# Drug Repurposing as a Strategy to Uncover Effective Antibacterial Compounds using *Salmonella* as a model

**A dissertation presented for the degree of**

**Doctor of Philosophy**

**May 2021**

by


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**Declaration of Authorship**

I, Daniela Alves Ferreira, certify that the experimentation recorded herein represents my own work, unless otherwise stated in the text and has not been previously presented for a higher degree at this or any other university.

Chapter 2: Figure 2.8. Electrophoretic evaluation of DNA double-strand cleavage by the both Zn(II) phendione compounds (TS262 and TS267) and Figure 2.9 were performed by our collaborators at UCIBIO, Departamento Ciências da Vida, Faculdade de Ciências e Tecnologia (Campus de Caparica, Portugal).

Chapter 3: Table 3.3. Identification of protein spots was performed by UniMS – Mass Spectrometry Unit, ITQB/IBET (Oeiras, Portugal). Figure 3.13. Validation of 3x-FLAG strains were performed by Dr. Ciaran Finn.

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**Summary**

Without doubt, antimicrobial resistance (AMR) is one of the most serious global challenge for public, animals and environmental health. Tackling this huge issue that is threatening modern medicine, is an urgent priority to regulatory agencies such as the World Health Organization (WHO) and the Centre for Disease Control and Prevention (CDC). The gap in the discovery and development of new antibiotics has turned the attention to alternative approaches, such as the repurposing of already existing drugs. In this Thesis, two different families of compounds were assessed for their potential antimicrobial activity.

In the first section of this thesis, the antibacterial potential of two zinc- and one cobalt- metallic compounds (TS262, TS265 and TS267), previously reported as having antitumor properties, was investigated against Gram -positive and –negative bacteria. The three antitumor compounds had antimicrobial activity against the bacteria tested however, when tested against human cells had an impact on viability.

The second section of this work focused on the mechanism(s) of action of the antipsychotic from the family of phenothiazines, Thioridazine (TZ), using *Salmonella enterica* serovar Typhimurium ATCC14028S as a bacterium model. TZ has been widely studied worldwide due to its attractive antimicrobial activity however, the full mechanism of action of this drug in bacteria hasn't been fully understood. The results initially obtained in this Chapter, revealed that TZ does not seem to have a specific bacterial target. However, when tested *in vitro* against *Salmonella*, the studies conducted provide new insight(s) into the mechanism of action highlighting its effect(s) on the bacterial membrane and consequently in the generation of energy.

Expanding on the potential of TZ to treat *Salmonella*-infected macrophages, the last chapter of this Thesis focused on the effect that TZ has on these cells. It was observed that the drug was able to impact intracellular survival of *Salmonella*, reducing the numbers of internalised bacteria and enhancing the killing activity of the infected macrophage. Similar effect had been previously reported in studies conducted on *M. tuberculosis* and *S. aureus* infected macrophages. These pathogens adapt and live in an intracellular niche. Consequently, compounds capable of reaching the site of infection are of a huge importance to tackle AMR.

Taken together, the results obtained are promising and open an avenue for further studies using these compounds that can lead to the development of effective antimicrobials. Therefore, drug repurposing shows promise as an alternative strategy to fight infections caused by multidrug-resistant bacteria.

**“Science is not only a disciple of reason but also one of romance and passion.”**

**– Stephen Hawking**

**Acknowledgements**

There are a number of people that have been a key part of both this project, and my life the past three years, and none of this would have been possible without them.

Firstly, I would like to express my sincerest thanks to my supervisor Dr. Marta Martins for providing me with the opportunity of a lifetime to work in her lab, and for all her guidance and support throughout the course of my postgraduate studies. I can't express the level of gratitude I have for the endless support and encouragement you have given me these past years. Thank you for always believing in me.

I would also like to thank my PhD thesis committee, Dr. Joan Geoghegan, Dr. Sinéad Corr and Dr. Kim Roberts, for the useful discussions, advice and help during the last three years.

I would like to say a big thank you to our collaborators at Universidade Nova de Lisboa-Faculdade de Ciências e Tecnologias (FCT-UNL) in Portugal. Dr. Alexandra Fernandes and Dr. Pedro V. Baptista for kindly welcoming me into their "home" and allowing me to perform my proteomics work and cell viability assay. Dr. Catarina Roma-Rodrigues, THANK YOU for all your guidance, knowledge, support, friendship and patience during the three months I spent there. Couldn't have done this part of my work without you! A big thank you to all the members of this lab that supported me during the short time I spent with all of you: Luís, Pedrosa, Cynthia, Ana, Veigas, Margarida, Catarina Braz, Rita, Raquel and Andreia.

Thank you to Dr. Ciaran Finn for his insight, helpful Spotify playlist, chats, and advice, especially the knowledge about western blots.

A huge thank you to Jane Twohig for always looking after me, and keeping the lab stocked! To the incredible members of the prep room who work tirelessly: Gerry, Mags, Dave, Miriam, Ronan and Stephen! You keep the house running smoothly and keep everyone in good spirits! A special thank you to Stephen for always making sure my heart was beating. No diapers were needed, I won! Jayne, you're a star. Always ready to help out with anything! Thank you to mama Dee for all the moral support and joy you provided every day! Connie and Noreen, you've always had a kind word to brighten up my day. Thank you!

To my lab partner, Niamh Mohan. What could I say? Thanks, doesn't even begin to cover what I owe you. I know I couldn't have come so far without your support and friendship. From this experience I gained a colleague at first, then a friend and ended up being housemates! Thank you for always listening, letting me cry when I need to let it out of my system and for the unconditional support. My Irish sister!

Amy and Aisling: my ride or die on this journey! Never left my side during these 3 years. This hierarchy is falling apart and soon to be Amy will be on top (RUN!!!!). You have made my days so much better during the last stressful year of this journey. Too much chocolate cookies involved in this process. To Michelle and Marina, two lovely pets to whom I am very grateful! So much to say and I am legit speechless.

To my ex- work husband, Eoin Hurley! You silly, duck. You were one of my biggest rocks here, I hope you know that. Missing our scone breaks!

Stefani, my girl! One of my biggest companies at the weekend in the Moyne! I miss you during this "last stretch". Thank you for all the help, ideas and support.

To rest of all my moynesters: Oisin, Roberto, Marty, Thaina, Mary, Chanelle, German, Brenda, Moh, Mick, Ali, Cândida: Ahhhhh guys THANK YOU! It was an absolute joy sharing the good and bad with all of you during these three years. You made being away from home so much easier. What a wonderful and CRAZY family I got here. I am forever thankful for your support and belief in me.

TJ, my biggest surprise at the end of this adventure! Where were you all this time?! ahah

To my friends back home. Thank you for all the support during this time even when you don't understand what I have being doing. You guys are amazing.

Finally, last but not last: my wonderful and beloved family! You were always there for me with unconditional support, patience, and love (tons of it!). Thank you for always raising my spirits, making me believe in myself and teaching me to not give up. Thank you for always trying to have all the food I love when I go home. Mother you are such a force of nature. There are not enough words in the world to let you know how much I am thankful for all you have done for me, for us! It hasn't been easy for us the last few years especially now that we're apart. BUT WE MADE

IT uhuh. Thank you for never, ever, leaving my side. Francisco, my little baby, my brother, thank you! Dad, I hope one day I can make you feel proud of me. There are lessons on the downside of life. For that, thank you.

To my amazing friend, João. I miss you and I wish you could be here to share this achievement with me. Your support, and joy are missed so much, there are no words in the world that can explain that. Thank you for always making sure I was ok at the beginning of this journey and for always, always making me smile. I hope you are looking down at me and raising a glass with me! I love you and you will always be in my heart ** saudade.

From the bottom of my heart, thank you all!

# Os últimos são sempre os primeiros: a ti família, à minha maravilhosa e gigante família: MIL OBRIGADAS. Obrigada pela paciência, apoio e amor (paletes dele!!) incondicional. Um viva às sardinhas fora de época (e os camarões !!!!) que vocês arranjam só para diminuir a saudade quando vos vou visitar. Sem vocês era dificil manter o astral em cima. Obrigada por sempre me ensinarem "cabeça erguida e siga para frente que é o caminho "! Aos meus avós, o meu coração aperta longe de vocês mas obrigada pelo carinho enorme que eu recebo sempre que me sento ao pé de vocês. Aos meus primos, aos meu eternos protectores, aos mê ricos meninos! Sem vocês e sem o tronco de natal nada seria igual. Aos meus tios e tias (segundos pais e mães), vocês são os maiores 'pá! "Com este copo na mão, eu brindo e brindarei ...." ! Mãe, minha mãezinha, minha Lulu, és um furacão, uma força da natureza inabativel que me inspira todos os dias. Nunca vai haver palavras suficientes neste universo que deêm para expressar o quão eu estou agradecida por todo o teu apoio e pelo que fazes por mim. Estes últimos anos têm sido dificeis, especialmente agora que estamos mais longe uma da outra mas conseguimos! Mais uma batalha vencida uhuh Obrigada por nunca me deixares desistir quando essa era a opção que parecia mais fácil e mais ainda por nunca me deixares sozinha ! Francisco, meu bebé, obrigada maninho por seres a calma do minha tempestade de pessoa. Adoro-te ! Pai, a vida atirou pedras no nosso caminho mas mesmas das lições mais duras da vida temos de estar agracedidos. Espero que um dia consigas te orgulhar de mim.

Do fundo do meu coração, obrigada a todos !

## Preface

This dissertation consists of five chapters, mainly focusing on ways to uncover effective antimicrobials using non-antibiotic compounds.

Chapter 1 provides a review of different compounds that have anti-tumour and antipsychotic activity and the drug repurposing of those compounds as a strategy to fight AMR. It also presents *Salmonella* as a bacterial model of infection that can be used to test the activity of these repurposed compounds.

Chapter 2 focuses on assessing the potential antimicrobial activity of anti-tumour metallic compounds against Gram-positive and –negative bacteria.

Chapter 3 explores the mechanism(s) of action of the antipsychotic Thioridazine (TZ) from the phenothiazines family, using *Salmonella enterica* serovar Typhimurium ATCC14028S as a model bacterium.

Chapter 4 focuses on the effect of TZ on human monocytes and macrophages. The effect of TZ on *Salmonella*-infected human macrophages and the possible mechanism associated to the enhanced killing of these cells was assessed.

Lastly, Chapter 5 concludes this dissertation and discusses final conclusions that can be drawn from the work conducted, as well as suggestions for future work.

## Publications and Communications in Scientific Conferences

### Publications in Peer Reviewed Journals

**Ferreira DA**, Martins LMDRS, Fernandes AR, Martins M. (2020) A Tale of Two Ends: Repurposing Metallic Compounds from Anti-Tumour Agents to Effective Antibacterial Activity. Antibiotics 9, 321. doi:10.3390/antibiotics9060321

Baptista PV, McCusker MP, Carvalho A, **Ferreira DA**, Mohan NM, Martins M and Fernandes AR. (2018) NanoStrategies to Fight Multidrug Resistant Bacteria - "A Battle of the Titans". Front. Microbiol. 9:1441. doi: 10.3389/fmicb.2018.01441

McCusker MP, **Ferreira DA**, Cooney D, Alves BM, Fanning S, Pagès JM, Martins M, Davin-Regli A. (2018) Modulation of antibiotic resistance in clinical isolates of *Enterobacter aerogenes* - a strategy combining antibiotics and chemosensitisers. J. Glob. Antimicrob. Res. 16:187-198 doi.org/10.1016/j.jgar.2018.10.009

### Conferences in Scientific Meetings

#### Oral presentations

**Alves Ferreira, D**., Roma-Rodrigues, C., C., Finn, Baptista, P.V., Fernandes, A.F., Martins, M. Finding gold on old drugs: repurposing of Thioridazine as an effective antimicrobial (2021). European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Vienna, Austria.

**Alves Ferreira, D**., Roma-Rodrigues, C., Finn, C.E., Baptista, P.V., Fernandes, A.F., Martins, M. When "old becomes new" – Drug repurposing to uncover effective antimicrobial (2020). Dublin Academy of Pathogenomics and Infection Biology Meeting, Dublin, Ireland.

**Alves Ferreira, D**., Roma-Rodrigues, C., Baptista, P.V., Fernandes, A.F., Martins, M. When "old becomes new" - Drug Repurposing to uncover effective

antimicrobial (2019). European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Amsterdam, Netherlands.

**Posters**

**Alves Ferreira, D**., Martins, M. Drug repurposing as an effective strategy to treat multidrug-resistant infections: “Teaching old drugs new tricks” (2021). European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Vienna, Austria.

**Alves Ferreira, D**., Roma-Rodrigues, C., Baptista, P.V., Fernandes, A.F., Martins, M. Drug repurposing as an effective strategy to treat multidrug resistant infections – “Teaching old drugs new tricks” (2020). European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Vienna, Austria.

**Alves Ferreira, D**., Roma-Rodrigues, C., Baptista, P.V., Fernandes, A.F., Martins, M. Repurposing Old Drugs to do New Tricks – The use of Thioridazine to treat multi-drug resistant infections (2020). Dublin Academy of Pathogenomics and Infection Biology Meeting, Dublin, Ireland.

**Alves Ferreira, D**., Roma-Rodrigues, C., Baptista, P.V., Fernandes, A.F., Martins, M. Repurposing Old Drugs to do New Tricks – The use of Thioridazine to treat multi-drug resistant infections (2019). Early Career Microbiologists’ Forum Summer Conference, Dublin, Ireland.

**Alves Ferreira, D**., Roma-Rodrigues, C., Baptista, P.V., Fernandes, A.F., Martins, M. Old drugs learn new tricks (2019). Microbiology Society Annual Conference 2019, Belfast, UK.

**Alves Ferreira, D**., Roma-Rodrigues, C., Baptista, P.V., Fernandes, A.F., Martins, M. New tricks for old drugs - Uncovering the mechanism of action of thioridazine in *Salmonella* Typhimurium (2018). ESCMID/ASM Conference on Drug Development to Meet the Challenge of Antimicrobial Resistance, Lisbon, Portugal.

**Alves Ferreira, D**., Martins, L.M.D.R.S., Fernandes, A.F., Martins, M. Drug Repurposing of Zinc and Cobalt Organometallic compounds to uncover effective

antibacterial (2018). ESCMID/ASM Conference on Drug Development to Meet the Challenge of Antimicrobial Resistance, Lisbon, Portugal.

**Alves Ferreira, D**., Martins, L.M.D.R.S., Fernandes, A.F., Martins, M. Drug Repurposing of Zinc and Cobalt Organometallic compounds to uncover effective antibacterial (2018). European Congress of Clinical Microbiology and Infectious Diseases, Madrid, Spain.

**Alves Ferreira, D**., Martins, L.M.D.R.S., Fernandes, A.F., Martins, M. Antibacterial activity of repurposed zinc organometallic compounds – from anticancer to effective antimicrobial against *E. coli* (2017). Focused Meeting 2017 Microbiology Society: Antimicrobial Resistance and One Health, Maynooth, Ireland.

**Alves Ferreira, D**., Martins, M. New tricks for old drugs – Revealing the mechanism of action of thioridazine in *Salmonella* (2017). Focused Meeting 2017 Microbiology Society: Antimicrobial Resistance and One Health, Maynooth, Ireland.

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**List of Abbreviations**

| | | |
|---|---|---|
| ° | °C | Degrees Celsius |
| µ | µM | Micromolar |
| 2 | 2-DE | Two - dimensional electrophoresis |
| A | AMP | Ampicillin |
| | AMR | Antimicrobial resistance |
| | ATCC | American Type Culture Collection |
| | ATP | Adenosine triphosphate |
| | ATR | Acid tolerance response |
| B | Bp | Base pair |
| | BTEC | Bronchial/tracheal epithelial cells |
| C | CCB | Calcium Channel Blockers |
| | CCCP | Carbonyl cyanide *m*-chlorophenylhydrazone |
| | CE | Common Era |
| | CFU | Colony forming units |
| | CGM | Complete growth media |
| | CHQ | Chloroquine |
| | CLSI | Clinical Laboratory Standards Institute |
| | $CO_2$ | Carbon dioxide |
| | CPZ | Chlorpromazine |
| | CXCL | Chemokine (C-X-C motif) ligand |
| E | EEA | European economic area |
| | ELISA | Enzyme-Linked Immunosorbent Assay |
| | EPs | Efflux pumps |
| | EPIs | Efflux pump inhibitors |
| | EU | European Union |
| F | FDA | Food and Drug Administration |
| G | GDP | Gross domestic product |
| H | HBSS | Hank's Balanced Salt Solution |
| | HGT | Horizontal gene transfer |
| | HRP | Horseradish peroxidase |
| I | IFN | Interferon |
| | Ig | Immunoglubolin |

| | | |
|---|---|---|
| | IL | Interleukin |
| K | Kan | Kanamycin |
| | kb | Kilobase |
| L | LA | Luria-Bertani agar |
| | LB | Luria-Bertani broth |
| | LOX | Loxapine |
| | LPS | Lipopolysaccharides |
| M | MBC | Minimum Bactericidal Concentration |
| | MEM | Eagle's Minimal Essential Medium |
| | mg | Milligram |
| | MIC | Minimum Inhibition Concentration |
| | mL | Millilitre |
| | MOI | Multiplicity of Infection |
| | MRSA | Methicillin-resistant *Staphylococcus aureus* |
| | MTS | 3-(4,5-dimethylthiazol-2-yl)-5-(3-caarboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt |
| N | NO | Nitric oxide |
| | NTS | Nontyphoidal *Salmonella* |
| O | OD | Optical density |
| | OMP | Outer Membrane Protein |
| | p.i. | Post infection |
| P | PBMDMs | Peripheral blood monocyte-derived macrophages |
| | PBPs | Penicillin-binding proteins |
| | PMBC | Primary blood mononuclear cells |
| | PBS | Phosphate buffered saline |
| | PCR | Polymerase chain reaction |
| | pI | Isoelectric point |
| | PMF | Proton motive force |
| | PM-M | Phenotypic microarray – mammalian |
| R | ROS | Reactive oxygen species |
| | rpm | Rotations per minute |
| | RPMI | Roswell Park Memorial Institute |
| S | SCV | *Salmonella*-containing vacuole |
| | SD | Standard deviation |

| | | |
|---|---|---|
| | SDS | Sodium dodecyl sulphate |
| | SDS-PAGE | Sodium dodecyl sulphate poly-acrylamide electrophoresis |
| | SOB | Super optimal broth |
| | SPI | *Salmonella* Pathogenicity island |
| | STRING | String Tool for the Retrieval of Interacting Genes/Protein |
| T | T3SS | Type III secretion system |
| | TB | Tuberculosis |
| | TMB | 3,3',5,5'-Tetramethylbenzidine |
| | TNF | Tumour necrosis factor |
| | TZ | Thioridazine |
| V | VER | Verapamil |
| | VGCC | Voltage gate calcium channels |
| W | WHO | World Health Organization |
| | WT | Wild Type |
| X | XDR-TB | Extensively Drug-Resistant Tuberculosis |

# 1. General Introduction

## 1.1. Antibiotics and Antimicrobial Resistance (AMR)

### 1.1.1. Definition of antimicrobial compound

Antimicrobial compounds are, by definition, a natural or synthetic substance with the capacity to kill (bactericidal) or inhibit (bacteriostatic) the growth of susceptible microorganism. These compounds can be classified as antivirals, antifungal, antiparasitic and antibacterial (antibiotics). The term antibiotic can be traced back to 1890. Paul Vuillemin used the word *antibiose* to describe the biological relationship in which "one living organism kills another to ensure its own existence" (Vuillemin, 1889). Later, Selman Waksman in 1941 introduced the term "antibiotic" as "any small molecule, produced by a microbe, with antagonistic properties on the growth of other microbe" (Clardy, Fischbach & Currie, 2009). Nowadays, the term has a broader meaning, in the sense that it includes naturally produced or synthetic compounds that fight bacterial infections in humans and animals.

### 1.1.2. Origin of antimicrobial compounds

Antimicrobial compounds are probably one of the most successful forms of chemotherapy in the history of medicine. They have been used to treat humans since primordial times. There is historical evidence that earliest civilisations used natural available treatments for infections such as moulds and plant extracts (Nelson *et al*., 2010; Pećanac *et al*., 2013). Traces of tetracycline have been found in human skeleton remains from ancient Sudanese Nubia dating back to 350-550 CE[1] - pre-antibiotic era (Bassett *et al*., 1980).

It wasn't until the late 19$^{th}$ century that scientists began to observe the action of antimicrobial compounds. The first antimicrobial compound described was a chemical compound synthesised by Paul Ehrlich in 1909. Arsphenanime (Salvarsan®) was an arsenic derivative, active against syphilis, a disease caused by the bacterium *Treponema pallidum* (Ehrlich & Bertheim, 1912; Ferrie, 2014). Until the discovery of penicillin, this was the most common prescribed compound at that time (Ferrie, 2014).

[1] CE = Common Era

The revolutionary era of modern medicine started with the discovery of penicillin by Sir Alexander Fleming in 1928 (Fleming, 1929). This accidental discovery made by Fleming was done when he was returning from his holidays and found a halo of inhibition of bacterial growth in a *Staphylococcus aureus* plate that was contaminated with a fungus (*Penicillium notatum*). This observation was very important since it demonstrated that some microorganisms could produce substances that inhibit the growth of other microorganisms. However, it was only in 1940, that Howard Florey and Ernest Chain were able to purify enough quantities of penicillin for clinical testing leading to mass production and commercialisation of the antibiotic. This revolutionary discovery had a huge impact on the number of casualties in World War II, making this the first war in which wound infections were not the major cause of amputations and death (Quinn, 2013). The introduction of this antibiotic into treatment initiated the "Golden Age" of antibiotics, recognised as one of the greatest advances in modern medicine. The "Golden Age" of antibiotics took place in the period between 1940 and the 1960 (Walsh & Wencewicz, 2014). During these glorious years the most important antibiotics were discovered, including penicillins, cephalosporins, tetracyclines, aminoglycosides, chloramphenicol, macrolides and glycopeptides (Aminov, 2010; Durand, Raoult & Dubourg, 2019). Since then, there has been a void on the development of new antibiotics with no new structural classes of antibiotics introduced in the market. The development of new drugs has been focused on extensive modification of existing natural drugs and complete chemical synthesis. It was only in 2000 that a few novel classes of antibiotics (natural and synthetic) started to be reported, namely, oxazolidinones (2000), lipopeptides (2003) (Alder, 2005), and diarylquinolines (2012) (Butler, Blaskovich & Cooper, 2013; Renwick *et al*., 2016). However, these three classes only target Gram-positive bacteria.

Based on their structure and mode of action, at least nine major groups of antibiotics have been described. These include β-lactams (inhibit cell wall synthesis), aminoglycosides (inhibit protein synthesis), macrolides (inhibit protein synthesis), tetracyclines (inhibit protein synthesis), daptomycin (affects the cell membrane function), platensimycin (inhibit fatty acid biosynthesis), fluoroquinolones (inhibit DNA synthesis), carbapenems and glycopeptides (inhibit cell wall synthesis).

Most of these antibiotics introduced within the last 20 years are semi-synthetically derived. Recently, a team from Hong Kong, China, reported a novel antimicrobial class candidate, Nusbiarylins, for the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) (Qiu *et al*., 2019). However, the research is in the stage of animal studies before being able to enter clinical trials on humans.

There are new antimicrobial compounds in the pipeline, however, the numbers of compounds being developed are not enough to replace the ones that no longer work (World Health Organization, 2019a,b,c). The lagging observed on the development pipeline for new antibiotics demonstrates how reluctant industry has become to the development of these drugs due to the significant obstacles encountered during the process.

### 1.1.3. Use of antimicrobial compounds, spread of bacterial resistance and the One Health Approach

Modern medicine brought to humanity a vast number of benefits, not only the increase of life expectancy but also benefits in the production of animals for human consumption and animal health. The extensive use of antimicrobials with different applications has been suggested as one of the issues for reported increase in antibiotic resistance, as the selective pressure induced in the environment is much stronger now than on previous times.

It is well recognised that the use of antimicrobials can be vital for both human and animal health. About 30 different antibiotics have been used in food and aquaculture. Antimicrobials are widely used in veterinary medicine; to accelerate and increase the size of animals (in certain countries, excluding the European Union); fish and seafood farming and also domestic uses (Meek, Vyas & Piddock, 2015). The misuse of antimicrobials for these different applications is often associated with potential risk of emergence and increased spread of AMR (**Figure 1.1**). Bacteria are constantly being exchanged between humans and animals. Over one hundred different antimicrobial compounds have been used in food-producing animals with different ends such as, therapy, growth promotion and disease prevention. The use of antibiotics to enhance livestock growth of animals was banned in Europe since early 2000s (Casewell *et al*., 2003), only being allowed to be used for veterinary

purposes. However, the United States and other countries still use antibiotics for this purpose.

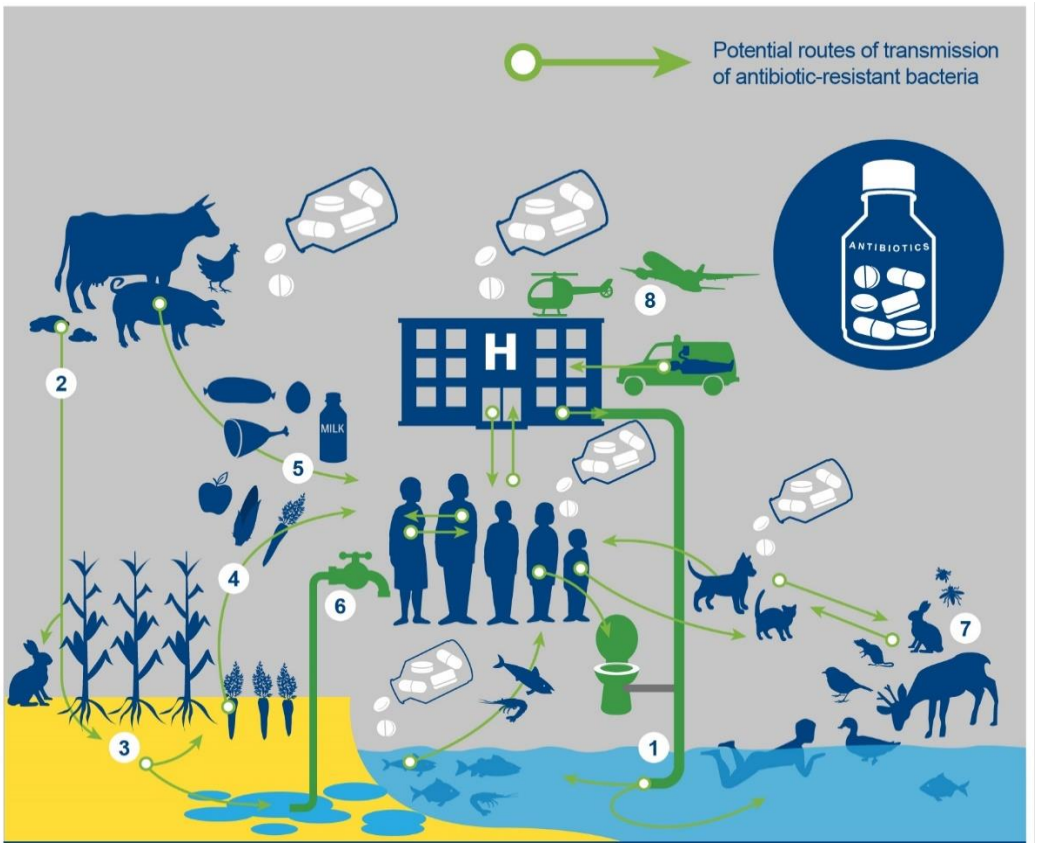


**Figure 1.1. Representation of diverse applications of antimicrobial compounds and their contribution to the spread of antimicrobial resistance (AMR).** When exposed to antibiotics bacteria evolve in order to survive. Then, they can (**1**) spread into the environment through different routes, for example, water sanitation systems and wastewater treatment facilities. (**2**) Misuse of antibiotics on animals contributes to the spread of resistance using (**3**) animal manure for agriculture applications and ultimately, being inserted in the food chain (**4 and 5**) for both animals and humans. (**6**) Wastewater treatment facilities are unable to entirely remove antibiotic resistant bacteria that constitute a route for the spread of resistance. Due to the antibiotic resistance in the environment, (**7**) wildlife is turned into a source and/or carrier of antibiotic resistant bacteria. (**8**) Hospital environment and discharges, tourism, migration and importation are reported to contribute to the spreading of this health issue. (Source: https://amr.biomerieux.com/en/challenges/from-farm-to-food-to-people-one-health/)

The One Health approach is a worldwide approach that aims to recognise that the human health is closely connected to the health of animals and our shared environment (World Health Organization, 2015). Despite the surveillance and regulation through the One Health approach, the use of antimicrobials in human and animal is still too excessive (Collignon, 2013; Meek, Vyas & Piddock, 2015; Van Boeckel *et al*., 2015).

Some zoonotic pathogens such as *Salmonella* are considered foodborne bacteria. The presence of resistant zoonotic bacteria in animals and food compromises the effective treatment of infectious diseases in humans. Data released from the European Centre for Disease Prevention and Control (ECDC) and the European Food Safety Authority (EFSA) in 2017, showed increasingly higher numbers of *Salmonella* resistant to fluoroquinolones. 28.3% of *Salmonella* Typhimurium in humans was found to be multidrug resistant and in animals too (EFSA (European Food Safety Authority) & ECDC (European Centre for Disease Prevention and Control), 2017). The One Health approach focus on reducing the number of antibiotics used in agriculture and food animals through regulations and laws.

Another application of antimicrobials is the use of heavy metals in animal food supplements and biocides on agricultural soils. Biocides and heavy metals contributed to the spread of AMR (Hernando-Amado *et al*., 2019). This contribution is due to the ability for co-selection of resistance to ampicillin, chloramphenicol and tetracycline (Stepanauskas *et al*., 2006; Peltier *et al*., 2010; Fang *et al*., 2016).

As it is known, not all antimicrobial compounds are metabolised by animals. Thus, some will be excreted through the urine and faeces of the animals entering wastewater treatment facilities. Similar events happen with human activity (FAO & WHO, 2019). Therefore, these types of water treatment facilities can constitute a source of resistant bacteria that will normally spread into the environment. Hospitals and their effluents have been reported as significant contributors to the spread of multidrug resistant bacteria (Berendonk *et al*., 2015; Hocquet, Muller & Bertrand, 2016). This type of effluent can be directly discharged in the sewage without any prior treatment, thus representing an important source of antibiotics in the wastewater treatment facilities (Carvalho & Santos, 2016). Although some countries have regulations for the pre-treatment of hospital effluents, the European Directive

91/271/EEC foresees no specific restrictions for discharges of this type of effluents (Council of the European Union, 1991). Taken together, effluents from different sectors (e.g. agriculture, hospitals, households) are directed to wastewater treatment facilities. These effluents contain large numbers of resistant bacteria adapted to sub-inhibitory concentrations of antimicrobial compounds, contributing to resistance. This is a global issue, although even more problematic in low income countries where sewage and water treatment infrastructures are usually lacking or if present are not well-developed due to the lack of main infrastructures and resources (Walsh *et al*., 2011; Nadimpalli *et al*., 2018).

Other factor that can drive resistance is the misuse of antibiotics, resulting in the widespread development of resistance by several bacterial species. In order to tackle AMR, it is vital to understand how bacteria can survive the action of antimicrobial compounds. Understanding the different applications and or uses of antimicrobial compounds in different settings will allow us to understand why there is a continually increase on AMR.

### 1.1.4. Mechanisms of antimicrobial resistance in bacteria

The development of resistance to antibiotics by bacteria should not have come as a surprise. On his Nobel Lecture in 1945, Sir Alexander Fleming, alerted for the possibility of the misuse of antibiotics and consequently, the associated resistance: “The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant.” Approximately 3.5 billion years of evolution have given bacteria the ability to evolve and fight back and this is a clear example of natural selection supporting Darwin’s theory of survival. During the late 1950s (during the “Golden Age” of antibiotics), resistance to several antimicrobial agents was reported for the very first time. This is one of the main problems the world faces with the treatment of infectious diseases since soon after a new antibiotic is introduced, resistance to it will arise (**Figure 1.2**).

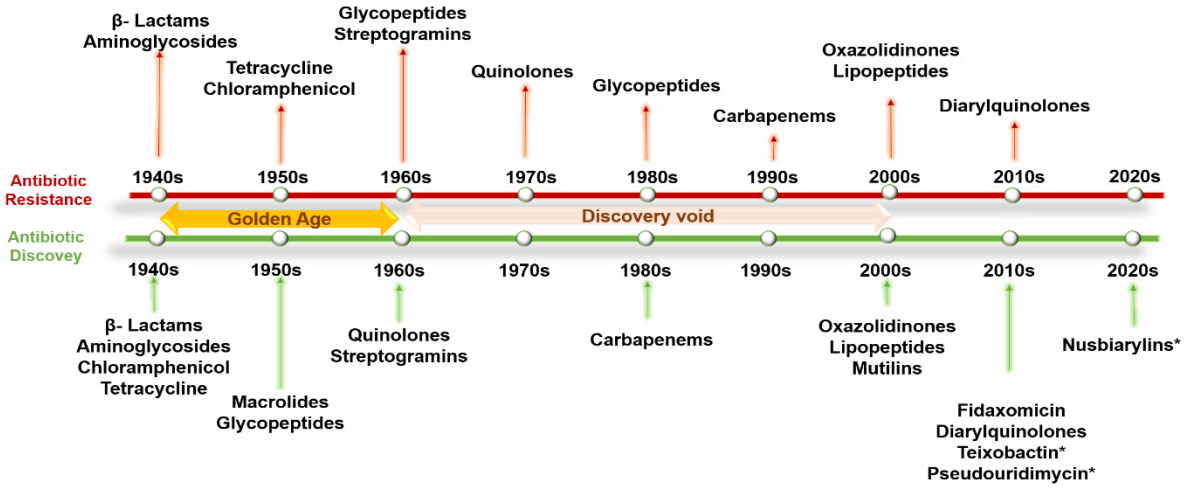


**Figure 1.2. Timeline of the 'Golden Age' of antibiotics and its reported resistance.** Green line indicates the discovery of a new class of antibiotics and red line indicates the first resistance described to each class of antibiotic. From 1960s to 2000s a discovery void was observed on the development of a new class of antibiotics and increase on antibiotic resistance observed to each antibiotic of each class. *waiting FDA approval. (Walsh & Wencewicz, 2014; Center for Disease Control and Prevention |CDC, 2019; Maffioli *et al*., 2019; Iyer, Madder & Singh, 2019).

The mechanism of action of an antimicrobial is associated with the mechanisms of resistance developed by the bacteria to counteract it (**Figure 1.3**). There are several fundamental mechanisms of resistance developed by bacteria and these can occur due to: (i) antibiotic modification or degradation; (ii) alteration of bacterial proteins that are antimicrobial targets; (iii) changes in the membrane permeability and/or overexpression of efflux pumps (EPs); and iv) horizontal gene transfer (HGT) (Allen *et al*., 2010; Boto & Martínez, 2011).

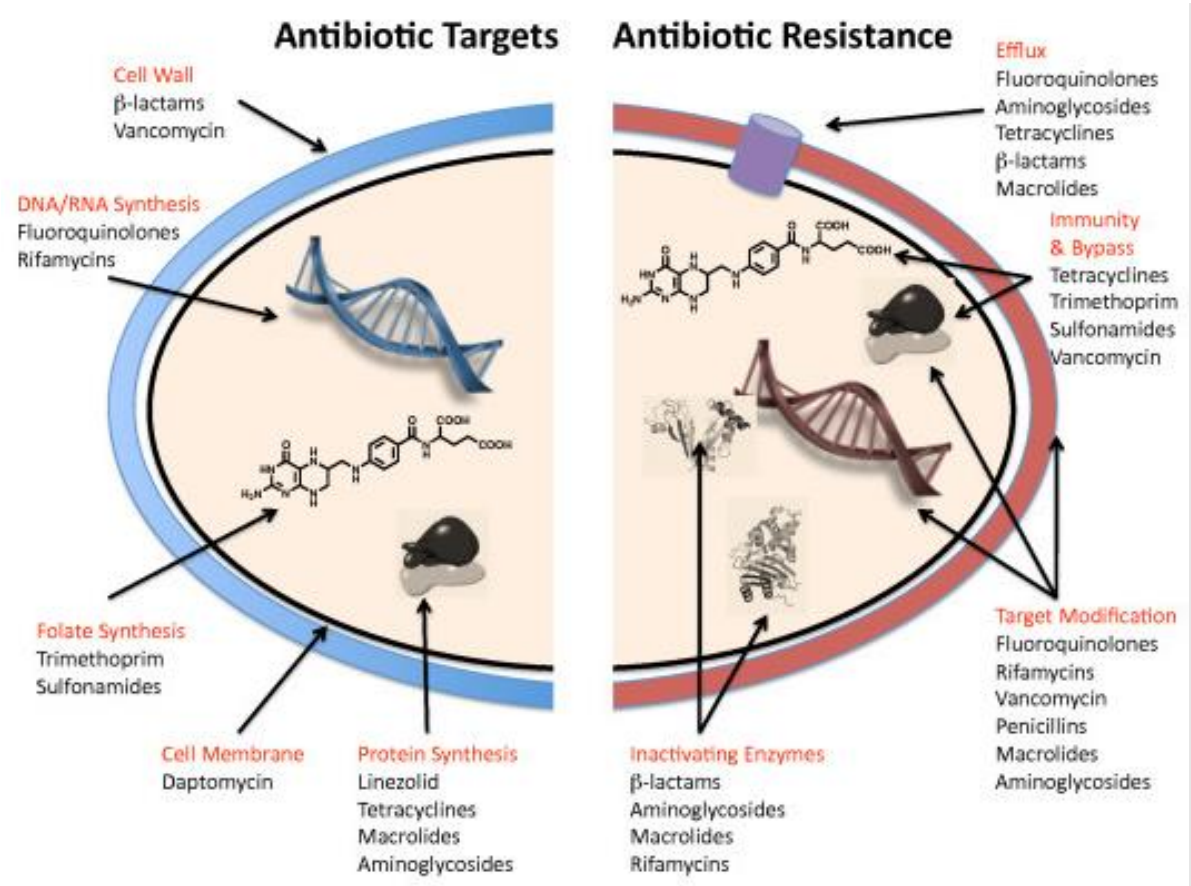


**Figure 1.3. Summary of antimicrobial targets and mechanisms of resistance in bacteria.** The blue side indicates the targets of each class of antibiotics and the red side corresponds to the respective bacterial mechanisms of resistance (Wright, 2010).

### (i) Antibiotic Modification or Degradation

Antibiotic modification or degradation is one of the most common strategies used by bacteria to adapt to the presence of the antimicrobial. The bacteria can destroy the antibiotic before it reaches its targets. This could be achieved by producing enzymes that chemically modify the antibiotic to a form that is unable to interact with its target. Some of the enzymes and the frequent biochemical reactions they catalyse include adenylation, phosphorylation and acetylation such as β-lactamases, aminoglycoside-modifying enzymes, or chloramphenicol acetyltransferases. Besides the chemical modification, enzymes can also destroy the antibiotics (Peterson & Kaur, 2018), for example through the production of extended-spectrum β-lactamases (ESBL), a common mechanism of resistance

against β-lactams antibiotics. This class of antibiotics is extremely important to treat bacterial infections and bacteria that produce ESBL are a major concern at the moment. Other approach that bacteria use is to modify their ribosome's components to avoid binding of the antibiotics to the ribosomes (Rao *et al*., 2008).

### (ii) Alteration of bacterial proteins that are antimicrobial targets

Bacteria have evolved different mechanisms to overcome the action of antibiotics. By modifying the target site, bacteria decrease the affinity of the antimicrobial for the target site. This can occur on several classes of antibiotics, including β-lactams, glycopeptides, macrolides, lincosamides, streptogramins (MLS) and aminoglycosides. These target modifications can consist of: i) enzymatic alterations of the binding site; ii) mutations in genes encoding the target site; and iii) replacement of the original target (Peterson & Kaur, 2018). For example, resistance to β-lactams can be due to alterations in the structure of penicillin-binding proteins (PBPs). PBPs have an important role on transpeptidation and transglycosylation of peptidoglycan units emerging from the bacterial cytoplasm. This is very common among strains of *Streptococcus pneumoniae*. In contrast, *S. aureus* acquires new low-affinity PBP, rather than altering the structure of existing PBPs. Methicilin resistance is due to the production of an additional PBP, designated PBP2a, which has a reduced affinity for penicillin and β-lactams in general. PBP2a acts as a substitute for the other PBPs, contributing to the transpeptidase activity. The low-affinity PBP2a is assumed to take over the cell wall biosynthetic functions of normal PBPs in the presence of β-lactam antibiotics, which rapidly acylate (and inactivate) each of the four native staphylococcal PBPs at concentrations that are far below the minimum needed to inhibit the growth of most MRSA strains (Alekshun & Levy, 2007; Fishovitz *et al*., 2014).

### (iii) Changes in the membrane permeability and/or overexpression of EPs

Bacteria have developed mechanisms to avoid the antibiotic from reaching its intracellular or periplasmic target. Majority of the antibiotics have intracellular targets, or these are located in the inner membrane (case of Gram-negative bacteria). This

mechanism is a key one for Gram-negative bacteria since the outer membrane acts as the first line of defence against the entrance of noxious compounds. By this manner, by decreasing the membrane permeability the bacteria are able to block the entry of the antibiotic. This influx is controlled by different type of porins (non-specific or specific channels or selective pores). Most porins involved in the transport of antibiotics belong to the outer membrane proteins (OMP) F and C subfamilies (also called "classic porins"). Alteration of porins in response to antibiotics could be achieved by 3 different processes: i) change in the level of the expression of the porin; ii) shift in the type of porins expressed; and iii) impairment of the porin function (Pagès, James & Winterhalter, 2008). This mechanism is important for Gram-negative bacteria offering an intrinsic mechanism for protection against hydrophilic antibiotics. For example, resistance to vancomycin (a glycopeptide) in Gram-negative bacteria results from the lack of penetration of this natural barrier (outer membrane) imposing a permeability barrier. Changes on the permeability often results in low-level resistance and are associated with other mechanisms, such as the over-expression of EPs.

EPs are transport proteins localised and imbedded in the plasma membrane of the bacterium that are involved in the extrusion of noxious compounds to the external environment. Several EPs have been described in Gram-negative and –positive bacteria, generally belonging to one of the five families or superfamilies: ATP-binding cassette (ABC) superfamily, Major Facilitator Superfamily (MFS), Resistance Nodulation Cell Division (RND) superfamily, Multidrug and Toxin compound Extrusion (MATE) family and Small Multidrug Resistance (SMR) family (Anes *et al*., 2015). EPs can utilise different sources of energy for their function. ABC transporters use adenosine triphosphate (ATP) as the energy source for extrusion of toxic compounds (Moitra *et al*., 2011; Du *et al*., 2018), whereas MATE pumps are driven by $Na^+/H^+$ drug antiport systems (Alvarez-Ortega *et al*., 2013). The MFS, SMR, and RND pumps are driven by proton motive force (PMF), which means that these types of EPs are dependent on the pH gradient (Pages, Amaral & Fanning, 2011; Du *et al*., 2018).

The most clinically relevant EPs in Gram-negative bacteria belong to the RND superfamily. The EPs of the RND superfamily are responsible for the extrusion of most noxious compounds and confer resistance to a range of antibiotics such as

tetracyclines, chloramphenicol, β-lactams and fluoroquinolones. Among the EPs from this family, the AcrAB-TolC system is one of the best studied. This system is classically found in *Escherichia coli*, *Salmonella enterica* serovar Typhimurium and *Enterobacter aerogenes* (Amaral *et al*., 2012a). It is composed of a transporter protein located in the inner membrane (AcrB); a linker protein located in the periplasmic space (AcrA); and a protein channel located in the outer membrane (TolC).

It is important to refer that antimicrobials as well as cellular metabolites that are prejudicial to the bacterium can be expelled through EPs (Martins, Hunyadi & Amaral, 2013; Blanco *et al*., 2016). EPs can be involved in bacterial resistance to these compounds at different levels. Some EPs are constitutively expressed at low levels and can contribute to intrinsic resistance. However, if EPs are overexpressed, this may promote higher levels of resistance. This overexpression can be transient, when in the presence of an effector, designated phenotypic resistance; or can be constitutive when mutants in regulatory elements of the expression of EPs are selected and this is referred to as acquired resistance. Hence, overexpression of EPs results in an MDR phenotype posing serious difficulties to the treatment of bacterial infections.

### (iv) Horizontal gene transfer (HGT)

Genes related with antibiotic resistance may be present on commensal bacteria or on environmental microbiota. The acquisition of foreign genetic material from other bacteria, *via* horizontal gene transfer (HGT) is directly involved on bacterial evolution. Although, a bacterium is capable to establish antibiotic resistance through spontaneous mutations, development of MDR would take a long time if it only relies on self-adaptive mutation (Salverda *et al*., 2017). Plasmids, bacteriophages, and extracellular DNA are the three primary drivers of HGT through the process of conjugation, transduction and transformation, respectively. Natural products found in the environment are the base for most of the antimicrobial compounds used in clinical practice (Boto & Martínez, 2011). Bacteria sharing the environment with these natural compounds shelters intrinsic genetic determinants of resistance. Therefore, the environment constitutes one of the biggest prolific sources

for acquisition of antibiotic genes in clinically relevant bacteria (Boto & Martínez, 2011). Conjugation is considered a very efficient and the main recognised mechanism responsible for genetic material transfer in bacteria, especially in hospital environments and aquaculture (Lerminiaux & Cameron, 2019). Conjugation involves cell-to-cell contact and occurs at high rates in the gastrointestinal tract of humans under antibiotic treatment.

### 1.1.5. Social and economic burden of antimicrobial resistance

The burden of AMR has significant impacts worldwide, namely in public health settings (O' Neil, 2014); economy and development (World Bank, 2017). In 2014, the United Kingdom (UK) Prime Minister, Mr. David Cameron MP, and the Wellcome Trust, commissioned a report to review the incidence of AMR and its impact on healthcare and gross domestic product (GDP). The British economist Jim O'Neil lead the team that performed this review. On this report it was estimated that unless action is taken, the burden of death from AMR could heighten to 10 million lives each year by 2050; more people than the ones that currently die from cancer (O' Neil, 2014). A study based on data from the European Antimicrobial Resistance Surveillance Network (EARS-Net), showed that 33,000 people die every year due to AMR (Cassini *et al*., 2019). This study also indicates, that 39% of the burden is caused by bacteria resistant to last-line antibiotics. This shows quite a worrying scenario for healthcare and economics not just in EU/EEA but worldwide. Economically, Jim O'Neill's prediction means a reduction by between 2 to 3.5% of the world GDP due to a reduction of population and of morbidity and this ultimately would cost up to 100$ trillion per year (O' Neil, 2014). EU expenditure on annual treatment and social costs are estimated at some €1.5 billion (EFSA (European Food Safety Authority) & ECDC (European Centre for Disease Prevention and Control), 2017). The societal burden of such events will have serious consequences wherein these untreated bacterial infections will exclude large numbers of working individuals from the workforce for extended periods of time. In addition, the increased burden of caring for those who are incapacitated will place additional burdens on their families, communities, and the healthcare system itself (Michael, Dominey-Howes & Labbate, 2014). Such events have a rippling societal and cultural effect that will ultimately have a global impact. By 2030, AMR could force up to 24 million people into extreme

poverty, as increased treatment costs drain funds, morbidity and mortality affect the possibility to make a living and both of these drive inequality (Interagency Coordination Group on Antimicrobial Resistance (IACG), 2019). It is also estimated that by the same year (2030) the use of antibiotics in food-producing animals will increase up to 67% in the most populated countries of the world (Brazil, China, India, Russia and South Africa (Boto & Martínez, 2011). The antibiotics used for veterinary medicine increased from 11.45$ billion (~10.38€ billion) over an 8-year period (2002-2010) to approximately 42$ billion in 2018, which contradicts the recommendations of major health agencies (O' Neil, 2014; Center for Disease Control and Prevention |CDC, 2019).

The development of new antimicrobials also has an economic impact on the society. The main costs for development of a new compound is estimated to be 500 – 800$ million (~ 453 -725€ million). Aside, the entire process of developing new compounds could go to 17 years (Norrby *et al*., 2005). More and more pharmaceutical industries are decreasing the number of groups researching new antibiotics. Large pharmaceutical industries including Merck, Aventis, GlaxoSmithKline, Bristol-Myers Squibb, Abbot Labs are some of the companies leaving or reducing research on this subject (Barrett, 2005). Small to medium-sized enterprises (SMEs) have done most of the contributions for the pre- and -clinical development for new antimicrobial drugs. Besides the challenge of developing novel drugs, unsatisfying commercial returns are making some pharmaceutical industry to re-direct their research from antibiotics to more profitable and rewarding areas. Some of these areas such as cancer; diabetes; *etc*, rely on drugs used for extended periods of time and not short-term treatments, such as the ones required for antibiotic therapy.

## 1.2. Drug repurposing as an alternative approach to uncover effective antimicrobials

The increasing emergence of AMR has led to an increased focus on novel antimicrobial compounds or approaches to overcome the threat of infectious diseases. Given the expense of developing a novel antibiotic, in some cases, drug repurposing has become a more attractive approach. Drug repurposing can also be called drug repositioning, reprofiling or re-tasking (Langedijk *et al*., 2015). This is a

strategy for identifying new uses for approved or investigational drugs that are outside the scope of the original medicinal indication. This approach has major advantages over *de novo* drug discovery since the safety, pharmacological properties and potential toxicity of the compounds are already tested and known. One of the most successful case that followed this approach is sildenafil. This drug was originally developed to treat hypertension and was then repurposed for treatment of erectile dysfunction and pulmonary arterial hypertension (Norrby *et al*., 2005). Another successful example is thalidomide, originally developed for morning sickness and gaining a new purpose for treatment of leprosy and myeloma (Norrby *et al*., 2005). These successful cases highlight that the repurposing of drugs could reduce the high costs involved in the development of new antibiotics. However, despite the advantages that this approach offers, only a few have been successfully approved for new purposes, such as anti-malarial, antifungal or antibacterial (Farha & Brown, 2019).

### 1.3. Metallic compounds as a source of new antibacterial to fight AMR

Metallic complexes are currently in clinical development for the treatment of cancer, malaria and neurodegenerative diseases and have been used prior to the antibiotic era in medicine and agriculture (Gasser, Ott & Metzler-Nolte, 2011). As an example, since the Persian kings, cobalt (Co) and silver (Ag) had been used in vessels for water disinfection and food preservation. Greeks, Romans, Egyptians and Phoenicians followed this practice too. Ag was used to prevent eye infections in newborns and also prevention of infection in surgical wounds (Aarestrup & Hasman, 2004; Lemire, Harrison & Turner, 2013). Metallic complexes are very different from conventional antibiotics. Antibiotics usually act on specific biochemical processes in the bacteria while metals Co, Ag, and others, target multiple cellular processes leading to pleiotropic effects on the bacteria (Lemire, Harrison & Turner, 2013). The metals that have been considered for antimicrobial agents are zinc (Zn), Co, copper (Cu), Ag, thallium (Tl), gallium (Ga), tungsten (W), mercury (Hg), chromium (Cr), nickel (Ni) and titanium (Ti) (Aarestrup & Hasman, 2004; Lemire, Harrison & Turner, 2013).

While some metal complexes and metal ions have been shown to possess excellent antimicrobial activity, the question remains whether metals offer any

significant advantages over purely organic compounds. Although metallic antimicrobial compounds might hold a promise, their potential toxicity for the host limits their current clinical use. Nevertheless, their toxicity can be advantageous in certain cases since this is the reason why these compounds are used in cancer therapy. Nevertheless, understanding the potential of these compounds for the design of novel drugs is important. These compounds can damage the cell wall of bacteria through the release of ions or/and, by i) direct transport and/or ii) co-transport with low-molecular-mass ligands. This can also result on oxidative stress and protein dysfunction (Lemire, Harrison & Turner, 2013).

One of the successful examples of this type of compounds is ferroquine. This compound is a derivative from chloroquine (CHQ) that due to its activity is an attractive antimalarial candidate. Studies conducted with this compound demonstrated activity against a CHQ-resistant strain of *Plasmodium falciparum* (*in vitro*) and *P. berghei* N. and *P. yoelli* Ns (*in vivo*) (Biot *et al*., 1997; Delhaes *et al*., 2002). In 2010, Tella *et al.*, reported a synthesis of metal complexes of sulphadimidine with antimicrobial activity against *E. coli*, *S. aureus* and *Klebsiella pneumoniae* but with no toxicity testing performed (Tella & Obaleye, 2010). Another example is Zn and its application on acrylic cement; a material commonly used to treat fractures in spine. In this study, Clarkin *et al*., developed a novel glass polyalkenoate cement containing Zn. The results showed antibacterial effect against *E. coli* and *P. aeruginosa*, bacteria relevant to infections of the spine (Clarkin *et al*., 2011). Recently, *Frei et al*., screened for antimicrobial activity of 906 metal complexes compounds through the Community for Open Antimicrobial Drug Discovery (CO-ADD). These authors reported that from the compounds tested, 88 had activity against most of the bacterial strains tested and with no toxicity to mammalian cell lines. Tested bacterial strains included pathogens from the ESKAPE pathogen list such as *E. coli*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and MRSA. These pathogens are the leading cause of healthcare-associated infections worldwide. The 88 compounds included on their structure manganese (Mn), Co, Zn, ruthenium (Ru), Ag, europium (Eu), iridium (Ir) and platinum (Pt) (Frei *et al*., 2020). Unfortunately, very little attention has been given to the development of metallic antibacterial compounds. Due to the toxicity obtained for some of these metallic compounds, its encapsulation (or vectorisation) into nanoparticles (NPs) may be a promising solution. NPs vehicles can enter host cells *via* endocytosis which

facilitates their intracellular entry. Interaction of NPs with bacteria generally triggers oxidative stress mechanisms, enzymatic inhibition, protein deactivation and changes in gene expression. Another approach being explored is the adsorption of antibiotics on the metallic NPs surface allowing for the delivery of active ingredients to target sites. Some of the antibiotics can also be conjugated onto the NPs shell to initiate potential bactericidal activity. NPs can be in the future a good approach to reduce the toxicity normally associated to some antibiotics or metal complexes (Gasser, Ott & Metzler-Nolte, 2011).

## 1.4. Phenothiazines as promising candidates for drug repurposing

Phenothiazines have been reported as an excellent family of compounds for drug repurposing as antimicrobials. The phenothiazines are an important class of synthetic and heterocyclic compounds widely used in medicinal chemistry as they exhibit a wide range of biological effects. They have two aromatic rings (pheno) linked by a third ring that contains a sulphur atom (Thio; position 5) and a nitrogen atom (azo; position 10). The length of the linking alkyl connector, the terminal amine, as well as substituents at the carbon atom at position 2 (C-2) of the tricyclic ring, determines the activity of these compounds (Jaszczyszyn *et al*., 2012). More precisely, at the nitrogen position 10, the attached side chain determines the activity and the substituent at the C-2 has an impact of its efficacy. Phenothiazines have a strong affinity to lipid bilayers of the cell membranes due to the high degree of lipophilicity of the phenothiazine ring (Michalak *et al*., 2006).

Phenothiazines are subdivided into three groups (aliphatic, piperadines and piperazines) dependent on the substituent at the nitrogen atom (**Figure 1.4**). Phenothiazines have a broad range of indications such as antipsychotic, antimalarial, antihistaminic, antiparkinson, antimicrobial, immunopotentiating, antifungal, antihelminthic, antitumour and antiemetic. Due to this vast range of applications, phenothiazines are in the origin of most of the medicinal compounds used until today. Depending on the structure of the substituent in the side chain, this will impact on the intensity of the neuroleptic properties and these could be ordered as follows: piperazine > piperidine > aliphatic. These drugs have similar structures to histamine and dopamine. The antihistaminic and antipsychotic activities might derive from this similarity. Phenothiazines and its derivatives have been referred in

the literature as “dirty drugs” because of their ability to act on numerous receptors such as dopamine, serotonin, muscarinic, GABAergic and histamine receptors (Peroutka & Snyder, 1980; Mozrzymas *et al*., 1999).

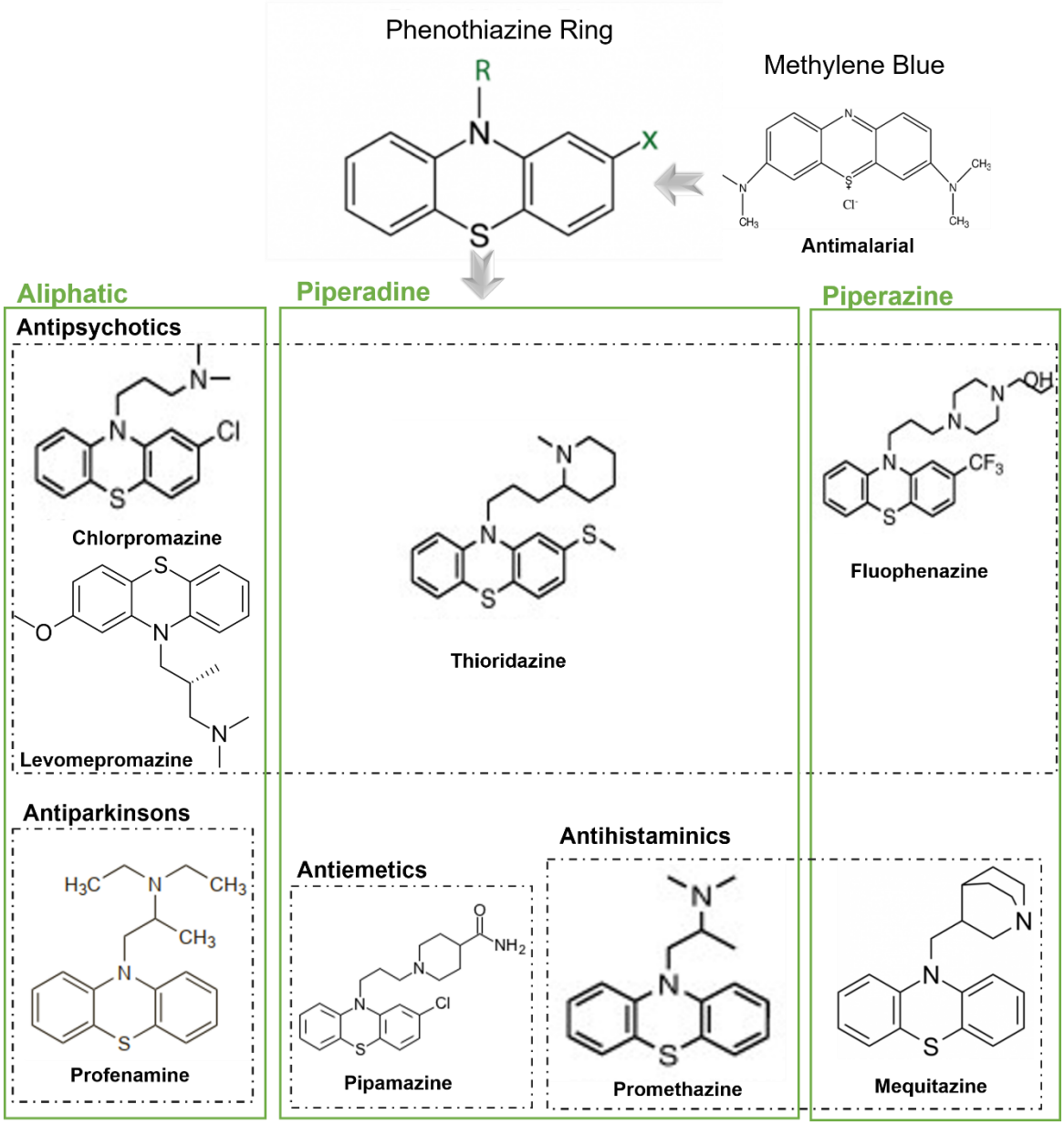


**Figure 1.4. Chemical structures and activity of some important phenothiazines.** Adapted from (Ohlow & Moosmann, 2011; Varga *et al*., 2017).

### 1.4.1. History of Phenothiazines

The history of this family of compounds goes back to the 19th century and it's development is linked with the history of dyes. Phenothiazines are derived from the reaction of diphenylamine with sulphur and were synthesised for the first time in 1883 by Heinrich August Bernthsen when studying the chemical structure of the dye, methylene blue (Bernthsen, 1883; Varga *et al*., 2017). Bernthsen showed that methylene blue was built up around the phenothiazine ring. This dye, whose chemical structure is a phenothiazine was first synthesized by Heinrich Caro in 1876, without knowledge of its constitution.

The medical properties of methylene blue were revealed in 1886 when Paul Ehrlich demonstrated that the dye was suitable for blood smear staining and caused reduced movement of *P. falciparum* (Ehrlich & Guttman, 1891). Due to its antimalarial properties, methylene blue was successfully used to treat two malaria-infected patients at the Moabit hospital in Berlin (Ohlow & Moosmann, 2011; Lu *et al*., 2018). This was the first time that a synthetic drug was ever administered to humans. This phenothiazine had a massive impact on the development of new antimalarial drugs (pamaquine and mepacrine). Pamaquine and Mepacrine were extensively used and became indispensable during World War II. Influenced by the studies conducted by Ehrlich, in 1899, Bodoni performed experiments using methylene blue in humans (Bodoni, 1899). He reported that the oral administration of methylene blue caused the patients to become calmer and more lethargic and indifferent to environmental stimuli (Amaral, Viveiros & Kristiansen, 2001). However, the dye produced a slightly blue coloration of the skin turning methylene blue unsuitable for the therapy of neurological diseases.

Fifty years and extensive research work later, Paul Charpentier brought attention to the first colourless phenothiazine, promethazine. at the Rhône-Poulenc laboratories in Paris (Charpantier, 1947). The French army surgeon, Henri-Marie Laborit, discovered that phenothiazines could be used in therapy of shock and in the treatment of pain after surgery (López-Muñoz *et al*., 2005; Ramachandraiah, Subramaniam & Tancer, 2009). Laborit started using promethazine as general anaesthesia in his surgery being quite successful during World War II. Based on this work, the laboratory synthesised more than 4000 phenothiazines. Thereafter,

Rhône-Poulenc laboratories synthesised and made available Chlorpromazine (CPZ), with the trade name Largactil. However, Laborit struggled to persuade the medical community that phenothiazines would revolutionize the field of psychiatry (Kunz, 2014). It was only at The University of Birmingham (UK) in 1954 that Elkes and Elkes demonstrated that CPZ was a viable clinical option for the treatment of psychosis (Kunz, 2014). However, the vast use of this phenothiazine revealed that the prolonged administration of this treatment produced many severe and toxic side effects. Those effects involved akathisia (restlessness), lethal neuroleptic malignant syndrome, hypotension, cardiotoxicity (prolongation of the QT interval, *i.e.* irregularity of the electrical activity of the heart that poses patients at risk for ventricular arrhythmias), arrhythmias and even sudden death (Elkes & Elkes, 1954; Amaral & Kristiansen, 2000; Amaral *et al*., 2001).

### 1.4.2. Antibacterial activity of Phenothiazines

Phenothiazines were shown to have antimicrobial activity against a wide range of microorganisms such as *Mycobacterium tuberculosis*, *S. aureus*, *Pseudomonas aeruginosa*, *Enterococcus* spp., *Streptococcus* spp., *Salmonella* spp., *Klebsiella pneumoniae*, *Shigella* spp., *Vibrio cholerae* and others (Hendricks, Butterworth & Kristiansen, 2003a; Ordway *et al*., 2003; Martins *et al*., 2004, 2007b; Kristiansen *et al*., 2007) for over a century and since the time of Paul Ehrlich. Since phenothiazines were discovered during the “Golden Age” of antibiotics and its antipsychotic properties were of great interest to the biomedical community, the drugs failed to receive attention as antimicrobials (**Figure 1.5**).

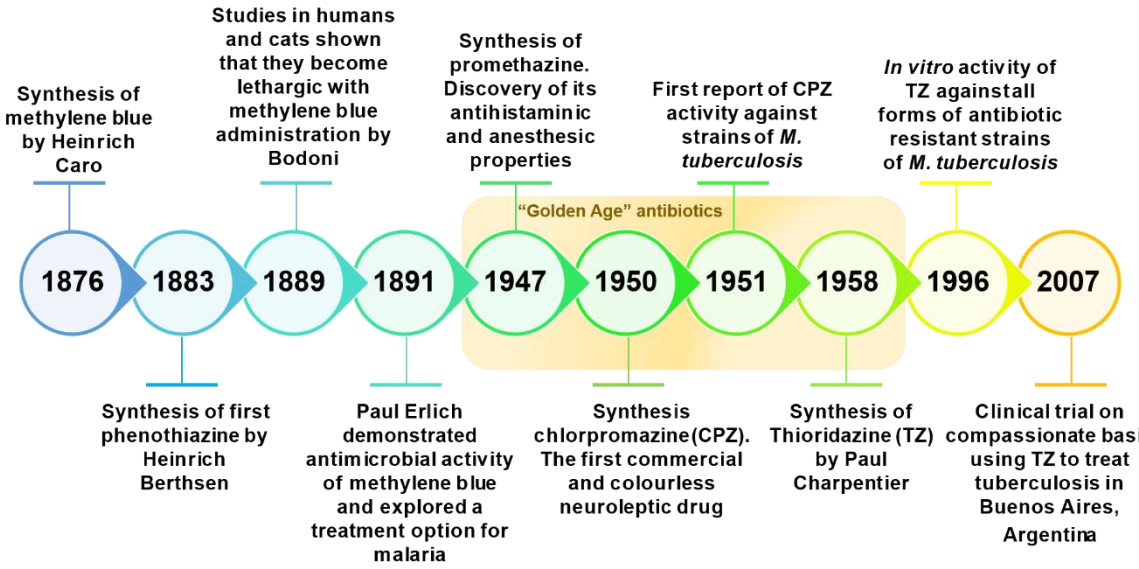


**Figure 1.5. Timeline of the most important developments of phenothiazines as antimicrobials.** (Kristiansen & Amaral, 1997; Abbate *et al*., 2007; Ohlow & Moosmann, 2011)

The activity of phenothiazines reported during the so-called "Golden Age" was not restricted to plasmodia. Insecticidal activity against fruit pests was also reported but never became very popular at the time (Nelson & Smith, 1942), despite the fact that fruits treated with phenothiazines showed delayed rotting. However, other activities such as antifungal and antibacterial were also reported.

Beside the side effects seen in patients treated with phenothiazines, positive effects were reported on patients under treatment with Largactil. Lower incidence of bacterial infections, especially tuberculosis (TB) where observed in psychiatric patients (Kristiansen & Vergmann, 1986; Kristiansen & Amaral, 1997; Amaral *et al*., 2011). Studies conducted by Amaral and his team demonstrated that TZ was very similar to CPZ with respect to its *in vitro* antimycobacterial properties but with less reported side effects and toxicity than CPZ. This was observed regardless the antibiotic susceptibility status of the bacteria (Amaral *et al*., 1996). However, the *in vitro* concentration of phenothiazines that usually inhibit microbial growth (e.g. 25 mg/L against *M. tuberculosis*) cannot be clinically achieved (maximum plasma concentration of 0.5 – 1 mg/L) (Ordway *et al*., 2003; Thanacoody, 2007). Interestingly, TZ and CPZ are concentrated within macrophages. Early studies

reported that phenothiazines diffuse across lysosomal membranes in their unionised form and, once in the acidic lysosomal environment, protonation of the amine moiety prevents the drugs from passive back-diffusion across the lysosomal membrane (Guth & Spirtes, 1964; Hu & Curry, 1989; Wójcikowski & Daniel, 2002; Daniel, 2003; Kornhuber *et al*., 2010). Since lysosomes then fuse with the phagosome, conditions by which the phagocytosed bacteria enter in contact with higher concentrations of phenothiazines (similar to the ones obtained *in vitro*) are created (Daniel, Bickel & Honegger, 1995; Daniel & Wójcikowski, 1999; Martins *et al*., 2009). This effect was shown by Crowle *et al*. in 1992 (Crowle, Douvas & May, 1992), where by exposing macrophages containing phagocytosed *M. tuberculosis* to clinically relevant concentrations of CPZ enhanced the killing of the intracellular bacteria. This enhancement of the killing activity of the macrophage was then speculated to be due to the concentration of phenothiazines inside the human cell. The same study reported that the human macrophage, as well as other cells rich in lysosomes, was able to concentrate phenothiazines as much as 100 times of the concentration initially added to the culture media. This would mean that these compounds could reach their *in vitro* minimum inhibitory and/or bactericidal concentrations (MIC/MBC) inside the macrophages without the need for the patient to take high doses of the phenothiazines, which otherwise would be toxic (Daniel, Bickel & Honegger, 1995; Daniel & Wójcikowski, 1999; Martins *et al*., 2009).

