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# ABBREVIATIONS

3D-QSAR – 3 Dimensional Quantitative Structure-Activity Relationship

**A**

AD - Anno Domini

AMR – Antimicrobial Resistance

Ar - Aromatic Region

AUC – Area Under the Curve

**C**

CADD - Computer-Aided Drug Design

**D**

DHFR - Dihydrofolate Reductase

DHFS - Dihydrofolate Synthase

DHNA - 7,8-Dihydroneopterin Aldolase

DHPS - Dihydropteroate Synthase

DNA - Deoxyribonucleic Acid

DS – Discovery Studio

DUD-E - Database of Useful Decoys-Enhanced

**E**

ECFP - Extended Connectivity Fingerprint

EF – Enrichment Factor

EU – European Union

**F**

FN - False Negative

**G**

GC – Guanylate Cyclase

GIT - Gastrointestinal Tract

GTP - Guanosine Triphosphate

GTPCHI - GTP Cyclo Hydrolase I

**H**

HAI - Hospital Acquired Infections

HBA – Hydrogen Bond Acceptor

HBD – Hydrogen Bond Donor

HPPK - 6-Hydroxymethyl-7,8-Dihydropterin Pyrophospho Kinase

HR – Hit Rate

HTS - High Throughput Screening

HY - Hydrophobic

HYD – Hydrophobic Moieties

HYPO – Pharmacophore Hypothesis

**I**

iNOS – Inducible Nitric Oxide

**L**

LAAA - Light Activated Antimicrobial Agent

LBDD - Ligand-Based Drug Design

**M**

MBC – Minimum Bactericidal Concentration

MDR – Multidrug Resistance

MHA - Mueller-Hinton Agar

MHB – Mueller-Hinton Broth

MIC - Minimum Inhibitory Concentration

MAO – Monoamine Oxidase

mRNA – Messenger RNA

MRSA - Methicillin-Resistant *Staphylococcus aureus*

**O**

OD – Optical Density

**P**

pABA - para-Aminobenzoic Acid

PBP - Penicillin Binding Protein

PDT - Photodynamic Therapy

PI – Positive Ionisable

**Q**

QSAR - Quantitative Structure-Activity Relationship

**R**

RA – Ring Aromatic

RNA - Ribonucleic Acid

rRNA - Ribosomal RNA

ROC - Receiver Operator Characteristic

**S**

SBDD - Structured-Based Drug Design

Se - Sensitivity

spp. –Species

STEG - Shiga Toxin-Producing

STEC - Shiga Toxin-Producing *Escherichia coli*

**T**

THF - Tetrahydrofolic Acid

TP - True Positive

tRNA – transfer RNA

**U**

USA – United States of America

UTI - Urinary Tract Infection

**V**

VRE - Vancomycin-Resistant *Enterobacter*

VS - Virtual Screening

**W**

WHO – World Health Organization

**Z**

ZOI – Zone of Inhibition

# AFKORTINGS

**M**

MBK - Minimum Bakterieedodende Konsentrasie

MIK - Minimum Inhiberende Konsentrasie

**O**

OOE-AOK - Ontvanger operateur-eienskap-Oppervlakte Onder Die Kurwe

**T**

TK - Trefkoers

**V**

VF - Verrykingsfaktor

# CHAPTER 1: INTRODUCTION

## 1.1 GENERAL BACKGROUND

### 1.1.1 Discovery of antibiotics

Antibiotics have been used since 350 AD, where traces of tetracycline were found while studying ancient bones (Sohn, 2010). Another example was the topical application of mouldy bread in parts of Egypt, Greece and China to treat skin infections. The modern era of antibiotics started with Paul Erich, who studied dyes for their antibacterial effects and discovered Salvarsan, which was used to treat syphilis (Gould, 2016:572). The discovery of penicillin in 1928 by Alexander Fleming, however is considered to be the start of the modern antibiotic era (Sengupta *et al.*, 2013:1). The vast majority of antibiotics used today were discovered between the 1920s and 1960s and is seen by many as the golden era of antibiotics (Davies, 2006:287).

In 1946 Alexander Fleming warned that bacteria could become resistant to antibiotics (Bartlett *et al.*, 2013:1445). As new antibiotics were discovered resistant strains soon followed thereafter (Ventola, 2015:277). Common infections that were once easily treated are becoming resistant, with standard treatment regimens ineffective (WHO, 2020). Antimicrobial resistance (AMR) not only increases a patient's morbidity and mortality but also the healthcare costs of treating them. Antibiotic resistance is responsible for more than 48 000 deaths per year in the USA and EU alone (CDC, 2019). In addition to the development of AMR, only two new antibiotic classes have been developed and approved in the last two decades, further exacerbating the problem (Tacconelli *et al.*, 2018). Limited research in the development of new antibiotics can be ascribed to; poor investment return due to the increased costs of drug development, rapid drug resistance leading to reduced sales and increased safety demands during clinical trials (Norrby *et al.*, 2005:2).

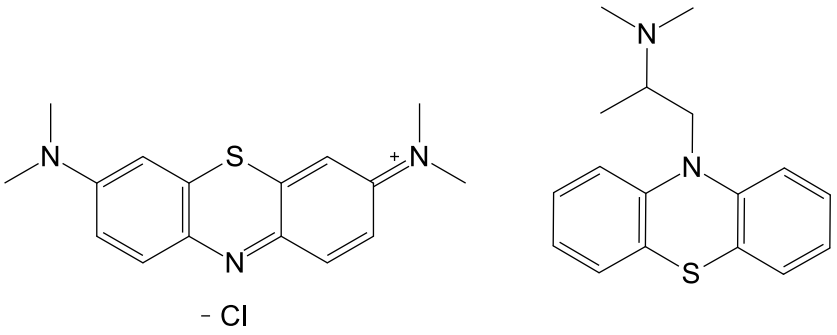
### 1.1.2 Resistance mechanisms

Bacteria have developed numerous mechanisms of resistance towards antibiotics, *i.e.* inactivation of the drug (*e.g.* production of ß-lactamase), decreased concentration of the drug compound (*e.g*. upregulation of efflux pumps), alteration of the target site and reduced cellular uptake of the drug (Denyer *et al.*, 2011:218). Factors that increase the probability to acquire these mechanisms are incorrect use of antibiotics, misdiagnosing an infection and misuse of antibiotics in the veterinary and agricultural sectors. Resistance genes are acquired through

different origins such as horizontal acquisition between cells and natural selection (Gillings & Stokes, 2012:348; Munita & Arias, 2016:483).

### 1.1.3 Methylene blue and derivatives

Methylene blue was developed as a dye for the textile industry, after which Rober Koch used the dye to treat tuberculosis in 1870 (Lo *et al*., 2014:670). Since then it has been used as treatment for numerous conditions, *e.g.* malaria, cyanide poisoning, methemoglobinemia, ifosfamide-induced encephalopathy and vasoplegia accompanied septic shock (Kwok & Howes, 2006:362; Lo *et al*., 2014:670; Patel, 2006:302; Schirmer *et al.,* 2003:272; Vutskits *et al*., 2008:684). Methylene blue also has antimicrobial properties and has activity against a variety of organisms, *e.g*. *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Candida albicans*, *Klebsiella pneumoniae* and *Aspergillus niger* (Ash *et al*., 2006:17, Li *et al.*, 2016:13615). The antimicrobial use of methylene blue dates back to the late 19$^{th}$ century, its antibacterial mechanism of action is still a topic of debate (Edwards, 2016:14). The phenothiazines, which were developed from methylene blue, also has antibacterial activity. Like methylene blue, their precise mechanism of action is still not clear although it is believed they might bind to the penicillin binding proteins (PBP) (figure 1.1) (Amaral *et al*., 2004:725; Dastidar *et al*., 2013:59-60; Varga *et al*., 2017:5987).




Methylene blue Promethazine

**Figure 1.1: Structure of methylene blue and promethazine (Amaral *et al.,* 2001:1016; Varga *et al*., 2017:5986).**

### 1.1.4 Pharmacophore modelling

The use of computers in pharmaceutical research is termed computer-aided drug design (CADD) and is increasingly being used in lead discovery and optimisation. Two main types of

CADD exists, namely structure-based drug design (SBDD) and ligand-based drug design (LBDD) (Aparoy *et al*., 2012:3765; Kapetanovic, 2008:166). SBDD is used when the exact 3-dimensional structure of the target is known allowing medicinal chemists to dock compounds into the active site and determine how well they will interact with the target. When the biological target of a group of compounds is unknown, *e.g*. methylene blue, LBDD is used. LBDD uses the structures of compounds with known activity to create a pharmacophore model which defines all the characteristics necessary for interaction with the target (Baig *et al*., 2016:572). This pharmacophore model can then be used to screen online libraries of compounds to identify compounds which might have similar activity against the target.

Each pharmacophore model remains a theoretical *in silico* prediction and needs to be properly validated to ensure reliable results (Adane *et al*., 2010:637; Pascual *et al*., 2019:4). The enrichment factor (EF), hit rate (HR) and receiver operator characteristic (ROC) curve are three reliable metrics that are often used to validate pharmacophore models. All three metrics validate the pharmacophores' ability to differentiate between active and decoy compounds (Hawkins *et al.*, 2007:75). Additionally, the ROC curve also determines what threshold should be used to differentiate between an active and decoy compound (Triballeau *et al.,* 2005:2536).

## 1.2 HYPOTHESIS OF THIS STUDY

There is an urgent need for developing novel antimicrobial drugs (WHO, 2019). Methylene blue and its analogues have antimicrobial activity, however few studies have determined their minimum inhibitory concentration (MIC) against pathogens and their exact mode of action is still not clear. Pharmacophore models created by computer modelling programs can assist researchers not only to explain the experimentally determined activity of a group of compounds but can also be used to screen online databases of organic compounds to identify novel compounds with similar activity compared to the known compounds. However, CADD does have its limitations and needs to be properly validated (Kandakatla *et al*. 2014:1097; Lee *et al*. 2018:9; Qing *et al.,* 2014:87).

## 1.3 AIMS AND OBJECTIVES OF THIS STUDY

This study will be divided into an *in vitro* and an *in silico* stage. The aim of the *in vitro* stage will be to determine the antibacterial activity of various methylene blue analogues against a panel of pathogens, in an attempt to identify possible novel lead compounds. The aim of the *in silico* stage will be to create a pharmacophore model of the compounds that had *in vitro* activity against the tested pathogens, and to use statistically sound methods to validate the pharmacophore model. The validated pharmacophore model can not only be used to identify

important features necessary for antimicrobial activity, but can also be used to identify other compounds that might share a similar antimicrobial mechanism of action.

**The objectives of this study are:**

***In vitro* stage:**

- To use the Kirby-Bauer (disc diffusion method) to determine if the methylene blue analogues have antimicrobial activity against the relevant pathogens.
- To use the broth microdilution method to determine the MIC of the analogues that have been identified to have activity against the relevant pathogens.
- To determine the minimum bactericidal concentration (MBC) of the analogues against the relevant pathogens.

***In silico* stage:**

- To use the structures of the active compounds that have been identified during the *in vitro* stage to create a training set and generate a pharmacophore model.
- To use a test set of active compounds to determine the ability of the pharmacophore model to predict the *in vitro* activity.
- To determine and use the EF, HR and ROC curve to validate the pharmacophore model.
- To use the validated pharmacophore model to identify compounds which might have similar activity against the pathogens.

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# CHAPTER 2: LITERATURE STUDY

## 2.1 BACKGROUND

Antibiotics have drastically improved the morbidity and mortality of mankind since its discovery in the early 20th century. In America for example, death rates due to bacterial illness decreased drastically over the span of seventy years, *i.e*. 1930 (247.7 per 100 000 population), 1936 (216.3 per 100 000 population), 1952 (59.7 per 100 000 population), 1960 (50.4 per 100 000 population) and 2002 (38.1 per 100 000 population). This is mainly attributed to the introduction of antibiotics (Gottfried, 2005:19). Antibiotics also allowed major advancements in the treatment of non-infectious diseases, *e.g*. heart disease, cancer, organ transplant *etc*., as these conditions were treated knowing that secondary infection could be prevented/treated (Rice, 2008:1079).

The modern era of antibiotic drugs began when Paul Ehrlich started the search for compounds that can selectively kill micro-organisms without having a toxic effect on the host (Gaynes, 2017:849). His efforts led to the discovery of arsphenamine (trade name: Salvarsan) in 1909, which was initially used to treat syphilis and trypanosomiases (Zaffiri *et al*., 2012:67). In 1929 Alexander Fleming discovered that a substance made by a mould from the *Penicillium* genus was able to kill various pyogenic cocci and diphtheria bacilli. He extracted the active compound from broth cultures of the *Penicillium* mould and named the active agent penicillin (Fleming, 1929:236; Gaynes, 2017:849). However, due to its instability and difficulty in isolating the active compound, penicillin was not used therapeutically until 1941 (Abraham *et al*., 1941:185; Zaffiri *et al*., 2012:70). In the thirteen years it took to purify penicillin, prontosil was discovered in 1932, a drug that was widely used because of its antibacterial properties. Prontosil was later proven to be the first sulphonamide antibiotic, as its antibacterial properties could be ascribed to its sulphonamide moiety (Zaffiri *et al*., 2012:68). Between 1930 and 1962, twenty new antibiotic classes were discovered (Coates *et al*., 2002:896; Coates *et al*., 2011:184). However, since 1962 only two new antibiotic classes have been marketed, *i.e*. oxazolidinones and cyclic lipopeptides, with almost all new antibiotics being merely derivatives of existing classes (Coates *et al*., 2011:184). Quinolines was the last antibacterial class discovered against Gram-negative bacteria in 1962, as both oxazolidinones and cyclic lipopeptides are only active against Gram-positive bacteria (Coates *et al*., 2011:190, Tacconelli *et al*., 2018:318).

Most antibiotics were firstly isolated from microbes and thus are of natural origin (Newman *et al.*, 2003:1025; Peláez, 2006:981). As mentioned above, the first antibiotic

isolated from a microbe was penicillin, produced in mass after extraction from the fungus *Penicillium notatum* (Wyatt, 1990:322). In 1943 the antibiotic streptomycin was isolated from the bacteria *Streptomyces griseus* and was one of the first antibacterial drugs to have activity against Gram-negative organisms (Gottfried, 2005:6; Waksman *et al*., 1948:259). Further investigation into the antibacterial activity of different bacteria species led to the discovery of both chloramphenicol (*Streptomyces* spp.) and chlortetracycline (*Streptomyces aureofaciens*) (Brock, 1961:32; Gottfried, 2005:7; Nelson & Levy, 2011:19).

Soon after the development of antibiotics, Alexander Fleming himself cautioned that misuse of antibiotics might eventually lead to the development of resistant pathogens (Aminov, 2010:2). After each discovery and utilisation of an antibiotic, resistance developed towards the antibiotic (CDC, 2013:28). Indeed, the utility of antibiotics have led to their widespread application and misuse resulting in numerous antimicrobial resistant bacteria strains (Aminov, 2010:3, Zaman *et. al.,* 2017:2). Almost 100 years after Fleming's prediction it is estimated that 700 000 people die annually as a direct result of antimicrobial resistance (AMR), and is projected to globally increase to a staggering 10 million people by 2050 surpassing the projected annual deaths caused by cancer. In addition to the increased mortality rate, AMR also increase healthcare costs and is projected to cost the world 100 trillion US dollars by 2050 (O'Neill, 2014:5). An increase in AMR and the decrease in the number of novel antibiotic drugs classes highlights the necessity for the search of novel antibacterial drugs.

## 2.2 BACTERIA

### 2.2.1 Cell structure

Organisms are classified into two main groups, namely prokaryotic and eukaryotic organisms. Prokaryotic organisms (bacteria and archaea) do not contain membrane-bound organelles and a defined nucleus, whereas eukaryotic organisms (fungi, protozoa, and algae) contain both a nucleus and membrane-bound organelles. Another significant difference is size, prokaryotic cells are much smaller than eukaryotic cells and their cell walls consist of peptidoglycan (Ingraham *et al.*, 2000:6).

Most bacteria are unicellular and possess simple shapes, *e.g.* rod-shaped (bacilli), spheres (cocci), comma-shaped (vibrios), and helicoidal (spirilla) (Margolin, 2009:812; Zapun *et al.*, 2008:345). Another rarer morphological form*, i.e*. actinomycetes, are rigid bacteria that grow as lengthy branched filaments resembling fungi. The cell wall is mainly responsible for maintaining these unique shapes. These different shapes influence each bacteria’s ability to

thrive in their environment *e.g*. the amount of nutrient intake, cell division, attachment, predation on other cells, motility and cell differentiation (Young, 2006:665).

Bacteria are further divided into three groups, namely Gram-positive, Gram-negative and mycoplasmas. Gram-negative bacteria contain an outer membrane, cytoplasmic membrane, and a periplasm matrix that links both membranes together, while in Gram-positive bacteria the outer membrane is absent. Mycoplasmas lack both the cell wall and outer membrane. Gram staining, using crystal violet, is used to distinguish between these groups. Due to a thicker peptidoglycan cell wall, Gram-positive bacteria will absorb the dye and stain purple, while Gram-negative bacteria will stain pink. Mycoplasmas is stained light purple when using the Giemsa stain (Harnett *et al.,* 1974:70). Because of the outer membrane, Gram-negative bacteria tend to be more pathogenic and less susceptible to antibiotics (Zgurskaya *et al.,* 2015:512).

### 2.2.2 Bacteria in human pathology

Many bacteria form part of the healthy human flora without becoming pathogenic. Areas where these micro-organisms are found, include the skin, nails, eyes, oropharynx, genitalia and the gastrointestinal tract (GIT). They perform various functions in which some may aid the host (*e.g.* competing for essential nutrients more effectively than other pathogens) or may cause harm (*e.g.* tooth decay). *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Salmonella enterica* all form part of the human skin flora but may spread to other areas other parts of the body (Davis, 1996:1).

Gram-negative bacteria cause numerous diseases, *e.g.,* bloodstream, urinary tract and upper/lower respiratory infections, most of which have a high mortality rate. The most common diseases caused by these bacteria are urinary tract infections (UTI) (Peleg & Hooper, 2010:1804). During the last decade, research mainly focused on multidrug-resistant (MDR) micro-organisms, focussing more on Gram-positive bacteria, *e.g.* methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *Enterobacter (*VRE). Still, infections caused by Gram-negative bacteria have become a growing problem (Giske *et al.*, 2008:813). Gram-negative bacteria predominate in hospital-acquired UTIs, almost all of which are associated with urethral catheterization (Tenke *et al.*, 2008:68).

*S. aureus* and *S. epidermidis* form part of the genus *Staphylococci*. Both of these bacteria are categorised as Gram-positive, however *S. aureus* is a coagulase-positive coccus whereas *S. epidermidis* is coagulase-negative. This enables *S. aureus* to convert fibrinogen to fibrin, which forms a coating around the surface of the cell, thus increasing the durability and survivability in the host (McAdow *et al.*, 2012:141). Besides causing local skin infections, *S.*

*aureus* can cause various invasive diseases, *e.g.* osteomyelitis, endocarditis, and toxic shock syndrome. It is also the leading cause of hospital-acquired infections (HAI) (Foster, 1996:1). *S. epidermidis*, on the other hand, is also a significant problem in hospital settings and is the number one cause of infections with regards to indwelling medical devices (*e.g*. prosthetic devices, intravascular catheters, *etc*.). Examples of these types of infections are shunt infection, bacteraemia and endophthalmitis (Huebner & Goldmann, 1999:223). Treating infections caused by these bacteria are becoming extremely difficult due to the development of MDR strains, *e.g.* MRSA (Taylor & Unakal, 2019:1; Thottacherry & Hassoun, 2020:1). MRSA in the United States now causes roughly 60% of staphylococcal infections in the intensive care units, and this percentage continues to rise (Rice, 2006:11). Recent reports indicated that methicillin resistance account for 44% of *S. aureus* bloodstream isolates from intensive care unit infections (Wisplinghoff *et al.*, 2004:313).

*Enterobacter aerogenes*, along with *Salmonella* spp.*, Klebsiella* spp.*, Escherichia* spp., fall under the *Enterobactericiae* genus, which is classified as Gram-negative, facultative-anaerobic bacteria (Chester & Moskowitz, 1987:440; Davin-Regli, 2015:1). These pathogens are notorious for rapidly acquiring drug resistance, which limits the number of viable antibacterial drugs health practitioners can use and is one of the explanations why infections caused by *E. aerogenes* are so challenging to control (Santajit & Indrawattana, 2016:1).

*E. coli* is classified as Gram-negative, facultative-anaerobic bacteria and can be found in the GIT where it forms part of the healthy microflora (Clements *et al.*, 2012:71; von Wulffen *et al.*, 2016:1). *E. coli* infections are usually the result of consuming contaminated food. *E. coli* does not always have to be consumed to become pathogenic. A specific strain known as the 'Shiga toxin-producing *E.coli* (STEC) strain produce Shiga toxins that cause severe enteric and systemic diseases (ECDC, 2017). Generally, *E. coli* infections are mild and can be easily treated however, in some cases *E. coli* can cause life-threatening infections, *e.g*. haemolytic uremic syndrome (WHO, 2018).

*K. pneumoniae,* firstly known as Friedlander's bacillus until 1886, was first discovered in 1882 by Carl Friedlander. *K. pneumoniae* is Gram-neagtive, facultative-anaerobic bacteria, and small traces of non-pathogenic *K. pneumoniae* is commonly found in the GIT (22%) and oropharynx surface (4%) (Goldberg & Hanau, 1980:745). Common infections caused by *K. pneumoniae* include UTI, nosocomial pneumonia and intra-abdominal infections (Ko *et al.*, 2002:160).

As mentioned above, *Salmonella spp*. (*e.g. S.entirica*) are part of the *Enterobacteriaceae* genus, which is responsible for numerous infections with high mortality rates. *Salmonella spp*.

are Gram-negative, facultative-anaerobic bacteria and are ordinarily present in the gut flora of humans and farm animals. Infections mostly occur when the bacteria are consumed, usually together with drinking water contaminated by human/animal feces (Andino & Hanning, 2015:1). Salmonellosis causes different symptoms that are categorised into three clinical forms: gastroenteritis, septicemia and enteric fever. Two different types of enteric fevers exist, *i.e*. typhoid and paratyphoid fever. Both of these types can be caused by *S.* enterica (Cabral, 2010:3663). Resistant Salmonella strains have already been documented since the early 1980s with even second and third-line antibiotics becoming less effective (Klemm *et al.*, 2018:2).

*Enterococcus faecium, S. aureus, K. pneumoniae, Acinetobacter baumannii, P. aeruginosa,* and *Enterobacter spp.,* have collectively been designated as the ESKAPE pathogens, and are notorious for being the leading cause of nosocomial infections. All of them have MDR isolates, causing a major obstacle in clinical practice. MDR is a significant threat to global public health, and factors contributing to this are excessive drug usage, inappropriate use of antimicrobials and substandard pharmaceuticals (Santajit & Indrawattana, 2016:1).

### 2.3 Antibiotics and antibiotic resistance

Antibiotics can be categorised according to their mode of action, *i.e*. how they interact with the bacteria either by inhibiting the growth and thus preventing further cell division (bacteriostatic), or by killing the cells (bactericidal). Currently, there are four main modes of action: cell wall synthesis inhibitors; nucleic acid synthesis inhibitors; protein synthesis inhibitors and metabolic compound inhibitors. These four main modes of action can be subdivided into different classes as described below in paragraphs 2.3.1 to 2.3.4 (Kırmusaoğlu *et al.*, 2019:1).

Unfortunately, numerous bacteria have developed resistance against antibiotics. Resistance can be described as either intrinsic or acquired. Intrinsic resistance is when an organism naturally does not contain the relevant drug target making it immune to a drug. Acquired resistance on the other hand is when a once susceptible organism becomes resistant by acquiring a mechanism by which the antibacterial mode of action is circumvented (Dowling *et al.*, 2017:536). Acquired resistance can be categorised into four main classes: efflux pumps (removing the antimicrobial compounds from the target site of action); enzymatic degradation of the antimicrobial drug, alteration to the cell wall permeability (slowing or preventing the antimicrobial from reaching the target site); and target site alterations that prevent the antimicrobial from binding. Organisms can exhibit multiple resistance mechanisms, which may grant resistance to different classes of antimicrobials (Chellat *et al.*, 2016:6602; Kulengowski, 2019:5).

### 2.3.1 Cell wall synthesis inhibitors

The cell wall of the majority of bacteria contains peptidoglycan (also known as murein) which assist in maintaining the cell structure, preventing lysis as a result of the internal pressure (Scheffers & Pinho, 2005:586). The peptidoglycan biosynthetic pathway is similar for most bacteria and consists of coupling N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) to form long glycan chains that are cross linked with short peptides (Barreteau *et al*., 2008:168). The final step in the synthesis of peptidoglycan is catalysed by the enzyme transpeptidase, also known as penicillin-binding protein (PBP), that is responsible for cross-linking the long glycan chains with short peptides. PBP is the target for the β-lactam antibiotics which inhibits the PBPs (Dowling *et al.*, 2017:536). The cell wall synthesis inhibitors are bactericidal and consist of the β-lactams (penicillins, cephalosporins, carbapenems and monobactams), glycopeptides and cyclic lipopeptides (Eagle & Musselman, 1948:99; Palzkill, 2013:1).

#### 2.3.1.1 β-Lactam antibiotics

Penicillin was the first β-lactam antibiotic and was discovered by Alexander Fleming in 1929 by observing that mould from the *Penicillium* genus was able to kill pyogenic cocci and diphtheria bacilli (figure 2.1) (Fleming, 1929:236; Gaynes, 2017:849). Numerous other penicillin antibiotics were subsequently derived from the parent penicillin structure, *e.g*. methicillin and ampicillin (Kong *et al.*, 2010:1). Penicillins have a broad spectrum of antibiotic activity and are used to treat various Gram-negative and Gram-positive infections, *e.g*. respiratory tract infections, gastrointestinal infections and septicaemia (Peechakara *et al.*, 2019:1).

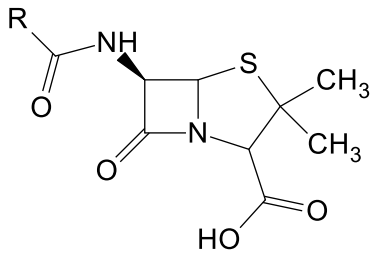



**Figure 2.1: Pharmacophore of penicillins (Palzkill, 2013:17).**

Cephalosporin C, the precursor of all cephalosporins, was first isolated in 1961 by Edward Abraham and Guy Newton from *Cephalosporium acremonium.* From 1961 until now, five generations of cephalosporins have emerged each generation active against a different

spectrum of bacteria (figure 2.2). The first-generation cephalosporins (cephalothin, cephapirin, cefazolin, cephalexin, cephradine and cefadroxil) are mainly active against Gram-positive cocci (except enterococci and methicillin-resistant staphylococci) and are moderately active against Gram-negative rods (Fernandes *et al.*, 2013:9). The second generation cephalosporins (cefamandole, cefuroxime, cefonicid, ceforanide, cefoxitin and cefmetazole) have the same activity as the first generation with extended activity against Gram-negative organisms (Fernandes *et al.*, 2013:9). Third generation cephalosporins (cefotaxime, ceftizoxime, ceftriaxone, ceftazidime, cefoperazone and cefixime) are less active against Gram-positive cocci, however they have excellent activity against Gram-negative rods (Fernandes *et al.*, 2013:9). The fourth generation cephalosporins (cefepime and cefpirome) have enhanced activity against *Enterobacter* and *Citrobacter* species that are resistant to the third generation. The fifth generation cephalosporins (ceftobiprole and ceftaroline) were designed to target highly resistant bacterial strains *e.g.* MRSA (Fernandes *et al.*, 2013:9).

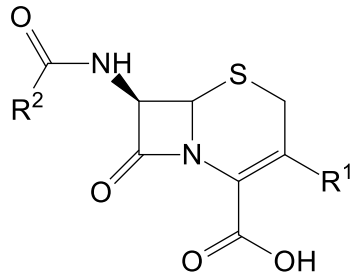



**Figure 2.2: Pharmacophore structure of cephalosporins (Palzkill, 2013:17).**

The first carbapenem, thienamycin, was discovered in 1976 after extraction from *Streptomyces cattleya*. Carbapenems (thienamycin, imipenem, meropenem, doripenem and ertapenem) have a broad spectrum of activity and are reserved for use against MDR pathogens, especially cephalosporin resistant organism (figure 2.3) (Elshamy & Aboshanab, 2020:439).

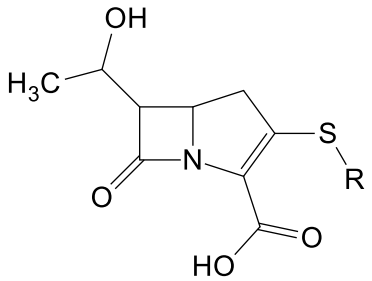



**Figure 2.3: Pharmacophore structure of carbapenem (Palzkill, 2013:17).**

Monobactam antibiotics were initially isolated from a variety of bacteria, *i.e*. *Chromobacterium, Gluconobacter, Acetobacter, Pseudomonas, Agrobacterium* and *Flexibacter* (Sykes & Bonner, 1985:579). The first monobactam, aztreonam, was approved for clinical use in 1986 against infections caused by *Enterobacteriaceae* and *P. aeruginosa* (figure 2.4) (Biedenbach *et al*., 2015:4239). Monobactams (aztreonam, carumonam and tigemonam) were later found to also be active against Gram-negative rods (Fernandes *et al.*, 2013:11, Fuchs *et al.*, 1988:346).

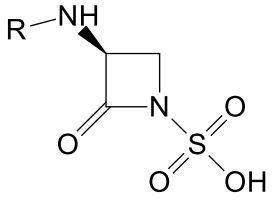



**Figure 2.4: Pharmacophore structure of monobactams (Palzkill, 2013:17).**

There are three main mechanisms of resistance that afflict the β-lactam antibiotics; the production of β-lactamases, decreased cell wall penetration and decreased binding affinity to the PBP (Dowling *et al.*, 2017:539). β-lactamases are enzymes capable of inactivating β-lactam antibiotics by binding to the β-lactam ring and hydrolysing the core (Worthington & Melander, 2013:1). β-lactamase inhibitors, *e.g*. clavulanic acid, sulbactam or tazobactam, can be used in conjunction with antibiotics to counteract this mechanism. β-lactamases can be divided into four different classes, *i.e*. class A, B, C and D. Class A (also known as penicillinases) is susceptible to β-lactamase inhibitors, class B (also known as metallo-β-lactamases) is resistant to β-lactamase inhibitors, class C (also known as cephalosporinases) is resistant only to clavulanic acid and class D (also known as oxacillin hydrolysing enzymes) is weakly inhibited by clavulanic acid (Kapoor *et al.*, 2017:7). In addition to the production of β-lactamases, bacteria can also reduce the amount of antibiotic capable of penetrating the cell membrane. The diminished penetration has been ascribed to mutations in cell membrane porins that are responsible for passive diffusion of antibiotics (Bush *et al*., 1985:559). Lastly, numerous bacteria develop resistance by altering the target PBP. Instead of expressing the normal PBP target resistant bacteria expresses PBP2a, an altered PBP that is capable of functioning as a normal PBP but have reduced affinity to most β-lactam antibiotics (Fuda *et al.*, 2004:40802, Munita & Arias, 2016:17).

#### 2.3.1.2 Glycopeptides

Apart from the β-lactams, there are another group of antibiotics, known as the glycopeptides, which also act as cell wall synthesis inhibitors, *e.g.* vancomycin and teicoplanin (Tenover,

2006:4). Vancomycin (figure 2.5), the first approved glycopeptide, was discovered in 1953 by Eli Lilly and Company and was extracted from *Amycolatopsis orientalis*, while teicoplanin was extracted from *Actinoplanes teichomyceticus* in 1978 (Binda *et al.*, 2014:573).

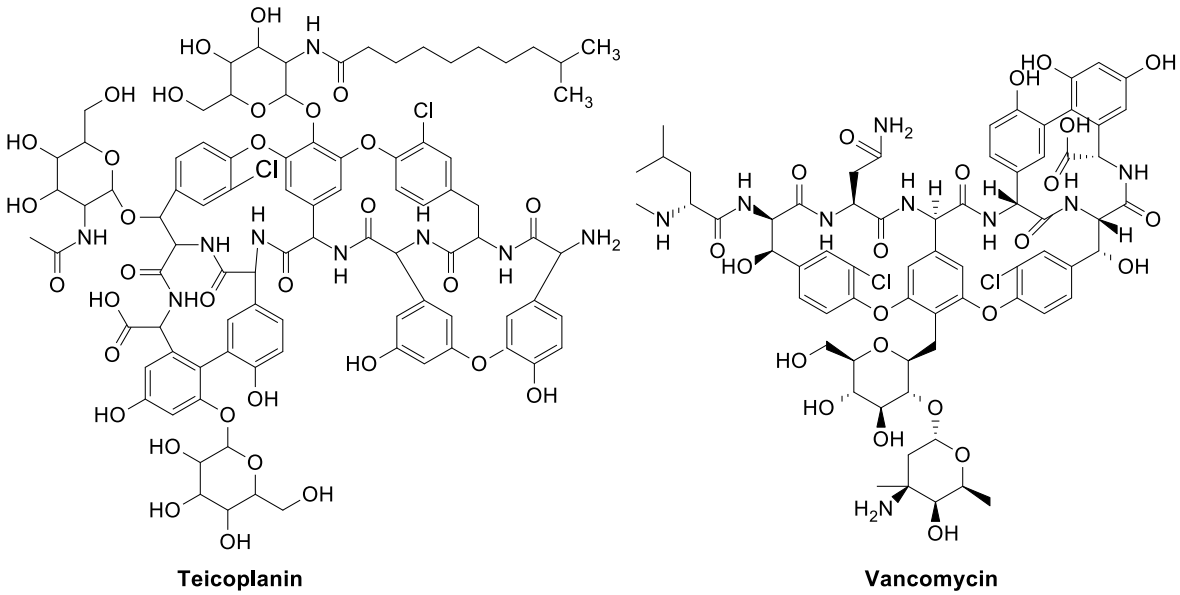



**Figure 2.5: Structures of teicoplanin and vancomycin (Economou *et al*., 2013:521, NLM, 2020).**

As a standalone drug, vancomycin is used for the treatment of MDR Gram-positive bacterial infections such as endophthalmitis. In combination with other drugs, *i.e*. trimethoprim, vancomycin has been found to penetrate the cell membrane of Gram-negative bacteria (Khera *et al.*, 2013:1; Zhou *et al.*, 2015:276). Resistance towards glycopeptides are mainly caused by the expression of resistant genes (*van*) that modifies the cell wall biosynthesis. By integrating an ester linkage in place of the amide of D-Ala-D-Ala, D-Ala-D-lactate or D-Ala-D-serine are produced instead of the normally occurring peptidyl-D-alanyl-D-alanine. These precursors have been shown to have a binding affinity 6-1000 times less than the normal D-Ala-D-Ala compound towards vancomycin. Currently there are six different vancomycin-resistance phenotypes (Arthur & Quintiliani, 2001:375; Binda *et al.*, 2014:577; Sujatha & Praharaj, 2012:1). Bacteria also alter their cell wall structure to prevent glycopeptides from entering, by either changing the net cell surface charge or by thickening the cell wall (Bionda *et al.*, 2013).

### 2.3.1.3 Cyclic lipopeptides

Lastly, cyclic lipopeptides (daptomycin) were discovered in the 1980s by Eli Lilly and Company and was developed to help combat MDR pathogens such as MRSA and VRE (Tally & DeBruin, 2000:523). Daptomycin is used to treat skin and soft skin infections, bacteraemia and right-sided endocarditis, usually caused by Gram-positive bacteria including MRSA (figure 2.6) (Arbeit *et al.*, 2004:1673; Baltz, 2009:144; Fowler Jr *et al.*, 2006:653). Details of daptomycin's exact mode of action is still not clear. It appears that daptomycin is incorporated into the bacterial cell membrane because of its lipid like structure, causing distortions in the cell curvature. This distortion is incorrectly recognised by cell division proteins as a site of cell division, leading to local changes in peptidoglycan biogenesis including inhibition of cell wall biosynthesis (Pogliano *et al.,* 2012:4502).

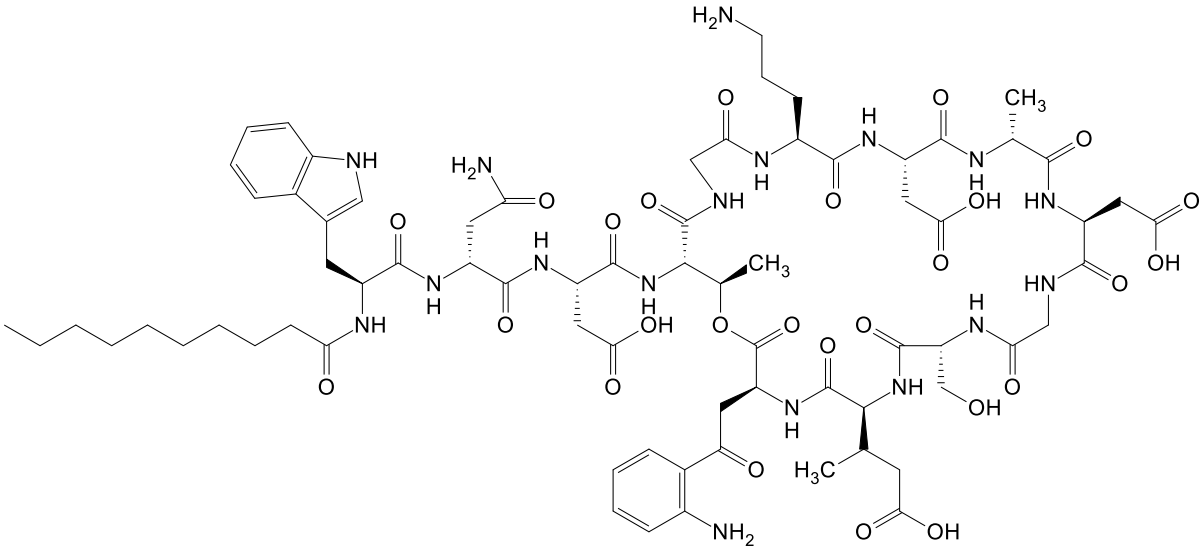


**Figure 2.6: Structure of daptomycin (Taylor & Palmer, 2016:6254).**

Unfortunately, *in vitro* resistance against daptomycin has already been observed in Staphylococcal and Enterococcal clinical isolates. It also appears that there is cross-resistance between daptomycin and vancomycin (Bionda *et al.,* 2013:1312). It seems that resistance against daptomycin is the results of multiple mechanisms resulting in changes to the cell surface charge and cell fluidity leading to a reduced affinity towards daptomycin. Cell thickening has also been observed in *S. aureus* resulting in reduced concentrations of daptomycin (Bionda *et al.,* 2013:1317).

### 2.3.2 Protein synthesis inhibitors

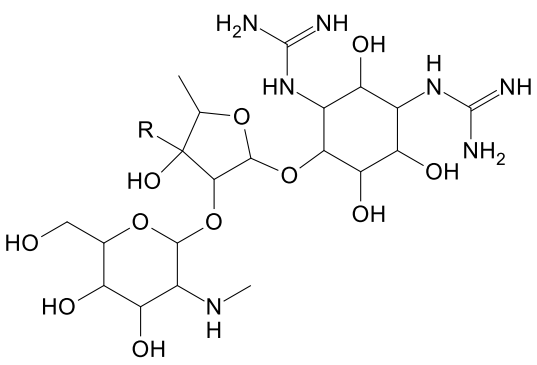
Ribosomes play a vital role in all living cells. Although they have many functions, their primary function is to translate genetic information encoded in the base sequence of messenger RNA (mRNA) into the amino acid sequence of polypeptides, which is required for protein synthesis. On each ribosome there are three binding sites namely the site-A, accepts the incoming transfer RNA (tRNA), site-P (binds to the tRNA) and the site-E (a role not wholly understood, but may serve as an "exit" point) (Sergiev *et al.*, 2005:6048). Two different ribosome subunits exist, *i.e*. the 30S- and 50S subunit. Protein synthesis begins with the smaller subunit (30S) interacting with the mRNA. The 16S ribosomal RNA (rRNA), which is one of 21 different proteins of the 30S subunit, helps to stabilize and align the mRNA when binding to the subunit. The 50S subunit is mostly responsible for the peptidyl transferase and translocase activities. Peptidyl transferase is responsible for peptide-bond formation by catalysing the amino acid residue. Translocation exports the final protein structure out of the cell through the cell membrane (Berg *et al.*, 2002:1; Sengupta et al., 2001:11991; Moore & Steitz, 2003:155; Stiller *et al.*, 2016:2).

Both prokaryotic and eukaryotic cells are dependent on protein synthesis as a means of replicating however, the type of subunit differs from each other. Prokaryotic cells utilise 30S and 50S ribosome subunits for protein synthesis, whereas eukaryotic cells use 40S and 60S subunits providing the perfect opportunity for selectively targeting bacteria cells. The protein synthesis inhibitors are bacteriostatic and selectively target bacteria by interfering with the normal protein synthesis and can be divided into two groups, *i.e*. those who target the 30S subunit (aminoglycosides and tetracyclines) or the 50S subunit (chloramphenicol, macrolides, lincosamides, streptogramins and oxazolidinones) (Kotra *et al.*, 2000:3249; Morar, *et al.*, 2009:1649; Shakil *et al.,* 2008:6; Shutter & Akhondi, 2019:1;Tariq *et al.,* 2018:5788).

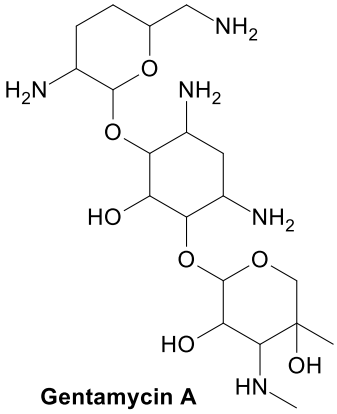
#### 2.3.2.1 Aminoglycosides

Between 1944 and 1972, aminoglycosides were extracted from multiple strains of bacteria belonging to the order actinomycetes. The first aminoglycoside was streptomycin derived from *Streptomyces griseus* followed by neomycin, kanamycin, gentamicin and tobramycin derived from *S. fradiae*, *S. kanamyceticus, Micromonospora purpurea* and *S tenebrarius* respectively. Netilmycin and amikacin were synthetically manufactured from sisomicin and kanamycin. Aminoglycosides bind to site-A of the bacterial 30S ribosome subunit making the ribosome unavailable for translocation, leading to cell death (Park, 2009:57). Aminoglycosides are relatively toxic, prompting clinicians to use other antibiotics however, as a result of emerging

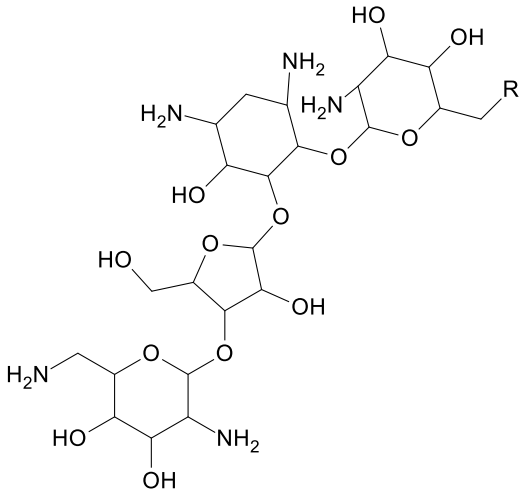
drug resistance they are again gaining popularity (figure 2.7) (Krause *et al.*, 2016:1).



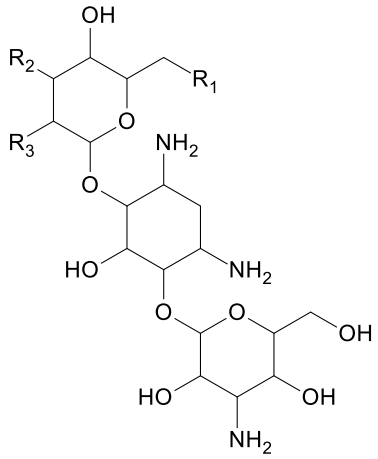
**Streptomycin (R=CHO)**
**Dihydrostreptomycin (R=$CH_2OH$)**



**Gentamycin A**



**Neomycin B ($NH_2$)**
**Paramomycin (OH)**



**Kanamycin A ($R^1$=$NH_2$; $R^2$=OH; $R^3$=OH)**
**Kanamycin B ($R^1$=$NH_2$; $R^2$=OH; $R^3$=$NH_2$)**
**Kanamycin C ($R^1$=OH; $R^2$=OH; $R^3$=$NH_2$)**
**Tobramycin NH ($R^1$=$NH_2$; $R^2$=H; $R^3$=$NH_2$)**

**Figure 2.7: General structures of aminoglycosides (Křen & Řezanka, 2008:860).**

Aminoglycosides are bacteriostatic and have a broad spectrum of antibacterial activity, being used for infections caused by either Gram-negative (*Enterobacteriaceae spp*.) or Gram-positive bacteria (*S. aureus*) (Aggen *et al.*, 2010:4636; Karlowsky *et al.*, 2003:1681; Krause *et al.*, 2016:3; Landman *et al.*, 2010:2123). Resistance towards aminoglycosides are categorised into three main mechanisms; efflux pumps and limiting drug uptake, modification of target ribosomes, and aminoglycoside-modifying enzymes (Chellat *et al.*, 2016:6607; Garneau-Tsodikova & Labby, 2016:11). The first two resistance mechanism are less common, whilst the latter is the main mechanism bacteria use to circumvent the aminoglycosides' bacteriostatic action (Chellat *et al.*, 2016:6607).

### 2.3.2.2 Tetracyclines

The tetracycline class was developed from the late 1940s until the early 1990s (figure 2.8). The first tetracyclines (chlortetracycline, oxytetracycline) were all derived from several *Streptomyces* spp. and by 1961 the first synthetic drugs were developed (lymecycline and methacycline). All tetracyclines are bacteriostatic and exhibit a broad spectrum towards both Gram-negative and Gram-positive bacteria (Chopra & Roberts, 2001:233; Jelić & Antolović, 2016:2; Shutter & Akhondi, 2019:1). They specifically target the 30S ribosome subunit preventing the coupling of aminoacyl-tRNA to the acceptor site on the mRNA-ribosome complex, thus inhibiting replication (Shutter & Akhondi, 2019:1). The prevalence of tetracycline resistance varies greatly between different geographical locations. By the mid-1970s however, resistance against tetracyclines was common among *Enterobacteriaceae*, staphylococci and streptococci (Chopra & Roberts, 2001:251). Widespread resistance is one of the main factors ascribed to the decline in the use of tetracyclines. Tetracycline resistance is similar to that of aminoglycosides, *i.e*. efflux pumps and limiting drug uptake, modification of target ribosomes, and enzymatic inactivation of tetracycline (Chopra & Roberts, 2001:237; Speer *et al.*, 1992:389).

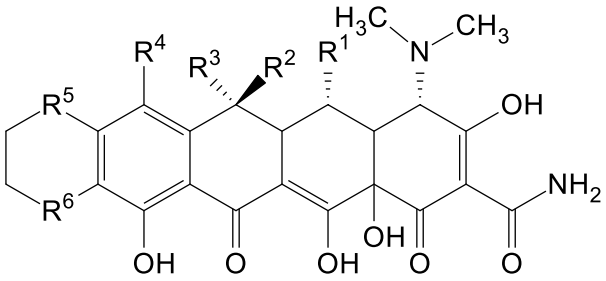
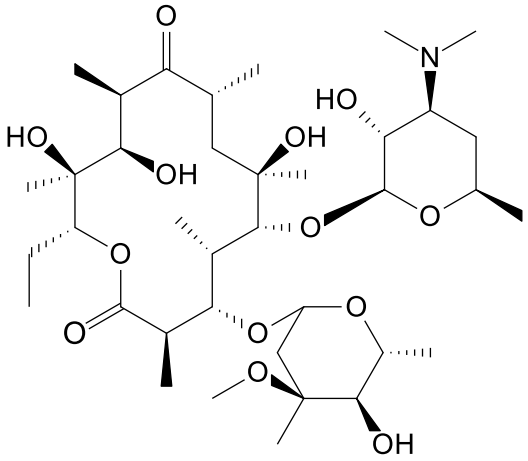



**Figure 2.8: Pharmacophore of tetracyclines (Fuoco, 2012:7).**

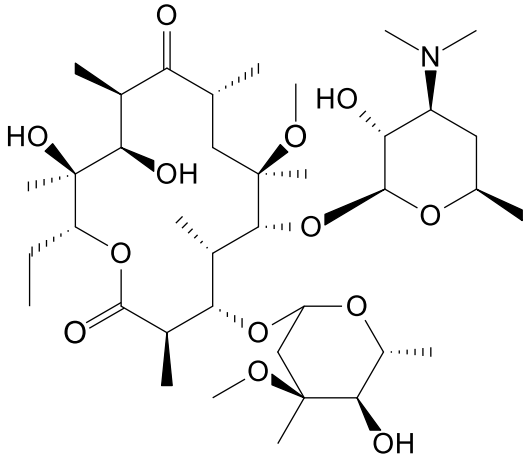
### 2.3.2.3 Macrolides

The first macrolide was discovered in 1950 and was named pikromycin due to the bitter tasted it had when taken orally (Brockmann & Henkel, 1951:284). Macrolides are generally categorised according to the size of their macrocyclic lactone ring (Dinos, 2017:2968). The most well-known macrolides that are still being used today are erythromycin, azithromycin and clarithromycin. Erythromycin was discovered in 1952 and was extracted from *Streptomyces erythreus* (Weber *et al.*, 1985:425). Both azithromycin and clarithromycin were synthetically produced in 1980 from Erythromycin A (Hirsch, 2010:193; Jelić & Antolović, 2016:4).

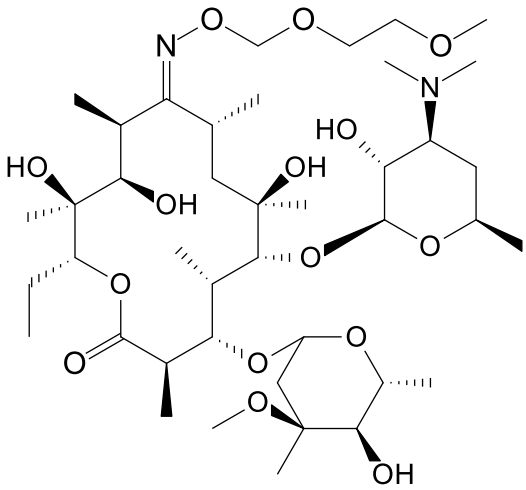
Macrolides are bacteriostatic and display a broad spectrum of antibacterial activity against both Gram-positive and Gram-negative bacteria (figure 2.9).



**Erythromycin**



**Clarithromycin**



**Roxithromycin**

**Figure 2.9: Structures of the most common macrolide antibiotics (Dinos, 2017:2968).**

They remain a valuable option for the treatment of *Mycoplasma pneumoniae, Haemophilus influenza*, *Helicobacter pylori*, *Chlamydia spp.*, *Rickettsia* and many other mycobacteriums (Kanfer *et al.*, 1998:256). Unfortunalty, there are increased reports of resistance against macrolides especially by Gram-positive bacteria (Leclercq, 2002:482). The three main mechanisms of resistance against macrolides are similar to aminoglycosides, *i.e*. efflux pumps, target modification and drug inactivating enzymes however, the majority of resistance

against macrolides is as a result of alteration in the target ribosomal subunit structure (Chellat *et al.*, 2016:6607; Leclercq, 2002:483).

#### 2.3.2.4 Chloramphenicol

Chloramphenicol was discovered in 1947 and extracted from *Streptomyces venezuelae* (figure 2.10). Chloramphenicol binds onto the 23S rRNA of the 50S ribosome subunit, which inhibits peptidyl transferase preventing cell growth (Finch *et al.*, 2010:14). Chloramphenicol is bacteriostatic and is classified as a broad-spectrum antibiotic, however chloramphenicol is rarely used in modern times due to its adverse effects, *e.g*. neurotoxicity and hematologic disorders (Dinos *et al.*, 2016:1). The most common method of resistance against chloramphenicol is enzymatic inactivation by acetylation of the drug. Other mechanisms include target site mutations, decreased outer membrane permeability and efflux pumps (Fernández *et al.,* 2012:1001; Shaw *et al.,* 1970:1095).

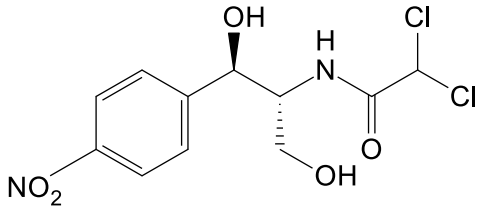
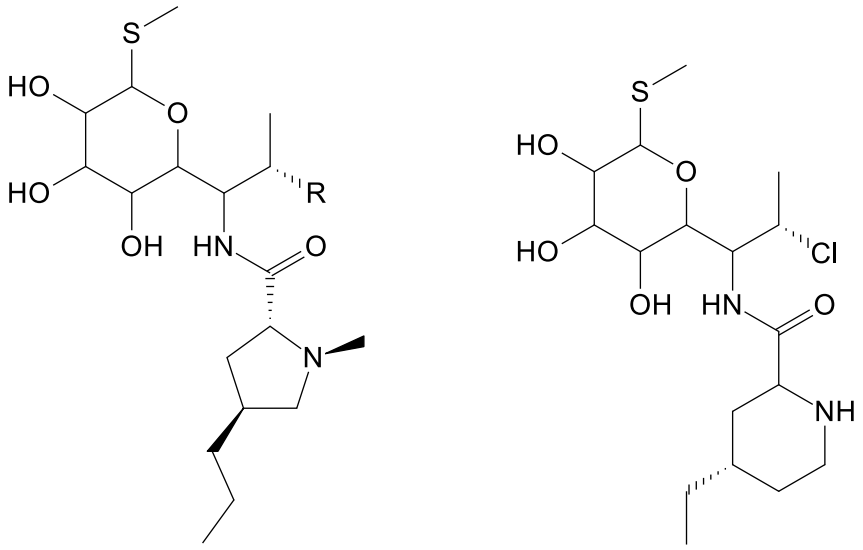



**Figure 2.10: Structure of chloramphenicol (Dinos *et al.,* 2016:7).**

#### 2.3.2.5 Lincosamides

The only two lincosamides that are clinically used are lincomycin and clindamycin (figure 2.11), the former was firstly isolated from *Streptomyces lincolnensis* whereas the latter is produced semi synthetically (Spížek & Řezanka, 2004:455). This class was firstly identified in 1960 and is mainly used to treat Gram-positive bacteria, however they can also be used to treat selected Gram-negative anaerobe bacteria and certain protozoa (Morar *et al.*, 2009:1649). The rapid development of drug resistance and gastrointestinal side effects have led to a decline in the use of lincosamides. The two most common methods of resistance are the enzymatic deactivation of lincosamides as well as modification of the ribosomal target (Morar *et al.*, 2009:1649).



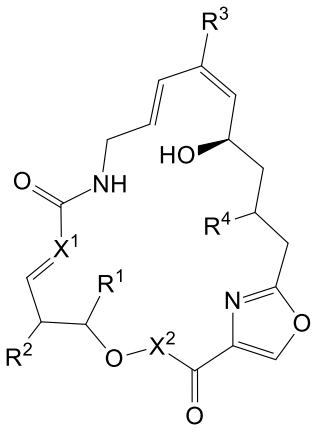

**Lincomycin (R:OH)**
**Clindamycin (R:Cl)**

**Pirlimycin**

**Figure 2.11: Structures of lincosamide, clindamycin and pirlimycin (Morar *et al.,* 2009:1650).**

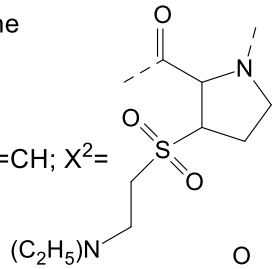
### 2.3.2.6 Streptogramins

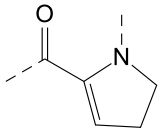
Streptogramins consists of two subtypes, *i.e*. streptogramin A (*e.g*. griseoviridin, dalfopristin and virginiamycin $M_1$) and streptogramin B (*e.g*. pristinamycin IA, virginiamycin S and quinupristin), and are produced by the genus *Streptomyces* (figure 2.12). When used separately both types are bacteriostatic, however when used together they are bactericidal (Cocito *et al*., 1997:8). Because of their similar mechanism of action, macrolides, lincosamides and streptogramins have the same spectrum of antibacterial activity (Spížek & Řezanka, 2004:455). Most Gram-negative bacteria are resistant to streptogramins, mainly as a result of decreased permeability of the drug compounds across the Gram-negative cell wall (Cocito *et al*., 1997:8). The three main resistance mechanisms against the streptogramins are, decreased concentration at the site of action, antibiotic inactivation and changes in the target structure (Cocito *et al*., 1997:8).