The findings from this study are interesting and relevant to the treatment of intracellular infections, since hundreds of compounds are able to inhibit the *in vitro* growth of bacteria, but sometimes these compounds are not pursued further, since these concentrations are too high and considered non-physiologically relevant to conduct infection studies with human macrophages. A study from Ordway *et al.,* published in 2002 showed that human monocyte-derived macrophages recovered from the whole blood (PBMDMs) and THP-1 human macrophages (cell line) were able to kill *S. aureus* when the culture medium contained TZ 0.01 µg/mL (MIC for *S. aureus* = 15 µg/mL) (Ordway *et al*., 2002a). In 2003, the same team, Ordway *et al*. demonstrated that TZ was also able to enhance the killing of PBMDM and THP-1 human macrophages infected with antibiotic susceptible and -resistant *M. tuberculosis*. Complete killing was observed by the end of 3 days post infection at the concentration of 0.1 µg/mL regardless of the antibiotic susceptibility status (Ordway *et al*., 2003).

Following this, a study conducted by Martins *et al*., in 2007 demonstrated that TZ could cure mice infected with antibiotic susceptible and resistant *M. tuberculosis* (Martins *et al*., 2007b)

### 1.4.3. Phenothiazine Thioridazine

Thioridazine is an alkylpiperadine phenothiazine and a well-known dopamine-antagonist compound. It was developed shortly after the discovery of CPZ in 1950s. Synthesis of TZ is one-step process by alkylating 2-methylthiophenothiazine with 2-(2-chloroethyl)-1-methylpiperidine and is mediated by $NaNH_2$ (Kleemann, 2012). TZ has been extensively used since 1959 as an antipsychotic compound for the treatment of psychosis and schizophrenia (commercial name Mellaril®). Contrary to CPZ, the side effects reported for TZ, such as lethargy, are milder (Amaral *et al*., 2001; Abbate *et al*., 2007; Thanacoody, 2007). The use of TZ through the years has proven to be therapeutically safe for the treatment of psychosis. However, Mellaril® (manufacturer Novartis) was withdrawn from the market in 2005 due to potential long-term effects, although it is still available in the generic version. A study on "compassionate basis" was conducted in Buenos Aires (Argentina, 2007) using TZ as an adjuvant (in combination with antibiotics) (Abbate *et al*., 2007) to treat TB patients. In this study, the treatment was administrated to non-responsive extensively drug-resistant (XDR)-TB patients. These patients showed lethargy as a side effect and the study was ended for this reason, despite the fact that within a few weeks of treatment the quality of life of these patients had improved significantly with the patients showing gaining of weight and reporting improvement in symptoms such as fever, fatigue and breathless. This study and others conducted in the last few years have attracted interest towards TZ as a potential candidate for development as an antimicrobial compound.

#### 1.4.3.1. Antibacterial activity of TZ

##### 1.4.3.1.1. *In vitro* activity of TZ

Over 15 years ago, the *in vitro* effect of TZ on the pathogen *M. tuberculosis* was reported by Amaral *et al*. (Amaral *et al*., 1996). Although exhaustive evidence

of the antimicrobial activity of TZ has been described in the literature, both *in vitro* an *in vivo*, the exact mechanism of action of TZ in bacteria remains unknown.

The possible mechanism of action by which TZ acts on bacteria has been studied *in vitro* by researchers, mostly in *M. tuberculosis*. In these studies, it is hypothesised that phenothiazines have their primary effects on the plasma membrane (prokaryotes, eukaryotes) where EPs are located and it is also able to interfere with energy sources and ultimately, with the bacterial growth. The first contact between the bacteria and TZ takes place at the surface of the cell envelope and is known to increase the permeability and changes in cellular morphology in both Gram-negative and -positive bacteria (Galeazzi *et al*., 1986; Amaral *et al*., 2000; Martins *et al*., 2004). Increased permeability ensures that TZ reaches the DNA and interferes with DNA-based processes (Stolze & Mason, 1991; Rohs & Sklenar, 2004; Bonde *et al*., 2011).

A study conducted by Keijzer and colleagues showed that in the presence of TZ there is a differential abundance of 16 proteins that are involved in the maintenance of the cell envelope permeability of *M. tuberculosis* (de Keijzer *et al*., 2016). This study also confirmed previous results from a different research group (Dutta, Mehra & Kaushal, 2010). TZ has been described in the literature as an efflux pump inhibitor (EPI) in different species, *M. tuberculosis*, *S. aureus*, *E. coli* and others (Kaatz *et al*., 2003; Klitgaard *et al*., 2008; Machado *et al*., 2012; Spengler *et al*., 2012). It is known that EPs have a major role in the development of drug resistance. When overexpression of EPs occurs, bacteria become resistant to a variety of antibiotics changing its phenotype to MDR. TZ is known to inhibit EPs of bacteria and reverse MDR phenotypes to susceptible (to antibiotics the bacteria was initially resistant to) (Viveiros *et al*., 2005; Kristiansen *et al*., 2006, 2007). Studies from Viveiros and his team demonstrated that TZ reversed the resistance of *E. coli* to tetracycline by inhibition of over-expressed EPs (Viveiros *et al*., 2005). In 2006, Kristiansen *et al.,* also reported that TZ was able to reverse the resistance to oxacillin in methicillin-resistant *Staphylococcus aureus* (MRSA) by inhibiting EPs in this bacteria (Kristiansen *et al*., 2006). The same was observed when a large number of clinical isolates of *M. tuberculosis* were induced to high level resistance to isoniazid (INH) and this resistance was reversed using TZ (Viveiros *et al*., 2002). Furthermore, other studies showed - and confirmed the previous observations - that this reversed

resistance interfered with the over-expressed EPs genes of *M. tuberculosis Rv2459, RV1258c, efpA, mmr, p55, mmpL7* (Machado *et al*., 2012). Most of these studies were performed with concentrations of TZ that did not completely inhibit replication of the bacteria. Studies conducted on *S.* Typhimurium indicated that CPZ may interact with the AcrAB-TolC system, acting on the bacteria similarly to an EPI (Yamasaki *et al*., 2016). It is hypothesised that TZ can have a similar interaction. Some EPs, including the AcrAB-TolC system, require energy generated by the PMF, to be able to extrude noxious compounds from the bacterial cell. The PMF is one of the ways by which cellular energy is created and is dependent on the membrane potential. The PMF is generated across the cell membrane by the extrusion of protons by the electron transport chain resulting in an electrochemical gradient of protons. The PMF is subsequently necessary for the synthesis of ATP by the F1F0-ATPase and for the transport of solutes. This force is the result of the sum of two parameters namely, the electric potential (ΔΨ) and the transmembrane proton gradient (ΔpH). Therefore, the PMF is critical for bacterial survival and could constitute a good target for the action of new antimicrobials.

Nevertheless, concerns regarding toxicity presented by this type of inhibitors of the PMF has stalled main research on this area. Despite these concerns ionophores such as monensin are known to dissipate the PMF through disruption of the flow of ions across the membrane and have been extensively used as antibacterial by cattle and poultry industries (Farha *et al*., 2013).

TZ and other phenothiazines have been reported to interfere with the flux of ion across the bacterial membrane (Kaatz *et al*., 2003; Rao *et al*., 2008). Interference with this flux across the membrane can alter the electric potential resulting on depolarisation of the bacterial membrane (Wassmann *et al*., 2018). Furthermore, processes such as flagellar motility, efflux, cell division and ATP synthesis will also be affected since PMF is essential for those.

Some studies in *M. tuberculosis* have also shown that TZ interferes with the respiratory chain by inhibiting type II- NADH-menaquinone oxidoreductase (NDH-2) - which is a key component of the respiratory chain of *M. tuberculosis* (Weinstein *et al*., 2005; Yano *et al*., 2006; Thanacoody, 2007; Teh, Yano & Rubin, 2007). Electron transport is initiated through the activity of various NADH dehydrogenases (NDH) and succinate dehydrogenases (SDH), which transfer electrons to menaquinone, a

lipophilic redox carrier. During this reaction, NDH-2 transfers two electrons from NADH to menaquinone, which is reduced to menaquinol. Yano *et al.* demonstrated that phenothiazines block NADH-dependent oxygen consumption by *M. tuberculosis* membranes and specifically inhibited purified recombinant *M. tuberculosis* NDH-2 and *M. tuberculosis* NDH-2A (Yano *et al*., 2006). This study suggested TZ as an attractive compound against replicative and dormant mycobacteria.

TZ and other phenothiazines are also able to inhibit the binding of calcium ($Ca^{2+}$) to $Ca^{2+}$-binding proteins (Martins *et al*., 2011). In eukaryotes, the PMF is dependent of $Ca^{2+}$-enzyme systems, such as ATP synthase (Klee *et al*., 1986; Mousavizadeh, Ghafourifar & Sadeghi-Nejad, 2002; Martins *et al*., 2008).

#### 1.4.3.1.2. *Ex-vivo* activity of TZ

Non-antibiotic compounds, such as TZ, are able to stimulate the host response to infection, which could be considered beneficial. TZ is able to inhibit the binding of $Ca^{2+}$ to $Ca^{2+}$-binding proteins such as calmodulin in mammalian cells (Martins *et al*., 2008). This inhibition results in the subsequent inhibition of certain enzyme systems that are dependent of $Ca^{2+}$, like the those involved in generating cellular energy, such as calmodulin-dependent protein kinases. This effect disturbs the $Ca^{2+}$ signalling, which is a mechanism that is essential for most of the biological processes, namely the phagocytosis of bacteria by the macrophage (Hoyal, Giron-Calle & Forman, 1998; Amaral & Viveiros, 2012). Besides the $Ca^{2+}$ channels, TZ (and other phenothiazines) is also capable of inhibiting potassium ($K^+$) ion channels (Ordway *et al*., 2003; Martins, Viveiros & Amaral, 2008). These ions have an important role for enhancing the killing activity of the host cell (Wittekindt *et al*., 2006). The concentration of TZ inside the macrophage might result on an *in situ* inhibitory effect in the phagocytosed bacteria. This is of great importance to treat infections caused by intracellular pathogens such as *M. tuberculosis* and *S.* Typhimurium.

#### 1.4.3.1.3. *In vivo* studies

To evaluate the *in vivo* efficacy of TZ, several studies have been published. In 2007, Martins and her colleagues (Martins *et al*., 2007b) evaluated the effectiveness of treating female BALB/C mice with different doses of TZ. These mice

had been infected intraperitoneally with a high dose ($1x10^6$ CFU/kg tissue) of *M. tuberculosis* ATCC H37Rv during 30 days after treatment started with TZ. The concentrations of TZ used were equivalent to those usually used in humans and ranged from 0.05 to 0.5 mg/day. In this study, Martins *et al.,* demonstrated that the concentration of 0.5 mg/day of TZ decreased the colony forming units (CFU) in the lungs of the animals within one month of treatment. The treatment of the animals finished at the end of 300 days showing numbers of mycobacteria in the lung noticeably low ($1x10^2$ CFU/Kg tissue). In 2010, van Soolingen and his team (van Soolingen *et al*., 2010) used the same mouse model to evaluate the effect of TZ when combined with standard first-line drugs (rifampicin, INH and pyrazinamide) to treat susceptible and MDR *M. tuberculosis*-infected mice. This treatment was done for two months and animals had a daily oral administration of TZ at 32 and 70 mg/kg. The authors observed a significant reduction in the CFU recovered from both groups of animals after two months of treatment. A reduction of -4.4 log CFU and -2.4 log CFU was obtained for the susceptible and MDR groups, respectively. A significant synergistic effect was also observed when TZ was used in combination with first-line drugs (usually used for TB treatment) in the susceptible group (-6.2 *vs.* -5.9 log CFU). In 2014, Dutta *et al* (Dutta, Pinn & Karakousis, 2014a,b) also observed effectiveness of TZ in murine models in combination with first-line drugs to treat TB. The same effectiveness was observed in *in vivo* models using *Salmonella* Typhimurium (Dasgupta *et al*., 2010). In the same year, Hahn and colleagues (Hahn & Sohnle, 2014) demonstrated that TZ was effective in treating cutaneous staphylococcal infections in two mouse models of *S. aureus* skin infections. In these models and after the inoculation of the skin surface, TZ significantly suppressed dissemination from the skin to the spleen and kidney and the size of abscesses produced by the intradermal injection. Although, TZ didn't affect the infections parameters in the skin itself.

In Argentina, a study conducted with TB patients under "compassionate basis" (mentioned previously) reported that the results previously observed with *M. tuberculosis* in the mouse model, were reproducible in humans (Amaral et al., 2010a). This study was conducted using TZ in a combination therapy with linezolid and moxifloxacin in 14 XDR-TB patients. Linezolid was included in the drug regimens of all patients. Moxifloxacin alone and/or combined with TZ were included in the regimens of the 14 patients and these remained hospitalised for ≥2 months or until

bacteriologically negative. 10 out of the 14 XDR-TB patients were cured when TZ was used in combination with the antibiotics to which the XDR-TB patients were non-responsive. The authors observed that TZ improved the quality of life of the patients and contributed to a longer life span (Abbate *et al*., 2007, 2012). However, TZ wasn't able to restore loss of pulmonary tissue and the patients succumbed to the disease. Another study conducted in India, showed similar results (Udwadia, Sen & Pinto, 2011). In both studies, patients were under cardiac monitoring and TZ had no significant effect on the QT intervals or any other cardiac complication (Abbate *et al*., 2007, 2012; Amaral *et al*., 2011; Udwadia, Sen & Pinto, 2011).

### 1.5. Other EPIs as antimicrobials

Given the importance of multidrug efflux mechanisms, EPIs have been given some attention as potential new antimicrobial compounds. The use of EPIs in combination with antibiotics (adjuvant therapy) has shown interesting results in overcoming resistance of bacteria to antibiotics, when efflux is the main mechanism of resistance reported (Bohnert & Kern, 2005). Since EPs are dependent on energy, the disruption of energy and efflux activity is the mechanism of action for some EPIs. Carbonyl cyanide-m-chlorophenylhydrazone (CCCP) is perhaps one of the most well know EPIs and it is usually used for the screening of activity of EPs of the RND family. Another well described inhibitor of RND EPs is Phenylalanine-Arginine-β-naphthylaminde (PaβN). This compound has not only been considered an EPI as it has been reported as being successful in reverting the resistance to antibiotics such as tetracycline and macrolides in *E. coli*, when compared to other known EPIs (Kern *et al*., 2005).

Other compounds such as $Ca^{2+}$ channel blockers have also been explored due to their potential activity in infected cells (namely macrophages). One such example is Verapamil (VER). This compound is a voltage dependent $Ca^{2+}$ channel blocker that inhibits several bacterial EPs. It has been reported as enhancer of the macrophage killing activity against several strains of *M. tuberculosis* (Martins *et al*., 2006; Machado *et al*., 2016). Indeed, it has been reported as not only showing *in vitro* activity against *M. tuberculosis* but also been able to reduce the bacterial load during macrophage infection, *i.e.,* numbers of CFU recovered from inside the infected macrophage. Due to its capacity to inhibit $Ca^{2+}$ flux on infected

macrophages, this type of compounds can be explored as activators of infected cells or alternatively, can be used as parental molecules for the design of the next-generation of $Ca^{2+}$ channel blockers.

Compounds able to target the intracellular niche of some bacterial pathogens are vital to strengthen the fight of AMR. However, despite some of these compounds being well known it's potential to treat macrophages infected with bacteria is still not sufficiently explored and understood. One of the main focus of this thesis is to study TZ and other potential $Ca^{2+}$ channel blockers on *Salmonella*-infected macrophages.

## 1.6. *Salmonella enterica* serovar Typhimurium

### 1.6.1. Overview of *Salmonella* infections

Members of the genus *Salmonella* belong to the *Enterobacteriaceae* family and represent the most common foodborne pathogens. *Salmonella* are Gram-negative, rod-shaped, motile flagellates, facultative anaerobes and intracellular pathogens. The *Salmonella* genus diverged from a common ancestor with *E. coli* between 100 and 150 million years ago (Fàbrega & Vila, 2013). The genus *Salmonella* can be subdivided into two species: *Salmonella bongori* (*S. bongori*) and *Salmonella enterica* (*S. enterica*). Based on biochemical and genomic modifications, *S. enterica* can be divided into six subspecies, *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV) and *indica* (VI). Currently, there are more than 2600 serovars within the entire *Salmonella* genus, and 1531 of these belong to the *Salmonella enterica* subsp. *enterica*. The species and subspecies of *S. enterica* subsp. *enterica* serovars are commonly omitted, for example *Salmonella enterica* subsp. *enterica* serovar Typhimurium is usually shortened to *Salmonella* Typhimurium (Brenner *et al*., 2000; Grimont & Weill, 2007). *Salmonella* have a broad host range but *Salmonella enterica* subspecies *enterica* is most commonly associated with human infections and other warm-blooded animals (Cheng, Eade & Wiedmann, 2019). *S. bongori* is commonly found in environmental sources and cold-blooded vertebrates such as reptiles and amphibians (Desai *et al*., 2013).

The two main clinical manifestations associated with *Salmonella enterica* infections are gastroenteritis and enteric fever, also known as Typhoid fever. Typhoid fever is caused by the human host-restricted serovar, Typhi. A clinically similar,

however, often less severe disease, Paratyphoid fever is caused by *S.* Paratyphi A, B and C (Gal-Mor, Boyle & Grassl, 2014). *S.* Typhi and *S.* Paratyphi are typically transmitted through faecal contamination of water sources or food. *S.* Typhi remains relevant today as it causes approximately 21 million infections annually. Resulting in 200,000 - 600,00 deaths especially in countries with poor economic development (Crump, Luby & Mintz, 2004). The early stages of this disease might go unseen, as typhoidal *Salmonella* strains do not trigger a pro-inflammatory response or gastroenteritis. The average incubation period for typhoidal serovars is 14 days with symptoms persisting for up to 3 weeks.

In contrast, infections with non-typhoidal *Salmonella* (NTS) usually results on production of inflammatory response and gastroenteritis that is cleared by the host within 4-7 days (Gal-Mor, Boyle & Grassl, 2014). This is observed typically with cases of *S.* Typhimurium and *S.* Enteritidis. A typical NTS infection rarely requires hospitalisation or treatment. However, these serovars can also cause serious and often fatal systemic infections, particularly in immunocompromised hosts (Ohl & Miller, 2001). Similar to Typhi and Paratyphi, NTS strains are commonly transmitted through ingestion of contaminated food and water. Unlike typhoidal serovars, NTS are zoonotic pathogens. There are an estimated 93.8 million cases of gastroenteritis leading to 155,000 deaths per year (Majowicz *et al*., 2010; Stanaway *et al*., 2019). However, this burden is not equally distributed per geographic regions. Eastern Mediterranean regions have the highest incidence of NTS strains comparing with the European region. African regions represent a concerning region with the highest death rate from non-typhoidal salmonellosis comparing to the rest of the world (Kirk *et al*., 2015). NTS are easily disseminated through agriculture and subsequently, contaminating food such as beef, poultry and eggs.

Thus, *Salmonella* infections represent a considerable concern to public health, animals and the food industry worldwide.

### 1.6.2. Pathogenesis of *S.* Typhimurium infection

Intracellular pathogens are capable of infect and survive within eukaryotic cells. This enables them to escape innate immune defences and antimicrobials compounds and overcome immune responses by residing and multiplying inside host immune cells.

The development of infection by *S.* Typhimurium involves a series of stressful conditions for the bacteria that are meant to protect the host (**Figure 1.6**). Following ingestion of contaminated food, *S.* Typhimurium will experience the first obstacle within the host, the acidic pH of the stomach. At the same time, an upshift in the temperature from the outside environment to approximately 37°C in humans, will also take place. To protect itself in response to acid shock, *Salmonella* activates an acid tolerance response (ATR). ATR prepares the bacterial cells for further acidic conditions of the intracellular environment promoting survival. *Salmonella* will colonise the small intestine to reach the preferential site for invasion, the ileum (Galan & Curtiss, 1989).

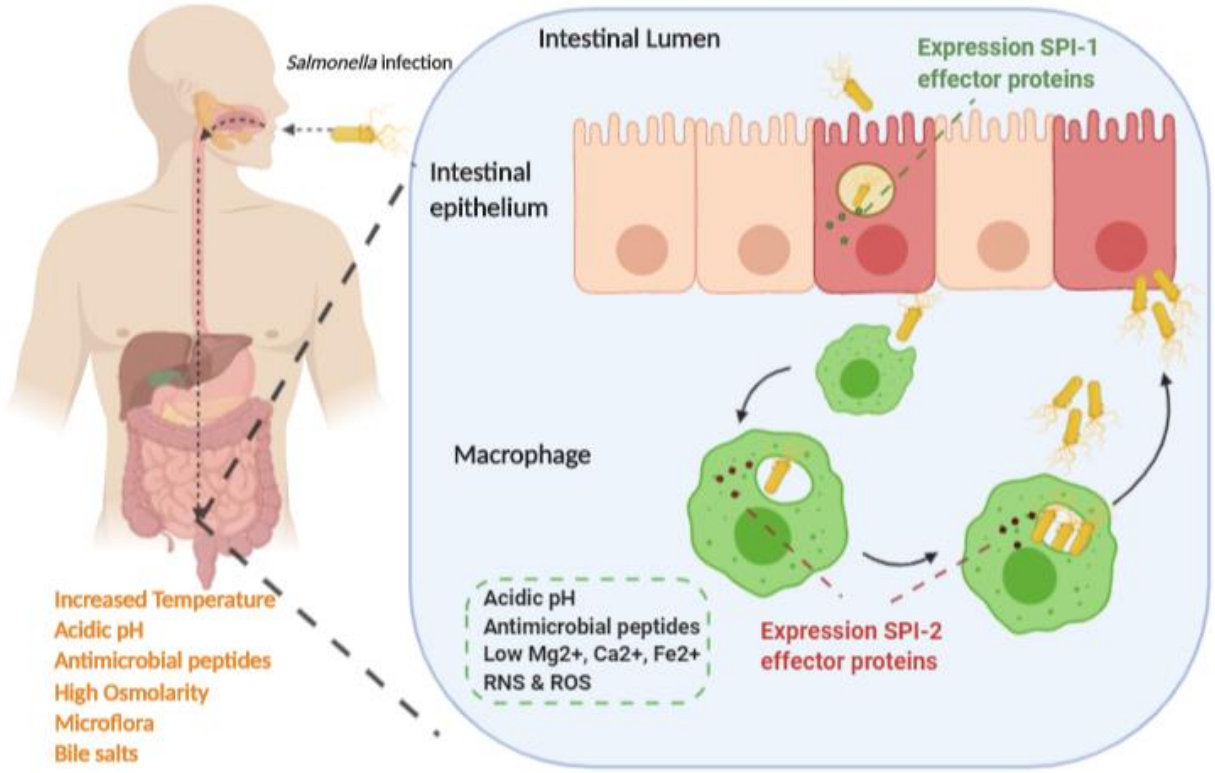


**Figure 1.6. Overall pathogenesis of S. Typhimurium.** Bacteria is ingested from contaminated food or water sources and passes through the mouth, esophagus, and stomach to the small intestine. While descending through the gastrointestinal tract S. Typhimurium encounters a variety of stressful conditions (labelled in orange) as part of the host first line of defence. Zoomed panel: S. Typhimurium invades epithelial cells of the intestinal epithelium. Environmental signals within the gut induce expression of SPI-1 T3SS which secretes effector proteins (in green) into the host epithelial cell that trigger intestinal inflammation and bacterial endocytosis. S. Typhimurium survives and replicates intracellularly within a Salmonella-containing vacuole (SCV). In susceptible hosts, S. Typhimurium becomes engulfed by macrophages and survives and replicates within the SCV, leading to bacterial dissemination and systemic infection. Environmental conditions within the macrophage induce the secretion of effector proteins of SPI-2 T3SS (in red), which are necessary for the intracellular lifestyle. Adapted from (Urdaneta & Casadesús, 2017).

A functional motility and chemotaxis systems is a prerequisite for *Salmonella* to approach and encounter the intestinal epithelium (Stecher *et al*., 2008). Adherence of *Salmonella* to the surface of epithelial cells induces significant host cell cytoskeletal rearrangement and membrane ruffling resulting in the engulfment of *Salmonella* (Francis, Starnbach & Falkow, 1992). Following attachment, *Salmonella* type III secretion system (T3SS)-encoded on the pathogenicity Island 1 (SPI-1) translocate effector proteins into the cytosol of non-phagocytic cells. This actively induces host cell cytoskeletal rearrangement, membrane ruffling and ultimately uptake of the bacterium into a membrane-derived vacuole (Steele-Mortimer *et al*., 1999; Srikanth *et al*., 2011). Some of these effector proteins (for e.g. SipA, SipB) are encoded by genes located on SPI-1 of the bacterial chromosome. Other effectors, such as SopA, SopB, SopD, SopE, SopE2, *etc*, are encoded by genes located around the *Salmonella* chromosome. SopB, SopE and SopE2 are essential for invasion of *S*. Typhimurium as mutants defective in any of these effectors are incapable of inducing actin cytoskeleton rearrangements (Galán & Zhou, 2000; Srikanth *et al*., 2011) The effectors SipA and SipC are responsible for membrane ruffling and engulfment (Srikanth *et al*., 2011). Thus, allowing *Salmonella* to exploit the intracellular niche and to interfere with the physiology of the host cells. Detection of the invading bacteria by the host immune system leads to a pro-inflammatory immune response, resulting in the inflammation of the gut, secretion of fluids and symptoms of gastroenteritis. This inflammatory response involves the production of tumour necrosis factor (TNF) alpha and interleukin-8 (IL-8).

S. Typhimurium cells that successfully invade host epithelial cells are enclosed in a membrane-derived vesicle termed the spacious phagosome (SP). The SP later fuses with lysosomes, acidifies and shrinks to become adherent around one or more bacteria creating the *Salmonella*-containing vacuole (SCV) which biogenesis resembles a phagosome (Steele-Mortimer, 2008). Maturation of the SCV and survival of bacterial cells is dependent upon proteins encoded on *Salmonella* pathogenicity island 2 (SPI-2). The SPI-2 T3SS translocates effector proteins across the phagosomal membrane to allow intracellular survival and replication. Formation of the SCV requires altering the host cell endocytic trafficking pathway to avoid normal phagosome maturation and fusion with lysosomes (Fàbrega & Vila, 2013). As the SCV matures, *Salmonella* faces additional stressors including a further

reduction of pH and magnesium, phosphate and iron starvation (Srikanth *et al*., 2011).

The mature SCV migrates to the Golgi-apparatus and simultaneously *Salmonella*-induced filaments (SIFs) are formed. SIFs are tubular filamentous structures which extend from the SCV and form complex networks throughout the cell to facilitate interactions with host organelles (Beuzón *et al*., 2000; Knuff & Finlay, 2017). *Salmonella* can also proliferate in epithelial cells outside of their SCV. A significant portion of internalised bacteria within the SCV lyses their phagosome and escapes to the cytosol of the host cell. These subpopulation, designated cytosolic *Salmonella,* evades destruction by autophagic mechanisms of the host epithelial cell, and survive to hyper-replicate later on during infection using SPI-1 encoded proteins (Knodler, Nair & Steele-Mortimer, 2014; Klein, Powers & Knodler, 2017; Finn *et al*., 2017).

*Salmonella* is able to invade both non-phagocytic and phagocytic cells. After exiting the intestinal tract, *Salmonella* is phagocytosed by resident and recruited immune cells, including macrophages. SCVs transcytose to the basolateral membrane of epithelial cells and are subsequently engulfed by phagocytic cells, primarily macrophages. This bacterial internalisation ultimately results in an intra-macrophage SCV. *Salmonella* located within intra-macrophage SCVs trigger similar host cell pathways to those triggered by intra-epithelial cell SCVs, avoiding phagosome maturation and allowing the bacteria to proliferate (Fàbrega & Vila, 2013). There are common characteristics but also some important differences between the SCV of epithelial cells and macrophages. While nutrient starvation and acidic pH is a feature of both intracellular vacuoles, the intra-macrophage SCV is more bactericidal in nature (Hautefort *et al*., 2008). However, *Salmonella* and other bacteria have encountered ways to overcome this by remodelling the protein, carbohydrate and membrane components of the envelope. This way, bacteria are able to resist the action of antimicrobial peptides and reactive oxygen and nitrogen species (ROS, RNS) that can damage their cells (Hautefort *et al*., 2008).

Known signals of SPI-2 induction are acidic pH, limitation of inorganic phosphate ($P_i$), $Mg^{2+}$ deprivation, high osmolarity and the presence of antimicrobial peptides. These are signals thought to mimic the environmental cues found in the SCV (Löber *et al*., 2006). The SPI-2 effector SsaB (SpiC), which also functions as

part of the SPI-2 apparatus, is translocated into the host cytosol where it inactivates the mammalian protein Hook3, resulting in a blocking of the fusion of the SCV and the lysosome (Buchmeier & Heffron, 1991; Freeman *et al*., 2002; Kujat Choy *et al*., 2004). SpiC interferes with endosomal trafficking upon expression in mammalian cells and in *in vitro* assays (Uchiya *et al*., 1999). SPI-2 effectors SseG and SseF play a role in maintaining the SCV near the nucleus and Golgi apparatus, while the SifA protein contributes by limiting the amount of kinesin recruited to the SCV site, thus avoiding displacement of the SCV to the cell periphery (Kuhle & Hensel, 2004; Abrahams & Hensel, 2006). SifA was shown to promote intracellular survival within macrophages and to be required for systemic infection. The ability of SPI-2-secreted effector proteins to modulate the motility of infected macrophages plays an important role in bacterial dissemination and the establishment of systemic infection within the liver and spleen (Fabrega & Vila, 2013).

The presence of *Salmonella* within the macrophages leads to an immune response through cytokine production, inflammatory reaction or programmed cell death. During the process of internalisation as well as inside the host macrophage, *Salmonella* activates signalling of the host pattern recognition receptors (PRRs) like Toll-like receptors (TLRs) and NOD-like receptors (NLRs). Phagocytic receptors on the plasma membrane recognise structural determinants on the surface of pathogens (opsonin-independent receptors) whereas others recognise host opsonins bound to the pathogen (opsonin-dependent receptors). Both types of receptor can greatly enhance particle uptake but also initiate the signalling cascades required for efficient microbicidal activity, antigen processing, and production of cytokines and chemokines (Stuart & Ezekowitz, 2005)

The equilibrium between pro- and anti-inflammatory cytokines wheels the infection, preventing damage to the host. Macrophages can undergo apoptosis, and *Salmonella* will escape the cell to basolaterally reinvade epithelial cells or other cells such as phagocytic, causing bacterial dissemination and establishing systemic infection within the liver and spleen.

## 1.7. Aims of the study

The primary aim of this study was to explore the approach of drug repurposing as an effective way to uncover potential new antimicrobials compounds.

The first chapter of this study explored the antimicrobial activity of three different metallic complexes synthesised previously as antitumor compounds and tested against a range of Gram-positive and -negative bacteria.

The second experimental chapter is focused on uncovering the mechanism of action of the phenothiazine, thioridazine, using *Salmonella* Typhimurium as a model bacterium for intracellular pathogens. Since AMR is one of the major concerns of the 21$^{st}$ century, the development of new approaches and new antimicrobial compounds has become an urgent priority. TZ and other phenothiazines have been identified as potential antimicrobials against *M. tuberculosis*, however, the mechanism(s) of action of these compounds hasn't been fully understood. In this chapter, we explore the potential *in vitro* activity and mode of action of TZ against *Salmonella* Typhimurium.

The final chapter of results expands on the *ex-vivo* effect of TZ in human macrophages infected with *Salmonella* Typhimurium. The effect of TZ on *Salmonella* internalised by human macrophages was investigated initially by quantifying the intracellular bacteria. The cytokine response of *Salmonella*-infected human macrophages treated and non-treated with TZ-treated as well as the potential effect on the $Ca^{2+}$ flux of the macrophage was also assessed.

In summary, the work developed in this thesis, focused on the drug repurposing of TZ to treat *Salmonella* infections. By contributing to uncover the *in vitro* mechanism of action of TZ in *Salmonella* and providing novel data regarding the use of TZ on *Salmonella*-infected macrophages, the data generated in this thesis supports the use of this type of compounds to tackle AMR. Understanding the mechanism of action of TZ and other similar compounds on *Salmonella*-infected macrophages is an important step forward for future research on new antimicrobials active against antibiotic resistant bacteria.

# 2. A tale of two ends: repurposing metallic compounds from anti-tumour agents to effective antibacterial

Data presented in this Chapter is published in the Special Issue: "New Insights into Antibacterial Compounds: From Synthesis and Discovery to Molecular Mechanisms of Action" of the Journal Antibiotics.

**Alves Ferreira, D.**; Martins, L.M.D.R.S.; Fernandes, A.R.; Martins, M. A Tale of Two Ends: Repurposing Metallic Compounds from Anti-Tumour Agents to Effective Antibacterial Activity. Antibiotics 2020, 9, 321. doi:10.3390/antibiotics9060321

**Abstract**

The rise in antibiotic resistance coupled with the gap in the discovery of active molecules has driven the need for more effective antimicrobials while focusing the attention into the repurpose of already existing drugs. Here, we evaluated the potential antibacterial activity of two zinc- and one cobalt-organometallic compounds previously reported as having anticancer properties. Compounds were tested against a range of Gram-positive and -negative bacteria. The determination of the minimum inhibitory and bactericidal concentrations (MIC/MBC) of the drugs, were used to assess their potential antibacterial activity and their effect on the bacterial growth. Motility assays were conducted by exposing the bacteria to sub-MIC of each of the compounds. The effect of sub-MIC of the compounds on the membrane permeability was measured by the Ethidium Bromide (EtBr) accumulation assay. Cell viability assays were performed in THP-1 cells. Compound TS262 was the most active against the range of bacteria tested. No effect was observed on the motility or accumulation of EtBr for any of the bacteria tested. Cell viability assays demonstrated that at the MIC, the compounds showed decrease on cell viability. These results are promising and further studies on these compounds can lead to the development of new effective antimicrobials.

## 2.1. Introduction

According, to the World Health Organization (WHO), antimicrobial resistance (AMR) is one of the most complex threats in the twenty-first century, not only to human health but also to economics. The extensive misuse of antibiotics, among other factors, has contributed to the emergence of multidrug resistant bacteria and consequently, leading to the development of hard-to-treat infections. Jim O'Neil and his team wrote an extensive review on AMR commissioned by the British Government – Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations (O' Neil, 2014). One of the review's main findings is that annual deaths attributable to antimicrobial resistant infections would reach approximately 10 million cases each year by 2050 unless action is taken (O' Neil, 2014). Adding to this predictions, the European Centre for Disease Prevention and Control reported that nowadays more than 670 000 infections occur in the EU/EEA and 33 000 people die per year in the EU/EEA, mostly due to bacterial infections (Cassini *et al*., 2019; European Centre for Disease Prevention and Control, 2019). These numbers correspond to an incidence rate of 131 infections per 100 000 population with an attributable mortality rate of 6.44 deaths per 100 000 population (Cassini et al., 2019; Ouakrim et al., 2019).

The discovery and development of novel antibiotics is stagnating and the urge for new approaches is increasing to meet the challenges posed by the rapid development of bacterial infections resistant to common antimicrobial drugs. Several novel approaches, such as the use of combination of antibiotics (Tyers & Wright, 2019), bacteriophage therapy (Qadir & Chauhdary, 2018), antibacterial antibodies (Hua *et al*., 2014), probiotics (Kotzampassi & Giamarellos-Bourboulis, 2012), nanomaterials (Baptista *et al*., 2018), antimicrobial peptides (Mohan *et al*., 2019), among others, have been tested. Adding to this, the repurposing of existing drugs is becoming more attractive as a new strategy to fight AMR. This approach presents some advantages, namely due to the leverage of these drugs been already approved for use in humans and therefore, this reduces the timelines required for the drugs to be effectively available for treatment. The most successful and classical case is sildenafil (brand names: Revatio, Viagra). This drug was originally designed to treat hypertension and then repurposed to treat erectile dysfunction and pulmonary arterial hypertension (Goldstein *et al*., 1998). However, this drug was not repurposed as an antimicrobial. Drug repurposing has been used advantageously in cancer

therapy to access previously approved non-cancer drugs and direct them for cancer treatment (Moroney *et al*., 2011). Therefore, the repurposing of anti-tumour compounds such as metallic compounds (Patra, Gasser & Metzler-Nolte, 2012) could be of interest as a source of new antibacterials. Although the research of these compounds is mainly focused on antitumor purposes, to our knowledge, data available on the antimicrobial activity of these compounds is quite limited. As an example of a very active antimicrobial compound described a long time ago and used for the treatment of syphilis is salvarsan, an orgaoarsenical compound (Patra *et al*., 2015).

In the present study, Zinc- and Cobalt- metallic compounds, with previously reported anti-tumour properties (Silva *et al*., 2013; Luís *et al*., 2014) were tested for their potential antimicrobial activity against a range of bacteria. These zinc and cobalt metallic compounds were selected from an initial screening of 17 new molecules synthesised and previously tested for their potential anti-tumour activity. From this initial screening for antimicrobial activity, three compounds (identified as TS262, TS265, and TS267) showed the best activity, i.e., lower minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values against Gram-negative and -positive bacteria. On the basis of these results, we decided to investigate these three TS compounds further regarding their potential mode of action on bacteria. For that, we tested the effect of these compounds on the growth kinetics, motility, and permeability of the bacteria. There are several studies published regarding the effect that metallic compounds have on the bacterial membrane, by inducing damage to this structure and indirectly collapsing efflux systems (Lemire, Harrison & Turner, 2013; Viganon *et al*., 2017; Frei, 2020). Some bacterial efflux systems are dependent on the proton motive force and therefore any compound acting on this gradient will have an impact on the bacteria itself (Mahamoud *et al*., 2007; Sharma, Gupta & Pathania, 2019). If a compound affects the proton motive force it will also impact bacterial motility since bacterial flagella are driven by a rotary motor that uses free energy stored in the electrochemical proton gradient across the cytoplasmic membrane to do mechanical work (Meister, Lowe & Berg, 1987). Therefore, the assessment of bacterial phenotypes such as increased permeability of the bacterial membrane and motility can be of importance when analysing the potential mode of action of these metallic compounds.

## 2.2. Materials and methods

### 2.2.1. Compounds

Three metallic compounds coupled with Zn and Co were used in this study. The compounds were identified as follows: [Zn(phendione)$_2$] Cl$_2$ (phendione = 1,10-phenanthroline-5,6-dione) - TS262; Co(II) coordination compound CoCl(H$_2$O)(phendione)$_2$][BF$_4$] (phendione = 1,10-phenanthroline-5,6-dione) - TS265 and [ZnCl(κO-PTA=O)(phendione)]]BF$_4$] (phendione = 1,10-phenanthroline-5,6-dione) - TS267 (Silva *et al*., 2013). The structure of the compounds, as well as their main characteristics are described in more detail in **Figure 2.1**; **Table 2.1**. These compounds were previously synthesised by Silva *et al.* (Silva *et al*., 2013).

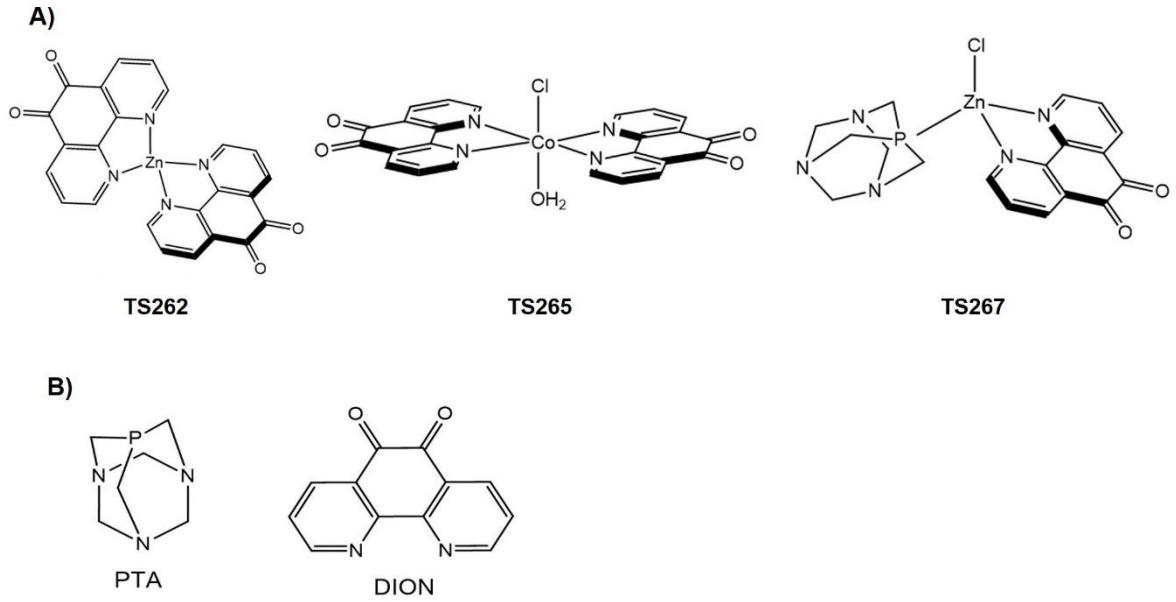


**Figure 2.1. Chemical Structure of the compounds A) and their ligands B).** Ligands: DION (phendione); PTA (1,3,5-Triaza-7-phosphaadamantane).

**Table 2.1. Chemical formula, molecular weight, and solvent of the three metallic compounds and their ligands**

| Compound | Chemical formula | Molecular Weight (g. mol$^{-1}$) | Solvent |
|---|---|---|---|
| **TS262** | $ZnC_{24}H_{12}N_4O_4Cl_2$ | 556,67 | $H_2O$ |
| **TS265** | $CoC_{24}H_{14}N_4O_5BF_4$ | 620,59 | $H_2O$ |
| **TS267** | $ZnClC_{18}H_{18}N_5O_2PBF_4$ | 570,99 | $H_2O$ |
| **DION** | $C_{12}H_6N_2O_2$ | 210,2 | $H_2O$ |
| **PTA** | $C_6H_{12}N_3P$ | 157,2 | $H_2O$ |

**Legend.** Ligands: DION (phendione); PTA (1,3,5-Triaza-7-phosphaadamantane).

### 2.2.2. Bacterial strains

*Listeria monocytogenes* EGDe, *Staphylococcus aureus* ATCC25923, *Acinetobacter baumannii* ATCC19606, *Escherichia coli* NCTC19200, *Klebsiella pneumoniae* ATCC70063, *Salmonella* Typhimurium ATCC14028S and *Pseudomonas aeruginosa* ATCC27853 were used in this study. The strains were grown in Mueller-Hinton (MH) broth at 37°C with shaking.

### 2.2.3. Antibacterial activity

The minimum inhibitory concentration (MIC) was determined by using the broth microdilution method in a 96-well plate according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute, 2013). Briefly, overnight cultures were diluted in sterilized phosphate buffered saline (PBS) solution to ~$10^5$ colony forming units (CFU)/mL. Aliquots of 10 µL were then transferred to separate wells in a 96-well plate that contained 100 µL of each compound at varying concentrations in Mueller-Hinton (MH) broth. Metallic compounds were tested at concentrations ranging from 0.2 µM to 104 µM (TS262 and TS265) and 0.2 µM to 120µM (TS267). After incubation at 37°C for 18 hours, the MIC was determined as the lowest concentration of compound were no visible growth of the bacteria was obtained. The determination of the minimum bactericidal

concentration (MBC) was performed by replica transfer of the MIC plate into a new 96-well plate with compound-free media. Plates were incubated at 37°C and the MBC results recorded after 18 hours. The MBC was defined as the minimum concentration of the compounds were no bacterial growth was obtained. The assays were performed with three biological replicates.

### 2.2.4. Bacterial growth in the presence of anti-tumour compounds

A 96-well plate was prepared with different concentrations of the three metallic compounds in MH broth. Diluted overnights cultures of the bacteria (~$10^5$ CFU/mL) were added to each well, except to the control wells (sterility) where only media was added. The microplate was then incubated in a microplate reader (Synergy™ HT multi-mode), and the bacterial absorbance (Optical density; $OD_{600nm}$) measured over a period of 24 hours at 37°C with shaking. The assays were performed with three biological replicates.

### 2.2.5. Motility assays

To assess bacterial motility, swim and swarm assays were performed on all isolates in the absence/presence of the compounds, as previously described (Wang *et al*., 2007). Briefly, for the swim assays, culture plates were previously prepared with MH broth with 0.4% (w/v) agar; Sigma, UK) and stab inoculated with bacterial cultures grown overnight in MH broth. These plates were then incubated for 8 hours at 37°C. The region of visible colony spread on the agar was measured (in millimetres - mm) with a ruler. Swarm motility assays were also performed with bacterial cultures grown overnight in MH broth. One microliter of each cell suspension was spotted directly onto a motility plate (MH broth with 0.6% (w/v) agar with 0.5% glucose; Sigma, UK) and incubated at 37°C overnight for 24 hours. The region of visible colony spread on the agar was then recorded.

### 2.2.6. Membrane permeability

Accumulation of ethidium bromide (EtBr) was measured as an indication of the ability of the compounds to permeabilise the bacterial membrane. To determine the EtBr concentration that did not exceed the ability of the efflux systems to extrude

EtBr, initial accumulation assays were performed in the presence of increasing concentrations of EtBr, as previously described (Rodrigues, Viveiros & Aínsa, 2015). The bacterial strains were grown in MH broth until mid-log phase. Bacterial cells were washed with PBS and the OD adjusted to 0.3 at 600 nm. Aliquots of this suspension with EtBr were transferred into a 96-well black plate with flat bottom (Costar, Sigma-Aldrich, St. Louis, Missouri, US). Heat-inactivated (30 minutes at 90°C) bacteria were used as a control for maximum fluorescence. Fluorescence was recorded using a Synergy™ HT multimode microplate reader for 30 minutes using excitation and emission filters of 515 and 600 nm, respectively. The compounds were then added at sub-MIC. The technical duplicates were averaged and plotted and compared based on their average fluorescence units. The assays were performed with three biological replicates.

### 2.2.7. Electrophoretic analysis of DNA-metal compound interaction

The interactions between DNA binding assays were conducted to determine if compound TS262 was able to cleave plasmid DNA. Plasmid DNA (pDNA) was obtained from *E. coli* transformed cells and grown overnight (o.n.) in LB liquid medium (Applichem, Darmstadt, Germany) with 100 µg mL−1 ampicillin (Bioline, London, UK) at 37°C with stirring. Plasmid extractions were performed using the Invisorb Spin Plasmid Mini Two Kit (Invitek, Berlin, Germany) and DNA were quantified by spectrophotometry with NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA). The interactions between TS262 and pBluescript II SK(+) (pBSKII) DNA (Agilent Technologies, Santa Clara, CA, USA) were determined as previously described (Luís *et al*., 2014). For the concentration-dependent studies, we incubated pBSK II (200 ng in a final volume of 20 µL) in the presence (25–100 µM) or absence of the tested compound for 24 h at 37°C in reaction buffer (5 mM Tris-HCl, 50 mM NaCl (pH 7.02)).

### 2.2.8. Mammalian cell culture and cell viability

Primary bronchial/tracheal epithelial cells (BTEC) and primary peripheral blood mononuclear cells (PBMC) were purchased from the American Type Culture Collection (ATCC) (www.atcc.org) and cultured according to the manufacturer's

specifications. BTEC were maintained in Airway Epithelial Cell Basal medium (ATCC) supplemented with bronchial epithelial cell growth kit (ATCC), 33 µmol/L Phenol Red (Sigma), and a mixture of 100 U/mL penicillin and 100 µg/mL streptomycin (ThermoFisher Scientific, Waltham, MA, USA) at 37°C and 5% $CO_2$ (as described in (Alves-Barroco *et al*., 2019). PBMC have a limited lifespan in culture and should only be thawed immediately prior to their use and maintained in Roswell Park Memorial Institute (RPMI) 1640 (Sigma) supplemented with 10% foetal bovine serum (FBS; Sigma), 2 mM glutamine (Sigma), 1% nonessential amino acids (Sigma), 1% sodium pyruvate (Sigma), and 50 U/mL penicillin and streptomycin (ThermoFisher Scientific). One day before treatment, cells were seeded at a density of $2 \times 10^4$ cells per well on a 96-well plate containing the respective media at 37°C, 5% (v/v) of $CO_2$, and an atmosphere of 99% (v/v) humidity for 24 hours. Cell viability was assessed after exposure to concentrations between 0 and 40 µM of TS262. MTS assay (CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay, Promega, Madison, Wisconsin, USA) was performed 24 hours after initial stimulus (at 37°C) using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and inner salt (MTS) as previously described (Pedrosa *et al*., 2018). Briefly, MTS was added to each well and cells were incubated during 45 minutes at 37°C. Following incubation, the absorbance of the soluble formazan product (brown) was measured in a microplate reader (TECAN, GE) at 490 nm. The formazan product is directly proportional to the number of living cells. The assays were performed with three biological replicates.

### 2.2.9. Statistical analysis

Statistical analyses were performed using Prim GraphPad software version 8.0.2. *, **, *** and **** represent p-values of < 0.05, < 0.01, <0.001 and < 0.0001 respectively. A $p < 0.05$ was considered statistically significant and highly significant when ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. One-way ANOVA was used to compare the mean values of at least three independent samples, where there was one independent variable in the experimental procedure. Allowing to determine any statistically significant difference between the samples' means.

## 2.3. Results

### 2.3.1. Antibacterial activity of anti-tumour compounds

The antimicrobial activity and effect on bacterial growth of three metallic compounds with Zn and Co as metal centre were assessed against a range of Gram-positive and -negative bacteria. The activity of the three metallic compounds against bacteria is summarised in **Table 2.2** (**Table S2.1** corresponds to the µg/mL activity of the three compounds). These compounds were initially selected from a group of 17 new molecules previously tested for their potential anti-tumour activity (for more details on these molecules, please see **Table S2.2**.

From the three compounds tested, Zn compound, TS262 was the most active against all the seven strains tested [MIC range from 0.9 (0.5 µg/mL) to 14.4 µM (8 µg/mL)]. The compound that showed lower antibacterial activity was TS265 (MIC range from 1.6 (1 µg/mL) to 25.8 µM (16 µg/mL)). TS262 showed good activity against *Acinetobacter baumannii* ATCC19606 (MIC of 0.9 µM– 0.5 µg/mL). The other two compounds, TS265 and TS267, also showed good activity against this bacterium, with MIC of 1.6 µM–1 µg/mL and 1.8 µM–1µg/mL, respectively. All compounds showed good activity against *A. baumannii* ATCC19606, with low concentrations not only inhibiting bacteria growth but also inducing its complete killing, indicating that they also have a good bactericidal activity. The three compounds were less active against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, which is not totally unexpected, since Gram-negative bacteria are almost impermeable to new drugs because majority of these drugs are unable to penetrate the bacterial cell wall. Adding to this, they also possess other mechanisms of resistance and efflux systems that are involved in the extrusion of the drugs from the cell to the external environment. In general, the majority of the compounds showed bactericidal activity at the same level as the MIC, with the exceptions of TS262 tested against *A. baumannii* (MIC = 0.9 µM–0.5 µg/mL and MBC = 1.8 µM–1 µg/mL) and TS267 against *P. aeruginosa* (MIC = 14 µM–8 µg/mL and MBC = 328 µM–16 µg/mL).

**Table 2.2. Antibacterial activity of the metallic compounds against a range of Gram-positive and -negative bacteria**

| Bacteria | TS 262 | | TS 265 | | TS 267 | |
|---|---|---|---|---|---|---|
| | MIC | MBC | MIC | MBC | MIC | MBC |
| | μM | | | | | |
| ***S. aureus* ATCC25923** | 1.8 | 1.8 | 3.2 | 3.2 | 3.5 | 3.5 |
| ***L. monocytogenes* EGDe** | 3.6 | 3.6 | 6.5 | 6.5 | 7 | 7 |
| ***E. coli* NCTC12900** | 3.6 | 3.6 | 6.5 | 6.4 | 3.5 | 3.5 |
| ***S.* Typhimurium ATCC14028S** | 3.6 | 3.6 | 6.5 | 6.5 | 7 | 7 |
| ***A. baumannii* ATCC19606** | 0.9 | 1.8 | 1.6 | 1.6 | 1.8 | 1.8 |
| ***K. pneumoniae* ATCC70063** | 14.4 | 14.4 | 25.8 | 25.8 | 14 | 14 |
| ***P. aeruginosa* ATCC27853** | 14.4 | 14.4 | 25.8 | 25.8 | 14 | 28 |

**Legend:** MIC, Minimum inhibitory concentration; MBC, Minimum bactericidal concentration; μM – micromolar. [Zn(phendione)$_2$] Cl$_2$ (phendione = 1,10-phenanthroline-5,6-dione) - TS262; Co(II) coordination compound CoCl(H$_2$O)(phendione)$_2$][BF$_4$] (phendione = 1,10-phenanthroline-5,6-dione) - TS265 and [ZnCl(κO-PTA=O)(phendione)]]BF$_4$] (phendione = 1,10-phenanthroline-5,6-dione) - TS267.

The growth of the bacterial strains in the presence of the MIC and sub-MICs of the compounds was monitored in rich media, namely, Mueller–Hinton (MH) broth (**Figures 2.2** and **2.3**). Data obtained for Gram-positive bacteria (**Figure 2.2A–F**) indicated that growth was similar to the untreated (control) at the lowest concentration tested (corresponding to one-quarter MIC of each strain tested) of each compound, with no significant changes being observed, with the exception of *S. aureus* (**Figure 2.2A–C**). A slight delay on the lag phase was observed when *S. aureus* was cultured in the presence of one-quarter MIC of the three compounds (**Table 2.3**). Concerning the Gram-negative bacteria (**Figure 2.3A–O**), a similar effect was observed, with the exception of *P. aeruginosa* (**Figure 2.3M– O**), where a slight extension of the lag phase was observed when the bacteria were grown in the presence of the three compounds. At one-half MIC of the three compounds, all

the strains demonstrated an extension of the lag phase (**Table 2.3**). This effect was also observed on the Gram- negative bacteria (**Figure 2.3A–O**). This effect is not unexpected since it is known that bacteria require time to adapt to the presence of toxic compounds, in this case, the anti-tumour compounds.

**Table 2.3. Summary of the effect of the three metallic compounds on bacterial lag phase.**

| Lag phases (in hours) | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | TS 262 | | TS 265 | | TS 267 | |
| | | ¼ MIC | ½ MIC | ¼ MIC | ½ MIC | ¼ MIC | ½ MIC |
| ***S. aureus* ATCC25923** | 4.75 | 6 | 7.5 | 7.5 | 16.5 | 6.5 | 11.5 |
| ***L. monocytogenes* EGDe** | 3.25 | 3.2 | 6 | 4 | 7.3 | 3.75 | 8.25 |
| ***E. coli* NCTC12900** | 3 | 4.5 | 8.25 | 4.5 | 8.5 | 4 | 5.25 |
| ***S.* Typhimurium ATCC14028S** | 3.75 | 4 | 6 | 5 | 8 | 4 | 9 |
| ***A. baumannii* ATCC19606** | 3.75 | 5.25 | 9 | 7.25 | 10.5 | 6 | 10.25 |
| ***K. pneumoniae* ATCC70063** | 3 | 4.25 | 11.25 | 5 | 12 | 4 | 5.5 |
| ***P. aeruginosa* ATCC27853** | 5 | 8 | 15.25 | 9 | 15 | - | 10 |

**Legend:** MIC, Minimuminhibitory concentration; µM – micromolar. $[Zn(phendione)_2]Cl_2$ (phendione = 1,10-phenanthroline-5,6-dione) - TS262; Co(II) coordination compound $CoCl(H_2O)(phendione)_2][BF_4]$ (phendione = 1,10-phenanthroline-5,6-dione) - TS265 and $[ZnCl(\kappa O\text{-}PTA{=}O)(phendione)]]BF_4]$ (phendione = 1,10-phenanthroline-5,6-dione) - TS267.

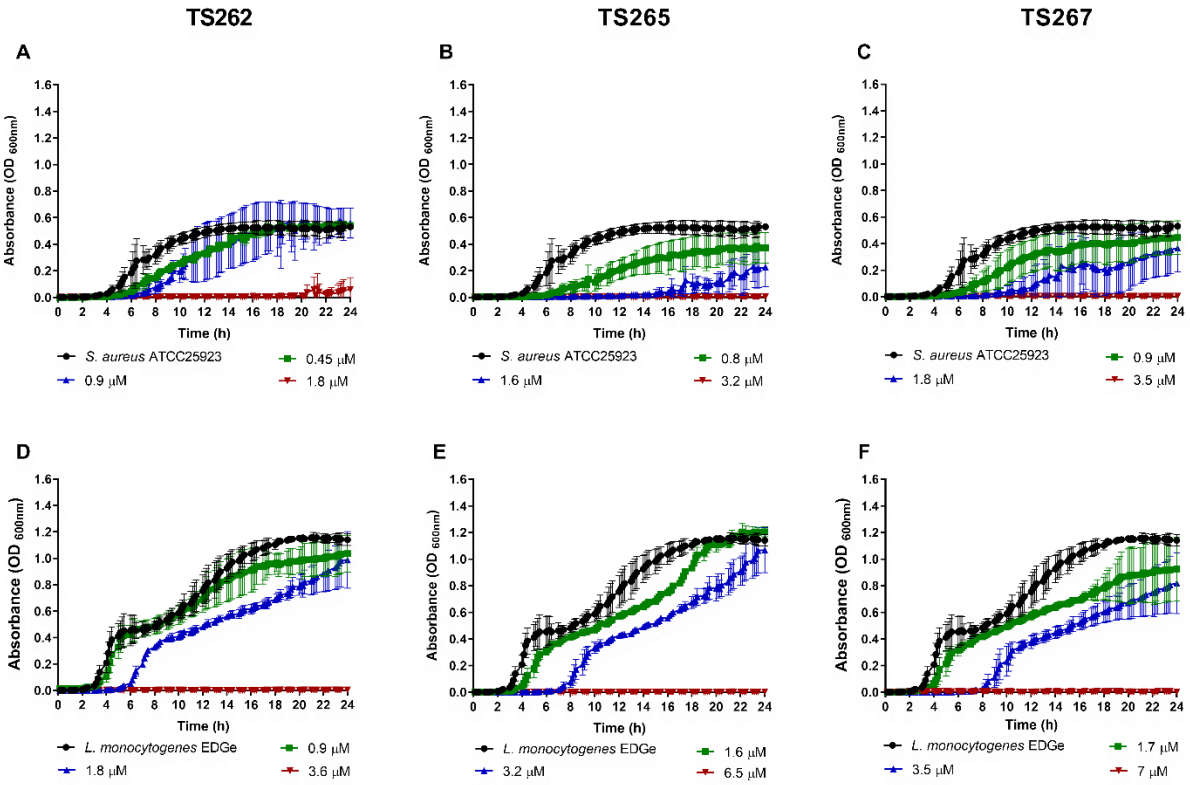


**Figure 2.2. Growth kinetics of Gram-positive bacteria in the presence of the three metallic compounds**.

Effect of the metallic compounds on the growth of *S. aureus* ATCC25923 (**A-C**), and *L. monocytogenes* EDGe (**D-F**) in Mueller-Hinton (MH) broth. The results correspond to the average of 3 independent experiments ± standard deviation (SD).

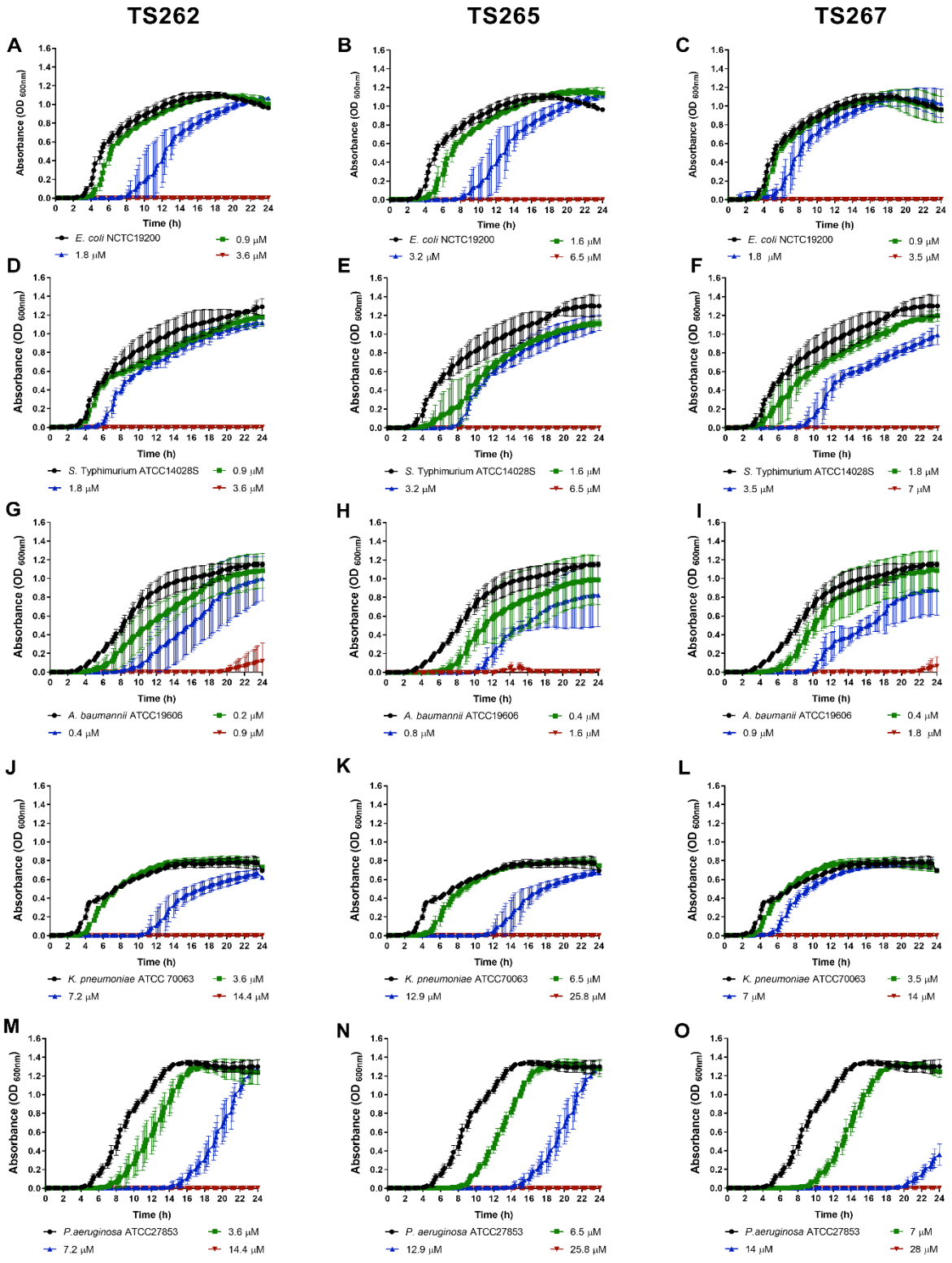


**Figure 2.3. Growth kinetics of Gram-negative bacteria in the presence of the three metallic compounds.** Effect of metallic compounds on the growth of *E. coli* (A-C), *S. Typhimurium* (D-F), *A. baumannii* (G-I), *K. pneumoniae* (J-L) and *P. aeruginosa* (M-O) in MH broth. The results presented correspond to the average of 3 independent experiments ± standard deviation (SD).

### 2.3.2. Effect on the bacterial motility

The swimming activity phenotype of each strain tested in this study was assessed in the presence of TS262 (**Figure 2.4.A, D, G, J, M**), TS265 (**Figure 2.4.B, E, H, K, N**) and TS267 (**Figure 2.4.C, F, I, L, O**). When the swimming activity of *A. baumannii* was tested in the presence of TS265 and TS267, a significant reduction on the swim activity of this strain was obtained when compared to the untreated control. In the presence of the three compounds, *L. monocytogenes* showed a slightly higher swim activity (but not significant) when compared with the untreated bacteria. For the other strains, no effect was obtained when in the presence of the compounds in comparison with the untreated strain. *K. pneumoniae* and *S. aureus* strains were not included in this experiment due to lack of classical motility ability.

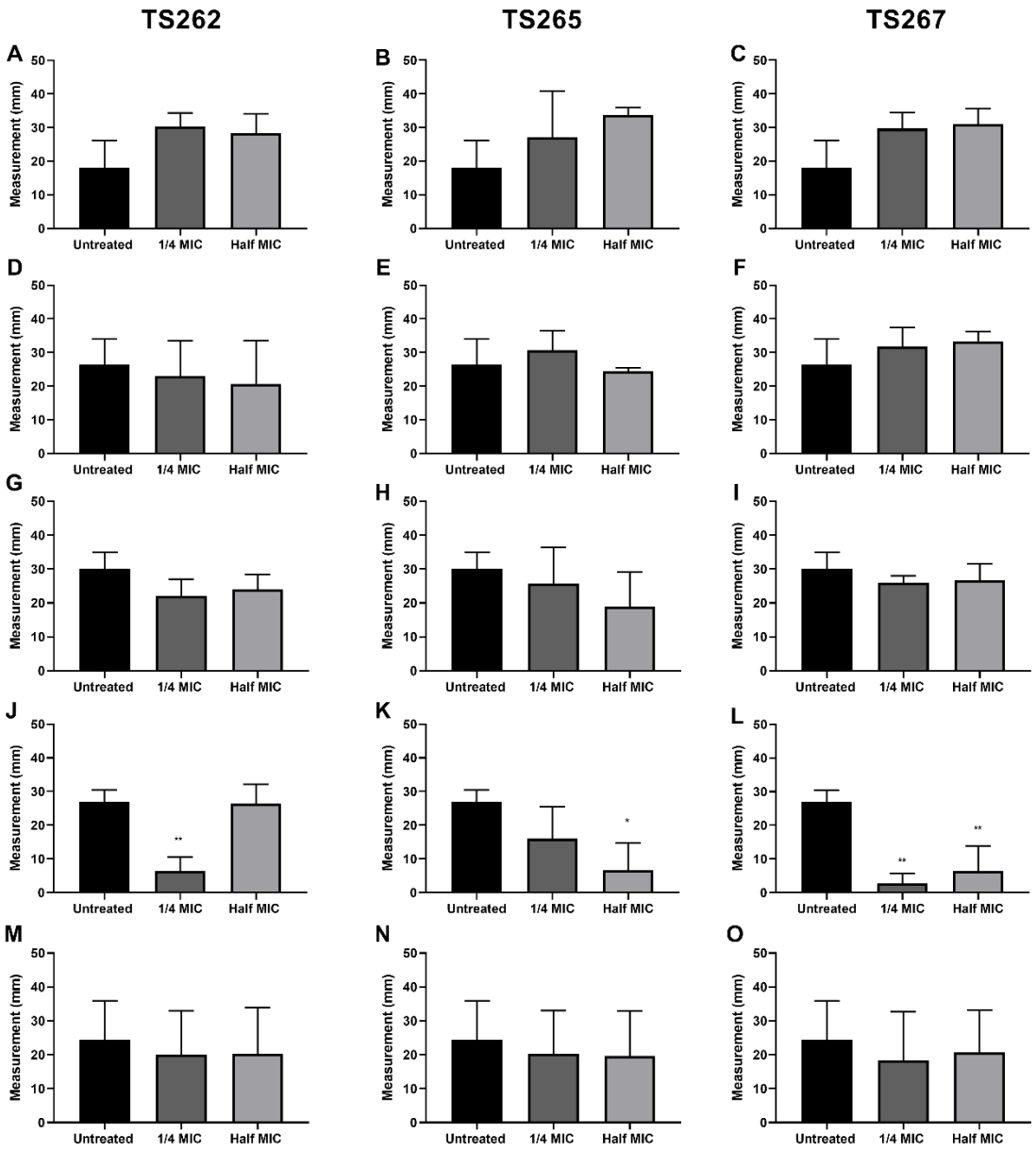


**Figure 2.4. Effect of the anti-tumour compounds on the bacterial swimming.** The motility of *L. monocytogenes* (**A-C**), *E. coli* (**D-F**), *S.* Typhimurium (**G-I**), *A. baumannii* (**J-L**) and *P. aeruginosa* (**M-O**) was assessed in 0.4% MH agar. The results presented correspond to the average of 3 independent experiments ± standard deviation (SD). *$p < 0.005$ was considered significant and highly significant when **$p < 0.01$ (one-way ANOVA test).

### 2.3.3. Study of the effect of the anti-tumour compounds on the bacterial membrane permeability

The seven strains were tested for their ability to accumulate EtBr in the presence of sub-MIC concentrations of each compound. EtBr has been used to assess permeability of the bacterial membrane. It is a DNA-intercalating agent that enters and binds the DNA when the bacterial membrane integrity is compromised, and this results in an increase in the fluorescence emitted. Initially, the concentration of EtBr at which the influx of the dye equals its efflux (equilibrium) and do not affect bacterial viability was determined. The accumulation of EtBr started at concentrations above 0.315 µM for *S. aureus, L. monocytogenes* and *K. pneumoniae;* 0.625 µM for *E. coli*, *P. aeruginosa* and *S.* Typhimurium and 1.25 µM for *A. baumannii* (**Figure 2.5**). Using these concentrations, determined previously, the potential of these three metallic compounds to permeabilise the membrane of Gram-positive and -negative bacteria at sub-inhibitory concentrations (1/2 and ¼ MIC) was assessed (**Figure 2.6. A- F** and **7. A-O**). Heat-inactivated bacteria were used as control for maximum fluorescence. When the bacteria were incubated with EtBr and the compounds (TS262, 265 and 267), no accumulation of EtBr was obtained. These results indicate that the Zn and Co anti-tumour compounds had no effect on the permeabilisation of the bacterial membrane both in Gram-positive and -negative bacteria.