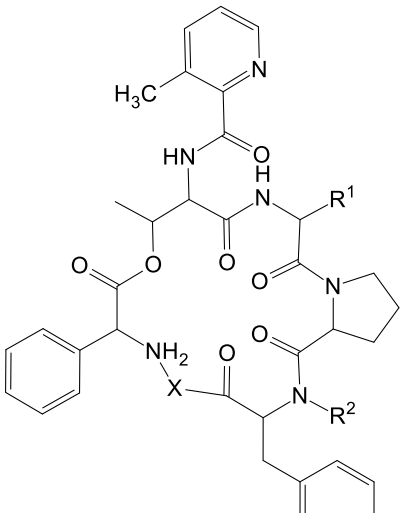


**Streptogramin A**

Griseoviridin: $R^1$=$CH_3$; $R^2$=H; $R^3$=H; $R^4$=OH; $X^1$=C; $X^2$=Cysteine

Dalfopristin: $R^1$=$CH_2CH(OH)CH_2$; $R^2$=$CH_3$; $R^3$=$CH_3$; $R^4$=O; $X^1$=CH; $X^2$= 

Virginiamycin $M_1$: $R^1$=$CH_2CH(OH)CH_2$; $R^2$=$CH_3$; $R^3$=$CH_3$; $R^4$=O; $X^1$=CH; $X^2$= 



**Streptogramin B**

Pristinamycin IA: $R^1$=$CH_2CH_3$; $R^2$=$CH_3$; $R^3$=$N(CH_3)_2$; X=4-Oxopipecolic acid

Virginiamycin S: $R^1$=$CH_2CH_3$; $R^2$=H; $R^3$=H; X=4-Oxopipecolic acid

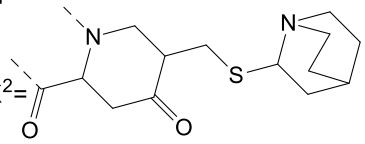
Quinupristin: $R^1$=$CH_2CH_3$; $R^2$=$CH_3$; $R^3$=$N(CH_3)_2$; $R^4$=O; $X^1$=CH; $X^2$= 

**Figure 2.12: Structures of streptomycin A and B (Mukhtar & Wright, 2005:532).**

#### 2.3.2.7 Oxazolidinones

Oxazolidinones differ in the way they prevent bacteria cell growth when comparing them to the rest of the protein cell inhibitor drug classes (figure 2.13). Linezolid became the first approved oxazolidinone for clinical use in 2000 and is still used today (Tsuji *et al.*, 2005:223). After linezolid, only one other drug from this class was released for clinical use, *i.e.* tedizolid phosphate (Burdette & Trotman, 2015:1315). Both drugs share the same method of action by binding to the 23S ribosomal RNA of the 50S subunit preventing the formation of the 70S initiation complex, thus inhibiting protein synthesis (Bozdogan & Appelbaum, 2004:115). Linezolid is bacteriostatic and its primary indications are the treatment of MDR bacterial strains such as MRSA, VRE and penicillin-resistant streptococci (PR-*Streptococci)*. Examples of infections caused by these bacteria include pneumonia, skin and soft tissue infections (Bozdogan & Appelbaum, 2004:113; Diekema & Jones, 2001:1975; Moellering Jr, 2003:135; Moise *et al.*, 2002:1017; Pandit *et al.*, 2012:1; Peppard & Weigelt, 2006:357; Prasad, 2007:454). Oxazolidinone resistance is still rare however, some bacteria are capable of altering the target site of action, reducing the binding affinity of the rug compound (Chellat *et al*., 2016:6605).

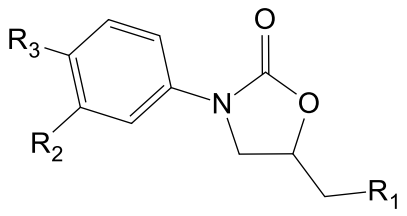



**Figure 2.13: Pharmacophore of oxazolidinones (Saini, 2018:14).**

### 2.3.3 Nucleic acid synthesis inhibitors

Some antibiotics exert their antibacterial action by inhibiting the DNA synthesis, replication or transcription. These include quinolones, metronidazole and isoniazid (Kirmisaoğlu *et al.*, 2019:570).

#### 2.3.3.1 Quinolones

The quinolones inhibit bacterial DNA synthesis by targeting bacterial type II topoisomerase, *i.e.* gyrase and topoisomerase IV, which regulates supercoiling during bacterial DNA synthesis (Pham *et al.* 2019:1719). Inhibition of DNA gyrase correlates with Gram-negative bacterial activity, while DNA topoisomerase IV inhibition correlates with Gram-positive bacterial activity (Oliphant *et al*., 2002:455). Topoisomerases are enzymes responsible for catalysing

chromosomal supercoiling, a process necessary for DNA synthesis, mRNA transcription and cell division (Kohanski *et al*., 2010:2). Nalidixic acid was the first antibacterial quinolone, and was discovered as a by-product during the synthesis of chloroquine (Pham *et al.*, 2019:1720). The antibacterial spectrum of quinolones was greatly expanded by the development of fluoroquinolones, which also have better pharmacokinetic properties (Pharm *et al*., 2019:1719). They are classified as bactericidal drugs and used to treat numerous infections, *e.g.* uncomplicated UTIs, pyelonephritis, skin and soft tissue infections and bronchitis (Bush *et al.,* 2011:179; King *et al.,* 2000:2743; Kohanski *et al.,* 2010:1).

Since their discovery many derivatives of the original quinolones have been synthesised in an effort to decrease their toxicity, increase their activity and broaden the spectrum of bacteria against which they are active (figure 2.14). As a result the quinolone antibiotics can be divided into four generations, *i.e*. the first generation (nalidixic acid, oxolinic acid *etc*.) which has poor oral bioavailability, the second generation (norfloxacin, ciprofloxacin, ofloxacin *etc*.) which has increased oral bioavailability and Gram-negative activity, the third generation (levofloxacin, sparfloxacin, moxifloxacin etc.) which has expanded activity against Gram-positive bacteria and the fourth generation (trovafloxacin) which adds activity against anaerobes (King *et al*., 2000:2741; Oliphant *et al*., 2002:456, Pham *et al.*, 2019:1721).

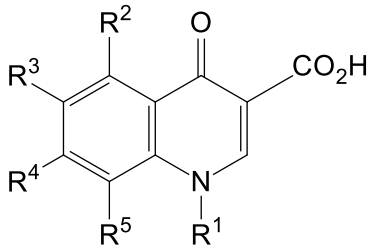



**Figure 2.14: Pharmacophore of quinolones (Pham *et al.*, 2019:1723).**

Resistance against quinolines have increased, limiting their use (Pham *et al.*, 2019:1729). Quinolone resistance can broadly be grouped into three categories, *i.e*. target-, plasmid- and chromosome mediated resistance (Aldred *et al*., 2014:1569). Target mediated resistance consists of alterations of the target enzyme, resulting in a weakened drug-enzyme interaction (Drlica *et al*. 2009:982). Plasmid-mediated resistance entails the horizontal transference of resistance (through bacterial conjugation), which can result in either decreased enzyme-DNA binding, enzymatic alteration of the drug compounds, or an increase in the prevalence of efflux pumps. Chromosome mediated resistance results in either under excretion of porins leading to decreased drug concentration or over expression of efflux pump, leading to decreased drug retention (Alfred *et al*., 2014:1570).

### 2.3.3.2 Isoniazid

Isoniazid was firstly synthesised in 1912 but had little activity against most bacteria (figure 2.15). The drug however gained popularity in the 1950s when it was found to have notable activity against tuberculosis (Fernandes *et al.,* 2017:298). Isoniazid's mechanism of action is still a topic of debate, however it appears that isoniazid is a prodrug and once activated its bactericidal action is a result of lipid cell wall inhibition and nucleic acid synthesis inhibition (Timmins & Deretic 2006:1220). Some bacteria have developed resistance against isoniazid which is the result of a mutation in the *inhA* gene, preventing the conversion of the prodrug into its active form (Fernandes *et al.,* 2017:299).

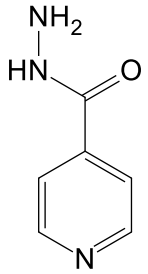



**Figure 2.15: Structure of isoniazid (Kaur *et al.*, 2019:2).**

### 2.3.3.3 Metronidazole

Metronidazole was also discovered in the 1950s and was initially used to treat the protozoan infection *Trichomonas vaginalis*. It was later discovered that metronidazole displayed bactericidal activity against numerous Gram-negative and Gram-positive bacteria (figure 2.16) (Freeman *et al.*, 1997:680; Ralph & Kirby, 1975:409). The precise mechanism of action of metronidazole is still not clearly understood, however it is believed that reduction of the nitro group facilitates entry into the organism and subsequently causes DNA strand breakage (Edwards, 1980:285). In common with other antibiotics, resistance against metronidazole can be ascribed to reduced drug uptake, increased removal of the drug (efflux pumps) as well as a reduction in drug activation (Smith, 2018:403).

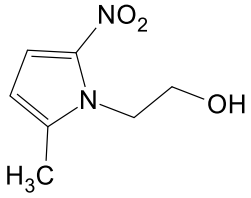



**Figure 2.16: Structure of metronidazole (NLM, 2020).**

### 2.3.4 Metabolic compound inhibitors

Folic acid, the base structure of vitamin B, is an essential substrate for all living organisms. Not only is it necessary for the proper functioning of living cells but also the prevention of various illnesses (Lucock, 2000:121). Folic acid is essential in various biosynthetic pathways, *e.g*. the synthesis of proteins, nucleic acids, amino acids, purine and pyrimidine nitrogenous bases (Fernández-Villa *et al.*, 2019:2; Fowler, 2001:221; Zheng & Cantley, 2019:253). Human cells obtain folate directly from their surroundings *via* transport proteins, whilst bacteria cells must synthesise folate *de novo* making the folate biosynthetic pathway a perfect target for selectively inhibiting bacteria (Griffith *et. al.* 2018:2). The bacterial folate biosynthesis pathway is depicted in figure 2.18. The important steps in this pathway with regards to the folic acid inhibitors are the conversion of hydroxymethyl-7, 8-dihydropterin pyrophosphate to dihydropteroate by the enzyme dihydropteroate synthase (DHPS), as well as the conversion of dihydrofolate to tetrahydrofolic acid (THF) by the enzyme dihydrofolate reductase (DHFR) (Bermingham & Derrick, 2002:637; Dias *et al*., 2018:935). The sulphonamide class of antibiotics is bacteriostatic and inhibit the DHPS enzyme by directly competing with its substrate, para-aminobenzoic acid (pABA), preventing pABA to bind to the active site on DHPS (figure 2.17) (Griffith, *et. al.* 2018). Trimethoprim is the only clinically relevant antibiotic that inhibits DHFR and is mostly used in combination with sulfamethoxazole (Hawser *et al.*, 2006:941). There is a great number of sulphonamide antibiotics which can be categorised as either short acting (sulfathiazole, sulfasomidine, *etc.*), medium acting (sulfasimazole, sulfamethoxazole, *etc.*) or long acting (sulfamethazine, sulfamethoxypyridazine, *etc.*) (Sreeremya, 2019:17, Tačić *et al*., 2017:58). They are used as treatment for several diseases, *e.g.* cancer, psoriasis, rheumatoid arthritis, UTI's, *etc*. (Bowen *et al.,* 2017:1; Weinblatt, 2003:16; Wood Jr, 1942:369).

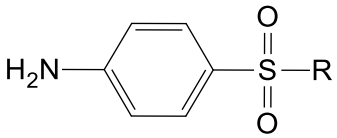



**Figure 2.17: Pharmacophore structure of sulphonamide (Griffith *et al.*, 2018:6).**

Since their introduction, resistance against the sulfonamide antibiotics developed rapidly (Davies, 2010:418). It has been shown that certain bacteria acquire resistance against sulphonamide antibiotics through spontaneous mutation of the *folp* gene, resulting in a single base pair substitution changing a phenylalanine moiety to either an isoleucine or a leucine at

position 28 in the amino acid sequence of the enzyme (Sköld, 2000:156). The change in position 28 of the amino acid chain results in a small structural change in the target DHPS enzyme that drastically decrease its affinity for sulphonamide antibiotics, whilst barely affecting its affinity for the pABA substrate (Sköld, 2000:156).

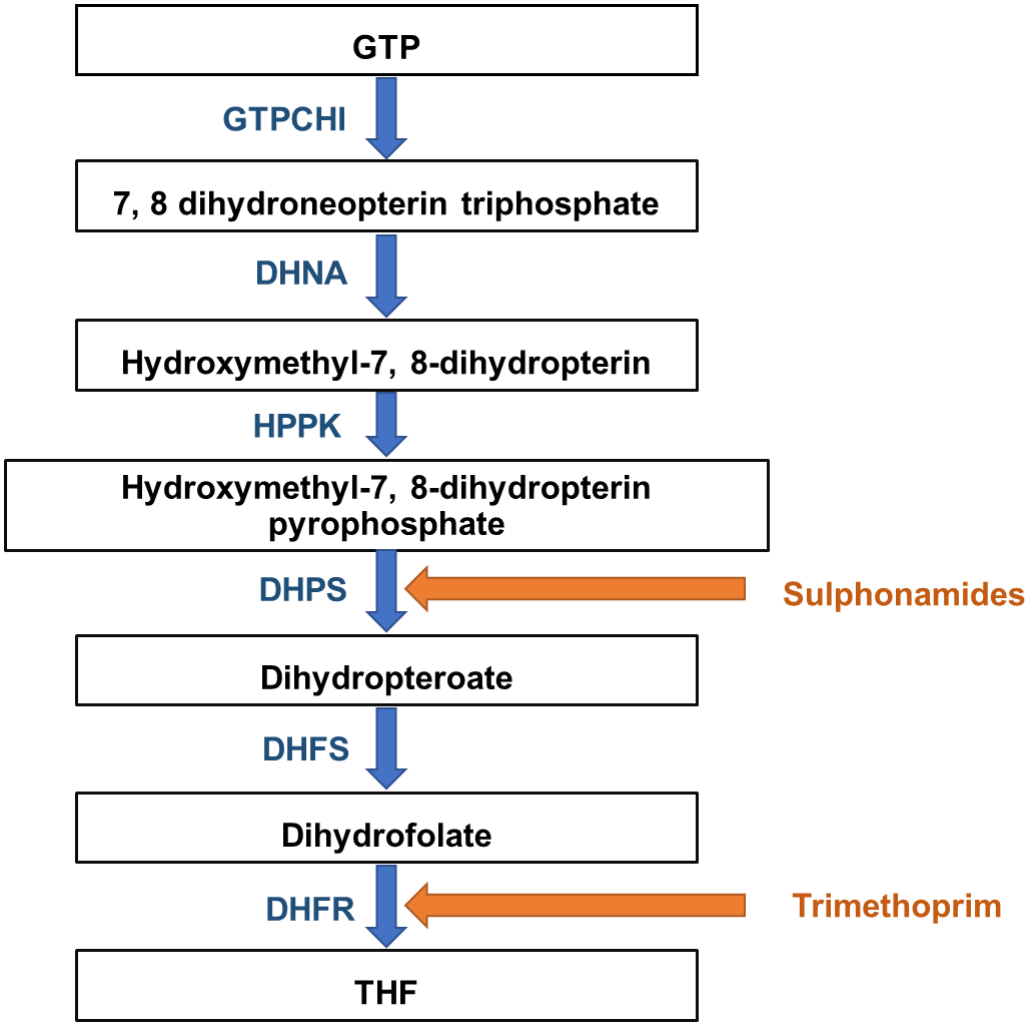



**Figure 2.18:** **Bacterial folate biosynthetic pathway (Bermingham & Derrick, 2002:637; Dias *et al*., 2018:935;). Abbreviations: guanosine triphosphate (GTP), GTP cyclo hydrolase I (GTPCHI), 7,8-dihydroneopterin aldolase (DHNA), 6-hydroxymethyl-7,8-dihydropterin pyrophospho kinase (HPPK), dihydropteroate synthase (DHPS), dihydrofolate synthase (DHFS), tetrahydrofolic acid (THF).**

## 2.4 Methylene blue analogous

Methylene blue (figure 2.19) is a cationic thiazine dye that was firstly developed in the textile industry in 1876 and was also used as a staining agent to visualise tuberculosis bacilli in

microscopy (Lo *et al*., 2014:670). In 1891 Paul Ehrlich, the father of chemotherapy, successfully treated malaria with methylene blue which was used throughout the end of World War 2 (Lo *et al*., 2014:670; Titford, 2010:497). Since its discovery methylene blue has found applications in numerous fields, *e.g*. biology, chemistry and medicine (Vutskits *et al*., 2008:684). Methylene blue is widely accepted to be the first synthetic drug to be used for its medicinal purpose and was used as a lead compound in the development of quinolines (antimalaria) and phenothiazines (neuroleptic) (Lo *et al*., 2014:670; Wainwright, 2009:1500).

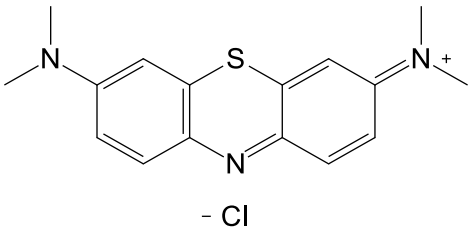



**Figure 2.19: Methylene blue structure (Amaral *et al.,* 2001:1016).**

In medicine methylene blue is commonly used to treat methemoglobinemia, ifosfamide-induced encephalopathy and vasoplegia accompanied septic shock (Kwok & Howes, 2006:362; Patel, 2006:302; Vutskits *et al*., 2008:684). In the past methylene blue has also been used to treat cyanide poisoning, as an antidepressant and an antimalarial drug (Lo *et al*., 2014:670). As a result of the increased resistance against commonly used antimalarials, methylene blue is once again considered as a possible treatment option (Schirmer *et al*. 2003:273). In surgery, methylene blue is also used to stain certain tissue, *e.g*. parathyroid glands (Dudley, 1971:680). Although neurotoxic side-effects have been reported, methylene blue is considered safe at a dose of 7.5 mg/kg showing no toxic effects in humans (Kuriloff & Sanborn, 2004:616; Vutskits *et al*., 2008:691).

Methylene blue also has antimicrobial properties and was first used as an antibacterial agent at the end of the 19$^{th}$ century (Oz *et al.*, 2011:1). On its own or in combination with other compounds methylene blue has been shown to have activity against *E. coli, Pseudomonas aeruginosa, Enterococcus faecalis, S. aureus, S. epidermidis, Candida albicans, K. pneumonia* and *Aspergillus niger* (Ash *et al*., 2006:17, Li *et al*., 2016:13615). Methylene blue is also used as a bactericidal agent in foam dressings to assist with prolonged wound healing (Edwards, 2016:11). In most cases the antibacterial activity of methylene blue has been proven in *in vitro* studies or has been utilised for external use. Methylene blue is also known to be a light activated antimicrobial agent (LAAA) that is often used in photodynamic therapy (PDT) to kill bacteria in the presence of light. PDT involves the topical or systemic

administration of an LAAA and the subsequent illumination with visible light. The LAAA is activated resulting in localised cell death of the target cell, leaving the host cells unharmed. The systemic use of PDT is limited to the upper layers of the skin (1-5 mm) depending on the penetrating power of the light wave used (Salva, 2002:571). The exact mechanism of PDT is not within the scope of this dissertation, however it involves activation of the LAAA allowing it to form an excited singlet or triplet state which interacts with surrounding molecules and oxygen atoms killing the target cells (Salva, 2002:571).

Although numerous studies have determined the *in vitro* and *in vivo* antimicrobial activity of methylene blue few have determined its MIC, using different methods instead. The MIC is considered the gold standard for susceptibility testing and is defined as the lowest concentration of an antimicrobial compound that will visibly inhibit growth of an organism after overnight incubation (Andrews, 2001:5). Using standardised methods for measuring antimicrobial activity is important, as it allows for comparison of results across different laboratories (Hannan, 2000:375; Wu *et al*., 2015:664). Some studies have determined the MIC of methylene blue against pathogenic organisms and found that it was active against *C. albicans* (100 µg/ml) (Ansari *et al*., 2016:16) as well as *S. aureus* (10 mg/ml) (Shatti & Authman, 2015:77).

The mode of action of methylene blue depends on its indication. When used as a treatment for methemoglobinemia, methylene blue prevents the oxidation of haemoglobin to methaemoglobin which is incapable of binding to oxygen (Haouzi *et al*., 2018:844; McDonagh *et al.,* 2013:502). Methylene blue's mode of action against cyanide poisoning is still unclear, but many believe it may be because of its redox properties. (Haouzi *et al.,* 2018:836-837). To treat ifosfamide toxicity, methylene blue is believed to have different modes of actions, *e.g.* electron acceptor, oxidation of NADH and inhibition of monoamine oxidases (Patel, 2006:300; Pelgrims *et al.,* 2000:293). When used to treat vasoplegia during shock, methylene blue inhibits inducible nitric oxide and guanylate cyclase, which in turns prevents vasodilation (Hosseinian *et al.,* 2016:194; Kwok & Howes, 2006:359). Methylene blue's antibacterial mechanism of action is still not properly understood. Many suggest that methylene blue's mode of action is based on its redox properties that interfere with various electron transport pathways (Edwards, 2016:14).

Research conducted on methylene blue led to the discovery of phenothiazines, *e.g*. promethazine, chlorpromazine, levomepromazine and thioridazine. As can be seen in figure 2.20, phenothiazines share similar structural properties compared to methylene blue. The main clinical indications of phenothiazines are as antipsychotics, antiemetics and hypno-sedatives, although they can also be used to treat vertigo, migraine, hiccups and motion

disorders (Dyer & Woolf, 1999:82). Phenothiazines have been found to have marked antibacterial properties, with MICs ranging from 2-10 µg/ml against most Gram-positive bacteria (Dastidar *et al*., 2013:59). Phenothiazines also have activity against Gram-negative rods as well as Mycobacteria (Amaral *et al*., 2004:725). The precise antibacterial mechanism of action is still a topic of debate although phenothiazines have been found to inhibit DNA base processes by intercalating DNA bases (Varga *et al*., 2017:5987). It has also been postulated that phenothiazines cause bactericidal haemolysis by interfering with potassium transport through the bacterial cell membrane (Dastidar *et al*., 2013:60). Researchers have also proven that phenothiazines are capable of binding to PBPs and have used this knowledge to design and synthesise phenothiazine derivatives capable of inhibiting PBP (Amaral *et al*., 2004:726; Onoabedje *et al*., 2016:2).

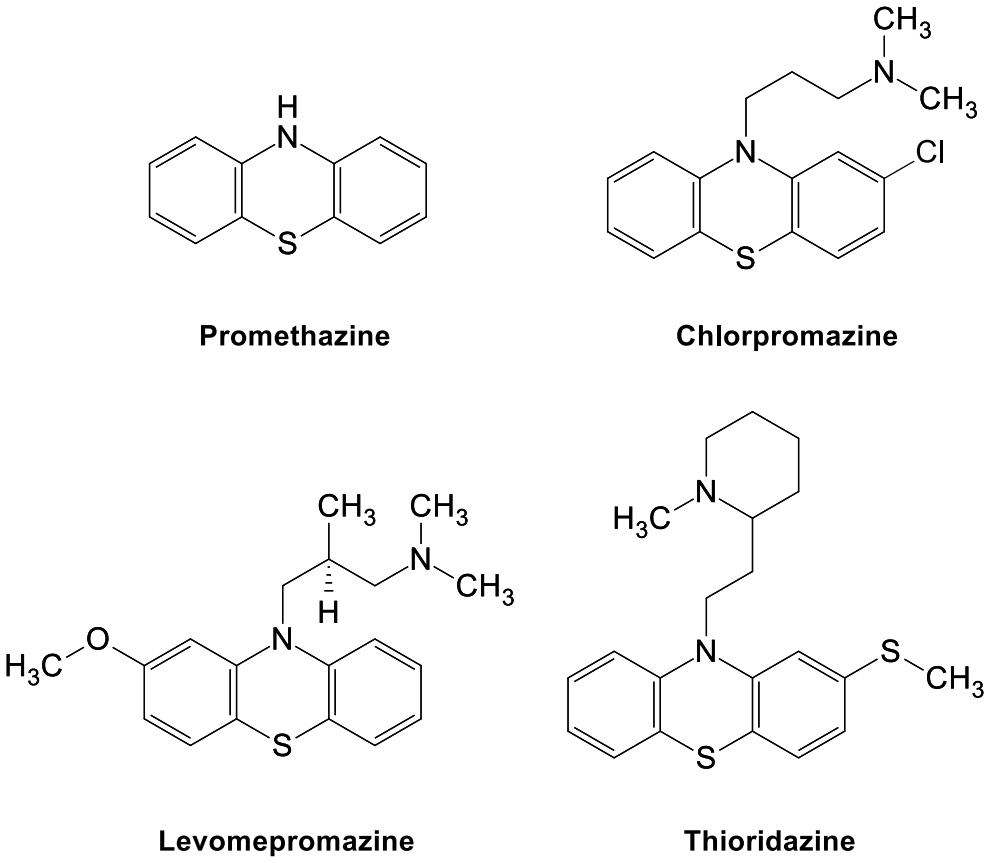



**Figure 2.20: Examples of phenothiazines developed from methylene blue (Varga *et al*., 2017:5984).**

## 2.5 Computer-aided drug design

Computer-aided drug design (CADD) is a rapidly growing field wherein studies that would have previously been conducted *in vitro* or *in vivo*, are firstly done *in silico* in an effort to save time and money (Kapetanovic, 2008:165). CADD is a broad term used to describe the mathematical manipulation and quantification of potential drug candidate properties and can be done by a range of publicly and commercially available software packages (Yu & MacKerell, 2017:4). Two types of CADD exists, namely structure-based drug design (SBDD) and ligand-based drug design (LBDD) (Aparoy *et al.*, 2012:3765; Kapetanovic, 2008:166). SBDD uses a known target receptor/enzyme to identify key sites of interaction between the target and drug compound. SBDD can however only be used when the target's structure has been elucidated by either nuclear magnetic resonance or X-ray crystallography (Batool *et al*., 2019:4; Huang *et al*., 2010:624). If the target structure is not available, the protein structure can be predicted using computational methods such as homology modelling. In contrast, LBDD does not need a target receptor instead it attempts to establish a correlation between the physiochemical properties of ligands and their activity against the unknown target. Examples of chemical features are hydrogen bond acceptors, hydrogen bond donors, hydrophobic groups, negatively ionisable groups, positively ionisable groups and aromatic rings (Dixon *et al*., 2006:650). Both SBDD and LBDD can be used for either hit identification or hit optimisation studies (Yu & MacKerell, 2017:5). Because the antimicrobial target of methylene blue is still unknown, LBDD will be used in this study.

### 2.5.1 Ligand-based drug design

LBDD is used to either create a quantitative structure-activity relationship (QSAR) model or a common feature pharmacophore model (Aparoy *et al.*, 2012:3766). Either one of these two types of models contain information regarding the feature type, position, direction and constraints required for a ligand to show activity against the target (Lee *et al*., 2011:5306). Once a pharmacophore model has been generated by either technique, it can be used to either screen a drug database for compounds that have a high probability of exhibiting the same activity against the target or to predict possible mechanisms of action of the test compound (Wu *et al.,* 2016:3372). This process is often called virtual screening (VS) and is employed by pharmaceutical companies to reduce the costs of traditional high-throughput screening (HTS) techniques to identify novel "hit" compounds (Yang, 2010:447). To create either a QSAR- or a common feature pharmacophore model, two data sets of compounds are needed, *i.e*. the training- and test set. The training set is used to create the model while the test set is used to validate the model. For both techniques the size of the training- and test set should be as large as possible however, to create a common feature pharmacophore

model fewer compounds are generally needed (John *et al.,* 2011:2; Rathee et al., 2017:114). Another key difference between these two models are that the exact activity of the ligands needs to be known to create a QSAR model, while only their degree of activity needs to be known to create a common feature pharmacophore model, *e.g*. active, moderately active or inactive (Adane *et al*., 2010:636; Liu *et al.*, 2020:6). Because we have a small number of compounds we will use common feature pharmacophore modelling in this study.

#### 2.5.1.1 Validation

To validate each pharmacophore model, internal and external validations methods are used (Lee *et al*., 2011:5309). As internal validation the computer program uses the training set to create and score several pharmacophore hypotheses. Each hypothesis contains a rank score and a fit value which are used to determine which hypotheses can accurately predict the activity of each compound in the training set. A high rank score indicates a smaller chance that the training set compounds mapped the hypothesis by chance correlation (Adane *et al*., 2010:637; Sakkiah *et al*., 2012:870). Each hypothesis will also fit each compound of the training set to a certain degree. The higher the fit value, the greater the activity of the compound against the target. The fit value should ideally show some correlation to the known *in vitro* activity. The fit value, together with the rank score, is used to determine which hypothesis is the most accurate. As an external validation method, a test set is used to validate the chosen pharmacophore model (Pascual *et al.*, 2019:4). The test set contains a small number of compounds with known activity against the target and a larger number of compounds that are known to not have activity against the target, *i.e*. decoy compounds (Vuorinen *et al*., 2014:5997). The pharmacophore model is then used to screen this database and its ability to identify the active compounds within this database is judged using three metrics, *i.e*. the enrichment factor (EF), the hit rate (HR) and the receiver operator characteristic (ROC) curve (described in paragraphs 3.5.1.2 and 3.5.1.3) (Aparoy *et al.*, 2012:3772; Pal *et al.*, 2019:291; Pascual *et al*., 2019:5).

The choice of decoy compounds within the test set is important, as selecting compounds with structures differing too much from the active compounds will result in biased positive results and selecting compounds with structures resembling the active compounds to closely will result in inaccurate results as these compounds themselves might elicit an *in vitro* response (Réau *et al*., 2018:1). There are a number of online sources where inactive decoy compounds can be downloaded, *e.g.* ZINC15 and the Database of Useful Decoys-Enhanced (DUD-E) (Mysinger, *et. al*., 2012:6582; Sterling & Irwin, 2015:2324). In this study we will use the DUD-E database to generate 50 decoy compounds for every active compound (Mysinger, *et. al*., 2012:6592). The DUD-E database uses an extensive set of criteria to select the decoy

compounds which is reviewed elsewhere (Mysinger, *et. al*., 2012:6583). In short, the database selects compounds that are similar to the active compounds with regards to their molecular weight, estimated water-octanol partition coefficient, rotatable bonds, hydrogen bond acceptors, hydrogen bond donors and net charge. The database will then generate the extended connectivity fingerprint (ECFP) of all the active compounds as well as the ECFP of all the potential decoys, then only select the most dissimilar 25% of the potential decoys. This is done to minimise the number of decoy compounds that might themselves elicit a response. Lastly, for every active compound only fifty decoys are selected (Mysinger, *et. al*., 2012:6592). The active and decoy compounds are combined to form the test set, which will be used to validate the pharmacophore models as described below.

#### 2.5.1.2 Enrichment factor and hit rate

Each pharmacophore model is used to screen and rank the test set from most to least active. From these results both the EF and HR are calculated. The EF is defined as the ratio between the percentage of active compounds in the selected subset and the percentage in the entire test set (Chen *et. al*., 2006:409). The subset is a percentage of the whole database, *e.g*. 1%. The EF can be determined using equation 1 (Chen *et al*., 2006:409).

Enrichment factor (EF) = $(Hits_{sel} / Hits_{tot}) \times (NC_{tot} / NC)$ ..... Equation 1

$Hits_{sel}$ is the number of active compounds in the sampled subset while $Hits_{tot}$ is the total number of active compounds in the database. $NC_{tot}$ is the total number of compounds (active and decoy) in the whole test set, while NC is the total number of compounds (active and decoy) in the selected subset. One drawback of the EF is that the method is dependent on the ratio between the number of active compounds and the total number of compounds screened. Because of this the EF can only be used to compare different pharmacophore hypotheses that were used to screen the same databases and should not be used to compare pharmacophore hypotheses used to screening different databases (Huang *et al*., 2006:6; Wei *et al*., 2002:341).

The HR is the ratio of active compounds identified at a certain percentage of the total database screened *vs*. all the known active compounds in the entire test set and is calculated by equation 2 (Hawkins *et al*., 2007:75).

Hit rate (HR) = $(Hits_{sel} / Hits_{tot}) \times 100$ ..... Equation 2

$Hits_{sel}$ is the total number of active compounds in the selected subset, and $Hits_{tot}$ refer to the total number of active compounds in the entire database (Chen *et al*., 2009:1697).

### 2.5.1.3 Receiver operator characteristic curve

The ROC curve is a well-recognised metric used in different fields of study and can be used as an objective way to evaluate the ability of a program to discriminate between active and decoy compounds. The ROC curve also determines what threshold should be used to differentiate between an active and decoy compound, *e.g*. the fit value. A ROC curve can be visualised as depicted in figure 2.21, where the sensitivity and specificity are plotted against each other. Sensitivity refers to the percentage of truly active compounds correctly identified by the program, while specificity refers to the percentage of decoy compounds correctly identified by the computer program. Sensitivity and specificity are calculated using equations 3 and 4 respectively (Triballeau *et al*., 2005:2536).

Se = TP / (TP+FN) ..... Equation 3

Sp = TN / (TN+FP) ..... Equation 4

Se refers to sensitivity, TP to true positive and FN to false negative. Sp refers to specificity, TN to true negative and FP to false positive (Triballeau *et al*., 2005:2536).

The area under the ROC curve (ROC-AUC) is used to determine the performance of a computer docking program (Lätti *et al*., 2016:1; Triballeau *et al*., 2005:2537). The ROC-AUC can be interpreted as follows; 0.9 ≤ AUC ≤ 1.0 is excellent, 0.8 ≤ AUC < 0.9 is good, 0.7 ≤ AUC < 0.8 is fair and 0.5 ≤ AUC < 0.6 is poor (Braga & Andrade, 2016:6).

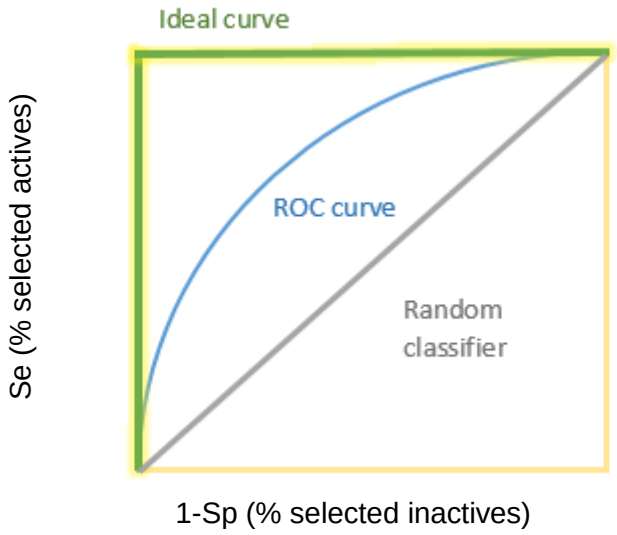



**Figure 2.21: Example of a ROC curve and its variables (Triballeau *et al.*, 2005:2536).**

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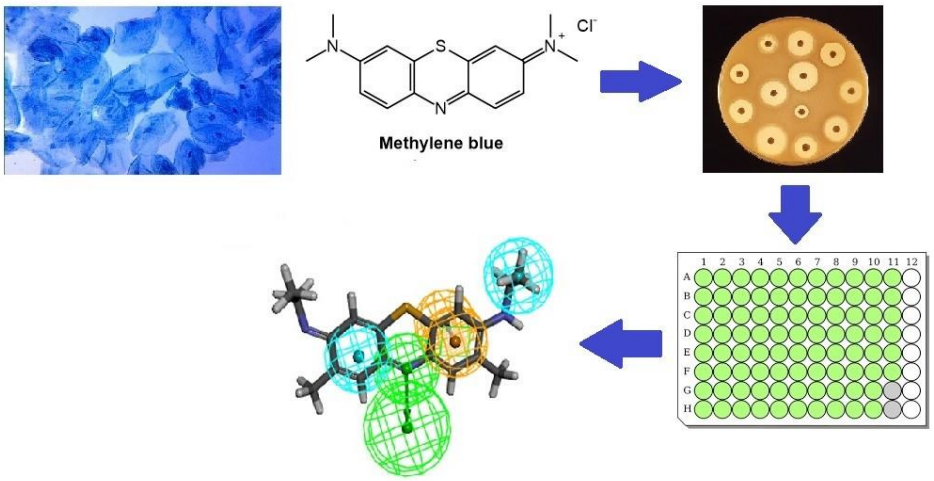
# CHAPTER 3: ARTICLE

The following article has been published in the European Journal of Pharmaceutical Sciences (Thesnaar *et al*., 2020). The manuscript is written as a research article and is presented according to the author notes as stipulated by the European journal of Pharmaceutical Sciences (EJPC, 2020). The preprint version of the article is shared in accordance with the publisher's policy (Elsevier, 2020). All co-authors provided permission to use this manuscript as part of L. Thesnaar's MSc dissertation as indicated in the letter of permission above. For the published article, please see annexure A.

**Graphical abstract**




**Abstract**

It has been shown that methylene blue has antimicrobial properties although few studies have determined its minimum inhibitory concentration (MIC), which is the gold standard used to measure antimicrobial activity. The exact antimicrobial mode of action of methylene blue is still unclear and to our knowledge no pharmacophore model has yet been created to investigate methylene blue's mode of action. The aim of this study was to determine the MIC of methylene blue and a number of its analogous against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella enterica* and *Candida albicans*, and to use these data to develop and validate a common feature pharmacophore model. Three statistical metrics, *i.e*. the enrichment factor (EF), hit rate (HR) and the area under the curve of a receiver operator characteristic (ROC-AUC), were used to determine the pharmacophore model's ability to differentiate between active/decoy compounds. The validated pharmacophore model was used to map similar antibacterial

compounds in an attempt to elucidate methylene blue's antimicrobial mode of action. Most test compounds only had activity against *S. aureus*, *S. epidermidis* and *K. pneumoniae*. Dimethyl methylene blue, new methylene blue and acriflavine proved to be the most active compounds against *S. aureus* [1 µg/ml (dimethyl methylene blue); 4 µg/ml (new methylene blue); 8 µg/ml (acriflavine)], *S. epidermidis* [1 µg/ml (dimethyl methylene blue); 4 µg/ml (new methylene blue); 2 µg/ml (acriflavine)] and *K. pneumoniae* [8 µg/ml (dimethyl methylene blue); 0.5 µg/ml (new methylene blue); 2 µg/ml (acriflavine)]. A common feature pharmacophore model was created (rank score: 26.664, max. fit value: 4), which was able to accurately identify active methylene blue analogous out of the test set ($EF^{2\%}$: 51, $HR^{2\%}$: 100%, ROC-AUC: 1.00 ± 0.00). Six phenothiazine derivatives with known antibacterial activity had high fit values when mapped with the validated pharmacophore model, *i.e*. >3.4. Dimethyl methylene blue, new methylene blue and acriflavine had potent antibacterial activity against *S. aureus*, *S. epidermidis* and *K. pneumoniae*. The MIC data will allow other researchers to compare the activity across different laboratories. The common feature pharmacophore model was able to identify methylene blue analogous with known *in vitro* antibacterial activity out of a database containing active/decoy compounds and highlighted the importance of two hydrophobic features, an aromatic feature and a hydrogen bond acceptor. The validated pharmacophore model also correctly mapped six phenothiazine derivatives with known antibacterial activity suggesting that they may share a common antibacterial mechanism of action.

**Key words:** Discovery Studio; Methylene blue; Antimicrobial activity; Common feature pharmacophore modelling.

# Methylene blue analogues: *In vitro* antimicrobial minimum inhibitory concentrations and *in silico* pharmacophore modelling

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## 3.1 Introduction

Since the discovery of arsphenamine (1910) by Paul Ehrlich and penicillin (1929) by Alexander Fleming, antibiotics have saved countless lives and drastically accelerated advancements in the field of medicine (Fleming, 1929; Gaynes, 2017; Rice, 2008). The utility of antibiotics has led to their widespread misuse resulting in numerous antimicrobial resistant bacteria strains (Aminov, 2010). It is estimated that 700 000 people die annually as a direct result of antimicrobial resistance (AMR), and this is projected to globally increase to a staggering 10 million people by 2050 (O'Neill, 2014). In addition to the development of AMR, only two new antibiotic classes have been developed and approved in the last two decades further exacerbating the problem (Tacconelli et al., 2018). There is an urgent need to develop new antimicrobial drugs to combat this emerging threat.

Methylene blue is a cationic thiazine dye that has found application in numerous fields, *e.g.* biology, chemistry and medicine (Vutskits et al., 2008). It was developed in 1876 as a dye in

the textile industry and later became the first synthetic drug used in medicine (Lo et al., 2014). Since then methylene blue has also been used as a lead compound in the development of quinolines (antimalaria) and phenothiazines (neuroleptic) (Wainwright, 2003). Figure 3.1 depicts the similarities between methylene blue and the quinoline and phenothiazine pharmacophores.

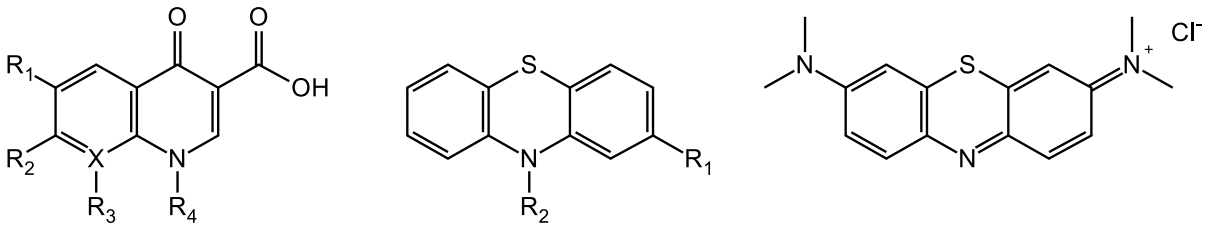


**Figure 3.1:** The structure of methylene blue and the pharmacophores of quinolines and phenothiazines.

In medicine, methylene blue is often used to treat methemoglobinemia, ifosfamide-induced encephalopathy and vasoplegia accompanied septic shock (Kwok and Howes, 2006; Patel, 2006; Vutskits et al., 2008). Historically, methylene blue has also been used to treat cyanide poisoning, as an antidepressant and an antimalarial drug (Lo et al., 2014). During surgery methylene blue may also be used as a dye to effectively visualise certain tissue, *e.g*. parathyroid glands (Dudley, 1971). Methylene blue is generally considered safe for *in vivo* use, with doses of 7.5 mg/kg showing no toxic effects in humans (Kuriloff and Sanborn, 2004). However, some studies have suggested dose dependant neurotoxic effect on the central nervous system (Vutskits et al., 2008).

Methylene blue is known to have antibacterial activity. Numerous studies have found that methylene blue has activity on its own or in combination with various other compounds. For example, Steczko and co-workers showed that in combination with sodium citrate methylene blue had *in vitro* activity against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis, Candida albicans* and *Aspergillus niger* (Steczko et al., 2009). Li and co-workers showed that methylene blue alone, or in combination with silver nanoparticles, had *in vitro* activity against *E. coli*, *Klebsiella pneumoniae* and *P. aeruginosa* (Li et al., 2016). Methylene blue is also known to be a light activated antimicrobial agent (LAAA) that is often used in photodynamic therapy (PDT) to kill bacteria in the presence of light. For example, Piccirillo and co-workers showed that methylene blue has substantial activity against both *E. coli* and *S. epidermidis* when used as an LAAA (Piccirillo et al., 2009). It has also been shown that methylene blue can be used in PDT, or even alone, to kill *Helicobacter pylori* (Choi et al., 2010).

Although methylene blue has proven antimicrobial activity most studies have not determined its minimum inhibitory concentration (MIC), using different methods instead. The MIC is defined as the lowest concentration of an antimicrobial compound that will visibly inhibit growth of an organism after overnight incubation and is considered the "gold standard" for susceptibility testing (Andrews, 2001). Using standardised methods for measuring antimicrobial activity is important, as it allows for comparison of results across different laboratories (Hannan, 2000; Wu et al., 2015). Some studies have determined the MIC of methylene blue against pathogenic organisms and found that it was active against *C. albicans* (100 µg/ml) (Ansari et al., 2016) as well as *S. aureus* (10 mg/ml) (Shatti and Authman, 2015).

Depending on the indication, methylene blue has different biological targets. When used to treat methemoglobinemia methylene blue prevents the oxidation of haemoglobin to form methaemoglobin (McDonagh et al., 2013). The mechanism by which methylene blue alleviates ifosfamide-induced encephalopathy remains unclear, however it is believed that it may act as an electron acceptor to decrease the inhibition of flavoproteins by ifosfamide metabolites (Patel, 2006). To treat vasoplegia during septic shock, methylene blue is used to inhibit inducible nitric oxide (iNOS) and guanylate cyclase (GC), thereby preventing widespread vasodilatation (Kwok and Howes, 2006). The mechanism with which methylene blue treats cyanide poisoning is poorly understood, but its activity is believed to be the result of methylene blue's unique redox properties (Haouzi et al., 2018). Methylene blue is also capable of inhibiting monoamine oxidase (MAO) A, which may explain its antidepressant effects (Delport et al., 2017; Naylor et al., 1986; Ramsay et al., 2007).

With regards to its antimicrobial properties, methylene blue has been used since the 19$^{th}$ century as an antimalarial compound (Lu et al., 2018; Schirmer et al., 2003). Its mechanism of action may be based on the prevention of heme polymerisation in the parasite preventing heme detoxification, or the interruption of the mitochondrial electron transport chain, although the latter mechanism has been questioned (Atamna et al., 1996; Ehrhardt et al., 2013). Methylene blue's antibacterial mechanism of action is still a topic of debate. Studies hypothesise that its mechanism of actions is dependent on its peculiar redox properties that interfere with numerous electron transport pathways in the bacteria (Dubos, 1929; Edwards, 2016; Hoffmann and Rahn, 1944; Woo and Heil, 2017). Additionally, phenothiazines (methylene blue analogues) are believed to exert their antibacterial effect by either binding or interfering with the penicillin binding protein (PBP) (Amaral et al., 2004). Numerous phenothiazine derivatives, *i.e*. flupenthixol, fluphenazine, methdilazine, prochlorperazine, promazine, promethazine, thioridazine, trifluoperazine, triflupromazine and trimeprazine have proven antibacterial activity (Das Gupta et al., 2008). In photodynamic therapy (PDT) methylene blue absorbs light and create a singlet oxygen ($^1O_2$). This extremely electrophilic

entity oxidises double bonds in biological molecules and can lead to the formation of hydroxyl radicals, lipid hydroperoxides or the breaking of nucleic acid strands (Oz et al., 2011). Red light with a wavelength of either 630 nm or 700 nm is commonly used, although other wavelengths can have the same effect (Mahmoudi et al., 2018; Salva, 2002). It has also been stated that visible light illumination may have the potential to photosensitize methylene blue (Oz et al., 2011).

When the mode of action of a set of ligands is unknown, ligand-based drug design (LBDD) uses the structures of ligands with known activity to create a common feature pharmacophore model or a 3-dimensional quantitative structure-activity relationship (3D-QSAR) model. The generated model contains information regarding the feature type, position, direction and possible steric constraints that ligands require to be active against the unknown target (Lee et al., 2011). Examples of chemical features are hydrogen bond acceptors (HBA), hydrogen bond donors (HBD), hydrophobic moieties (HYD) and aromatic regions (Ar) (Vuorinen et al., 2014).

Numerous compounds that have known activity is used to create a pharmacophore model; these compounds are termed the training set. The optimum number of compounds needed in the training set differs for 3D-QSAR and common feature pharmacophore models, with the former needing a large number of compounds and the later needing less (Arooj et al., 2013; John et al., 2011; Liu et al., 2020; Rathee et al., 2017; Thangapandian et al., 2011). In this study common feature pharmacophore models will be used.

Using the training set, a computer program creates and ranks several possible pharmacophore model hypotheses of which the most accurate should be chosen. A higher rank score indicates a smaller chance that the training set compounds mapped the hypothesis by chance correlation (Adane et al., 2010; Sakkiah et al., 2012). Each hypothesis will also fit each training set compound to a certain degree, with a higher fit value indicating greater activity. The rank score together with the fit value are used to determine which pharmacophore hypothesis is the most accurate. Common feature pharmacophore models can be used to make accurate predictions of how an active pharmacophore will look, but it still has limitations and needs to be properly validated before reliable results can be obtained. A test set, containing known active and decoy compounds, is used to validate the pharmacophore models. The enrichment factor (EF), hit rate (HR) and receiver operator characteristic (ROC) curve are three metrics commonly used to validate the ability of a pharmacophore model to identify active compounds within the test set (Pascual et al., 2019).

The EF is defined as the ratio between the percentage of active compounds in a selected subset and the percentage in the entire database (Chen et al., 2006). The results can be expressed by calculating the EF with equation 1 (Wei et al., 2002).

$$\text{Enrichment factor (EF)} = (Hits_{sel} / Hits_{tot}) \times (NC_{tot} / NC) \quad \text{Equation 1}$$

$Hits_{sel}$ is the number of active compounds selected by the docking program at a specific percentage of the total dataset. $Hits_{tot}$ is the total number of active compounds for the target in question, $NC_{tot}$ is the total number of molecules (active and decoy) screened in the database and NC is the total number of compounds (active and decoy) in the specific percentage of the database (Chen et al., 2006; Wei et al., 2002). The specific percentage, as mentioned above, is selected by the researcher and is usually between 0.1 – 10 %, depending on the size of the total dataset (Chen et al., 2006; Wei et al., 2002).

The HR is the ratio of active compounds identified at a certain percentage of the total database screened *vs*. all the known active compounds in the entire data base and is calculated by equation 2 (Chen et al., 2009).

$$\text{Hit rate (HR)} = (Hits_{sel} / Hits_{tot}) \times 100 \quad \text{Equation 2}$$

The ROC curve is a well-recognised metric used as an objective way to evaluate the ability of a program to discriminate between active and decoy compounds. The ROC curve also allows the researcher to determine what threshold should be used to differentiate between an active and decoy compound, *e.g*. the fit value. The area under the ROC curve (ROC-AUC) is a practical way of determining the performance of a computer docking program (Lätti et al., 2016; Triballeau et al., 2005). The ROC-AUC can be interpreted as follows; $0.9 \leq AUC \leq 1.0$ is excellent, $0.8 \leq AUC < 0.9$ is good, $0.7 \leq AUC < 0.8$ is fair and $0.5 \leq AUC < 0.6$ is poor (Braga and Andrade, 2013).

Because of the drastic increase in antimicrobial resistance and the lack of novel antibiotic drug classes, there is a great need to develop new drugs with antimicrobial activity. One of the oldest synthetic drugs, methylene blue, is known to have antimicrobial properties however few studies have determined its MIC against pathogens making comparisons between different laboratories difficult. In addition, its antimicrobial mechanism of action is still not clear. This study aims to determine the MIC of methylene blue and methylene blue analogues against

*S. aureus*, *S. epidermidis*, *E. coli*, *K. pneumoniae*, *Salmonella enterica* and *C. albicans*. This data will then be used to develop and validate a pharmacophore model which can, not only be used to identify lead compounds with similar activity but might also give insight into methylene blue's mechanism of action.

### 3.2 Study design

The test compounds were firstly screened with the Kirby Bauer method to determine their *in vitro* activity against *S. aureus*, *S. epidermidis*, *E. coli*, *K. pneumoniae*, *S. enterica* and *C. albicans*. Using the broth microdilution method, the MIC of the test compounds were determined only against the pathogens which proved susceptible during the Kirby Bauer method. With regards to the *in silico* LBDD study, the test compounds were divided into a training- and a test set. The test set was supplemented with 249 inactive compounds downloaded from the directory of useful decoys enhanced (DUD-E) database. The training set was used to create pharmacophore models for *S. aureus*, *S. epidermidis* and *K. pneumoniae*. The best pharmacophore hypothesis was chosen based on its rank score and fit value. Each hypothesis was also validated using a test set, after which the $EF^{2\%}$, $HR^{2\%}$ and ROC-AUC metrics were used as an objective way of determining which hypothesis was the most accurate. Finally, we used the best pharmacophore model to map phenothiazine derivatives with known antibacterial activity to ascertain if they might share a common mode of action.

### 3.3 Materials and methods

The *in vitro* antimicrobial activities of methylene blue, methylene green, neutral red, new methylene blue, azure B, dimethyl methylene blue, methylene violet, cresyl violet, acriflavine and nile blue were determined (Sigma-Aldrich, United States). The *in vitro* activity of a compound previously synthesised by our group, *i.e*. ethylthioninium chloride, was also determined (Delport et al., 2014). The test compounds were evaluated against *S. aureus* (ATCC 43300), *S. epidermidis* (ATCC 12228), *E. coli* (ATCC 10536), *K. pneumoniae* (ATCC 10031), *S. enterica* (ATCC 9150) and *C. albicans* (ATCC 14053) (Virginia, United States). During the experiments, the optical density of the pathogens was measured with a Biochrom, Novaspec II spectrophotometer (Gemini, Netherlands). All non-sterile equipment was autoclaved at 121 °C for 15 min using a CL-40M autoclave (ALP Co. Ltd) (Lasec, South Africa).

### 3.3.1 Kirby Bauer method

Muller-Hinton agar (MHA), Muller-Hinton broth (MHB) (Sigma-Aldrich, South Africa) and potato dextrose agar (PDA) (Merck, South Africa) were prepared according to the manufacturer's instructions. The MHA and PDA were used to prepare petri dishes (90 x 15 mm) (Lasec, South Africa) containing agar with a depth of 4 mm. The MHA-petri dishes were used for the bacteria and the PDA-petri dishes for the fungi. To create each inoculum two to three morphological similar colonies of each pathogen were suspended in 10 ml MHB and incubated over night at 35 ± 2 °C (bacteria) or 25 ± 2 °C (fungi) in a walk-in incubator (T&S Refrigeration, South Africa). All pathogens were handled in a level 2 biosafety cabinet (EuroClone S.p.A, Italy). Ampicillin (Sigma-Aldrich, South Africa) and amphotericin B (Sigma-Aldrich, South Africa) were used as positive controls against the bacteria and fungi, respectively. Ampicillin, methylene blue, methylene green, neutral red, new methylene blue, azure B, dimethyl methylene blue, ethylthioninium chloride and acriflavine were dissolved in distilled water ($DH_2O$) to give a concentration of 10 mg/ml, amphotericin B was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, South Africa) and methylene violet, cresyl violet and nile blue were dissolved in a 1:1 mixture of $DH_2O$ and DMSO to give a concentration of 10 mg/ml. The relevant solvent of each compound was used as negative control. Each solution was filter sterilised (0.2 µm) (Lasec, South Africa) and was used within 12 hrs of preparation.

A lawn of bacteria/fungi was created on each agar petri dish by evenly spreading 100 µl of the above-mentioned inoculum. Dry discs with a diameter of 6 mm was created from Whatman grade 4 filter paper (Lasec, South Africa) with a paper punch. Five discs were place on each agar petri dish, *i.e.* tests compound ($n$ = 3), a positive control ($n$ = 1) and a negative control ($n$ = 1). Of each test compound, 5 µl (resulting in 50 µg) were added to each disc ($n$ = 3) and 10 µg of the positive control were added to each disc ($n$ = 1). Of each negative control, 5 µl solution were added to each disc ($n$ = 1). The petri dishes were incubated at either 35 ± 2 °C bacteria)) or 25 ± 2 °C (fungi) for 18 hrs. After incubation the zone of inhibition (ZOI) of each disc was measured with a ruler (table 3.1). If a compound had a ZOI > 6 mm it was considered active.

**Table 3.1:** ZOI of each test compound against the relevant pathogen.

| Test compound | ZOI (mm) | | | | | |
|---|---|---|---|---|---|---|
| | *S. aureus* | *S. epidermidis* | *E. coli* | *K. pneumoniae* | *S. enterica* | *C. albicans* |
| **Methylene blue** | 9.7 ± 0.5 | 14.0 ± 1.4 | 6.0 ± 0.0 | 15.3 ± 0.5 | 6.0 ± 0.5 | 6.0 ± 0.0 |
| **Methylene green** | 10.7 ± 1.7 | 14.5 ± 1.5 | 6.7 ± 0.9 | 14.0 ± 0.0 | 6.7 ± 0.5 | 7.3 ± 0.5 |
| **New methylene blue** | 9.7 ± 0.5 | 10.7 ± 0.5 | 6.0 ± 0.0 | 15.7 ± 0.5 | 6.0 ± 0.0 | 6.0 ± 0.0 |
| **Dimethyl methylene blue** | 9.7 ± 0.5 | 12.7 ± 0.5 | 6.0 ± 0.0 | 15.3 ± 0.5 | 6.0 ± 0.0 | 6.0 ± 0.0 |
| **Methylene violet** | 8.7 ± 0.5 | 8.3 ± 0.5 | 6.0 ± 0.0 | 8.7 ± 0.5 | 6.0 ± 0.0 | 6.0 ± 0.0 |
| **Cresyl violet** | 8.0 ± 0.8 | 7.7 ± 0.5 | 6.0 ± 0.0 | 8.0 ± 0.8 | 6.0 ± 0.0 | 6.0 ± 0.0 |
| **Nile blue** | 7.0 ± 0.0 | 7.0 ± 0.0 | 6.0 ± 0.0 | 7.7 ± 0.5 | 6.0 ± 0.0 | 6.0 ± 0.0 |
| **Acriflavine** | 7.7 ± 0.5 | 16.7 ± 1.9 | 13.3 ± 0.5 | 11.7 ± 0.5 | 10.0 ± 0.8 | 6.3 ± 0.5 |
| **Azure B** | 9.7 ± 0.5 | 6.0 ± 0.0 | 6.0 ± 0.0 | 14.0 ± 0.8 | 6.0 ± 0.0 | 6.0 ± 0.0 |
| **Neutral red** | 6.0 ± 0.0 | 7.3 ± 0.5 | 6.0 ± 0.0 | 6.0 ± 0.0 | 6.0 ± 0.0 | 6.0 ± 0.0 |
| **Ethylthioninium chloride** | 8.3 ± 0.5 | 14.0 ± 0.8 | 7.7 ± 0.5 | 10.5 ± 0.5 | 7.0 ± 0.0 | 6.0 ± 0.0 |
| **Positive control*** | 8.7 ± 1.6 | 8.8 ± 2.5 | 15.5 ± 2.7 | 6.5 ± 0.7 | 20.2 ± 7.0 | 15.5 ± 3.1 |
| **Negative control†** | 6.0 ± 0 | 6.1 ± 0.3 | 6.0 ± 0 | 6.0 ± 0 | 6.0 ± 0 | 6.1 ± 0.3 |

* Bacteria (ampicillin) and *C. albicans* (amphotericin B).
† Ampicillin ($DH_2O$), methylene violet, cresyl violet and nile blue [1 ($DH_2O$) : 1 (DMSO)] and amphotericin B (DMSO).