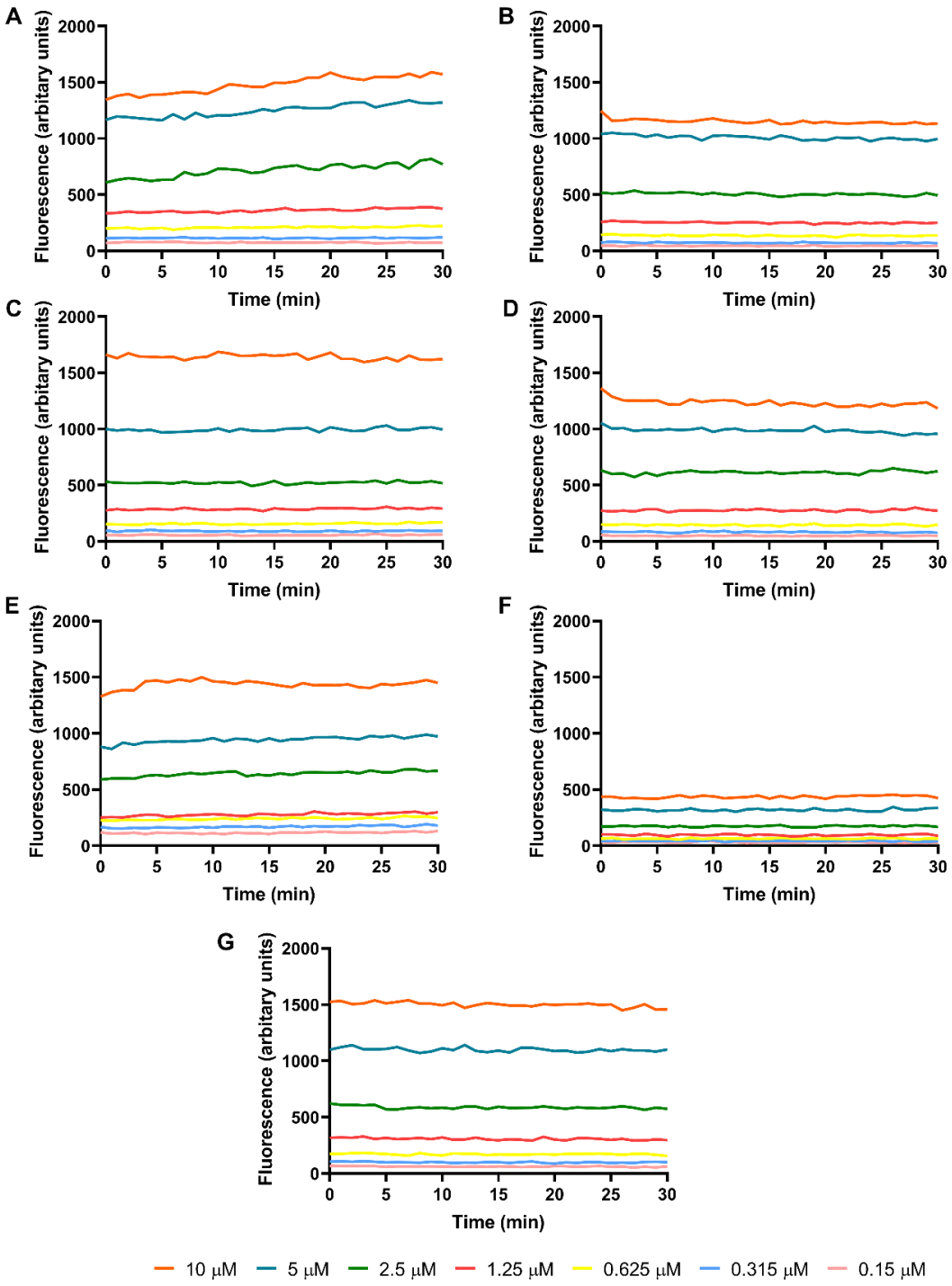


**Figure 2.5. Determination of the equilibrium concentration of ethidium bromide (EtBr) by *S. aureus* (A), *L. monocytogenes* (B), *E. coli* (C), *S.* Typhimurium (D), *A. baumannii* (E), *K. pneumoniae* (F) and *P. aeruginosa* (G).**

Bacteria were incubated with a range of concentrations of EtBr during 30 minutes at 37°C. The fluorescence intensity was recorded at excitation and emission wavelength of 515 and 600 nm. The results presented correspond to the average of 3 independent experiments.

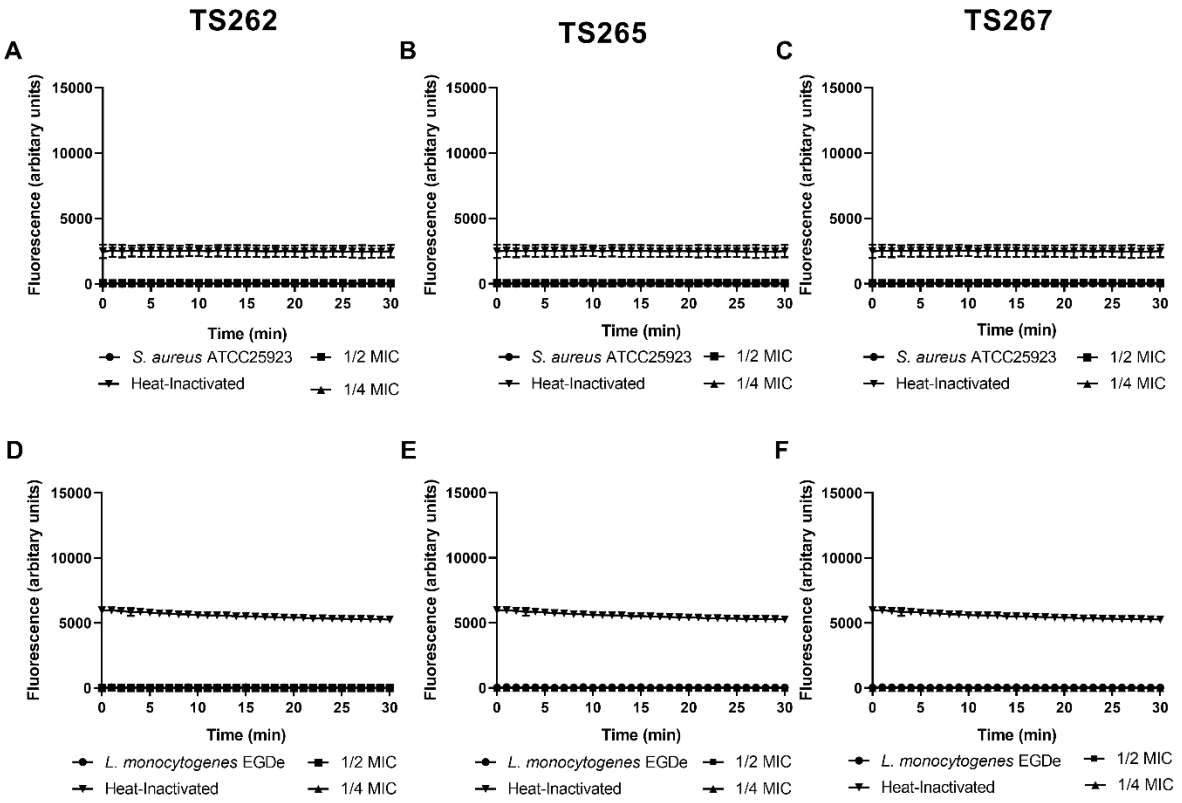


**Figure 2.6.Effect of the metallic compounds on the accumulation of EtBr by Gram-positive bacteria.**

*S. aureus* (**A-C**) and *L. monocytogenes* (**D-F**) were incubated with EtBr in the presence and absence of TS262, TS265 and TS267 for 30 minutes. Heat-inactivated bacteria were incubated with EtBr and run in parallel as a control for maximum fluorescence. The fluorescence intensity was recorded at excitation and emission wavelength of 515 and 600 nm. The results presented correspond to the average of 3 independent experiments.

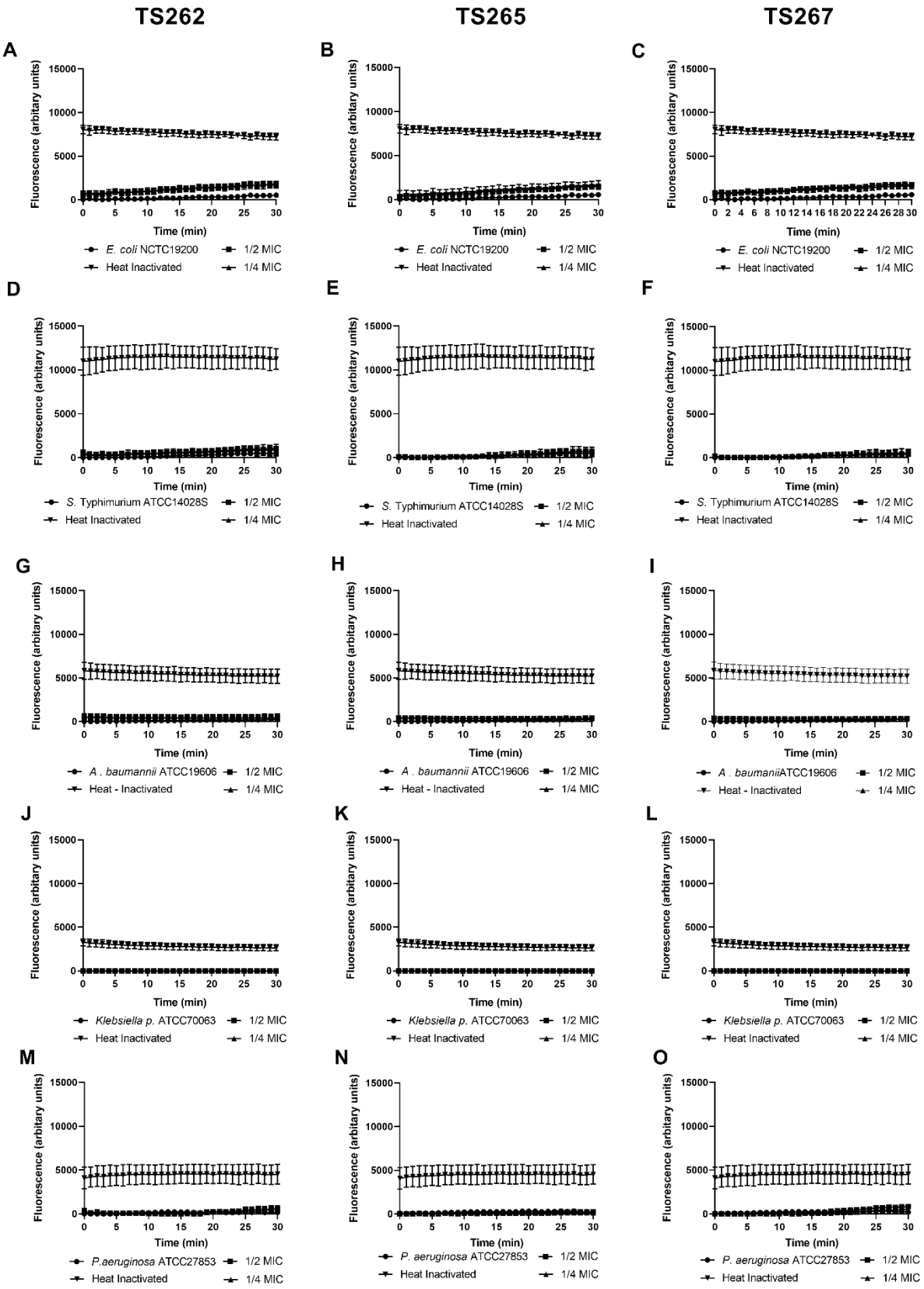
TS262
TS265
TS267
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
Fluorescence (arbitary units)
Time (min)
E. coli NCTC19200
Heat Inactivated
1/2 MIC
1/4 MIC
S. Typhimurium ATCC14028S
A . baumannii ATCC19606
A . baumanniiATCC19606
Heat - Inactivated
Klebsiella p. ATCC70063
P. aeruginosa ATCC27853

**Figure 2.7. Effect of the metallic compounds on the accumulation of EtBr by Gram-negative bacteria.**

*E. coli* (**A-C**), *S.* Typhimurium (**D-F**), *A. baumannii* (**G-I**), *K. pneumoniae* (**J-L**) and *P. aeruginosa* (**M-O**) were incubated with EtBr in the presence and absence of TS262, TS265 and TS267, for 30 minutes. Heat-inactivated bacteria were incubated with EtBr and run in parallel as a control for maximum fluorescence. The fluorescence intensity was recorded at excitation and emission wavelength of 515 and 600 nm. The results presented correspond to the average of 3 independent experiments.

### 2.3.4. DNA-Metal Compound Interaction

Previously work conducted by Luís *et al*. demonstrated that TS265 was able to interact with DNA and induce plasmid DNA (pDNA) cleavage, as well as being able to produce double-strand breaks in a concentration-dependent manner (Luís *et al*., 2014). In order to provide information concerning the capability of TS262 and TS267 to cleave pDNA, pDNA was incubated with increasing concentrations of each compound (separately) (**Figure 2.8**). As observed, neither compound was able to cleave pDNA or change the migration of supercoiled isoform, indicating that contrary to the Co(II) compound (TS265), these two Zn(II) compounds do not act in pDNA.

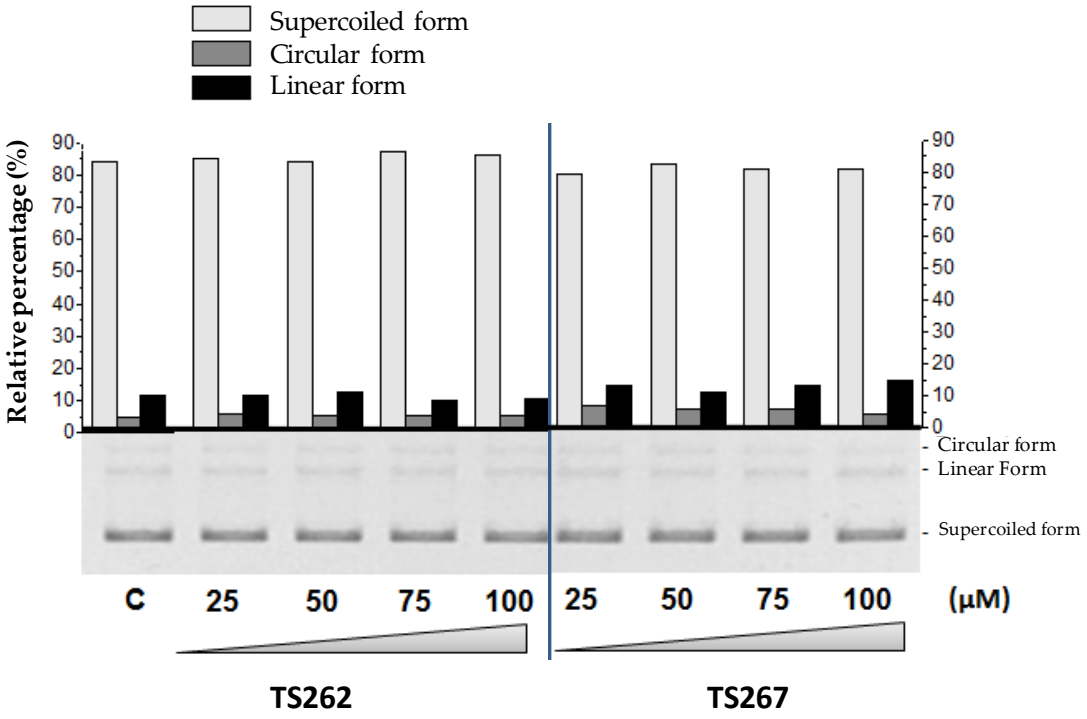


**Figure 2.8. Electrophoretic evaluation of DNA double-strand cleavage by both Zn(II) phendione compounds (TS262 and TS267).** Bottom: electrophoretic distribution of the three plasmid forms in an agarose gel (0.7% w/v) as a result of exposure to 25-100 μM of the compounds. All reactions were conducted in 5mM Tris-HCL, 50 mM NaCl pH 7.02 for 24h at 37°C. Plasmid DNA pBluescript II SK(+) (pBSKII): (C) untreated. Top: densiometric quantification of plasmid forms using the image analysis software GelAnalyzer 2010a. Light grey bars represent the supercoiled form of pDNA; dark grey bars the circular form; and black bars the linear form of pDNA. Results are expressed as mean ± SD of 3 independent experiments.

### 2.3.5. Cell viability

Due to the promising result shown by the TS262 compound concerning *in vitro* antimicrobial activity against Gram-positive and -negative bacteria, its effect on the cell viability of human primary blood mononuclear cells (PBMC) and primary bronchial/tracheal epithelial cells (BTEC) was assessed, and these results were compared to the inhibitory concentrations previously obtained *in vitro*. After 24 hours of exposure, the viability of both cell lines was assessed using the Cell Titer 96® AQueous One solution. Briefly, the conversion of a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] into an aqueous formazan product is accomplished by dehydrogenase enzymes found in metabolically active cells (Berridge & Tan, 1993).

As shown in **Figure 2.9**, there was a decrease of cell viability in a concentration dependent manner. The exposure of the cells to the Zn compound (TS262) at 2 µM caused a decrease in viability by 55%, only showing 45% of viable PBMC. When the effect in BTEC was analysed, only for concentrations higher than 5 µM, a reduction of viability of 50% was observed (**Figure 2.9**). Indeed, at 5 µM, a decreased of viability was observed by 55% with 45% of viable BTEC.

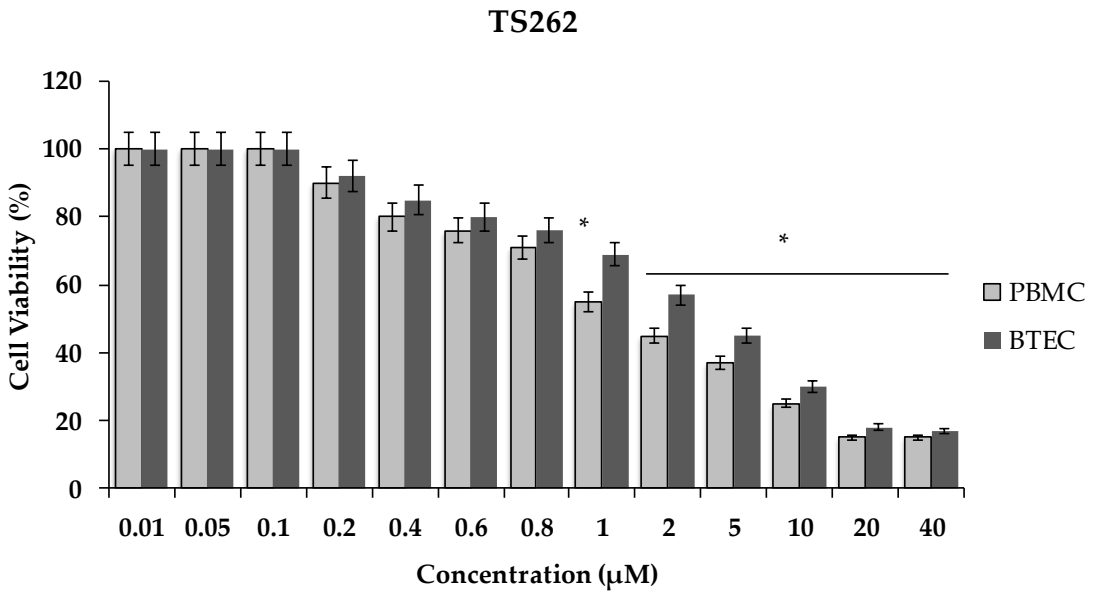


**Figure 2.9. Effect of compound TS262 on the cellular viability of human primary blood mononuclear cells (PBMC) (black bar) and primary bronchial/tracheal epithelial cells (BTEC) (grey bar).**

Cells were exposed to the compound for 24 hours at 37°C, 5% $CO_2$. The results presented correspond to the average of three independent experiments ± standard deviation (SD); percentage compared to controls from three independent biological replicates. Results were considered significant when * $p < 0.01$ (one-way ANOVA test).

## 2.4. Discussion

The escalating numbers of antibiotic-resistant bacteria worldwide raises the urgency for novel classes of antimicrobial compounds. Treatment options that rely on existing antibiotics are becoming less effective, and therefore re-purposing of existing non-antibiotic compounds, such as metallic anti-tumour compounds, would be a valuable approach. Here, we demonstrated that Zn and Co compounds have *in vitro* antimicrobial activity against Gram-positive and -negative bacteria. These Zn and Co anti-tumour compounds were tested for their potential effect on the growth kinetics, motility, and permeability of the bacteria. *In vitro*, compound TS262 was the most active against all the seven bacterial strains tested with an MIC range from 0.9 to 14.4 µM. This compound showed good activity against *A. baumannii* ATCC19606, with an MIC of 0.9 µM. The other two compounds, TS265 and TS267, also showed good activity against this bacterium, with MIC of 1.6 µM and 1.8 µM, respectively. These results are very encouraging since *A. baumannii* is one of the main pathogens related to hospital-acquired infections and is considered a critical pathogen (in the case of resistance to carbapenems) in the WHO priority pathogens list for research and development of new antibiotics. When the swimming activity of *A. baumannii* was tested in the presence of TS265 and TS267, a significant reduction was obtained when compared to the untreated control. When these compounds were tested for their potential effect on the permeabilisation of the bacteria, no effect was obtained. Therefore, these Zn and Co anti-tumour compounds did not seem to damage the cell wall of Gram-positive or -negative bacteria, which is in opposition to other published studies that tested different compounds with effects on the bacterial membrane (Cannon *et al*., 2009; Patra *et al*., 2015; Kazemizadeh *et al*., 2016; Viganon *et al*., 2017).

Here, it is also shown that contrary to the Co (II) compound TS265, which was able to cleave DNA in a redox-dependent manner (Luís *et al*., 2014), Zn (II) compounds were not able to cleave pDNA. On the basis of these results, I can only speculate that the potential cellular targets of these compounds might be related with protein dysfunction and/or impaired enzyme activity due to oxidative protein damage or exchange of a structural or catalytic metal. Since Co (II) compound TS265 was able to cleave DNA in a redox-dependent manner, it can be speculated that the production of reactive oxygen species and antioxidant depletion demonstrated in various other metal toxicity studies, particularly in the case of

compounds complexed with iron and copper, could be a possibility. It can be speculated that due to the structure of these compounds, they could also interfere with nutrient uptake and assimilation by the bacteria, which can directly affect gene expression and signalling mechanisms of quorum sensing on the bacterial population (Lemire, Harrison & Turner, 2013a; Viganon *et al*., 2017; Frei, 2020).

One important aspect to be considered in the design of a new antibacterial is the possibility of the bacteria becoming resistant to these compounds through well-known mechanisms such as active efflux. To address this, efflux studies were conducted on *E. coli* in the presence and absence of these compounds at varying concentrations. These compounds showed no direct effect in the efflux of the bacteria and therefore we do not anticipate these compounds to be extruded from the bacterial cell, therefore giving rise to resistance. However, future studies will address this question in more detail by using bacterial strains that have well-characterised mechanism of resistance to metals, such as Hg and Cu, among others (Mindlin *et al*., 2001; Abou-Shanab, van Berkum & Angle, 2007; Torres-Urquidy & Bright, 2012; Lima de Silva *et al*., 2012; Giovanella *et al*., 2016; Peters *et al*., 2018).

Considering the more interesting results of compound TS262, particularly with *A. baumannii* ATCC19606, its effect on the viability of PBMC and BTEC normal primary cells was tested. These compounds seem to have a negative effect on the viability of these cells at very low concentrations. However, it is important to stress that the concentrations needed to achieve a reduction of viability over 50% were 2 µM and 5 µM for PBMC and BTEC, respectively. Interestingly, these results agree with previous data obtained by Fernandes *et al*. on normal epithelial cells (Luís *et al*., 2014). Indeed, the half-maximal inhibitory concentration (IC50) obtained for this epithelial cell line (5.14 ± 0.01) µM was more than 6.9 times higher than that determined for a tumorigenic breast cell line MCF7 (0.73 µM) (Luís *et al*., 2014). Considering the MIC of 0.9 µM, a therapeutic window might exist (of 2.5× for PBMC, 6.25× for BTEC, and 6.9× for epithelial cells).

Nevertheless, for human administration, the vectorisation of these compounds using for instance gold nanoparticles (Pedrosa *et al*., 2019) might be an interesting approach that can be considered for future work.

On a broader discussion, these metallic compounds have been previously tested against different cell lines, such as human fibroblast and epithelial cell lines (Silva *et al*., 2013). Similar results were obtained with those cell lines, *i.e.*, the three compounds caused a reduction on the cell viability. Fibroblast cells lines had a reduction on cell viability higher than 50% at 1 µM, for the three compounds. Regarding the epithelial cell lines, HCT116, HepG2, and MCF-7 cells were tested. HepG2 and MCF-7 showed similar reduction on cell viability at concentration above 1 µM, for the three compounds. An exception to this was the effect of TS267 (5 µM) on MCF-7 cells, where a reduction of around 50% on the cell viability was obtained. Regarding the epithelial cell line HCT116, it showed decreased cell viability at 0.4 and 0.6 µM for TS262 and TS265, and TS267, respectively.

Due to the promising activity that these compounds reported *in vitro*, they can be an alternative to the available arsenal of antibacterial compounds. Additionally, these compounds can constitute good parental molecules to re-design derivatives of these molecules, presenting different metal centres. These approaches support the repurposing of drugs as an alternative source of new antimicrobial compounds to fight infections caused by antibiotic-resistant bacteria.

## 2.5. Supplementary Information

**Table S 2.1. Antibacterial activity (µg/mL) of TS262, TS265 and TS267 compounds against a range of Gram-positive and -negative bacteria.**

| Bacteria | TS 262 | | TS 265 | | TS 267 | |
|---|---|---|---|---|---|---|
| | MIC | MBC | MIC | MBC | MIC | MBC |
| | µg/mL | | | | | |
| ***S. aureus* ATCC25923** | 1 | 1 | 2 | 2 | 2 | 2 |
| ***L. monocytogenes* EGDe** | 2 | 2 | 4 | 4 | 4 | 4 |
| ***E. coli* NCTC12900** | 2 | 2 | 4 | 4 | 2 | 2 |
| ***S.* Typhimurium ATCC14028S** | 2 | 2 | 4 | 4 | 4 | 4 |
| ***A. baumannii* ATCC19606** | 0.5 | 1 | 1 | 1 | 1 | 1 |
| ***K. pneumoniae* ATCC70063** | 8 | 8 | 16 | 16 | 8 | 8 |
| ***P. aeruginosa* ATCC27853** | 8 | 8 | 16 | 16 | 8 | 16 |

**Legend:** MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; µM-micromolar. [Zn(phendione)2] Cl2 (phendione = 1,10-phenanthroline-5, 6-dione) - TS262; Co(II) coordination compound CoCl(H2O)(phendione)2][BF4] (phendione = 1, 10-phenanthroline-5,6-dione)—TS265; and [ZnCl(κO-PTA = O)(phendione)]]BF4] (phendione = 1, 10-phenanthroline-5,6-dione)—TS267

**Table S 2.2. Antibacterial activity of metallic compounds against Gram – positive and – negative bacteria**

| Compound ID | Solvent | Metal | *S. aureus* (µg/mL) | | *E. coli* (µg/mL) | |
|---|---|---|---|---|---|---|
| | | | MIC | MBC | MIC | MBC |
| **I.6**[a] | DMSO | Cu | 32 | 64 | >128 | >128 |
| **II.4**[a] | DMSO | Cu | >128 | >128 | >128 | >128 |
| **ComeOH**[a] | $H_2O$ | Cu | >128 | >128 | >128 | 128 |
| **TS119**[a] | $H_2O$ | Cu | >128 | >128 | >128 | >128 |
| **TS217**[b] | $H_2O$ | Co(II) | >128 | >128 | >128 | >128 |
| **TS232**[c] | $H_2O$ | Co | >128 | >128 | >128 | >128 |
| **TS236**[d] | DMSO | Co | >128 | >128 | >128 | 128 |
| **TS253**[c] | $H_2O$ | Co(II) | >128 | >128 | >128 | >128 |
| **TS254.1**[e] | $H_2O$ | V | 8 | 8 | 8 | 8 |
| **TS262.1**[b] | **$H_2O$** | **Zn(II)** | **1** | **1** | **2** | **2** |
| **TS265.1**[b] | **$H_2O$** | **Co(II)** | **2** | **2** | **4** | **4** |
| **TS267.1**[b] | **$H_2O$** | **Zn(II)** | **2** | **2** | **2** | **2** |
| **TS293**[d] | DMSO | Co | >128 | >128 | >128 | >128 |
| **RTS218.1**[e] | **$H_2O$** | V | >128 | >128 | >128 | >128 |
| **JL653 F2**[f] | DMSO | Pt(II) | >128 | >128 | >128 | >128 |
| **ZM2**[a] | DMSO | Cu | 32 | 32 | 64 | 64 |
| **ZM5**[g] | DMSO | Cu | 16 | 32 | 64 | 64 |

**Legend:** MIC, Minimum inhibitory concentration; MBC, Minimum bactericidal concentration. DMSO - Dimethyl sulfoxide; Cu - Copper; Co - Cobalt; V – Vanadium; Zn - Zinc; Pt - Platinum; [Zn(phendione)2] Cl2 (phendione = 1,10-phenanthroline-5,6-dione) - TS262; Co(II) coordination compound CoCl(H2O)(phendione)2][BF4] (phendione = 1,10-phenanthroline-5,6-dione) - TS265 and [ZnCl(κO-PTA=O)(phendione)]]BF4] (phendione = 1,10-phenanthroline-5,6-dione) - TS267. Bacterial strains used were Methicillin Resistant *S. aureus* ATCC43300 and *E. coli* ATCC25922. The structures, details of the synthesis and characterisation of all the compounds were previously published (a-g) (please see references below).

(a) **Ma, Z.; Zhang, B.; Guedes da Silva, M.F.C.; Silva, J.; Mendo, A.S.; Baptista, P.V.; Fernandes, A.R.; Pombeiro, A.J.L**. 2016. Synthesis, characterization, thermal properties and antiproliferative potential of copper(ii) 4′-phenyl-terpyridine compounds. *Dalton Trans.* **2016**, 5339-5355.

(b) **Silva, T.F.S.; Smoleński, P.; Martins, L.M.D.R.S.; Guedes da Silva, M.F.C.; Fernandes, A.R.; Luis, D.; Silva, A.; Santos, S.; Borralho, P.M.; Rodrigues, C.M.P.;** *et al*. Cobalt and Zinc Compounds Bearing 1,10-Phenanthroline-5,6-dione or 1,3,5-Triaza-7-phosphaadamantane Derivatives - Synthesis, Characterization, Cytotoxicity, and Cell Selectivity Studies. *Eur. J. Inorg. Chem.* **2013**, 3651–3658.

(c) **Silva, T.F.S.; Martins, L.M.D.R.S.; Guedes da Silva, M.F.C.; Fernandes, A.R.; Silva, A.; Borralho, P.M.; Santos, S.; C.M.P.; Pombeiro, A.J.L.** Cobalt complexes bearing scorpionate ligands: synthesis, characterization, cytotoxicity and DNA cleavage. *Dalton Trans.* **2012**, 12888-12897.

(d) **Silva, T.F. S.; Martins, L.M.D.R.S.; Guedes da Silva, M.F.C.; Kuznetsov, M.L.; Fernandes, A.R.; Silva, A.; Pan, C-J; Lee, J-; Hwang, B-J; Pombeiro, A.J.L.** Cobalt Complexes with Pyrazole Ligands as Catalyst Precursors for the Peroxidative Oxidation of Cyclohexane: X-ray Absorption Spectroscopy Studies and Biological Applications. *Chemistry, an Asian Journal* **2014**, 1132-1143.

(e) **Sutradhar, M.; Fernandes, A.R.; Silva, J.; Mahmudov, K.T.; Guedes da Silva, M.F.C.; Pombeiro, AJ.L.** Water soluble heterometallic potassium-dioxidovanadium(V) complexes as potential antiproliferative agents. *J. Inorg. Biochem.* **2016**, 17-25.

(f) **Silva, J.; Rodrigues, A.S.; Videira, P.A.; Lasri, J.; Charmier, A.J.; Pombeiro, A.J.L.; Fernandes, A.R.** Characterization of the antiproliferative potential and biological targets of a trans ketoimine platinum complex. *Inorganica Chimica Acta* **2014**, 156-167.

(g) **Mendo, A.S.; Figueiredo, S.; Roma-Rodrigues, C.; Videira, P.A.; Ma, Z.; Diniz, M.; Larguinho, M.; Costa, P.M.; Lima, J.C.; Pombeiro, A.J.L.; *et al*.** Characterization of antiproliferative potential and biological targets of a copper compound containing 4′-phenyl terpyridine. *J. Biol. Inorg. Chem.* **2015**, 935–948.

# 3. Old drugs learn new tricks – uncovering the mechanism of action of thioridazine in *Salmonella*

Data presented in this Chapter is submitted for publication in:

**Alves Ferreira, D.**; Finn, C.; Roma-Rodrigues, C.; Baptista, P.V.; Fernandes, A.R.; Martins, M. "Old drugs learn new tricks" – uncovering the *in vitro* mechanism of action of thioridazine in *Salmonella*. mSphere: *under review*.

**Abstract**

**Background:** Antibiotic resistance has become one of the main health crises of the 21st century. Due to the lack of new molecules, the repurpose of "old" drugs used to treat different conditions is becoming attractive. The antipsychotic Thioridazine (TZ) has been reported as an efflux pump inhibitor (EPI) with demonstrated activity against *Mycobacterium tuberculosis*, *Acinetobacter baumannii*, *etc*. but its mechanism of action has never been fully described. The main aims of this study were to uncover the mechanism of action of TZ in *Salmonella* Typhimurium (model bacterium). **Materials/methods:** The initial *in vitro* antibacterial activity of TZ was determined based on its minimum inhibitory concentration (MIC). Membrane permeability assays were performed using the Ethidium-Bromide (EtBr) accumulation assay. The effects on the membrane potential and the cell wall were assessed by flow cytometry and Transmission Electron Microscopy, respectively. ATP level production was assessed using a luminescence assay. Efflux activity was measured using an EtBr real-time measurement method. Effects on the bacterial proteome were assessed through 2D gel electrophoresis. Cytotoxicity was determined in THP-1 cells. **Results:** The MIC of TZ against *Salmonella* was 200 µg/mL. *In vitro* data demonstrates that TZ mechanism(s) of action involves primarily *Salmonella*'s membrane by affecting its permeability and potential after 15 minutes of exposure to TZ. At half of the MIC, and only after 15 minutes, TZ disrupts the bacterial membrane leading to leakage of the cellular contents and lysis of *Salmonella*. Proteomic profiling revealed 75 upregulated and 62 downregulated proteins, among which were TolC and AtpD, involved in efflux and cell energetics. TZ demonstrated no cytotoxicity on human cells at clinically relevant concentrations. **Conclusions:** The results obtained in this study suggest that TZ may act by targeting the bacterial cell envelope. Due to its probable mode of action, and lack of toxicity to human cells at clinically relevant concentrations, TZ can be considered a useful adjuvant to current therapeutic regimens used to treat multidrug resistant infections.

## 3.1. Introduction

Antimicrobial resistance (AMR) is one of the main public health challenges worldwide currently leading us into a “post-antibiotic” era, where a common infection may lead to death (World Health Organization, 2019d). The misuse and over prescription of antibiotics constitute a major health issue worldwide, by contributing to the increase of bacterial resistance. Adding to this, the discovery of new classes of antibiotics lags behind the continuing increase of bacterial resistance to current available therapy (Butler, Blaskovich & Cooper, 2017). Considering that the last novel antibiotic class discovered and approved for human use was in 1962, this highlights the need for novel drugs. Although new classes of antibiotics, such as teixobactin and pseudourimycin, have been discovered in more recent years, these have not yet been approved by the FDA for treatment of infections in humans. Factors that contributed to this devoid in drug discovery are the high costs associated with the research and development that, in the majority of the studies, ends up with high failure rates due to safety or toxicity issues (Hughes & Karlén, 2014). For that reason, researchers have been focusing their studies and effort on finding new antimicrobials and also in developing novel therapeutic strategies to overcome AMR. Some of these approaches include the development of new molecules through modification of the structures of existing antibiotics (Ashburn & Thor, 2004); the use of bacteriophages (Qadir & Chauhdary, 2018); the use of antimicrobial peptides (Mohan *et al*., 2019) or the repurposing of drugs. Drug repurposing is described as the process of creating new medical uses for existing approved drugs (Ashburn & Thor, 2004).

A good candidate for this is a family of compounds named phenothiazines. Phenothiazines are commercially available and it's *in vitro* antimicrobial activity has been described for decades (Crowle, Douvas & May, 1992; Martins *et al*., 2004). Thioridazine (TZ) belongs to the phenothiazine family and was originally developed as a neuroleptic drug to treat psychosis and schizophrenia. It has a reported effect against Gram -positive and -negative bacteria. The antimicrobial effect of TZ has been subject of numerous studies to date by several researchers in different locations of the world, but majority of the studies were performed in *Mycobacterium tuberculosis* (Ordway *et al*., 2003; de Keijzer *et al*., 2016) .

One of the main mechanism of action proposed in the literature for phenothiazines, including TZ, is the potential inhibition of efflux pumps of bacteria (Viveiros *et al*., 2005; Rodrigues *et al*., 2011). Efflux pumps are membrane proteins, involved in the export of toxic substances from within the bacterial cell into the external environment. TZ has been reported to block efflux pumps indirectly, *i.e.* by interfering with the bioenergetics of the bacterial cell. TZ is known to reverse MDR phenotypes making bacteria susceptible to antibiotics that they were initially resistant to (Viveiros *et al*., 2005; Kristiansen *et al*., 2006, 2007; Machado *et al*., 2016). Studies from Viveiros and his team demonstrated that TZ reversed the resistance of *Escherichia coli* to tetracycline by inhibition of over-expressed efflux pumps (Viveiros *et al*., 2005). In 2006, Kristiansen *et al*. also reported that TZ was able to reverse the resistance of *Staphylococcus aureus* to methicillin by inhibiting efflux pumps in methicillin-resistant *Staphylococcus aureus* (MRSA) (Kristiansen *et al*., 2006). The same was observed when a large number of clinical isolates of *M. tuberculosis* were induced to high level resistance to isoniazid (INH) and this resistance was reversed by using TZ (Viveiros *et al*., 2002). Besides the number of studies reporting TZ as an efflux pump inhibitor, the mechanism of how TZ interacts with efflux pumps hasn't been described yet.

Although there is a significant number of studies reporting the antimicrobial activity of TZ, both *in vitro* and *in vivo,* the fully mechanism of action of this compound has never been fully understood. The aim of the present study was to understand the *in vitro* mechanism of action of TZ using *Salmonella* Typhimurium as a model bacterium.

## 3.2. Materials and Methods

### 3.2.1. Bacterial strain and culture conditions

The strains included in the study was mainly *Salmonella* Typhimurium 14028S (**Table 3.1**). The strain was grown aerobically in Lysogeny Broth (LB) Miller overnight at 37°C with shaking. A subculture was prepared from the overnight bacterial suspension by diluting it to an optical density at $600_{nm}$ ($OD_{600nm}$) of ~0.05 in fresh media and re-incubating for 2-3 h until logarithmic phase was reached ($OD_{600nm}$ ~0.5). Bacterial cultures were then adjusted to the desired concentration for assay in sterile phosphate buffer (PBS) solution.

**Table 3.1. Bacterial strains used in this study.**

| Strain Name | Relevant Genotype | Resistance | Original Source |
|---|---|---|---|
| *S.* Typhimurium 14028S | Wild type | None | ATCC |
| | *atpD*-3x FLAG | Kan | In this study (*Dr. C. Finn) |
| | *tolC*-3x FLAG | Kan | In this study (*Dr. C. Finn) |
| | ΔacrA::kan | Kan | Martins Lab (Dr. C. Finn) |
| | ΔacrB::kan | Kan | Martins Lab (Dr. C. Finn) |
| | ΔacrAB::kan | Kan | Martins Lab (Dr. C. Finn) |
| | ΔtolC::kan | Kan | Martins Lab (Dr. C. Finn) |

**Legend:** Kan, Kanamycin.

### 3.2.2. TZ and other reagents

TZ and CCCP were purchased from Sigma-Aldrich (St. Louis, MO, USA). TZ stock solutions were prepared in sterile distilled water to a stock of 50 mg/mL, filtered, aliquoted and protected from light. These aliquots were kept frozen at -20°C. On the day of the experiment the aliquots were defrosted, and a working solution of 5 mg/mL was prepared and then diluted to the desired concentrations. CCCP stock solutions were prepared in Dimethyl sulfoxide (DMSO) to a stock of 5 mg/mL, filtered, aliquoted and protected from light. These aliquots were kept frozen at -20°C. On the day of the experiment the aliquots were defrosted, and a working solution of 1 mg/mL was prepared and then diluted to the desired concentrations. Ethidium Bromide (EtBr) stock solutions were freshly prepared on the days of the experiments in sterile distilled water and protected from light.

### 3.2.3. Antibacterial activity

The minimum inhibitory concentration (MIC) was determined by using the microdilution broth method in a 96-well plate according to the CLSI guidelines (Clinical and Laboratory Standards Institute, 2013) with slight modifications. Briefly, bacterial overnight cultures were diluted in sterile PBS solution to ~$10^5$ CFU/mL. Aliquots of 10 µL were then transferred to separate wells in a 96-well plate that contained 100 µL of TZ at varying concentrations in LB broth. TZ was tested at concentrations ranging from 1.5 to 800 µg/mL. The MIC was determined as being the lowest concentration of the compound at which no visible growth was observed after 18 hours of incubation at 37°C. Determination of the minimum bactericidal concentration (MBC) was performed by replica transfer of the MIC plate into a 96-well plate with compound-free media. Plates were incubated at 37°C and the MBC results recorded after 18 hours. The MBC was defined as the lowest concentration compound where no bacterial growth was obtained. The assays were performed in triplicate.

### 3.2.4. Growth kinetics of *Salmonella* in the presence of TZ

Bacterial growth was monitored in LB broth with or without TZ at different concentrations. Briefly, overnight cultures were diluted in sterile PBS to ~$10^5$ CFU/mL. Aliquots of 5 µL were then transferred to separate wells in a 96-well plate that contained 100 µL of each compound at varying concentrations in LB broth. TZ was tested at concentrations ranging from 50 to 200 µg/mL and $OD_{600nm}$ readings taken every 15 minutes for a 24 hours period using a Synergy™ HT multi-mode microplate reader (Biotek, Winooski, Vermont, US) at 37°C with orbital shaking. In parallel, a manual growth curve was performed. Briefly, the bacteria were cultured until the culture reached mid-log phase; at this stage the culture was split into two conditions (treated with 100 µg/mL TZ and non-treated). Measurements of the $OD_{600nm}$ were conducted and samples taken for CFU enumeration. The assays were performed with three biological replicates.

### 3.2.5. Thermostability

The thermostability of TZ was assessed by pre-incubating TZ 200 µg/mL for 24 hours at 37°C with shaking prior to inoculation with *Salmonella* Typhimurium. Using a microplate reader (Synergy™ HT multi-mode), the bacterial growth was monitored over a period of 24 hours at 37°C with shaking. A 96-well plate was then prepared with different concentrations of TZ in LB media. Diluted overnights cultures (10 µL - ~$10^5$ CFU/mL) were added each well. The plate was incubated for 18 hours at 37°C and the MIC/MBC values recorded and compared with the ones obtained from the MIC/MBC determined previously (Section 3.2.3).

### 3.2.6. Time-kill kinetics

The determination of the killing activity of TZ was analysed by time-kill assays. This was done according to a previously published protocol (Pillai, Moellering Jr & Eliopoulos, 2005), with slight modifications. Briefly, bacterial overnight cultures were diluted in sterile PBS to ~$10^5$ CFU/mL and 500 µL added to test tubes containing LB Miller broth and TZ at the desired concentration. TZ was added to each tube to achieve the final concentrations of 4x to 0.5x the MIC (previously determined). A compound-free control was included in the assay to

monitor the normal growth of the strain. Cultures were sampled for CFU determination after 0,15, 30, 60, 120 minutes of incubation at 37°C with shaking. Agar plates were then incubated at 37°C for 18 hours and CFU counted. The assays were performed in triplicate in three separate days.

### 3.2.7. Bacterial motility

To assess bacterial motility, swim and swarm assays were performed in the absence/presence of TZ. Briefly, for the swim assays, culture plates were previously prepared (LB broth with 0.3% (w/v) agar) and stab inoculated (Mariconda, Wang & Harshey, 2006). Motility assays were performed with 1 µL of an overnight culture and plates allowed to dry for 5 minutes. Followed by incubation for 8 hours at 37°C. The region of visible colony spread on the agar was measured (in mm) with a ruler. Swarm motility assays were also performed with bacterial cultures grown overnight in LB broth. 1 µL of the overnight bacterial suspension was spotted directly onto a motility plate (LB broth with 0.6% (w/v) agar with 0.5% glucose) and incubated at 37°C overnight for 24 hours (Wang *et al*., 2004). The region of visible colony spread on the agar was then recorded.

### 3.2.8. Membrane Permeability Assay

The potential effect of TZ on the membrane permeability was performed as previously described (Coldham *et al*., 2010). *Salmonella* 14028S were cultured overnight at 37°C with shaking and used to inoculate fresh medium LB broth that was incubated for 2 hours at 37°C with shaking. Bacterial cells were collected by centrifugation at 4000x g and resuspended in PBS. The $OD_{600nm}$ was adjusted to 0.3 in PBS. Aliquots of 180 µL of bacterial suspension were transferred to wells of a black flat bottom 96-well plate and Ethidium Bromide (EtBr) was added to each well (20 µL) at a final concentration of 1 µg/mL and incubated for 30 minutes at 37°C using a Synergy™ HT multi-mode microplate reader. After incubation, TZ was added to the wells of the microplate at final concentrations of 25, 50 and 100 µg/mL. The fluorescence was measured from the top of the wells using excitation and emission filters of 515 nm and 600 nm, respectively; readings were taken every 60 seconds for 30 minutes.

### 3.2.9. Membrane Potential Assay

The effect of TZ on the membrane potential of *Salmonella* 14028S was measured using the BacLight™ Bacterial Membrane Potential Kit (Molecular Probes, Life Technologies) in combination with flow cytometry according to the manufacturer's instructions. Briefly, bacteria cells were collected in mid exponential phase, washed three times with PBS, and resuspended at a final concentration of $1x10^6$ CFU/mL in PBS. TZ (50 and 100 µg/mL final concentrations) and CCCP (100 µg/mL final concentration ) were added to the cell suspension and incubated for 15 minutes at 37°C. Untreated cells (no compound added) were used as the live control and cells heat treated for 90 minutes at 90°C as "dead" control (maximum fluorescence). These samples were used to establish the initial gate between live and dead populations (data not shown). All samples were prepared to a final volume of 1 mL before the addition of the dyes. After this, 30 µM of DiOC2(3) was added to the mixture and the samples were incubated for 10-15 minutes. The samples were then analysed using a Flow cytometry Accuri™ C6. For each sample 10,000 cells were analysed using a 488 nm laser. Emission fluorescence was detected using the green fluorescence filter 533/30 and the red fluorescence filter 670LP. As a measure of the membrane potential, the ration of the mean red fluorescence intensity compared to the mean green fluorescence intensity was calculated. CCCP was used as a positive control, since it inhibits the proton motive force, eliminating the membrane potential. The assays were performed in triplicate.

### 3.2.10. Measurement of intracellular ATP levels

The ATP levels were measured using the EnLiten® ATP assay system (Promega, Ireland) according to the manufacturer's instructions. Briefly, *Salmonella* cultures were diluted to approximately $1x10^6$ CFU/mL in PBS. TZ was added to the bacterial suspension at concentrations from 50 to 100 µg/mL and incubated for 15 minutes at 37°C. After that, bacteria were inactivated by heating and immediately deep-frozen. The cell lysates were transferred into a white flat bottom 96 well plate and the ATP content measured using a Synergy™ HT multi-mode plate reader. CCCP was included as a control for comparison.

### 3.2.11. Evaluation of efflux activity by real-time fluorometry

The detection of ethidium bromide (EtBr) was performed as described previously (Rodrigues, Viveiros & Aínsa, 2015) with some modifications performed for *Salmonella* Typhimurium. Briefly, bacteria were grown until an $OD_{600nm}$ of 0.6 at 37°C with shaking. *Salmonella* Typhimurium 14028S were cultured overnight at 37°C with shaking and used to inoculate fresh LB broth that was incubated for 2 hours at 37°C with shaking. $OD_{600nm}$ of the bacterial cell suspension was adjusted to 0.6 by adding PBS, allowing the assay to run with a final OD of 0.3 After this, bacterial cells were collected by centrifugation and the pellet washed in PBS. To evaluate the effect of EPIs on the accumulation of EtBr, the assay was prepared with a range of concentrations of TZ and CCCP for a final volume of 100 µL.

For the efflux assays, 0.125x MIC was used in order to guarantee that the effects of the compound on the inhibition of efflux were not due to any antimicrobial effect of the compound itself. Briefly, *Salmonella* was exposed to conditions that promoted maximum accumulation of EtBr (*i.e.*, EtBr at the equilibrium concentration; no glucose; presence of the EPI) and incubated at room temperature for 1 hour. After that, cells were centrifuged, and resuspended in cold PBS. The assays were prepared on a black flat bottom 96-well plate to a final volume of 100 µL containing 50 µL of bacterial suspension plus 50 µL of EtBr and the compounds at a final concentration of 0.125x MIC. Control wells with only cells and cells with and without 0.4% glucose were included. The fluorescence was then measured in a Synergy™ HT multi-plate reader (excitation, 530 nm; emission 590 nm) at 37°C every 60 seconds for 30 minutes. The efflux activity was quantified by comparing fluorescence data obtained under conditions that promote efflux (presence of glucose and absence of EPIs) with data from bacteria under conditions of no efflux (presence of an EPIs and no energy source). The relative fluorescence corresponded to the ratio of the fluorescence that remained per unit of time, relatively to the EtBr-loaded bacterial cells.

### 3.2.12. Transmission electron microscopy (TEM)

*Salmonella* 14028S was grown to early exponential phase as described in the previous sections. The cultures were exposed to TZ 100 µg/mL for 15, 30 and 60 minutes at 37°C after which cells were collected by centrifugation and washed with

PBS. Pellets were fixed with PBS and electron microscope grade glutaraldehyde (2.5%) at room temperature with frequent inversions. An untreated control was also included. After washing the cells with PBS, they were suspended in PBS, embedded in agarose (2%) and then fixed with osmium tetroxide (2%). The cells were then dehydrated in increasing concentrations of ethanol and embedded in epoxy resin (Agar Scientific). Polymerisation of the resin was performed at 60°C for 24 hours. Sections were then cut with an ultra-microtome and cells were contrasted with 0.5% uranyl acetate and Reynold's lead citrate. The sections were examined on a JEOL JEM 1400 transmission electron microscope operated at 120 kV. These procedures were conducted at the Trinity Imaging core facility

### 3.2.13. Protein extraction for two-dimensional (2-D) electrophoresis

Bacterial cells were allowed to grow in the presence or absence of TZ 100 µg/mL for 15 minutes and processed for total protein extraction. Briefly, cells were washed with PBS, collected and lysed using lysis buffer (50mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM EDTA) supplemented with 0.1% dithiothreitol (DTT), 1% Nonidet P-40, 1x Halt Protease and Phosphatase Inhibitor Cocktail. Then, the lysates were centrifuged at 12,000 g for 10 minutes, at 4°C. Total proteins in the supernatant were recovered and stored at -80ºC. Protein concentrations were determined using the Bio-Rad protein assay kit according to the manufacturer's instructions.

### 3.2.14. Two-dimensional electrophoresis

For isoelectric focusing (IEF), 100 µL of the total crude extract was treated with 2-D Clean-Up Kit (GE Healthcare) for removal of IEF interfering molecules (*e.g.* lipids and salts), according to the manufacturer's instructions. Precipitated proteins were resuspended in 150 µL of rehydration buffer (2% (v/v) NP-40 (GE Healthcare), 7 M urea (GE Healthcare), 2 M thiourea (GE Healthcare), 1x protease inhibitors (cOmplete ULTRA Tablets, Mini, Ethylenediaminetetraacetic acid (EDTA)-free, EASYpack Protease Inhibitor Cocktail, Roche), 1x phosphatase inhibitors (PhosSTOP, Roche), 1 mM phenylmethylsulfonyl fluoride (PMSF) (GE Healthcare), 0.5% (w/v) DTT (GE Healthcare), 0.5% (v/v) Destreak rehydration solution (GE

Healthcare), 0.5% (v/v) IPG electrolytes 3-10 NL (GE Healthcare), traces of bromophenol blue (GE Healthcare), and incubated at room temperature for 2 hours. After protein quantification using the Pierce 660 nm protein assay reagent (Thermo Scientific), samples with 200 µg of protein in a total volume of 125 µL of rehydration buffer were used to actively rehydrate Immobiline Drystrip (IPG strips, GE Healthcare) with 7 cm and 3-10 non-linear pH in an Ettan IPGphor instrument (GE Healthcare), followed by the IEF using a maximum voltage of 5000 V. The IPG strips were incubated for 15 minutes in equilibration buffer 1 (50 mM tris (hydroxymethyl) aminomethane (THAM) hydrochloride (Tris-HCl) pH 8.8, 2% (w/v) sodium dodecyl sulphate (SDS), 6 M urea, 30% (v/v) glycerol, 1% (w/v) DTT and traces of bromophenol blue), followed by another incubation for 15 minutes in equilibration buffer 2 (50 mM Tris-HCl pH 8.8, 2% (w/v) SDS, 6 M urea, 30% (v/v) glycerol, 2.5% (w/v) iodoacetamide and traces of bromophenol blue) and placed on top of a 10% SDS-polyacrylamide gel for second dimension. After SDS-polyacrylamide gel electrophoresis (PAGE), gels were incubated in PhastGel Blue R-350 (GE Healthcare) according to manufacturer's instructions. Gel images were acquired in an Image Scanner II from GE Healthcare. Gels were performed in duplicate for each condition.

### 3.2.15. Analysis of protein expression levels

The analysis of gel images was performed using Melanie 7.0 software (Geneva Bioinformatics, Geneva, Switzerland). Protein spot detection was first performed automatically using the software, followed by manual confirmation. For protein expression analysis, the volume of each individual spot normalised against the total volume of spots of the respective gel was considered. The average of the normalised volume of the matched protein spot in duplicated gels of the same condition was then used to calculate the fold variation between different conditions. The protein expression was considered altered if the fold between two conditions was lower than 0.7 (lower protein expression) or higher than 1.5 (increased protein expression).

### 3.2.16. Protein spot identification

Additionally, to confirm the correct translations from the reference map and to identify other proteins of interest, protein spots were manually excised from the gel and identified in the UniMS – Mass Spectrometry Unit, ITQB/IBET (Oeiras, Portugal) using Peptide Mass fingerprint. The groups of altered proteins between conditions were analysed in STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database to infer possible protein-protein interactions, sub-cellular locations, and major altered pathways.

### 3.2.17. Epitope Tagging

The *Salmonella* strains carrying a chromosomal FLAG-tagged *atpD* or *tolC* gene were generated by the λ Red recombinase system, as described previously using gene-specific primer pairs, as shown in **Table 3.2** (Datsenko & Wanner, 2000; Uzzau *et al*., 2001). Briefly, 3x FLAG tags preceding the Kanamycin (Kan) resistance cassette were amplified from the pSUB11 plasmid using primers with 40 base pair (bp) homology to the 3' end of the gene to be tagged. The PCR products were integrated into the bacterial chromosome by transformation of a strain carrying the pKD46 plasmid by homologous recombination. Transformants carrying the FLAG-tagged gene were selected for on LB agar plates supplemented with Kan. and the presence of the tag was confirmed by sequencing. Tagged genes were transferred into a fresh *Salmonella* 14028S strain backgrounds by phage P22 transduction and confirmed by Polymerase Chain Reaction (PCR).

**Table 3.2.Primers used for construction of FLAG-tag strains in this study**

| Primers | Sequence (5'– 3') | Expected PCR Product Size (bp) | |
|---|---|---|---|
| | | 3xFLAG Construct | WT |
| *atpD*-3xFLAG – F | CGGTTCCATCGACGAAGCCGTGGAAAAA GCCAAAAAACTTGACTACAAAGA CCATGACGG | 1996 | 503 |
| *atpD*-3xFLAG – R | GTGGTAAGTCATTGCCATATCACCCTCCG ATTAAGGCGCATATGAATATCCTCCTTA GT | | |
| *tolC*-3xFLAG – F | CTCGCCAACAGCAATAACGGCAATCCATT CCGGCATGACTACAAAGACCATGACGG | 2083 | 590 |
| *tolC*-3xFLAG – R | GGGCACAGGTCTGATAAGCGCAGCGCCA GCGAATAACCATATGAATATCCT CCT TAG T | | |
| *atpD*-3xFLAG_Scrn– F | AAGTATTTACCGGTTCTCCGGG | | |
| *atpD*-3xFLAG_Scrn – R | AGCTCTTAATGTGCTCTTCGGC | | |
| *tolC*-3xFLAG_Scrn – F | GTATGATGCCAAGCAGCAACTG | | |
| *tolC*-3xFLAG_Scrn – R | AGCATAAAAACAGCCGTAACCG | | |

**Legend.** WT, Wild type. F, forward; R, reverse; bp, base pairs; Scrn, screen.

### 3.2.18. Proteomics data validation by Immunoblot analysis

Protein samples were normalized according to the optical density ($O.D._{600nm}$) of the cultures. Gel electrophoresis was carried out according to the method of Laemmli (Laemmli, 1970) using SDS-10% polyacrylamide gels and transferred onto Immobilon-P (Millipore). Membranes were blocked with blocking buffer (5% (w/v) skimmed milk powder, PBS, 0.05% Tween-20) and incubated at 4°C overnight on a rocker. The primary antibodies, monoclonal anti-FLAG M2 (Sigma) and monoclonal anti-DnaK (*E. coli*, Enzo Life Sciences) were diluted in blocking buffer 1:10,000 and 1:30,000 respectively, and incubated for 1 hour at room temperature.

DnaK was used as a loading control for protein levels for Western blotting. This allowed to determine if general protein levels were expressed evenly between different samples. Membranes were washed in PBST (PBS, 0.05% Tween-20) three times for a total of 30 min before incubation with the secondary antibody anti-mouse immunoglobulin G (IgG) horseradish peroxidase (HRP) linked antibody (Cell Signalling®) Technology) diluted 1:25,000 in blocking buffer for 45 minutes at room

temperature on a rocker. Membranes were washed an additional 3 times in PBST for a total 30 min. For protein detection, membranes were processed using ECL chemiluminescent HRP substrate (Thermo Fisher). A ratio of 1:1 of luminol and peroxide were mixed and used to flood the membrane and incubated in the dark for 5 minutes. Light emission occurs as a by-product of the oxidation of luminol by HRP and this was detected using the ImageQuant LAS4000 (GE Healthcare). The relative intensities of protein bands were quantified using the ImageJ software version 1.52n.

### 3.2.19. Cell Viability against human monocytes

THP-1 human monocytes were grown in suspension in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco) in a humidified 37°C, 5% $CO_2$. THP-1 cells were seeded at a density of $2.5x10^5$ per well in a 96-well, cell culture treated microplate. Cells were then treated with TZ at concentrations ranging from 0.1 µg/mL to 50 µg/mL and incubated for 24 hours at 37°C with 5% $CO_2$. Viability assay was performed using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions.

### 3.2.10. Statistical analysis

Statistical analyses were performed using Prim Graphpad software version 8.0.2. *, **, *** and **** represent p-values of $< 0.05$, $< 0.01$, $<0.001$ and $< 0.0001$ respectively. A $p < 0.05$ was considered statistically significant and highly significant when ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. One-way ANOVA was used to compare the mean values of at least three independent samples, where there was one independent variable in the experimental procedure. Allowing to determine any statistically significant difference between the samples' means. Two-way ANOVA was used to compare the mean values of at least two or more samples where there were also two or more independent variables are considered in the experimental assay.

## 3.3. Results

### 3.3.1. Antibacterial activity

To assess the antimicrobial activity of TZ, antimicrobial susceptibility assays including MIC/MBC determinations and monitoring of bacterial growth were performed. The MIC and MBC of TZ against *Salmonella* Typhimurium 14028S was obtained at the same concentration of 200 µg/mL (**Table 3.3**). *Salmonella* strains carrying a chromosomal FLAG-tagged *atpD* or *tolC* gene had an MIC (and MBC) of 400 µg/mL, one-fold higher comparing with the wild type. However, this difference on the MIC was not considered significant since the method used was broth microdilution, following CLSI guidelines.

**Table 3.3. Minimum Inhibitory and Bactericidal Concentrations (MIC/MBC) against *Salmonella* Typhimurium.**

| Bacterial strains | TZ | | EtBr | | CCCP | |
|---|---|---|---|---|---|---|
| | MIC (µg/mL) | MBC (µg/mL) | MIC (µg/mL) | MBC (µg/mL) | MIC (µg/mL) | MBC (µg/mL) |
| *Salmonella* Typhimurium ATCC14028S | 200 | 200 | >1000 | >1000 | 20 | 20 |
| *atpD*::3xFLAG | 400 | 400 | - | - | - | - |
| t*olC*::3xFLAG | 400 | 400 | - | - | - | - |

**Legend.** TZ, Thioridazine; EtBr, Ethidium Bromide; CCCP, Carbonyl Cyanide 3-Chlorophenylhydrazone

### 3.3.2. Growth kinetics of *Salmonella* in the presence of TZ.

As shown in **Figure 3.1**, the effect of TZ in the growth of *Salmonella* is concentration dependent. During the first 6 to 8 hours of exposure to TZ at half of the MIC (100 µg/mL), the growth of *Salmonella* was inhibited. After this extension of the lag phase, bacteria grew at a similar rate to that of the control (no TZ). When *Salmonella* was exposed to the MIC of TZ no bacterial growth was obtained, as expected, since this value also corresponds to the MBC determined previously. A similar effect was observed in relation to the CFU/mL when *Salmonella* was cultured in the presence of half of the MIC of TZ (**Figure 3.2**).

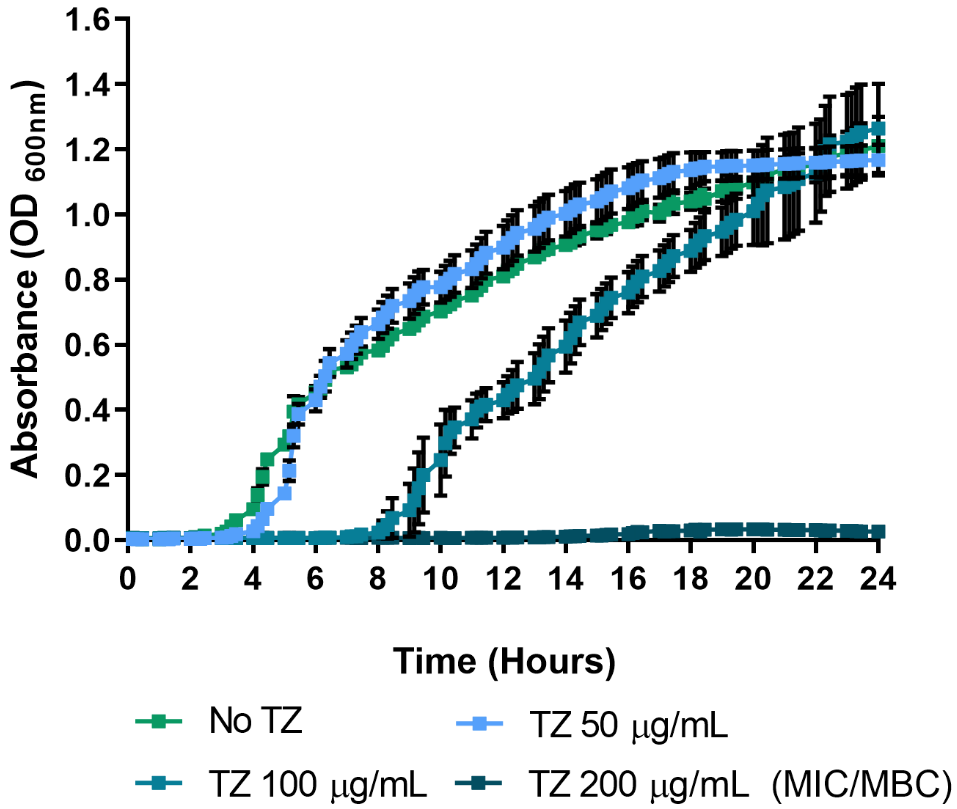


**Figure 3.1. The effect of different concentrations of TZ against *Salmonella* Typhimurium.** The growth of *Salmonella* Typhimurium was monitored by measuring $OD_{600nm}$ for 24 hours in LB broth in the presence and absence of TZ at 37°C with orbital shaking. The results presented correspond to the average of 3 independent experiments plus ± standard deviations (SD).

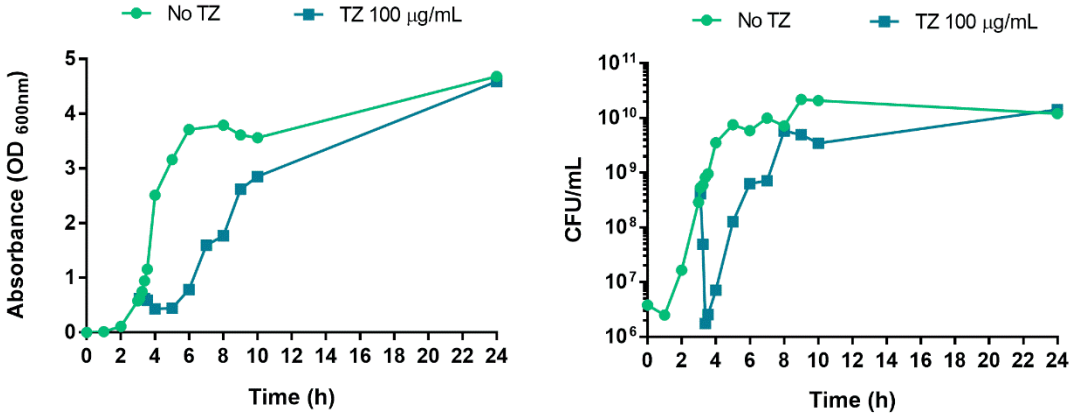


**Figure 3.2. Representative images of *Salmonella* Typhimurium of the effect of TZ 100 µg/mL on bacterial growth and CFU/mL.** Growth of *Salmonella* Typhimurium for 24 h in LB broth in the presence of TZ at 37°C with orbital shaking. The growth was monitored by reading the $OD_{600nm}$ every 15 minutes for 24 hours. Results are representative of 4 independent experiments. TZ, Thioridazine; OD, optical density.

### 3.3.3. Thermostability

To assess the stability of TZ over the culture period, TZ 200 µg/mL was pre-incubated in LB media for 24 hours at 37°C with shaking, prior to inoculation with *Salmonella*. Pre-incubated TZ was compared with freshly prepared TZ 200 µg/mL. As shown on **Figure 3.3** there was no difference observed in terms of bacterial growth when *Salmonella* was cultured in media containing TZ that was freshly prepared and media containing TZ pre-incubated. This was the same for the three concentrations of TZ tested (50, 100 and 200 µg/mL) indicating that TZ is thermostable during the timescale of the studies. A similar result was reported by Keijzer *et al.,* when assessing the growth rate of *M. tuberculosis* for 21 days with freshly prepared media and media containing pre-incubated TZ (de Keijzer *et al*., 2016).

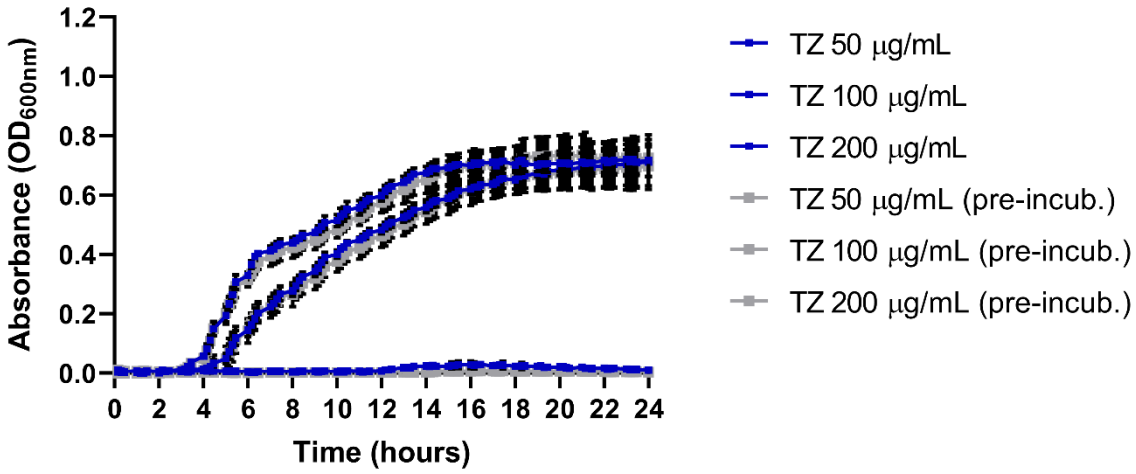


**Figure 3.3. Growth kinetics of *Salmonella* Typhimurium in media containing 50, 100 and 200 µg/mL of TZ pre-incubated (pre-incub.) in media for 24 hours at 37°C (stability) and media that had TZ freshly prepared.** The results presented correspond to the average of 3 independent experiments plus ± standard deviation (SD).

### 3.3.4. Time-kill kinetics

To characterize the effect of TZ in the bacterial killing, its activity against *Salmonella* 14028S was measured through time-kill studies. As shown in **Figure 3.4**, the rate of bacterial killing was concentration dependent. At half of the MIC of TZ (0.5x MIC), a reduction of approximately 1 log compared with the untreated control was observed and maintained until 120 minutes (endpoint of the experiment). Exposure to 200 µg/mL of TZ (MIC) completely eliminated *Salmonella* (0 CFU/mL) after 60 minutes. However, when this concentration was increased to 2x MIC total bacterial clearance was observed after 15 minutes of exposure to the compound. These results show that the killing activity of TZ is very rapid, reaching total bacterial clearance at the MIC and MBC values.

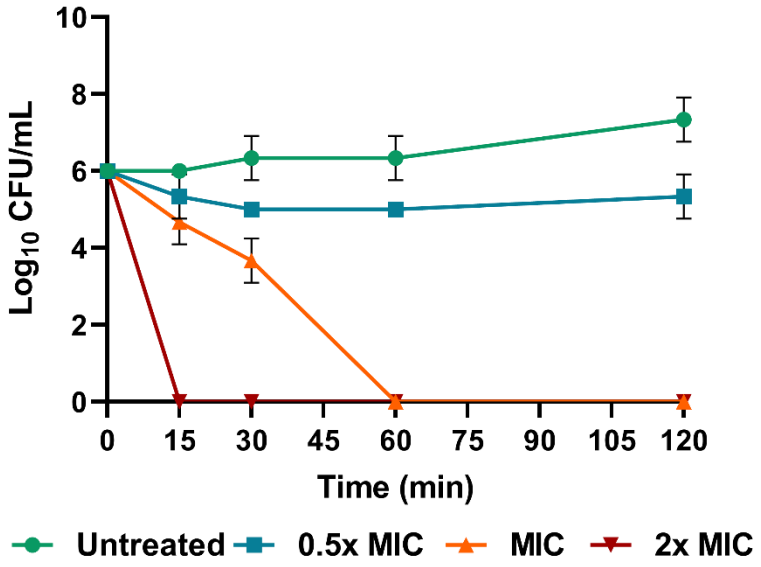


**Figure 3.4. Killing kinetics of TZ against *Salmonella* Typhimurium.** TZ was added at concentrations ranging from 0.5x MIC (100 µg/mL) to 2x MIC (400 µg/mL) and bacterial growth monitored through plating of bacteria for colony forming units enumeration for 120 minutes. The CFU/mL was calculated at the timepoints, 0, 15, 30, 60 and 120 minutes. The results presented correspond to the average of 3 independent experiments plus ± standard deviations (SD).

### 3.3.5. Bacterial motility was reduced in the presence of TZ

Swim (individual movement) and swarm (multicellular movement) motility is associated with the regulation and rotation of the flagella within liquid and solid surfaces, respectively. For both swimming and swarming assays, when *Salmonella* was exposed to TZ a decrease on bacterial motility was observed as the concentration of the compound increased, as shown in **Figure 3.5**. *Salmonella* 14028S exposed to the MIC concentration of TZ no swimming ability is observed (**Figure 3.5A** and **Figure 3.5B iv** top). In the presence of 100 µg/mL of TZ (0.5x MIC), bacterial swimming decreased almost to half comparing with the control (**Figure 3.5. A** and **Figure 3.5B iii top**). At the 50 µg/mL, a similar swimming ability was observed as seen at **Figure 3.5A** and **Figure 3.5Bi and ii**. Regarding the swarming ability of *Salmonella* 14028S in the present of TZ, a similar effect to swimming was observed (**Figure 3.5. B**) where at half of the MIC (**Figure 3.5.Biii**) the swarming ability was very reduce comparing the control (**Figure 3.5Bi** bottom panel. Concerning the MIC, no swimming or swarming was observed as shown in the **Figure 3.5. Biv**.

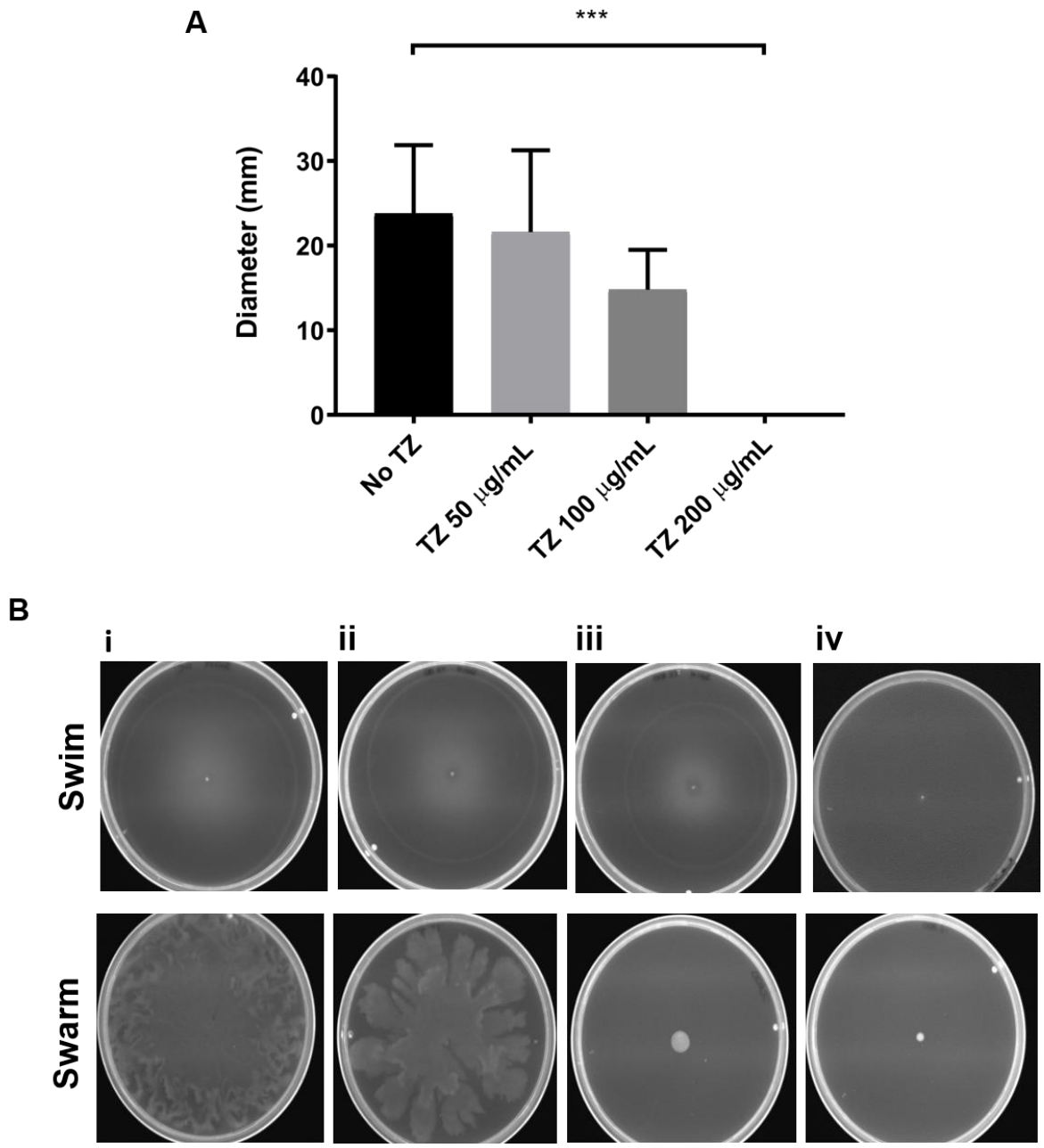


**Figure 3.5. Effect of different concentrations of TZ on bacterial motility.** (**A**) Swimming ability of *Salmonella* Typhimurium in the presence of 0, 50, 100 and 200 µg/mL of TZ. Error bars represent standard deviation; *** indicate P< 0.001 (one-way ANOVA). (**B**) Representative images of *Salmonella* Typhimurium swimming and swarming in the absence of TZ (**i**) and in the presence of TZ 50 µg/mL (**ii**), TZ 100 µg/mL (**iii**) and TZ 200 µg/mL (**iv**). The results presented correspond to the average of 3 independent experiments.

### 3.3.6. Membrane Permeability after exposure to TZ

The effect of TZ on the membrane permeability was assessed by exposing *Salmonella* Typhimurium 14028S to different concentrations of TZ (50 and 100 µg/mL). EtBr has been used as a marker to assess the contribution of efflux pumps and outer membrane proteins, to the cellular accumulation of compounds, by different bacteria. It is known that EtBr changes its fluorescence in different environments, namely when it penetrates the bacterial cell wall and binds DNA. Therefore, accumulation of EtBr was measured, as a means of indicating the efficacy of TZ to permeabilise the bacterial membrane. The results obtained showed that TZ had the ability to permeabilise the cell membrane obtained by increasing fluorescence of the dye (**Figure 3.6**) in a concentration dependent manner. In the presence of 100 µg/mL of TZ, an increase on the fluorescence of EtBr after addition of TZ was observed comparing with the control (drug free). The effect of TZ was also compared with CCCP (a known uncoupler of the proton motive force). These results clearly indicate an increased permeability of the *Salmonella* membrane in response to the exposure of the bacteria to TZ.

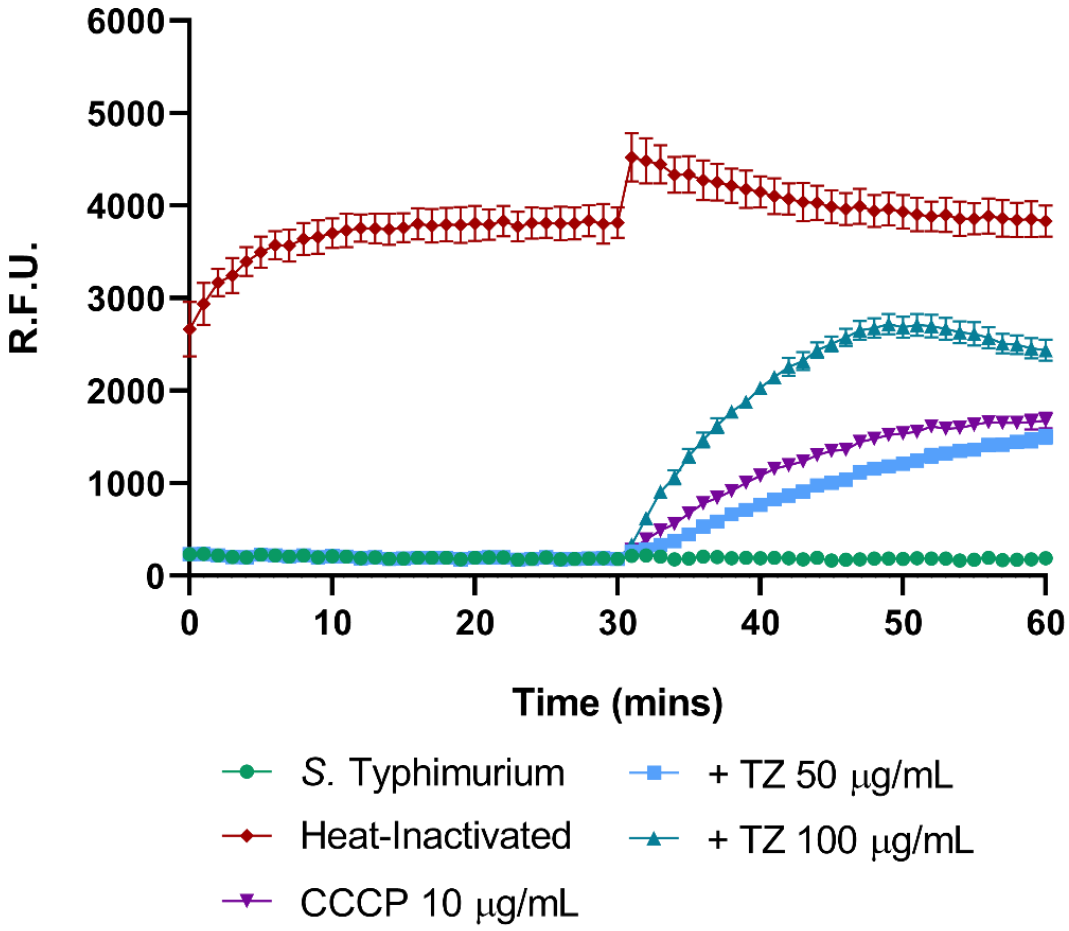


**Figure 3.6. Accumulation of EtBr by *Salmonella* Typhimurium in the presence of TZ.** *Salmonella* Typhimurium and heat-inactivated *Salmonella* (control for maximum fluorescence) were incubated with EtBr for 30 minutes. After that, TZ and CCCP were added to the cultures. The fluorescence was recorded at excitation and emission wavelengths of 515 and 600 nm. The results presented correspond to the average of 3 independent experiments plus ± standard deviations (SD). R.F.U., Relative Fluorescence Units; TZ, thioridazine; CCCP, Carbonyl Cyanide 3-Chlorophenylhydrazone.

### 3.3.7. Effect of TZ in the depolarisation of the cytoplasmic membrane in *Salmonella*

It has been reported in the literature that TZ is able to interfere with the bacterial energy metabolism (Weinstein *et al*., 2005; Yano *et al*., 2014; Wassmann *et al*., 2018). To test this hypothesis, the effect of TZ on the membrane potential of *Salmonella* Typhimurium was evaluated using the BacLight kit (Invitrogen). In this experiment, TZ was used at 50 and 100 µg/mL (0.25x and 0.5x MIC, respectively) and CCCP was used at a final concentration of 10 µg/mL (0.5x MIC) or 15 min. The red/green fluorescence ratio for *Salmonella* untreated and treated with TZ and CCCP is shown in **Figure 3.7**. CCCP, an uncoupler of the proton-motive force, can indirectly affect proton pump activity by depolarising the plasma membrane potential and reducing ATP production. This compound was used as a positive control. As expected, CCCP reduced the membrane potential when compared with the control (*Salmonella* only). A similar depolarising effect was observed when *Salmonella* exposed to TZ 100 µg/mL was compared with *Salmonella* (alone). A concentration dependent effect was observed since *Salmonella* in the presence of TZ 50 µg/mL shown similar values with *Salmonella* not exposed to TZ. These results suggest that TZ depolarises the membrane of *Salmonella* 14028S by interfering with the gradient of protons flowing on the cell membrane.

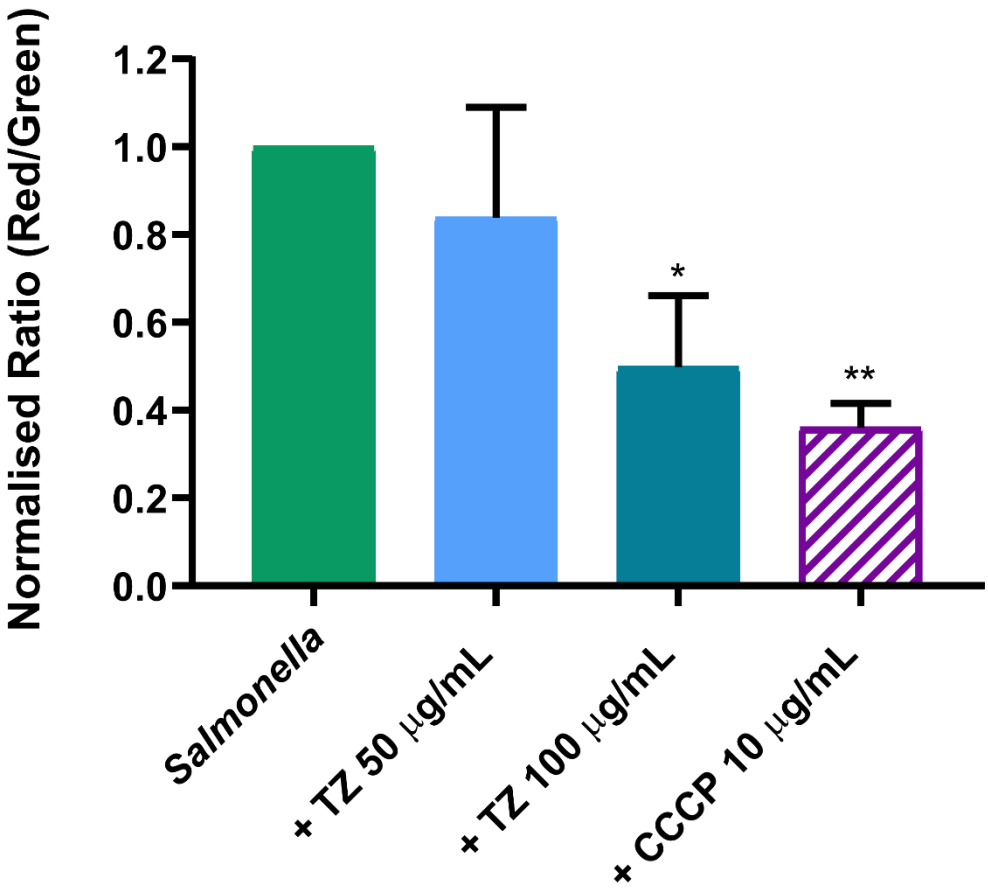


**Figure 3.7. Assessment of the effect of TZ in the membrane depolarisation of *Salmonella* Typhimurium by flow cytometry.** Bar-graph showing red/green fluorescence intensity ratio where a low ratio indicates a depolarised membrane. CCCP is an uncoupler of the proton-motive force used as a positive control for depolarisation of the membrane potential. Data were normalised against an untreated control (no TZ). The results presented correspond to the average of 3 independent experiments plus ± standard deviations (SD); * and ** indicate $P < 0.05$ and 0.01, respectively (one-way ANOVA). TZ, Thioridazine; CCCP, Carbonyl Cyanide 3-Chlorophenylhydrazone.

### 3.3.8. Effect of TZ on the levels of ATP production

To evaluate if the destabilisation of the membrane influences the ATP production in the bacteria, ATP levels were measured after 15 minutes of exposure to TZ. When *Salmonella* was exposed to 0.25x MIC, the ATP levels remained similar to the drug-free levels (**Figure 3.8**). At half of the MIC, a reduction (approximately of 50.5%) of the ATP levels occurs. This reduction upon exposure to TZ correlates with the reduction on bacterial viability (**Figure 3.4**). CCCP showed a reduction of approximately 63.1% compared with the drug free *Salmonella.* These results highlight the early effect promoted by TZ, most probably due to the rapid energy depletion. The energy generated as PMF is utilized by ATP synthase in the production of ATP. Hence, this result is concordant with the depolarisation of membrane potential of *Salmonella* in the presence of TZ.

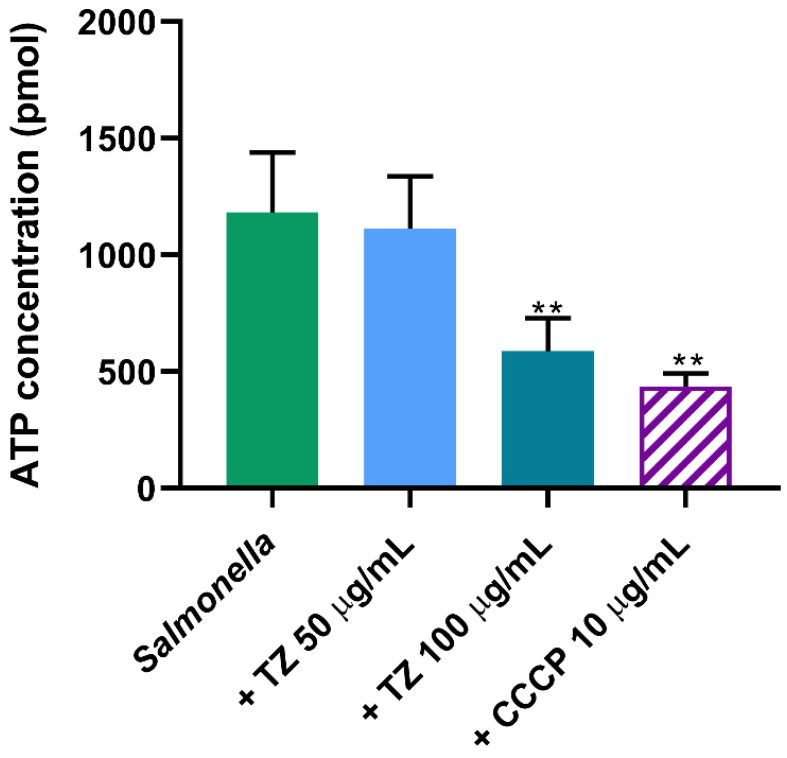


**Figure 3.8. Effect of TZ on the production of ATP in *Salmonella* Typhimurium.** *Salmonella* was exposed to TZ at 0.25x MIC and 0.5x MIC for 15 minutes. The ATP levels were quantified using a luciferin-luciferase bioluminescence detection assay. The results presented correspond to the average of 3 independent experiments plus ± standard deviations (SD); * and ** indicates $P < 0.05$ and 0.01, respectively (one-way ANOVA). TZ, Thioridazine. CCCP, Carbonyl Cyanide 3-Chlorophenylhydrazone

### 3.3.9. Inhibition of efflux of EtBr demonstrated the role of TZ as an efflux pump inhibitor

To confirm the ability of TZ to interfere, on a real-time basis, with the efflux activity of *Salmonella* Typhimurium the EtBr efflux assay was used. The main efflux system of *Salmonella* AcrAB-TolC is dependent on energy. EtBr is a broad efflux pump substrate (Viveiros *et al*., 2010). When *Salmonella* was incubated in the presence of TZ and CCCP at 0.125x MIC, no accumulation of EtBr was obtained (**Figure 3.9**). This result demonstrates that this concentration was ideal to evaluate efflux activity since there was no direct antimicrobial effect of the compounds itself.

*Salmonella* was then subject to conditions that promote significant EtBr accumulation (as mentioned on section 3.2.11). After the maximum accumulation of EtBr was reached, EtBr was washed out and *Salmonella* was subsequently re-suspended in fresh PBS with glucose and TZ or CCCP. As shown in **Figure 3.10**, when *Salmonella* is in the presence of an energy source, there is a decrease of fluorescence, indicating that efflux is taking place.

When *Salmonella* is in the presence of TZ and CCCP, inhibition of the efflux activity was observed. To a lesser extent in the presence of CCCP. Overall, these results show that the efflux of EtBr is considerably inhibited by TZ even when an energy source is present in the media to promote active efflux by re-energizing the cells.

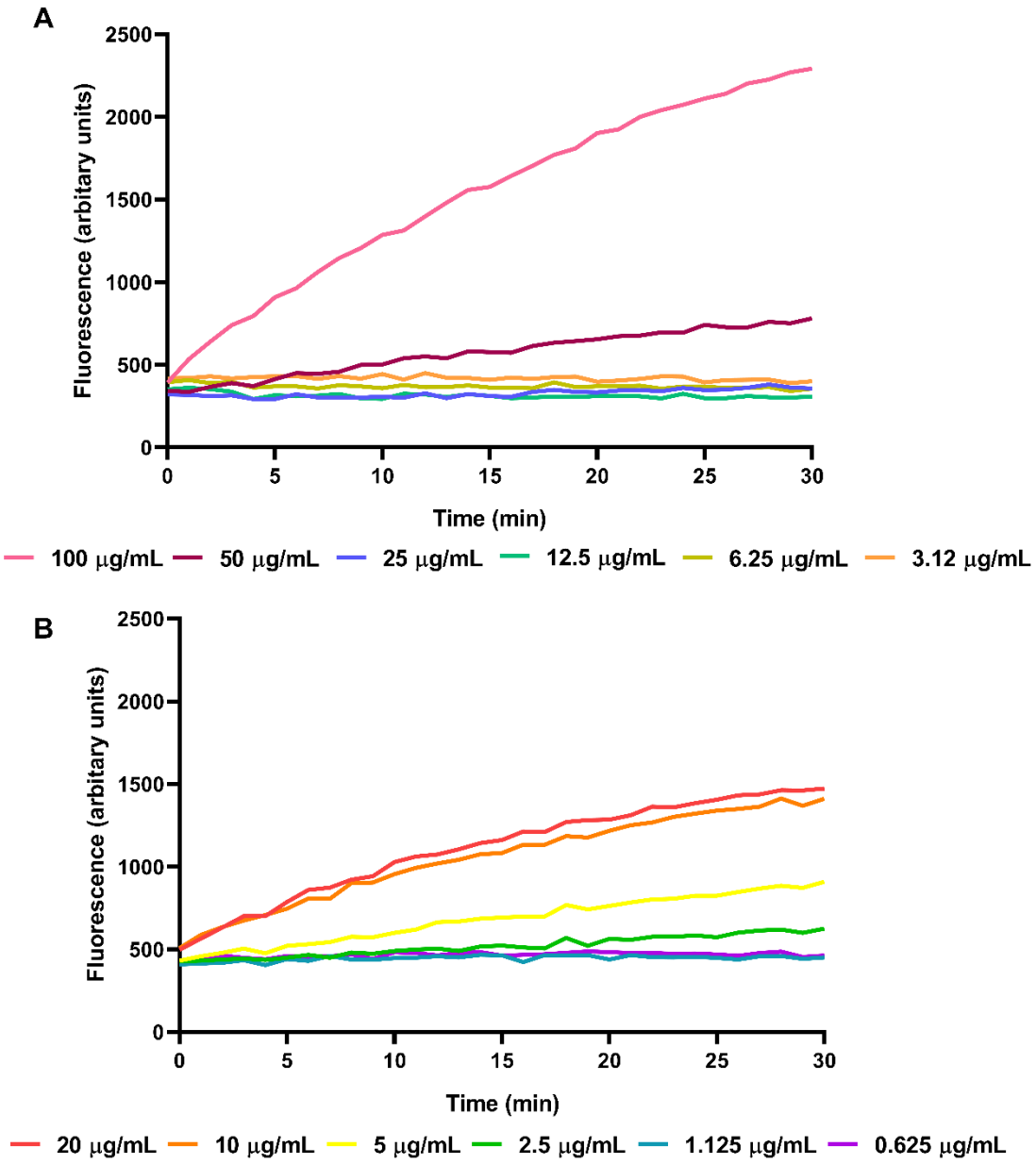


**Figure 3.9. Effect of efflux inhibitors on the accumulation of EtBr by *Salmonella* Typhimurium.** TZ (**A**) and CCCP (**B**) were tested at a range of concentrations, from 0.625x MIC to MIC correspondent of each compound. For both compounds 0.125x MIC were selected. Those concentration shown to not compromise bacterial cellular viability. The assays were performed using a semi-automated fluorometric method at 37°C. The results presented correspond to the average of 3 independent experiments plus ± standard deviations (SD).

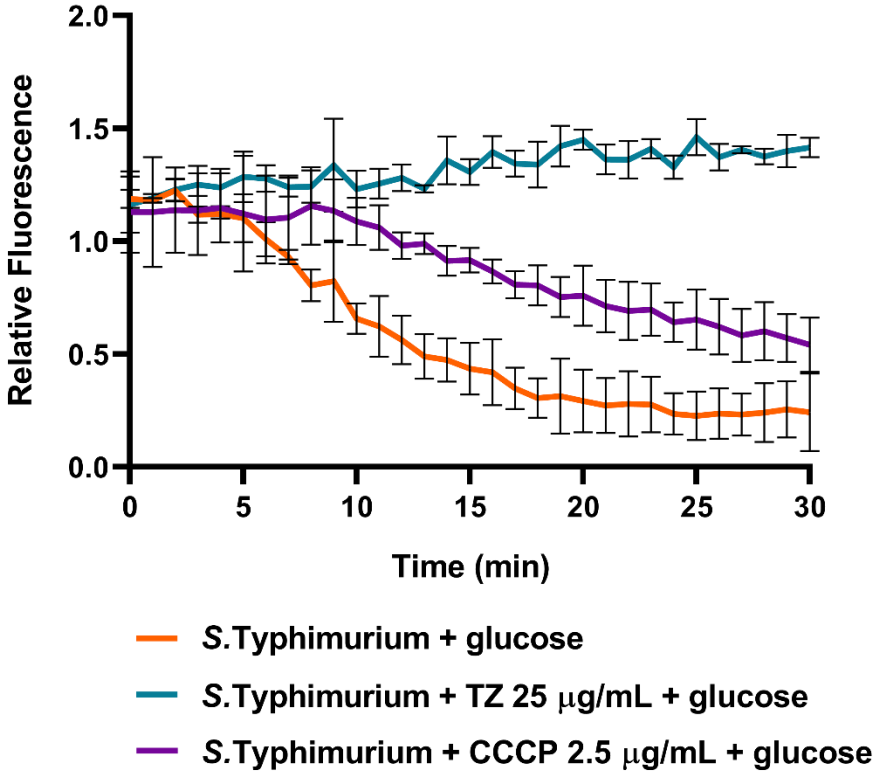


**Figure 3.10. Effect of TZ and CCCP on the efflux of EtBr by *Salmonella* Typhimurium.** The assays were performed using a semi-automated fluorometric method at 37°C in the presence and absence of glucose. The concentrations used correspond to 0.125x MIC of each compound in order to guarantee that the real-time efflux inhibition measured were not due to any antimicrobial effect of these compounds. The results presented correspond to the average of 3 independent experiments plus ± standard deviations (SD); TZ, Thioridazine; CCCP, Carbonyl Cyanide 3-Chlorophenylhydrazone

### 3.3.10. *In vitro* effect of TZ on the ultra-structure of *Salmonella* Typhimurium

To understand if exposure to TZ leads to morphological changes on the bacterium, *Salmonella* 14028S was treated with 100 µg/mL (0.5x MIC) at three different time points (15, 30 and 60 minutes) in the exponential growth phase and compared to an untreated sample (control). Samples were fixed with glutaraldehyde and processed for Transmission Electron Microscopy (TEM) (as described previously in the Materials and Methods section). Results show that *Salmonella* exposed to TZ exhibited discernable morphological changes in particular at the level of the bacterial membrane (**Figure 3.11. B-D**) comparing with the unexposed *Salmonella* (**Figure 3.11.A**). The morphological changes observed include: blebbing of the cell wall (**Figure 3.11. B.i**); partial loss of the cell wall with leakage of the intracellular components (**Figure 3.11.C.iii-iv**); continued production of cross-walls without subsequent separation of the replicated cells (**Figure 3.11.B.ii**); lysis of the cells. These morphological changes, namely cytoplasmic condensation and cell content leakage were observed only after 15 minutes of exposure to the compound (**Figure 3.11.B**). After 30 and 60 minutes (**Figure 3.11.C** and **3.10.D**) these changes appeared to be more evident, namely with the appearance of 'ghost cells' (**Figure 3.11.D.v**). These results confirm the previously obtained ones that were indicative of membrane permeabilisation when *Salmonella* Typhimurium was exposed to TZ.

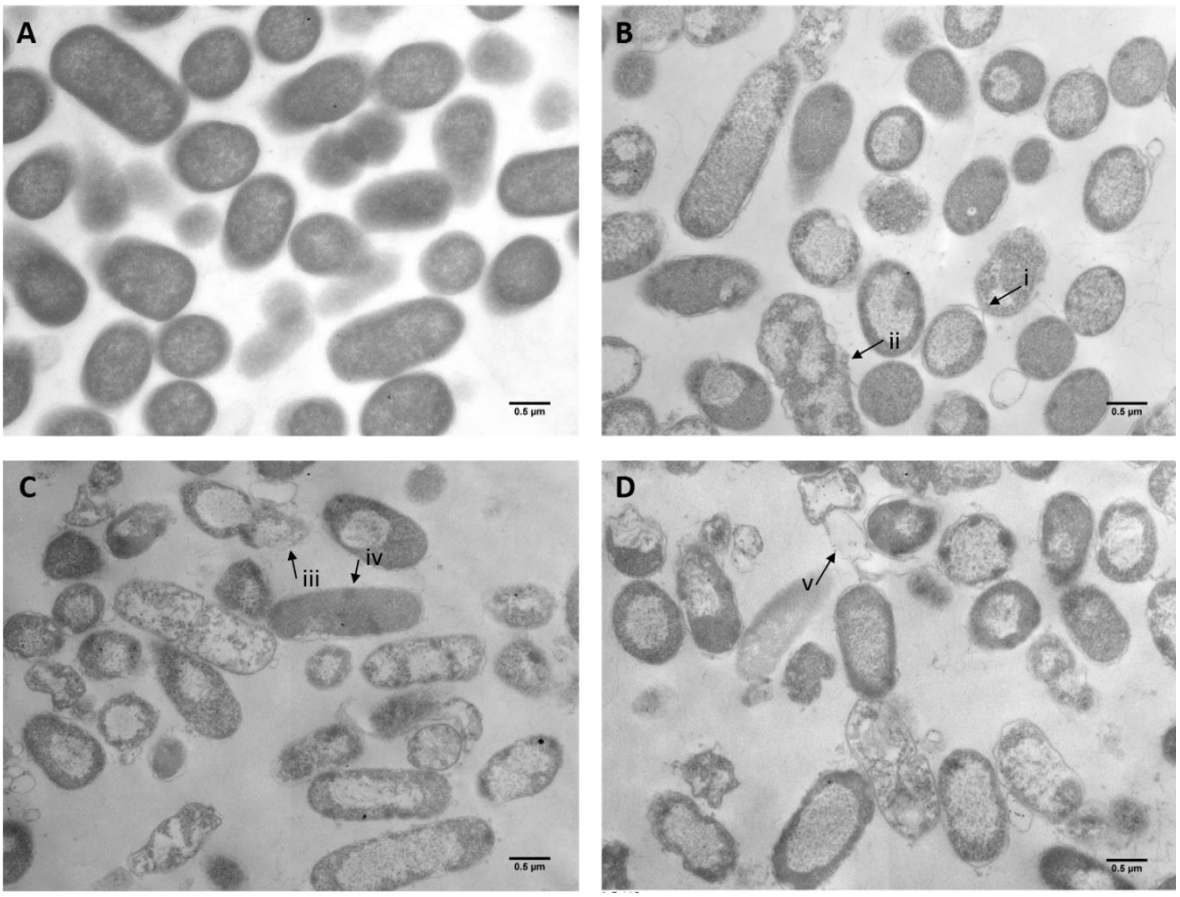


**Figure 3.11. Transmission electron micrographs of *Salmonella* Typhimurium exposed to 100 µg/mL of TZ.** Images were collected for the control (**A**) no TZ and after 15 minutes (**B**), 30 minutes (**C**) and 60 minutes (**D**) of exposure to the compound. Note: Blebbing of cell wall (**i**); continued production of cross-walls without subsequent separation of the replicated cells (**ii**); partial loss of cell wall with leakage of the intracellular components (**iii** and **iv**); and bacterial lysis (**v**). The images were magnified 5,000X.

### 3.3.11. Identification of differently expressed proteins in the presence of TZ

Protein extracts from *Salmonella* non-exposed and exposed to TZ for 15 minutes were subjected to proteomic analysis to identify potential targets involved in the mechanism of action of TZ. Bacterial proteome analysis was based on two-dimensional electrophoresis (2-DE) focused on the most abundant proteins with isoelectric points (pI) between 3 and 10. Representative 2-DE gels of *Salmonella* (drug-free) and *Salmonella* exposed to 100 µg/mL of TZ is provided in **Figure 3.12**. A and B, respectively. Protein spots were identified *via* matrix-assisted laser desorption/ionisation-time of flight (Maldi-TOF) mass spectrometry and compared with the proteome of untreated *Salmonella* Typhimurium, under the same conditions. These experiments reproducibly shown approximately 373 spots corresponding to individual proteins. The volume percentage of each protein spot in each condition is described in **Table S3.1**. To identify the differences between protein patterns across the conditions, the fold variation between samples untreated and treated with TZ was calculated. A variation was considered substantial if the fold-change was < 0.7 (underexpressed) or > 1.5 (overexpressed) (Mendo *et al*., 2015) compared to the *Salmonella* drug-free control. Based on the fold variation calculated (and SD), 60 protein spots showed to be overexpressed and 67 showed to be underexpressed when *Salmonella* was exposed to TZ (**Table S3.1**).

From the total altered proteins, 40 proteins were identified based on fold expression (**Figure 3.12**). The protein spot reference number, protein name, functional annotation (NCBI database), and expression in the proteome of *Salmonella* Typhimurium (control) and treated with TZ 100 µg/mL are summarised in **Table 3.3**. The proteins identified in the proteome included mainly cell envelope and outer membrane proteins, and those involved in a wide range of cellular functions including, transport; enzymes involved with energy production; protein synthesis and chaperones. The presence of these proteins is consistent with those reported to be present in *Salmonella*'s extracts prepared by others (Qi, Moir & O' Connor, 1996; Correia *et al*., 2017).

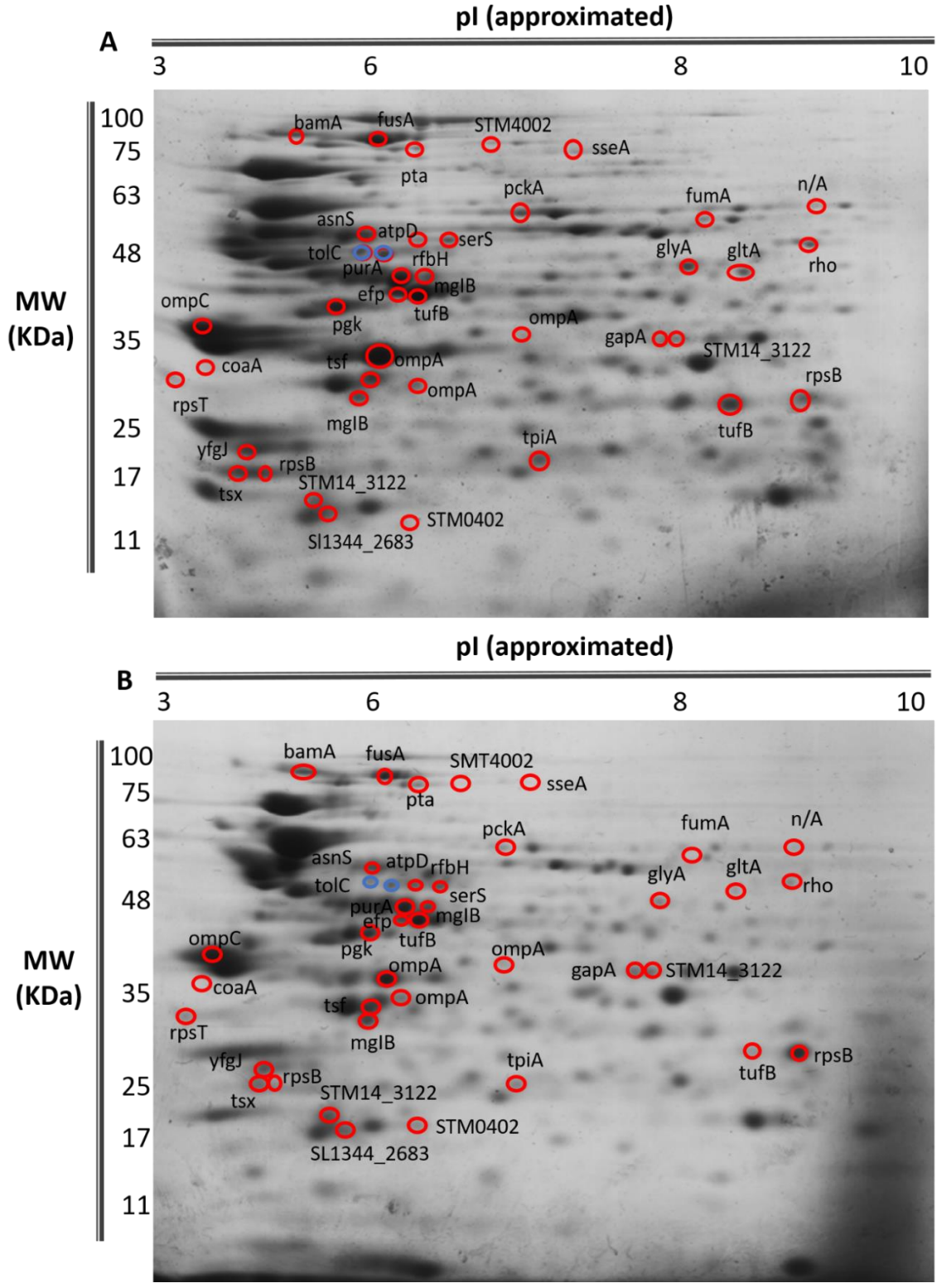


**Figure 3.12. Two-dimensional gel electrophoresis analysis of *Salmonella* Typhimurium after 15 minutes of exposure to TZ.** Proteins of *Salmonella* Typhimurium exposed to TZ for 15 minutes were separated by 2-DE (IEF (pI 3-10 non-linear)) in the first dimension and SDS-PAGE in the second dimension and visualised by staining with Coomasie brilliant blue R-350. (**A**) Representative 2-DE

gels untreated and (**B**) treated with TZ 100 µg/mL for 15 minutes. Identified proteins are listed in **Table 3.4**. Grey spots represent proteins detected in the 2-DE gels. Red circles represent identified proteins spots. Blue circles represent the selected targets for this project. These are representative images of *Salmonella* Typhimurium 14028S 2-DE gels. Gels were performed in duplicate for each condition tested. pI, isoelectric points; MW, molecular weight; KDa, Kilo Dalton.

**Table 3.4. Summary of the expression analysis by 2D gel electrophoresis of 40 selected protein spots of the proteome of *Salmonella* Typhimurium following treatment with TZ (100 µg/mL).**

| Spot nº | Gene | Functional annotation | Protein expression (% vol.) | | Fold Variance |
|---|---|---|---|---|---|
| | | | No TZ mean ± SD | TZ 100 µg/mL mean ± SD | |
| 8 | *ompC* | Outer membrane protein C | 5.33±0.34 | 2.13±0.52 | 0.40±0.09 |
| 14 | *bamA* | Outer membrane protein assembly factor BamA | 0.19±0.07 | 0.29±0.03 | 1.51±0.17 |
| 22 | *fusA* | Elongation factor G | 1.41±0.82 | 0.56±0.20 | 0.40±0.14 |
| 33 | *ompA* | Membrane protein | 2.37±1.49 | 1.57±0.63 | 0.66±0.26 |
| 35 | *mglB* | Galactose transport protein | 0.21±0.04 | 0.41±0.03 | 1.95±0.16 |
| 36 | *tsf* | Elongation factor Ts | 0.10±0.01 | 0.48±0.02 | 5.02±0.17 |
| 40 | *pgk* | Phosphoglycerate kinase | 0.25±0.03 | 0.47±0.04 | 1.86±0.16 |
| 48 | *Rfbh* | CDP-6-deoxy-D-xylo-4-hexulose-3-dehydrase | 0.09±0.01 | 0.23±0.09 | 2.46±0.96 |
| 50 | *serS* | Serine tRNA ligase | 0.12±0.07 | 0.25±0.04 | 2.01± 0.3 |
| 52 | *mglB* | Adenylosuccinate synthetase | 0.23±0.10 | 0.39±0.16 | 1.71±0.71 |
| 53 | *tufB* | Elongation factor Tu | 4.36±2.90 | 0.89±0.18 | 0.20±0.04 |
| 54 | *purA* | Adenylosuccinate synthetase | 1.70±0.11 | 0.94±0.12 | 0.55±0.07 |
| 59 | *atpD* | ATP synthetase subunit beta | 0.30±0.04 | 0.14±0.01 | 0.46±0.04 |
| 61 | *rpsB* | 30S ribosomal protein S2 | 0.16±0.20 | 0.66±0.21 | 4.06±1.29 |
| 65 | *pta* | Phosphate acetyltransferase | 0.06±0.01 | 0.14±0.05 | 2.54±0.87 |
| 76 | *gapA* | Glyceraldehyde-3-phosphate dehydrogenase | 0.13±0.08 | 0.21±0.03 | 1.59±0.23 |
| 77 | STM14 3122 | Uncharacterised protein | 0.10±0.02 | 0.23±0.08 | 2.28±0.77 |
| 88 | *glyA* | Serine hydroxymethyltransferase | 0.27±0.01 | 0.19±0.04 | 0.69±0.14 |
| 94 | *tolC* | Outer membrane channel | 0.02±0.00 | 0.07±0.00 | 3.12±0.09 |
| 95 | *asnS* | Asparagine tRNA ligase | 0.17±0.06 | 0.73±0.3 | 4.26±1.75 |
| 114 | *ompA* | Membrane protein | 0.06±0.01 | 0.17±0.05 | 2.79±0.82 |

| | | | | | |
|---|---|---|---|---|---|
| 123 | *rpsT* | 30S ribosomal protein S20 | 0.04±0.00 | 0.11±0.03 | 2.68±0.79 |
| 130 | *pckA* | Phosphoenolpyruvate carboxykinase (ATP) | 0.17±0.06 | 0.11±0.03 | 0.64±0.17 |
| 134 | *rho* | Transcription termination factor Rho | 0.17±0.05 | 0.09±0.02 | 0.54±0.10 |
| 137 | *tpiA* | Triosephosphate isomerase | 0.42±0.01 | 0.29±0.09 | 0.70±0.18 |
| 156 | STM0402 | Putative thiol-alkyl hypedroperoxide reductase | 0.05±0.02 | 0.27±0.00 | 4.94±0.01 |
| 159 | *ompA* | Outer membrane protein A | 0.07±0.03 | 0.16±0.05 | 2.37±0.76 |
| 166 | *gltA* | Citrate synthase | 0.13±0.01 | 0.06±0.01 | 0.47±0.07 |
| 220 | *N/A* | Shufflon protein B | 0.03±0.00 | 0.06±0.01 | 1.68±0.35 |
| 278 | *coaA* | Pantothenate kinase | 0.07±0.07 | 0.03±0.01 | 0.46±0.08 |
| 298 | *tsx* | Nucleoside-specific channel-forming protein tsx | 0.20±0.14 | 0.08±0.03 | 0.40±0.16 |
| 315 | *fumA* | Fumarate hydratase class I | 0.05±0.01 | 0.02±0.00 | 0.35±0.05 |
| 328 | SMT4002 | Putative cytoplasmic protein | 0.02±0.00 | 0.07±0.00 | 3.12±0.09 |
| 333 | *sseA* | Sulfurtransferase | 0.06±0.01 | 0.03±0.00 | 0.47±0.01 |
| 334 | *yfgJ* | Putative cytoplasmatic protein (Fragment) | 1.43±0.49 | 2.80±0.35 | 1.96±0.24 |
| 340 | *rpsB* | 30S ribosomal S2 | 0.14±0.03 | 0.32±0.02 | 2.33±0.13 |
| 346 | *tufB* | Elongation factor Tu | 0.14±0.03 | 0.26±0.07 | 1.88±0.52 |
| 366 | *efp* | Elongation factor P | 0.38±0.15 | 0.89±0.18 | 0.20±0.04 |
| 369 | SMT14_3122 | Uncharacterized protein | 0.20±0.00 | 0.46±0.01 | 2.33±0.05 |
| 373 | SL1344_2683 | Hypothetical conserved bacteriophage protein | 0.10±0.03 | 0.39±0.09 | 4.06±0.91 |

These 40 proteins were analysed using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins), functional protein network database version 10.5 (http://string-db.org, accessed in January 2019). Protein-Protein interaction network analysis revealed a significant interaction between 25 proteins as shown in **Figure 3.13**. Among the interacting proteins, proteins related with cell envelope (Cellular Component GO pathway number 0030313) as shown in **Figure 3.13**- Red nods, including Outer membrane protein C (OmpC), Membrane protein (OmpA), Outer membrane protein assembly factor BamA (BamA) and Nucleoside-specific channel-forming protein Tsx (Tsx) were identified. An alteration on the proteins involved in the ATP metabolic process was observed (Biological Process GO pathway number 0046034) on **Figure 3.13**-Blue nods, namely ATP synthetase subunit beta (AtpD), glyceraldehyde-3-phosphate dehydrogenase (GapA), triosephosphate isomerase (TpiA) and phosphoglycerate kinase (Pgk). Some proteins, particularly the products encoded by *ompA* gene, were found in multiple forms (spots 33, 114 and 159), *mglB* (spots 35 and 52) and *rpsB* (spots 61 and 340). The presence of such isoforms can be indicative of post-translational modifications or a result of sample preparation. The outer membrane channel (TolC) protein was found to have an increased expression (3.12-fold variance).

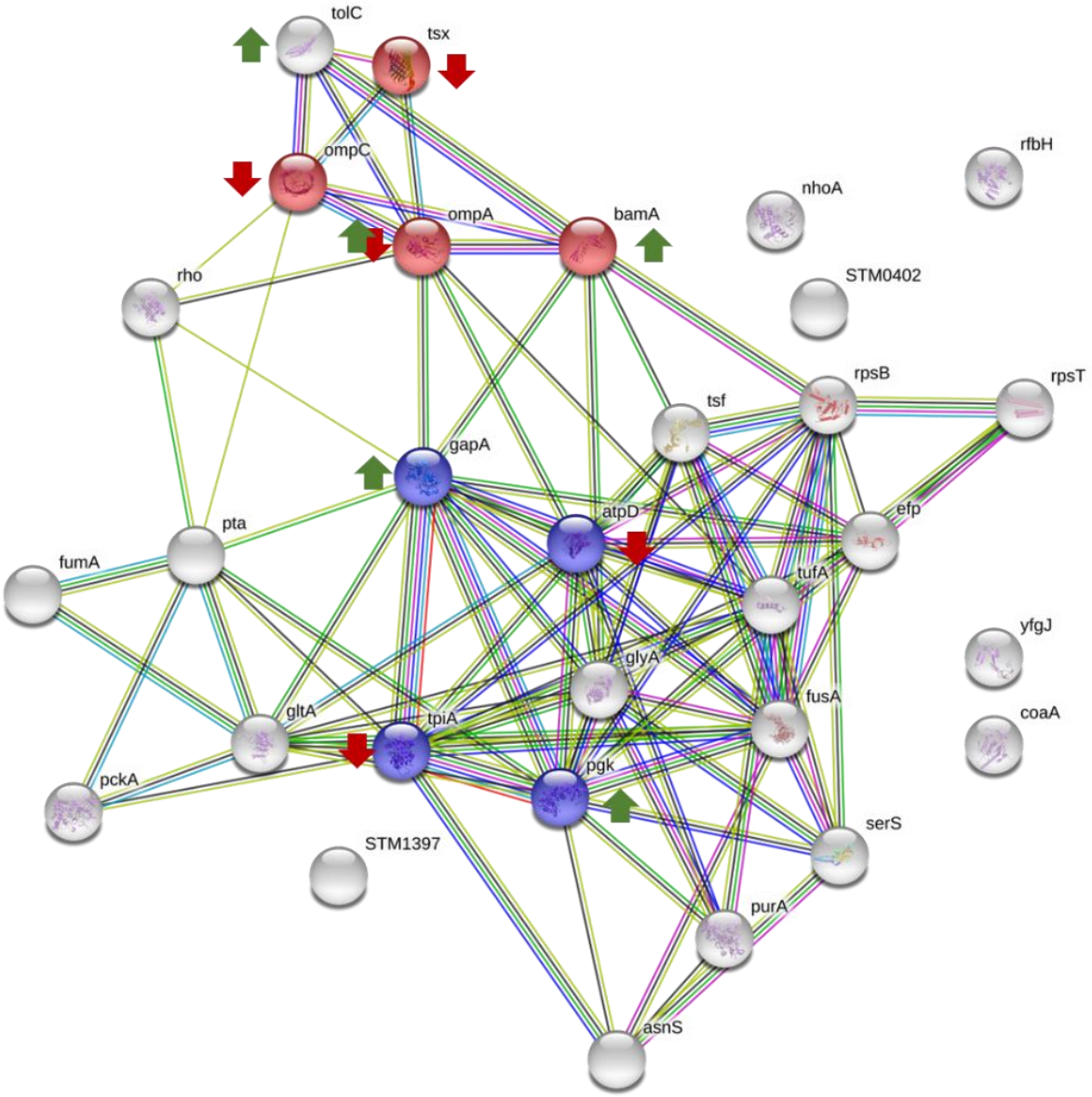


**Figure 3.13. Protein-protein network with altered expression in *Salmonella* Typhimurium in the presence of TZ.** Nodes represent proteins and lines connecting nodes indicate direct, or indirect interactions between proteins. Red nodes represent proteins involved in *Salmonella*'s cell envelope (Cellular component GO:0030313) and blue nodes represent proteins involve in the ATP metabolic process (Biological process GO:0046035). Red arrows represent proteins that were down-regulated, while green arrows represent proteins that were up-regulated in the presence of TZ.

### 3.3.12. Validation of the *Salmonella* expressed proteins, AtpD and TolC in the presence of TZ

The results obtained from the protein-protein network suggest that the proteins to be modulated by exposure to TZ are involved in the cell envelope and ATP metabolic process, as previously mentioned. In order to validate these results, an immunoblotting on *Salmonella* untreated and treated in the same conditions (exposure to TZ) was performed (**Figure 3.15**). For this validation, AtpD (**Figure 3.14A**) and TolC (**Figure 3.14.B**) proteins were selected as the main targets. *Salmonella* strains with a 3xFLAG-tagged *atpD* or *tolC* were generated using the Datsenko and Wanner method (as described in Materials and Methods).

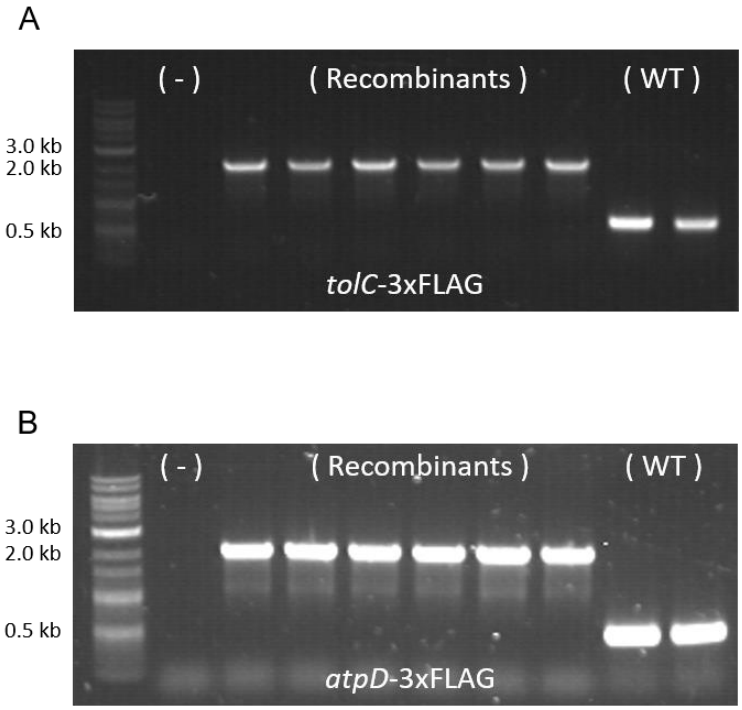


**Figure 3.14. Confirmation of *Salmonella* Typhimurium 3xFLAG strains by PCR.** (**A**) Confirmation of *Salmonella tolC* 3xFLAG and (**B**) *atpD* 3xFLAG strains. Confirmation of the 3x-FLAG strains were performed by Dr. Ciaran Finn at the Martins lab in the Department of Microbiology, Trinity College Dublin, Dublin, Ireland.

The MIC (400 µg/mL; **Table 3.2**) for both strains was determined previous to the immunoblotting assay. Based on the densiometry immunoblotting results it is shown that AtpD was downregulated and TolC upregulated in *Salmonella* when the strain was in the presence of half of the MIC of TZ for 15 minutes. These results combined with the previous ones clearly show that TZ interferes with the cell-envelope and energy sources on the bacteria.

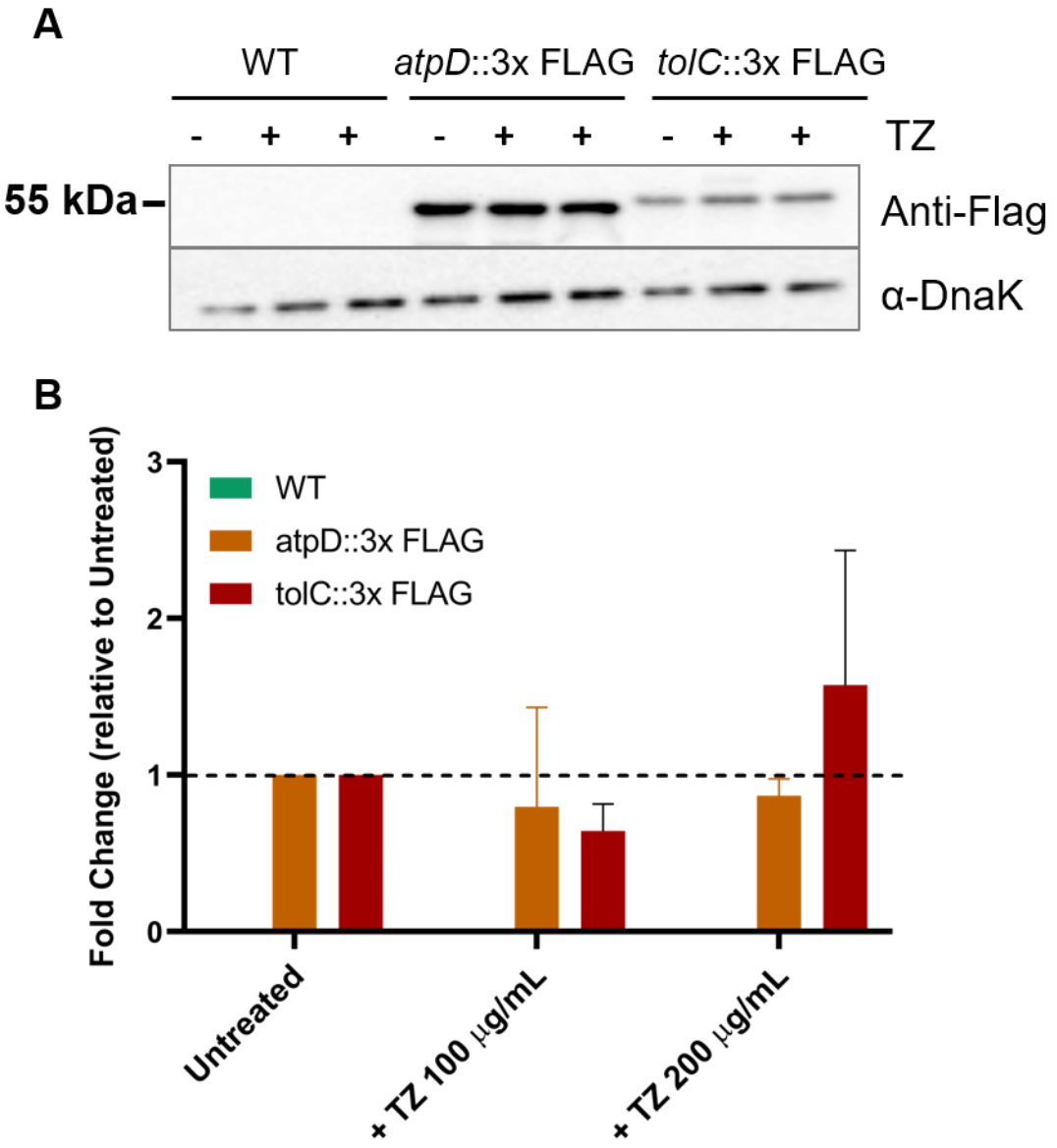


**Figure 3.15. Expression of AtpD and TolC proteins after *Salmonella* Typhimurium was exposed to TZ.** (**A**) *Salmonella* cultures with *atpD*-3xFLAG::kan and *tolC*-3xFLAG::kan were grown to mid-log phase in LB broth and then exposed to ½ MIC of  for 15 minutes, replicating proteomic conditions. Protein samples were normalised according to the optical density ($O.D._{600nm}$) of the cultures and separated on SDS-PAGE. Membrane were probed with anti-FLAG M2 and anti-DnaK. DnaK was used as a loading control and the results were normalised to this. (**B**) Blots were analysed for quantification by densitometry using ImageJ software. Values were normalised to the untreated values of each strain to DnaK, respectively. A representative blot is shown. The results presented correspond to the average of 3 independent experiments WT, wild type; TZ, thioridazine.

In addition to these results, MIC measurements of TZ against efflux transporter mutants *ΔacrA, ΔacrB, ΔacrAB* and *ΔtolC* were performed (**Table 3.5**). The MICs of TZ for these strains (25 µg/mL) showed a 3-fold decrease compared to the wild-type *Salmonella* (200µg/mL). These results confirm that when the main efflux pump system in *Salmonella* is interrupted the bacteria becomes more susceptible to TZ. Therefore, these results support that one of the main targets for TZ in *Salmonella* is the AcrAB-TolC system.

**Table 3.5. Susceptibility of *Salmonella* Typhimurium drug efflux transporter deleted strains to Thioridazine**

| Strain | MIC (µg/mL) |
|---|---|
| *S.* Typhimurium 14028S ΔacrA | 25 |
| *S.* Typhimurium 14028S ΔacrB | 25 |
| *S.* Typhimurium 14028S ΔacrAB | 25 |
| *S.* Typhimurium 14028S ΔtolC | 25 |

### 3.3.13. Effect of TZ on the viability of human monocytes

The cytotoxic potential of TZ in human monocytes was investigated by the quantification of cell viability after exposing the cells to a range of concentrations of TZ for 24 hours. After exposure of the cells to TZ, the viability of the monocytes was assessed by their ability to convert 3-(4,5-dimethylthiazol-2-yl)-5-(3-caarboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS). The conversion of MTS into an aqueous soluble formazan product is accomplished by dehydrogenase enzymes, that are found in metabolically active cells. TZ decreased significantly the viability of the monocytes at concentrations above 5 µg/mL (**Figure 3.16**). The exposure of the cells to 0.1 to 1 µg/mL of TZ only caused 7% -10% decrease on cell viability compared with non-exposed cells; at 5 µg/mL there was a highly significant decrease of 28.45% and decreases in the cell viability of 81.71% - 94.89% were obtained at higher concentrations, 10 and 50 µg/mL respectively. Therefore, it is confirmed that TZ is highly toxic but at only concentrations above 5

µg/mL and after 24 hours in culture. These results are in line with previous studies performed in macrophages showing a lethal concentration (LC 50) value of 3 µg/mL for TZ (Ordway *et al*., 2002a).

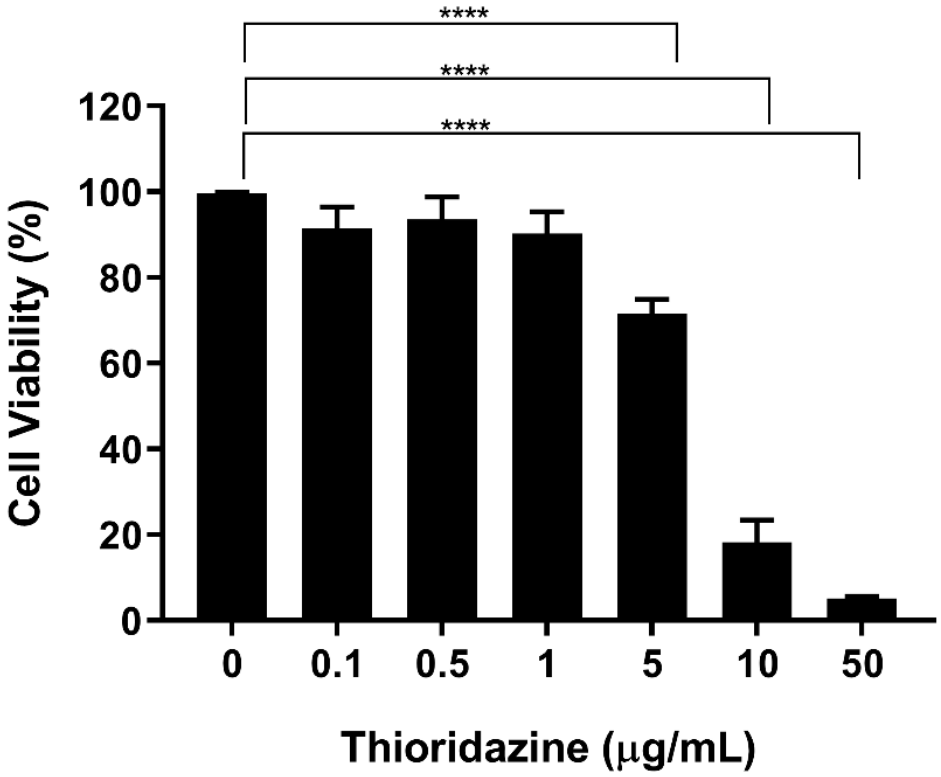


**Figure 3.16. The effect of TZ on the cellular viability of THP-1 monocytes.** Cells were exposed to different concentrations of TZ during 24 hours at 37°C, 5% $CO_2$. The results presented correspond to the average of three independent assays plus standard deviation (±SD); TZ, Thioridazine; **** indicate $P < 0.00001$ (one-way ANOVA test).

## 3.4 Discussion

AMR is a worldwide challenging public health issue as bacterial resistance mechanisms keep evolving and spreading globally, hampering the ability to treat bacterial infection (Kaul *et al*., 2019). This highlights the need to develop radically new approaches to combat antimicrobial resistance. Current therapy options that rely on existing antibiotics are not effective, therefore repurposing of existing non-antibiotic compounds, such as TZ would be valuable as a possible adjuvant treatment (combination therapy).

The possible mechanism(s) of action by which TZ acts against bacteria have been studied *in vitro* mainly in *M. tuberculosis* and *Staphylococcus aureus*. However, its mechanism of action hasn't been fully understood in these or other bacteria. What is the *in vitro* mechanism of action of TZ? A question that somehow remained to be answered for many years, until the present work was developed. This chapter focused on the details of the antibacterial activity of TZ against *Salmonella* Typhimurium used in this work as a model bacterium. *Salmonella* was used due to the fact it is a Gram-negative pathogen and a fast grower (contrary to *M. tuberculosis*). To understand the mechanism(s) of action of TZ against *Salmonella* several independent experimental approaches were used to assess the bacterial response after its exposure to sub-inhibitory concentrations of TZ.

TZ is an amphiphilic cation able to interact with the negatively charged phospholipids of the cytoplasmic membrane (Hendrich *et al*., 2002; Michalak *et al*., 2006). The hypothesis raised in this work was that the bacterial cell membrane might represent a target for the neuroleptic drug, TZ. This hypothesis was confirmed by several approaches including membrane permeability, membrane potential and proteomics. TZ effects on bacteria were observed in a concentration dependent manner. At half of the MIC, TZ disrupts the bacterial membrane leading to leakage of the cellular contents only after 15 minutes of exposure of the bacteria to the compound. This result demonstrates that TZ has a rapid bactericidal activity. This membrane destabilisation triggers effects on the bacterial inner membrane. The bacterial inner cell membrane is responsible for many essential functions such as, cell wall synthesis, respiration processes, *etc* (Brogden, 2005; Strahl & Hamoen, 2010). It is doubtless that for all these functions membrane integrity is absolutely essential, and its instabilities may direct or indirectly affect metabolic function and

consequently, cell death. Alteration of the membrane potential is an early indication of damage to the bacteria (Martins *et al*., 2008). In this work, it was demonstrated that at half of the MIC of TZ there was a significant depolarisation of the *Salmonella* membrane that disturbed the function of the electron transport chain. Subsequently, this led to a significant reduction on the production of ATP and the ATP levels. Similar results were obtained with the protonophore, CCCP. CCCP is an uncoupler of the proton motive force, reducing production of ATP and increasing membrane permeability, ultimately, causing cell death (Park & Ko, 2015; Ni *et al*., 2016). CCCP was used in this study solely as a positive control, with no therapeutic value clinically, but as an important comparison to validate the initial hypothesis.

TZ evidently demonstrated a real-time inhibitory effect on the efflux activity of *Salmonella*, as shown by the presence of an energy source in the presence and absence of the EPI. The decrease on the MIC of the efflux transporter strains indicates an increased susceptibility of the knockout strains to TZ. This further supports the results obtained in this work. The proteomic data confirmed that in the presence of TZ the cell envelope of *Salmonella* is altered, and the ATP generation processes are disturbed.