### 3.3.2 Broth microdilution method

Following the results of the Kirby Bauer method, the MIC of each test compound was determined against *S. aureus*, *S. epidermidis* and *K. pneumoniae* according the Clinical and Laboratory Standards Institute (CLSI) guidelines (Cockerill et al., 2012).

The test compounds were dissolved (8% DMSO) to a concentration of 256 µg/ml, which resulted in a final concentration of 128 µg/ml (4% DMSO) when the inoculum was added. Tetracycline (Sigma-Aldrich, South Africa) was dissolved ($DH_2O$) to a concentration of 64 µg/ml which resulted in a final concentration of 32 µg/ml. All stock solutions were filter sterilised (0.2 µm). Two-fold serial dilutions ($n$ = 11) were made of all the compounds. Each Corning flat bottom 96-well plate (Lasec, South Africa) was primed with all eleven concentrations of two test compounds (100 µl, $n$ = 3 rows each) and a positive control (100 µl, $n$ = 1 row). A sterility control (200 µl, $n$ = 8 wells), *i.e*. sterile MHB, and a growth control (100 µl, $n$ = 11 wells), *i.e.* $DH_2O$, was also added to each 96-well plate.

The inoculum of each bacteria was prepared by suspending two to three colonies in 10 ml sterile MHB and incubating the suspension at 35 ± 2 °C for 2 – 5 hrs while shaking (250 rpm). It was experimentally determined beforehand that all three bacteria would be in the mid-log growth phase after 3 – 5 hrs incubation (data not shown). Each inoculum was diluted with sterile MHB to an $OD_{600}$ of 0.35 corresponding to a bacterial concentration of $2.6 \times 10^8$ CFU/ml (*S. aureus*), $2.14 \times 10^8$ CFU/ml (*S. epidermidis*) and $3.0 \times 10^8$ CFU/ml (*K. pneumoniae*). The correlation between the $OD_{600}$ value and the bacteria concentration was experimentally determined beforehand (data not shown). Each inoculum was further diluted with sterile MHB to a concentration of $1 \times 10^6$ CFU/ml. Finally, 100 µl inoculum was added to all wells (except the sterility control) which resulted in a final concentration of $5 \times 10^5$ CFU/ml and a final volume of 200 µl. Each inoculum was used within 30 min to avoid changes in the bacterial cell count. Each plate was covered, placed in a plastic container and incubated at 35 ± 2 °C for 20 hrs. A beaker containing 250 ml hot water was also placed inside the plastic contained to increase the humidity and prevent evaporation. The bacterial growth was measured with a 96-well plate reader (BioTek Instrument Inc, USA) (ADP, South Africa) at a wavelength of 520 nm before and after incubation. Because of the colour of the test compounds, 520 nm gave more reliable results than 600 nm. The MIC was considered as the lowest concentration of a compound at which the growth was inhibited ≥ 80%. The average of the $OD_{520}$ values were used to determine the MIC value.

A viability count was conducted on each inoculum by suspending 10 µl from one growth control well in 10 ml sterile MHB. Of this suspension 100 µl were spread over a MHA petri dish ($n$ = 3) and incubated at 35 ± 2 °C for 20 hrs (EUCAST, 2003).

### 3.3.3 Common Feature Pharmacophore Modelling

The docking procedures were conducted using Accelrys Discovery Studio (DS) v3.1 (San Diego, United States). The ROC-AUC curve calculations were conducted using SPSS Statistics for Windows, version 26 (SPSS Inc., Chicago, Ill., USA). All structures were downloaded from the PubChem database (Kim et al., 2018).

The structures of all molecules were typed with the CHARMm force field, and then subjected to the *Prepare Ligands* protocol. Depending on the bacteria strain the following principal values were ascribed to the training set compounds, *i.e*. *S. aureus* [methylene green (1), new methylene blue (2), dimethyl methylene blue (2), cresyl violet (1)], *S. epidermidis* [methylene green (1), new methylene blue (2), dimethyl methylene blue (2), cresyl violet (0)] and *K. pneumoniae* [methylene green (2), new methylene blue (2), dimethyl methylene blue (2), cresyl violet (1)]. A maximum omit feature value of 0 was used for all training set compounds. The *Feature Mapping* protocol identified common chemical groups present in the training set, *i.e*. hydrogen bond acceptor (HBA), hydrogen bond donor (HBD), hydrophobic (HY), positive ionisable (PI) and ring aromatic (RA). To obtain a diverse range of conformations the *Common Feature Pharmacophore Generation* protocol considered the BEST 200 conformations within a 20 kcal/mol threshold and a maximum interference distance of 2 Å. The final pharmacophore models were ranked by fit value.

Acriflavine was omitted from the modelling protocols, as it consists of a mixture of two compounds (figure 3.2). The eight remaining test compounds were divided into a training set, *i.e*. methylene green, new methylene blue, dimethyl methylene blue, cresyl violet, and a test set, *i.e*. methylene blue, azure B, ethylthioninium chloride, neutral red. As part of the test set, we included 249 decoy compounds that were downloaded from the directory of useful decoys enhanced (DUD-E) database (Mysinger et al., 2012). The way in which the DUD-E data base selects decoy compounds is described in detail elsewhere (Mysinger et al., 2012). In short, the database selects molecules from the ZINC database that are similar to the active compounds with regards to their molecular weight, estimated water-octanol partition coefficient, rotatable bonds, hydrogen bond acceptors, hydrogen bond donors and net charge (Mysinger et al., 2012; Sterling and Irwin, 2015). DUD-E then generates the extended connectivity fingerprint (ECFP) of all the test compounds as well as the ECFP of all the potential decoys, then only select the most dissimilar 25% of the potential decoys to minimise the number of decoys that might have activity against the target (Mysinger et al., 2012).

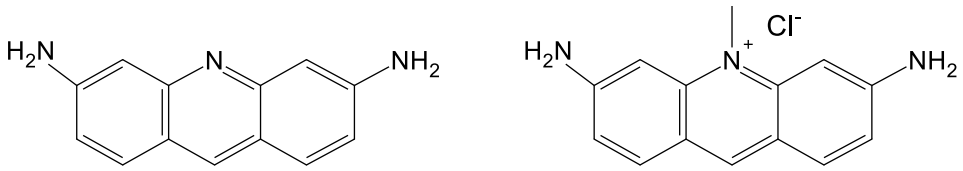


**Figure 3.2:** Structures of the acriflavine mixture.

The entire test set was docked into each pharmacophore model created for *S. aureus*, *S. epidermidis* and *K. pneumoniae* with the *Ligand Profiler* protocol using the same settings described above. A maximum omit feature of 0 was chosen for all compounds. This data was used to calculate the $EF^{2\%}$, $HR^{2\%}$ and the AUC of each ROC curve for each pharmacophore model.

## 3.4 Results

### 3.4.1 Kirby Bauer method

The results of the Kirby Bauer method are shown in table 3.1. Against *S. aureus* most compounds showed activity, *i.e*. methylene blue, methylene green, new methylene blue, dimethyl methylene blue, methylene violet, cresyl violet, nile blue, acriflavine, azure B and ethylthioninium chloride. Only neutral red did not show activity. Against *S. aureus* ampicillin was also active. Against *S. epidermidis* most compounds showed activity, *i.e*. methylene blue, methylene green, new methylene blue, dimethyl methylene blue, methylene violet, cresyl violet, nile blue, acriflavine, neutral red and ethylthioninium chloride. Only azure B did not show activity. Against *S. epidermidis* ampicillin was also active. Against *K. pneumoniae* most compounds showed activity, *i.e*. methylene blue, methylene green, new methylene blue, dimethyl methylene blue, methylene violet, cresyl violet, nile blue, acriflavine, azure B and ethylthioninium chloride. Only neutral red did not show activity. Against *K. pneumoniae* ampicillin was also active. Against *E. coli*, *S. enterica* and *C. albicans* none of the test compounds had activity expect acriflavine, methylene green and ethylthioninium chloride. Ampicillin was active against *E. coli* and *S. enterica*, while amphotericin B was active against *C. albicans*. The MIC of the test compounds were determined against *S. aureus*, *S. epidermidis* and *K. pneumoniae* as they proved to be susceptible (described below).

### 3.4.2 Broth microdilution method

The MIC results are given in table 3.2.

**Table 3.2:** MIC values of the test compounds against *S. aureus*, *S. epidermidis* and *K. pneumoniae.*

| Test compound | MIC (µg/ml) | | |
|---|---|---|---|
| | *S. aureus* | *S. epidermidis* | *K. pneumoniae* |
| **Methylene blue** | 16 | 8 | 4 |
| **Methylene green** | 16† | 16 | 8 |
| **New methylene blue** | 4 | 4 | 0.5 |
| **Dimethyl methylene blue** | 1 | 1 | 8 |
| **Methylene violet** | > 128* | > 128* | > 128* |
| **Cresyl violet** | 16 | 32 | 16‡ |
| **Nile blue** | 128* | > 128* | > 128* |
| **Acriflavine** | 8 | 2 | 2 |
| **Azure B** | 16 | 16 | 8 |
| **Neutral red** | 128 | 128 | 128 |
| **Ethylthioninium chloride** | 16 | 16 | 16‡ |
| **Tetracycline** | 0.5 | 1 | 0.5 |

* Did not dissolve properly. Actual MIC values might differ.
† Bacterial concentration was 142.5 ± 10.6 CFU/ml.
‡ Bacterial concentration was 165 ± 21.2 CFU/ml.

Both methylene violet and nile blue did not dissolve properly during the experiment which may result in inaccurate MIC values. Both compounds will not be discussed further. The viability count showed that the bacterial concentration was higher than expected for methylene green (*S. aureus*), cresyl violet and ethylthioninium chloride (*K. pneumoniae*) and is reported as such in table 3.2.

A compound was defined as active if it had an MIC ≤ 16 µg/ml. As with the Kirby Bauer method most test compounds showed activity against *S. aureus*, *i.e*. methylene blue, methylene green, new methylene blue, dimethyl methylene blue, cresyl violet, acriflavine, azure B and ethylthioninium chloride. Only neutral red did not show activity. Tetracycline was also active against *S. aureus*. As with the Kirby Bauer method most test compounds showed activity against *S. epidermidis*, *i.e*. methylene blue, methylene green, new methylene blue, dimethyl methylene blue, acriflavine, azure B and ethylthioninium chloride. Both cresyl violet and neutral red did not show activity. Tetracycline was also active against *S. epidermidis*. As with

the Kirby Bauer method most test compounds showed activity against *K. pneumoniae*, *i.e*. methylene blue, methylene green, new methylene blue, dimethyl methylene blue, cresyl violet, acriflavine, azure B and ethylthioninium chloride. Only neutral red did not show activity. Tetracycline was also active against *K. pneumoniae*.

### 3.4.3 Common Feature Pharmacophore Modelling

Ten pharmacophore hypotheses were created for *S. aureus*, *S. epidermidis* and *K. pneumoniae*. A summary of each pharmacophore hypothesis (hypo.) is given in table 3.3 (*S. aureus*), table 3.4 (*S. epidermidis*) and table 3.5 (*K. pneumoniae*). The ten hypotheses created for *S. aureus* had a high degree of similarity compared to its corresponding hypothesis *vs*. *S. epidermidis* and *K. pneumoniae*. As such only the *S. aureus* data will be reported however, where there was a difference it will be indicated. The ranking scores differed from 26.664 – 22.057 (*S. aureus* and *K. pneumoniae*) and 21.753 - 18.298 (*S. epidermidis*). For *S. aureus*, *S. epidermidis* and *K. pneumoniae* the different hypotheses contained either ring aromatics (R), hydrophobic features (H) or hydrogen bond acceptors (A) or different combinations thereof. All hypotheses had a maximum fit value of 4.

**Table 3.3:** Summary of the pharmacophore hypotheses created for *S. aureus.*

| Hypothesis | Features* | Rank score | Direct hit† | Partial hit‡ | Maximum fit |
|---|---|---|---|---|---|
| 01 | RHHA | 26.664 | 1111 | 0000 | 4 |
| 02 | RHHA | 26.664 | 1111 | 0000 | 4 |
| 03 | HHHA | 25.547 | 1111 | 0000 | 4 |
| 04 | RHHA | 25.315 | 1111 | 0000 | 4 |
| 05 | RHHA | 25.315 | 1111 | 0000 | 4 |
| 06 | RHHA | 24.642 | 1111 | 0000 | 4 |
| 07 | HHHA | 24.305 | 1111 | 0000 | 4 |
| 08 | RHHA | 23.795 | 1111 | 0000 | 4 |
| 09 | HHHA | 23.305 | 1111 | 0000 | 4 |
| 10 | HHHA | 22.057 | 1111 | 0000 | 4 |

* R = Ring aromatic, H = Hydrophobic feature, A = Hydrogen bond acceptor.
† Indicates whether (1) or not (0) a molecule is mapped.
‡ Indicates whether (1) or not (0) a molecule mapped all but one feature.

Comparing the best fit value of the training set compounds between the different hypotheses the following was observed. For hypo. 1 (new methylene blue > cresyl violet > methylene green > dimethyl methylene blue), hypo. 2 (new methylene blue > cresyl violet > methylene green > dimethyl methylene blue), hypo. 3 (new methylene blue > methylene green > dimethyl methylene blue > cresyl violet), hypo. 4 (new methylene blue > cresyl violet > methylene green > dimethyl methylene blue), hypo. 5 (new methylene blue > cresyl violet > methylene green > dimethyl methylene blue), hypo. 6 (new methylene blue > cresyl violet > dimethyl methylene blue > methylene green), hypo. 7 (new methylene blue > methylene green > cresyl violet > dimethyl methylene blue), hypo. 8 (new methylene blue > cresyl violet > dimethyl methylene blue > methylene green), hypo. 9 (new methylene blue > cresyl violet > dimethyl methylene blue > methylene green) and hypo. 10 (new methylene blue > cresyl violet > dimethyl methylene blue > methylene green) (table 3.6).

The pharmacophore models' hypotheses were validated by their ability to correctly identify active compounds from a test set containing active and decoy compounds. Comparing the $EF^{2\%}$ and $HR^{2\%}$ of each hypothesis, both hypo. 1 ($EF^{2\%}$ = 51; $HR^{2\%}$ = 100%) and hypo. 2 ($EF^{2\%}$ = 51; $HR^{2\%}$ = 100%), had the most promising results (table 3.7). Comparing the AUC of each hypothesis' ROC curve again hypo. 1 (1.00 ± 0.00) and hypo. 2 (0.99 ± 0.01) had the most promising results.

After mapping ten phenothiazine derivatives with the most promising pharmacophore model, *i.e*. hypo. 1, each derivative had the following fit value; flupenthixol (3.49), fluphenazine (3.49), methdilazine (2.02), prochlorperazine (3.47), promazine (0.48), promethazine, (2.18) thioridazine (3.65), trifluoperazine (3.66), triflupromazine (3.46) and trimeprazine (2.16) (table 3.8).

**Table 3.4:** Summary of the pharmacophore hypotheses created for *S. epidermidis.*

| Hypothesis | Features* | Rank score | Direct hit† | Partial hit‡ | Maximum fit |
|---|---|---|---|---|---|
| 01 | RHHA | 21.753 | 111 | 000 | 4 |
| 02 | RHHA | 21.753 | 111 | 000 | 4 |
| 03 | HHHA | 20.915 | 111 | 000 | 4 |
| 04 | RHHA | 20.741 | 111 | 000 | 4 |
| 05 | RHHA | 20.741 | 111 | 000 | 4 |
| 06 | RHHA | 20.237 | 111 | 000 | 4 |
| 07 | HHHA | 19.984 | 111 | 000 | 4 |
| 08 | RHHA | 19.601 | 111 | 000 | 4 |
| 09 | HHHA | 19.234 | 111 | 000 | 4 |
| 10 | HHHA | 18.298 | 111 | 000 | 4 |

* R = Ring aromatic, H = Hydrophobic feature, A = Hydrogen bond acceptor.
† Indicates whether (1) or not (0) a molecule is mapped.
‡ Indicates whether (1) or not (0) a molecule mapped all but one feature.

**Table 3.5:** Summary of the pharmacophore hypotheses created for *K. pneumoniae.*

| Hypothesis | Features* | Rank score | Direct hit† | Partial hit‡ | Maximum fit |
|---|---|---|---|---|---|
| 01 | RHHA | 26.664 | 1111 | 0000 | 4 |
| 02 | RHHA | 26.664 | 1111 | 0000 | 4 |
| 03 | HHHA | 25.547 | 1111 | 0000 | 4 |
| 04 | RHHA | 25.315 | 1111 | 0000 | 4 |
| 05 | RHHA | 25.315 | 1111 | 0000 | 4 |
| 06 | RHHA | 24.642 | 1111 | 0000 | 4 |
| 07 | HHHA | 24.305 | 1111 | 0000 | 4 |
| 08 | RHHA | 23.795 | 1111 | 0000 | 4 |
| 09 | HHHA | 23.305 | 1111 | 0000 | 4 |
| 10 | HHHA | 22.057 | 1111 | 0000 | 4 |

* R = Ring aromatic, H = Hydrophobic feature, A = Hydrogen bond acceptor.
† Indicates whether (1) or not (0) a molecule is mapped.
‡ Indicates whether (1) or not (0) a molecule mapped all but one feature.

**Table 3.6:** Best fit value of the training set against *S. aureus*, *S. epidermidis* and *K. pneumoniae.*

| Compound | Fit value | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Hypo. 1** | **Hypo. 2** | **Hypo. 3** | **Hypo. 4** | **Hypo. 5** | **Hypo. 6** | **Hypo. 7** | **Hypo. 8** | **Hypo. 9** | **Hypo. 10** |
| **Methylene green** | 1.78 | 1.66* | 2.40 | 1.81 | 1.83 | 0.56 | 1.50 | 2.08 | 1.01 | 1.83 |
| **New methylene blue** | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 |
| **Dimethyl methylene blue** | 0.89 | 0.88 | 1.43 | 1.62 | 1.61 | 1.85 | 0.35 | 2.60 | 2.31‡ | 3.01 |
| **Cresyl violet** | 2.46 | 2.61 | 1.41 | 3.42 | 3.42† | 2.08 | 1.26 | 2.69 | 2.48 | 3.37 |

* *K. pneumoniae* had a fit value of 1.65.
† *K. pneumoniae* had a fit value of 3.43.
‡ *K. pneumoniae* had a fit value of 2.32.

**Table 3.7:** The $EF^{2\%}$, $HR^{2\%}$ and ROC-AUC results for each pharmacophore hypotheses created for *S. aureus, S. epidermidis* and *K. pneumoniae.*

| Hypothesis | $EF^{2\%}$ | $HR^{2\%}$ | ROC-AUC values |
|---|---|---|---|
| 01 | 51 | 100% | 1.00 ± 0.00 |
| 02 | 51 | 100% | 0.99 ± 0.01 |
| 03 | 0 | 0% | 0.77 ± 0.06* |
| 04 | 13 | 25% | 0.68 ± 0.11 |
| 05 | 13 | 25% | 0.68 ± 0.11† |
| 06 | 25¶ | 50%¶ | 0.97 ± 0.03 |
| 07 | 0 | 0% | 0.36 ± 0.12‡ |
| 08 | 0 | 0% | 0.64 ± 0.08 |
| 09 | 38 | 75% | 0.82 ± 0.16§ |
| 10 | 0 | 0% | 0.82 ± 0.05 |

* *K. pneumoniae* had a value of 0.79 ± 0.06.
† *K. pneumoniae* had a value of 0.69 ± 0.11.
‡ *K. pneumoniae* had a value of 0.34 ± 0.12.
§ *K. pneumoniae* had a value of 0.81 ± 0.16.
¶ *S. epidermidis* had an $EF^{2\%}$ and $HR^{2\%}$ of 38 and 75% respectively.

**Table 3.8:** Fit values of phenothiazine derivatives mapped with the hypo. 1.

| Derivative | Fit Value |
|---|---|
| Flupenthixol | 3.49 |
| Fluphenazine | 3.49 |
| Methdilazine | 2.02 |
| Prochlorperazine | 3.47 |
| Promazine | 0.48 |
| Promethazine | 2.18 |
| Thioridazine | 3.65 |
| Trifluoperazine | 3.66 |
| Trifluprom azine | 3.46 |
| Trimeprazine | 2.16 |

## 3.5 Discussion

### 3.5.1 Kirby Bauer method

It should be noted that the Kirby Bauer test was used as a screening method in this study, and the standard interpretation of the results, *i.e*. susceptible "S", intermediate "I" and resistant "R", could not be used as the initial bacteria concentration was not determined. The Kirby Bauer method found that all compounds were active against *S. aureus, S. epidermidis* and *K. pneumoniae* except neutral red which was inactive against *S. aureus* and *K. pneumoniae*, and azure B that was inactive against *S. epidermidis*. The test compounds were inactive against *E. coli*, *S. enterica* and *C. albicans* except acriflavine and ethylthioninium chloride that were also active against *E. coli* and *S. enterica*. Methylene green also had weak activity against *E. coli*, *S. enterica* and *C. albicans*. The methylene blue analogue synthesised by our group, *i.e*. ethylthioninium chloride, had better activity compared to most of the other test compounds. Although both acriflavine and ethylthioninium chloride had activity against all pathogens except *C. albicans*, their MIC values were not determined against *E. coli*, *S. enterica* and *C. albicans* as the subsequent *in silico* experiments require more than one active compound per bacterial strain.

As with previous studies, methylene blue was active against both *S. aureus* and *K. pneumoniae* (Li et al., 2016; Shatti and Authman, 2015). As reported by Gunics and co-workers methylene blue did not have activity against *E. coli*, however another study did report activity against a different strain of *E. coli* (Gunics et al., 2000; Li et al., 2016). In this study methylene blue had good activity against *S. epidermidis* whereas others found no activity using a clinical isolate (Gunics et al., 2000). While another study found that methylene blue had activity against *C. albicans* this study did not (Ansari et al., 2016). This may be because of a different strain of *C. albicans* that was used in this study. As reported by others, this study also did not find activity against *S. enterica* (Nishino et al., 2009). Acriflavine is another dye with well-known antimicrobial properties. Our results were similar to other studies which found that acriflavine had activity against *S. aureus* (Kawai and Yamagishi, 2009), *E. coli* (Nakamura, 1966), *K. pneumoniae* (Mondal et al., 2016) and *S. enterica* (Hayashi-Nishino et al., 2012) (table 3.1).

### 3.5.2 Broth microdilution method

For the broth microdilution results, an MIC value differing by one two-fold dilution is considered comparable (Barry et al., 1978). All test compounds showed activity against *S. aureus* except neutral red. Dimethyl methylene blue was the most active compound, followed by new methylene blue then acriflavine. Methylene blue, methylene green, cresyl violet, azure B and ethylthioninium chloride were all equally active. Compared to the positive control, dimethyl methylene blue had

comparable activity. All test compounds showed activity against *S. epidermidis* except cresyl violet and neutral red. Dimethyl methylene blue was again the most active compound, followed by acriflavine then new methylene blue. Methylene blue had comparable activity to methylene green, ethylthioninium chloride and azure B. The activity of cresyl violet was comparable to methylene green, ethylthioninium chloride and azure B, however this compound was less active compared to methylene blue. Again, compared to the positive control dimethyl methylene blue was equally active. All test compounds showed activity against *K. pneumoniae*, except neutral red. In this instance, new methylene blue was the most active compound. Both acriflavine and methylene blue were less active than new methylene blue. Methylene green, dimethyl methylene blue and azure B were equally active. The activities of both cresyl violet and ethylthioninium chloride were comparable to that of methylene green, dimethyl methylene blue and azure B. Compared to the positive control new methylene blue was equally active. Overall, new methylene blue, dimethyl methylene blue and acriflavine had the highest activity against all three pathogens, and in some instances the activities were comparable to that of tetracycline (table 3.2).

### 3.5.3 Common Feature Pharmacophore Modelling

Hypo. 1 – 10 for *S. aureus*, *S. epidermidis* and *K. pneumoniae* are similar. This can be expected as the *in vitro* activity and the principal values of the training set compounds did not differ to a great extent.

To determine which pharmacophore hypothesis was the most accurate, the rank score and fit values of each hypothesis were firstly considered. For *S. aureus* (table 3.3)*, S. epidermidis* (table 4) and *K. pneumoniae* (table 3.5) hypo. 1 and 2 had the highest rank scores, however hypo. 1 and 2 for *S. aureus* and *K. pneumoniae* were higher than hypo. 1 and 2 of *S. epidermidis*. The fit values for hypo. 1 and 2 were the same and correctly determined new methylene blue would be highly active and methylene green and cresyl violet would be less active (table 3.6). Both hypotheses however determine dimethyl methylene blue would be the least active which was incorrect considering the MIC values for *S. aureus*, *S. epidermidis* and *K. pneumoniae*. Some hypotheses did rank dimethyl methylene blue higher, *i.e*. hypo. 3, 6, 8, 9 and 10, however their rank scores were lower than hypo. 1 and 2. To further validate each hypothesis, the $EF^{2\%}$, $HR^{2\%}$ and ROC-AUC results were also considered. Again, both hypo. 1 and 2 showed the most promising results each with an $EF^{2\%}$ of 51, an $HR^{2\%}$ of 100% and a ROC-AUC score of 1.00 ± 0.00 and 0.99 ± 0.01, respectively (table 3.7). Hypo. 9 also had promising results with an $EF^{2\%}$ of 38, an $HR^{2\%}$ of 75% and a ROC-AUC score of 0.82 ± 0.16. Tanking all the metrics into consideration both hypo. 1 and 2 were considered equally accurate and are depicted in figure 3.3.

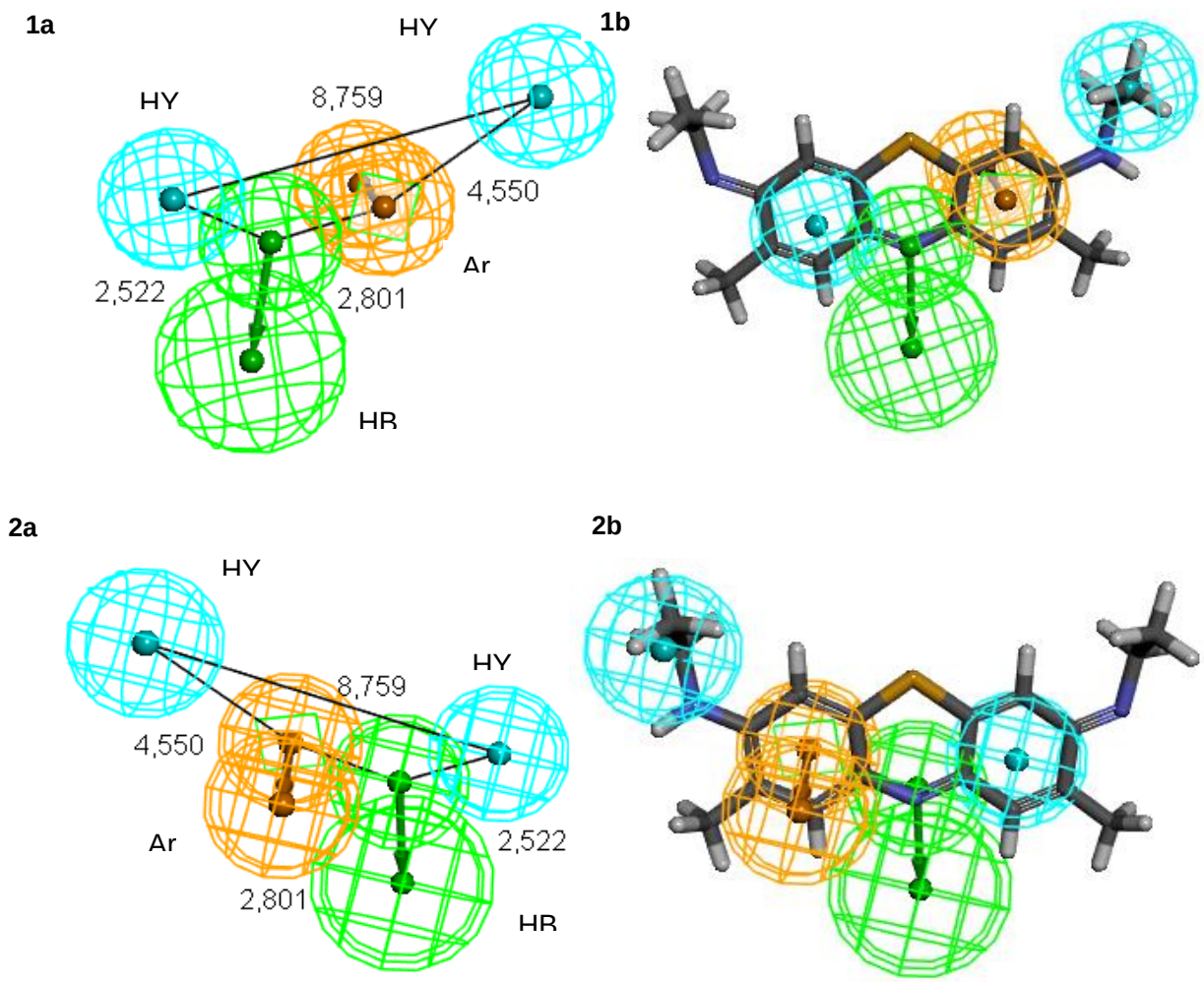


**Figure 3.3:** Hypothesis 1 (a) with interfeature distance constraints and (b) with new methylene blue mapped, and hypothesis 2 (a) with interfeature distance constraints and (b) with new methylene blue mapped. The colours of the pharmacophore features are; green (HBA), blue (HYD) and orange (Ar).