Bacteria usually encounters toxic compounds that are released from the host. In order to overcome the action of these compounds and be able to survive intracellularly, bacteria activate efflux systems. Some of these efflux systems rely on the use of the proton motive force. TolC is part of the main AcrAB-TolC efflux pump, one of the main efflux pumps of *Salmonella* spp. (Amaral *et al*., 2012a) and *Escherichia coli*. This channel makes the connection between the periplasmic space and the cell exterior, through the outer membrane. Overexpression of AcrAB-TolC has been associated with fluoroquinolone resistant *Salmonella enterica* (Swick *et al*., 2011). This efflux pump is not only responsible for extruding noxious compounds but has also a role in virulence. Several studies have shown that inactivation of AcrAB – TolC decreases bacterial virulence (Stone & Miller, 1995; Baucheron *et al*., 2005). In the present study, an increased expression of TolC (outer membrane channel) was observed. TolC is part of the main AcrAB-TolC efflux pump, one of the main efflux pumps of *Salmonella*. This is a possible mechanism for *Salmonella* Typhimurium overcome the cell-envelope damage induced by the exposure to TZ. This channel makes the connection between the periplasmic space and the cell. The

vast majority of the studies *in vitro* on TZ are performed on mycobacteria, especially *Mycobacterium tuberculosis.* Previous studies demonstrated TZ as an EPI and interfering with the cell envelope of mycobacteria (Amaral *et al*., 2000; Martins *et al*., 2004; de Keijzer *et al*., 2016; Wassmann *et al*., 2018).

The results obtained in the present study, are in agreement with the observations on mycobacteria. As far as we are aware, this is the first time reporting the effect of TZ not only as efflux pump inhibitor but also, interfering with the energy sources of bacteria, particularly *Salmonella* Typhimurium

Our study also demonstrated that TZ interferes with the energy sources. Therefore, this chapter emphasises how inhibiting with the activity of the efflux pumps would be a good approach against multidrug-resistant pathogenic bacteria. These observations go in line with previous published studies demonstrating that TZ affects firstly the cell envelope (Amaral *et al*., 2000; Martins *et al*., 2004; de Keijzer *et al*., 2016; Wassmann *et al*., 2018). Taken all these results into account, a hypothetical model for TZ's mechanism of action is illustrated in **Figure 3.17**. Firstly, TZ increases the membrane permeability causing its destabilisation (**Figure 3.17.1**) leading to a pleiotropic effect. This disruption leads to reduction of the membrane potential (**Figure 3.17.2**) causing reduction of the ATP levels (**Figure 3.17.3**). Interference with the bacteria's energy levels, will interfere with the efflux activity (**Figure 3.17.4**) and with the bacterial motility (**Figure 3.17.5**) which are dependent on energy derived from the proton motive force. The events (**Figure 3.17;1-6**) proposed on this hypothetical model are not reflective of a sequence but of targets and metabolic processes that may be affected when the bacteria are exposed to TZ.

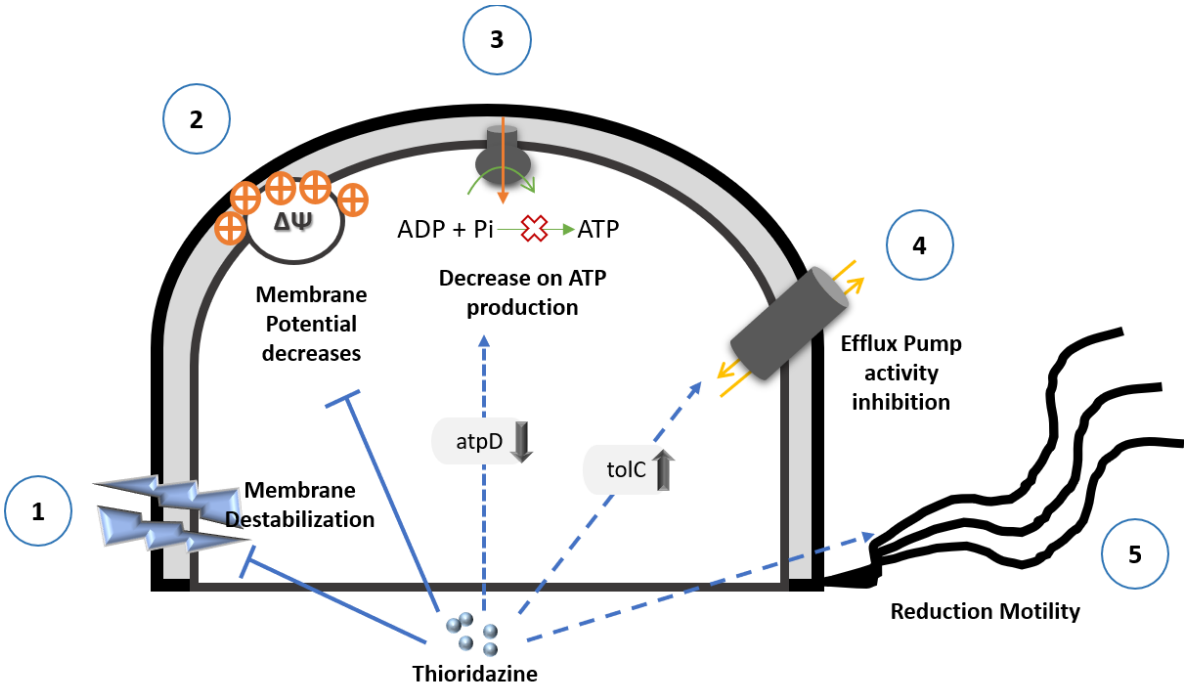


**Figure 3.17. Proposed of events describing the mechanism of action of TZ against *Salmonella* Typhimurium.** The mechanism of action of TZ involves a cascade of effects, namely, an increased membrane permeability (**1**) resulting in decreased bacterial membrane potential (**2**) leading to a decrease in the production of ATP (**3**) and inhibition of efflux activity dependent of energy (**4**) and reduction of motility (**5**). ΔΨ, membrane potential.

TZ may have lost its shine as an antipsychotic, but its potential as an antimicrobial can no longer be ignored, least of all in the vast areas of the world plagued by endemic infectious diseases. Therefore, identifying bacterial targets and the mechanism of action of TZ against bacteria is an important step forward. Since combination therapy of antibiotics and outer membrane permeabilising agents (such as TZ), have become more attractive over the last decade, the use of TZ may be of interest for the future treatment of antibiotic resistant bacteria. Adding to this, the antimicrobial activity of TZ *in vitro* (MIC 200 µg/mL) was produced with concentrations that were hundreds of times greater than those that could be clinically achieved safely (maximum of 0.5-1 mg/L of plasma) (Ordway *et al*., 2003; Thanacoody, 2007). However, it is known that the human macrophage (and other cells rich in lysosomes) are able to concentrate phenothiazines in the medium as much as 100 times (Crowle, Douvas & May, 1992) reaching the *in vitro* MIC or MBC

intracellularly, which otherwise would be toxic to the host cells. Taken this into account, the findings in this study were very significant since hundreds of compounds have been able to inhibit the *in vitro* growth of bacteria, but majority don't progress to studies involving their activity against intracellular pathogens. That is not the case of TZ that demonstrated minimal toxicity against human monocytes at concentrations below 5 µg/mL and that can constitute a good candidate for testing in infected macrophages.

### 3.5. Supplementary Information

**Table S3.1. Summary of the expression analysis by 2D gel electrophoresis of protein spots in the *Salmonella* Typhimurium proteome following treatment with TZ 100 µg/mL for 15 minutes**

| Gel Spot Ref. Nr. | No TZ % Vol. | | TZ 100 µg/mL % Vol. | | Fold variance TZ *vs.* No TZ | |
|---|---|---|---|---|---|---|
| | mean | SD | mean | SD | mean | SD |
| 1 | 8.23 | 0.12 | 6.32 | 0.22 | 0.77 | 0.026 |
| 2 | 5.54 | 1.22 | 9.50 | 3.49 | 1.71 | 0.630 |
| 3 | 3.05 | 0.42 | 2.94 | 0.44 | 0.97 | 0.144 |
| 4 | 1.29 | 0.30 | 1.00 | 0.15 | 0.78 | 0.118 |
| 5 | 2.72 | 2.53 | 2.67 | 0.15 | 0.98 | 0.054 |
| 6 | 0.96 | 0.94 | 1.77 | 0.77 | 1.84 | 0.802 |
| 7 | 5.02 | 0.03 | 5.62 | 1.27 | 1.12 | 0.253 |
| 8 | 5.33 | 0.34 | 2.13 | 0.52 | 0.40 | 0.097 |
| 9 | 0.24 | 0.13 | 0.84 | 0.23 | 3.56 | 0.998 |
| 10 | 0.34 | 0.23 | 0.30 | 0.05 | 0.91 | 0.143 |
| 11 | 0.35 | 0.38 | 0.27 | 0.05 | 0.76 | 0.152 |
| 12 | 0.05 | 0.01 | 0.09 | 0.02 | 1.86 | 0.464 |
| 13 | 0.12 | 0.12 | 0.17 | 0.10 | 1.47 | 0.853 |
| 14 | 0.19 | 0.07 | 0.29 | 0.03 | 1.51 | 0.170 |
| 15 | 0.10 | 0.08 | 0.18 | 0.03 | 1.81 | 0.285 |
| 16 | 0.02 | 0.00 | 0.09 | 0.05 | 3.66 | 2.210 |
| 17 | 0.32 | 0.13 | 0.21 | 0.07 | 0.66 | 0.223 |
| 18 | 0.06 | 0.01 | 0.14 | 0.06 | 2.51 | 1.120 |
| 19 | 0.04 | 0.00 | 0.08 | 0.03 | 1.89 | 0.733 |
| 20 | 0.05 | 0.03 | 0.08 | 0.02 | 1.64 | 0.419 |
| 21 | 0.24 | 0.11 | 0.21 | 0.10 | 0.87 | 0.412 |
| 22 | 1.41 | 0.82 | 0.56 | 0.20 | 0.40 | 0.144 |
| 23 | 0.47 | 0.09 | 0.46 | 0.20 | 0.99 | 0.429 |
| 24 | 0.26 | 0.07 | 0.23 | 0.11 | 0.87 | 0.412 |
| 25 | 0.29 | 0.04 | 0.21 | 0.09 | 0.72 | 0.318 |
| 26 | 0.17 | 0.05 | 0.18 | 0.05 | 1.05 | 0.310 |
| 27 | 0.10 | 0.08 | 0.11 | 0.07 | 1.03 | 0.685 |
| 28 | 0.20 | 0.13 | 0.18 | 0.11 | 0.92 | 0.541 |
| 29 | 0.07 | 0.01 | 0.12 | 0.08 | 1.74 | 1.246 |
| 30 | 0.50 | 0.19 | 0.68 | 0.68 | 1.37 | 1.361 |
| 31 | 0.07 | 0.01 | 0.31 | 0.27 | 4.27 | 3.694 |
| 32 | 1.48 | 0.25 | 1.26 | 0.38 | 0.85 | 0.259 |
| 33 | 2.37 | 1.49 | 1.57 | 0.63 | 0.66 | 0.265 |
| 34 | 0.79 | 0.48 | 0.78 | 0.15 | 1 | 0.184 |
| 35 | 0.21 | 0.04 | 0.41 | 0.03 | 1.95 | 0.164 |
| 36 | 0.10 | 0.01 | 0.48 | 0.02 | 5.02 | 0.168 |
| 37 | 0.26 | 0.03 | 0.47 | 0.11 | 1.81 | 0.405 |
| 38 | 0.35 | 0.25 | 0.70 | 0.38 | 1.98 | 1.069 |
| 39 | 0.75 | 0.06 | 0.84 | 0.14 | 1.12 | 0.188 |
| 40 | 0.25 | 0.03 | 0.47 | 0.04 | 1.86 | 0.160 |
| 41 | 0.50 | 0.26 | 0.35 | 0.05 | 0.71 | 0.102 |
| 42 | 0.18 | 0.00 | 0.34 | 0.06 | 1.89 | 0.363 |
| 43 | 0.06 | 0.02 | 0.1 | 0.01 | 1.81 | 0.208 |
| 44 | 0.08 | 0.10 | 0.48 | 0.31 | 5.72 | 3.635 |
| 45 | 0.18 | 0.08 | 0.20 | 0.09 | 1.14 | 0.518 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 46 | 0.13 | 0.08 | 0.10 | 0.05 | 0.74 | 0.363 |
| 47 | 0.23 | 0.03 | 0.07 | 0.07 | 0.30 | 0.294 |
| 48 | 0.09 | 0.01 | 0.23 | 0.09 | 2.46 | 0.957 |
| 49 | 0.29 | 0.36 | 0.31 | 0.06 | 1.06 | 0.210 |
| 50 | 0.12 | 0.07 | 0.25 | 0.04 | 2.01 | 0.299 |
| 51 | 0.90 | 0.37 | 0.67 | 0.07 | 0.74 | 0.082 |
| 52 | 0.23 | 0.10 | 0.39 | 0.16 | 1.71 | 0.705 |
| 53 | 4.36 | 2.90 | 0.89 | 0.18 | 0.20 | 0.042 |
| 54 | 1.70 | 0.11 | 0.94 | 0.12 | 0.55 | 0.072 |
| 55 | 0.39 | 0.18 | 0.96 | 0.10 | 2.43 | 0.245 |
| 56 | 0.84 | 0.39 | 0.90 | 0.06 | 1.07 | 0.07 |
| 57 | 0.29 | 0.03 | 0.41 | 0.05 | 1.42 | 0.187 |
| 58 | 0.34 | 0.15 | 0.21 | 0.02 | 0.60 | 0.072 |
| 59 | 0.30 | 0.04 | 0.14 | 0.01 | 0.46 | 0.040 |
| 60 | 1.02 | 0.25 | 0.96 | 0.07 | 0.94 | 0.073 |
| 61 | 0.16 | 0.20 | 0.66 | 0.21 | 4.06 | 1.287 |
| 62 | 0.51 | 0.15 | 0.48 | 0.04 | 0.94 | 0.079 |
| 63 | 0.88 | 0.23 | 0.93 | 0.06 | 1.07 | 0.063 |
| 64 | 0.15 | 0.04 | 0.13 | 0.01 | 0.86 | 0.042 |
| 65 | 0.06 | 0.01 | 0.14 | 0.05 | 2.55 | 0.868 |
| 66 | 0.19 | 0.13 | 0.05 | 0.03 | 0.22 | 0.172 |
| 67 | 0.06 | 0.02 | 0.04 | 0.03 | 0.81 | 0.475 |
| 68 | 0.23 | 0.02 | 0.27 | 0.06 | 1.20 | 0.243 |
| 69 | 0.17 | 0.07 | 0.17 | 0.03 | 1.00 | 0.146 |
| 70 | 0.12 | 0.05 | 0.13 | 0.01 | 1.13 | 0.080 |
| 71 | 0.10 | 0.03 | 0.10 | 0.04 | 0.92 | 0.346 |
| 72 | 0.10 | 0.05 | 0.11 | 0.00 | 1.11 | 0.041 |
| 73 | 0.11 | 0.03 | 0.11 | 0.01 | 1.06 | 0.087 |
| 74 | 0.34 | 0.24 | 0.36 | 0.17 | 1.07 | 0.509 |
| 75 | 0.44 | 0.23 | 0.35 | 0.12 | 0.79 | 0.272 |
| 76 | 0.13 | 0.08 | 0.21 | 0.03 | 1.59 | 0.235 |
| 77 | 0.10 | 0.02 | 0.23 | 0.08 | 2.28 | 0.766 |
| 78 | 0.12 | 0.01 | 0.20 | 0.00 | 1.68 | 0.004 |
| 79 | 0.26 | 0.05 | 0.33 | 0.20 | 1.29 | 0.771 |
| 80 | 0.13 | 0.12 | 0.47 | 0.08 | 3.70 | 0.597 |
| 81 | 0.63 | 0.03 | 0.44 | 0.18 | 0.71 | 0.289 |
| 82 | 0.21 | 0.05 | 0.30 | 0.12 | 1.38 | 0.554 |
| 83 | 0.16 | 0.07 | 0.12 | 0.02 | 0.71 | 0.126 |
| 84 | 0.24 | 0.05 | 0.26 | 0.07 | 1.07 | 0.286 |
| 85 | 0.24 | 0.02 | 0.32 | 0.09 | 1.31 | 0.379 |
| 86 | 0.19 | 0.02 | 0.15 | 0.00 | 0.79 | 0.015 |
| 87 | 0.15 | 0.02 | 0.11 | 0.03 | 0.71 | 0.215 |
| 88 | 0.27 | 0.01 | 0.19 | 0.04 | 0.69 | 0.141 |
| 89 | 0.08 | 0.01 | 0.09 | 0.01 | 1.13 | 0.092 |
| 90 | 0.20 | 0.11 | 0.11 | 0.08 | 0.56 | 0.414 |
| 91 | 0.15 | 0.10 | 0.22 | 0.07 | 1.47 | 0.452 |
| 92 | 0.11 | 0.08 | 0.09 | 0.01 | 0.86 | 0.090 |
| 93 | 0.19 | 0.21 | 0.13 | 0.04 | 0.70 | 0.211 |
| 94 | 0.02 | 0.00 | 0.07 | 0.00 | 3.12 | 0.099 |
| 95 | 0.02 | 0.06 | 0.73 | 0.30 | 4.26 | 1.752 |
| 96 | 0.32 | 0.22 | 0.32 | 0.01 | 1.00 | 0.024 |
| 97 | 0.15 | 0.15 | 0.31 | 0.22 | 2.06 | 1.442 |
| 98 | 0.15 | 0.15 | 0.12 | 0.02 | 0.79 | 0.144 |
| 99 | 0.10 | 0.02 | 0.21 | 0.06 | 2.01 | 0.558 |
| 100 | 0.14 | 0.05 | 0.13 | 0.07 | 0.93 | 0.483 |

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|---|---|---|---|---|---|---|
| 101 | 0.08 | 0.03 | 0.11 | 0.00 | 1.32 | 0.004 |
| 102 | 0.09 | 0.01 | 0.14 | 0.07 | 1.64 | 0.826 |
| 103 | 0.08 | 0.00 | 0.06 | 0.03 | 0.78 | 0.448 |
| 104 | 0.08 | 0.03 | 0.09 | 0.06 | 1.18 | 0.8812 |
| 105 | 0.13 | 0.10 | 0.08 | 0.02 | 0.63 | 0.119 |
| 106 | 0.19 | 0.15 | 0.20 | 0.11 | 1.07 | 0.585 |
| 107 | 0.16 | 0.11 | 0.40 | 0.26 | 2.50 | 1.632 |
| 108 | 0.31 | 0.33 | 0.22 | 0.11 | 0.72 | 0.354 |
| 109 | 0.44 | 0.53 | 0.2 | 0.04 | 0.40 | 0.100 |
| 110 | 0.13 | 0.01 | 0.20 | 0.05 | 1.52 | 0.416 |
| 111 | 0.03 | 0.00 | 0.16 | 0.11 | 4.81 | 3.534 |
| 112 | 0.03 | 0.01 | 0.05 | 0.00 | 1.50 | 0.032 |
| 113 | 0.02 | 0.00 | 0.05 | 0.01 | 1.95 | 0.375 |
| 114 | 0.06 | 0.01 | 0.17 | 0.05 | 2.79 | 0.809 |
| 115 | 0.11 | 0.08 | 0.14 | 0.03 | 1.25 | 0.287 |
| 116 | 0.08 | 0.09 | 0.23 | 0.02 | 2.72 | 0.267 |
| 117 | 0.13 | 0.04 | 0.17 | 0.02 | 1.31 | 0.117 |
| 118 | 0.24 | 0.05 | 0.27 | 0.03 | 1.12 | 0.117 |
| 119 | 0.07 | 0.00 | 0.10 | 0.04 | 1.41 | 0.632 |
| 120 | 0.07 | 0.03 | 0.04 | 0.03 | 0.60 | 0.386 |
| 121 | 0.08 | 0.05 | 0.04 | 0.03 | 0.50 | 0.406 |
| 122 | 0.07 | 0.01 | 0.04 | 0.03 | 1.08 | 0.809 |
| 123 | 0.04 | 0.00 | 0.09 | 0.02 | 2.68 | 0.784 |
| 124 | 0.12 | 0.00 | 0.09 | 0.02 | 0.76 | 0.132 |
| 125 | 0.05 | 0.01 | 0.04 | 0.02 | 0.77 | 0.346 |
| 126 | 0.17 | 0.17 | 0.08 | 0.03 | 0.46 | 0.176 |
| 127 | 0.79 | 0.35 | 0.92 | 0.42 | 1.16 | 0.531 |
| 128 | 0.56 | 0.08 | 0.37 | 0.14 | 0.66 | 0.247 |
| 129 | 0.19 | 0.05 | 0.22 | 0.05 | 1.17 | 0.264 |
| 130 | 0.17 | 0.06 | 0.11 | 0.03 | 0.64 | 0.173 |
| 131 | 0.68 | 0.11 | 0.74 | 0.35 | 1.10 | 0.511 |
| 132 | 0.41 | 0.12 | 0.29 | 0.09 | 0.71 | 0.221 |
| 133 | 0.12 | 0.02 | 0.16 | 0.01 | 1.32 | 0.115 |
| 134 | 0.17 | 0.05 | 0.09 | 0.02 | 0.54 | 0.101 |
| 135 | 0.09 | 0.00 | 0.09 | 0.03 | 1.06 | 0.315 |
| 136 | 0.33 | 0.09 | 0.19 | 0.18 | 0.58 | 0.540 |
| 137 | 0.42 | 0.01 | 0.29 | 0.08 | 0.70 | 0.181 |
| 138 | 0.32 | 0.18 | 0.37 | 0.07 | 1.14 | 0.224 |
| 139 | 0.18 | 0.07 | 0.17 | 0.04 | 0.97 | 0.209 |
| 140 | 0.31 | 0.04 | 0.31 | 0.02 | 0.98 | 0.067 |
| 141 | 0.14 | 0.03 | 0.15 | 0.02 | 1.11 | 0.169 |
| 142 | 0.16 | 0.08 | 0.17 | 0.02 | 1.10 | 0.110 |
| 143 | 0.21 | 0.08 | 0.23 | 0.03 | 1.12 | 0.149 |
| 144 | 0.13 | 0.02 | 0.17 | 0.02 | 1.33 | 0.126 |
| 145 | 0.26 | 0.33 | 0.14 | 0.00 | 0.53 | 0.007 |
| 146 | 0.13 | 0.00 | 0.15 | 0.05 | 1.17 | 0.394 |
| 147 | 0.15 | 0.00 | 0.09 | 0.04 | 0.60 | 0.252 |
| 148 | 0.13 | 0.09 | 0.15 | 0.11 | 1.19 | 0.843 |
| 149 | 0.17 | 0.15 | 0.13 | 0.13 | 0.77 | 0.777 |
| 150 | 0.14 | 0.04 | 0.10 | 0.06 | 0.72 | 0.468 |
| 151 | 0.23 | 0.22 | 0.20 | 0.19 | 0.90 | 0.837 |
| 152 | 0.11 | 0.08 | 0.10 | 0.07 | 0.91 | 0.594 |
| 153 | 0.07 | 0.03 | 0.04 | 0.04 | 0.60 | 0.553 |
| 154 | 0.12 | 0.08 | 0.07 | 0.02 | 0.58 | 0.164 |
| 155 | 0.11 | 0.12 | 0.11 | 0.06 | 1.03 | 0.596 |

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|---|---|---|---|---|---|---|
| 156 | 0.05 | 0.01 | 0.27 | 0.00 | 4.94 | 0.011 |
| 157 | 0.06 | 0.01 | 0.07 | 0.04 | 1.25 | 0.624 |
| 158 | 0.17 | 0.06 | 0.12 | 0.02 | 0.71 | 0.119 |
| 159 | 0.07 | 0.03 | 0.16 | 0.05 | 2.37 | 0.762 |
| 160 | 0.10 | 0.02 | 0.12 | 0.14 | 1.17 | 1.342 |
| 161 | 0.21 | 0.05 | 0.23 | 0.07 | 1.05 | ‘.314 |
| 162 | 0.10 | 0.08 | 0.06 | 0.01 | 0.54 | 0.100 |
| 163 | 0.34 | 0.15 | 0.21 | 0.13 | 0.61 | 0.391 |
| 164 | 0.15 | 0.05 | 0.16 | 0.03 | 1.01 | 0.177 |
| 165 | 0.07 | 0.01 | 0.08 | 0.03 | 1.07 | 0.476 |
| 166 | 0.13 | 0.01 | 0.06 | 0.01 | 0.47 | 0.076 |
| 167 | 0.05 | 0.01 | 0.07 | 0.00 | 1.40 | 0.018 |
| 168 | 0.07 | 0.05 | 0.12 | 0.03 | 1.68 | 0.463 |
| 169 | 0.10 | 0.01 | 0.10 | 0.06 | 1.01 | 0.579 |
| 170 | 0.10 | 0.00 | 0.14 | 0.08 | 1.45 | 0.820 |
| 171 | 0.09 | 0.06 | 0.09 | 0.01 | 0.92 | 0.078 |
| 172 | 0.07 | 0.03 | 0.06 | 0.01 | 0.90 | 0.166 |
| 173 | 0.08 | 0.08 | 0.17 | 0.02 | 2.16 | 0.295 |
| 174 | 0.07 | 0.00 | 0.08 | 0.00 | 1.16 | 0.003 |
| 175 | 0.12 | 0.07 | 0.05 | 0.01 | 0.42 | 0.105 |
| 176 | 0.06 | 0.04 | 0.06 | 0.05 | 0.89 | 0.805 |
| 177 | 0.07 | 0.06 | 0.09 | 0.00 | 1.39 | 0.021 |
| 178 | 0.08 | 0.09 | 0.06 | 0.01 | 0.84 | 0.148 |
| 179 | 0.17 | 0.09 | 0.20 | 0.01 | 1.15 | 0.038 |
| 180 | 0.19 | 0.16 | 0.15 | 0.02 | 0.83 | 0.092 |
| 181 | 0.03 | 0.00 | 0.08 | 0.06 | 3.16 | 2.333 |
| 182 | 0.10 | 0.09 | 0.11 | 0.01 | 1.08 | 0.057 |
| 183 | 0.04 | 0.03 | 0.08 | 0.03 | 2.01 | 0.882 |
| 184 | 0.12 | 0.10 | 0.12 | 0.04 | 0.96 | 0.288 |
| 185 | 0.14 | 0.08 | 0.19 | 0.20 | 1.34 | 1.385 |
| 186 | 0.06 | 0.03 | 0.07 | 0.04 | 1.14 | 0.697 |
| 187 | 0.11 | 0.06 | 0.08 | 0.01 | 0.73 | 0.078 |
| 188 | 0.04 | 0.02 | 0.03 | 0.01 | 0.62 | 0.202 |
| 189 | 0.02 | 0.01 | 0.03 | 0.00 | 1.69 | 0.041 |
| 190 | 0.07 | 0.09 | 0.06 | 0.02 | 0.82 | 0.281 |
| 191 | 0.02 | 0.02 | 0.02 | 0.01 | 0.75 | 0.382 |
| 192 | 0.05 | 0.01 | 0.09 | 0.01 | 1.89 | 0.244 |
| 193 | 0.14 | 0.02 | 0.12 | 0.05 | 0.88 | 0.363 |
| 194 | 0.02 | 0.00 | 0.06 | 0.03 | 3.57 | 1.619 |
| 195 | 0.04 | 0.00 | 0.02 | 0.01 | 0.48 | 0.312 |
| 196 | 0.11 | 0.04 | 0.17 | 0.06 | 1.64 | 0.552 |
| 197 | 0.60 | 0.18 | 0.54 | 0.26 | 0.90 | 0.427 |
| 198 | 0.21 | 0.01 | 0.36 | 0.16 | 1.70 | 0.732 |
| 199 | 0.22 | 0.01 | 0.23 | 0.01 | 1.07 | 0.026 |
| 200 | 0.12 | 0.01 | 0.24 | 0.14 | 2.04 | 1.167 |
| 201 | 0.03 | 0.00 | 0.05 | 0.04 | 1.46 | 1.166 |
| 202 | 0.05 | 0.01 | 0.08 | 0.08 | 1.57 | 1.578 |
| 203 | 0.07 | 0.04 | 0.06 | 0.05 | 0.81 | 0.718 |
| 204 | 0.05 | 0.04 | 0.02 | 0.01 | 0.45 | 0.219 |
| 205 | 0.03 | 0.02 | 0.06 | 0.01 | 1.73 | 0.377 |
| 207 | 0.09 | 0.00 | 0.06 | 0.01 | 0.69 | 0.138 |
| 208 | 0.11 | 0.02 | 0.06 | 0.04 | 0.61 | 0.367 |
| 209 | 0.07 | 0.03 | 0.06 | 0.01 | 0.84 | 0.075 |
| 210 | 0.15 | 0.09 | 0.29 | 0.17 | 1.96 | 1.126 |
| 211 | 0.07 | 0.01 | 0.16 | 0.07 | 2.21 | 1.036 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 212 | 0.08 | 0.01 | 0.13 | 0.06 | 1.58 | 0.718 |
| 213 | 0.10 | 0.01 | 0.12 | 0.02 | 1.21 | 0.248 |
| 214 | 0.22 | 0.17 | 0.10 | 0.02 | 0.47 | 0.100 |
| 215 | 0.04 | 0.02 | 0.10 | 0.03 | 2.76 | 0.815 |
| 216 | 0.27 | 0.25 | 0.05 | 0.01 | 0.19 | 0.036 |
| 217 | 0.21 | 0.08 | 0.16 | 0.07 | 0.19 | 0.036 |
| 218 | 0.31 | 0.18 | 0.44 | 0.14 | 1.44 | 0.462 |
| 219 | 0.07 | 0.03 | 0.06 | 0.03 | 0.83 | 0.379 |
| 220 | 0.03 | 0.00 | 0.06 | 0.01 | 1.68 | 0.353 |
| 221 | 0.04 | 0.01 | 0.04 | 0.01 | 1.04 | 0.202 |
| 222 | 0.03 | 0.01 | 0.04 | 0.00 | 1.13 | 0.025 |
| 223 | 0.04 | 0.01 | 0.03 | 0.00 | 0.69 | 0.059 |
| 224 | 0.12 | 0.06 | 0.15 | 0.05 | 1.20 | 0.366 |
| 225 | 0.13 | 0.09 | 0.09 | 0.02 | 0.63 | 0.139 |
| 226 | 0.12 | 0.08 | 0.10 | 0.03 | 1.10 | 0.263 |
| 228 | 0.06 | 0.02 | 0.05 | 0.02 | 0.88 | 0.325 |
| 229 | 0.10 | 0.05 | 0.06 | 0.03 | 0.59 | 0.295 |
| 230 | 0.07 | 0.06 | 0.14 | 0.01 | 1.91 | 0.183 |
| 231 | 0.05 | 0.03 | 0.06 | 0.00 | 1.17 | 0.074 |
| 232 | 0.05 | 0.03 | 0.08 | 0.04 | 1.55 | 0.676 |
| 233 | 0.05 | 0.03 | 0.08 | 0.04 | 1.54 | 0.674 |
| 234 | 0.07 | 0.02 | 0.03 | 0.04 | 0.47 | 0.516 |
| 235 | 0.06 | 0.02 | 0.05 | 0.00 | 0.78 | 0.048 |
| 236 | 0.06 | 0.01 | 0.05 | 0.01 | 0.85 | 0.096 |
| 237 | 0.02 | 0.01 | 0.03 | 0.02 | 1.36 | 0.776 |
| 238 | 0.02 | 0.00 | 0.02 | 0.02 | 1.18 | 0.922 |
| 239 | 0.10 | 0.01 | 0.08 | 0.03 | 0.76 | 0.260 |
| 240 | 0.05 | 0.00 | 0.04 | 0.03 | 0.91 | 0.579 |
| 241 | 0.03 | 0.01 | 0.07 | 0.01 | 2.05 | 0.401 |
| 242 | 0.03 | 0.00 | 0.06 | 0.00 | 2.00 | 0.004 |
| 243 | 0.02 | 0.02 | 0.06 | 0.03 | 2.63 | 1.169 |
| 244 | 0.06 | 0.06 | 0.06 | 0.03 | 0.96 | 0.444 |
| 245 | 0.04 | 0.00 | 0.01 | 0.01 | 0.24 | 0.251 |
| 246 | 0.03 | 0.01 | 0.01 | 0.00 | 0.22 | 0.118 |
| 247 | 0.02 | 0.01 | 0.06 | 0.03 | 3.12 | 0.189 |
| 248 | 0.38 | 0.48 | 0.08 | 0.09 | 0.21 | 0.235 |
| 249 | 0.18 | 0.19 | 0.05 | 0.04 | 0.30 | 0.241 |
| 250 | 0.01 | 0.00 | 0.04 | 0.02 | 3.51 | 2.010 |
| 251 | 0.03 | 0.01 | 0.03 | 0.01 | 0.93 | 0.348 |
| 252 | 0.06 | 0.06 | 0.05 | 0.02 | 0.77 | 0.309 |
| 253 | 0.08 | 0.01 | 0.10 | 0.04 | 1.19 | 0.515 |
| 254 | 0.14 | 0.05 | 0.09 | 0.05 | 0.63 | 0.375 |
| 255 | 0.02 | 0.01 | 0.07 | 0.08 | 4.27 | 4.703 |
| 256 | 0.02 | 0.00 | 0.03 | 0.00 | 1.23 | 0.007 |
| 257 | 0.03 | 0.03 | 0.05 | 0.03 | 1.51 | 0.892 |
| 258 | 0.04 | 0.00 | 0.11 | 0.03 | 2.75 | 0.891 |
| 259 | 0.05 | 0.05 | 0.13 | 0.05 | 2.66 | 0.923 |
| 260 | 0.10 | 0.04 | 0.13 | 0.03 | 1.33 | 0.270 |
| 261 | 0.09 | 0.01 | 0.07 | 0.03 | 0.82 | 0.322 |
| 262 | 0.10 | 0.04 | 0.34 | 0.06 | 3.38 | 0.638 |
| 263 | 0.03 | 0.01 | 0.03 | 0.02 | 1.20 | 0.629 |
| 264 | 0.02 | 0.01 | 0.01 | 0.00 | 0.49 | 0.018 |
| 265 | 0.07 | 0.00 | 0.10 | 0.00 | 1.48 | 0.015 |
| 266 | 0.12 | 0.05 | 0.07 | 0.03 | 0.59 | 0.246 |
| 267 | 0.03 | 0.00 | 0.04 | 0.03 | 1.39 | 1.014 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 268 | 0.16 | 0.02 | 0.13 | 0.02 | 0.83 | 0.101 |
| 269 | 0.16 | 0.09 | 0.20 | 0.01 | 1.25 | 0.077 |
| 270 | 0.06 | 0.04 | 0.08 | 0.03 | 1.51 | 0.456 |
| 271 | 0.04 | 0.00 | 0.08 | 0.07 | 2.09 | 1.651 |
| 272 | 0.32 | 0.22 | 0.15 | 0.01 | 0.47 | 0.019 |
| 273 | 0.07 | 0.05 | 0.04 | 0.04 | 0.61 | 0.601 |
| 274 | 0.04 | 0.01 | 0.04 | 0.01 | 1.03 | 0.283 |
| 275 | 0.05 | 0.03 | 0.10 | 0.03 | 1.90 | 0.599 |
| 276 | 0.19 | 0.12 | 0.10 | 0.03 | 0.50 | 0.143 |
| 277 | 0.16 | 0.20 | 0.20 | 0.04 | 1.26 | 0.243 |
| 278 | 0.07 | 0.07 | 0.03 | 0.01 | 0.46 | 0.080 |
| 279 | 0.10 | 0-02 | 0.09 | 0.02 | 0.95 | 0.194 |
| 280 | 0.05 | 0.01 | 0.13 | 0.06 | 2.73 | 1.214 |
| 281 | 0.08 | 0.01 | 0.07 | 0.00 | 0.84 | 0.017 |
| 282 | 0.02 | 0.01 | 0.01 | 0.00 | 0.55 | 0.034 |
| 283 | 0.04 | 0.02 | 0.01 | 0.00 | 0.24 | 0.135 |
| 284 | 0.06 | 0.04 | 0.01 | 0.00 | 0.13 | 0.080 |
| 285 | 0.03 | 0.03 | 0.06 | 0.03 | 1.90 | 0.778 |
| 286 | 0.04 | 0.00 | 0.08 | 0.05 | 2.00 | 1.200 |
| 287 | 0.05 | 0.01 | 0.03 | 0.01 | 0.57 | 0.326 |
| 288 | 0.06 | 0.01 | 0.07 | 0.03 | 1.27 | 0.551 |
| 289 | 0.06 | 0.01 | 0.04 | 0.01 | 0.64 | 0.181 |
| 290 | 0.02 | 0.01 | 0.05 | 0.05 | 2.13 | 1.949 |
| 291 | 0.26 | 0.31 | 0.22 | 0.15 | 0.85 | 0.559 |
| 292 | 0.09 | 0.07 | 0.12 | 0.19 | 1.96 | 2.132 |
| 293 | 0.03 | 0.03 | 0.07 | 0.05 | 2.03 | 1.371 |
| 294 | 0.05 | 0.03 | 0.04 | 0.01 | 0.85 | 0.231 |
| 295 | 0.07 | 0.04 | 0.05 | 0.03 | 0.70 | 0.473 |
| 296 | 0.03 | 0.01 | 0.07 | 0.01 | 2.84 | 0.215 |
| 297 | 0.26 | 0.28 | 0.07 | 0.01 | 1.39 | 0.365 |
| 298 | 0.20 | 0.14 | 0.08 | 0.03 | 0.40 | 0.164 |
| 299 | 0.08 | 0.01 | 0.05 | 0.00 | 0.57 | 0.053 |
| 300 | 0.56 | 0.70 | 0.41 | 0.10 | 0.74 | 0.171 |
| 301 | 0.06 | 0.01 | 0.09 | 0.00 | 1.47 | 0.061 |
| 302 | 0.09 | 0.09 | 0.04 | 0.00 | 0.47 | 0.024 |
| 303 | 0.13 | 0.04 | 0.06 | 0.02 | 0.46 | 0.167 |
| 304 | 0.15 | 0.02 | 0.40 | 0.16 | 2.76 | 1.093 |
| 305 | 0.16 | 0.03 | 0.11 | 0.15 | 0.69 | 0.917 |
| 306 | 0.06 | 0.01 | 0.10 | 0.03 | 1.63 | 0.460 |
| 307 | 0.07 | 0.04 | 0.04 | 0.01 | 2.74 | 1.093 |
| 308 | 0.26 | 0.11 | 0.13 | 0.09 | 0.52 | 0.363 |
| 309 | 0.04 | 0.00 | 0.04 | 0.00 | 0.97 | 0.094 |
| 310 | 0.11 | 0.05 | 0.01 | 0.01 | 0.13 | 0.091 |
| 311 | 0.04 | 0.02 | 0.03 | 0.00 | 0.77 | 0.096 |
| 312 | 0.02 | 0.00 | 0.02 | 0.02 | 0.89 | 0.741 |
| 313 | 0.07 | 0.01 | 0.08 | 0.02 | 1.10 | 0.276 |
| 314 | 0.03 | 0.01 | 0.04 | 0.01 | 1.50 | 0.380 |
| 315 | 0.05 | 0.01 | 0.02 | 0.00 | 0.35 | 0.049 |
| 316 | 0.13 | 0.05 | 0.08 | 0.01 | 0.66 | 0.099 |
| 317 | 0.04 | 0.01 | 0.08 | 0.02 | 1.74 | 0.494 |
| 318 | 0.05 | 0.01 | 0.04 | 0.03 | 0.85 | 0.579 |
| 319 | 0.05 | 0.02 | 0.03 | 0.01 | 0.56 | 0.159 |
| 320 | 0.75 | 0.29 | 0.20 | 0.02 | 0.27 | 0.031 |
| 321 | 0.20 | 0.020 | 0.04 | 0.04 | 0.22 | 0.177 |
| 322 | 0.23 | 0.003 | 0.22 | 0.01 | 0.98 | 0.049 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 323 | 0.02 | 0.01 | 0.23 | 0.01 | 1.54 | 0.640 |
| 324 | 0.13 | 0.12 | 0.03 | 0.00 | 0.27 | 0.027 |
| 325 | 0.018 | 0.12 | 0.03 | 0.01 | 0.17 | 0.068 |
| 326 | 0.11 | 0.07 | 0.02 | 0.04 | 0.35 | 0.411 |
| 327 | 0.02 | 0.01 | 0.02 | 0.01 | 1.33 | 0.592 |
| 328 | 0.01 | 0.00 | 0.06 | 0.00 | 3.99 | 0.328 |
| 329 | 0.02 | 0.00 | 0.03 | 0.01 | 1.53 | 0.668 |
| 330 | 0.03 | 0.02 | 0.03 | 0.02 | 1.00 | 0.637 |
| 331 | 0.04 | 0.03 | 0.04 | 0.02 | 0.97 | 0.431 |
| 332 | 0.02 | 0.00 | 0.03 | 0.01 | 1.57 | 0.485 |
| 333 | 0.06 | 0.01 | 0.03 | 0.00 | 0.47 | 0.010 |
| 334 | 1.43 | 0.49 | 2.80 | 0.35 | 1.96 | 0.242 |
| 335 | 0.70 | 0.74 | 1.20 | 0.03 | 1.71 | 0.048 |
| 336 | 0.13 | 0.02 | 1.17 | 0.01 | 0.76 | 0.112 |
| 337 | 0.10 | 0.07 | 0.11 | 0.04 | 1.11 | 0.453 |
| 338 | 0.05 | 0.03 | 0.05 | 0.00 | 0.98 | 0.107 |
| 339 | 0.14 | 0.06 | 0.28 | 0.12 | 1.95 | 0.813 |
| 340 | 0.14 | 0.03 | 0.32 | 0.03 | 2.33 | 0.129 |
| 341 | 0.10 | 0.05 | 0.08 | 0.02 | 2.33 | 0.129 |
| 342 | 0.07 | 004 | 0.07 | 0.03 | 0.91 | 0.439 |
| 343 | 0.05 | 0.03 | 0.02 | 0.00 | 0.41 | 0.069 |
| 344 | 0.08 | 0.02 | 0.04 | 0.00 | 0.43 | 0.036 |
| 345 | 0.20 | 0.22 | 0.38 | 0.08 | 1.91 | 0.404 |
| 346 | 0.14 | 0.03 | 0.26 | 0.07 | 1.88 | 0.516 |
| 347 | 0.19 | 0.17 | 0.16 | 0.03 | 0.85 | 0.132 |
| 348 | 0.21 | 0.14 | 0.15 | 0.03 | 0.71 | 0.166 |
| 349 | 0.15 | 0.06 | 0.12 | 0.02 | 0.79 | 0.128 |
| 350 | 0.04 | 0.02 | 0.13 | 0.01 | 0.31 | 0.166 |
| 351 | 0.14 | 0.03 | 0.2.4 | 0.04 | 1.76 | 0.267 |
| 352 | 0.06 | 0.06 | 0.15 | 0.03 | 2.33 | 0.453 |
| 353 | 0.04 | 0.02 | 0.02 | 0.01 | 0.60 | 0.322 |
| 354 | 0.03 | 0.02 | 0.03 | 0.01 | 1.07 | 0.266 |
| 355 | 0.04 | 0.01 | 0.06 | 0.00 | 1.74 | 0.014 |
| 356 | 0.03 | 0.02 | 0.01 | 0.01 | 0.53 | 0.298 |
| 357 | 0.10 | 0.04 | 0.03 | 0.03 | 0.32 | 0.260 |
| 358 | 0.05 | 0.001 | 0.09 | 0.05 | 1.66 | 1.011 |
| 359 | 0.20 | 0.06 | 0.19 | 0.02 | 0.95 | 0.097 |
| 360 | 0.04 | 0.02 | 0.02 | 0.01 | 0.51 | 0.148 |
| 361 | 0.05 | 0.02 | 0.04 | 0.03 | 0.72 | 0.509 |
| 362 | 0.33 | 0.04 | 0.17 | 0.11 | 0.51 | 0.339 |
| 363 | 0.07 | 0.01 | 0.04 | 0.02 | 0.63 | 0.365 |
| 364 | 0.07 | 0.01 | 0.06 | 0.01 | 0.84 | 0.097 |
| 365 | 0.13 | 0.07 | 0.06 | 0.02 | 0.44 | 0.152 |
| 366 | 0.38 | 0.15 | 0.15 | 0.02 | 0.40 | 0.040 |
| 367 | 0.57 | 0.06 | 0.60 | 0.07 | 1.05 | 0.124 |
| 368 | 0.84 | 0.08 | 0.86 | 0.06 | 1.03 | 0.069 |
| 369 | 0.20 | 0.00 | 0.46 | 0.01 | 2.33 | 0.049 |
| 371 | 0.25 | 0.05 | 0.31 | 0.01 | 1.28 | 0.042 |
| 373 | 0.10 | 0.03 | 0.39 | 0.09 | 4.06 | 0.908 |
| 374 | 0.10 | 0.04 | 0.06 | 0.04 | 0.65 | 0.436 |
| 375 | 0.10 | 0.08 | 0.02 | 0.01 | 0.22 | 0.091 |
| 376 | 0.11 | 0.02 | 0.04 | 0.00 | 0.36 | 0.024 |

# 4. Effect of TZ on *Salmonella* infected human macrophages

**Abstract**

**Background.** One of the main obstacles to the treatment of infections caused by pathogens such as *Salmonella* Typhimurium, is the fact that these pathogens are adapted to a host; able to live and replicate in an intracellular niche; and able to circumvent and modulate the immune responses of the host. Therefore, new antimicrobial compounds able to reach the site of infection, such as the inside of the macrophage, are urgently needed. Here, the aim of this study was to explore the effect of TZ on *Salmonella*-infected human macrophages. **Materials/methods.** THP-1 cells were used to study the effect of TZ on monocytes and macrophages. Initially, the effect of TZ on the metabolism of human monocytes was assessed using phenotypic microarray plates. ELISAs were performed to explore the possible effect of TZ on the secretion of cytokines. The effect of TZ on the activity of caspases of human uninfected macrophages was performed using the FluoFire Kit. Cell viability assays were performed using THP-1 monocytes. Infection assays were performed in THP-1 macrophages treated and non-treated with TZ, verapamil (VER) and Loxapine (LOX). Supernatants of the infection assays were collected and analysed for nitric oxide production using Griess reagent and for cytokine production by ELISA. Intracellular calcium was measured using Cal-520™ dye on non-treated and treated *Salmonella*-infected macrophages. **Results:** Uninfected monocytes and macrophages treated with TZ showed no difference on the immune response when exposed to TZ. *Salmonella*-infected macrophages treated with sub-MIC of TZ showed a significant decrease in intracellular CFU/mL and this was a concentration dependent effect. TZ did not impact on the secretion of cytokines or the production of nitric oxide by infected macrophages in comparison with the controls. When TZ was added to cultures of *Salmonella*-infected macrophages a significant decrease in the intracellular calcium levels of the macrophages was observed. **Conclusions:** These results suggest that TZ may enhance the killing activity of infected macrophages by interfering with the calcium levels of the infected cells. Due to its effect on infected macrophages, TZ may be considered a potential adjuvant to antibiotic therapy to treat multidrug-resistant bacterial infections.

## 4.1. Introduction

Bacterial pathogens that can survive within host cells are a cause for concern due to the failure of current therapeutic regimens and their ability to evade and modulate the immune response of the host. One of the main obstacles to treating infections caused by pathogens such as *Salmonella spp.* and *M. tuberculosis* is the fact that these pathogens display exquisite host adaptations that facilitate survival and replication in an intracellular niche by evading and modulating the immune responses of the host (Martins *et al*., 2007a; Diacovich *et al*., 2017). This constitutes an additional obstacle to the development of novel antimicrobial compounds, since, in order to be effective, these compounds must cross the host cell membrane, in addition to maintaining activity once inside the macrophage (Martins *et al*., 2009).

TZ has been widely studied and its antibacterial activity reported: i) *in vitro* against *M. tuberculosis* (Ordway *et al*., 2003; Martins *et al*., 2007a), *S. aureus* (Wassmann *et al*., 2018), *Salmonella* spp., *Pseudomonas aeruginosa* (Hendricks, Butterworth & Kristiansen, 2003b) ii) *ex-vivo* in *M. tuberculosis* infected macrophages (Ordway *et al*., 2003); and iii) *in vivo* by curing of mice infected with *Salmonella* (Dasgupta *et al*., 2010) or *M. tuberculosis* (van Soolingen *et al*., 2010). *In vitro* antimicrobial activity of TZ is achieved using concentrations that vastly exceed those considered safe for clinical use (maximum of 0.5-1 mg/L of plasma) (Thanacoody, 2007). However, studies performed by Crowle *et al.* (Crowle, Douvas & May, 1992) showed that exposure of macrophages containing phagocytosed *M. tuberculosis* to clinically relevant concentrations of the phenothiazine chlorpromazine (CPZ), enhanced killing of intracellular *M. tuberculosis* (Crowle, Douvas & May, 1992). Years later, Ordway *et al*. (Ordway *et al*., 2003) also demonstrated that both CPZ and TZ could effectively kill intracellular *M. tuberculosis* at clinically relevant concentrations. Studies conducted by Daniel (1995) and Wojcikowski (Daniel, Bickel & Honegger, 1995; Daniel & Wójcikowski, 1999) have reported that this success is linked with the ability of cells rich in lysosomes (e.g., macrophages) to concentrate phenothiazines to levels 100 times greater than their initial starting concentrations. Accordingly, this effect leads to intracellular concentrations been comparable to those achieved *in vitro*. Accumulation of phenothiazines inside these lysosomal rich cells results in inhibition of intracellular bacterial replication and enhanced killing activity of the phagocytic cell towards the internalised bacteria.

A direct mechanism of action for TZ in infected macrophages has been previously inferred for cells infected with *M. tuberculosis.* (Martins *et al*., 2009; Machado *et al*., 2012, 2016). After binding of the infecting bacterium to a receptor on the macrophage plasma membrane, and subsequent uptake into a phagosome, this compartment matures along the endocytic pathway, eventually fusing with lysosomes to form the phagolysosome unit. The plasma membrane of macrophages contains $Ca^{2+}$ channels (L-type), which are inhibited in the presence of TZ. This leads to the indirect acidification of the phagolysosome compartment and subsequent activation of hydrolytic enzymes. The action of these hydrolytic enzymes results in killing of the entrapped bacterium. However, conclusive evidence for this hypothesis is lacking and, to this day, the *ex-vivo* mode of action of TZ is still not fully understood.

Further studies have indicated that phenothiazines, including TZ, inhibit the binding of calcium ($Ca^{2+}$) to $Ca^{2+}$-binding proteins (e.g., calmodulin) in mammalian cells (Santos *et al*., 2007; Martins, Viveiros & Amaral, 2008; Machado *et al*., 2016; Thomas & Timson, 2018). Together, $Ca^{2+}$ and calmodulin play a vital role in regulating a variety of cellular functions, including maturation of the phagolysosome. Importantly, macrophages require efflux of $Ca^{2+}$ from the phagolysosome for killing of bacteria within this intracellular compartment. Additionally, interaction of bacteria with host cells has been shown to induce oscillation of intracellular $Ca^{2+}$. Bacterial structural components such as LPS or flagellin, induce $Ca^{2+}$ influx (Pace, Hayman & Galán, 1993; He *et al*., 2019; Frión-Herrera *et al*., 2020). *Salmonella* induces a transient increase in intracellular $Ca^{2+}$ to direct reorganization of the host cell cytoskeleton and this way facilitate bacterial uptake and control subsequent intracellular trafficking. The important role played by $Ca^{2+}$ in bacterial infections has led to great interest in the use of inhibitors and antagonists of $Ca^{2+}$ release as possible treatments for infectious disease (Machado *et al*., 2016; Lee *et al*., 2021).

Calcium channel blockers (CCBs) represent a group of diverse chemical structures that block $Ca^{2+}$-selective channels in the plasma membrane of a variety of cells, including macrophages, and have been widely used in eukaryotic systems to elucidate the mechanism and mode of action of $Ca^{2+}$ channels. CCBs are used in a clinical setting, usual as part of treatments for hypertension. One such compound, Verapamil (VER) is a known $Ca^{2+}$-channel blocker in eukaryotic cells, namely of L-type channels. These channels are key transducers of membrane potential

variations into intracellular $Ca^{2+}$, which initiates numerous physiological events. VER inhibits plasma membrane-mediated transport of $K^+$ into the macrophage by inhibiting $Ca^{2+}$-dependent pumps. This drug has been widely studied as a potential adjuvant therapy for *M. tuberculosis* and *S. aureus* infection (Martins *et al*., 2006; Gupta *et al*., 2013; Machado *et al*., 2016).

The Gram-negative bacterium *Salmonella* is currently listed by the WHO as a priority pathogen. Globally, non-typhoidal *Salmonella* (NTS) infections affect 93.8 millions of people and cause 681,316 deaths, annually (Stanaway *et al*., 2019). The highest incidence of these infections is observed in sub-Saharan Africa, where resistance to ampicillin, trimethoprim-sulfamethoxazole and chloramphenicol is currently widespread (Neuert *et al*., 2018; Amuasi & May, 2019). This resistance is a serious concern as all three antibiotics are categorised by the WHO model list medicine as “Access” and therefore recommended as an empiric first-choice treatment for invasive infections. Also, resistance to third-generation cephalosporin and fluoroquinolones has been reported in various countries in sub-Saharan Africa, and use of these antibiotics poses a high risk of selecting for bacterial resistance (World Health Organization, 2016; Gilchrist & MacLennan, 2019). These represent a challenge for treatment of *Salmonella* infections causing a significant public health treat. Therefore, the importance of exploring new approaches to treat *Salmonella* infection, such as drug repurposing, cannot be overstated.

In the field of *Salmonella* infection biology, a widely accepted cell type for studying the interaction of the bacterium with mammalian phagocytes is the THP-1 monocyte cell line. THP-1 cells have been widely used to study monocyte/macrophage functions, cellular mechanisms, signalling pathways and nutrient and drug transport (Auwerx, 1991; Chanput, Mes & Wichers, 2014) However, to be used as an *ex-vivo* model mimicking human macrophages, THP-1 cells must undergo a process of differentiation. Various approaches to differentiation of THP-1 cells have been studied, which include treatment with phorbol 12-myristate-12-acetate (PMA), 1 α, 25-dihydroxyvitamin D3 (vD3), or macrophage colony stimulating factor (M-CSF). Several protocols have been tested (Tsuchiya *et al*., 1982; Takashiba *et al*., 1999; Qin, 2012; Lund *et al*., 2016; Starr *et al*., 2018) however, a consensus protocol remains to be adopted by all researchers in the field. Generally, the use of PMA is the preferred method for differentiating THP-1

monocytes into a macrophage-like cell type “THP-1 macrophages” (Chanput, Mes & Wichers, 2014; Starr *et al*., 2018). After exposure to PMA, THP-1 monocytes start to adhere to culture plates and alter their morphology which is an indication for differentiation into macrophages; these morphological changes involve cells that are flat and amoeboid in shape with well-developed Golgi apparatuses, rough endoplasmic reticula and large numbers of ribosomes in the cytoplasm (Tsuchiya *et al*., 1982). Also, this differentiation also leads to a more mature phenotype with higher levels of adherence, a lower rate of proliferation and a higher rate of phagocytosis (Aldo *et al*., 2013). Across the various protocols adopted, a range of PMA concentrations (10 ng/mL –200 ng/mL) and exposure duration (1 days – 5 days) have been studied. A recent study by Starr *et al.,* (Starr *et al*., 2018) compared several conditions of THP-1 macrophage differentiation using PMA and how the various protocols affected the ability of the cells to interact with *S.* Typhimurium. The results showed that THP-1 cells differentiated at lower PMA concentrations (20 ng/mL) are more bactericidal than those differentiated with higher concentrations (≥100 ng/mL). Direct comparison of these cells with human monocyte derived macrophages suggest that they have a similar ability to control intracellular *S.* Typhimurium. Interestingly, the study showed that THP-1 cells differentiated in high concentrations of PMA (≥ 100 ng/mL) undergo rapid cell death following infection, whereas those differentiated in low concentrations of PMA survived. Importantly, the study also showed that, in THP-1 macrophages, *Salmonella* trafficking is similar to that seen in human monocyte derived macrophages. Since these were the conditions already used in the Martins lab for differentiation of human macrophages, , the *Starr et al.* protocol continued to be followed in this study.

Using THP-1 monocytes differentiated (from here on designated as “THP-1 macrophages”) as per Starr *et al*., as a model for intracellular *Salmonella* infection, the work conducted in this chapter aimed to: i) investigate the efficacy of TZ in treating *Salmonella* infection, ii) determine any changes to the immune response mounted by *Salmonella*-infected THP-1 macrophages; and iii) undertake initial investigations into the mechanism of action of TZ inside the infected macrophage.

## 4.2. Materials and Methods

### 4.2.1. Bacterial strain

The strain included in the study was *Salmonella enterica* serovar Typhimurium 14028S. The strain was grown in LB-Miller broth at 37°C with shaking.

### 4.2.2. TZ and other reagents

Unless otherwise stated, all reagents were purchased from Sigma- Aldrich (Sigma-Aldrich Ireland Limited, Wicklow, IE). TZ and VER stock solutions were prepared in sterile distilled water to a concentration of 50 mg/mL and 25 mg/mL, respectively. LOX was prepared at 25 mg/mL in DMSO. The three compounds were filtered, aliquoted and protected from light. These aliquots were frozen and stored at -20°C. Lipopolysaccharides (LPS - from *Salmonella enterica)* and human interferon-gamma (IFN-γ) stock solutions were prepared in sterile distilled water to a concentration of 5 mg/mL and 1 mg/mL, respectively. LPS and IFN-γ were stored at -4°C and -20°C, respectively. On the day of the experiment, aliquots were defrosted, and a working solution of 50 µg/mL and 100 µg/mL (respectively) were prepared to the desired concentrations. PMA was prepared in sterile distilled water to a stock of 1 mg/mL and kept frozen at -20°C.

### 4.2.3. Human cell line

Human THP-1 cells (Sigma-Aldrich, UK) differentiated with PMA were used as a macrophage model and prior to passage 14. These cells were grown in suspension in a humidified incubator at 37°C, 5% $CO_2$ in RPMI-1640 media containing Glutamax and supplemented with FBS (10% (v/v)). THP-1 monocytes were differentiated in 24-well plates (Corning® Costar ®, New York, US) or 96-well plates (Corning® Costar®, New York, US) in the presence of 20 ng/mL of PMA for 5 days prior to conducting assays with macrophages, as previously described (Starr *et al*., 2018). After exposure to PMA, nearly all THP-1 cells start to adhere to culture plates accompanied by differentiation into a macrophage-like phenotype with marked morphological changes, which mimic primary human macrophages. LPS and IFN-γ were used to stimulate the macrophages and activate the innate immune response. LPS was used at 50 ng/mL and IFN-γ at 100 ng/mL (final concentrations).

As a quality control, THP-1 cells were regularly tested for the presence of mycoplasma using 4′,6-diamidino-2-phenylindole (DAPI) staining and cells observed on a fluorescence microscope (Nikon fluorescent microscope ECLIPSE E400, Minato City, Tokyo, Japan). No mycoplasma contamination was detected on these cells during all the time of the passages and experiments performed (**Figure S4.1**).

### 4.2.4. Minimum Inhibitory and bactericidal concentrations (MIC/MBC)

The minimum inhibitory concentration (MIC) was determined by using the broth microdilution method in a 96-well plate according to the CLSI guidelines (Clinical and Laboratory Standards Institute, 2013). Briefly, overnight cultures of *Salmonella* were diluted in sterile phosphate buffered saline (PBS) solution to ~$10^5$ Colony Forming Units (CFU)/mL. Aliquots of 10 µL were then transferred to separate wells of a 96-well plate containing 100 µL of LB Miller and varying concentrations of each compound. VER, and LOX were added at a range of concentrations of 19.5 to 5000 µg/mL and 1.5 to 800 µg/mL, respectively. Plates were then incubated at 37° for 18 hours. After this time, plates were read by eyeball. The MIC was defined as the lowest concentration at which no visible bacterial growth was observed after incubation. Determination of the minimum bactericidal concentration (MBC) was performed by replica transfer of the MIC plate into a 96-well plate with compound-free media. Plates were incubated at 37°C and the MBC results recorded after 18 hours. MBC was defined as the lowest concentration of compound where no bacterial growth was obtained. The assays were performed in triplicate on three separate occasions.

### 4.2.5. Cell Viability

THP-1 monocytes were exposed to VER and LOX at a range of 0 to 160 µg/mL and 0 to 200 µg/mL, respectively. Effect on cell viability of THP-1 monocytes in the presence of TZ was shown in the previous chapter (Chapter 3, section 3.3.13). Cells were incubated with the compounds for 24 hours at 37°C with 5% $CO_2$. Viability assays were performed using the CellTiter 96™ Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, 3-(4,5-dimethylthiazol-2-yl)-5-(3-caarboxymethoxyphenyl)-2-(4-

sulfophenyl)-2H-tetrazolium, inner salt (MTS)/phenazine metasulfate (PMS) was added to each well and cells were incubated during 1 hour at 37°C. Following incubation, the absorbance measurements of the soluble formazan product (brown) were measured in a microplate reader (Synergy™ HT multimode microplate) at $490_{nm}$. The formazan product is directly proportional to the number of living cells. The assays were performed with three independent biological replicates.

### 4.2.6. Metabolic profiling – effect of TZ in human monocytes

The metabolic profiling of THP-1 monocytes in the presence and absence of TZ was conducted using the Biolog™ phenotype microarray platform. It is known that phenotyping allows for the identification of properties that can change due to variations on the cell. These variations canoccur at the genetic or environmental levels. One of the advantages of performing phenotypic analysis is the ability to track live cells over the course of their growth rather than having to conduct repeated sampling during time-course experiments as the ones conducted in common molecular analyses techniques, such as transcriptomics and proteomics. Therefore, the Biolog™ phenotype microarray platform used on this study relies on a high-throughput phenotyping technology that uses live cellular analysis. Using this platform, it is possible to measure hundreds of phenotypes simultaneously across multiple cell types using pre-prepared 96-wells microtiter plates. These pre-prepared plates are referred to as phenotype microarrays. Each plate allows for the analyses of a different set of assays with each well containing a different substrate that comprise, carbon sources, nitrogen substrates, ions, hormones, cytokines, *etc*. The automated Biolog™ system monitors respiration output of the cells when these are in the presence of various nutrient sources or substrates and allows to test for possible chemical sensitivities. This is measured *via* the principle of colorimetric analysis of energy production, by adding a purple redox tetrazolium dye that changes colour proportionally to the energy production. It is known that viable cells will generate energy rich NADH that reduces the redox dye, therefore promoting a colour change of the dye. This colour change (indicative of the cells' metabolism) can then be monitored and read with the Biolog OmniLog automated incubator-reader or as an alternative by using a spectrophotometer (end-point readings). In this study, the following phenotypic microarray plates PM-Mammalian (PM-M) panels were tested:

PM-M1-M4 for carbon and energy sources; PM-M5 for ions; and PM-M6 and 7 containing hormones and metabolic effectors, respectively.

All Phenotype Microarray (PM-M) assay reagents were purchases from Biolog, Inc (Hayward, CA, USA). [2]

Assays were performed according to the manufacturer's instructions. Specifically, monocytes were seeded at 4 x $10^5$ cells/mL in MC-0 assay medium (no glucose, low glutamine (0.3 mM) and low FBS 5% (v/v); Hayward, CA, USA) for PM-M1-4 and RPMI-1640 for PM-M5-7. Plates were incubated at 37°C, 5% $CO_2$ and 95% humidity for 24 hours. Subsequently, TZ was added to the plates (10 µL/ well) at a final concentration of 1 µg/mL plus Biolog Redox Dye Mix MA (10µL/well). After 24 hours, tetrazolium reduction (resulting in the formation of a purple colour) was measured using a Synergy™ HT multi-mode microplate reader. Absorbance was measured at $590_{nm}$ and $750_{nm}$ for absorbance correction as recommended by the manufacturer. Data were adjusted by subtracting average values of three negative control wells from all other samples of each plate. The effect of TZ exposure on the metabolism of the monocytes in comparison with untreated monocytes was compared using heatmaps.

### 4.2.7. Caspases -3/7 activity measurement.

Caspase-3/7 activation was measured using a FluoFire Caspase-3/7 fluorescent assay kit according to manufacturer's instructions (Molecutools, Dublin, Ireland). Prior to commencing the assay, THP-1 monocytes were differentiated with PMA for five days, as previously described (Starr *et al*., 2018). Subsequently, the complete media was removed, and the cells were washed with 100 µL of PBS. Serum depleted media (0.5% FBS (v/v)) was added, and the cells incubated for 24 hours. Cells were then treated with TZ (1 µg/mL) and incubated for a further 24 hours. The fluorescence was then measured in a Synergy™ HT multi-plate reader at 37°C (excitation, $485_{nm}$; emission $538_{nm}$).

[2]https://www.biolog.com/products-portfolio-overview/phenotype-microarrays-for-mammalian-cells/

### 4.2.8. Macrophage Infection assays.

Differentiated THP-1 monocytes ("THP-1 macrophages") were infected with *Salmonella* 14028S grown to stationary phase (~ 18 hours incubation at 37°C with shaking in LB Miller), as previously described (Starr *et al*., 2018). For a MOI ~ 20:1 (20 bacteria:1 macrophage), bacteria were opsonised with 15% (v/v) heat-inactivated human serum (Sigma-Aldrich, Ireland) for 30 minutes at 37°C, 5% $CO_2$. THP-1 macrophages were allowed to phagocytose *Salmonella* for 30 minutes at 37°C, 5% $CO_2$ before treatment with 100 µg/mL gentamicin for 1 hour at 37°C, 5% $CO_2$ (to kill extracellular bacteria), followed by incubation with 10 µg/mL gentamicin and 0.5, 1 and 2 µg/mL of TZ for the remainder of the experiment. VER (80 µg/mL) and LOX (25 µg/mL) were also tested in these macrophage cultures. For enumeration of bacteria, infected monolayers were solubilised in lysis buffer (0.2% (w/v) sodium deoxycholate in PBS) and serially diluted in PBS before plating on LB agar plates. Possible synergy between TZ and gentamicin was assessed to guarantee that the effect observed in macrophage cultures was due to the compound of interest alone (TZ, VER or LOX) (**Table 4.2**).

### 4.2.9. Effect of TZ on the activation of infected macrophages

Nitric Oxide (NO) and its degradation products, namely, reactive nitrogen species, contribute to the innate immune response to intracellular pathogens, such as *Salmonella*. *Salmonella* is exposed to these reactive species during its infectious cycle in the host. Products of the *Salmonella* pathogenicity island 1 type III secretion system and *Salmonella*-associated molecular patterns are able to stimulate transcription of inducible NO synthase (iNOS) by cells of the mononuclear phagocytic cell lineage. The resulting NO, or products that arise from its interactions with oxygen ($O_2$) or iron and low-molecular weight thiols, are preferentially bacteriostatic against *Salmonella*, while reaction of NO and superoxide ($O_2^-$) generates the bactericidal compound peroxynitrite ($ONOO^-$). Therefore, the quantification of NO is an indirect measure of activation of the infected macrophage.

Supernatants were collected from uninfected and *Salmonella*-infected THP-1 macrophages treated and untreated with TZ 1 µg/mL. The presence of NO was measured indirectly by assaying the presence of nitrite ($NO_2^-$) using a commercially available Griess reagent kit (Biotium inc.), according to the manufacturer's protocol.

In brief, 150 µL of supernatant was incubated with 20 µL of Griess reagent and 130 µL of deionised water for 30 minutes at room temperature in a 96-well plate. The samples were photometrically measured at a wavelength of 548$_{nm}$ using a Synergy™ HT multi-mode microplate reader. The assays were performed with at least three biological replicates.

### 4.2.10. Quantification of cytokine production in uninfected and *Salmonella*-infected THP-1 macrophages

Supernatants were collected from uninfected and *Salmonella*-infected THP-1 macrophages cultures, untreated and treated with TZ 1 µg/mL. The secretion of cytokines was measured by ELISA using the R&D systems DuoSet kit (R&D systems, Minneapolis, USA) in Nunc MaxiSorp 96-well plates (ThermoFisher, Massachusetts, USA), according to the manufacturer's instructions. Plates were washed with PBS containing 0.05% (v/v) Tween 20 (PBST), and reagent diluent containing 1% (w/v) of bovine serum albumin in PBS. Optical density was read using a Synergy™ HT multi-mode microplate reader at 450$_{nm}$ immediately after stopping the reaction of 3,3',5,5'-Tetramethylbenzidine (TMB) with 2 N sulfuric acid. Results were calculated by interpolation from a standard curve generated from the assay, and cytokine levels represented as picograms per millilitre (pg/mL).