The similar results obtained with both hypo. 1 and 2 are explained by figure 3.3 which shows that hypo. 1 and 2 are identical, only differing in the way new methylene blue is orientated. The pharmacophore model consists of two hydrophobic features, an aromatic feature and a hydrogen bond acceptor. According to the pharmacophore model, the amine in the heterocyclic ring is important and acts as a hydrogen bond acceptor. One of the adjacent cyclic carbon rings act as a hydrophobic region, whilst the other is important because of its aromatic interactions. The substituent on the carbon rings also seems to influence activity as a result of its hydrophobic interactions.

Figure 3.4 shows the top six phenothiazine derivatives with the best fit values, mapped with the validated pharmacophore model.

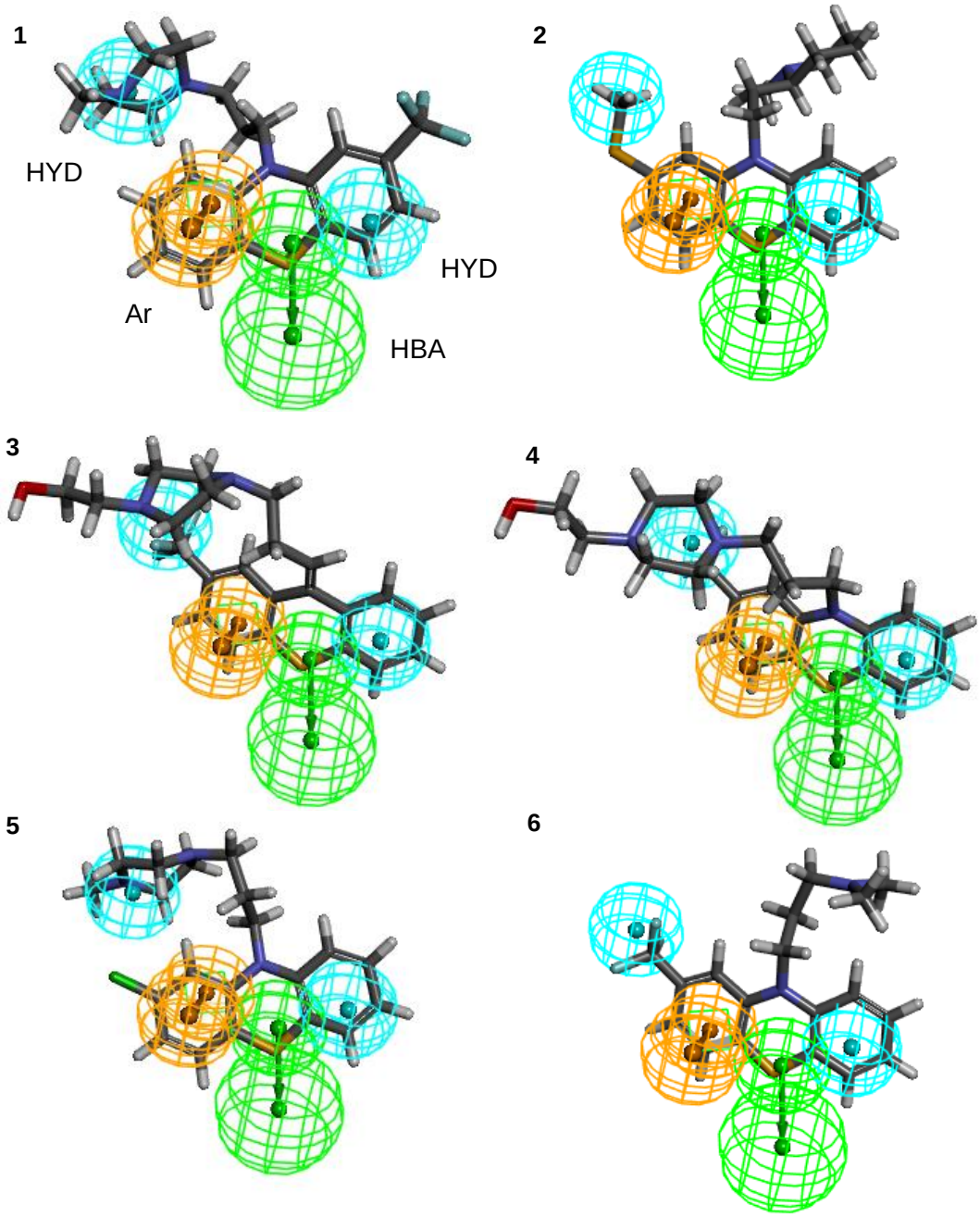


**Figure 3.4:** Trifluoperazine (1), thioridazine (2), flupenthixol (3), fluphenazine (4), prochlorperazine (5) and triflupromazine (6) mapped with the validated pharmacophore model (hypo. 1). The colours of the pharmacophore features are; green (HBA), blue (HYD) and orange (Ar).

These derivatives had fit values >3.4 (table 3.8) indicating that there is a high probability they are active according to the ROC curve analysis (data not shown). Because of the substitution on the heterocyclic amine, the sulphur group acts as the hydrogen bond acceptor rather than the amine. As with the methylene blue analogues, one of the adjacent carbon rings acts as the hydrophobic

moiety and the other as the aromatic region. According to the pharmacophore model there is a possibility that the methylene blue analogues and the phenothiazine derivatives have the same antibacterial mode of action.

### 3.6 Conclusion

Because of the rapid and widespread increase in AMR and the lack of novel antibacterial drug classes, there is an urgent need to develop new antimicrobial drugs. Methylene blue and its analogues are known to have antibacterial activity, although few studies have determined their MICs which is the gold standard used to measure and compare antimicrobial activity. Despite methylene blue being the oldest synthetic drug, its antibacterial mechanism of action is still not clear.

In this study methylene blue and a number of its analogues were firstly screened for antimicrobial activity against *S. aureus*, *S. epidermidis*, *E. coli*, *K. pneumoniae*, *S. enterica* and *C. albicans*. Most analogues had activity against *S. aureus*, *S. epidermidis* and *K. pneumoniae*. The MIC of each analogue were determined against *S. aureus*, *S. epidermidis* and *K. pneumoniae* and it was found that dimethyl methylene blue, new methylene blue and acriflavine were the most active compounds. The *in vitro* activities of the analogues were used to create a common feature pharmacophore model for *S. aureus*, *S. epidermidis* and *K. pneumoniae*, which were assessed by their rank scores and vit values. Each hypothesis was also validated by determining their $ER^{2\%}$, $HR^{2\%}$ and ROC-AUC, after which it was concluded that hypothesis 1 (of *S. aureus* and *K. pneumoniae*) was the best pharmacophore model. The pharmacophore model identified two hydrophobic features, an aromatic feature and a hydrogen bond acceptor as being important for antibacterial activity. Finally, ten phenothiazine derivatives with known antibacterial activity were mapped with the pharmacophore model. High fit values of six derivatives indicate that they may share a common antibacterial mode of action with the methylene blue analogues.

The MIC data reported here will allow other researchers to compare the activity of the methylene blue analogues across different laboratories. The validated pharmacophore model can be used to identify lead compounds with a similar mode of action to methylene blue. The possibility that methylene blue analogues and phenothiazine derivatives have the same antibacterial mode of action needs to be confirmed with additional experiments.

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## Conflict of interest

The authors declare no conflict of interest.

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# CHAPTER 4: BACTERIAL GROWTH AND BACTERICIDAL TESTS

This chapter contains the materials and methods, results and discussion for the bacterial growth curve determinations as well as the MBC determination for the methylene blue analogues. The microbial growth curves were not included in the article submitted to the European Journal of Pharmaceutical Sciences because this type of experiment does not fall within the journal's scope, and the bactericidal tests was not included because the results were inconclusive (see section 4.2.1.1).

## 4.1 Materials and methods

During all experiments, both the MHB as well as MHA (Sigma-Aldrich, South Africa) was prepared according the manufacturer's instructions. All non-sterile equipment was autoclaved at 121 °C for 15 min using a CL-40M autoclave (ALP Co. Ltd) (Lasec, South Africa). All pathogens were handled in a level 2 biosafety cabinet (EuroClone S.p.A, Italy).

### 4.1.1 Bacterial growth curves

Bacterial growth curves were only determined for *S. aureus, S. epidermidis* and *K. pneumoniae* because the MICs of the analogues were determined against these pathogens. To create each inoculum two to three morphological similar colonies of each pathogen were suspended in 10 ml sterile MHB and incubated overnight at 35 ± 2 °C whilst being agitated at 250 rpm in a walk-in incubator (T&S Refrigeration, South Africa).

Each inoculum was diluted with sterile MHB to an $OD_{600}$ of 0.1 ± 0.02, corresponding to a 0.5 McFarland standard (Hudzicki, 2009:5). Each inoculum (200 µl), as well as the sterility control (200 µl sterile MHB), was added to a row ($n$ = 12) of the 96 well plate. To measure the bacterial growth, the 96-well plate was incubated at 35 ± 2 °C for 24 hours while the absorbance was measured ($OD_{600}$) every 60 minutes with a plate reader (BioTek Instrument Inc., USA).

### 4.1.2 Bactericidal tests

The MBC of the analogues was determined against *S. aureus* (ATCC 43300), *S. epidermidis* (ATCC 12228) and *K. pneumoniae* (ATCC 10031). During the method development the MBC was determined only for the compounds of which we had sufficient quantities. A standard method for determining the MBC was firstly used (section 4.1.2.1) (Hacek et al., 1999:1881; Peterson & Shanholtzer, 1992:422; Hacek *et al*., 1999:1881). This method however delivered inconsistent

results, where after a second less common method was used (section 4.1.2.2) (Cui *et al*., 2015:3). Both methods however proved to be unable to provide consistent results and was omitted from the article. Both methylene violet and nile blue did not dissolve properly during the experiments, which may result in inaccurate results. Both compounds will not be discussed further.

#### 4.1.2.1 Standard bactericidal test

This bactericidal test is discussed in detail by others (Hacek *et al.*, 1999:1883; Peterson & Shanholtzer, 1992:422;). After the MIC values were determined four dilutions above the MIC, one dilution below the MIC as well as the MIC dilution were included in the bactericidal test. As each compound was tested in triplicate during the MIC assay, this resulted in a total number of 18 wells being included in the bactericidal test. Each well was gently mixed by flushing the contents a few times with a micropipette, after which 100 µl of the content was lawned onto a MHA-petri dish. The lawned plates were incubated for 24 hours at 35 ± 2 °C. The number of colonies was counted after incubation, and the values for each concentration were expressed as the average including the standard deviation. The MBC is defined as the concentration where 99.9% of the original bacteria has been killed. This is determined by the formula, $n + 2\sqrt{n}$, where $n$ is 0.1% of the original bacterial concentration as determined by the viability count during the MIC assay. This formula gives the quantitative endpoint that has a 95% confidence interval. If the MBC value of one of the three repeats vary with one dilution, the least active result is reported. If the MBC values of the three repeats differ more than 1 dilution, the results are considered discrepant and the experiment should be repeated (Hacek *et al.*, 1999:1883; Peterson & Shanholtzer, 1992:425).

#### 4.1.2.2 Spot inoculation bactericidal test

A less common bactericidal test was adapted from Cui and co-workers (2015:2). MHA-petri dishes were divided, by drawing 0.5 x 0.5 cm squares on the back of the MHA-petri dish. After the MIC values were determined, sterilised toothpicks were used to inoculate the agar in a square. The contents of each well was inoculated in triplicate. As each compound was tested in triplicate during the MIC assay, this resulted in a total number of 9 repeats for each concentration. The MHA-petri dishes were incubated for 24 hours at 35 ± 2 °C. The MBC was defined as the concentration where ≤ 1 (≤ 11%) of the inoculation areas showed visible growth.

## 4.2 Results

### 4.2.1 Bacterial growth curves

All bacteria were in their log growth phase from the start of the experiment (figure 4.1). *K. pneumoniae* was the first bacterium to reach the stationary phase at roughly 11 hours after the start of incubation. Both *S. aureus* and *S. epidermidis* reached their stationary growth phases at roughly 19 hours after the start of incubation. None of the bacteria reached their death phase during the span of the experiment. No growth was observed in the sterility control throughout the experiment.

#### 4.2.1.1 Standard bactericidal test

The results of the standard bactericidal test are depicted in table 4.1. Because of limited availability, only methylene blue, neutral red, new methylene blue, azure B, dimethyl methylene blue and acriflavine were included in the method development. A compound is considered bactericidal if its MBC is not more than four times its MIC (French, 2006:1109).

Against *S. aureus*, acriflavine (16 µg/ml) was the most active, followed by methylene blue (32 µg/ml), azure B (64 µg/ml) then neutral red (> 128 µg/ml). The MBC results for both new methylene blue and dimethyl methylene blue were inconclusive as one or more of the wells in the concentration range appeared free of bacterial growth, whilst other wells with higher antibacterial concentrations had bacterial growth. This phenomenon is called “skipped wells” and is discussed further in section 4.3.2. Acriflavine [16 µg/ml (MBC) *vs*. 8 µg/ml (MIC)], methylene blue [32 µg/ml (MBC) *vs*. 16 µg/ml (MIC)] and azure B [64 µg/ml (MBC) *vs*. 16 µg/ml (MIC)] are thus considered bactericidal against *S. aureus* (chapter 3, table 3.2).

Against *S. epidermidis*, acriflavine (8 µg/ml) was the most active, followed by azure B (128 µg/ml) then neutral red (> 128 µg/ml). The MBC results for methylene blue, new methylene blue and dimethyl methylene blue were inconclusive, again because of skipped wells. Acriflavine [8 µg/ml (MBC) *vs*. 2 µg/ml (MIC)] is considered bactericidal, whilst azure B [128 µg/ml (MBC) *vs*. 16 µg/ml (MIC)] is considered bacteriostatic against *S. epidermidis* (chapter 3, table 3.2).

Against *K. pneumoniae*, methylene blue (16 µg/ml) was the most active, followed by azure B (32 µg/ml), new methylene blue (>64 µg/ml) then neutral red (> 128 µg/ml). The MBC results for dimethyl methylene blue and acriflavine were inconclusive because of skipped wells. Methylene blue [16 µg/ml (MBC) *vs*. 4 µg/ml (MIC)] and azure B [32 µg/ml (MBC) *vs*. 8 µg/ml (MIC)] are considered bactericidal against *K. pneumoniae* (chapter 3, table 3.2).

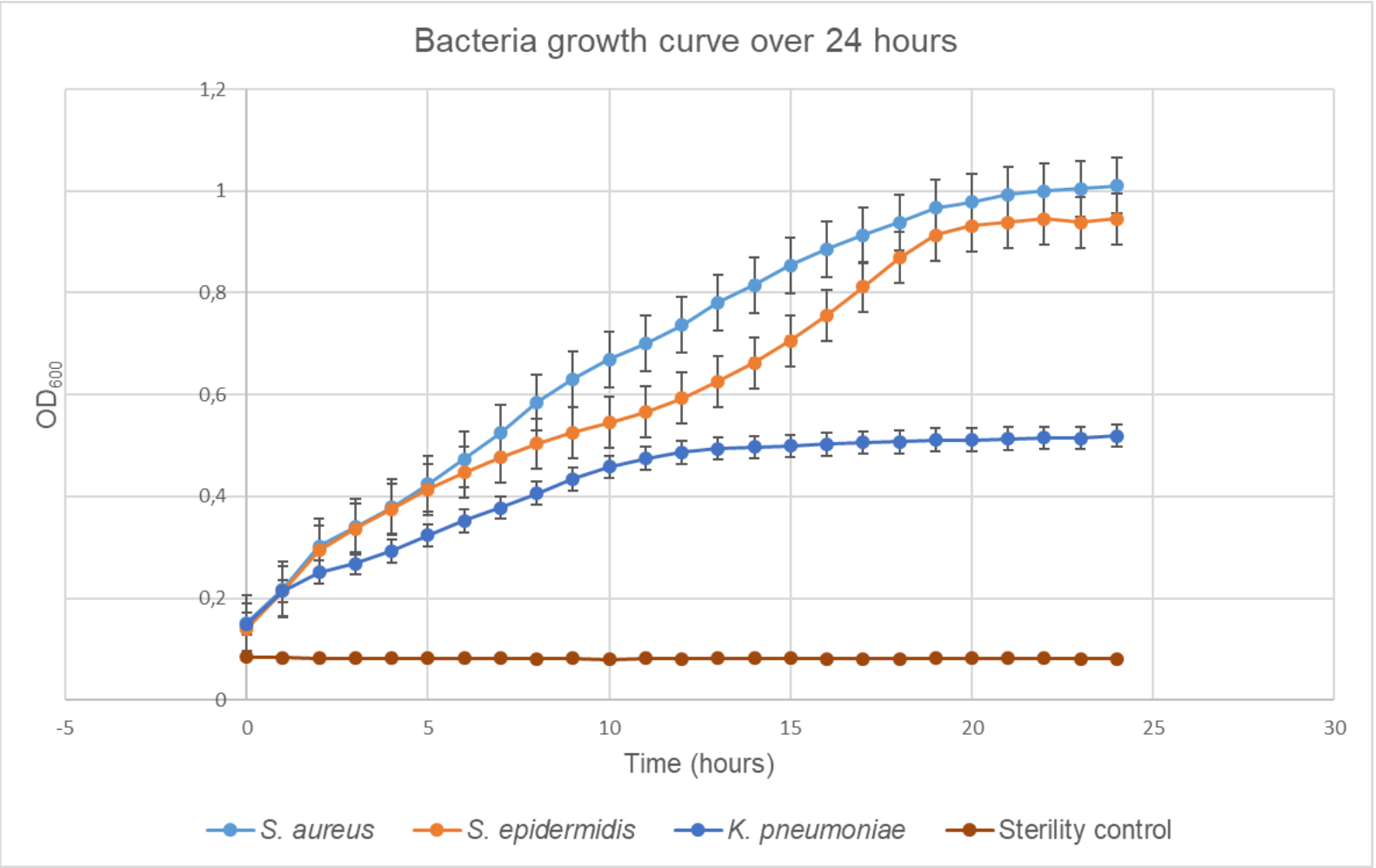


**Figure 4.1:** **Growth curves of *S. aureus, S. epidermidis* and *K. pneumoniae* over 24 hours.**

**Table 4.1: Standard bactericidal test results against *S. aureus*, *S. epidermidis* and *K. pneumoniae.***

| Test compound | MBC (µg/ml) | | |
|---|---|---|---|
| | *S. aureus* | *S. epidermidis* | *K. pneumoniae* |
| **Methylene blue** | 32 | INC* | 16 |
| **Neutral Red** | >128 | >128 | >128 |
| **New Methylene Blue** | INC* | INC* | >64 |
| **Azure B** | 64 | 128 | 32 |
| **Dimethyl methylene blue** | INC* | INC* | INC* |
| **Acriflavine** | 16 | 8 | INC* |

* Inconclusive.

#### 4.2.1.2 Spot inoculation bactericidal test

The results of the spot inoculation bactericidal test are depicted in table 4.2. Because of limited availability, only methylene blue, azure B and acriflavine were compared against the standard bactericidal test.

Against *S. aureus* acriflavine had an MBC of 16 µg/ml. The MBC results for both methylene blue and azure B were inconclusive because of skipped wells. Interestingly, acriflavine's MBC result was exactly the same compared to the standard bactericidal test. Again, this indicates that acriflavine [16 µg/ml (MBC) *vs*. 8 µg/ml (MIC)] is bactericidal against *S. aureus* (chapter 3, table 3.2).

Against *S. epidermidis* methylene blue had an MBC of 64 µg/ml. The MBC results for both azure B and acriflavine were inconclusive because of skipped wells. Methylene blue's standard bactericidal test result was inconclusive (table 4.1), making it impossible to compare the spot inoculation results. This indicates that methylene blue [64 µg/ml (MBC) *vs*. 8 µg/ml (MIC)] is bacteriostatic against *S. epidermidis* (chapter 3, table 3.2).

Against *K. pneumoniae* methylene blue (16 µg/ml) and acriflavine (16 µg/ml) were equally active, whilst azure B (32 µg/ml) was the least active. Interestingly, methylene blue and azure B had

exactly the same bactericidal test results compared to the standard bactericidal test. This indicates that methylene blue [16 µg/ml (MBC) *vs*. 4 µg/ml (MIC)] and azure B [32 µg/ml (MBC) *vs*. 8 µg/ml (MIC)] had bactericidal activity and acriflavine [16 µg/ml (MBC) *vs*. 2 µg/ml (MIC)] bacteriostatic activity against *K. pneumoniae* (chapter 3, table 3.2).

**Table 4.2: Spot inoculation bactericidal test results against *S. aureus*, *S. epidermidis* and *K. pneumoniae.***

| Test compound | MBC (µg/ml) | | |
|---|---|---|---|
| | *S. aureus* | *S. epidermidis* | *K. pneumoniae* |
| **Methylene blue** | INC* | 64 | 16 |
| **Azure B** | INC* | INC* | 32 |
| **Acriflavine** | 16 | INC* | 16 |

* Inconclusive.

## 4.3 Discussion

### 4.3.1 Bacterial growth curve

All bacteria have four growth phases, namely the lag phase, exponential phase, stationary phase and the death phase (Rolfe *et al.,* 2012:686). It is vital that the inoculum must be in the exponential phase to ensure accurate results (Kim & Anthony, 1981:1076; Taylor *et al.*, 1983:144).

No lag growth phase was observed for any of the bacteria, instead all bacteria immediately achieved the exponential growth phase. This might be because the inoculum was created in the same medium as in which the experiment was conducted, *i.e*. MHB. Also, each bacterial inoculum was freshly prepared and thus did not need to acclimatise to the new medium and could instead continue to grow as normal. The data showed that the micro broth dilution assays used to determine the MIC of the test compounds can be conducted immediately after an inoculum is prepared, as each bacteria would immediately be in the exponential growth phase. The bacteria should however not be incubated for longer than 11 hours as *K. pneumoniae* enters the stationary growth phase at this time point.

### 4.3.2 Bactericidal tests

Using the standard bactericidal test against *S. aureus*, acriflavine was the most active, followed by methylene blue then azure B. Comparing these results to their MIC's all three are considered bactericidal. The bactericidal test was unable to deliver conclusive results for methylene blue and dimethyl methylene blue. The spot inoculation bactericidal test gave precisely the same result for acriflavine as compared to the standard bactericidal test, but was inconclusive for both methylene blue and azure B. Using the standard bactericidal test against *S. epidermidis*, acriflavine was the most active, followed by azure B. Comparing these results to their MIC results, acriflavine is considered bactericidal and azure B bacteriostatic. The standard bactericidal test was unable to deliver conclusive results for methylene blue, new methylene blue and dimethyl methylene blue. The spot inoculation bactericidal test was only able to deliver a result for methylene blue. Because of the inconclusive results, the standard and spot inoculation bactericidal test could not be compared to one another. Using the standard bactericidal test against *K. pneumoniae*, methylene blue was the most active, followed by azure B. Comparing these results to their MIC results both are considered bactericidal. The standard bactericidal test was unable to deliver conclusive results for dimethyl methylene blue and acriflavine. The spot inoculation bactericidal test gave precisely the same results for both methylene blue and azure B as compared to the standard bactericidal test.

For each compound where both the standard- and the spot inoculation bactericidal tests gave valid results, the results were exactly the same, *i.e*. acriflavine (*S. aureus*), methylene blue (*K pneumoniae*) and azure B (*K pneumoniae*). Although there was some correlation between these two methods, the inconclusive results made both methods unreliable. The skipped well phenomenon was observed with both methods and is defined by one or more of the wells in a series of dilutions which appears to be free of bacterial growth, whereas one or more wells in the dilution series containing a higher concentration of the antibacterial agent have bacterial growth (Peterson & Shanholtzer, 1992:421). The exact reason for this skipped well phenomenon is unclear. It is believed that it is caused by persisters which is defined as metabolic inactive bacterial cells that survive the lethal antibacterial action, and which regrowth after being plated on a MHA-petri dish (Gunnison *et al.*, 1963:335).

Bactericidal tests are notorious for being unreliable, and can be influenced by a myriad of variables including; the type of test medium, the bacterial growth phase, incubation time and temperature, whether the 96-well plates were shaken, the volume of the wells, the volume of subculture, antibacterial compound inactivation at subculture, and the bacterial isolate storing

conditions before testing (Peterson & Shanholtzer, 1992:421). During the experiment discussed in the dissertation, the utmost care was taken to keep all variables constant, however anyone of the above mentioned variables could have caused the skipped well phenomenon.

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# CHAPTER 5: CONCLUSION

Antibiotics not only change the way we control infections but also accelerate the advancements of medical technology and surgery, making previously impossible procedures possible (Gould & Bal, 2013:185). Since the discovery of penicillin, the average life expectancy of people living in the United States has risen from 59.2 to 77.6 years (Shrestha, 2005:3,20).

A few years after the introduction of penicillin, bacteria had developed resistance and this pattern continued after the release of any new antibiotics (Abraham & Chain, 1940:837; Lobanovska & Pilla, 2017:140). The lack of novel antibiotics places further strain on the existing resistance problem due to old and current antibiotics being overused (Wright *et al.,* 2014:8855).

The lack of novel antibiotics as well as the increase in the development of resistant pathogens, underlines the need for novel antibiotics. One of the oldest synthetic drugs, methylene blue, is known to have antibacterial activity although few studies have determined its MIC against most human pathogens. Despite methylene blue being the oldest synthetic drug, its antibacterial mechanism of action is still not clear.

In this study, we firstly determined the antimicrobial activity of methylene blue and methylene blue analogues against *S. aureus, S. epidermidis, K. pneumoniae, S. enterica, E. coli* and *C. albicans* using the Kirby Bauer disk diffusion method. A few analogues had activity against either *S. enterica* (acriflavine and ethylthioninium chloride), *E. coli* (acriflavine and ethylthioninium chloride) and *C. albicans* (methylene green). Most compounds had activity towards *S. aureus, S. epidermidis* and *K. pneumoniae*, *i.e*. acriflavine, dimethyl methylene blue, new methylene blue, methylene blue, methylene green, methylene violet, nile blue, cresyl violet and ethylthioninium chloride. Azure B had no activity towards *S. epidermidis* while neutral red displayed no activity towards either *S. aureus* or *K. pneumoniae*.

Due to most of the analogues displaying activity towards *S. aureus, S. epidermidis* and *K. pneumoniae*, the MICs were determined only against these three bacteria. Overall, acriflavine, dimethyl methylene blue and new methylene blue were the most active of all the analogues tested. Methylene blue, methylene green, azure B and cresyl violet had some activity against each of the bacteria. Neutral red had poor activity against each bacterium. Methylene violet and nile blue had solubility problems and were excluded from the discussion.

As part of the aim of this study a standard method was used to determine the MBC of each analogue against *S. aureus*, *S. epidermidis* and *K. pneumoniae.* As part of the method development the MBC of methylene blue, new methylene blue, neutral red, azure B, dimethyl

methylene blue and acriflavine was firstly determined. During this development stage inconclusive data was obtained with regards to *S. aureus* (new methylene blue and dimethyl methylene blue), *S. epidermidis* (methylene blue, new methylene blue and dimethyl methylene blue) and *K. pneumoniae* (dimethyl methylene blue and acriflavine). For comparison, the MBCs of methylene blue, azure B and acriflavine were determined with a less common spot inoculation method. This method gave inconclusive results with regards to *S. aureus* (azure B and methylene blue) and *S. epidermidis* (azure B and acriflavine). Both methods gave the same results for acriflavine (*vs. S. aureus*), methylene blue (*vs*. *K pneumoniae*) and azure B (*vs*. *K pneumoniae*). Acriflavine can thus be considered bactericidal against *S. aureus*, and both methylene blue and azure B can also be considered bactericidal against *K. pneumoniae*. Bactericidal tests are known for being unreliable, and can be influenced by a number of factors.