### 4.2.11. Measurement of intracellular calcium in infected macrophages

The levels of intracellular calcium were measured using the Screen Quest™ Calbryte-520 Probenecid-Free and Wash-Free Calcium Assay Kit (Assay Genie, Dublin, Ireland) according to the manufacturer's instructions with slight modifications. Briefly, THP-1 macrophages were infected with *Salmonella* 14028S (as described in section 4.2.9) in a black, clear bottom 96-well plate. Following infection, macrophages were treated with TZ (1 µg/mL) and VER (80 µg/mL) for 18 hours [21.5 hours post infection (p.i.]. Post treatment, media was removed and Cal-520™ AM dye-loading solution was added into the cell plate and the plate incubated for 1 hour at 37℃, 5% $CO_2$. Cells were washed and Hank's Buffer Solution with HEPES (HHBS) added to the plate. The calcium flux assay was run in a Synergy™ HT multi-

mode microplate reader (excitation 490nm and emission 525nm). The assay was performed with three biological replicates.

### 4.2.12. Statistical analysis

Statistical analyses were performed using Prism Graphpad software version 8.0.2. *, **, *** and **** represent p-values of < 0.05, < 0.01, <0.001 and < 0.0001, respectively. A $p < 0.05$ was considered statistically significant and highly significant when ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. One-way ANOVA was used to compare the mean values of at least three independent samples, where there was one independent variable in the experimental procedure, allowing determination of any statistically significant difference between the samples' means. Two-way ANOVA was used to compare the mean values of at least two or more samples where there were also two or more independent variables in the experimental assay.

## 4.3. Results

### 4.3.1. Minimum Inhibitory and bactericidal concentrations (MIC/MBC)

In this study, three compounds were used: thioridazine (TZ), verapamil (VER) and loxapine (LOX). VER, previously described as a $Ca^{2+}$ channel blocker in eukaryotic cells (Machado *et al*., 2016) was used as a main comparison for the possible effect of TZ on the $Ca^{2+}$ flux of THP-1 macrophages. LOX, an antipsychotic compound presenting clinical similarity to phenothiazines, has been previously reported as having activity against *Salmonella*-infected cells but it's mode of action in infected macrophages was not investigated in detail (Singh *et al*., 2003; Yang *et al*., 2019). Due to the similarity between this compound and TZ, this compound was included on this work and studied in parallel. Accordingly, prior to commencing studies involving infections with THP-1 macrophages, the MIC and MBC for each compound against *Salmonella* Typhimurium was determined. The MIC and MBC values for these two compounds (VER and LOX) against *Salmonella* Typhimurium are presented in **Table 4.1**. The MIC and MBC of VER was 2500 µg/mL, indicating no relevant *in vitro* antibacterial activity against *Salmonella.* In contrast, LOX showed an inhibitory and bactericidal effect at 400 µg/mL.This compound had similar antibacterial activity to TZ.

**Table 4.1. Minimum inhibitory and bactericidal concentrations of TZ, VER and LOX against *Salmonella* Typhimurium 14028S.**

| Class of compound | Compounds | MIC | | MBC | |
|---|---|---|---|---|---|
| | | µg/mL | µM | µg/mL | µM |
| Phenothiazines (antipsychotic) | TZ | 200 | 491 | 200 | 491 |
| Calcium channel blocker | VER | 2500 | 5091 | 2500 | 5091 |
| Antipsychotic | LOX | 400 | 897 | 400 | 897 |

**Legend.** TZ, Thioridazine; VER, Verapamil; LOX, Loxapine; MIC, Minimum Inhibitory Concentration; MBC, Minimum Bactericidal Concentration.

### 4.3.2. Effect of TZ on the metabolism of human monocytes

Monocytes are a crucial cell type of the innate immune system. This cell type is recruited to inflamed tissues and can differentiate into macrophages that produce inflammatory mediators. Production of these inflammatory mediators is influenced by various metabolic pathways within the immune cell, and alterations within these pathways can directly impact on immune cell function (Ganeshan & Chawla, 2014). Some studies performed with antipsychotic drugs have previously showed that these drugs seem to interfere with metabolic regulation (De Hert *et al*., 2012; Gonçalves, Araújo & Martel, 2015). In order to address if TZ does or does not interfere with the general metabolism of THP-1 monocytes and consequently the immune response of the host, Biolog phenotype MicroArrays™ assays were performed. **Table 4.2** shows a summary of the PM-M panels tested. In total 367 substrates were analysed.

**Table 4.2. Summary of Panels tested using Phenotype Microarrays™ Plates**

| Phenotype MicroArray Plate | Panel Tested |
|---|---|
| PM-M1 – M4 | Carbon and Engergy Sources/Nitrogen Sources |
| PM-M5 | Ions |
| PM-M6-8 | Hormones and Metabolic Effectors |

The heatmaps obtained were plotted on a scale from 1.5 to -1.5, where a positive and negative values correspond to increased or decreased use of the substrates by the cells, when in the presence of TZ. No breakpoint was considered for this experiment as, for some plates only one independent experiment was performed. Results for PM-M1 and M5-M7 correspond to two independent experiments, while PM-M2-M4 and -M8 correspond to one independent experiment.

As shown in **Figure 4.1**, the presence of TZ does not potentiate any effect on the utilisation of carbon and energy sources compared with untreated THP-1 monocytes (drug-free control). In the presence of the substrate D-Sorbitol there was a slight difference (**Figure 4.1**), but this was not significantly relevant (0.02 for untreated *versus* -0.145 reduction of tetrazolium for cells exposed to TZ).

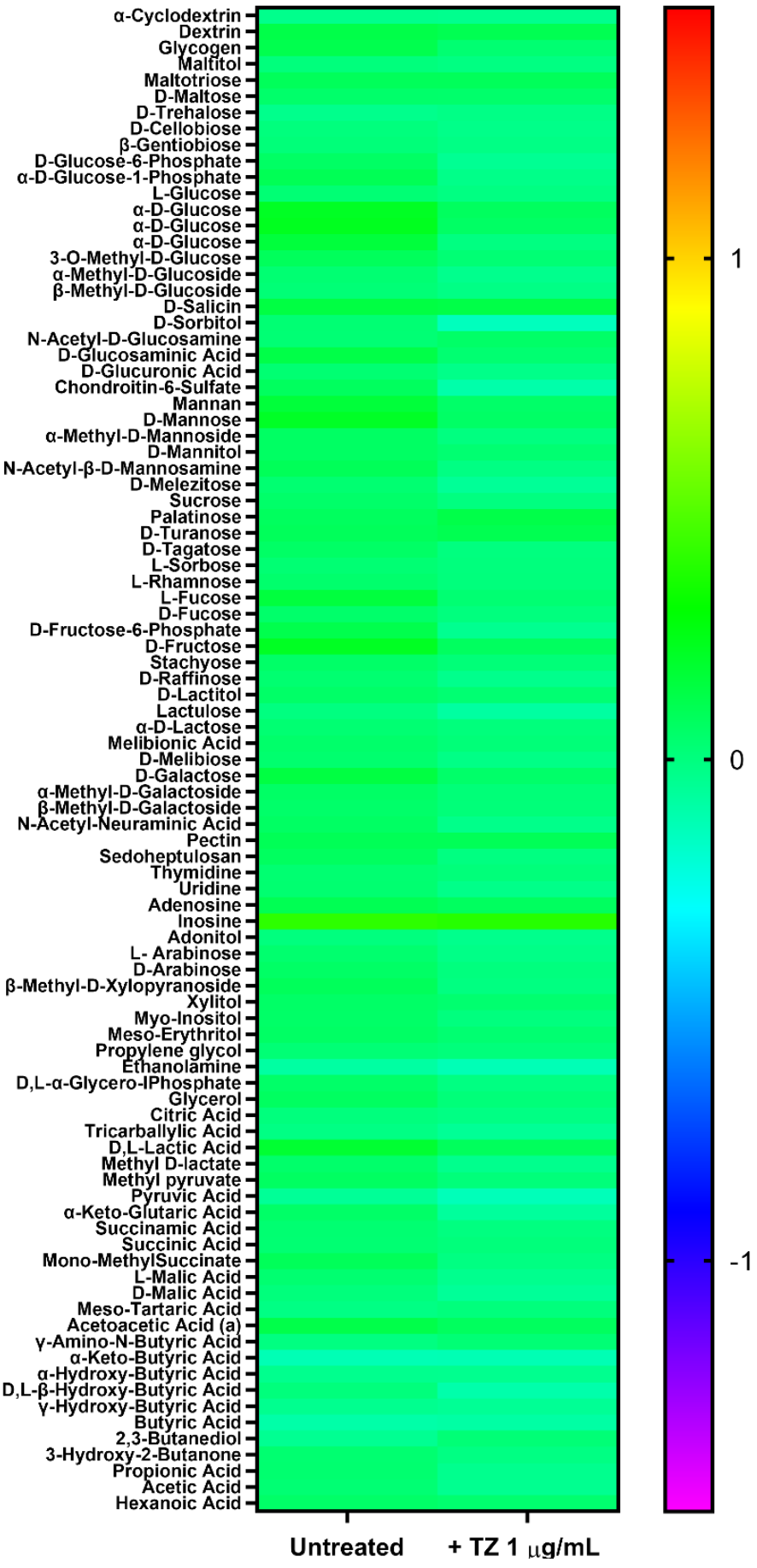

α-Cyclodextrin
Dextrin
Glycogen
Maltitol
Maltotriose
D-Maltose
D-Trehalose
D-Cellobiose
β-Gentiobiose
D-Glucose-6-Phosphate
α-D-Glucose-1-Phosphate
L-Glucose
α-D-Glucose
α-D-Glucose
α-D-Glucose
3-O-Methyl-D-Glucose
α-Methyl-D-Glucoside
β-Methyl-D-Glucoside
D-Salicin
D-Sorbitol
N-Acetyl-D-Glucosamine
D-Glucosaminic Acid
D-Glucuronic Acid
Chondroitin-6-Sulfate
Mannan
D-Mannose
α-Methyl-D-Mannoside
D-Mannitol
N-Acetyl-β-D-Mannosamine
D-Melezitose
Sucrose
Palatinose
D-Turanose
D-Tagatose
L-Sorbose
L-Rhamnose
L-Fucose
D-Fucose
D-Fructose-6-Phosphate
D-Fructose
Stachyose
D-Raffinose
D-Lactitol
Lactulose
α-D-Lactose
Melibionic Acid
D-Melibiose
D-Galactose
α-Methyl-D-Galactoside
β-Methyl-D-Galactoside
N-Acetyl-Neuraminic Acid
Pectin
Sedoheptulosan
Thymidine
Uridine
Adenosine
Inosine
Adonitol
L- Arabinose
D-Arabinose
β-Methyl-D-Xylopyranoside
Xylitol
Myo-Inositol
Meso-Erythritol
Propylene glycol
Ethanolamine
D,L-α-Glycero-lPhosphate
Glycerol
Citric Acid
Tricarballylic Acid
D,L-Lactic Acid
Methyl D-lactate
Methyl pyruvate
Pyruvic Acid
α-Keto-Glutaric Acid
Succinamic Acid
Succinic Acid
Mono-MethylSuccinate
L-Malic Acid
D-Malic Acid
Meso-Tartaric Acid
Acetoacetic Acid (a)
γ-Amino-N-Butyric Acid
α-Keto-Butyric Acid
α-Hydroxy-Butyric Acid
D,L-β-Hydroxy-Butyric Acid
γ-Hydroxy-Butyric Acid
Butyric Acid
2,3-Butanediol
3-Hydroxy-2-Butanone
Propionic Acid
Acetic Acid
Hexanoic Acid
1
0
-1
Untreated
+ TZ 1 μg/mL

**Figure 4.1. Effect of TZ on human THP-1 monocytes in the presence of different carbon and energy sources/nitrogen sources.** Heat maps comparing substrate utilisation by THP-1 monocytes (in the presence and absence of TZ) on plates PM-M1. THP-1 monocytes were dispensed into PM panel M1 (20,000 cells per well) in RPM1-1640 media lacking phenol red and glucose supplemented with FBS (5%) and reduced levels of glutamine (0.3 mM). Plates were incubated at 37°C, 5% $CO_2$ for 24 hours. After this incubation, TZ and Biolog Redox dye Mix MA were added to achieve 1x final concentration and cells were incubated for an additional 24 hours at 37°C, 5% $CO_2$. End point measurements were taken by measuring the reduction of the tetrazolium. dye. This was done by measuring the optical density at 590$_{nm}$.The y-axes correspond to the substrates in the wells of the 96-well plates (PMM plates). α-D-Glucose was used as a positive control. TZ; Thioridazine.

There seems to be a difference regarding the use of tween (20, 40 and 80) by the monocytes, when in the presence of TZ (**Figure 4.2**; below). Tween (also known as polysorbate) is a hydrophilic non-ionic surfactant that is usually used in formulations of pharmaceutical products. It can act as an emulsifier and also as an excipient to stabilise aqueous formulations of medications for parenteral administration or vaccinations. For example, patent (WO2003002153A1; Composition and method for reducing adverse interactions between phenothiazine derivatives and plasma using cyclodextrins) refers to the use of polysorbate to improve solubility of the phenothiazine promethazine when used in injectable formulations. Therefore, it is possible that some chemical interaction with thioridazine is taking place and that interaction can impact on the use of this substrate by the monocytes.

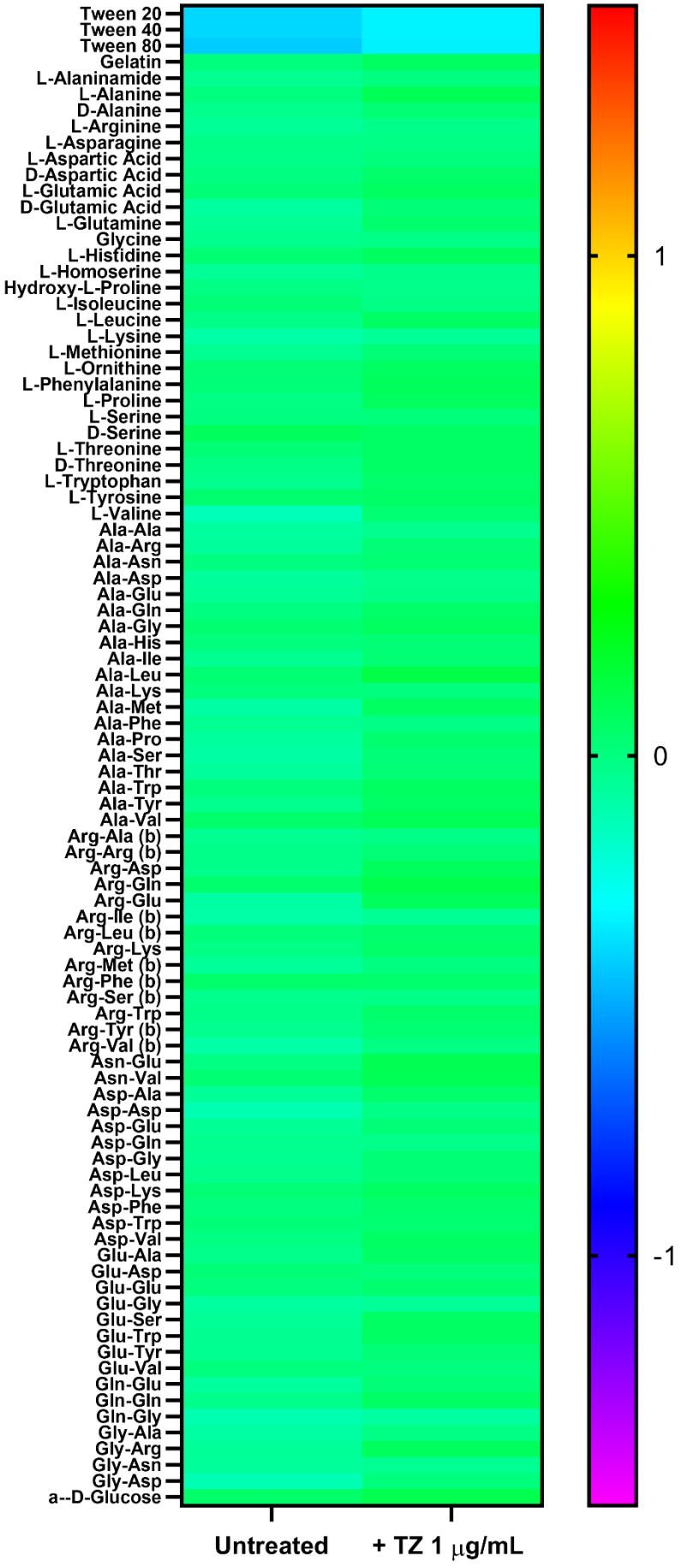

Tween 20
Tween 40
Tween 80
Gelatin
L-Alaninamide
L-Alanine
D-Alanine
L-Arginine
L-Asparagine
L-Aspartic Acid
D-Aspartic Acid
L-Glutamic Acid
D-Glutamic Acid
L-Glutamine
Glycine
L-Histidine
L-Homoserine
Hydroxy-L-Proline
L-Isoleucine
L-Leucine
L-Lysine
L-Methionine
L-Ornithine
L-Phenylalanine
L-Proline
L-Serine
D-Serine
L-Threonine
D-Threonine
L-Tryptophan
L-Tyrosine
L-Valine
Ala-Ala
Ala-Arg
Ala-Asn
Ala-Asp
Ala-Glu
Ala-Gln
Ala-Gly
Ala-His
Ala-Ile
Ala-Leu
Ala-Lys
Ala-Met
Ala-Phe
Ala-Pro
Ala-Ser
Ala-Thr
Ala-Trp
Ala-Tyr
Ala-Val
Arg-Ala (b)
Arg-Arg (b)
Arg-Asp
Arg-Gln
Arg-Glu
Arg-Ile (b)
Arg-Leu (b)
Arg-Lys
Arg-Met (b)
Arg-Phe (b)
Arg-Ser (b)
Arg-Trp
Arg-Tyr (b)
Arg-Val (b)
Asn-Glu
Asn-Val
Asp-Ala
Asp-Asp
Asp-Glu
Asp-Gln
Asp-Gly
Asp-Leu
Asp-Lys
Asp-Phe
Asp-Trp
Asp-Val
Glu-Ala
Glu-Asp
Glu-Glu
Glu-Gly
Glu-Ser
Glu-Trp
Glu-Tyr
Glu-Val
Gln-Glu
Gln-Gln
Gln-Gly
Gly-Ala
Gly-Arg
Gly-Asn
Gly-Asp
a--D-Glucose
1
0
-1
Untreated
+ TZ 1 μg/mL

**Figure 4.2. Effect of TZ in human THP-1 monocytes in the presence of different carbon and energy sources/nitrogen sources.** Heat maps comparing substrate utilisation by THP-1 monocytes (in the presence and absence of TZ) on plates PM-M2. THP-1 monocytes were dispensed into PM panel M2 (20,000 cells per well) in RPM1-1640 media lacking phenol red and glucose and these were supplemented with FBS (5%) and reduced levels of glutamine (0.3 mM). Plates were incubated at 37°C, 5% $CO_2$ for 24 hours. After this incubation, TZ and Biolog Redox dye Mix MA were added to achieve 1x final concentration and incubated for additional 24 hours at 37°C, 5% $CO_2$. End point measurements were taken by measuring the reduction of the dye, tetrazolium. This was done by measuring the optical density at 590 nm. The y-axes correspond to the substrates in the wells of the 96-well plates (PMM plates). α-D-Glucose was use as a positive control. TZ; Thioridazine.

As previously discussed, the presence of TZ does not seem to potentiate any effect on the utilisation of carbon and energy sources compared with untreated THP-1 monocytes (drug-free control). (**Figures 4.3. and 4.4**). However, it should be noted some differences in the biological replicates on PM-1, PM5-M7 but those were not significant. A third replicate would be is required to confirm these potential differences.

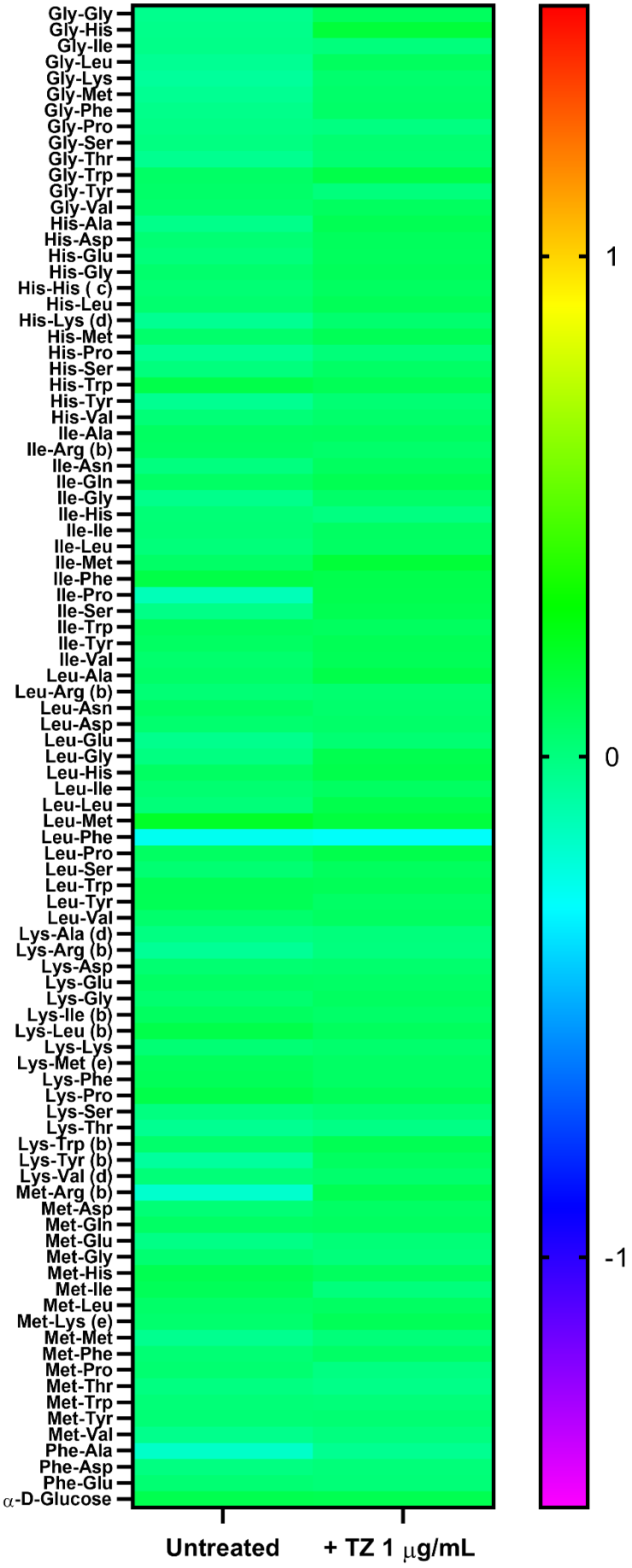

Gly-Gly
Gly-His
Gly-Ile
Gly-Leu
Gly-Lys
Gly-Met
Gly-Phe
Gly-Pro
Gly-Ser
Gly-Thr
Gly-Trp
Gly-Tyr
Gly-Val
His-Ala
His-Asp
His-Glu
His-Gly
His-His ( c)
His-Leu
His-Lys (d)
His-Met
His-Pro
His-Ser
His-Trp
His-Tyr
His-Val
Ile-Ala
Ile-Arg (b)
Ile-Asn
Ile-Gln
Ile-Gly
Ile-His
Ile-Ile
Ile-Leu
Ile-Met
Ile-Phe
Ile-Pro
Ile-Ser
Ile-Trp
Ile-Tyr
Ile-Val
Leu-Ala
Leu-Arg (b)
Leu-Asn
Leu-Asp
Leu-Glu
Leu-Gly
Leu-His
Leu-Ile
Leu-Leu
Leu-Met
Leu-Phe
Leu-Pro
Leu-Ser
Leu-Trp
Leu-Tyr
Leu-Val
Lys-Ala (d)
Lys-Arg (b)
Lys-Asp
Lys-Glu
Lys-Gly
Lys-Ile (b)
Lys-Leu (b)
Lys-Lys
Lys-Met (e)
Lys-Phe
Lys-Pro
Lys-Ser
Lys-Thr
Lys-Trp (b)
Lys-Tyr (b)
Lys-Val (d)
Met-Arg (b)
Met-Asp
Met-Gln
Met-Glu
Met-Gly
Met-His
Met-Ile
Met-Leu
Met-Lys (e)
Met-Met
Met-Phe
Met-Pro
Met-Thr
Met-Trp
Met-Tyr
Met-Val
Phe-Ala
Phe-Asp
Phe-Glu
α-D-Glucose
1
0
-1
Untreated
+ TZ 1 μg/mL

**Figure 4.3. Effect of TZ on human THP-1 monocytes in the presence of different carbon and energy sources/nitrogen sources.** Heat maps for comparing substrate utilisation by THP-1 monocytes (in the presence and absence of TZ) on plates PM-M3. THP-1 monocytes were dispensed into PM panel M3 (20,000 cells per well) in RPM1-1640 media lacking phenol red and glucose and these were supplemented with FBS (5%) and reduced levels of glutamine (0.3 mM). Plates were incubated at 37°C, 5% $CO_2$ for 24 hours. After this incubation, TZ and Biolog Redox dye Mix MA were added to achieve 1x final concentration and cells incubated for additional 24 hours at 37°C, 5% $CO_2$. End point measurements were taken by measuring the reduction of the tetrazolium dye, tetrazolium. This was done by measuring the optical density at $590_{nm}$.The y-axes correspond to the substrates in the wells of the 96-well plates (PMM plates). α-D-Glucose was use as a positive control. TZ; Thioridazine.

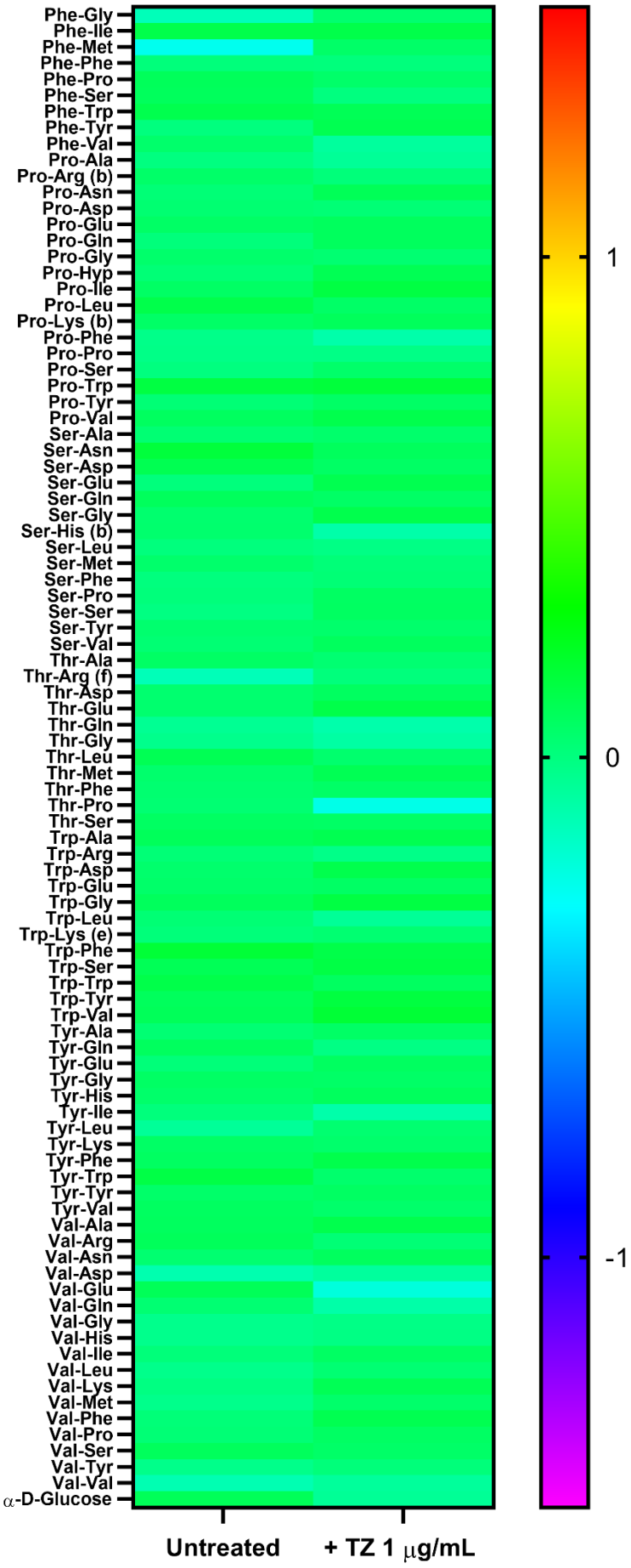

Phe-Gly
Phe-Ile
Phe-Met
Phe-Phe
Phe-Pro
Phe-Ser
Phe-Trp
Phe-Tyr
Phe-Val
Pro-Ala
Pro-Arg (b)
Pro-Asn
Pro-Asp
Pro-Glu
Pro-Gln
Pro-Gly
Pro-Hyp
Pro-Ile
Pro-Leu
Pro-Lys (b)
Pro-Phe
Pro-Pro
Pro-Ser
Pro-Trp
Pro-Tyr
Pro-Val
Ser-Ala
Ser-Asn
Ser-Asp
Ser-Glu
Ser-Gln
Ser-Gly
Ser-His (b)
Ser-Leu
Ser-Met
Ser-Phe
Ser-Pro
Ser-Ser
Ser-Tyr
Ser-Val
Thr-Ala
Thr-Arg (f)
Thr-Asp
Thr-Glu
Thr-Gln
Thr-Gly
Thr-Leu
Thr-Met
Thr-Phe
Thr-Pro
Thr-Ser
Trp-Ala
Trp-Arg
Trp-Asp
Trp-Glu
Trp-Gly
Trp-Leu
Trp-Lys (e)
Trp-Phe
Trp-Ser
Trp-Trp
Trp-Tyr
Trp-Val
Tyr-Ala
Tyr-Gln
Tyr-Glu
Tyr-Gly
Tyr-His
Tyr-Ile
Tyr-Leu
Tyr-Lys
Tyr-Phe
Tyr-Trp
Tyr-Tyr
Tyr-Val
Val-Ala
Val-Arg
Val-Asn
Val-Asp
Val-Glu
Val-Gln
Val-Gly
Val-His
Val-Ile
Val-Leu
Val-Lys
Val-Met
Val-Phe
Val-Pro
Val-Ser
Val-Tyr
Val-Val
α-D-Glucose
1
0
-1
Untreated
+ TZ 1 μg/mL

**Figure 4.4. Effect of TZ on human THP-1 monocytes in the presence of different carbon and energy sources/nitrogen sources.** Heat maps for comparing substrate utilisation by THP-1 monocytes (in the presence and absence of TZ) on plates PM-M4. THP-1 monocytes were dispensed into PM panel M4 (20,000 cells per well) in RPM1-1640 media lacking phenol red and glucose; supplemented with FBS (5%) and reduced levels of glutamine (0.3 mM). Plates were incubated at 37°C, 5% $CO_2$ for 24 hours. After this incubation, TZ and Biolog Redox dye Mix MA were added to achieve 1x final concentration and incubated for additional 24 hours at 37°C, 5% $CO_2$. End point measurements were taken by measuring the reduction of the dye, tetrazolium. This was done by measuring the optical density at 590$_{nm}$.The y-axes correspond to the substrates in the wells of the 96-well plates (PMM plates). α-D-Glucose was used as a positive control. TZ; Thioridazine.

Regarding the use of different ions by THP-1 monocytes when these are in the presence of TZ, no significant differences were obtained (**Figure 4.5**).

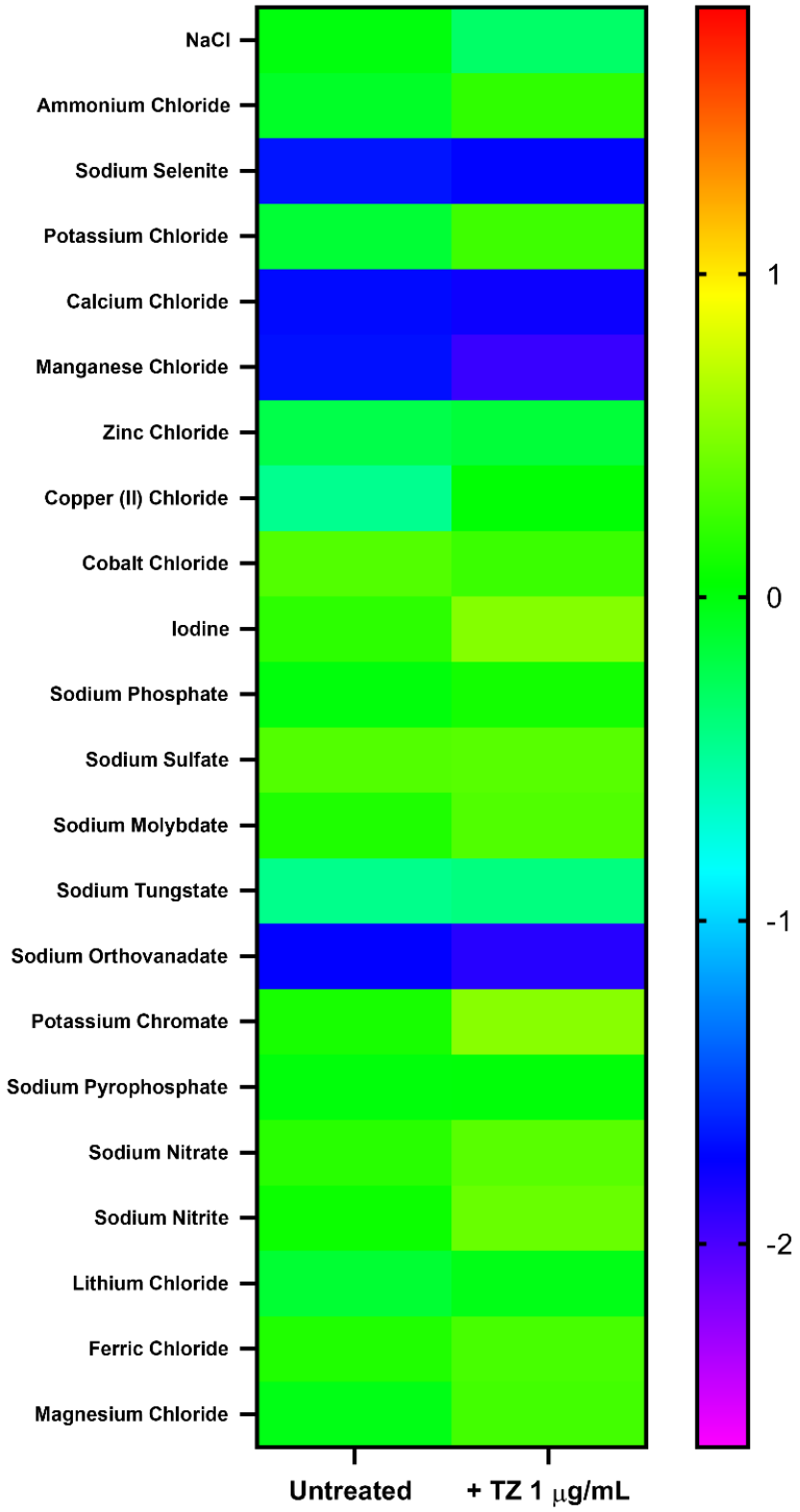



**Figure 4.5. Effect of TZ on the metabolism of human THP-1 monocytes in the presence of different ions.** Heat maps for comparing substrate utilisation by

THP-1 monocytes (in the presence and absence of TZ) on plates PM-M5. THP-1 monocytes were dispensed into PM panel M5 (20, 000 cells per well) in RPMI-1640 media lacking phenol red and glucose and incubated with TZ at 37°C, 5% $CO_2$ for 24 hours. Biolog Redox dye Mix A was then added to the plates to achieve 1x final concentration. Tetrazolium reduction was measured after an additional 24 hours. End point measurements were taken by measuring the reduction of the dye, tetrazolium. This was done by measuring the optical density at 590$_{nm}$. The y-axes correspond to the substrates in the wells of the 96-well plates (PMM plates). The results presented correspond to the average of two independent experiments. TZ; Thioridazine.

Thioridazine seems to interfere slightly with the use of creatine, progesterone and beta-estradiol by THP-1 monocytes (**Figure 4.6**). Creatine is an organic compound found in vertebrates and known to facilitate recycling of ATP, mainly in the muscles and brain tissue. This "recycling" process can be achieved by conversion of ADP into ATP via donation of phosphate groups (Bredahl *et al*., 2021)(Bredahl *et al*., 2021). Based on the *in vitro* results from the previous chapter, TZ interferes with the production of ATP in *Salmonella*, therefore this result would not be totally surprising, even if in eukaryotic cells. However, it should be kept in mind that this effect is concentration dependent, so concentrations of TZ that affect human monocytes may be quite different from the ones that are required *in vitro* to impact on *Salmonella*. Regarding progesterone, this hormone regulates the growth and development of human tissues, including the reproductive system and breasts. Receptors for this hormone are important indicators for the clinical prognosis of breast cancer as well as other various reproductive cancers. Several studies have reported the selective activity of TZ against tumour cells, namely, inhibition of human breast cancer proliferation (Strobl *et al*., 1990; Sachlos *et al*., 2012).

β-estradiol, more especifically 17 β-estradiol has a direct role in the modulation of monocyte and macrophage immune function. β-estradiol signals are transduced through estrogen receptors (Murphy *et al*., 2009). We cannot disregard that TZ has the ability to interfere with some of these hormones and its receptors. That was also observed with the metabolism of glucagon, a peptide hormone that is produced by alpha cells of the pancreas (**Figure 4.7**). However, for these observations to be confirmed, additional replicates would have to be performed to ensure significance of these results.

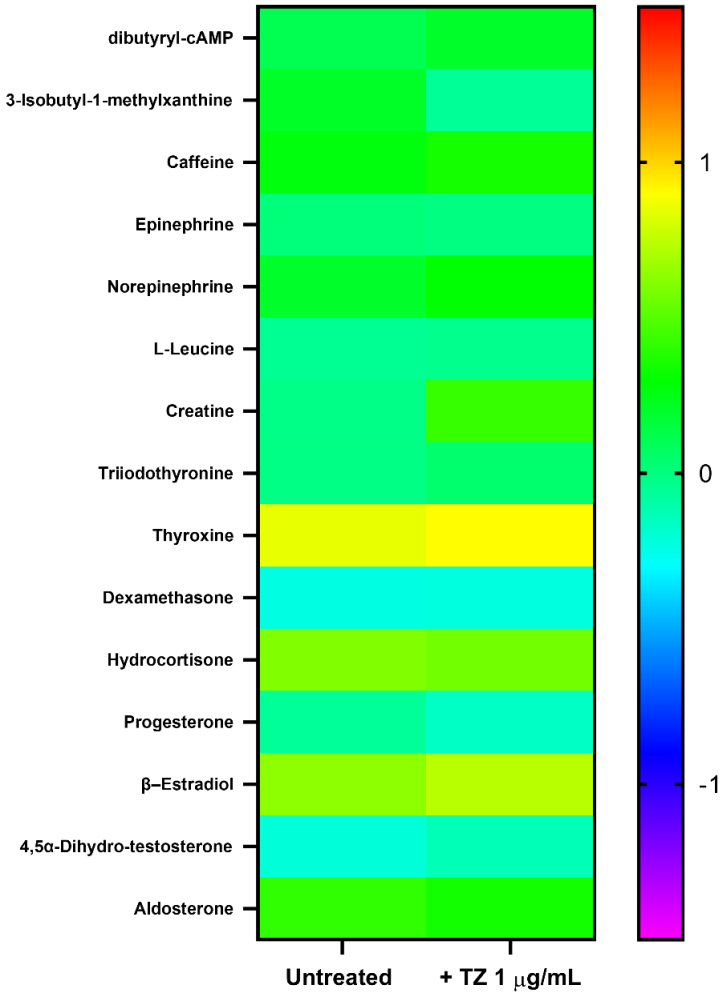



**Figure 4.6. Effect of TZ on the metabolism of hormones and metabolic effectors by human THP-1 monocytes.** Heat maps for comparing substrate utilisation by THP-1 monocytes (in the presence and absence of TZ) on plates PM-M6. THP-1 monocytes were dispensed into PM panel M6 (20, 000 cells per well) in RPMI-1640 media lacking phenol red and glucose and incubated with TZ at 37°C, 5% $CO_2$ for 24 hours. Biolog Redox dye Mix A was then added to achieve 1x final concentration. Tetrazolium reduction was measured after an additional 24 hours. End point measurements were taken by measuring the reduction of the dye, tetrazolium. This was done by measuring optical density values at 590 nm. The y-axes correspond to the substrates in the wells of the 96-well plates (PMM plates). The results presented correspond to the average of two independent experiments. TZ; Thioridazine.

In the presence of TZ, there were slight differences (not significant) on the metabolism of the THP-1 monocytes in the presence of the interleukins, IL-2, Il-6 and IL-8 (**Figure 4.7**). IL-2 is secreted by T-cells and it able to induce monocyte functions, including tumor cytotoxicity, $H_2O_2$ production, and release of tumour necrosis factor alpha and IL-1 beta (Scheibenbogen *et al*., 1992). It is known that human monocytes, when activated with LPS, exhibited rapid expression of mRNA for IL-1 beta, TNF-alpha, and IL-8, which is usually followed by IL-6 (Agarwal *et al*., 1995). Therefore, these cytokines are important markers of infection. When assessing the potential of using TZ on human monocytes it is important to guarantee that the compound does not interfere with the normal production of cytokines by these cells. That doesn't seem to be the case, but more replicates would have to be performed to fully support this statement.

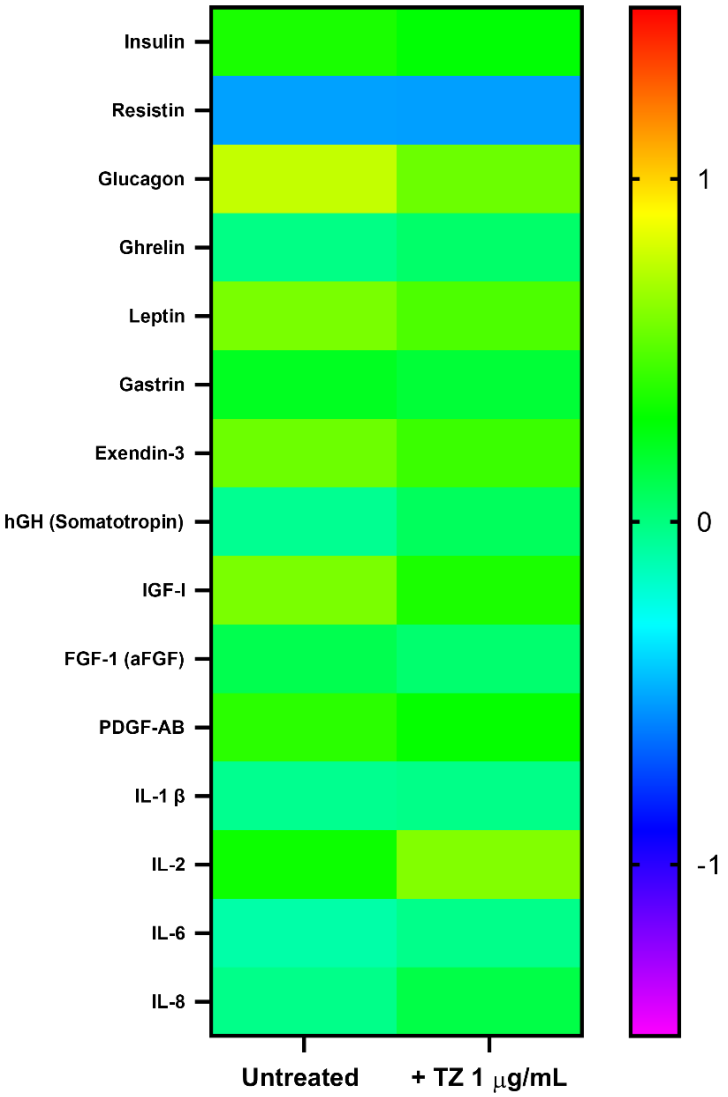



**Figure 4.7. Effect of TZ on the metabolism of human THP-1 monocytes in the presence of hormones and metabolic effectors.** Heat maps for comparing substrate utilisation by THP-1 monocytes (in the presence and absence of TZ) on plates PM-M7. THP-1 monocytes were dispensed into PM panel M7 (20, 000 cells per well) in RPMI-1640 media lacking phenol red and glucose and incubated with TZ at 37°C, 5% $CO_2$ for 24 hours. Biolog Redox dye Mix A was then added to achieve 1x final concentration. Tetrazolium reduction was measured after an additional 24 hours. End point measurements were taken by measuring the reduction of the dye, tetrazolium. This was done by measuring the optical density at 590nm. The y-axes correspond to the substrate in the wells of the 96-well plates (PMM plates). The results presented correspond to the average of two independent experiments. TZ; Thioridazine.

In the presence of TZ, a small increase in the reduction of tetrazolium was observed for adenosine (**Figure 4.8**). This result as previously discussed can be concordant with previous *in vitro* data where TZ was shown to interfere with the production of ATP. However, this difference was not significant. All together, these results indicate that TZ does not appear to alter the metabolism of uninfected THP-1 monocytes.

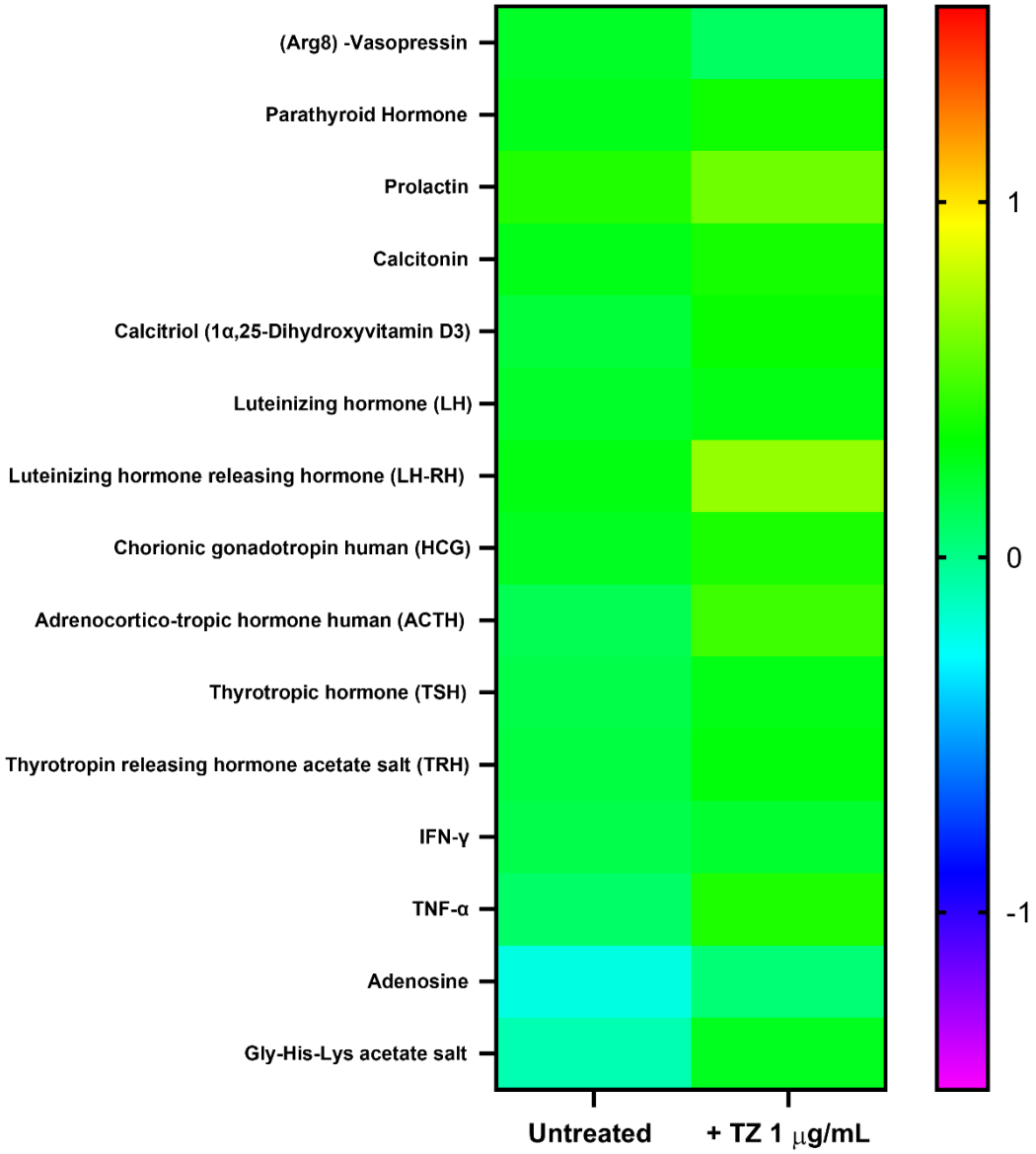



**Figure 4.8. Effect of TZ on the metabolism of human THP-1 monocytes in the presence of hormones and metabolic effectors.** Heat maps for comparing substrate utilisation by THP-1 monocytes (in the presence and absence of TZ) on plates PM-M8. THP-1 monocytes were dispensed into PM panel M8 (20, 000 cells per well) in RPMI-1640 media lacking phenol red and glucose and incubated with TZ at 37°C, 5% $CO_2$ for 24 hours. Biolog Redox dye Mix A was then added to achieve 1x final concentration. Tetrazolium reduction was measured after an additional 24 hours. End point measurements were taken by measuring the reduction of the tetrazolium dye. This was done by measuring the optical density at 590nm. The y-axes correspond to the substrate in the wells of the 96-well plates (PMM plates). TZ; Thioridazine.

### 4.3.3. Effect of TZ on the production of cytokines by uninfected THP-1 macrophages

As shown in section 4.3.4, no major differences were observed on the metabolism of THP-1 monocytes when these cells were exposed to TZ compared with unexposed THP-1 monocytes. Monocytes migrate from the bloodstream into tissues in response to inflammation, differentiating into macrophages. Macrophages are considered professional phagocytes, which engulf pathogens invading the body (Hirayama, Iida & Nakase, 2018). Besides being a phagocytic cell, macrophages release cytokines and chemokines as a major component of the innate immune response in response to danger signals or pathogens (Hume, 2006).

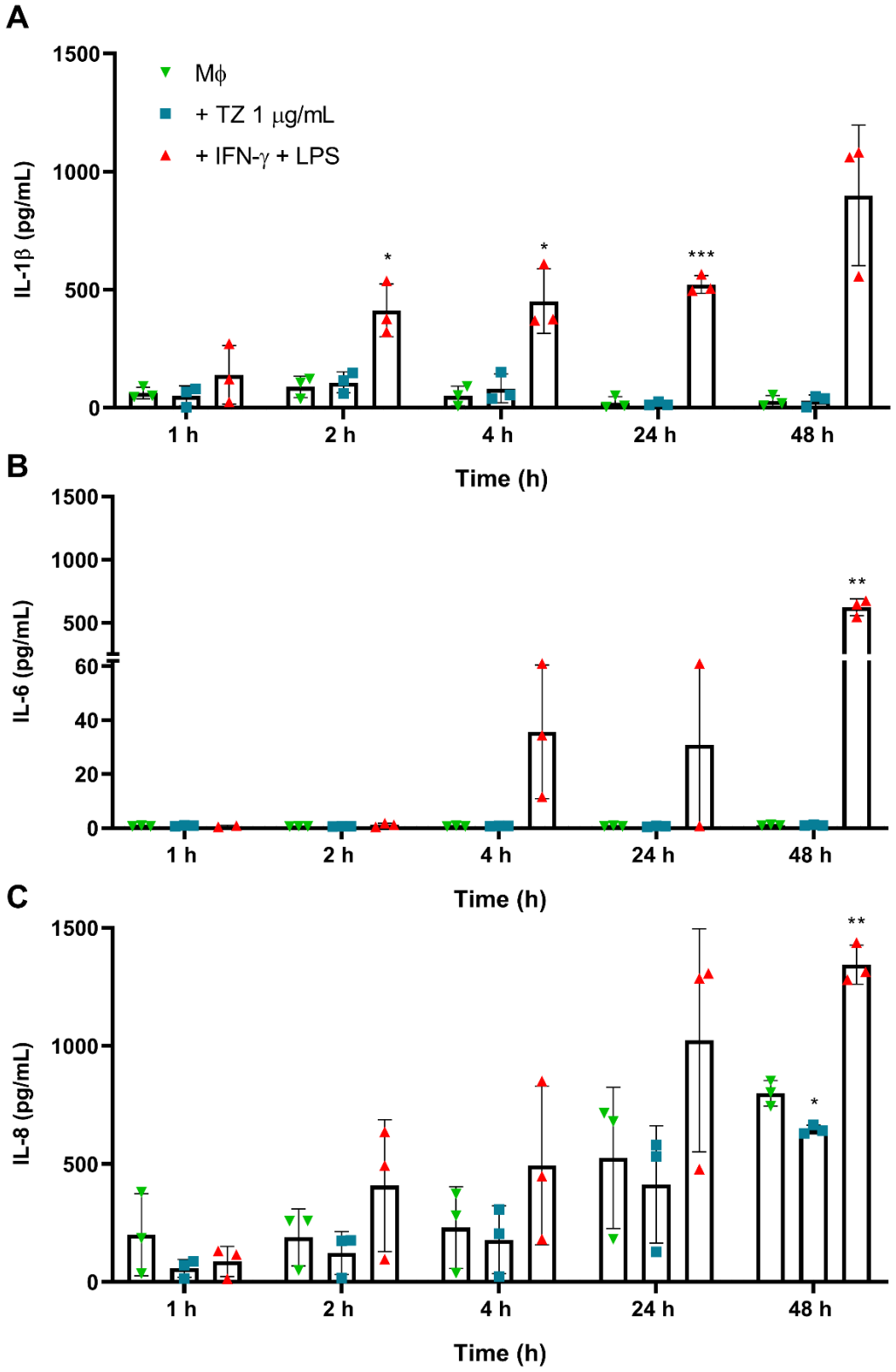
To further investigate if TZ had any effect on the immune response produced by the phagocytic cells, the cytokine response of uninfected, differentiated THP-1 monocytes ("THP-1 macrophages") was assessed in the presence of TZ (1 µg/mL).

TZ-treated THP-1 macrophages were compared with cells stimulated with IFN-γ and LPS (positive control) and untreated, i.e. resting-state macrophages. Based on the previous results obtained on the phenotypic MicroArrays™ plates, the secretion of a range of Interleukins (IL), (IL-1β, -6, -8, -10, -12 and tumour necrosis factor (TNF)-α) by THP-1 macrophages were tested at different time-points by ELISA. Macrophages exposed to IFN-γ or LPS were also individually tested. However, for simplicity and since in the presence of IFN-γ or LPS no significant differences were obtained, these were not included in the figures. As expected, non-activated THP-1 macrophages showed no secretion of any of the cytokines measured. These results were expected since there were no stimuli to activate the macrophages and produce an immune response to protect the host. When IFN- γ and LPS are present, a significant increase in secretion of IL-1β, IL-6, IL-8 and TNF-α was observed (**Figure 4.9** and **Figure 4.10**). IL-1β secretion was significantly increased at 2, 4 and 24h. IL-6 and IL-8 showed a significant increase at 48 h and TNF-α at 24h and 48h.

As seen in **Figures 4.9** and **4.10**, when TZ was added to THP-1 macrophages, no cytokine response was obtained at different timepoints (1, 2, 4, 24, and 48 hours) except for IL-8 and TNF- α. A significant decrease in IL-8 (644.794 pg/mL) was observed at 48h of exposure when compared with THP-1 macrophages with no stimuli (798.869 pg/mL). At 2h of exposure, TNF-α had a significant decreased (1.33

pg/mL) compared with the THP-1 macrophages with no stimuli (2.83 pg/mL). Regarding TNF-α, no significant changes were observed throughout the rest of the timepoints measured. This is concordant with what was observed for THP-1 monocytes in the Biolog phenotype MicroArrays™ (section 4.3.2), where only a small increase in TNF-αbut this difference was not significant.

These results indicate that, in general, TZ does not affect general immunological responses in THP-1 macrophages; therefore, further studies examining bacterial survival in infected THP-1 macrophages were pursued.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IL-1β (pg/mL)
Mϕ
+ TZ 1 μg/mL
+ IFN-γ + LPS
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*
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24 h
48 h
Time (h)
B
1500
1000
500
60
40
20
0
IL-6 (pg/mL)
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1 h
2 h
4 h
24 h
48 h
Time (h)
C
1500
1000
500
0
IL-8 (pg/mL)
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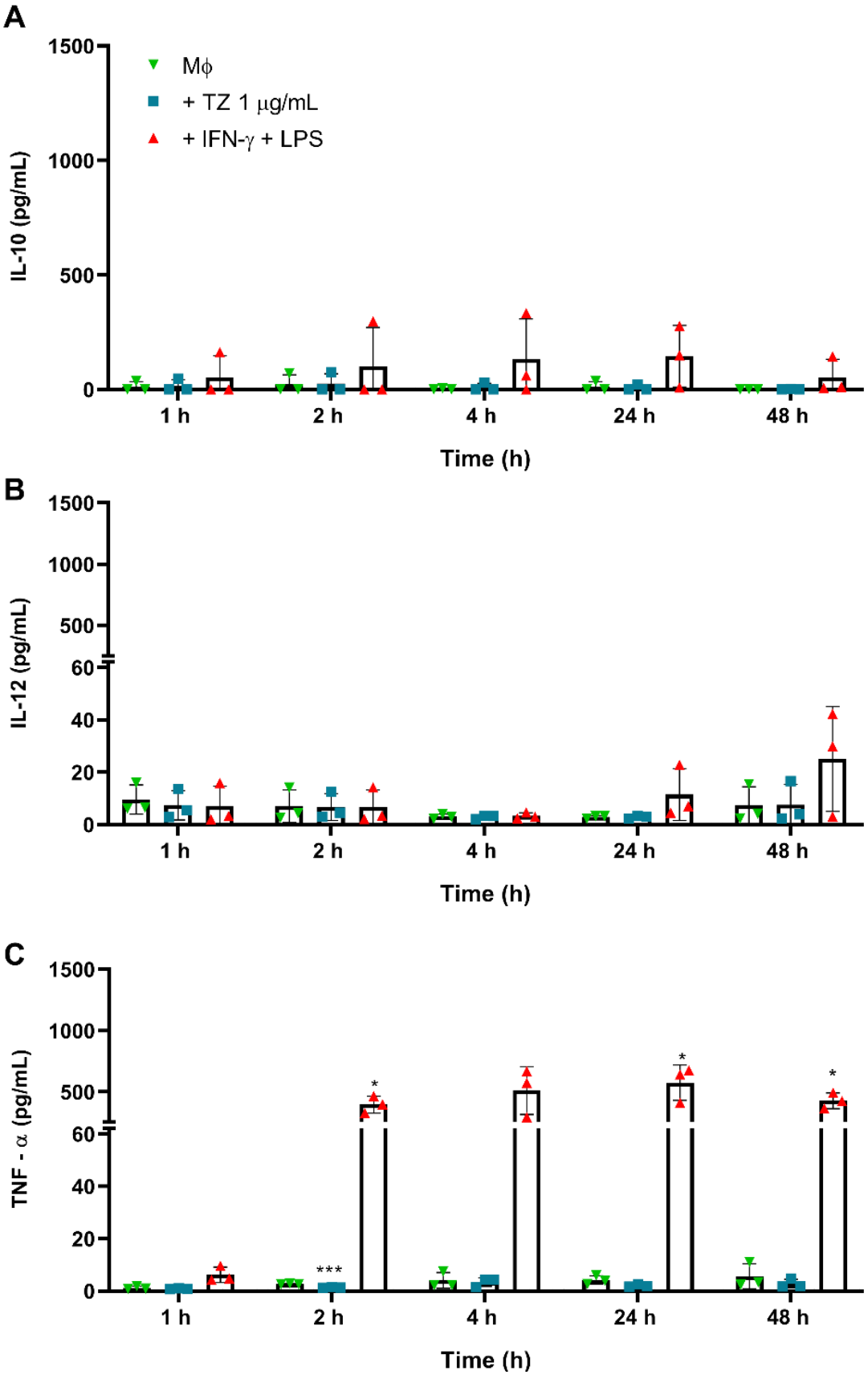
*
1 h
2 h
4 h
24 h
48 h
Time (h)

**Figure 4.9. TZ does not induce a cytokine response in THP-1 macrophages.** Differentiated macrophages were incubated with TZ (1 µg/mL), IFN-γ + LPS (100 ng/mL + 50 ng/mL, respectively) for 1, 2, 4, 24 and 48 hours at 37°C, 5% $CO_2$. Levels of (A) IL-1β, (B) IL-6, (C) IL-8 in the supernatants of cultures of uninfected THP-1 macrophages treated with TZ were measured by ELISA. The results presented correspond to the average of three independent assays ± standard deviation (SD); IFN, interferon; IL, interleukins; LPS, Lipopolysaccharides; Mϕ, Macrophages M0; TZ, Thioridazine. * and ** indicate $P < 0.05$ and $P < 0.01$, respectively (two-way ANOVA multiple comparisons, comparing with Mϕ (control)).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IL-10 (pg/mL)
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+ TZ 1 μg/mL
+ IFN-γ + LPS
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1 h
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4 h
24 h
48 h
Time (h)

**Figure 4.10. TZ does not induce a cytokine response in human THP-1 macrophages.** Differentiated macrophages were incubated with TZ 1 µg/mL, IFN-γ + LPS (100 ng/mL + 50 ng/mL, respectively) for 1, 2, 4, 24 and 48 hours at 37°C, 5% $CO_2$. Levels of (**A**) IL-10, (**B**) IL-12, (**C**) TNF-α in the supernatants of cultures of uninfected THP-1 macrophages treated with TZ, were measured by ELISA. The results presented correspond to the average of three independent assays ± standard deviation (SD); IFN, interferon; IL, interleukins; LPS, Lipopolysaccharides; Mϕ, Macrophages M0; TNF, tumour necrosis factor; TZ, Thioridazine. * and *** indicates $P < 0.05$ and $P < 0.001$, respectively (two-way ANOVA multiple comparisons, comparing with Mϕ (control)).

### 4.3.4. Effect of TZ on the apoptosis of human macrophages

Previous results in Chapter 3 (section 3.3.13) demonstrated that TZ had no effect on the viability of monocytes at concentrations below 5 µg/mL. While cell viability assays such as 3-(4,5-dimethylthiazol-2-yl)-5-(3-caarboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) provide a readout of the overall health of cells in a sample through measurement of metabolic activity, they do not distinguish between healthy, dividing cells and non-dividing cells, that may be undergoing cell death, e.g. *via* apoptosis. Apoptosis is a form of programmed cell death which is necessary to ensure that healthy cells maintain proper growth, development, and homeostasis. Apoptosis can also occur in response to cellular stress. Apoptosis markers can also be used to assess and confirm cell viability.

To confirm that TZ did not contribute to THP-1 macrophages apoptosis, the activity of caspase-3/7 was examined in THP-1 macrophages in the presence or absence of TZ (1 µg/mL) and IFN-γ (100 ng/mL) plus LPS (50 ng/mL; positive control for macrophage activation). Caspases are enzymes that are typically inactive zymogens which when activated initiate apoptosis. Therefore, detection of activated caspase-3 and -7 is considered a reliable marker for cells undergoing apoptosis (McIlwain, Berger & Mak, 2013).

As shown in **Figure 4.11**, addition of TZ (1 µg/mL) to THP-1 macrophages had no effect on activation of caspases-3/7. This result correlates with the cell viability assay performed in Chapter 3 (section 3.3.13 - **Figure 3.16**) with THP-1 monocytes and whereno decrease in cell viability was obtained in the presence of TZ, at the same concentration.

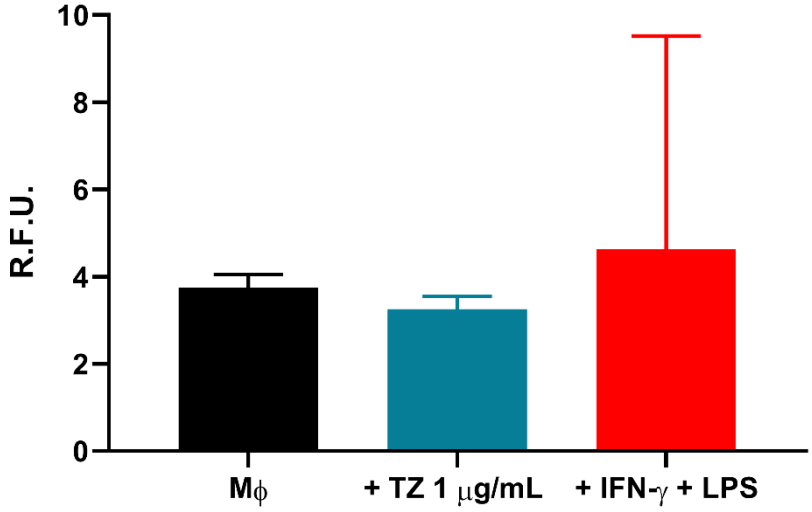


**Figure 4.11. TZ does not induce apoptosis in THP-1 macrophages.** Caspase-3/7 activity was measured using FluoFire Caspase-3/7 fluorescent assay. The results presented correspond to the average of two independent assays ± standard deviation (SD); Mϕ, Macrophages M0; TZ, Thioridazine; IFN, interferon; LPS, Lipopolysaccharides.

### 4.3.5. Effect of TZ on the intracellular survival of *Salmonella* in infected THP-1 macrophages

To assess the effect of TZ on the intracellular survival of *Salmonella* phagocytosed by human macrophages, infection assays were conducted in THP-1 macrophages (PMA 20 ng/ml, 5 days) according to the same protocol used by (Starr *et al*., 2018).Prior to commencing experiments, the MIC of TZ against *Salmonella* was determined using RPMI-1640 media (**Table 4.2**), to verify that the different media did not impact on the MIC of TZ. No difference in MIC value was observed. Additionally, potential synergy between TZ and gentamicin (used in the infection assay to kill extracellular bacteria) was evaluated to guarantee that any reduction in recoverable CFUs following TZ treatment was not due to synergy between TZ and gentamicin. The MIC values obtained indicated no synergy between the two compounds (**Table 4.3**).

**Table 4.3. Antibacterial activity of Gentamicin and TZ in different media assays and synergistic effects**

| | MIC (µg/mL) | | |
|---|---|---|---|
| **Assay media** | **Gentamicin** | **TZ** | **Gent. (+ TZ)** |
| **LB Miller Broth** | 3.125 | 200 | 3.125 |
| **RPMI 1640 – Glutamax** | 3.125 | 200 | 3.125 |

**Legend:** LB, Lysogeny broth; MIC, minimum inhibitory concentration; TZ, Thioridazine.

THP-1 macrophages were initially infected with ~2.2x106 CFU/mL (~6 log) *Salmonella,* after macrophage uptake and Gentamicin treatment, approximately ~4 log was recovered, as shown at **Figure 4.12** at 1.5 h p.i.. THP-1 macrophages were exposed to TZ for a period of 1, 3, 6, 8 and 20 hours (corresponding to 1.5, 2.5, 7.5, 9.5 and 21.5 hours p.i.). Upon treatment of *Salmonella*-infected macrophages with TZ (0.5, 1 and 2 µg/mL), a concentration dependent effect was observed (**Figure**

**4.12**). In the presence of the three concentrations of TZ, there was a significant decrease in the viability of the internalised *Salmonella* treated with 1 and 2 µg/mL TZ compared with the control sample (untreated infected THP-1 macrophages). This falls in line with the previous *in vitro* results (Chapter 3), where a concentration dependent effect was observed too. When infected THP-1 macrophages were treated with 0.5 µg/mL of TZ and throughout the infection assay, no replication of *Salmonella* was observed. The log CFU for this concentration is ~4 Log through the experiment assay.

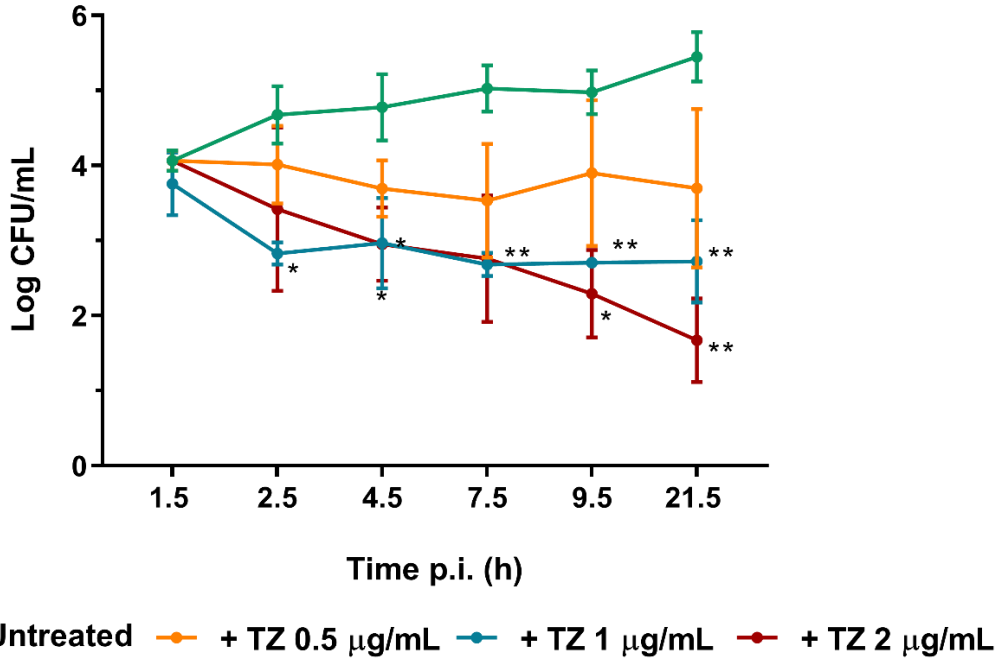


**Figure 4.12. Concentration dependent effect of TZ on replication of *Salmonella* in THP-1 macrophages.** *Salmonella*-infected THP-1 macrophages were exposed to different concentrations of TZ (0.5, 1 and 2 µg/mL). TZ was added at 1.5 hours p.i. (after bacterial uptake - gentamicin treatment for one hour at 100 µg/mL) and maintained throughout the remainder of the assay. The results correspond to the average of 3 independent experiments ± standard deviation (SD). Untreated corresponds to *Salmonella*-infected macrophages drug-free; p.i., post infection; TZ, Thioridazine; * and ** indicates $P < 0.05$ and $P < 0.01$, respectively (two-way ANOVA multiple comparisons, comparing with untreated (control)).

*Salmonella*-infected THP-1 macrophages were treated with 1 µg/mL [2.5 µM (100 times lower than the active *in vitro* concentration previously determined 100 µg/mL)] of TZ. As shown in **Figure 4.13**., following 2.5 hours p.i. (corresponding to 1 hour of exposure of the macrophages to TZ) a significant decrease in intracellular bacteria was observed when compared with untreated, infected macrophages (~ Log 2 reduction in CFU/mL). Throughout the time-course of *Salmonella* infection, exposure of the human macrophages to TZ resulted in a significant decrease in recoverable intracellular bacteria compared with untreated infected macrophages.

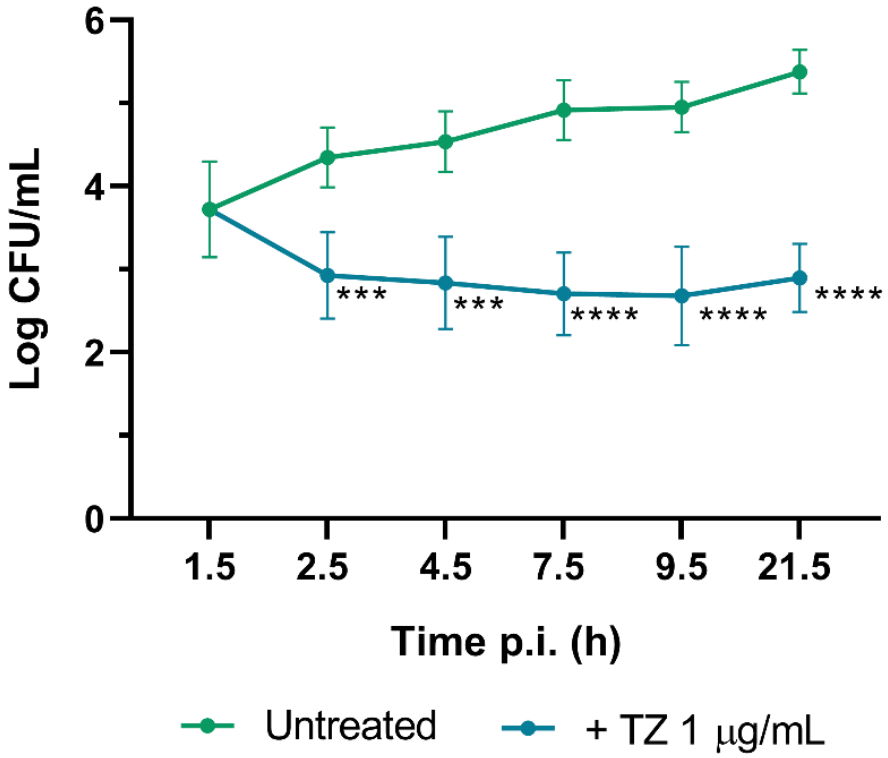


**Figure 4.13. Activity of TZ against *Salmonella*-infected THP-1 macrophages.** Effect of TZ (1 µg/mL) on the intracellular survival of *Salmonella* Typhimurium phagocytosed by human macrophages. THP-1 monocytes were differentiated with PMA (20 ng/mL) for 5 days. TZ was added at 1.5h (after bacterial uptake- gentamicin treatment for one hour at 100 µg/mL) to final concentrations of 1 µg/mL and maintain throughout the remainder of the experimental assay. The results correspond to the average of eight independent experiments ± standard deviation (SD); p.i., post infection; TZ, Thioridazine; *** and **** indicates $P < 0.001$ and $P < 0.0001$, respectively (two-way ANOVA multiple comparisons, comparing with untreated (control)).

### 4.3.6. Effect of TZ on the production of nitric oxide by *Salmonella*-infected THP-1 macrophages

Macrophages have an important role in the first line of defence of innate immunity, in which they phagocytose pathogens and produce microbicidal molecules, which kill the internalised pathogen. One such molecule is Nitric Oxide (NO). To assess whether the observed reduction in intracellular survival of *Salmonella* in THP-1 macrophages in the presence of TZ could be linked with enhanced killing activity of the macrophage, the accumulation of nitrite ($NO_2^-$), resulting from the response to *Salmonella* infection, was measured. Supernatants from THP-1 macrophage cultures infected with *Salmonella* (with or without TZ treatment) were collected and nitrite production assessed. As shown in **Figure 4.14**, the concentration of $NO_2^-$ produced and secreted into the culture supernatant in the presence of TZ was low (<0.8 µM). A similar result was seen in untreated THP-1 macrophages and macrophages activated with IFN-γ+ LPS over the full time-course of the assay. These results would suggest that TZ has no effect on nitric oxide production during *Salmonella*-infected THP-1 macrophages.

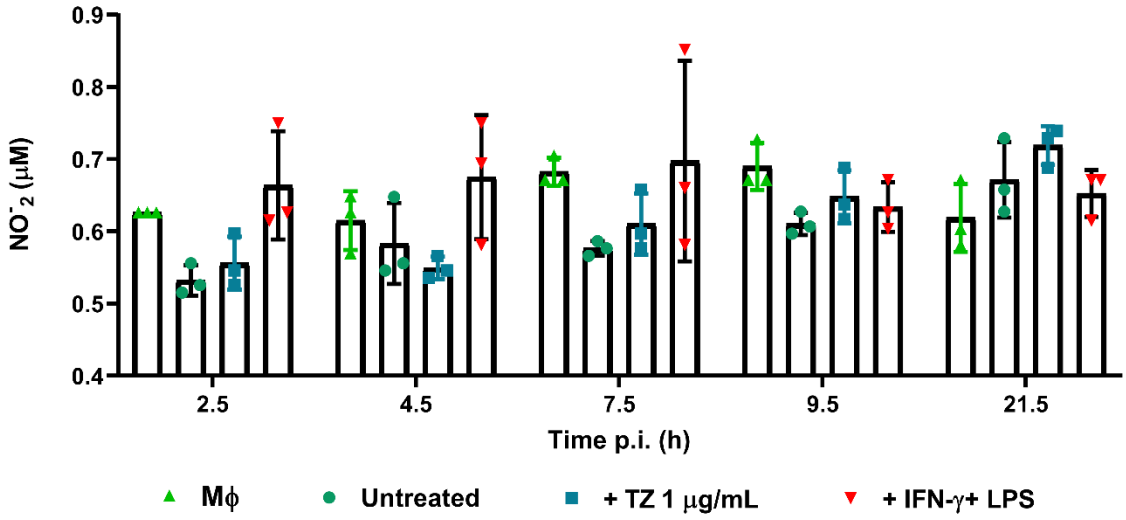


**Figure 4.14. Effect of TZ in the production of nitrite ($NO_2^-$) by *Salmonella*-infected THP-1 macrophages.** Nitrite concentrations were determined in the supernatants of *Salmonella*-infected THP-1 macrophages treated and untreated with TZ (1 µg/mL); uninfected macrophages activated with IFN-γ (100 ng/mL) and LPS (50 ng/mL) and uninfected macrophages. The concentration of $NO_2^-$ was assessed by the Griess method after 2.5, 4.5, 7.5, 9.5, and 21.5 hours post-infection. The results correspond to the average of 3 independent experiments ± standard deviation (SD). Mϕ, Macrophages (control); p.i., post-infection; TZ, thioridazine; IFN-γ, Interferon-gamma; LPS, lipopolysaccharides.

### 4.3.7. Effect of VER and LOX on infected human macrophages

It has previously been reported that enhanced killing activity of human macrophages upon exposure to TZ may be linked with TZ's ability to interfere with $Ca^{2+}$ signalling (Martins, Viveiros & Amaral, 2008; Martins *et al*., 2009; Machado *et al*., 2016). Thus, it is possible that the observed reduction in the log CFU from *Salmonella* recovered from TZ-treated THP-1 macrophages is linked to TZ-inducing alterations in the $Ca^{2+}$ signalling of the infected cells. To explore this hypothesis, a known $Ca^{2+}$ channel blocker, VER, was employed to determine the effect of altered $Ca^{2+}$ signalling on *Salmonella* replication in THP-1 macrophages. Additionally, to evaluate whether other anti-psychotic drugs, similar to TZ, could enhance the macrophage killing activity, LOX was tested under the same conditions.

VER demonstrates antibacterial activity against *Salmonella* at considerably higher concentrations than those obtained for TZ (2500 µg/mL *vs.* 200 µg/mL: **Table 4.1**). LOX showed activity against *Salmonella* at concentrations comparable to that of TZ (400 µg/mL *vs.* 200 µg/mL). Before commencing cell infection assays, viability assays were conducted to ensure that these concentrations of VER and LOX would not be toxic to host cells. As shown in **Figure 4.15.A**, VER significantly affected cell viability of THP-1 monocytes at a concentration of 160 µg/mL (89.7% reduction in cell viability). At half this concentration (80 µg/mL), a decrease of ~21.5% in cell viability was observed. At higher concentrations of LOX (50, 100, 200 µg/mL), a significant decrease in cell viability was observed. (≥95%; **Figure 4.15.B**). However, a decrease in cell viability of ~20% was observed at a LOX concentration of 25 µg/mL. Based on these results, concentrations of 80 µg/mL (162 µM) and 25 µg/mL (56 µM) for VER and LOX, respectively, were tested against *Salmonella*-infected THP-1 macrophages. In the presence of both VER and LOX, replication of intracellular *Salmonella* Typhimurium was significantly impaired compared with untreated infected macrophages (**Figure 4.15.C** and **D**). LOX appears to have a more significant effect compared to VER throughout the time-course of the assay. Comparing the effect of the three compounds (TZ, VER and LOX) against intracellular *Salmonella*, TZ (**Figure 4.15**) was the compound that showed a more pronounced effect on the viability of the internalised bacteria. The concentration of TZ needed to reduce intracellular *Salmonella* (1 µg/mL = 2.45 µM) was lower than that used for either VER or LOX.

Together, these results show that TZ, VER and LOX promote a significant decrease in the intracellular survival of *Salmonella* in infected macrophage. Furthermore, this effect on the infected macrophage took place at clinically relevant concentrations. Adding to this, these compounds also exhibited low toxicity in human macrophages.

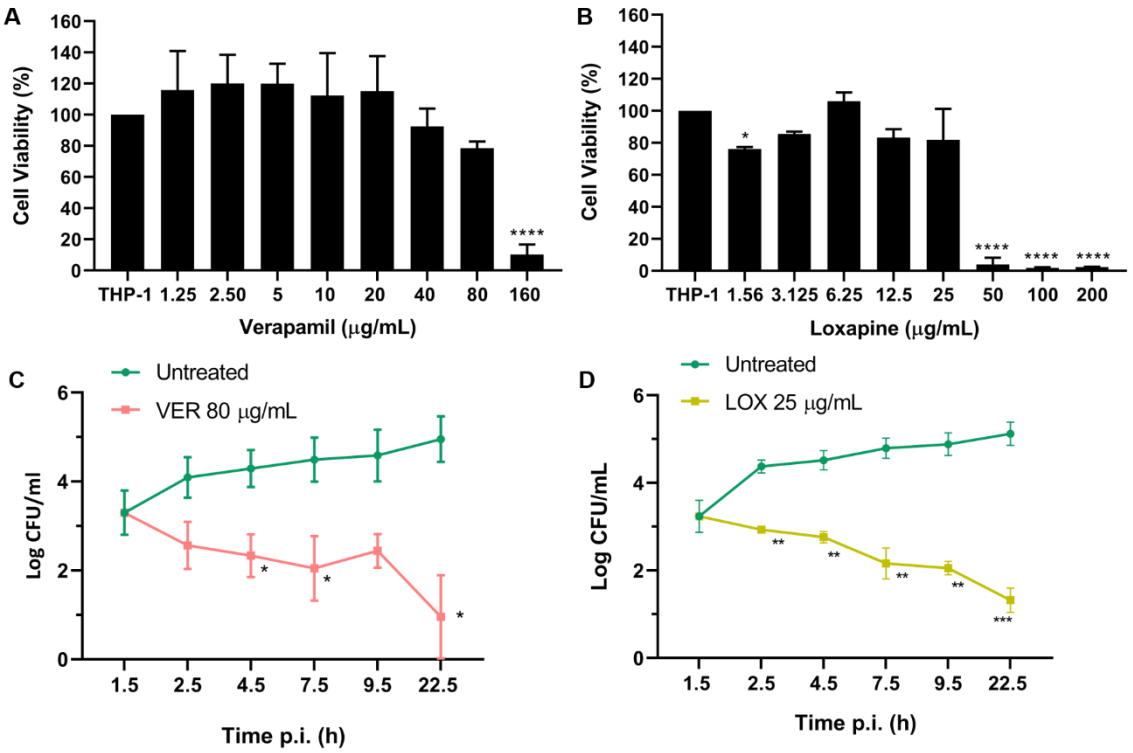


**Figure 4.15. Effect of the $Ca^{2+}$ channel blocker VER and the antipsychotic LOX in THP-1 infected macrophages.** (**A**) Cytotoxicity of VER and **(B)** LOX in human THP-1 monocytes (**C**) Effect of VER and LOX (**D**) on killing of intracellular *Salmonella* in THP-1 macrophages. Cell viability was assessed using the MTS cell viability assay for 24 hours (A and B). Results correspond to the average of 3 independent experiments ± standard deviation (SD). Intracellular survival results (**C** and **D**) correspond to the average of 4 independent experiments for VER and 3 independent experiments for LOX ± SD. "Untreated" corresponds to *Salmonella*-infected THP-1 macrophages in drug-free conditions; THP-1, human monocytic cell line; p.i., post infection; LOX, Loxapine; VER, Verapamil; *, **, **** indicates $P < 0.05$, $P < 0.01$ and $P < 0.0001$, respectively (one-way ANOVA, comparing with untreated).

### 4.3.8. Effect of TZ on the production of cytokines by *Salmonella*- infected THP-1 macrophages

Human macrophages produce a range of cytokines in response to bacterial infection. Assessment of cytokine production following exposure of differentiated THP-1 macrophages to TZ (24h, 1 µg/mL; Section 4.3.3) revealed no significant alterations to cytokine levels, suggesting that TZ itself does not stimulate cytokine production.

To examine whether exposure of *Salmonella*-infected macrophages to TZ elicited alterations in cytokine production, the profile of secreted cytokines in the culture of supernatants of infected THP-1 macrophages was examined. In parallel, the effect of VER on cytokine production was also measured. Cell culture supernatants were collected at the end of the infection assay (21.5 hours p.i). The *in vitro* assay is a closed system (24-well plate), and later time points were chosen to allow for a more accurate measurement of differences between the conditions tested (untreated and treated with compounds). Untreated THP-1 macrophages and macrophages stimulated with LPS and INF-γ were used as negative and positive controls, respectively.

The range of cytokines measured was IL-1β, IL-6, IL-8, IL-10, IL-12 and TNF-α (**Table 4.4**).

**Table 4.4.4. Summary of cytokines tested and their main characteristics.**

| Cytokine Tested | |
|---|---|
| **IL-1β** | Potent pro-inflammatory cytokine secreted by activated macrophages in response to the presence of LPS. |
| **IL-6** | Anti- and pro-inflammatory molecule and it is secreted by macrophages upon recognition of LPS to stimulate an immune response. |
| **IL-8 (CXCL8)** | Chemokine secreted by macrophages and other cell types. It functions as a chemotactic factor, guiding neutrophils to the site of infection and inflammation. |
| **IL-10** | Anti-inflammatory cytokine with a pleiotropic effect, playing a crucial role in infection by limiting the immune response to pathogens and thereby preventing damage to the host. |
| **IL-12** | Induced subsequent to intracellular infection and has been shown to have a role in clearing *Salmonella* infections. |
| **TNF-α** | Multifunctional, pro-inflammatory cytokine mainly secreted by macrophages and involved in systemic infection. |

**Legend.** IL, Interleukins; CXCL, C-X-C motif chemokine; TNF, Tumour necrosis factor; LPS, Lipopolysaccharide. Adapted from (Kincy-Cain, Clements & Bost, 1996; Lopez-Castejon & Brough, 2011; Arango Duque & Descoteaux, 2014).

Comparing untreated, infected THP-1 macrophages with TZ and VER treated *Salmonella*-infected THP-1 macrophages, no significant changes were observed (**Figures 4.16** and **4.17**). A slight increase in the secretion of IL-1β was seen upon treatment with TZ and VER (**Figure 4.16**), 205.7 and 170.4 pg/mL respectively, in comparison with the 152.21 pg/mL obtained from untreated *Salmonella*-infected THP-1 macrophages; however, this increase was not significant. Lower levels of IL-6 were detected for both TZ- and VER-treated infected THP-1 macrophages (**Figure 4.16**), but this was not significant.

As shown in **Figure 4.17**, secretion of IL-10, IL-12 and TNF-α was similar between untreated and treated infected macrophages. As expected, the presence of LPS and IFN-γ (positive control) resulted in secretion of all cytokines tested compared with the uninfected and non-activated macrophages (negative control) (**Figure 4.16** and **4.17**).

Altogether, the results suggest that the mechanism by which TZ impacts intracellular survival of *S.* Typhimurium does not involve an immunomodulatory effect on the macrophage.

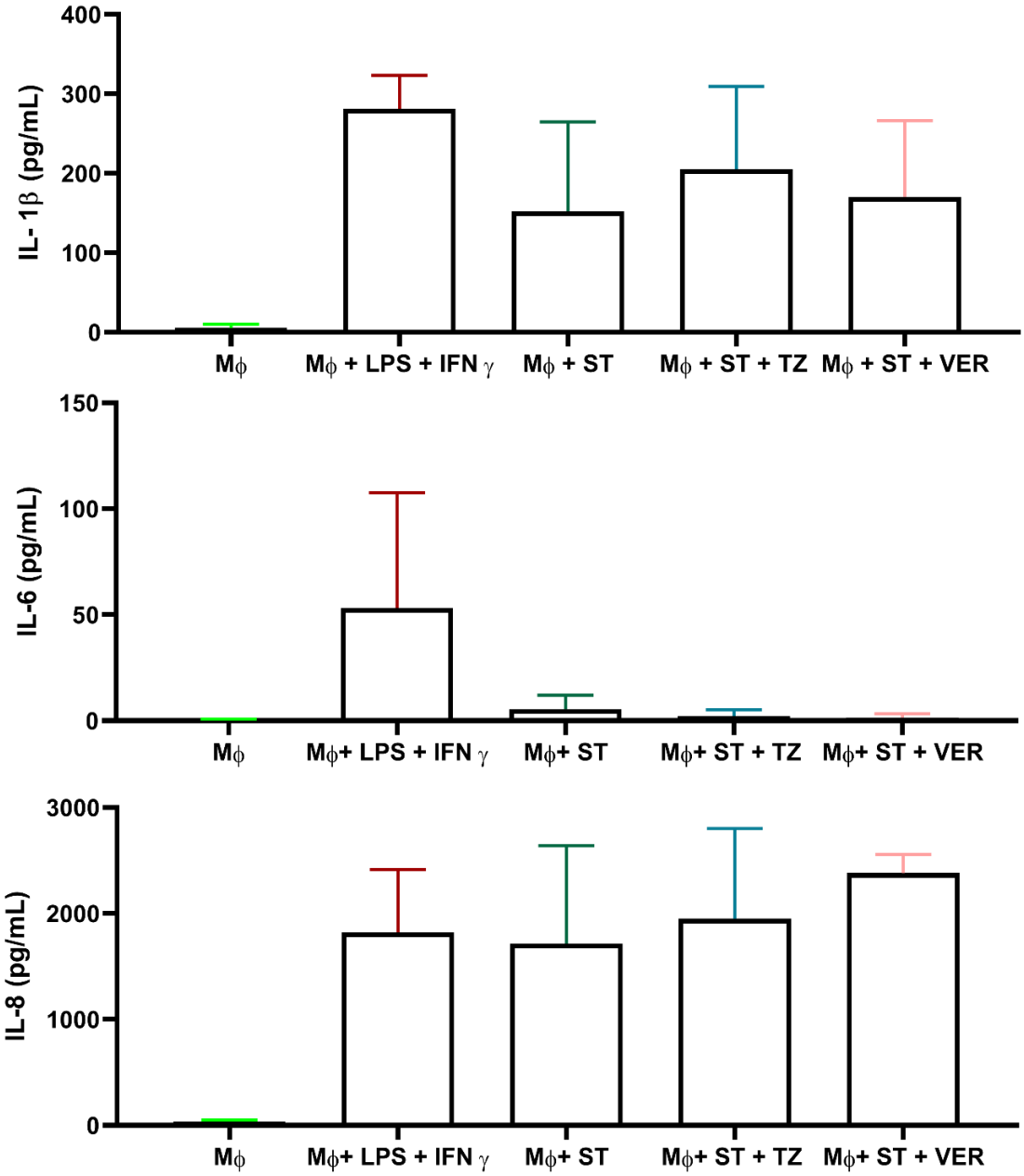


**Figure 4.16. Effect of TZ on cytokine secretion by *Salmonella*-infected macrophages in cell culture supernatants.**

Cell culture supernatants from infections assays were collected at 21.5 hours p.i.. Levels of IL-1β, IL-6, IL-8 in the supernatants of cultures of infected macrophages untreated and treated with TZ and VER were measured by ELISA. Mϕ and stimulation with LPS plus IFN-γ were used as internal controls. Results correspond to the average of three independent assays ± standard deviation (SD); IFN, interferon; IL, interleukins; LPS, Lipopolysaccharides; Mϕ, Macrophages M0; ST, *Salmonella* Typhimurium; TZ, Thioridazine. two-way ANOVA multiple comparisons, comparing with Mϕ + ST, no significant statistic determined.

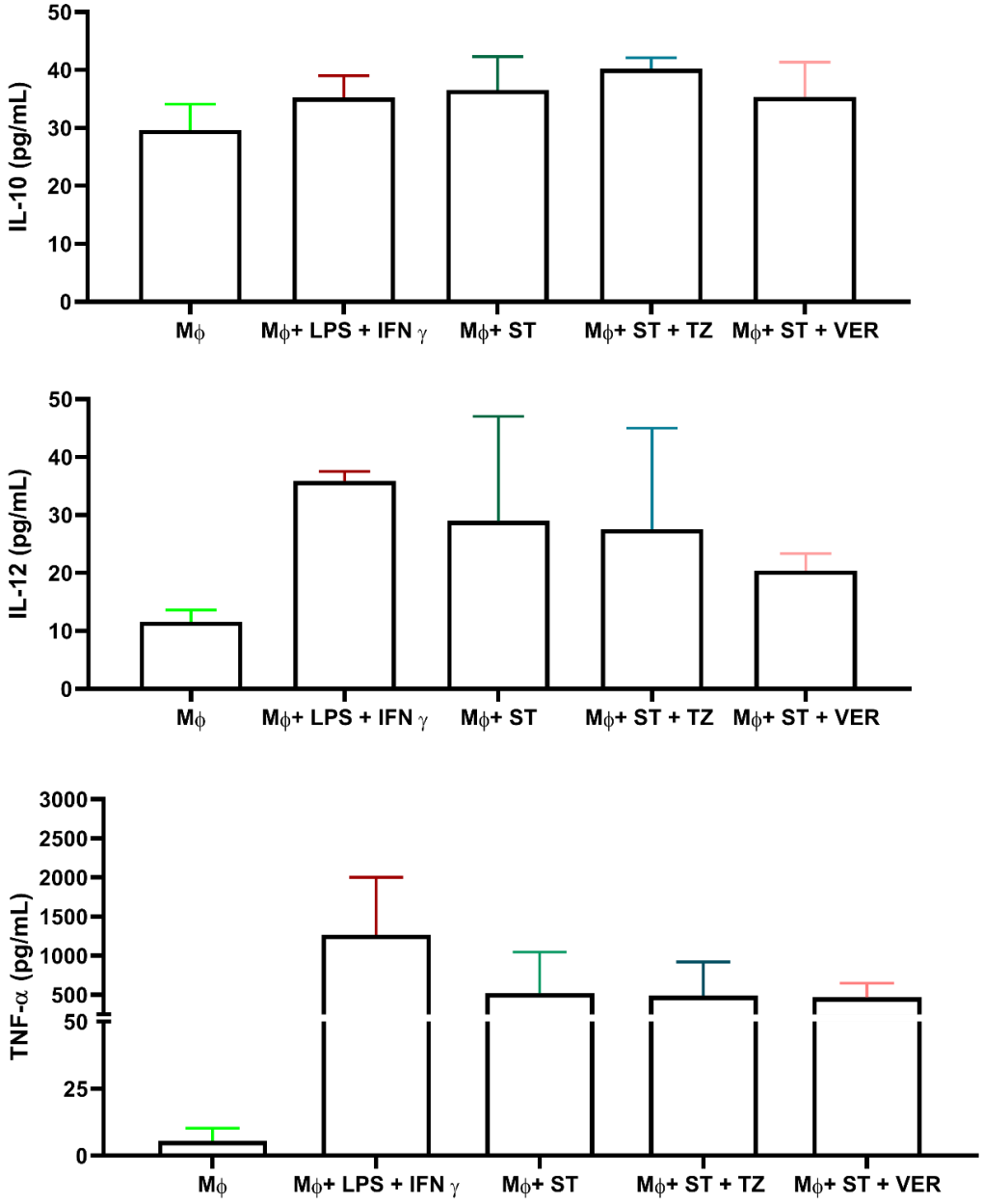


**Figure 4.17. Effect of TZ on cytokine secretion by *Salmonella*-infected macrophages in cell culture supernatants.**

Cell culture supernatants from infections assays were collected at 21.5 hours p.i.. Levels of IL-10, IL-12p40, TNF-α in the supernatants of cultures of infected macrophages untreated and treated with TZ and VER were measured by ELISA. Mϕ and stimulation with LPS plus IFN-γ were used as internal controls. Results correspond to the average of three independent assays ± standard deviation (SD); TNF, Tumor necrosis factor; IFN, interferon; IL, interleukins; LPS, Lipopolysaccharides; Mϕ, Macrophages M0; ST, *Salmonella* Typhimurium; TZ, Thioridazine. two-way ANOVA multiple comparisons, comparing with Mϕ + ST, no significant statistic determined.

### *4.3.9.* Effect of TZ on intracellular calcium levels of *Salmonella* - infected macrophages

As mentioned previously, one of the hypotheses posed for the mechanism of action of TZ on macrophages is the potential inhibition of calcium channels on infected macrophages.

To evaluate the effect of TZ on the $Ca^{2+}$ levels of *Salmonella*-infected macrophages, VER, a well known calcium channel blocker, was used in parallel. Macrophages and macrophages stimulated with IFN-γ plus LPS were used as internal controls for this assay.

As observed in **Figure 4.18**, THP-1 macrophages stimulated with IFN-γ plus LPS and *Salmonella*-infected THP-1 showed similar relative fluorescence values (RFU), 830.17 and 852.83, respectively. Upon treatment with the calcium channel blocker VER, the intracellular levels of calcium significantly decreased (467.5 RFU) in comparison with the untreated *Salmonella*-infected THP-1 macrophages. A similar effect to VER was observed on TZ treated infected THP-1 macrophages (577 RFU) compared with the untreated infected THP-1 macrophages.

The results of this assay confirm that TZ interferes with calcium on infected THP-1 macrophages, namely by decreasing the calcium levels. This effect was significantly different ($P<0.05$) from *Salmonella*-infected macrophage. Phagocytosis of bacteria by macrophage is a complex, physiological process critical for defence against pathogens and consequently for innate immunity. The common unfolding of phagocytosis includes pathogens recognition by specific receptors, actin cytoskeleton rearrangement, and protein clustering leading to particle internalisation. Calcium and sodium ions are known to play an important role in the different steps of phagocytosis including acidification of phagolysosomes. A localised cytosolic $Ca^{2+}$ gradient is required to generate the signals necessary for phagocytosis mediated by specific receptors (Fcγ).Therefore, compounds such as TZ and VER that are able to interfere with the levels of calcium may be able to trigger important signals for phagosome and lysosome maturation.

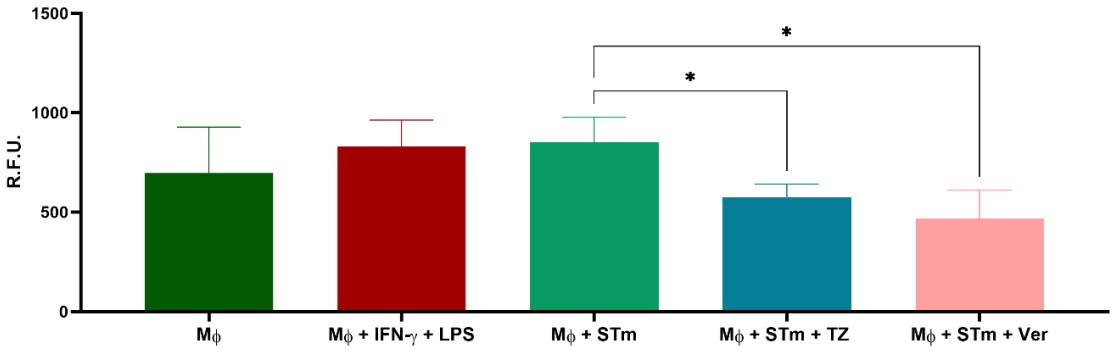


**Figure 4.18. Effect of TZ on the level of intracellular $Ca^{2+}$ on *Salmonella*-infected macrophages.** Intracellular $Ca^{2+}$ was measured by fluorescence using a membrane-permeable calcium indicator, Cal-520™ on TZ and VER untreated and treated infected macrophages at 21.5 hour p.i.. The results presented correspond to the average of three independent assays ± standard deviation (SD); Mϕ, Macrophages M0, IFN, interferon; LPS, lipopolysaccharides; STm, *Salmonella* Typhimurium, TZ, thioridazine; VER, verapamil. * indicate $P< 0.05$ (one-way ANOVA, comparing to Mϕ + STm (control)).

## 4.4. Discussion

Traditionally, bacterial infections have been controlled by antibiotics since the so-called “Golden Age”. Although antibiotics contributed to a reduction in the global burden of bacterial infections, in the background they also triggered a higher burden, the development of AMR. To tackle the increasing emergence of AMR, alternative approaches need to be designed and explored with the objective of overcoming this global health issue. A major concern linked with AMR is the continuing number of infections that are difficult to treat and caused by intracellular bacteria. Pathogens such as *Salmonella* spp. and *M. tuberculosis* have evolved and adapted to host immune cells. Both reside within a host vacuole, which they modify to create a favourable intracellular niche that promotes pathogen survival and replication. Furthermore, both pathogens can interfere with trafficking within host cells, directing essential nutrients to these modified vacuoles (Singh *et al*., 2003; Liss *et al*., 2017).

In this chapter, studies were conducted to investigate the effect of TZ on human monocytes and macrophages uninfected and subsequently infected with *Salmonella*. Due to the results obtained from the infection assays, the role of TZ on the survival of intracellular *Salmonella* within macrophages was investigated further in order to understand the possible mechanism by which TZ impacts viability or replication of intracellular *Salmonella*. It has been previously shown that phenothiazines can be concentrated by organs or tissues with high populations of macrophages. This concentration can achieve levels that are clinically significant (0.5 mg/L – 1 mg/L) (Ordway *et al*., 2003). Within cells that are rich in lysosomes, e.g. macrophages, these organelles play a significant role in the concentration of phenothiazines. In infected macrophages, where the intracellular bacterium is residing within a phagosome, fusion of phenothiazine-loaded lysosomes with this compartment can serve to concentrate the phenothiazine, creating conditions by which the phagocytosed bacteria is exposed to a concentration of the compound that is at least equal to that required for its *in vitro* killing activity.

In Chapter 3 – section 3.3.13, it was determined that, at 1 µg/mL, TZ had no effect on the cell viability of THP-1 monocytes. Thus, this was the concentration selected for *ex-vivo* studies since it corresponds to the sub-MIC determined *in vitro*. Firstly, it was investigated whether TZ had any effect on uninfected THP-1 monocytes and macrophages, potentially triggering an immune response that could

interfere or influence the intracellular survival of *Salmonella* upon exposure to TZ. When TZ is administrated in long-term psychosis treatment, side effects are obtained (Harrigan *et al*., 2004) and this has put TZ aside to be considered as a potential antimicrobial. However we must keep in mind that if TZ was to be used as an antimicrobial, shorter courses of treatment are needed, thus lower toxicity, therefore TZ could be taken into consideration for these cases. In the case of drug repurposing, a potential candidate drug has already been subjected to safety and toxicological evaluation for the original indication. Hence, a demonstration of efficacy for the new indication could be assessed in a cell model system (Farha & Brown, 2019). When monocytes were exposed to clinically relevant concentrations of TZ (1 µg/mL) no alterations on the basal metabolism of the cells were obtained. This is a very interesting result as in some cases, antipsychotic drugs have been linked with dysregulated peripheral metabolism and increase of hormone production such as glucagon and leptin associated with metabolic dysfunction (Gonçalves, Araújo & Martel, 2015).

Since no effect of TZ was observed *per se* in these cells, monocytes were then differentiated to macrophages and further studies were carried to validate the potential use of TZ *in vitro* as antimicrobial to treat intracellular bacteria. Cytokine levels and caspases markers were evaluated. As expected, no cytokine response was obtained when exposing THP-1 macrophages to TZ on a non-infection setting. Furthermore, no apoptotic markers on THP-1 macrophages were detected after 24 hours of exposure to TZ. These are quite promising results showing that TZ can be safely used for the treatment of intracellular infections.

Phenothiazines, especially TZ, have been reported to enhance the killing activity of macrophages infected with *M. tuberculosis* (Amaral, Martins & Viveiros, 2007; Machado *et al*., 2016) and *S. aureus* (Ordway *et al*., 2002a; Martins *et al*., 2004). Here, it was demonstrated that TZ has a concentration dependent effect on the survival and replication of intracellular *Salmonella* within macrophages. At sub-MIC concentrations, a significant reduction on *Salmonella* survival was observed at all timepoints sampled during the infection assay. At the end of the assay, ~ 5.4 $\log_{10}$ CFU/mL were recovered from untreated- infected THP-1 macrophages while only 2.8 $\log_{10}$ CFU/mL were recovered after treatment with TZ 1 µg/mL. However, the question remains. How does TZ enhances the killing of intracellular pathogens?

Also, is this effect specific to TZ or could other compounds of the same class also present a similar effect in infected macrophages?

Previous work from the lab conducted with infected macrophages indicated that TZ can potentially interfere and have an effect on the flux of $Ca^{2+}$, however this was never confirmed by measuring the levels of calcium in *Salmonella i*nfected macrophages treated with TZ. To confirm the hypothesis the intracellular $Ca^{2+}$ levels of the infected macrophage were quantified. One additional compound was introduced on subsequent assays, VER, a well-known L-type voltage-gated $Ca^{2+}$ channel (VGCC) blocker with demonstrated ability in reducing intracellular survival of *M. tuberculosis* (Gupta *et al*., 2009, 2013; Adams, Szumowski & Ramakrishnan, 2014; Machado *et al*., 2016).

Another antipsychotic drug (LOX) was used to clarify if this effect was only due to TZ or if other drugs from this class (antipsychotics) could also have this same effect on reducing intracellular bacterial survival. There is no clear evidence in the literature regarding its possible accumulation on macrophages at the same level as TZ or its specific mode of action on infected macrophages. When tested on *Salmonella*-infected THP-1 macrophages, these two compounds, VER and LOX, lead to a significant decrease in the intracellular bacterial load at concentrations that don't interfere with macrophage viability. However, the concentrations used, especially for VER, were quite different from TZ, as a much higher amount of VER (162 µM) and LOX (56 µM) was needed comparing TZ (2.45 µM).

As TZ treatment significantly decreased *Salmonella* survival inside macrophages, investigating the possible mechanism by which TZ exerts its effect in infected cells was studied further. Possible mechanisms include: i) alterations of the levels of nitric oxide; ii) immunomodulatory effects on immune response namely on the secretion of cytokines and iii) alteration of the intracellular levels of $Ca^{2+}$.

When investigating whether TZ affected the production of nitric oxide, a low production of nitrite in all conditions tested was obtained, including in the supernatants of macrophages previously stimulated with IFN-γ and LPS. This stimulus (IFN-γ and LPS) was added to THP-1 macrophages as a positive control for activation of these cells. Human macrophages are not robust NO producers *in vitro* as compared to mouse cell line and this is observed here in the study. A Study

by Gross *et al*, concluded that, contrary to murine macrophages, human macrophages produce little detectable inducible NOS (iNOS) and NO in response to inflammatory stimuli due to epigenetic regulation around the nitric oxide synthase (NOS) - 2 transcription start site (Gross *et al*., 2014). Ozleyen *et al*. reported that macrophage-like THP-1 released 0.30 ± 0.10 μM of NO in the culture medium and the level of NO was found almost identical (0.35 ± 0.01 μM) in the medium of LPS-treated cells. Different concentrations (0.05, 1, and 10 μg/mL) of LPS treatment were tested in differentiated THP-1 cells, however, at even the highest concentration of LPS, the amount of NO release was not significantly different than the one detected in the non-treated control group (Ozleyen, Yilmaz & Tumer, 2021). The results from the study of Ozleyen *et al.* are concordant with the results obtained in the present chapter with THP-1 macrophages.

After measuring the levels of NO, the cytokine production by untreated and treated *Salmonella*-infected THP-1 macrophage supernatants was also assessed through ELISA. The cytokines tested in this study were IL-1β, -6, -8, -10, -12 and TNF-α (same cytokines tested previously in non-infected macrophages). It is known that the response of macrophages to *Salmonella* infection involves secretion of cytokines mainly IL-1β, IL-8 (CXCL8), IL-12, TNF-α (Vitiello *et al*., 2004; Hurley *et al*., 2014; Lathrop *et al*., 2015; Starr *et al*., 2018). No significant differences were detected between untreated and treated conditions with both compounds, TZ and VER. A slight increase was observed for IL-1β and IL-8 in infected macrophages treated with TZ and VER; however, this was not significant. This could be related with the low intracellular bacteria recovered at this time post infection and after treatment with both compounds (section 4.3.7) during the infection assays. These results would suggest that the effect seen on *Salmonella* replication in THP-1 macrophages upon treatment with TZ is not due to an immunomodulatory activity of the compound on the infected THP-1 macrophages, therefore, not interfering with the levels of cytokine production.

In *M. tuberculosis*-infected macrophages it has been postulated that TZ presents a mediated inhibition of ion channels in infected macrophage (Amaral, Martins & Viveiros, 2007; Machado *et al*., 2016). Some studies also report that TZ has the ability to inhibit efflux pumps, not only in bacteria but also in human cells (Amaral et al., 2010b, 2012b; Shen et al., 2017). Due to these characteristics, it has

been reported that TZ inhibits $Ca^{2+}$ channels (Mousavizadeh, Ghafourifar & Sadeghi-Nejad, 2002). This inhibition could also impact efflux of ions of the phagolysosomal compartment. Studies by Martins *et al*., (Martins *et al*., 2006, 2009; Martins, Viveiros & Amaral, 2008) have hypothesised on the possible activity of TZ on the efflux pumps of macrophages and its potential link to the inhibition of $Ca^{2+}$ channels on these same cells. However, an experiment targeting $Ca^{2+}$ on the infected macrophages was never conducted. In this study, the effect of TZ on the calcium flux of *Salmonella*-infected macrophages was performed in order to clarify this question.

When *Salmonella*-infected macrophages were treated with TZ and VER, a significant decrease in intracellular calcium was obtained. It is important to refer that this significant decrease in the intracellular calcium can be due to a possible accumulation of $Ca^{2+}$ inside the phagolysosome. To the best of my knowledge, this is the first study that measured $Ca^{2+}$ flux on *Salmonella*-infected macrophages that were treated with TZ. It is known that the plasma membrane of macrophages is rich in ion channels that transport $Ca^{2+}$ and $K^{+}$ into the cell (Feske, Wulff & Skolnik, 2015). Following phagocytosis, the intracellular bacterium is contained within a vacuolar compartment termed a 'phagosome'. In the case of *Salmonella*, maturation of this compartment is directed by the bacterium itself to establish a favourable intracellular niche known as the SCV. Hence, when on a phagolysosome these ions ($Ca^{2+}$ and $K^{+}$) will be pumped to the cytoplasm instead of into the phagolysosome. The low concentrations of these ions prevent acidification of the phagolysosome machinery and the killing of the bacterium is avoided. Therefore, I can only speculate at this stage that TZ is able to revert this situation, allowing an increase of these ions on the phagolysosome (through the inhibition of the $Ca^{2+}$ channels) and subsequently, promoting killing of *Salmonella*.

A study from 2009, also verified that inhibition of L-type ion channels results in a significant decrease in intracellular $Ca^{2+}$ in macrophages leading to a reduction in *M. tuberculosis* survival in the presence of VER (Gupta *et al*., 2009). Using *M. bovis,* as a model, Machado *et al.* (Machado *et al*., 2016) elegantly demonstrated that in macrophages treated with TZ (2.5 µg/mL), there is an increase in the number of acidic endosomal vesicles. These results support the data obtained and presented in this chapter. However, further studies, such as measurement of phagolysosome acidification, need to be carried. During infection, *Salmonella* adapts to the

intracellular environment of the macrophage, ultimately residing within the modified SCV. *Salmonella* uses the initial acidic environment within the phagosome as a signal to induce expression of virulence factors encoded within *Salmonella* Pathogenicity Island 2 (SPI-2). The SPI2-encoded Type III Secretion System 2 (T3SS2) and its associated effector proteins control fusion of the phagosome with the lysosome, inhibiting formation of a canonical phagolysosome, which enables *Salmonella* to survive and replicate. Should TZ alter the dynamics of endosome acidification (either at the level of acidification itself or the concentration of acidified endosomal compartments within the cell), it can be envisaged that this would have a knock-on effect on maturation of the SCV. Future work will likely address the impact of TZ exposure on expression of SPI2 and associated virulence factors within the infected macrophage. Construction of a Transposon Directed Insertion Sequencing (TRaDIs) library has been completed; however, due to time constraints, experiments using this library to address the SPI2 question have not been completed.

The results obtained in this chapter, demonstrate that TZ has an effect on the killing of *Salmonella* in infected THP-1 macrophages and therefore could be a good candidate for use in adjuvant therapy to treat bacterial infections. A similar approach was reported on a study conducted in Argentina under “compassionate basis” (Abbate *et al*., 2007). In this study, patients diagnosed with extensively drug-resistant tuberculosis (XDR-TB) received combination therapy with TZ and two antibiotics (linezolid and moxifloxacin. The authors observed that TZ improved the quality of life of the XDR-TB patients and contributed to a longer life span. Similar results were also obtained in a study conducted in India on TB patients (Udwadia, Sen & Pinto, 2011). Taken together, the results from these studies are encouraging and support the relevance of studying TZ in more detail regarding its mode of action on infected cells.

It is estimated that *Salmonella* is the cause of approximately 1.35 million illnesses, 26,500 hospitalizations, and 420 deaths in the United States every year (Centers for Disease Control and Prevention | CDC, 2019). Beside the United States, *Salmonella* infections also constitute a major burden in certain European countries, parts of Asia and Africa. According to a study conducted in 2019, infections caused by non-typhoidal *Salmonella* are more prevalent in Africa in comparison to other

parts of the world (Balasubramanian *et al*., 2019). Therefore, the potential treatment of *Salmonella* infections is of benefit and with a crucial and immediate impact on public health. Since antibiotics are not advised for the treatment of *Salmonella* infections, the use of potential adjuvant compounds, such as TZ can be of interest.

Adding to this, TZ can also be used as a parental molecule for the design of new and more effective derivatives for the treatment of intracellular infections.

### 4.4.1. Model for activity of TZ in human macrophages infected with *Salmonella*.

From the results obtained in this study, a model for the activity of TZ in *Salmonella* infected THP- macrophages, was developed. This model (**Figure 4.19**) is that focused on the inhibition of $Ca^{2+}$ and considers a dual action (prokaryotic-eukaryotic targets) for TZ, as follows:

**(1)** Following phagocytosis, *Salmonella* resides in a modified phagosome, the SCV (**Figure 4.18.A**). Maturation of this intracellular compartment is directed by effector proteins delivered by Type III Secretion Systems encoded on SPI1 (early events) and, more importantly, SPI2. In the absence of TZ, genes encoded on SPI-2 are expressed, effector protein delivery *via* T3SS2 occurs, and *Salmonella* inhibits the formation of the phagolysosome by preventing fusion of lysosomes with the SCV (**Figure 4.18.B1-3**) (Hurley *et al*., 2014). In the presence of TZ, it can be hypothesised that TZ affects secretion of effector proteins encoded within SPI-2 or the T3SS2 apparatus, preventing establishment of the SCV and allowing formation of the canonical phagolysosome compartment.

**(2)** Upon treatment with TZ, vacuoles loaded with TZ fuse with the phagolysosome leading to the inhibition of voltage long lasting (L-Type) $Ca^{2+}$ channels and $K^+$ ion pumps of the phagolysosome, and as a result, there is an increase in the concentration of these ions inside the phagolysosome (Martins, Viveiros & Amaral, 2008; Martins *et al*., 2009; Machado *et al*., 2016). Subsequently, activation of vacuolar proton ($H^+$)- ATPases (V-ATPases) occurs to maintain the osmolarity of the cell and to compensate the hypertonicity of the organelle by pumping protons into the lumen (Harvey, 2009; Martins *et al*., 2009). This rise of protons is responsible for the decrease of pH within the phagolysosome unit and

therefore, consequent activation of the hydrolytic enzymes, promoting degradation of the intracellular bacterium (**Figure 4.19.B4-5**) (Martins, Viveiros & Amaral, 2008; Gupta *et al*., 2013; Machado *et al*., 2016).

This model illustrates the potential mode of action of TZ (and potentially other compounds, such as VER) in treating *Salmonella*-infected macrophages. Other compounds that have the same effect as TZ, on the host cell could be designed or repurposed to activate the killing activity of the host cell and reduce the intracellular bacterial load.

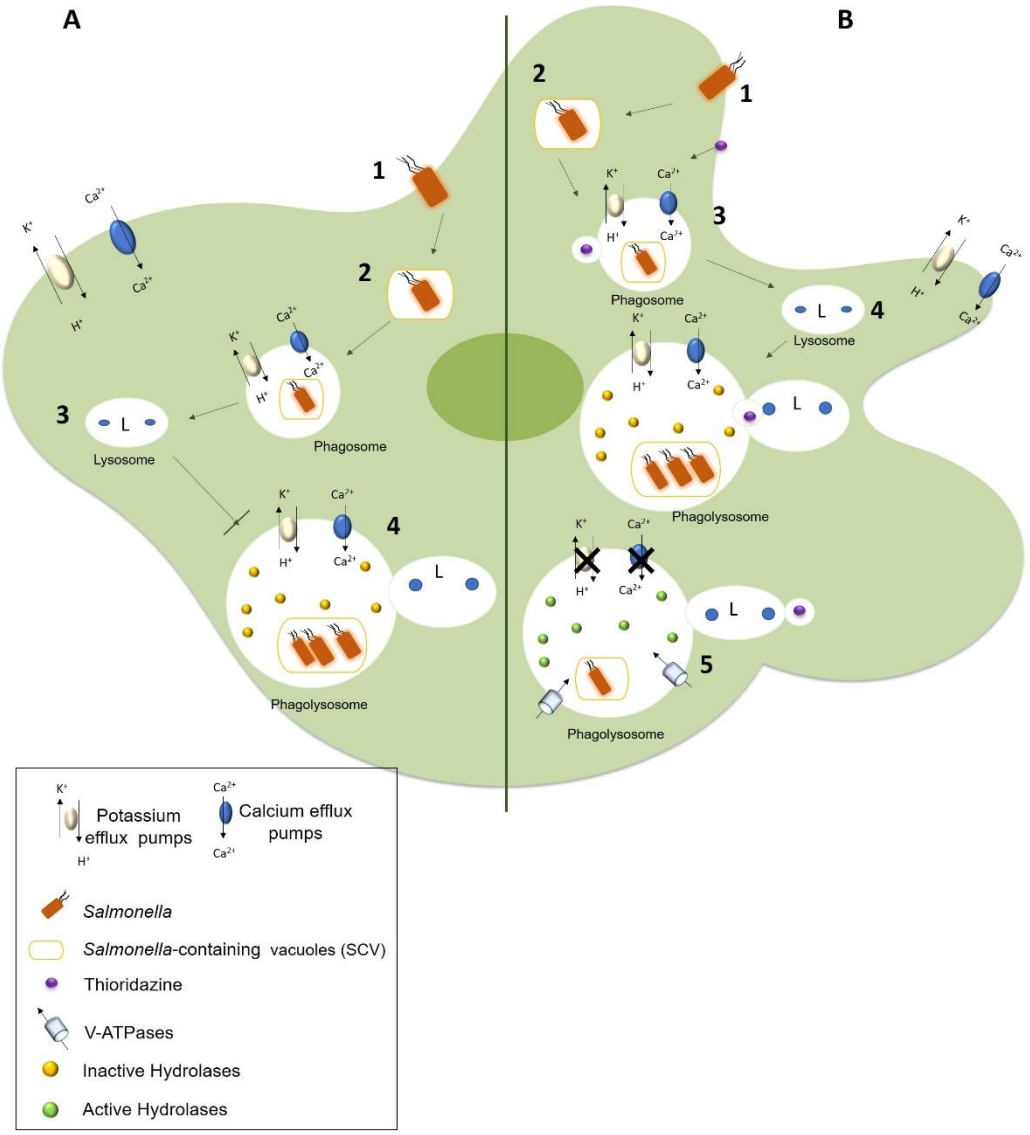


**Figure 4.19. Model proposed for the effect of TZ in the enhancement of the macrophage killing activity.** (**A**) Untreated infected macrophage. **(B)** Infected macrophage treated with thioridazine (TZ). (**A** and **B**1-2) *Salmonella* Typhimurium is phagocytosed by the macrophage and resides within the *Salmonella*-Containing Vacuole (SCV). (**B**3-4) Treatment with TZ will interfere with the secretion of effector proteins encoded within SPI-2 allowing fusion between lysosomes and SCVs and, forming the phagolysosome unit. (**B**5) TZ inhibits the $Ca^{2+}$-activated $K^+$ pumps and $Ca^{2+}$ channels on the phagolysosome membrane. The concentration of ions is increased by diffusion from the cytoplasm into the phagolysosome and the vacuolar proton ($H^+$)-ATPases (vATPases) are activated. This increase on the concentration of protons causes the decrease on the pH of the phagolysosome, activating hydrolases that kill the bacteria (Adapted from Martins *et al*., 2009; *L. Amaral et al., 2007).*

## 4.5. Supplementary Information

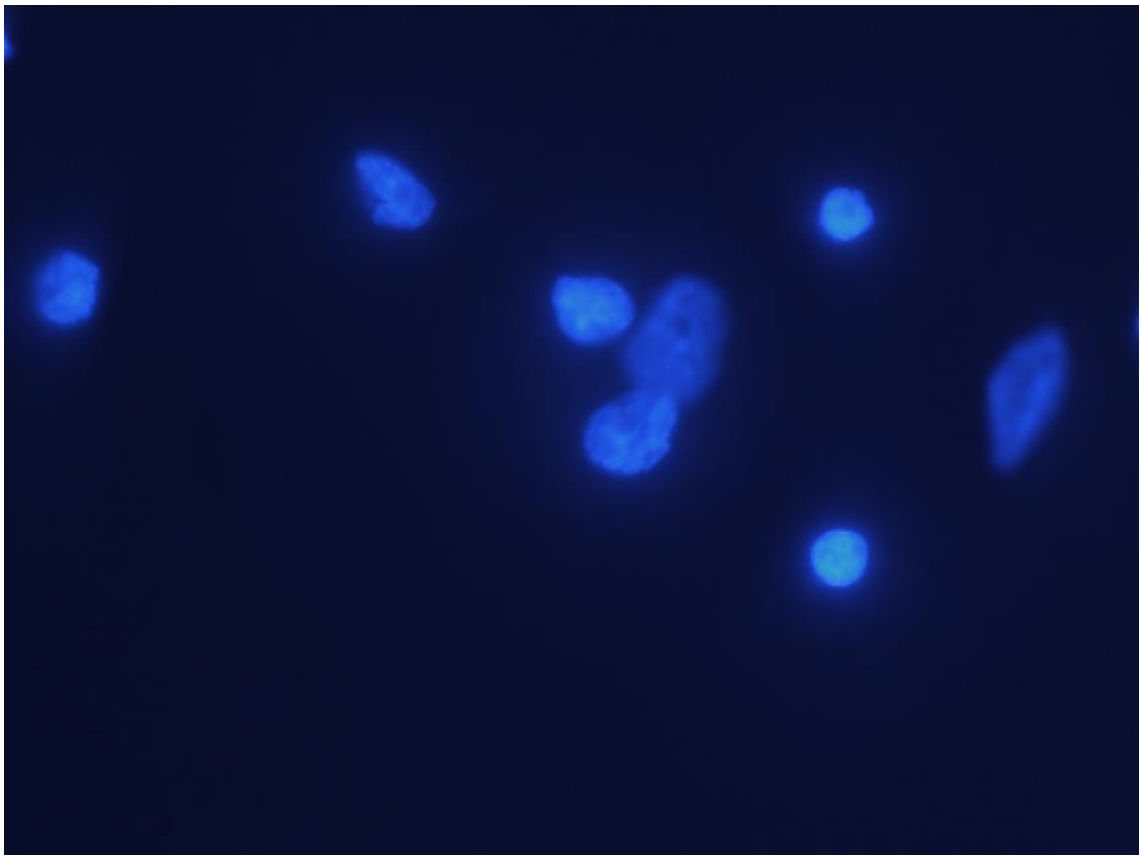

**Figure S4.1. DAPI staining of THP-1 macrophages.** DAPI staining was performed in THP-1 macrophages (as described in the Materials and Methods section) for Mycoplasma detection. Cells were fixed with 2.5% (w/v) paraformaldehyde for 20 minutes at 37°C and then washed with PBS. All subsequent steps were done at room temperature. Next, cells were permeabilised with 0.1% (w/v) saponin for 30 minutes. DAPI (1 µg/mL final concentration in water) was added for 20 minutes, and cells were washed twice with PBS and once with water, then overlaid with 30 µL mounting medium (Mowiol). Cells were observed using a fluorescence microscope NIKON ECLIPSE E400 at x100 lens with immersion oil.

# Chapter 5. General Discussion and Future perspectives

Since the discovery of the first antibiotic, penicillin, by Alexander Fleming in 1928, that many antibiotics have been discovered and further developed. This initial revolutionary discovery made hundreds of years ago provided an answer for the treatment of bacterial infections when a simple bacterial infection could mean a death sentence. However, the euphoria over the potential defeat of infectious diseases was short. Along with the development of antibiotics, bacteria responded by developing mechanisms of resistance. This cannot be stressed enough and have led us to 2020, where AMR is still one of the major's global public health issues that threatens the effective treatment of infectious diseases. According to the Director-General of the WHO, Dr. Tedros Adhanom Ghebreyesus: "Never has the threat of antimicrobial resistance been more immediate and the need for solutions more urgent". As referred previously, AMR is not only a public health problem but an economic one too. It is predicted that by 2050 AMR could be responsible for over 700,000 deaths annually while costing US$100 trillion worldwide (O' Neil, 2014). Over the past few decades, the number of research-based biopharmaceutical companies conducting AMR relevant research and development has significantly declined. Development of a new drug can take 10 to 17 years and the success rate of developing a new drug is usually around 2% (on average) (Yeu, Yoon & Park, 2015; Farha & Brown, 2019). Two new reports released in 2019 by the WHO, revealed that the current clinical pipeline for antibiotic agents in development was composed of 60 compounds (50 antibiotics and 10 biologicals). From this report, only one new anti-tuberculosis agent is being developed. The preclinical pipeline revealed that 252 agents are being developed to treat pathogens in the WHO priority list, including *M. tuberculosis*. It is estimated that only two to five products of these agents will reach the market within 10 years (World Health Organization, 2019a,b). In July 2020, an AMR Action Fund was created to invest in smaller biotech companies providing industry expertise to support the clinical development of novel antibiotics. The Fund aims to bring 2-4 new antibiotics to patients by 2030 (Clancy & Nguyen, 2020). Although this is an optimist scenario it is still not an efficient solution or sufficient for the problem we are facing nowadays.

This raises the question: But what if the antibiotics we now have do not fully work anymore? Will the world enter (already entering in some cases) a post-antibiotics era, where a simple paper cut could lead to death? The continuous battle

between humans and the multitude of microorganisms that cause infection and disease is still a reality nowadays and in the future.

Drug repurposing is one of the approaches being used for the discovery of new antibacterial drugs and to overcome this worldwide so-called “silent tsunami” - AMR. This approach will reduce time and cost usually linked to the early development of new drugs since repurposed drugs have already passed all clinical tests in Phase I, Phase II, and Phase III and been Approved by the U. S. Food and Drug Administration (FDA) or in Europe, by the European Medicines Agency. Reports of drug repurposing as a strategy to uncover new antimicrobial compounds has been increasing. Auranofin, an antirheumatoid arthritis drug, was repurposed as a broadspectrum antimicrobial in 2012 and has undergone a clinical trial for the treatment of gastrointestinal protozoa (Debnath *et al*., 2012). The repurpose of disulfiram, an anti-alcoholic drug, as an antibacterial against *Staphylococcus aureus* (Thakare *et al*., 2019) and non-tuberculous mycobacteria bacteria (Das *et al.*, 2019) has also be reported. Anti-tumour drugs such as, tamoxifen, miltefosine have been explored for repurposing as antifungal and anti-leishmania (visceral form), respectively (Krysan & Didone, 2008) as well as other anti-tumour drugs (Soo *et al*., 2017).

The work described in this research thesis is focused on the repurposing of different compounds (anti-tumour and neuroleptic drugs) with the main goal to potentially uncover new effective antimicrobials.

On Chapter 2, the antibacterial activity of three organometallic anti-tumour drugs complexed with Zn and Co were tested against a range of Gram-negative and -positive bacteria. These compounds were previously synthesised by collaborators (Silva *et al*., 2013). The three compounds showed antimicrobial activity against several ESKAPE pathogens namely *S. aureus*, *K. pneumoniae*, *A. baumannii* and *P. aeruginosa*. According to WHO, these pathogens are considered one of the most urgent MDR threats to human health.

All three anti-tumour compounds showed activity against *A. baumannii* with MIC values of 0.8 µM for TS262 and 1.6 µM for TS265 and TS267. In general, Zinc compound TS262 was the most active of the three. Although the antibacterial activity of these compounds against the bacteria tested was promising, the exact

mechanism of action of these three compounds wasn't fully investigated. This was due to the toxicity levels obtained when human monocytes were exposed to the compounds. When Zinc compound TS262 was tested at 2 µM on primary blood mononuclear cells (PBMC) and 5 µM on primary bronchial/tracheal epithelial cells a reduction of viability of more than 50% was obtained for both cell types (Alves Ferreira *et al*., 2020). These results are concordant with previous data obtained by our collaborators when this compound was tested on healthy epithelial cells (Luís *et al*., 2014). The half-maximal inhibitory concentration ($IC_{50}$) obtained for that epithelial cell line (5.14 ± 0.01) µM was more than 6.9 times higher than that determined for a tumorigenic breast cell line MCF7 (0.73 µM) (Luís *et al*., 2014). Considering the MIC of 0.9 µM obtained for *Acinetobacter baumannii*, a therapeutic window might exist (of 2.5× for PBMC, 6.25× for BTEC, and 6.9× for epithelial cells). Nevertheless, for human administration, the vectorisation of these compounds using gold nanoparticles might be an interesting approach that we can consider in the future (Pedrosa *et al*., 2019).

The following two chapters were focused on investigating TZ as a potential antimicrobial and to study it's *in vitro* mechanism of action against *Salmonella* Typhimurium (Chapter 3) or its effect on *Salmonella*-infected human macrophages (Chapter 4). Phenothiazines are heterocyclic compounds from which majority of clinical compounds have their origins (Mosnaim *et al*., 2006). A well-known example of this type of compounds is the dye methylene blue. TZ, another phenothiazine, is a neuroleptic drug that has called the attention of researchers worldwide because of its ability to inhibit and kill drug resistant bacteria. The antimicrobial properties of TZ (and other phenothiazines) are known since the "Golden Age" of antibiotics. During that time there was no need to pursue new antimicrobial compounds due to the abundance of antibiotics. Until today, the mechanism(s) of action by which this neuroleptic drug exert its effect as a powerful antimicrobial hasn't been fully understood.

On chapter 3, the *in vitro* effect of TZ against *Salmonella* Typhimurium was investigated. The studies conducted in this chapter, confirmed that TZ has antibacterial activity against *Salmonella* Typhimurium at concentrations of 200 µg/mL. This result is supported by other studies that reported that TZ has activity against a range of bacteria, namely Gram-negative (Viveiros *et al*., 2005; Martins *et*

*al*., 2008). Recently it has been reported that pathogen's cell membranes and bioenergetics represent effective therapeutic targets (Hurdle *et al*., 2011; Hasenoehrl, Wiggins & Berney, 2021). Here, it was also observed that at sub-inhibitory concentrations, TZ damages the outer membrane of *S.* Typhimurium, resulting on depolarisation of the membrane potential and leading to a reduction of the intracellular production of ATP. This interferes with the proton motive force and the bioenergetics of the bacterial cells, subsequently inhibiting the efflux activity that is dependent on energy. This interference with the proton motive force can also potentially impact on the reduced motility (both swimming and swarming) of *Salmonella* Typhimurium as it was observed. It is known that flagella are driven by a rotary motor that uses the free energy stored in the electrochemical proton gradient across the cytoplasmic membrane to do the mechanical work. Therefore, if TZ is able to interfere with this electrochemical proton gradient then several other bacterial systems can be impacted and not only efflux systems. The PMF is essential for a variety of critical bacterial processes, such as ATP synthesis and flagellar motility. Collapsing of PMF inhibits these important functions for bacteria and thus resulting in the loss of bacterial viability. A schematic (**Figure 3.17**; section 3.4; page 110) considering these effects on the bacteria was proposed. To note that the inhibition of efflux activity and motility may not occur on the exact order that is presented on the schematics, but for simplicity it was represented and discussed this way.

Here, it was demonstrated that in a first stage, TZ compromises the bacterial membrane. This initial step destabilises the bacterial membrane generating a cascade of events that involve significant decrease of energy production, affecting the activity of efflux pumps (dependent on energy), namely AcrAB-TolC, the main efflux system in *Salmonella*. Bacterial efflux pumps are membrane proteins which function is to decrease the intracellular concentrations of antibiotics, other antimicrobial compounds, dyes, *etc*, by extruding these out of the bacterial cells, thereby reducing their effectiveness. The use of compounds, such as TZ, that are able to interfere with the energy required for the efflux systems to function is therefore of interest. By interfering with the efflux systems and decreasing the ability of the bacteria to extrude noxious compounds, the antibiotics effectiveness can be maintained. This could be a promising combination therapy to revive "old antibiotics" that were set aside due to the lack of activity or increased toxicity. The use of TZ and

other possible adjuvants can in the future replace the conventional therapy mainly reliant on the use of antibiotics or combinations of thereof.

From the proteomics results, several bacterial targets were obtained, specifically the upregulation of TolC and the downregulation of AtpD. These two proteins are mainly involved in efflux and ATP binding. Clearly, the *in vitro* results presented in this thesis show that TZ has a pleiotropic effect on *Salmonella*. These results were to the best of my knowledge the first ones to show the broad effect of TZ in *Salmonella* Typhimurium, indicating an extended and more complex mechanism of action than the one initially described (only an EPI; with direct blocking of efflux pumps), Taken together, these are very promising results since Gram-negative bacteria are becoming more difficult to treat due to the composition of the cell wall and infections caused by these bacteria have dramatically increased in all parts of the world. The development of new drugs to target Gram-negative bacteria (in the WHO list of priority pathogens) is very low (World Health Organization, 2019b). Therefore, the discovery and development of new compounds to treat infections caused by Gram-negative bacteria commonly relies on targeting at least one membrane or often both membranes (outer and inner membrane) of the bacterial cell envelope. If an antimicrobial compound is able to penetrate one or both membranes it is subject to extrusion from the cell, promoted by numerous broadly acting efflux pumps. This scenario commonly renders the compound ineffective. Therefore, repurposing of TZ (or compounds with similar mechanism) would be an excellent approach to treat Gram-negative bacteria. The same approach could be applied to Gram-positive bacteria and ultimately to *M. tuberculosis.* Since TZ is able to penetrate and compromise the cell wall of the bacteria, it can represent a solution for the unsolved problem of difficult to treat bacterial infections that have a worldwide impact in health and economy, alike.

The results obtained *in vitro* correlated with the bacteria survival inside the host showing that when a *Salmonella*-infected macrophage is treated with TZ, there is a significant decrease in the phagocytosed bacteria recovered. It is known that *Salmonella* Typhimurium is able to make its metabolic survival inside macrophages by acquiring intracellular glucose and undergoing glycolysis and Tricarboxylic Acid Cycle (TCA). On Chapter 4 of this Thesis, the effect of TZ on *Salmonella*-infected human macrophages was investigated. The human THP-1 cell line was chosen for

this purpose for several reasons, as follows: 1) this is a well-established human model for testing of phenothiazine compounds (Ordway *et al*., 2002a,b, 2003; Martins *et al*., 2004; Machado *et al*., 2016); 2) the differentiation protocol with PMA that was followed for *Salmonella* infections is well established in the area by renowned labs on the field, such as the Steele-Mortimer lab; Laboratory of Bacteriology located at the National Institute of Allergy and Infectious Diseases (NIH), Rocky Mountain Laboratories, Hamilton, USA); 3) due to the purpose of the study in investigating the potential of using TZ as a macrophage activator; a cell line with low killing (such as THP-1) would be selected. 4) The use of TZ as a potential adjuvant to human therapy; therefore, the relevance of studying a human cell line (and not a murine one); 5) the fact that is a well-established model for macrophage function assays (Auwerx, 1991; Chanput, Mes & Wichers, 2014; Bosshart & Heinzelmann, 2016; Starr *et al*., 2018; Tedesco *et al*., 2018; Madhvi *et al*., 2019).

It was hypothesised that TZ might have a dual target on an *ex-vivo* macrophage model. TZ has been proposed to be a $Ca^{2+}$ modulator, able to enhance the killing activity of infected macrophages. In order to pursue this hypothesis, a well-known $Ca^{2+}$ modulator (VER) was used. Contrary to TZ, this $Ca^{2+}$ channel blocker does not have demonstrated *in vitro* antimicrobial activity at physiologically relevant concentrations (MIC = 2500 µg/mL). When TZ was added to *Salmonella*-infected macrophages a decrease (- 2.5 log CFU at 22.5 p.i.) on the numbers of phagocytosed bacteria was obtained. A similar decrease (- 3.9 log CFU at 22.5 p.i. was obtained in *Salmonella*(Martins *et al*., 2006; Adams, Szumowski & Ramakrishnan, 2014; Machado *et al*., 2016). Although these results seem to indicate that VER is more active than TZ since it showed a higher reduction (lower than 50%) on intracellular bacteria comparing with TZ (approximately 50%), in reality the amount of compound used was higher. When this effect is compared based on the molarity of the compounds, it is clear that VER was used at 162 µM while TZ was only used at 2.45 µM. Therefore, TZ is more effective in reducing the concentration of phagocytosed *Salmonella.*

Another antipsychotic (LOX) was also tested for potential activity in infected macrophages. In the same conditions, LOX showed a reduction on the growth of intracellular *S.* Typhimurium. A recent study from 2019, reported LOX activity against *S.* Typhimurium in murine infected macrophage (Yang *et al*., 2019). Comparing the

results obtained in this Thesis with that study, different concentrations of compound were used. The study from Yang *et al*., used a reduced concentration of 3.56 µM, while in this project 56 µM were used. However, it is important to refer that the *in vitro* MIC of LOX wasn't reported on the study conducted by Yang *et al*. This might suggest that LOX does not have a concentration dependent effect, as the one obtained with TZ. Comparing the activity of TZ, VER and LOX in this Thesis, TZ was the more effective compound for treating *Salmonella*-infected macrophages.