Using the *in vitro* MIC results, a common feature pharmacophore model was developed for *S. aureus, S. epidermidis* and *K. pneumoniae.* Each hypothesis was also validated by determining their $ER^{2\%}$, $HR^{2\%}$ and ROC-AUC, which showed that hypothesis 1 (of *S. aureus* and *K. pneumoniae*) was the best pharmacophore model. The pharmacophore model identified three regions that might be important for antibacterial activity, *i.e*. two hydrophobic features, an aromatic feature and a hydrogen bond acceptor. Lastly, ten phenothiazine derivatives with known antibacterial activity were mapped with the pharmacophore model. High fit values of six derivatives indicate that they might share a common antibacterial mode of action with the methylene blue analogues.

The MIC data reported in this study will allow other researchers to compare the antimicrobial activity of methylene blue analogues across different laboratories. The MIC data together with the pharmacophore model can be used to develop novel lead compounds with antibacterial activity. The phenothiazine derivatives and methylene blue analogues might share a common mode of action, and needs to be confirmed with additional experiments.

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# ANNEXURE A

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# Methylene blue analogues: *In vitro* antimicrobial minimum inhibitory concentrations and *in silico* pharmacophore modelling

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ABSTRACT

It has been shown that methylene blue has antimicrobial properties although few studies have determined its minimum inhibitory concentration (MIC), which is the gold standard used to measure antimicrobial activity. The exact antimicrobial mode of action of methylene blue is still unclear and to our knowledge no pharmacophore model has yet been created to investigate methylene blue's mode of action. The aim of this study was to determine the MIC of methylene blue and a number of its analogous against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella enterica* and *Candida albicans*, and to use these data to develop and validate a common feature pharmacophore model. Three statistical metrics, i.e. the enrichment factor (EF), hit rate (HR) and the area under the curve of a receiver operator characteristic (ROC-AUC), were used to determine the pharmacophore model's ability to differentiate between active/decoy compounds. The validated pharmacophore model was used to map similar antibacterial compounds in an attempt to elucidate methylene blue's antimicrobial mode of action. Most test compounds only had activity against *S. aureus*, *S. epidermidis* and *K. pneumoniae*. Dimethyl methylene blue, new methylene blue and acriflavine proved to be the most active compounds against *S. aureus* [1 μg/ml (dimethyl methylene blue); 4 μg/ml (new methylene blue); 8 μg/ml (acriflavine)], *S. epidermidis* [1 μg/ml (dimethyl methylene blue); 4 μg/ml (new methylene blue); 2 μg/ml (acriflavine)] and *K. pneumoniae* [8 μg/ml (dimethyl methylene blue); 0.5 μg/ml (new methylene blue); 2 μg/ml (acriflavine)]. A common feature pharmacophore model was created (rank score: 26.664, max. fit value: 4), which was able to accurately identify active methylene blue analogous out of the test set ($EF^{2\%}$: 51, $HR^{2\%}$: 100%, ROC-AUC: 1.00 ± 0.00). Six phenothiazine derivatives with known antibacterial activity had high fit values when mapped with the validated pharmacophore model, i.e. >3.4. Dimethyl methylene blue, new methylene blue and acriflavine had potent antibacterial activity against *S. aureus*, *S. epidermidis* and *K. pneumoniae*. The MIC data will allow other researchers to compare the activity across different laboratories. The common feature pharmacophore model was able to identify methylene blue analogous with known *in vitro* antibacterial activity out of a database containing active/decoy compounds and highlighted the importance of two hydrophobic features, an aromatic feature and a hydrogen bond acceptor. The validated pharmacophore model also correctly mapped six phenothiazine derivatives with known antibacterial activity suggesting that they may share a common antibacterial mechanism of action.

## 1. Introduction

Since the discovery of arsphenamine (disc. 1910) by Paul Ehrlich and penicillin (disc. 1929) by Alexander Fleming, antibiotics have saved countless lives and drastically accelerated advancements in the field of medicine (Fleming, 1929; Gaynes, 2017; Rice, 2008). The utility of antibiotics has led to their widespread misuse resulting in numerous antimicrobial resistant bacteria strains (Aminov, 2010). It is estimated that 700 000 people die annually as a direct result of antimicrobial resistance (AMR), and this is projected to globally increase to a staggering 10 million people by 2050 (O'Niell, 2014). In addition to the development of AMR, only two new antibiotic classes have been developed and approved in the last two decades further exacerbating the problem (Tacconelli et al., 2018). There is an urgent need to develop new antimicrobial drugs to combat this emerging threat.

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Methylene blue is a cationic thiazine dye that has found application in numerous fields, *e.g.* biology, chemistry and medicine (Vutskits et al., 2008). It was developed in 1876 as a dye in the textile industry and later became the first synthetic drug used in medicine (Lo et al., 2014). Since then methylene blue has also been used as a lead compound in the development of quinolines (antimalaria) and phenothiazines (neuroleptic) (Wainwright, 2003). Fig. 1 depicts the similarities between methylene blue and the quinoline and phenothiazine pharmacophores.

In medicine, methylene blue is often used to treat methemoglobinemia, ifosfamide-induced encephalopathy and vasoplegia accompanied septic shock (Kwok and Howes, 2006; Patel, 2006; Vutskits et al., 2008). Historically, methylene blue has also been used to treat cyanide poisoning, as an antidepressant and an antimalarial drug (Lo et al., 2014). During surgery methylene blue may also be used as a dye to effectively visualise certain tissue, *e.g.* parathyroid glands (Dudley, 1971). Methylene blue is generally considered safe for *in vivo* use, with doses of 7.5 mg/kg showing no toxic effects in humans (Kuriloff and Sanborn, 2004). However, some studies have suggested dose dependant neurotoxic effect on the central nervous system (Vutskits et al., 2008).

Methylene blue is known to have antibacterial activity. Numerous studies have found that methylene blue has activity on its own or in combination with various other compounds. For example, Steczko and co-workers showed that in combination with sodium citrate methylene blue had *in vitro* activity against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis, Candida albicans* and *Aspergillus niger* (Steczko et al., 2009). Li and co-workers showed that methylene blue alone, or in combination with silver nanoparticles, had *in vitro* activity against *E. coli, Klebsiella pneumoniae* and *P. aeruginosa* (Li et al., 2016). Methylene blue is also known to be a light activated antimicrobial agent (LAAA) that is often used in photodynamic therapy (PDT) to kill bacteria in the presence of light. For example, Piccirillo and co-workers showed that methylene blue has substantial activity against both *E. coli* and *S. epidermidis* when used as an LAAA (Piccirillo et al., 2009). It has also been shown that methylene blue can be used in PDT, or even alone, to kill *Helicobacter pylori* (Choi et al., 2010).

Although methylene blue has proven antimicrobial activity most studies have not determined its minimum inhibitory concentration (MIC), using different methods instead. The MIC is defined as the lowest concentration of an antimicrobial compound that will visibly inhibit growth of an organism after overnight incubation and is considered the "gold standard" for susceptibility testing (Andrews, 2001). Using standardised methods for measuring antimicrobial activity is important, as it allows for comparison of results across different laboratories (Hannan, 2000; Wu et al., 2015). Some studies have determined the MIC of methylene blue against pathogenic organisms and found that it was active against *C. albicans* (100 μg/ml) (Ansari et al., 2016) as well as *S. aureus* (10 mg/ml) (Shatti and Authman, 2015).

Depending on the indication, methylene blue has different biological targets. When used to treat methemoglobinemia methylene blue prevents the oxidation of haemoglobin to form methaemoglobin (McDonagh et al., 2013). The mechanism by which methylene blue alleviates ifosfamide-induced encephalopathy remains unclear, however it is believed that it may act as an electron acceptor to decrease the inhibition of flavoproteins by ifosfamide metabolites (Patel, 2006). To treat vasoplegia during septic shock, methylene blue is used to inhibit inducible nitric oxide (iNOS) and guanylate cyclase (GC), thereby preventing widespread vasodilatation (Kwok and Howes, 2006). The mechanism with which methylene blue treats cyanide poisoning is poorly understood, but its activity is believed to be the result of methylene blue's unique redox properties (Haouzi et al., 2018). Methylene blue is also capable of inhibiting monoamine oxidase (MAO) A, which may explain its antidepressant effects (Delport et al., 2017; Naylor et al., 1986; Ramsay et al., 2007).

With regards to its antimicrobial properties, methylene blue has been used since the 19$^{th}$ century as an antimalarial compound (Lu et al., 2018; Schirmer et al., 2003). Its mechanism of action may be based on the prevention of heme polymerisation in the parasite preventing heme detoxification, or the interruption of the mitochondrial electron transport chain, although the latter mechanism has been questioned (Atamna et al., 1996; Ehrhardt et al., 2013). Methylene blue's antibacterial mechanism of action is still a topic of debate. Studies hypothesize that its mechanism of actions is dependent on its peculiar redox properties that interfere with numerous electron transport pathways in the bacteria (Dubos, 1929; Edwards, 2016; Hoffmann and Rahn, 1944; Woo and Heil, 2017). Additionally, phenothiazines (methylene blue analogues) are believed to exert their antibacterial effect by either binding or interfering with the penicillin binding protein (PBP) (Amaral et al., 2004). Numerous phenothiazine derivatives, *i.e.* flupenthixol, fluphenazine, methdilazine, prochlorperazine, promazine, promethazine, thioridazine, trifluoperazine, triflupromazine and trimeprazine have proven antibacterial activity (Das Gupta et al., 2003). In photodynamic therapy (PDT) methylene blue absorbs light and create a singlet oxygen ($^1O_2$). This extremely electrophilic entity oxidises double bonds in biological molecules and can lead to the formation of hydroxyl radicals, lipid hydroperoxides or the breaking of nucleic acid strands (Oz et al., 2011). Red light with a wavelength of either 630 nm or 700 nm is commonly used, although other wavelengths can have the same effect (Mahmoudi et al., 2018; Salva, 2002). It has also been stated that visible light illumination may have the potential to photosensitize methylene blue (Oz et al., 2011).

When the mode of action of a set of ligands is unknown, ligand-based drug design (LBDD) uses the structures of ligands with known activity to create a common feature pharmacophore model or a 3-dimensional quantitative structure-activity relationship (3D-QSAR) model. The generated model contains information regarding the feature type, position, direction and possible steric constraints that ligands require to be active against the unknown target (Lee et al., 2011). Examples of chemical features are hydrogen bond acceptors (HBA), hydrogen bond donors (HBD), hydrophobic moieties (HYD) and aromatic regions (Ar) (Vuorinen et al., 2014).

Numerous compounds that have known activity is used to create a pharmacophore model; these compounds are termed the training set. The optimum number of compounds needed in the training set differs for 3D-QSAR and common feature pharmacophore models, with the former needing a large number of compounds and the later needing less (Arooj et al., 2013; John et al., 2011; Liu et al., 2020; Rathee et al., 2017; Thangapandian et al., 2011). In this study common feature pharmacophore models will be used.

Using the training set, a computer program creates and ranks several possible pharmacophore model hypotheses of which the most accurate should be chosen. A higher rank score indicates a smaller chance

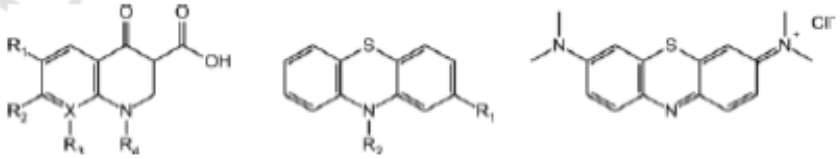


Fig. 1. The structure of methylene blue and the pharmacophores of quinolines and phenothiazines.

that the training set compounds mapped the hypothesis by chance correlation (Adane et al., 2010; Sakkiah et al., 2012). Each hypothesis will also fit each training set compound to a certain degree, with a higher fit value indicating greater activity. The rank score together with the fit value are used to determine which pharmacophore hypothesis is the most accurate. Common feature pharmacophore models can be used to make accurate predictions of how an active pharmacophore will look, but it still has limitations and needs to be properly validated before reliable results can be obtained. A test set, containing known active and decoy compounds, is used to validate the pharmacophore models. The enrichment factor (EF), hit rate (HR) and receiver operator characteristic (ROC) curve are three metrics commonly used to validate the ability of a pharmacophore model to identify active compounds within the test set (Pascual et al., 2019).

The EF is defined as the ratio between the percentage of active compounds in a selected subset and the percentage in the entire database (Chen et al., 2006). The results can be expressed by calculating the EF with equation 1 (Wei et al., 2002).

$$\text{Enrichment Factor (EF)} = \left(\text{Hits}_{sel}/\text{Hits}_{tot}\right) \times \left(\text{NC}_{tot}/\text{NC}\right) \quad (1)$$

$\text{Hits}_{sel}$ is the number of active compounds selected by the docking program at a specific percentage of the total dataset. $\text{Hits}_{tot}$ is the total number of active compounds for the target in question, $\text{NC}_{tot}$ is the total number of molecules (active and decoy) screened in the database and NC is the total number of compounds (active and decoy) in the specific percentage of the database (Chen et al., 2006; Wei et al., 2002). The specific percentage, as mentioned above, is selected by the researcher and is usually between 0.1 – 10 %, depending on the size of the total dataset (Chen et al., 2006; Wei et al., 2002).

The HR is the ratio of active compounds identified at a certain percentage of the total database screened *vs.* all the known active compounds in the entire data base and is calculated by equation 2 (Chen et al., 2009).

$$\text{Hit rate (HR)} = \left(\text{Hits}_{sel}/\text{Hits}_{tot}\right) \times 100 \quad (2)$$

The ROC curve is a well-recognized metric used as an objective way to evaluate the ability of a program to discriminate between active and decoy compounds. The ROC curve also allows the researcher to determine what threshold should be used to differentiate between an active and decoy compound, *e.g.* the fit value. The area under the ROC curve (ROC-AUC) is a practical way of determining the performance of a computer docking program (Lätti et al., 2016; Triballeau et al., 2005). The ROC-AUC can be interpreted as follows; $0.9 \leq \text{AUC} \leq 1.0$ is excellent, $0.8 \leq \text{AUC} < 0.9$ is good, $0.7 \leq \text{AUC} < 0.8$ is fair and $0.5 \leq \text{AUC} < 0.6$ is poor (Braga and Andrade, 2013).

Because of the drastic increase in antimicrobial resistance and the lack of novel antibiotic drug classes, there is a great need to develop new drugs with antimicrobial activity. One of the oldest synthetic drugs, methylene blue, is known to have antimicrobial properties however few studies have determined its MIC against pathogens making comparisons between different laboratories difficult. In addition, its antimicrobial mechanism of action is still not clear. This study aims to determine the MIC of methylene blue and methylene blue analogues against *S. aureus*, *S. epidermidis*, *E. coli*, *K. pneumoniae*, *Salmonella enterica* and *C. albicans*. This data will then be used to develop and validate a pharmacophore model which can, not only be used to identify lead compounds with similar activity but might also give insight into methylene blue's mechanism of action.

## 2. Study design

The test compounds were firstly screened with the Kirby Bauer method to determine their *in vitro* activity against *S. aureus*, *S. epidermidis*, *E. coli*, *K. pneumoniae*, *S. enterica* and *C. albicans*. Using the broth microdilution method, the MIC of the test compounds were determined only against the pathogens which proved susceptible during the Kirby Bauer method. With regards to the *in silico* LBDD study, the test compounds were divided into a training- and a test set. The test set was supplemented with 249 inactive compounds downloaded from the directory of useful decoys enhanced (DUD-E) database. The training set was used to create pharmacophore models for *S. aureus*, *S. epidermidis* and *K. pneumoniae*. The best pharmacophore hypothesis was chosen based on its rank score and fit value. Each hypothesis was also validated using a test set, after which the $EF^{2\%}$, $HR^{2\%}$ and ROC-AUC metrics were used as an objective way of determining which hypothesis was the most accurate. Finally, we used the best pharmacophore model to map phenothiazine derivatives with known antibacterial activity to ascertain if they might share a common mode of action.

## 3. Materials and methods

The *in vitro* antimicrobial activities of methylene blue, methylene green, neutral red, new methylene blue, azure B, dimethyl methylene blue, methylene violet, cresyl violet, acriflavine and nile blue were determined (Sigma-Aldrich, United States). The *in vitro* activity of a compound previously synthesized by our group, *i.e.* ethylthioninium chloride, was also determined (Delport et al., 2014). The test compounds were evaluated against *S. aureus* (ATCC 43300), *S. epidermidis* (ATCC 12228), *E. coli* (ATCC 10536), *K. pneumoniae* (ATCC 10031), *S. enterica* (ATCC 9150) and *C. albicans* (ATCC 14053) (Virginia, United States). During the experiments, the optical density of the pathogens was measured with a Biochrom, Novaspec II spectrophotometer (Gemini, Netherlands). All non-sterile equipment was autoclaved at 121°C for 15 min using a CL-40M autoclave (ALP Co. Ltd) (Lasec, South Africa).

### 3.1. Kirby Bauer method

Muller-Hinton agar (MHA), Muller-Hinton broth (MHB) (Sigma-Aldrich, South Africa) and potato dextrose agar (PDA) (Merck, South Africa) were prepared according to the manufacturer's instructions. The MHA and PDA were used to prepare petri dishes (90 × 15 mm) (Lasec, South Africa) containing agar with a depth of 4 mm. The MHA-petri dishes were used for the bacteria and the PDA-petri dishes for the fungi. To create each inoculum two to three morphological similar colonies of each pathogen were suspended in 10 ml MHB and incubated over night at 35 ± 2°C (bacteria) or 25 ± 2°C (fungi) in a walk-in incubator (T&S Refrigeration, South Africa). All pathogens were handled in a level 2 biosafety cabinet (EuroClone S.p.A, Italy). Ampicillin (Sigma-Aldrich, South Africa) and amphotericin B (Sigma-Aldrich, South Africa) were used as positive controls against the bacteria and fungi, respectively. Ampicillin, methylene blue, methylene green, neutral red, new methylene blue, azure B, dimethyl methylene blue, ethylthioninium chloride and acriflavine were dissolved in distilled water ($DH_2O$) to give a concentration of 10 mg/ml, amphotericin B was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, South Africa) and methylene violet, cresyl violet and nile blue were dissolved in a 1:1 mixture of $DH_2O$ and DMSO to give a concentration of 10 mg/ml. The relevant solvent of each compound was used as negative control. Each solution was filter sterilised (0.2 µm) (Lasec, South Africa) and was used within 12 hrs of preparation.

A lawn of bacteria/fungi was created on each agar petri dish by evenly spreading 100 µl of the above-mentioned inoculum. Dry discs with a diameter of 6 mm was created from Whatman grade 4 filter paper (Lasec, South Africa) with a paper punch. Five discs were place on each agar petri dish, *i.e.* tests compound ($n = 3$), a positive control ($n = 1$) and a negative control ($n = 1$). Of each test compound, 5 µl (resulting in 50 µg) were added to each disc ($n = 3$) and 10 µg of the positive control were added to each disc ($n = 1$). Of each negative control, 5 µl solution were added to each disc ($n = 1$). The petri dishes were incubated at either 35 ± 2°C (bacteria) or 25 ± 2°C (fungi) for 18 hrs. After incubation the zone of inhibition (ZOI) of each disc was

measured with a ruler (Table 1). If a compound had a ZOI > 6 mm it was considered active.

### 3.2. Broth microdilution method

Following the results of the Kirby Bauer method, the MIC of each test compound was determined against *S. aureus*, *S. epidermidis* and *K. pneumoniae* according the Clinical and Laboratory Standards Institute (CLSI) guidelines (Cockerill et al., 2012).

The test compounds were dissolved (8% DMSO) to a concentration of 256 μg/ml, which resulted in a final concentration of 128 μg/ml (4% DMSO) when the inoculum was added. Tetracycline (Sigma-Aldrich, South Africa) was dissolved ($DH_2O$) to a concentration of 64 μg/ml which resulted in a final concentration of 32 μg/ml. All stock solutions were filter sterilised (0.2 μm). Two-fold serial dilutions ($n = 11$) were made of all the compounds. Each Corning flat bottom 96-well plate (Lasec, South Africa) was primed with all eleven concentrations of two test compounds (100 μl, $n = 3$ rows each) and a positive control (100 μl, $n = 1$ row). A sterility control (200 μl, $n = 3$ wells), *i.e.* sterile MHB, and a growth control (100 μl, $n = 11$ wells), *i.e.* $DH_2O$, was also added to each 96-well plate.

The inoculum of each bacteria was prepared by suspending two to three colonies in 10 ml sterile MHB and incubating the suspension at 35 ± 2°C for 2 – 5 hrs while shaking (250 rpm). It was experimentally determined beforehand that all three bacteria would be in the mid-log growth phase after 3 – 5 hrs incubation (data not shown). Each inoculum was diluted with sterile MHB to an $OD_{600}$ of 0.35 corresponding to a bacterial concentration of $2.6 \times 10^8$ CFU/ml (*S. aureus*), $2.14 \times 10^8$ CFU/ml (*S. epidermidis*) and $3.0 \times 10^8$ CFU/ml (*K. pneumoniae*). The correlation between the $OD_{600}$ value and the bacteria concentration was experimentally determined beforehand (data not shown). Each inoculum was further diluted with sterile MHB to a concentration of $1 \times 10^6$ CFU/ml. Finally, 100 μl inoculum was added to all wells (except the sterility control) which resulted in a final concentration of $5 \times 10^5$ CFU/ml and a final volume of 200 μl. Each inoculum was used within 30 min to avoid changes in the bacterial cell count. Each plate was covered, placed in a plastic container and incubated at 35 ± 2°C for 20 hrs. A beaker containing 250 ml hot water was also placed inside the plastic contained to increase the humidity and prevent evaporation. The bacterial growth was measured with a 96-well plate reader (BioTek Instrument Inc, USA) (ADP, South Africa) at a wavelength of 520 nm before and after incubation. Because of the colour of the test compounds, 520 nm gave more reliable results than 600 nm. The MIC was considered as the lowest concentration of a compound at which the growth was inhibited ≥ 80%. The average of the $OD_{520}$ values were used to determine the MIC value.

A viability count was conducted on each inoculum by suspending 10 μl from one growth control well in 10 ml sterile MHB. Of this suspension 100 μl were spread over a MHA petri dish ($n = 3$) and incubated at 35 ± 2°C for 20 hrs (EUCAST, 2003).

### 3.3. Common Feature Pharmacophore Modelling

The docking procedures were conducted using Accelrys Discovery Studio (DS) v3.1 (San Diego, United States). The ROC-AUC curve calculations were conducted using SPSS Statistics for Windows, version 26 (SPSS Inc., Chicago, Ill., USA). All structures were downloaded from the PubChem database (Kim et al., 2018).

The structures of all molecules were typed with the CHARMm force field, and then subjected to the *Prepare Ligands* protocol. Depending on the bacteria strain the following principal values were ascribed to the training set compounds, *i.e. S. aureus* [methylene green (1), new methylene blue (2), dimethyl methylene blue (2), cresyl violet (1)], *S. epidermidis* [methylene green (1), new methylene blue (2), dimethyl methylene blue (2), cresyl violet (0)] and *K. pneumoniae* [methylene green (2), new methylene blue (2), dimethyl methylene blue (2), cresyl violet (1)]. A maximum omit feature value of 0 was used for all training set compounds. The *Feature Mapping* protocol identified common chemical groups present in the training set, *i.e.* hydrogen bond acceptor (HBA), hydrogen bond donor (HBD), hydrophobic (HY), positive ionizable (PI) and ring aromatic (RA). To obtain a diverse range of conformations the *Common Feature Pharmacophore Generation* protocol considered the BEST 200 conformations within a 20 kcal/mol threshold and a maximum interference distance of 2 Å. The final pharmacophore models were ranked by fit value.

Acriflavine was omitted from the modelling protocols, as it consists of a mixture of two compounds (figure 2). The eight remaining test compounds were divided into a training set, *i.e.* methylene green, new methylene blue, dimethyl methylene blue, cresyl violet, and a test set, *i.e.* methylene blue, azure B, ethylthioninium chloride, neutral red. As part of the test set, we included 249 decoy compounds that were downloaded from the directory of useful decoys enhanced (DUD-E) database (Mysinger et al., 2012). The way in which the DUD-E data base selects decoy compounds is described in detail elsewhere (Mysinger et al., 2012). In short, the database selects molecules from the ZINC database that are similar to the active compounds with regards to their molecular weight, estimated water-octanol partition coefficient, rotat-

**Table 1**
ZOI of each test compound against the relevant pathogen.

| Test compound | ZOI (mm) | | | | | |
|---|---|---|---|---|---|---|
| | *S. aureus* | *S. epidermidis* | *E. coli* | *K. pneumoniae* | *S. enterica* | *C. albicans* |
| **Methylene blue** | 9.7 ± 0.5 | 14.0 ± 1.4 | 6.0 ± 0.0 | 15.3 ± 0.5 | 6.0 ± 0.5 | 6.0 ± 0.0 |
| **Methylene green** | 10.7 ± 1.7 | 14.5 ± 1.5 | 6.7 ± 0.9 | 14.0 ± 0.0 | 6.7 ± 0.5 | 7.3 ± 0.5 |
| **New methylene blue** | 9.7 ± 0.5 | 10.7 ± 0.5 | 6.0 ± 0.0 | 15.7 ± 0.5 | 6.0 ± 0.0 | 6.0 ± 0.0 |
| **Dimethyl methylene blue** | 9.7 ± 0.5 | 12.7 ± 0.5 | 6.0 ± 0.0 | 15.3 ± 0.5 | 6.0 ± 0.0 | 6.0 ± 0.0 |
| **Methylene violet** | 8.7 ± 0.5 | 8.3 ± 0.5 | 6.0 ± 0.0 | 8.7 ± 0.5 | 6.0 ± 0.0 | 6.0 ± 0.0 |
| **Cresyl violet** | 8.0 ± 0.8 | 7.7 ± 0.5 | 6.0 ± 0.0 | 8.0 ± 0.8 | 6.0 ± 0.0 | 6.0 ± 0.0 |
| **Nile blue** | 7.0 ± 0.0 | 7.0 ± 0.0 | 6.0 ± 0.0 | 7.7 ± 0.5 | 6.0 ± 0.0 | 6.0 ± 0.0 |
| **Acriflavine** | 7.7 ± 0.5 | 16.7 ± 1.9 | 13.3 ± 0.5 | 11.7 ± 0.5 | 10.0 ± 0.8 | 6.3 ± 0.5 |
| **Azure B** | 9.7 ± 0.5 | 6.0 ± 0.0 | 6.0 ± 0.0 | 14.0 ± 0.8 | 6.0 ± 0.0 | 6.0 ± 0.0 |
| **Neutral red** | 6.0 ± 0.0 | 7.3 ± 0.5 | 6.0 ± 0.0 | 6.0 ± 0.0 | 6.0 ± 0.0 | 6.0 ± 0.0 |
| **Ethylthioninium chloride** | 8.3 ± 0.5 | 14.0 ± 0.8 | 7.7 ± 0.5 | 10.5 ± 0.5 | 7.0 ± 0.0 | 6.0 ± 0.0 |
| **Positive control*** | 8.7 ± 1.6 | 8.8 ± 2.5 | 15.5 ± 2.7 | 6.5 ± 0.7 | 20.2 ± 7.0 | 15.5 ± 3.1 |
| **Negative control†** | 6.0 ± 0 | 6.1 ± 0.3 | 6.0 ± 0 | 6.0 ± 0 | 6.0 ± 0 | 6.1 ± 0.3 |

* Bacteria (ampicillin) and *C. albicans* (amphotericin B).
† Ampicillin ($DH_2O$), methylene violet, cresyl violet and nile blue [1 ($DH_2O$) : 1 (DMSO)] and amphotericin B (DMSO).

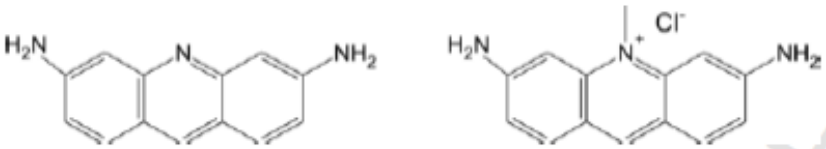


Fig. 2. Structures of the acriflavine mixture.

able bonds, hydrogen bond acceptors, hydrogen bond donors and net charge (Mysinger et al., 2012; Sterling and Irwin, 2015). DUD-E then generates the extended connectivity fingerprint (ECFP) of all the test compounds as well as the ECFP of all the potential decoys, then only select the most dissimilar 25% of the potential decoys to minimise the number of decoys that might have activity against the target (Mysinger et al., 2012).

The entire test set was docked into each pharmacophore model created for *S. aureus*, *S. epidermidis* and *K. pneumoniae* with the *Ligand Profiler* protocol using the same settings described above. A maximum omit feature of 0 was chosen for all compounds. This data was used to calculate the $EF^{2\%}$, $HR^{2\%}$ and the AUC of each ROC curve for each pharmacophore model.

## 4. Results

### 4.1. Kirby Bauer method

The results of the Kirby Bauer method are shown in Table 1. Against *S. aureus* most compounds showed activity, *i.e.* methylene blue, methylene green, new methylene blue, dimethyl methylene blue, methylene violet, cresyl violet, nile blue, acriflavine, azure B and ethylthioninium chloride. Only neutral red did not show activity. Against *S. aureus* ampicillin was also active. Against *S. epidermidis* most compounds showed activity, *i.e.* methylene blue, methylene green, new methylene blue, dimethyl methylene blue, methylene violet, cresyl violet, nile blue, acriflavine, neutral red and ethylthioninium chloride. Only azure B did not show activity. Against *S. epidermidis* ampicillin was also active. Against *K. pneumoniae* most compounds showed activity, *i.e.* methylene blue, methylene green, new methylene blue, dimethyl methylene blue, methylene violet, cresyl violet, nile blue, acriflavine, azure B and ethylthioninium chloride. Only neutral red did not show activity. Against *K. pneumoniae* ampicillin was also active. Against *E. coli*, *S. enterica* and *C. albicans* none of the test compounds had activity expect acriflavine, methylene green and ethylthioninium chloride. Ampicillin was active against *E. coli* and *S. enterica*, while amphotericin B was active against *C. albicans*. The MIC of the test compounds were determined against *S. aureus*, *S. epidermidis* and *K. pneumoniae* as they proved to be susceptible (described below).

### 4.2. Broth microdilution method

The MIC results are given in Table 2. Both methylene violet and nile blue did not dissolve properly during the experiment which may result in inaccurate MIC values. Both compounds will not be discussed further. The viability count showed that the bacterial concentration was higher than expected for methylene green (*S. aureus*), cresyl violet and ethylthioninium chloride (*K. pneumoniae*) and is reported as such in Table 2. A compound was defined as active if it had an MIC $\leq$ 16 μg/ml. As with the Kirby Bauer method most test compounds showed activity against *S. aureus*, *i.e.* methylene blue, methylene green, new methylene blue, dimethyl methylene blue, cresyl violet, acriflavine, azure B and ethylthioninium chloride. Only neutral red did not show activity. Tetracycline was also active against *S. aureus*. As with the Kirby Bauer method most test compounds showed activity against *S. epidermidis*, *i.e.* methylene blue, methylene green, new methylene blue, dimethyl methylene blue, acriflavine, azure B and ethylthioninium chloride. Both cresyl violet and neutral red did not show activity.

**Table 2**
MIC values of the test compounds against *S. aureus*, *S. epidermidis* and *K. pneumoniae*.

| Test compound | MIC (μg/ml) | | |
|---|---|---|---|
| | *S. aureus* | *S. epidermidis* | *K. pneumoniae* |
| **Methylene blue** | 16 | 8 | 4 |
| **Methylene green** | 16* | 16 | 8 |
| **New methylene blue** | 4 | 4 | 0.5 |
| **Dimethyl methylene blue** | 1 | 1 | 8 |
| **Methylene violet** | > 128* | > 128* | > 128* |
| **Cresyl violet** | 16 | 32 | 16‡ |
| **Nile blue** | 128* | > 128* | > 128* |
| **Acriflavine** | 8 | 2 | 2 |
| **Azure B** | 16 | 16 | 8 |
| **Neutral red** | 128 | 128 | 128 |
| **Ethylthioninium chloride** | 16 | 16 | 16‡ |
| **Tetracycline** | 0.5 | 1 | 0.5 |

* Did not dissolve properly. Actual MIC values might differ.
† Bacterial concentration was 142.5 ± 10.6 CFU/ml.
‡ Bacterial concentration was 165 ± 21.2 CFU/ml.

Tetracycline was also active against *S. epidermidis*. As with the Kirby Bauer method most test compounds showed activity against *K. pneumoniae*, *i.e.* methylene blue, methylene green, new methylene blue, dimethyl methylene blue, cresyl violet, acriflavine, azure B and ethylthioninium chloride. Only neutral red did not show activity. Tetracycline was also active against *K. pneumoniae*.

### 4.3. Common Feature Pharmacophore Modelling

Ten pharmacophore hypotheses were created for *S. aureus*, *S. epidermidis* and *K. pneumoniae*. A summary of each pharmacophore hypothesis (hypo.) is given in Table 3 (*S. aureus*), Table 4 (*S. epidermidis*) and Table 5 (*K. pneumoniae*). The ten hypotheses created for *S. aureus* had a high degree of similarity compared to its corresponding hypothesis *vs.* *S. epidermidis* and *K. pneumoniae*. As such only the *S. aureus* data will be

**Table 3**
Summary of the pharmacophore hypotheses created for *S. aureus*.

| Hypothesis | Features* | Rank score | Direct hit† | Partial hit‡ | Maximum fit |
|---|---|---|---|---|---|
| 01 | RHHA | 26.664 | 1111 | 0000 | 4 |
| 02 | RHHA | 26.664 | 1111 | 0000 | 4 |
| 03 | HHHA | 25.547 | 1111 | 0000 | 4 |
| 04 | RHHA | 25.315 | 1111 | 0000 | 4 |
| 05 | RHHA | 25.315 | 1111 | 0000 | 4 |
| 06 | RHHA | 24.642 | 1111 | 0000 | 4 |
| 07 | HHHA | 24.305 | 1111 | 0000 | 4 |
| 08 | RHHA | 23.795 | 1111 | 0000 | 4 |
| 09 | HHHA | 23.305 | 1111 | 0000 | 4 |
| 10 | HHHA | 22.057 | 1111 | 0000 | 4 |

* R = Ring aromatic, H = Hydrophobic feature, A = Hydrogen bond acceptor.
† Indicates whether (1) or not (0) a molecule is mapped.
‡ Indicates whether (1) or not (0) a molecule mapped all but one feature.

**Table 4**
Summary of the pharmacophore hypotheses created for *S. epidermidis*.

| Hypothesis | Features* | Rank score | Direct hit† | Partial hit‡ | Maximum fit |
|---|---|---|---|---|---|
| 01 | RHHA | 21.753 | 111 | 000 | 4 |
| 02 | RHHA | 21.753 | 111 | 000 | 4 |
| 03 | HHHA | 20.915 | 111 | 000 | 4 |
| 04 | RHHA | 20.741 | 111 | 000 | 4 |
| 05 | RHHA | 20.741 | 111 | 000 | 4 |
| 06 | RHHA | 20.237 | 111 | 000 | 4 |
| 07 | HHHA | 19.984 | 111 | 000 | 4 |
| 08 | RHHA | 19.601 | 111 | 000 | 4 |
| 09 | HHHA | 19.234 | 111 | 000 | 4 |
| 10 | HHHA | 18.298 | 111 | 000 | 4 |

* R = Ring aromatic, H = Hydrophobic feature, A = Hydrogen bond acceptor.
† Indicates whether (1) or not (0) a molecule is mapped.
‡ Indicates whether (1) or not (0) a molecule mapped all but one feature.

**Table 5**
Summary of the pharmacophore hypotheses created for *K. pneumoniae*.

| Hypothesis | Features* | Rank score | Direct hit† | Partial hit‡ | Maximum fit |
|---|---|---|---|---|---|
| 01 | RHHA | 26.664 | 1111 | 0000 | 4 |
| 02 | RHHA | 26.664 | 1111 | 0000 | 4 |
| 03 | HHHA | 25.547 | 1111 | 0000 | 4 |
| 04 | RHHA | 25.315 | 1111 | 0000 | 4 |
| 05 | RHHA | 25.315 | 1111 | 0000 | 4 |
| 06 | RHHA | 24.642 | 1111 | 0000 | 4 |
| 07 | HHHA | 24.305 | 1111 | 0000 | 4 |
| 08 | RHHA | 23.795 | 1111 | 0000 | 4 |
| 09 | HHHA | 23.305 | 1111 | 0000 | 4 |
| 10 | HHHA | 22.057 | 1111 | 0000 | 4 |

* R = Ring aromatic, H = Hydrophobic feature, A = Hydrogen bond acceptor.
† Indicates whether (1) or not (0) a molecule is mapped.
‡ Indicates whether (1) or not (0) a molecule mapped all but one feature.

reported however, where there was a difference it will be indicated. The ranking scores differed from 26.664 – 22.057 (*S. aureus* and *K. pneumoniae*) and 21.753 - 18.298 (*S. epidermidis*). For *S. aureus*, *S. epidermidis* and *K. pneumoniae* the different hypotheses contained either ring aromatics (R), hydrophobic features (H) or hydrogen bond acceptors (A) or different combinations thereof. All hypotheses had a maximum fit value of 4.

Comparing the best fit value of the training set compounds between the different hypotheses the following was observed. For hypo. 1 (new methylene blue > cresyl violet > methylene green > dimethyl methylene blue), hypo. 2 (new methylene blue > cresyl violet > methylene green > dimethyl methylene blue), hypo. 3 (new methylene blue > methylene green > dimethyl methylene blue > cresyl violet), hypo. 4 (new methylene blue > cresyl violet > methylene green > dimethyl methylene blue), hypo. 5 (new methylene blue > cresyl violet > methylene green > dimethyl methylene blue), hypo. 6 (new methylene blue > cresyl violet > dimethyl methylene blue > methylene green), hypo. 7 (new methylene blue > methylene green > cresyl violet > dimethyl methylene blue), hypo. 8 (new methylene blue > cresyl violet > dimethyl methylene blue > methylene green), hypo. 9 (new methylene blue > cresyl violet > dimethyl methylene blue > methylene green) and hypo. 10 (new methylene blue > cresyl violet > dimethyl methylene blue > methylene green) (Table 6).

The pharmacophore models' hypotheses were validated by their ability to correctly identify active compounds from a test set containing active and decoy compounds. Comparing the $EF^{2\%}$ and $HR^{2\%}$ of each hypothesis, both hypo. 1 ($EF^{2\%}$ = 51; $HR^{2\%}$ = 100%) and hypo. 2 ($EF^{2\%}$ = 51; $HR^{2\%}$ = 100%), had the most promising results (Table 7). Comparing the AUC of each hypothesis' ROC curve again hypo. 1 (1.00 ± 0.00) and hypo. 2 (0.99 ± 0.01) had the most promising results.

After mapping ten phenothiazine derivatives with the most promising pharmacophore model, *i.e.* hypo. 1, each derivative had the following fit value; flupenthixol (3.49), fluphenazine (3.49), methdilazine (2.02), prochlorperazine (3.47), promazine (0.48), promethazine, (2.18) thioridazine (3.65), trifluoperazine (3.66), triflupromazine (3.46) and trimeprazine (2.16) (Table 8).

## 5. Discussion

### 5.1. Kirby Bauer method

It should be noted that the Kirby Bauer test was used as a screening method in this study, and the standard interpretation of the results, *i.e.* susceptible "S", intermediate "I" and resistant "R", could not be used as the initial bacteria concentration was not determined. The Kirby Bauer method found that all compounds were active against *S. aureus*, *S. epidermidis* and *K. pneumoniae* except neutral red which was inactive against *S. aureus* and *K. pneumoniae*, and azure B that was inactive against *S. epidermidis*. The test compounds were inactive against *E. coli*, *S. enterica* and *C. albicans* except acriflavine and ethylthioninium chloride that were also active against *E. coli* and *S. enterica*. Methylene green also had weak activity against *E. coli*, *S. enterica* and *C. albicans*. The methylene blue analogue synthesized by our group, *i.e.* ethylthioninium chloride, had better activity compared to most of the other test compounds. Although both acriflavine and ethylthioninium chloride had activity against all pathogens except *C. albicans*, their MIC values were not determined against *E. coli*, *S. enterica* and *C. albicans* as the

**Table 6**
Best fit value of the training set against *S. aureus*, *S. epidermidis* and *K. pneumoniae*.

| | Fit value | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Compound | Hypo. 1 | Hypo. 2 | Hypo. 3 | Hypo. 4 | Hypo. 5 | Hypo. 6 | Hypo. 7 | Hypo. 8 | Hypo. 9 | Hypo. 10 |
| **Methylene green** | 1.78 | 1.66* | 2.40 | 1.81 | 1.83 | 0.56 | 1.50 | 2.08 | 1.01 | 1.83 |
| **New methylene blue** | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 | 4.00 |
| **Dimethyl methylene blue** | 0.89 | 0.88 | 1.43 | 1.62 | 1.61 | 1.85 | 0.35 | 2.60 | 2.31‡ | 3.01 |
| **Cresyl violet** | 2.46 | 2.61 | 1.41 | 3.42 | 3.42† | 2.08 | 1.26 | 2.69 | 2.48 | 3.37 |

* *K. pneumoniae* had a fit value of 1.65.
† *K. pneumoniae* had a fit value of 3.43.
‡ *K. pneumoniae* had a fit value of 2.32.

**Table 7**
The $EF^{2\%}$, $HR^{2\%}$ and ROC-AUC results for each pharmacophore hypotheses created for *S. aureus*, *S. epidermidis* and *K. pneumoniae*.

| Hypothesis | $EF^{2\%}$ | $HR^{2\%}$ | ROC-AUC values |
|---|---|---|---|
| 01 | 51 | 100% | 1.00 ± 0.00 |
| 02 | 51 | 100% | 0.99 ± 0.01 |
| 03 | 0 | 0% | 0.77 ± 0.06* |
| 04 | 13 | 25% | 0.68 ± 0.11 |
| 05 | 13 | 25% | 0.68 ± 0.11† |
| 06 | 25¶ | 50%¶ | 0.97 ± 0.03 |
| 07 | 0 | 0% | 0.36 ± 0.12‡ |
| 08 | 0 | 0% | 0.64 ± 0.08 |
| 09 | 38 | 75% | 0.82 ± 0.16§ |
| 10 | 0 | 0% | 0.82 ± 0.05 |

*K. pneumoniae had a value of 0.79 ± 0.06.
† K. pneumoniae had a value of 0.69 ± 0.11.
‡ K. pneumoniae had a value of 0.34 ± 0.12.
§ K. pneumoniae had a value of 0.81 ± 0.16.
¶ S. epidermidis had an $EF^{2\%}$ and $HR^{2\%}$ of 38 and 75% respectively.

**Table 8**
Fit values of phenothiazine derivatives mapped with the hypo. 1.

| Derivative | Fit Value |
|---|---|
| Flupenthixol | 3.49 |
| Fluphenazine | 3.49 |
| Methdilazine | 2.02 |
| Prochlorperazine | 3.47 |
| Promazine | 0.48 |
| Promethazine | 2.18 |
| Thioridazine | 3.65 |
| Trifluoperazine | 3.66 |
| Triflupromazine | 3.46 |
| Trimeprazine | 2.16 |

subsequent *in silico* experiments require more than one active compound per bacterial strain.

As with previous studies, methylene blue was active against both *S. aureus* and *K. pneumoniae* (Li et al., 2016; Shatti and Authman, 2015). As reported by Gunics and co-workers methylene blue did not have activity against *E. coli*, however another study did report activity against a different strain of *E. coli* (Gunics et al., 2000; Li et al., 2016). In this study methylene blue had good activity against *S. epidermidis* whereas others found no activity using a clinical isolate (Gunics et al., 2000). While another study found that methylene blue had activity against *C. albicans* this study did not (Ansari et al., 2016). This may be because of a different strain of *C. albicans* that was used in this study. As reported by others, this study also did not find activity against *S. enterica* (Nishino et al., 2009). Acriflavine is another dye with well-known antimicrobial properties. Our results were similar to other studies which found that acriflavine had activity against *S. aureus* (Kawai and Yamagishi, 2009), *E. coli* (Nakamura, 1966), *K. pneumoniae* (Mondal et al., 2016) and *S. enterica* (Hayashi-Nishino et al., 2012) (Table 1).

### 5.2. Broth microdilution method

For the broth microdilution results, an MIC value differing by one two-fold dilution is considered comparable (Barry et al., 1978). All test compounds showed activity against *S. aureus* except neutral red. Dimethyl methylene blue was the most active compound, followed by new methylene blue then acriflavine. Methylene blue, methylene green, cresyl violet, azure B and ethylthioninium chloride were all equally active. Compared to the positive control, dimethyl methylene blue had comparable activity. All test compounds showed activity against *S. epidermidis* except cresyl violet and neutral red. Dimethyl methylene blue was again the most active compound, followed by acriflavine then new methylene blue. Methylene blue had comparable activity to methylene green, ethylthioninium chloride and azure B. The activity of cresyl violet was comparable to methylene green, ethylthioninium chloride and azure B, however this compound was less active compared to methylene blue. Again, compared to the positive control dimethyl methylene blue was equally active. All test compounds showed activity against *K. pneumoniae*, except neutral red. In this instance, new methylene blue was the most active compound. Both acriflavine and methylene blue were less active than new methylene blue. Methylene green, dimethyl methylene blue and azure B were equally active. The activities of both cresyl violet and ethylthioninium chloride were comparable to that of methylene green, dimethyl methylene blue and azure B. Compared to the positive control new methylene blue was equally active. Overall, new methylene blue, dimethyl methylene blue and acriflavine had the highest activity against all three pathogens, and in some instances the activities were comparable to that of tetracycline (Table 2).

### 5.3. Common Feature Pharmacophore Modelling

Hypo. 1 – 10 for *S. aureus*, *S. epidermidis* and *K. pneumoniae* are similar. This can be expected as the *in vitro* activity and the principal values of the training set compounds did not differ to a great extent.

To determine which pharmacophore hypothesis was the most accurate, the rank score and fit values of each hypothesis were firstly considered. For *S. aureus* (Table 3), *S. epidermidis* (Table 4) and *K. pneumoniae* (Table 5) hypo. 1 and 2 had the highest rank scores, however hypo. 1 and 2 for *S. aureus* and *K. pneumoniae* were higher than hypo. 1 and 2 of *S. epidermidis*. The fit values for hypo. 1 and 2 were the same and correctly determined new methylene blue would be highly active and methylene green and cresyl violet would be less active (Table 6). Both hypotheses however determine dimethyl methylene blue would be the least active which was incorrect considering the MIC values for *S. aureus*, *S. epidermidis* and *K. pneumoniae*. Some hypotheses did rank dimethyl methylene blue higher, i.e. hypo. 3, 6, 8, 9 and 10, however their rank scores were lower than hypo. 1 and 2. To further validate each hypothesis, the $EF^{2\%}$, $HR^{2\%}$ and ROC-AUC results were also considered. Again, both hypo. 1 and 2 showed the most promising results each with an $EF^{2\%}$ of 51, an $HR^{2\%}$ of 100% and a ROC-AUC score of 1.00 ± 0.00 and 0.99 ± 0.01, respectively (Table 7). Hypo. 9 also had promising results with an $EF^{2\%}$ of 38, an $HR^{2\%}$ of 75% and a ROC-AUC score of 0.82 ± 0.16. Tanking all the metrics into consideration both hypo. 1 and 2 were considered equally accurate and are depicted in figure 3.

The similar results obtained with both hypo. 1 and 2 are explained by figure 3 which shows that hypo. 1 and 2 are identical, only differing in the way new methylene blue is orientated. The pharmacophore model consists of two hydrophobic features, an aromatic feature and a hydrogen bond acceptor. According to the pharmacophore model, the amine in the heterocyclic ring is important and acts as a hydrogen bond acceptor. One of the adjacent cyclic carbon rings act as a hydrophobic region, whilst the other is important because of its aromatic interactions. The substituent on the carbon rings also seems to influence activity as a result of its hydrophobic interactions.

Fig. 4 shows the top six phenothiazine derivatives with the best fit values, mapped with the validated pharmacophore model. These derivatives had fit values >3.4 (Table 8) indicating that there is a high probability they are active according to the ROC curve analysis (data not shown). Because of the substitution on the heterocyclic amine, the sulphur group acts as the hydrogen bond acceptor rather than the amine. As with the methylene blue analogues, one of the adjacent carbon rings acts as the hydrophobic moiety and the other as the aromatic region. According to the pharmacophore model there is a possibility

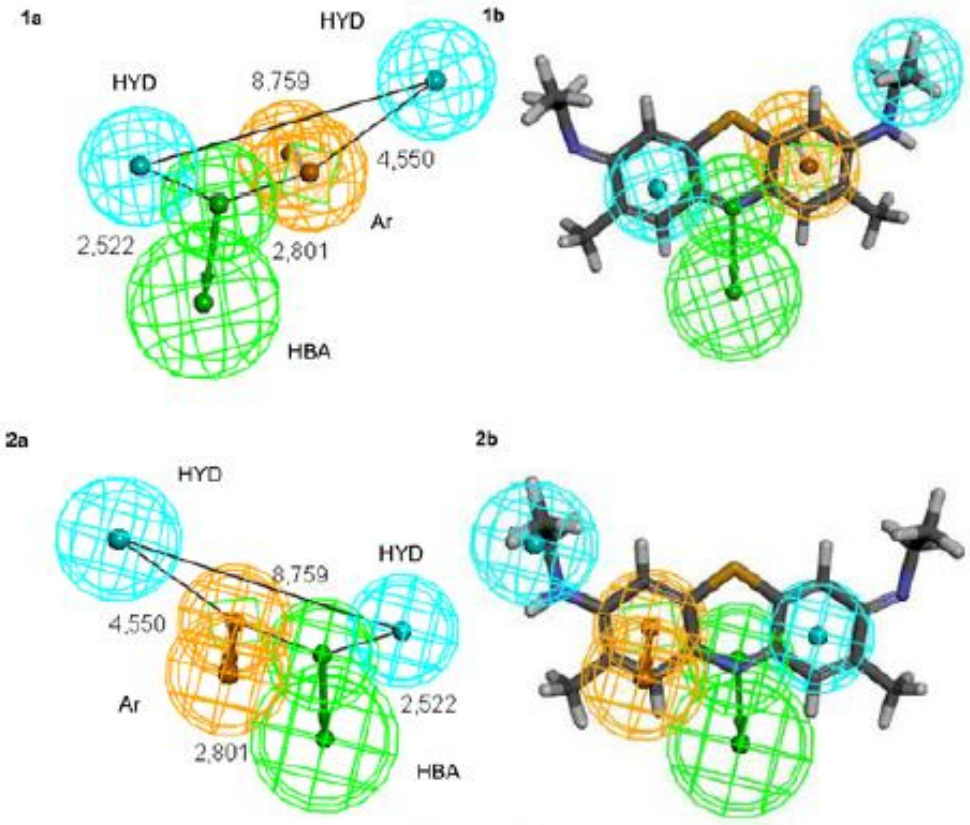


Fig. 3. Hypothesis 1 (a) with interfeature distance constraints and (b) with new methylene blue mapped, and hypothesis 2 (a) with interfeature distance constraints and (b) with new methylene blue mapped. The colours of the pharmacophore features are; green (HBA), blue (HYD) and orange (Ar).

that the methylene blue analogues and the phenothiazine derivatives have the same antibacterial mode of action.

## 6. Conclusion

Because of the rapid and widespread increase in AMR and the lack of novel antibacterial drug classes, there is an urgent need to develop new antimicrobial drugs. Methylene blue and its analogues are known to have antibacterial activity, although few studies have determined their MICs which is the gold standard used to measure and compare antimicrobial activity. Despite methylene blue being the oldest synthetic drug, its antibacterial mechanism of action is still not clear.

In this study methylene blue and a number of its analogues were firstly screened for antimicrobial activity against *S. aureus*, *S. epidermidis*, *E. coli*, *K. pneumoniae*, *S. enterica* and *C. albicans*. Most analogues had activity against *S. aureus*, *S. epidermidis* and *K. pneumoniae*. The MIC of each analogue were determined against *S. aureus*, *S. epidermidis* and *K. pneumoniae* and it was found that dimethyl methylene blue, new methylene blue and acriflavine were the most active compounds. The *in vitro* activities of the analogues were used to create a common feature pharmacophore model for *S. aureus*, *S. epidermidis* and *K. pneumoniae*, which were assessed by their rank scores and vit values. Each hypothesis was also validated by determining their $ER^{2\%}$, $HR^{2\%}$ and ROC-AUC, after which it was concluded that hypothesis 1 (of *S. aureus* and *K. pneumoniae*) was the best pharmacophore model. The pharmacophore model identified two hydrophobic features, an aromatic feature and a hydrogen bond acceptor as being important for antibacterial activity. Finally, ten phenothiazine derivatives with known antibacterial activity were mapped with the pharmacophore model. High fit values of six derivatives indicate that they may share a common antibacterial mode of action with the methylene blue analogues.

The MIC data reported here will allow other researchers to compare the activity of the methylene blue analogues across different laboratories. The validated pharmacophore model can be used to identify lead compounds with a similar mode of action to methylene blue. The possibility that methylene blue analogues and phenothiazine derivatives have the same antibacterial mode of action needs to be confirmed with additional experiments.

## Declaration of Competing Interest

The authors declare no conflict of interest.

## Acknowledgements

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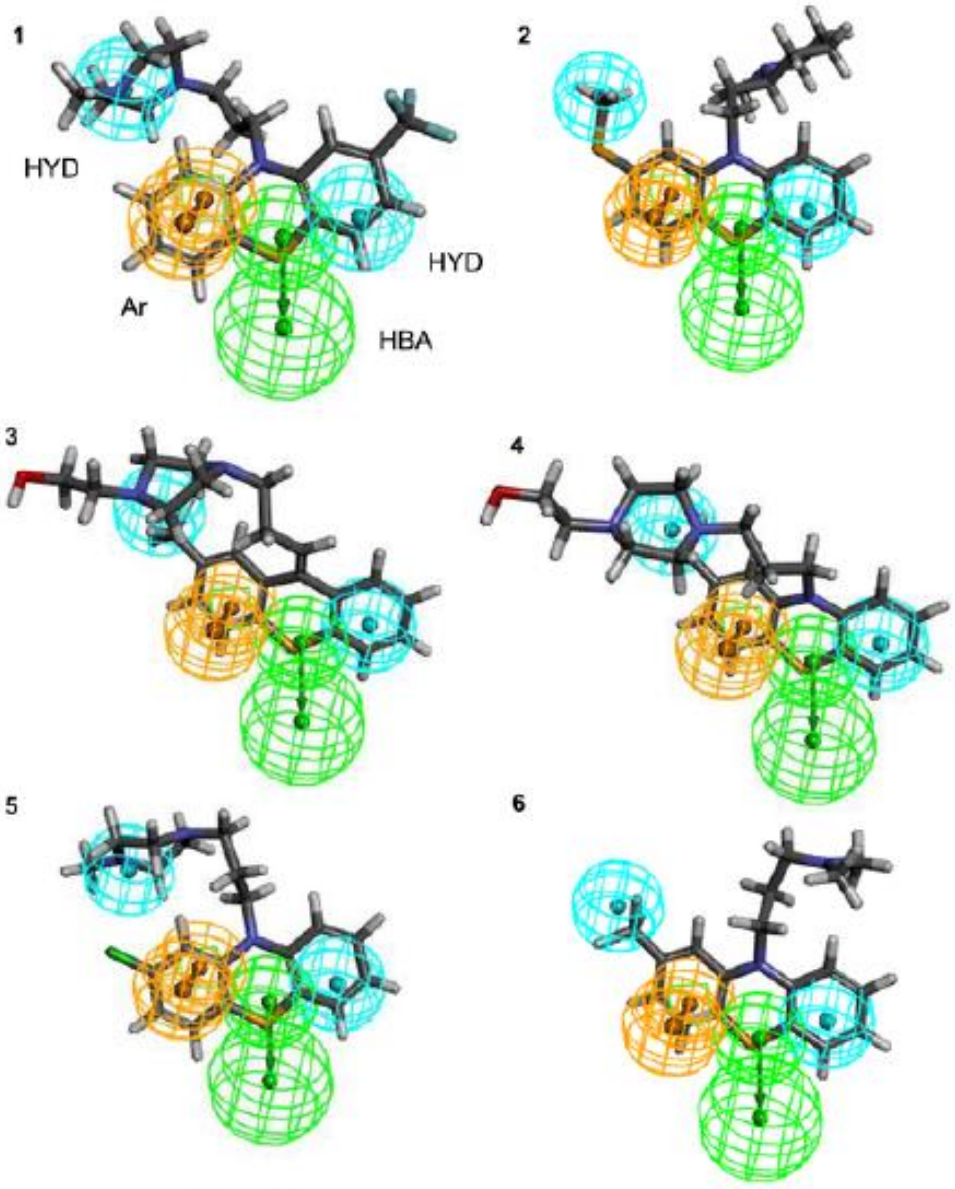


Fig. 4. Trifluoperazine (1), thioridazine (2), flupenthixol (3), fluphenazine (4), prochlorperazine (5) and triflupromazine (6) mapped with the validated pharmacophore model (hypo. 1). The colours of the pharmacophore features are; green (HBA), blue (HYD) and orange (Ar).

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