At the immune response level on *Salmonella*-infected macrophages after treatment with TZ, no changes were observed for the cytokines measured. This indicates that TZ had no direct immunomodulatory effect on infected macrophages that enhances the killing of intracellular *Salmonella*.

On the other side, when comparing the intracellular levels of $Ca^{2+}$ in a situation of infection, infected macrophages treated with TZ and VER showed significant reduced levels. As far as I am aware, this is the first time that it is demonstrated and quantified that TZ interferes with the $Ca^{2+}$ level on a macrophage infected with *Salmonella*. This result correlates with previous studies from Machado *et al*. and *Gupta et al.* on mycobacterial infected macrophages and phagolysosome acidification as a direct effect upon treatment with VER (Gupta *et al*., 2013; Machado *et al*., 2016).

Taken together all the previous results, TZ appears to be an excellent candidate for adjuvant therapy. However, some researchers and clinicians might raise concerns with the repurposing of this compound. TZ was removed from the market since 2005 due to evidence of connection between QTc prolongation (measure of delayed ventricular repolarisation, meaning that the heart muscle takes longer than normal to recharge between beats) and cardiac arrhythmias in patients with schizophrenia. However, for the past 60 years TZ has been the most used neuroleptic in clinical practice, and the most serious side effect (sudden death) is quite infrequent. One thing to consider when discussing the repurposing of TZ as an antimicrobial are the dosages and the duration of the treatment. The dosage administered to patients with QTc prolongations is usually quite high (300 mg) compared with dosages given at early managements of psychosis and schizophrenia. The treatment of these conditions with TZ is associated with a frequent administration of this compound. In a study published by Thanacoody it was

demonstrated that patients receiving treatment with TZ and who were free of cardiopathy showed no significant signs of danger (Thanacoody, 2007).

Further work will be necessary to confirm the full mechanism of action within infected macrophages, as for example, if the decrease of the level of calcium in the cytoplasm of the macrophage correlates with an increase on calcium present on the phagolysosome. Also, it can be speculated that if TZ interferes with AcrAB-TolC this can have an impact on *Salmonella* virulence factors. Understanding these interactions (TZ and infected macrophage) could change the way new compounds are designed and could provide some novel compounds useful in the battle against multidrug resistant infections, contributing to overcome the overall problem of AMR. Of important note is the low toxicity of TZ when tested in human monocytes. Concerns regarding TZ mode of action in eukaryotic cells, have also been raised since TZ may potentially interfere with the PMF on these cells. In eukaryotic cells, the respiratory chain and resulting PMF is in the mitochondrial membrane instead of the cytoplasmic membrane. This distinct localisation of the respiratory chain between eukaryotic and bacterial cells could partly explain the observed low toxicity (Wu *et al*., 1999). Also, these effects are concentration dependent and the concentrations used in this Thesis were shown to be devoid of toxicity to human monocytes and also clinically relevant.

In summary, this Thesis revealed new *in vitro* data regarding specific targets of TZ in *Salmonella* as well as the effect of TZ in reducing bacterial load on *Salmonella* infected macrophages. The results obtained in this Thesis also stress the potential use of TZ for adjuvant therapy of problematic antibiotic resistant infections, namely, the ones caused by intracellular pathogens.

# References

**Aarestrup FM, Hasman H. 2004.** Susceptibility of different bacterial species isolated from food animals to copper sulphate, zinc chloride and antimicrobial substances used for disinfection. *Veterinary Microbiology* 100:83–89.

**Abbate E, Vescovo M, Natiello M, Cufré M, García A, Ambroggi M, Poggi S, Simboli N, Ritacco V. 2007.** Tuberculosis extensamente resistente (XDR-TB) en Argentina: aspectos destacables epidemiológicos, bacteriológicos, terapéuticos y evolutivos. *Revista Argentica de Medicina Respiratória* 1:19–25.

**Abbate E, Vescovo M, Natiello M, Cufre M, Garcia A, Gonzalez Montaner P, Ambroggi M, Ritacco V, van Soolingen D. 2012**. Successful alternative treatment of extensively drug-resistant tuberculosis in Argentina with a combination of linezolid, moxifloxacin and thioridazine. *Journal of Antimicrobial Chemotherapy* 67:473–477.

**Abrahams GL, Hensel M. 2006**. Manipulating cellular transport and immune responses: Dynamic interactions between intracellular *Salmonella enterica* and its host cells. *Cellular Microbiology* 8:728–737.

**Adams KN, Szumowski JD, Ramakrishnan L. 2014**. Verapamil, and its metabolite norverapamil, inhibit macrophage-induced, bacterial efflux pump-mediated tolerance to multiple anti-tubercular drugs. *Journal of Infectious Diseases* 210:456–466.

**Agarwal S, Piesco NP, Johns LP, Riccelli AE. 1995**. Differential Expression of IL-1β, TNF-α, IL-6, and IL-8 in Human Monocytes in Response to Lipopolysaccharides from Different Microbes. *Journal of Dental Research* 74:1057–1065.

**Alder JD. 2005**. Daptomycin, a new drug class for the treatment of gram-positive infections. *Drugs of Today* 41:81–90.

**Aldo PB, Craveiro V, Guller S, Mor G. 2013.** Effect of culture conditions on the phenotype of THP-1 monocyte cell line. *American Journal of Reproductive Immunology* 70:80–86.

**Alekshun MN, Levy SB. 2007.** Molecular Mechanisms of Antibacterial Multidrug Resistance. *Cell* 128:1037–1050.

**Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J. 2010.** Call of the wild: Antibiotic resistance genes in natural environments. *Nature Reviews Microbiology* 8:251–259.

**Alves-Barroco C, Roma-Rodrigues C, Raposo LR, Brás C, Diniz M, Caço J, Costa PM, Santos-Sanches I, Fernandes AR. 2019.** Streptococcus dysgalactiae subsp. dysgalactiae isolated from milk of the bovine udder as emerging pathogens: *In vitro* and *in vivo* infection of human cells and zebrafish as biological models. *MicrobiologyOpen* 8:e00623.

**Alves Ferreira D, Martins LMDRS, Fernandes AR, Martins M. 2020.** A Tale of Two Ends: Repurposing Metallic Compounds from Anti-Tumour Agents to Effective Antibacterial Activity. *Antibiotics* 9:321.

**Amaral L, Boeree MJ, Gillespie SH, Udwadia ZF, van Soolingen D. 2010a.** Thioridazine cures extensively drug-resistant tuberculosis (XDR-TB) and the need for global trials is now! *International Journal of Antimicrobial Agents* 35:524–526.

**Amaral L, Kristiansen JE. 2000.** Phenothiazines: an alternative to conventional therapy for the initial management of suspected multidrug resistant tuberculosis. A call for studies. *International journal of antimicrobial agents* 14:173–6.

**Amaral L, Kristiansen JE, Abebe LS, Millett W. 1996**. Inhibition of the respiration of multi-drug resistant clinical isolates of *Mycobacterium tuberculosis* by thioridazine: potential use for initial therapy of freshly diagnosed tuberculosis. *Journal of Antimicrobial Chemotherapy* 38:1049–1053.

**Amaral L, Kristiansen JE, Frolund Thomsen V, Markovich B. 2000**. The effects of chlorpromazine on the outer cell wall of *Salmonella* typhimurium in ensuring resistance to the drug. *International journal of antimicrobial agents* 14:225–9.

**Amaral L, Kristiansen JE, Viveiros M, Atouguia J. 2001**. Activity of phenothiazines against antibiotic-resistant *Mycobacterium tuberculosis*: a review supporting further studies that may elucidate the potential use of thioridazine as anti-tuberculosis therapy. *The Journal of antimicrobial chemotherapy* 47:505–11.

**Amaral L, Martins A, Molnar J, Kristiansen JE, Martins M, Viveiros M,**

**Rodrigues L, Spengler G, Couto I, Ramos J, Dastidar S, Fanning S, McCusker M, Pages J-M. 2010b.** Phenothiazines, bacterial efflux pumps and targeting the macrophage for enhanced killing of intracellular XDRTB. *In vivo (Athens, Greece)* 24:409–24.

**Amaral L, Martins A, Spengler G, Martins M, Rodrigues L, McCusker M, Ntokou E, Cerca P, Machado L, Viveiros M, Couto I, Fanning S, Kristiansen J, Molnar J. 2012a.** Structure, Genetic Regulation, Physiology and function of the acrab-tolc efflux pump of *Escherichia coli* and *Salmonella*. In: *Antimicrobial Drug Discovery: Emerging Strategies*. CABI Publishing, 44–61.

**Amaral L, Martins M, Viveiros M. 2007.** Enhanced killing of intracellular multidrug-resistant *Mycobacterium tuberculosis* by compounds that affect the activity of efflux pumps. *Journal of Antimicrobial Chemotherapy* 59:1237–1246.

**Amaral L, Spengler G, Martins A, Armada A, Handzlik J, Kiec-Kononowicz K, Molnar J. 2012b.** Inhibitors of bacterial efflux pumps that also inhibit efflux pumps of cancer cells. *Anticancer research* 32:2947–57.

**Amaral L, Viveiros M. 2012.** Why thioridazine in combination with antibiotics cures extensively drug-resistant *Mycobacterium tuberculosis* infections. *International Journal of Antimicrobial Agents* 39:376–380.

**Amaral L, Viveiros M, Kristiansen JE. 2001.** Phenothiazines: potential alternatives for the management of antibiotic resistant infections of tuberculosis and malaria in developing countries. *Tropical Medicine and International Health* 6:1016–1022.

**Amaral L, Viveiros M, Molnar J, Kristiansen JE. 2011.** Effective therapy with the neuroleptic thioridazine as an adjunct to second line of defence drugs, and the potential that thioridazine offers for new patents that cover a variety of new uses. *Recent patents on anti-infective drug discovery* 6:84–7.

**Aminov RI. 2010.** A Brief History of the Antibiotic Era: Lessons Learned and Challenges for the Future. *Frontiers in Microbiology* 1:1–7.

**Amuasi JH, May J. 2019.** Non-typhoidal *Salmonella*: invasive, lethal, and on the loose. *The Lancet Infectious Diseases* 19:1267–1269.

**Arango Duque G, Descoteaux A. 2014.** Macrophage cytokines: involvement in immunity and infectious diseases. *Frontiers in immunology* 5:491.

**Ashburn TT, Thor KB. 2004**. Drug repositioning: Identifying and developing new uses for existing drugs. *Nature Reviews Drug Discovery* 3:673–683.

**Auwerx J. 1991.** The human leukemia cell line, THP-1: A multifacetted model for the study of monocyte-macrophage differentiation. *Experientia* 47:22–31.

**Balasubramanian R, Im J, Lee JS, Jeon HJ, Mogeni OD, Kim JH, Rakotozandrindrainy R, Baker S, Marks F. 2019.** The global burden and epidemiology of invasive non-typhoidal *Salmonella* infections. *Human Vaccines and Immunotherapeutics* 15:1421–1426.

**Baptista PV, McCusker MP, Carvalho A, Ferreira DA, Mohan NM, Martins M, Fernandes AR. 2018.** Nano-strategies to fight multidrug resistant bacteria-"A Battle of the Titans". *Frontiers in Microbiology* 9.

**Barrett JF. 2005.** Can biotech deliver new antibiotics? *Current Opinion in Microbiology* 8:498–503.

**Bassett EJ, Keith MS, Armelagos GJ, Martin DL, Villanueva AR. 1980.** Tetracycline-labeled human bone from ancient Sudanese Nubia (A.D. 350). *Science* 209:1532–1534.

**Baucheron S, Mouline C, Praud K, Chaslus-Dancla E, Cloeckaert A. 2005**. TolC but not AcrB is essential for multidrug-resistant *Salmonella enterica* serotype Typhimurium colonization of chicks. *Journal of Antimicrobial Chemotherapy* 55:707–712.

**Berendonk TU, Manaia CM, Merlin C, Fatta-Kassinos D, Cytryn E, Walsh F, Bürgmann H, Sørum H, Norström M, Pons MN, Kreuzinger N, Huovinen P, Stefani S, Schwartz T, Kisand V, Baquero F, Martinez JL. 2015.** Tackling antibiotic resistance: The environmental framework. *Nature Reviews Microbiology* 13:310–317.

**Bernthsen A. 1883.** Ueber das Methylenblau. *Berichte der deutschen chemischen Gesellschaft* 16:1025–1028.

**Berridge M V., Tan AS. 1993.** Characterization of the Cellular Reduction of 3-(4,5-

dimethylthiazol-2- yl)-2,5-diphenyltetrazolium bromide (MTT): Subcellular Localization, Substrate Dependence, and Involvement of Mitochondrial Electron Transport in MTT Reduction. *Archives of Biochemistry and Biophysics* 303:474–482.

**Beuzón CR, Méresse S, Unsworth KE, Ruíz-Albert J, Garvis S, Waterman SR, Ryder TA, Boucrot E, Holden DW. 2000.** *Salmonella* maintains the integrity of its intracellular vacuole through the action of SifA. *EMBO Journal* 19:3235–3249.

**Biot C, Glorian G, Maciejewski LA, Brocard JS, Domarle O, Blampain G, Millet P, Georges AJ, Lebibi J. 1997.** Synthesis and antimalarial activity *in vitro* and *in vivo* of a new ferrocene-chloroquine analogue. *Journal of Medicinal Chemistry* 40:3715–3718.

**Bodoni P. 1899.** Dell'azione sedative del blue di metilene in varie forme di psicosi. *Clinica Medica Italiana*:217–222.

**Van Boeckel TP, Brower C, Gilbert M, Grenfell BT, Levin SA, Robinson TP, Teillant A, Laxminarayan R. 2015.** Global trends in antimicrobial use in food animals. *Proceedings of the National Academy of Sciences of the United States of America* 112:5649–5654.

**Bohnert JA, Kern W V. 2005.** Selected arylpiperazines are capable of reversing multidrug resistance in *Escherichia coli* overexpressing RND efflux pumps. *Antimicrobial Agents and Chemotherapy* 49:849–852.

**Bonde M, Højland DH, Kolmos HJ, Kallipolitis BH, Klitgaard JK. 2011.** Thioridazine affects transcription of genes involved in cell wall biosynthesis in methicillin-resistant *Staphylococcus aureus*. *FEMS Microbiology Letters* 318:168–176.

**Bosshart H, Heinzelmann M. 2016.** THP-1 cells as a model for human monocytes. *Annals of Translational Medicine* 4.

**Boto L, Martínez JL. 2011.** Ecological and Temporal Constraints in the Evolution of Bacterial Genomes. *Genes* 2:804–828.

**Bredahl EC, Eckerson JM, Tracy SM, McDonald TL, Drescher KM. 2021**. The

role of creatine in the development and activation of immune responses. *Nutrients* 13:1–17.

**Brenner FW, Villar RG, Angulo FJ, Tauxe R, Swaminathan B. 2000**. *Salmonella* nomenclature. *Journal of Clinical Microbiology* 38:2465–2467.

**Brogden KA. 2005.** Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? *Nature Reviews Microbiology* 3:238–250.

**Buchmeier NA, Heffron F. 1991.** Inhibition of macrophage phagosome-lysosome fusion by *Salmonella* typhimurium. *Infection and Immunity* 59:2232–2238.

**Butler MS, Blaskovich MA, Cooper MA. 2013.** Antibiotics in the clinical pipeline in 2013. *Journal of Antibiotics* 66:571–591.

**Butler MS, Blaskovich MA, Cooper MA. 2017.** Antibiotics in the clinical pipeline at the end of 2015. *Journal of Antibiotics* 70:3–24.

**Carvalho IT, Santos L. 2016.** Antibiotics in the aquatic environments: A review of the European scenario. *Environment International* 94:736–757.

**Casewell M, Friis C, Marco E, McMullin P, Phillips I. 2003**. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. *Journal of Antimicrobial Chemotherapy* 52:159–161.

**Cassini A, Högberg LD, Plachouras D, Quattrocchi A, Hoxha A, Simonsen GS, Colomb-Cotinat M, Kretzschmar ME, Devleesschauwer B, Cecchini M, Ouakrim DA, Oliveira TC, Struelens MJ, Suetens C, Monnet DL, Strauss R, Mertens K, Struyf T, Catry B, Latour K, Ivanov IN, Dobreva EG, Tambic Andraševic A, Soprek S, Budimir A, Paphitou N, Žemlicková H, Schytte Olsen S, Wolff Sönksen U, Märtin P, Ivanova M, Lyytikäinen O, Jalava J, Coignard B, Eckmanns T, Abu Sin M, Haller S, Daikos GL, Gikas A, Tsiodras S, Kontopidou F, Tóth Á, Hajdu Á, Guólaugsson Ó, Kristinsson KG, Murchan S, Burns K, Pezzotti P, Gagliotti C, Dumpis U, Liuimiene A, Perrin M, Borg M. A, de Greeff SC, Monen JCM, Koek MBG, Elstrøm P, Zabicka D, Deptula A, Hryniewicz W, Caniça M, Nogueira PJ, Fernandes PA, Manageiro V, Popescu GA, Serban RI, Schréterová E, Litvová S, Štefkovicová M, Kolman J, Klavs I, Korošec A, Aracil B, Asensio A, Pérez-Vázquez M, Larsson S, Reilly JS, Johnson A, Hopkins S. 2019.** Attributable

deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. *The Lancet Infectious Diseases* 19:56–66.

**Center for Disease Control and Prevention |CDC. 2019**. *Antibiotic Resistance Threats in the United States 2019*. Altante, GA, US.

**Centers for Disease Control and Prevention | CDC. 2019.** *Salmonella* | *CDC*.

**Chanput W, Mes JJ, Wichers HJ. 2014.** THP-1 cell line: An *in vitro* cell model for immune modulation approach. *International Immunopharmacology* 23:37–45. DOI: 10.1016/j.intimp.2014.08.002.

**Charpantier P. 1947.** Chimie organique-sur la constitution dunne dimethilamino-propyl-N-phenothiazine. *Comptes Rendus Hebdomadaries des Seances I Acad des Sci*:306–308.

**Cheng RA, Eade CR, Wiedmann M. 2019.** Embracing diversity: Differences in virulence mechanisms, disease severity, and host adaptations contribute to the success of nontyphoidal *Salmonella*as a foodborne pathogen. *Frontiers in Microbiology* 10:1368.

**Clancy CJ, Nguyen MH. 2020.** Buying Time: The AMR Action Fund and the State of Antibiotic Development in the United States 2020. *Open Forum Infectious Diseases* 7.

**Clardy J, Fischbach MA, Currie CR. 2009.** The natural history of antibiotics. *Current Biology* 19.

**Clarkin O, Wren A, Thornton R, Cooney J, Towler M. 2011**. Antibacterial analysis of a zinc-based glass polyalkenoate cement. *Journal of Biomaterials Applications* 26:277–292.

**Clinical and Laboratory Standards Institute. 2013.** *Performance standards for antimicrobial susceptibility testing: twenty-third informational supplement. In: CLSI document M100-S23*.

**Coldham NG, Webber M, Woodward MJ, Piddock LJ V. 2010.** A 96-well plate fluorescence assay for assessment of cellular permeability and active efflux in *Salmonella enterica* serovar Typhimurium and *Escherichia coli*. *Journal of*

*Antimicrobial Chemotherapy* 65:1655–1663.

**Collignon P. 2013.** The importance of a one health approach to preventing the development and spread of antibiotic resistance. *Current Topics in Microbiology and Immunology* 366:19–36.

**Correia S, Hébraud M, Chafsey I, Chambon C, Viala D, Torres C, Caniça M, Capelo JL, Poeta P, Igrejas G. 2017.** Subproteomic signature comparison of *in vitro* selected fluoroquinolone resistance and ciprofloxacin stress in *Salmonella* Typhimurium DT104B. *Expert Review of Proteomics* 14:941–961.

**Council of the European Union. 1991**. Council Directive 91/271/EEC of 21 May 1991 concerning urban waste-water treatment. *EEC Council Directive*:10.

**Crowle AJ, Douvas GS, May MH. 1992.** Chlorpromazine: a drug potentially useful for treating mycobacterial infections. *Chemotherapy* 38:410–9.

**Crump JA, Luby SP, Mintz ED. 2004**. The global burden of typhoid fever. *Bulletin of the World Health Organization* 82:346–353.

**Daniel WA. 2003.** Mechanisms of cellular distribution of psychotropic drugs. Significance for drug action and interactions. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 27:65–73.

**Daniel WA, Bickel MH, Honegger UE. 1995.** The contribution of lysosomal trapping in the uptake of desipramine and chloroquine by different tissues. *Pharmacology & toxicology* 77:402–6.

**Daniel WA, Wójcikowski J. 1999.** The Role of Lysosomes in the Cellular Distribution of Thioridazine and Potential Drug Interactions. *Toxicology and Applied Pharmacology* 158:115–124.

**Das S, Garg T, Chopra S, Dasgupta A. 2019.** Repurposing disulfiram to target infections caused by non-tuberculous mycobacteria. *Journal of Antimicrobial Chemotherapy* 74:1317–1322.

**Dasgupta A, Mukherjee S, Chaki S, Dastidar SG, Hendricks O, Christensen JB, Kristiansen JE, Amaral L. 2010.** Thioridazine protects the mouse from a virulent infection by *Salmonella enterica* serovar Typhimurium 74. *International Journal of Antimicrobial Agents* 35:174–176.

**Datsenko KA, Wanner BL. 2000.** One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proceedings of the National Academy of Sciences of the United States of America* 97:6640–6645.

**Debnath A, Parsonage D, Andrade RM, He C, Cobo ER, Hirata K, Chen S, García-Rivera G, Orozco E, Martínez MB, Gunatilleke SS, Barrios AM, Arkin MR, Poole LB, McKerrow JH, Reed SL. 2012.** A high-throughput drug screen for Entamoeba histolytica identifies a new lead and target. *Nature Medicine* 18:956–960.

**Delhaes L, Biot C, Berry L, Delcourt P, Maciejewski LA, Camus D, Brocard JS, Dive D. 2002.** Synthesis of Ferroquine Enantiomers: First Investigation of Effects of Metallocenic Chirality upon Antimalarial Activity and Cytotoxicity. *ChemBioChem* 3:418–423.

**Desai PT, Porwollik S, Long F, Cheng P, Wollam A, Clifton SW, Weinstock GM, McClelland M. 2013.** Evolutionary genomics of *Salmonella enterica* subspecies. *mBio* 4.

**Diacovich L, ıa Lorenzi L, Tomassetti M, ephane eresse SM, Gramajo H. 2017.** The infectious intracellular lifestyle of *Salmonella enterica* relies on the adaptation to nutritional conditions within the *Salmonella*-containing vacuole. 8:975–992.

**Durand GA, Raoult D, Dubourg G. 2019.** Antibiotic discovery: history, methods and perspectives. *International Journal of Antimicrobial Agents* 53:371–382.

**Dutta NK, Mehra S, Kaushal D. 2010.** A *Mycobacterium tuberculosis* Sigma Factor Network Responds to Cell-Envelope Damage by the Promising Anti-Mycobacterial Thioridazine. *PLoS ONE* 5:e10069.

**Dutta NK, Pinn ML, Karakousis PC. 2014a.** Sterilizing activity of thioridazine in combination with the first-line regimen against acute murine tuberculosis. *Antimicrobial agents and chemotherapy* 58:5567–9.

**Dutta NK, Pinn ML, Karakousis PC. 2014b.** Reduced Emergence of Isoniazid Resistance with Concurrent Use of Thioridazine against Acute Murine Tuberculosis. *Antimicrobial Agents and Chemotherapy* 58:4048–4053.

**EFSA (European Food Safety Authority), ECDC (European Centre for Disease Prevention and Control). 2017.** *The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2017*.

**Ehrlich P, Bertheim A. 1912.** Über das salzsaure 3.3′-Diamino-4.4′-dioxy-arsenobenzol und seine nächsten Verwandten. *Berichte der deutschen chemischen Gesellschaft* 45:756–766.

**Ehrlich P, Guttman P. 1891.** Ueber die Wirkung des Methylenblau bei Malaria. *Berliner klinische Wochenschrift* 28:953–6.

**Elkes J, Elkes C. 1954.** Effect of chlorpromazine on the behaviour of chronically overactive psychotic patients. *British Medical Journal* 2:560–565.

**European Centre for Disease Prevention and Control. 2019**.Antimicrobial resistance in the EU/EEA (EARS-Net) - Annual Epidemiological Report for 2019. *Available at https://www.ecdc.europa.eu/en/publications-data/surveillance-antimicrobial-resistance-europe-2019* (accessed May 7, 2021).

**Fàbrega A, Vila J. 2013.** *Salmonella enterica* serovar Typhimurium skills to succeed in the host: Virulence and regulation. *Clinical Microbiology Reviews* 26:308–341.

**Fang L, Li X, Li L, Li S, Liao X, Sun J, Liu Y. 2016**. Co-spread of metal and antibiotic resistance within ST3-IncHI2 plasmids from *E. coli* isolates of food-producing animals. *Scientific Reports* 6:25312.

**FAO, WHO. 2019.** *Joint FAO/WHO Expert Meeting in collaboration with OIE on Foodborne Antimicrobial Resistance:Role of the Environment, Crops and Biocides - Meeting report*. Rome.

**Farha MA, Brown ED. 2019**. Drug repurposing for antimicrobial discovery. *Nature Microbiology* 4:565–577.

**Farha MA, Verschoor CP, Bowdish D, Brown ED. 2013.** Collapsing the Proton Motive Force to Identify Synergistic Combinations against *Staphylococcus aureus*. *Chemistry & Biology* 20:1168–1178.

**Ferrie JE. 2014.** Arsenic, antibiotics and interventions. *International Journal of Epidemiology*:977–982.

**Feske S, Wulff H, Skolnik EY. 2015**. Ion channels in innate and adaptive immunity. *Annual Review of Immunology* 33:291–353.

**Finn CE, Chong A, Cooper KG, Starr T, Steele-Mortimer O. 2017**. A second wave of *Salmonella* T3SS1 activity prolongs the lifespan of infected epithelial cells. *PLoS Pathogens* 13.

**Fishovitz J, Hermoso JA, Chang M, Mobashery S. 2014.** Penicillin-binding protein 2a of methicillin-resistant *Staphylococcus aureus*. *IUBMB Life* 66:572–577.

**Fleming A. 1929.** On the antibacterial action of cultures of a Penicillium, with special reference to theit use in the isolantion of B. influenzae. *The British Journal of Experimental Pathology* 10:226–236.

**Francis CL, Starnbach MN, Falkow S. 1992.** Morphological and cytoskeletal changes in epithelial cells occur immediately upon interaction with *Salmonella* typhimurium grown under low-oxygen conditions. *Molecular Microbiology* 6:3077–3087.

**Freeman JA, Rappl C, Kuhle V, Hensel M, Miller SI. 2002.** SpiC is required for translocation of *Salmonella* pathogenicity island 2 effectors and secretion of translocon proteins SseB and SseC. *Journal of Bacteriology* 184:4971–4980.

**Frei A. 2020.** Metal Complexes, an Untapped Source of Antibiotic Potential? *Antibiotics* 9:90.

**Frei A, Zuegg J, Elliott AG, Baker M V., Braese S, Brown C, Chen F, Dowson CG, Dujardin G, Jung N, King AP, Mansour AM, Massi M, Moat J, Mohamed HA, Renfrew A, Rutledge P, Sadler P. ., Todd MH, Willans CE, Wilson JJ, Cooper MA, Blaskovich M. 2020.** Metal Complexes as a Promising Source for New Antibiotics. *Chemical Science* 68:42–61.

**Frión-Herrera Y, Gabbia D, Scaffidi M, Zagni L, Cuesta-Rubio O, De Martin S, Carrara M. 2020.** Cuban brown propolis interferes in the crosstalk between colorectal cancer cells and m2 macrophages. *Nutrients* 12:1–16.

**Gal-Mor O, Boyle EC, Grassl GA. 2014**. Same species, different diseases: How

and why typhoidal and non-typhoidal *Salmonella enterica* serovars differ. *Frontiers in Microbiology* 5:391.

**Galan JE, Curtiss R. 1989**. Cloning and molecular characterization of genes whose products allow *Salmonella* typhimurium to penetrate tissue culture cells. *Proceedings of the National Academy of Sciences of the United States of America* 86:6383–6387.

**Galán JE, Zhou D. 2000.** Striking a balance: Modulation of the actin cytoskeleton by *Salmonella*. *Proceedings of the National Academy of Sciences of the United States of America* 97:8754–8761.

**Galeazzi L, Turchetti G, Grilli G, Groppa G, Giunta S. 1986.** Chlorpromazine as permeabilizer and reagent for detection of microbial peroxidase and peroxidaselike activities. *Applied and environmental microbiology* 52:1433–5.

**Ganeshan K, Chawla A. 2014.** Metabolic regulation of immune responses. *Annual Review of Immunology* 32:609–634.

**Gasser G, Ott I, Metzler-Nolte N. 2011.** Organometallic anticancer compounds. *Journal of Medicinal Chemistry* 54:3–25.

**Gilchrist JJ, MacLennan CA. 2019**. Invasive Nontyphoidal *Salmonella* Disease in Africa. *EcoSal Plus* 8.

**Goldstein I, Lue TF, Padma-Nathan H, Rosen RC, Steers WD, Wicker PA. 1998.** Oral Sildenafil in the Treatment of Erectile Dysfunction. *New England Journal of Medicine* 338:1397–1404.

**Gonçalves P, Araújo JR, Martel F. 2015**. Antipsychotics-induced metabolic alterations: Focus on adipose tissue and molecular mechanisms. *European Neuropsychopharmacology* 25:1–16.

**Grimont PAD, Weill FX. 2007.** *Antigenic formulae of the Salmonella serovars, 9th revision. WHO Collaborating Centre for Reference and Research on Salmonella*. Paris, France.

**Gross TJ, Kremens K, Powers LS, Brink B, Knutson T, Domann FE, Philibert RA, Milhem MM, Monick MM. 2014.** Epigenetic Silencing of the Human NOS2 Gene: Rethinking the Role of Nitric Oxide in Human Macrophage Inflammatory

Responses. *The Journal of Immunology* 192:2326–2338.

**Gupta S, Salam N, Srivastava V, Singla R, Behera D. 2009.** Voltage Gated Calcium Channels Negatively Regulate Protective Immunity to *Mycobacterium tuberculosis*. *PLoS ONE* 4:5305.

**Gupta S, Tyagi S, Almeida D V., Maiga MC, Ammerman NC, Bishai WR. 2013.** Acceleration of Tuberculosis Treatment by Adjunctive Therapy with Verapamil as an Efflux Inhibitor. *American Journal of Respiratory and Critical Care Medicine* 188:600–607.

**Guth PS, Spirtes MA. 1964.** The Phenothiazinetranquilizers: Biochemical and Biophysical Actions. *International Review of Neurobiology* 7:231–278.

**Hahn BL, Sohnle PG. 2014.** Effect of thioridazine on experimental cutaneous staphylococcal infections. *In vivo (Athens, Greece)* 28:33–8.

**Harrigan EP, Miceli JJ, Anziano R, Watsky E, Reeves KR, Cutler NR, Sramek J, Shiovitz T, Middle M. 2004.** A Randomized Evaluation of the Effects of Six Antipsychotic Agents on QTc, In the Absence and Presence of Metabolic Inhibition. *Journal of Clinical Psychopharmacology* 24:62–69.

**Harvey WR. 2009.** Voltage coupling of primary H+ V-ATPases to secondary Na+- or K+-dependent transporters. *Journal of Experimental Biology* 212:1620–1629.

**Hasenoehrl EJ, Wiggins TJ, Berney M. 2021.** Bioenergetic Inhibitors: Antibiotic Efficacy and Mechanisms of Action in *Mycobacterium tuberculosis*. *Frontiers in Cellular and Infection Microbiology* 10:815.

**Hautefort I, Thompson A, Eriksson-Ygberg S, Parker ML, Lucchini S, Danino V, Bongaerts RJM, Ahmad N, Rhen M, Hinton JCD. 2008**. During infection of epithelial cells *Salmonella enterica* serovar Typhimurium undergoes a time-dependent transcriptional adaptation that results in simultaneous expression of three type 3 secretion systems. *Cellular Microbiology* 10:958–984.

**He H, Arsenault RJ, Genovese KJ, Swaggerty CL, Johnson C, Nisbet DJ, Kogut MH. 2019.** Inhibition of calmodulin increases intracellular survival of *Salmonella* in chicken macrophage cells. *Veterinary Microbiology* 232:156–161.

**Hendrich AB, Lichacz K, Burek A, Michalak K. 2002.** Thioridazine induces

erythrocyte stomatocytosis due to interactions with negatively charged lipids. *Cellular & molecular biology letters* 7:1081–6.

**Hendricks O, Butterworth TS, Kristiansen JE. 2003a.** The in-vitro antimicrobial effect of non-antibiotics and putative inhibitors of efflux pumps on *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Int. J. Antimicrob. Agents* 22:263–264.

**Hendricks O, Butterworth TS, Kristiansen JE. 2003b**. The in-vitro antimicrobial effect of non-antibiotics and putative inhibitors of efflux pumps on *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *International Journal of Antimicrobial Agents* 22:262–264.

**Hernando-Amado S, Coque TM, Baquero F, Martínez JL. 2019.** Defining and combating antibiotic resistance from One Health and Global Health perspectives. *Nature Microbiology* 4:1432–1442.

**De Hert M, Detraux J, Van Winkel R, Yu W, Correll CU. 2012.** Metabolic and cardiovascular adverse effects associated with antipsychotic drugs. *Nature Reviews Endocrinology* 8:114–126.

**Hirayama D, Iida T, Nakase H. 2018.** The phagocytic function of macrophage-enforcing innate immunity and tissue homeostasis. *International Journal of Molecular Sciences* 19.

**Hocquet D, Muller A, Bertrand X. 2016.** What happens in hospitals does not stay in hospitals: antibiotic-resistant bacteria in hospital wastewater systems. *Journal of Hospital Infection* 93:395–402.

**Hoyal CR, Giron-Calle J, Forman HJ. 1998**. The alveolar macrophage as a model of calcium signaling in oxidative stress. *Journal of Toxicology and Environmental Health, Part B* 1:117–134.

**Hu OY, Curry SH. 1989**. Stability, human blood distribution and rat tissue localization of promazine and desmonomethylpromazine. *Biopharmaceutics & Drug Disposition* 10:537–548.

**Hua L, Hilliard JJ, Shi Y, Tkaczyk C, Cheng LI, Yu X, Datta V, Ren S, Feng H, Zinsou R, Keller A, O'Day T, Du Q, Cheng L, Damschroder M, Robbie G, Suzich J, Stover CK, Sellman BR. 2014.** Assessment of an anti-alpha-toxin

monoclonal antibody for prevention and treatment of *Staphylococcus aureus*-induced pneumonia. *Antimicrobial Agents and Chemotherapy* 58:1108–1117.

**Hughes D, Karlén A. 2014**. Discovery and preclinical development of new antibiotics. *Upsala Journal of Medical Sciences* 119:162–169.

**Hume DA. 2006.** The mononuclear phagocyte system. *Current Opinion in Immunology* 18:49–53.

**Hurdle JG, O'Neill AJ, Chopra I, Lee RE. 2011.** Targeting bacterial membrane function: An underexploited mechanism for treating persistent infections. *Nature Reviews Microbiology* 9:62–75.

**Hurley D, McCusker MP, Fanning S, Martins M. 2014.** *Salmonella*-host interactions - modulation of the host innate immune system. *Frontiers in immunology* 5:481.

**Interagency Coordination Group on Antimicrobial Resistance (IACG). 2019**. *No Time to wait: Securing the future from drug-resistance infections.*

**Iyer A, Madder A, Singh I. 2019.** Teixobactins: A new class of 21st century antibiotics to combat multidrug-resistant bacterial pathogens. *Future Microbiology* 14:457–460.

**Jaszczyszyn A, Gasiorowski K, Swiatek P, Malinka W, Cieslik-Boczula K, Petrus J, Czarnik-Matusewicz B. 2012.** Chemical structure of phenothiazines and their biological activity. *Pharmacol Rep.* 64:16–23.

**Kaatz GW, Moudgal V V., Seo SM, Kristiansen JE. 2003.** Phenothiazines and Thioxanthenes Inhibit Multidrug Efflux Pump Activity in *Staphylococcus aureus*. *Antimicrobial agents and chemotherapy* 47:719–726.

**Kaul G, Shukla M, Dasgupta A, Chopra S. 2019.** Update on drug-repurposing: is it useful for tackling antimicrobial resistance? *Future Microbiology* 14:892–831.

**de Keijzer J, Mulder A, de Haas PEW, de Ru AH, Heerkens EM, Amaral L, van Soolingen D, van Veelen PA. 2016.** Thioridazine Alters the Cell-Envelope Permeability of *Mycobacterium tuberculosis*. *Journal of Proteome Research* 15:1776–1786.

**Kern W V., Steinke P, Schumacher A, Schuster S, Baum H, Bohnert JA. 2005**.

Effect of 1-(1-naphthylmethyl)-piperazine, a novel putative efflux pump inhibitor, on antimicrobial drug susceptibility in clinical isolates of *Escherichia coli*. *Journal of Antimicrobial Chemotherapy* 57:339–343.

**Kincy-Cain T, Clements JD, Bost KL. 1996**. Endogenous and exogenous interleukin-12 augment the protective immune response in mice orally challenged with *Salmonella* dublin. *Infection and Immunity* 64.

**Kirk MD, Pires SM, Black RE, Caipo M, Crump JA, Devleesschauwer B, Döpfer D, Fazil A, Fischer-Walker CL, Hald T, Hall AJ, Keddy KH, Lake RJ, Lanata CF, Torgerson PR, Havelaar AH, Angulo FJ. 2015.** World Health Organization Estimates of the Global and Regional Disease Burden of 22 Foodborne Bacterial, Protozoal, and Viral Diseases, 2010: A Data Synthesis. *PLoS Medicine* 12.

**Klee CB, No WC, Draetta GF, Newton DL. 1986.** Different Modes of Interaction of Calmodulin with Its Target Enzymes. *Journal of Cardiovascular Pharmacology* 8:S52–S56.

**Kleemann A. 2012.** Antipsychotics. In: *Ullmann's Encyclopedia of Industrial Chemistry*. Weinheim, Germany,.

**Klein JA, Powers TSR, Knodler LA. 2017.** Measurement of *Salmonella enterica* internalization and vacuole lysis in epithelial cells. In: *Methods in Molecular Biology*. Humana Press Inc., 285–296.

**Klitgaard JK, Skov MN, Kallipolitis BH, Kolmos HJ. 2008.** Reversal of methicillin resistance in *Staphylococcus aureus* by thioridazine. *Journal of Antimicrobial Chemotherapy* 62:1215–1221.

**Knodler LA, Nair V, Steele-Mortimer O. 2014.** Quantitative Assessment of Cytosolic *Salmonella* in Epithelial Cells. *PLoS ONE* 9:84681.

**Knuff K, Finlay BB. 2017.** What the SIF Is happening—The role of intracellular *Salmonella*-induced filaments. *Frontiers in Cellular and Infection Microbiology* 7:335.

**Kornhuber J, Henkel AW, Groemer TW, Städtler S, Welzel O, Tripal P, Rotter A, Bleich S, Trapp S. 2010**. Lipophilic cationic drugs increase the permeability of

lysosomal membranes in a cell culture system. *Journal of Cellular Physiology* 224:152–164.

**Kotzampassi K, Giamarellos-Bourboulis EJ. 2012.** Probiotics for infectious diseases: More drugs, less dietary supplementation. *International Journal of Antimicrobial Agents* 40:288–296.

**Kristiansen JE, Amaral L. 1997.** The potential management of resistant infections with non-antibiotics. *The Journal of antimicrobial chemotherapy* 40:319–27.

**Kristiansen JE, Hendricks O, Delvin T, Butterworth TS, Aagaard L, Christensen JB, Flores VC, Keyzer H. 2007.** Reversal of resistance in microorganisms by help of non-antibiotics. *Journal of Antimicrobial Chemotherapy* 59:1271–1279.

**Kristiansen MM, Leandro C, Ordway D, Martins M, Viveiros M, Pacheco T, Molnar J, Kristiansen JE, Amaral L. 2006.** Thioridazine reduces resistance of methicillin-resistant *Staphylococcus aureus* by inhibiting a reserpine-sensitive efflux pump. *In vivo (Athens, Greece)* 20:361–6.

**Kristiansen JE, Vergmann B. 1986.** The antibacterial effect of selected phenothiazines and thioxanthenes on slow-growing mycobacteria. *Acta pathologica, microbiologica, et immunologica Scandinavica. Section B, Microbiology* 94:393–8.

**Krysan DJ, Didone L. 2008.** A high-throughput screening assay for small molecules that disrupt yeast cell integrity. *Journal of Biomolecular Screening* 13:657–664.

**Kuhle V, Hensel M. 2004.** Cellular microbiology of intracellular *Salmonella enterica*: Functions of the type III secretion system encoded by *Salmonella* pathogenicity island 2. *Cellular and Molecular Life Sciences* 61:2812–2826.

**Kujat Choy SL, Boyle EC, Gal-Mor O, Goode DL, Valdez Y, Vallance BA, Finlay BB. 2004.** SseK1 and SseK2 are novel translocated proteins of *Salmonella enterica* serovar Typhimurium. *Infection and Immunity* 72:5115–5125.

**Kunz E. 2014.** Henri laborit and the inhibition of action. *Dialogues in Clinical Neuroscience* 16:113–117.

**Laemmli UK. 1970.** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.

**Langedijk J, Mantel-Teeuwisse AK, Slijkerman DS, Schutjens MHDB. 2015.** Drug repositioning and repurposing: terminology and definitions in literature. *Drug Discovery Today* 20:1027–1034.

**Lathrop SK, Binder KA, Starr T, Cooper KG, Chong A, Carmody AB, Steele-Mortimer O. 2015.** Replication of *Salmonella enterica* serovar Typhimurium in human monocyte-derived macrophages. *Infection and Immunity* 83:2661–2671.

**Lee CC, Lee MTG, Hsu WT, Park JY, Porta L, Liu MA, Chen SC, Chang SC. 2021.** Use of Calcium Channel Blockers and Risk of Active Tuberculosis Disease: A Population-Based Analysis. *Hypertension* 77:328–337.

**Lemire JA, Harrison JJ, Turner RJ. 2013.** Antimicrobial activity of metals: mechanisms, molecular targets and applications. *Nature Reviews Microbiology* 11:371–384.

**Lerminiaux NA, Cameron ADS. 2019.** Horizontal transfer of antibiotic resistance genes in clinical environments. *Canadian Journal of Microbiology* 65:34–44.

**Liss V, Swart AL, Kehl A, Hermanns N, Zhang Y, Chikkaballi D, Böhles N, Deiwick J, Hensel M. 2017.** *Salmonella enterica* Remodels the Host Cell Endosomal System for Efficient Intravacuolar Nutrition. *Cell Host and Microbe* 21:390–402.

**Löber S, Jäckel D, Kaiser N, Hensel M. 2006.** Regulation of *Salmonella* pathogenicity island 2 genes by independent environmental signals. *International Journal of Medical Microbiology* 296:435–447.

**Lopez-Castejon G, Brough D. 2011.** Understanding the mechanism of IL-1β secretion. *Cytokine and Growth Factor Reviews* 22:189–195.

**López-Muñoz F, Alamo C, Cuenca E, Shen WW, Clervoy P, Rubio G. 2005.** History of the discovery and clinical introduction of chlorpromazine. *Annals of Clinical Psychiatry* 17:113–135.

**Lu G, Nagbanshi M, Goldau N, Mendes Jorge M, Meissner P, Jahn A, Mockenhaupt FP, Müller O. 2018.** Efficacy and safety of methylene blue in the treatment of malaria: A systematic review. *BMC Medicine* 16.

**Luís D V., Silva J, Tomaz AI, De Almeida RFM, Larguinho M, Baptista P V.,**

**Martins LMDRS, Silva TFS, Borralho PM, Rodrigues CMP, Rodrigues AS, Pombeiro AJL, Fernandes AR. 2014**. Insights into the mechanisms underlying the antiproliferative potential of a Co(II) coordination compound bearing 1,10-phenanthroline-5,6-dione: DNA and protein interaction studies. *Journal of Biological Inorganic Chemistry* 19:787–803.

**Lund ME, To J, O'Brien BA, Donnelly S. 2016**. The choice of phorbol 12-myristate 13-acetate differentiation protocol influences the response of THP-1 macrophages to a pro-inflammatory stimulus. *Journal of Immunological Methods* 430:64–70.

**Machado D, Couto I, Perdigão J, Rodrigues L, Portugal I, Baptista P, Veigas B, Amaral L, Viveiros M. 2012.** Contribution of Efflux to the Emergence of Isoniazid and Multidrug Resistance in *Mycobacterium tuberculosis*. *PLoS ONE* 7:e34538.

**Machado D, Pires D, Perdigão J, Couto I, Portugal I, Martins M, Amaral L, Anes E, Viveiros M. 2016.** Ion Channel Blockers as Antimicrobial Agents, Efflux Inhibitors, and Enhancers of Macrophage Killing Activity against Drug Resistant *Mycobacterium tuberculosis*. *PLOS ONE* 11:e0149326.

**Madhvi A, Mishra H, Leisching GR, Mahlobo PZ, Baker B. 2019.** Comparison of human monocyte derived macrophages and THP1-like macrophages as *in vitro* models for *M. tuberculosis* infection. *Comparative Immunology, Microbiology and Infectious Diseases* 67:101355.

**Maffioli SI, Sosio M, Ebright RH, Donadio S. 2019.** Discovery, properties, and biosynthesis of pseudouridimycin, an antibacterial nucleoside-analog inhibitor of bacterial RNA polymerase. *Journal of Industrial Microbiology and Biotechnology* 46:335–343.

**Mahamoud A, Chevalier J, Alibert-Franco S, Kern W V, Pagè J-M. 2007.** Antibiotic efflux pumps in Gram-negative bacteria: the inhibitor response strategy. *Journal of Antimicrobial Chemotherapy* 59:1223–1229.

**Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'Brien SJ, Jones TF, Fazil A, Hoekstra RM. 2010.** The Global Burden of Nontyphoidal *Salmonella* Gastroenteritis. *Clinical Infectious Diseases* 50:882–889.

**Mariconda S, Wang Q, Harshey RM. 2006.** A mechanical role for the chemotaxis system in swarming motility. *Molecular Microbiology* 60:1590–1602.

**Martins M, Bleiss W, Marko A, Ordway D, Viveiros M, Leandro C, Pacheco T, Molnar J, Kristiansen JE, Amaral L. 2004.** Clinical concentrations of thioridazine enhance the killing of intracellular methicillin-resistant *Staphylococcus aureus*: an *in vivo*, ex vivo and electron microscopy study. *In vivo (Athens, Greece)* 18:787–94.

**Martins M, Dastidar SG, Fanning S, Kristiansen JE, Molnar J, Pagès JM, Schelz Z, Spengler G, Viveiros M, Amaral L. 2008.** Potential role of non-antibiotics (helper compounds) in the treatment of multidrug-resistant Gram-negative infections: mechanisms for their direct and indirect activities. *International Journal of Antimicrobial Agents* 31:198–208.

**Martins A, Machado L, Costa S, Cerca P, Spengler G, Viveiros M, Amaral L. 2011.** Role of calcium in the efflux system of *Escherichia coli*. *International Journal of Antimicrobial Agents* 37:410–414.

**Martins M, Schelz Z, Martins A, Molnar J, Hajös G, Riedl Z, Viveiros M, Yalcin I, Aki-Sener E, Amaral L. 2007a**. *In vitro* and ex vivo activity of thioridazine derivatives against *Mycobacterium tuberculosis*. *International Journal of Antimicrobial Agents* 29:338–340.

**Martins M, Viveiros M, Amaral L. 2008**. Inhibitors of Ca2+ and K+ transport enhance intracellular killing of *M. tuberculosis* by non-killing macrophages. *In vivo (Athens, Greece)* 22:69–75.

**Martins M, Viveiros M, Couto I, Amaral L. 2009.** Targeting human macrophages for enhanced killing of intracellular XDR-TB and MDR-TB. *The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease* 13:569–73.

**Martins M, Viveiros M, Kristiansen JE, Molnar J, Amaral L. 2007b**. The curative activity of thioridazine on mice infected with *Mycobacterium tuberculosis*. *In vivo (Athens, Greece)* 21:771–5.

**Martins M, Viveiros M, Ordway D, Kristiansen JE, Molnar J, Amaral L. 2006.** Reserpine, ouabain and the calcium channel blocker verapamil, cause

intracellular killing of *Staphylococcus aureus*. *Research Journal of Microbiology* 1:203–209.

**McIlwain DR, Berger T, Mak TW. 2013.** Caspase functions in cell death and disease. *Cold Spring Harbor Perspectives in Biology* 5:1–28.

**Meek RW, Vyas H, Piddock LJ V. 2015.** Nonmedical Uses of Antibiotics: Time to Restrict Their Use? *PLoS Biology* 13:1–11.

**Meister M, Lowe G, Berg HC. 1987.** The proton flux through the bacterial flagellar motor. *Cell* 49:643–650.

**Mendo AS, Figueiredo S, Roma-Rodrigues C, Videira PA, Ma Z, Diniz M, Larguinho M, Costa PM, Lima JC, Pombeiro AJL, Baptista P V., Fernandes AR. 2015.** Characterization of antiproliferative potential and biological targets of a copper compound containing 4′-phenyl terpyridine. *Journal of Biological Inorganic Chemistry* 20:935–948.

**Michael CA, Dominey-Howes D, Labbate M. 2014.** The antimicrobial resistance crisis: Causes, consequences, and management. *Frontiers in Public Health* 2.

**Michalak K, Wesolowska O, Motohashi N, Molnar J, Hendrich AB. 2006**. Interactions of phenothiazines with lipid bilayer and their role in multidrug resistance reversal. *Current drug targets* 7:1095–105.

**Mohan NM, Zorgani A, Jalowicki G, Kerr A, Khaldi N, Martins M. 2019.** Unlocking NuriPep 1653 From Common Pea Protein: A Potent Antimicrobial Peptide to Tackle a Pan-Drug Resistant *Acinetobacter baumannii*. *Frontiers in Microbiology* 10.

**Moroney JW, Schlumbrecht MP, Helgason T, Coleman RL, Moulder S, Naing A, Bodurka DC, Janku F, Hong DS, Kurzrock R. 2011.** A phase I trial of liposomal doxorubicin, bevacizumab, and temsirolimus in patients with advanced gynecologic and breast malignancies. *Clinical Cancer Research* 17:6840–6846.

**Mosnaim AD, Ranade V V., Wolf ME, Puente J, Antonieta Valenzuela M. 2006.** Phenothiazine molecule provides the basic chemical structure for various classes of pharmacotherapeutic agents. *American Journal of Therapeutics*

13:261–273.

**Mousavizadeh K, Ghafourifar P, Sadeghi-Nejad H. 2002.** Calcium channel blocking activity of thioridazine, clomipramine and fluoxetine in isolated rat vas deferens: a relative potency measurement study. *The Journal of urology* 168:2716–9.

**Mozrzymas JW, Barberis A, Michalak K, Cherubini E. 1999**. Chlorpromazine inhibits miniature GABAergic currents by reducing the binding and by increasing the unbinding rate of GABA(A) receptors. *Journal of Neuroscience* 19:2474–2488.

**Murphy AJ, Guyre PM, Wira CR, Pioli PA. 2009.** Estradiol regulates expression of estrogen receptor ERα46 in human macrophages. *PLoS ONE* 4:e5539.

**Nadimpalli M, Delarocque-Astagneau E, Love DC, Price LB, Huynh BT, Collard JM, Lay KS, Borand L, Ndir A, Walsh TR, Guillemot D, De Lauzanne A, Kerleguer A, Tarantola A, Piola P, Chon T, Lach S, Ngo V, Touch S, Andrianirina ZZ, Vray M, Richard V, Seck A, Bercion R, Sow AG, Diouf JB, Dieye PS, Sy B, Ndao B, Seguy M, Watier L, Abdou AY. 2018.** Combating Global Antibiotic Resistance: Emerging One Health Concerns in Lower-and Middle-Income Countries. *Clinical Infectious Diseases* 66:963–969.

**Nelson ML, Dinardo A, Hochberg J, Armelagos GJ. 2010.** Brief communication: Mass spectroscopic characterization of tetracycline in the skeletal remains of an ancient population from Sudanese Nubia 350-550 CE. *American Journal of Physical Anthropology* 143:151–154.

**Nelson A, Smith LE. 1942.** Vapor Pressure of Phenothiazine The Vapor Pressure of Phenothiazine. *J. Am. Chem. Soc.* 64:3035–3037.

**Neuert S, Nair S, Day MR, Doumith M, Ashton PM, Mellor KC, Jenkins C, Hopkins KL, Woodford N, de Pinna E, Godbole G, Dallman TJ. 2018.** Prediction of phenotypic antimicrobial resistance profiles from whole genome sequences of non-typhoidal *Salmonella enterica*. *Frontiers in Microbiology* 9.

**Ni W, Li Y, Guan J, Zhao J, Cui J, Wang R, Liu Y. 2016.** Effects of efflux pump inhibitors on colistin resistance in multidrug-resistant gram-negative bacteria. *Antimicrobial Agents and Chemotherapy* 60:3115–3118.

**Norrby SR, Nord CE, Finch R, European Society of Clinical Microbiology and Infectious Diseases. 2005.** Lack of development of new antimicrobial drugs: a potential serious threat to public health. *The Lancet. Infectious diseases* 5:115–9.

**O' Neil J. 2014.** *Review on Antibiotic resisitance. Antimicrobial Resistance : Tackling a crisis for the health and wealth of nations*.

**Ohl ME, Miller SI. 2001.** *Salmonella*: A Model for Bacterial Pathogenesis. *Annual Review of Medicine* 52:259–274.

**Ohlow MJ, Moosmann B. 2011.** Phenothiazine: the seven lives of pharmacology's first lead structure. *Drug Discovery Today* 16:119–131.

**Ordway D, Viveiros M, Leandro C, Arroz MJ, Amaral L. 2002a**. Intracellular activity of clinical concentrations of phenothiazines including thioridiazine against phagocytosed *Staphylococcus aureus*. *International journal of antimicrobial agents* 20:34–43.

**Ordway D, Viveiros M, Leandro C, Arroz MJ, Molnar J, Kristiansen JE, Amaral L. 2002b.** Chlorpromazine has intracellular killing activity against phagocytosed *Staphylococcus aureus* at clinical concentrations. *Journal of Infection and Chemotherapy* 8:227–231.

**Ordway D, Viveiros M, Leandro C, Bettencourt R, Almeida J, Martins M, Kristiansen JE, Molnar J, Amaral L. 2003.** Clinical concentrations of thioridazine kill intracellular multidrug-resistant *Mycobacterium tuberculosis*. *Antimicrobial agents and chemotherapy* 47:917–22.

**Ouakrim DA, Hashiguchi TCO, Padget M, Cecchini M, Cassini A, Suetens C, Plachouras D, Monnet DL, Weist K. 2019.** *Antimicrobial Resistance Tackling the Burden in the European Union Briefing note for EU/EEA countries Contents*.

**Ozleyen A, Yilmaz YB, Tumer TB. 2021**. Dataset on the differentiation of THP-1 monocytes to LPS inducible adherent macrophages and their capacity for NO/iNOS signaling. *Data in Brief* 35:106786.

**Pace J, Hayman MJ, Galán JE. 1993.** Signal transduction and invasion of epithelial cells by S. typhimurium. *Cell* 72:505–514.

**Pagès JM, James CE, Winterhalter M. 2008.** The porin and the permeating antibiotic: A selective diffusion barrier in Gram-negative bacteria. *Nature Reviews Microbiology* 6:893–903.

**Park YK, Ko KS. 2015.** Effect of carbonyl cyanide 3-chlorophenylhydrazone (CCCP) on killing *Acinetobacter baumannii* by colistin. *Journal of Microbiology* 53:53–59.

**Patra M, Gasser G, Metzler-Nolte N. 2012.** Small organometallic compounds as antibacterial agents. *Dalton Transactions* 41:6350–6358.

**Patra M, Wenzel M, Prochnow P, Pierroz V, Gasser G, Bandow JE, Metzler-Nolte N. 2015.** An organometallic structure-activity relationship study reveals the essential role of a Re(CO)3 moiety in the activity against gram-positive pathogens including MRSA. *Chemical Science* 6:214–224.

**Pećanac M, Janjić Z, Komarcević A, Pajić M, Dobanovacki D, Misković SS. 2013**. Burns treatment in ancient times. *Medicinski pregled* 66:263–267.

**Pedrosa P, Corvo ML, Ferreira-Silva M, Martins P, Carvalheiro MC, Costa PM, Martins C, Martins LMDRS, Baptista P V., Fernandes AR. 2019.** Targeting Cancer Resistance via Multifunctional Gold Nanoparticles. *International Journal of Molecular Sciences* 20:5510.

**Pedrosa P, Mendes R, Cabral R, Martins LMDRS, Baptista P V, Fernandes AR. 2018.** Combination of chemotherapy and Au-nanoparticle photothermy in the visible light to tackle doxorubicin resistance in cancer cells. *Scientific Reports* 8.

**Peltier E, Vincent J, Finn C, Graham DW. 2010.** Zinc-induced antibiotic resistance in activated sludge bioreactors. *Water Research* 44:3829–3836.

**Peroutka SJ, Snyder SH. 1980.** Relationship of neuroleptic drug effects at brain dopamine, serotonin, α-adrenergic, and histamine receptors to clinical potency. *American Journal of Psychiatry* 137:1518–1522.

**Peterson E, Kaur P. 2018.** Antibiotic resistance mechanisms in bacteria: Relationships between resistance determinants of antibiotic producers, environmental bacteria, and clinical pathogens. *Frontiers in Microbiology* 9:2928.

**Pillai SK, Moellering Jr RC, Eliopoulos GM. 2005**. Antimicrobial Combinations. In: *Antibiotics in laboratory medicine*. Philadelphia, 365–440.

**Qadir MI, Chauhdary Z. 2018.** Antibacterial activity of novel strains of bacteriophages: An experimental approach. *Critical Reviews in Eukaryotic Gene Expression* 28:1–12.

**Qi SY, Moir A, O' Connor CD. 1996.** Proteome of *Salmonella* Typhimurium SL1344: Identification of novel abundant cell envelope proteins and assignment to a two-dimensional reference map. *Journal of Bacteriology* 178:5032–5038.

**Qin Z. 2012.** The use of THP-1 cells as a model for mimicking the function and regulation of monocytes and macrophages in the vasculature. *Atherosclerosis* 221:2–11.

**Qiu Y, Chan ST, Lin L, Shek TL, Tsang TF, Zhang Y, Ip M, Chan PK, Blanchard N, Hanquet G, Zuo Z, Yang X, Ma C. 2019**. Nusbiarylins, a new class of antimicrobial agents: Rational design of bacterial transcription inhibitors targeting the interaction between the NusB and NusE proteins. *Bioorganic Chemistry* 92.

**Quinn R. 2013.** Rethinking antibiotic research and development: World War II and the penicillin collaborative. *American Journal of Public Health* 103:426–434.

**Ramachandraiah CT, Subramaniam N, Tancer M. 2009.** The story of antipsychotics: Past and present. *Indian Journal of Psychiatry* 51:324–326.

**Rao SPS, Alonso S, Rand L, Dick T, Pethe K. 2008.** The protonmotive force is required for maintaining ATP homeostasis and viability of hypoxic, nonreplicating *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences of the United States of America* 105:11945–11950.

**Renwick MJ, Simpkin V, Mossialos E, Schippers E. 2016.** *Targeting innovation in antibiotic drug discovery and development The need for a One Health-One Europe-One World Framework*.

**Rodrigues L, A. Ainsa J, Amaral L, Viveiros M. 2011.** Inhibition of Drug Efflux in Mycobacteria with Phenothiazines and Other Putative Efflux Inhibitors. *Recent Patents on Anti-Infective Drug Discovery* 6:118–127.

**Rodrigues L, Viveiros M, Aínsa JA. 2015.** Measuring efflux and permeability in mycobacteria. *Methods in Molecular Biology* 1285:227–239.

**Rohs R, Sklenar H. 2004.** Methylene Blue Binding to DNA with Alternating AT Base Sequence: Minor Groove Binding is Favored over Intercalation. *Journal of Biomolecular Structure and Dynamics* 21:699–711.

**Sachlos E, Risueño RM, Laronde S, Shapovalova Z, Lee JH, Russell J, Malig M, McNicol JD, Fiebig-Comyn A, Graham M, Levadoux-Martin M, Lee JB, Giacomelli AO, Hassell JA, Fischer-Russell D, Trus MR, Foley R, Leber B, Xenocostas A, Brown ED, Collins TJ, Bhatia M. 2012.** Identification of drugs including a dopamine receptor antagonist that selectively target cancer stem cells. *Cell* 149:1284–1297.

**Salverda MLM, Koomen J, Koopmanschap B, Zwart MP, De Visser JAGM. 2017.** Adaptive benefits from small mutation supplies in an antibiotic resistance enzyme. *Proceedings of the National Academy of Sciences of the United States of America* 114:12773–12778.

**Santos PS, Saraiva DF, Costa DCF, Scofano HM, De Carvalho-Alves PC. 2007.** Trifluoperazine protects brain plasma membrane Ca2+-ATPase from oxidative damaging. *Experimental Brain Research* 177:347–357.

**Scheibenbogen C, Keilholz U, Richter M, Brado B, Andreesen R, Hunstein W. 1992.** The Interleukin-2 Receptor (CD25 and p75) in Human Monocytes and Macrophages. In: *Cytokines in Hemopoiesis, Oncology, and AIDS II*. Springer Berlin Heidelberg, 291–296. DOI: 10.1007/978-3-642-48715-6_38.

**Sharma A, Gupta VK, Pathania R. 2019.** Efflux pump inhibitors for bacterial pathogens: From bench to bedside. *Indian Journal of Medical Research* 149:129–145.

**Shen J, Ma B, Zhang X, Sun X, Han J, Wang Y, Chu L, Xu H, Yang Y. 2017.** Thioridazine has potent antitumor effects on lung cancer stem-like cells. *Oncology Letters* 13:1563–1568.

**Silva TFS, Smoleński P, Martins LMDRS, Guedes da Silva MFC, Fernandes AR, Luis D, Silva A, Santos S, Borralho PM, Rodrigues CMP, Pombeiro AJL. 2013.** Cobalt and Zinc Compounds Bearing 1,10-Phenanthroline-5,6-dione or

1,3,5-Triaza-7-phosphaadamantane Derivatives - Synthesis, Characterization, Cytotoxicity, and Cell Selectivity Studies. *European Journal of Inorganic Chemistry* 2013:3651–3658.

**Singh AN, Barlas C, Saeedi H, Mishra RK. 2003.** Effect of loxapine on peripheral dopamine-like and serotonin receptors in patients with schizophrenia. *Journal of psychiatry & neuroscience : JPN* 28:39–47.

**Soo V, Kwan B, Quezada H, Castillo-Juárez I, Pérez-Eretza B, García-Contreras S, Martínez-Vázquez M, Wood T, García-Contreras R. 2017.** Repurposing of Anticancer Drugs for the Treatment of Bacterial Infections. *Current Topics in Medicinal Chemistry* 17:1157–1176.

**van Soolingen D, Hernandez-Pando R, Orozco H, Aguilar D, Magis-Escurra C, Amaral L, van Ingen J, Boeree MJ. 2010.** The Antipsychotic Thioridazine Shows Promising Therapeutic Activity in a Mouse Model of Multidrug-Resistant Tuberculosis. *PLoS ONE* 5:e12640.

**Spengler G, Rodrigues L, Martins A, Martins M, McCusker M, Cerca P, Machado L, Costa SS, Ntokou E, Couto I, Viveiros M, Fanning S, Molnar J, Amaral L. 2012.** Genetic response of *Salmonella enterica* serotype Enteritidis to thioridazine rendering the organism resistant to the agent. *International Journal of Antimicrobial Agents* 39:16–21.

**Srikanth C V., Mercado-Lubo R, Hallstrom K, McCormick BA. 2011.** *Salmonella* effector proteins and host-cell responses. *Cellular and Molecular Life Sciences* 68:3687–3697.

**Stanaway JD, Parisi A, Sarkar K, Blacker BF, Reiner RC, Hay SI, Nixon MR, Dolecek C, James SL, Mokdad AH, Abebe G, Ahmadian E, Alahdab F, Alemnew BTT, Alipour V, Allah Bakeshei F, Animut MD, Ansari F, Arabloo J, Asfaw ET, Bagherzadeh M, Bassat Q, Belayneh YMM, Carvalho F, Daryani A, Demeke FM, Demis ABB, Dubey M, Duken EE, Dunachie SJ, Eftekhari A, Fernandes E, Fouladi Fard R, Gedefaw GA, Geta B, Gibney KB, Hasanzadeh A, Hoang CL, Kasaeian A, Khater A, Kidanemariam ZT, Lakew AM, Malekzadeh R, Melese A, Mengistu DT, Mestrovic T, Miazgowski B, Mohammad KA, Mohammadian M, Mohammadian-Hafshejani A, Nguyen CT, Nguyen LH, Nguyen SH, Nirayo YL, Olagunju**

**AT, Olagunju TO, Pourjafar H, Qorbani M, Rabiee M, Rabiee N, Rafay A, Rezapour A, Samy AM, Sepanlou SG, Shaikh MA, Sharif M, Shigematsu M, Tessema B, Tran BX, Ullah I, Yimer EM, Zaidi Z, Murray CJL, Crump JA. 2019.** The global burden of non-typhoidal *Salmonella* invasive disease: a systematic analysis for the Global Burden of Disease Study 2017. *The Lancet Infectious Diseases* 19:1312–1324.

**Starr T, Bauler TJ, Malik-Kale .P., Steele-Mortimer O. 2018.** The phorbol 12-myristate-13-acetate differentiation protocol is critical to the interaction of THP-1 macrophages with *Salmonella* Typhimurium. *PLOS ONE* 13:e0193601.

**Stecher B, Barthel M, Schlumberger MC, Haberli L, Rabsch W, Kremer M, Hardt WD. 2008.** Motility allows S. Typhimurium to benefit from the mucosal defence. *Cellular Microbiology* 10:1166–1180.

**Steele-Mortimer O. 2008.** The *Salmonella*-containing vacuole-Moving with the times. *Current Opinion in Microbiology* 11:38–45.

**Steele-Mortimer O, Meresse S, Gorvel J-P, Toh B-H, Finlay BB. 1999.** Biogenesis of *Salmonella* typhimurium-containing vacuoles in epithelial cells involves interactions with the early endocytic pathway. *Cellular Microbiology* 1:33–49.

**Stepanauskas ., Glenn TC, Jagoe CH, Tuckfield RC, Lindell AH, King CJ, McArthur J V. 2006.** Coselection for microbial resistance to metals and antibiotics in freshwater microcosms. *Environmental Microbiology* 8:1510–1514.

**Stolze K, Mason RP. 1991.** ESR spectroscopy of flow-oriented cation radicals of phenothiazine derivatives and phenoxathiin intercalated in DNA. *Chemico-Biological Interactions* 77:283–289.

**Stone BJ, Miller VL. 1995**. *Salmonella* enteritidis has a homologue of tolC that is required for virulence in BALB/c mice. *Molecular Microbiology* 17:701–712.

**Strahl H, Hamoen LW. 2010.** Membrane potential is important for bacterial cell division. *PNAS* 107:12281–12286.

**Strobl JS, Kirkwood KL, Lantz TK, Lewine MA, Peterson VA, Worley JF. 1990.** Inhibition of Human Breast Cancer Cell Proliferation in Tissue Culture by the

Neuroleptic Agents Pimozide and Thioridazine. *Cancer Research* 50.

**Stuart LM, Ezekowitz RAB. 2005.** Phagocytosis: Elegant complexity. *Immunity* 22:539–550.

**Swick MC, Morgan-Linnell SK, Carlson KM, Zechiedrich L. 2011.** Expression of Multidrug Efflux Pump Genes acrAB-tolC , mdfA , and norE in *Escherichia coli* Clinical Isolates as a Function of Fluoroquinolone and Multidrug Resistance. *Antimicrobial Agents and Chemotherapy* 55:921–924.

**Takashiba S, Van Dyke TE, Amar S, Murayama Y, Soskolne AW, Shapira L. 1999.** Differentiation of monocytes to macrophages primes cells for lipopolysaccharide stimulation via accumulation of cytoplasmic nuclear factor κB. *Infection and Immunity* 67:5573–5578.

**Tedesco S, De Majo F, Kim J, Trenti A, Trevisi L, Fadini GP, Bolego C, Zandstra PW, Cignarella A, Vitiello L. 2018.** Convenience versus biological significance: Are PMA-differentiated THP-1 cells a reliable substitute for blood-derived macrophages when studying *in vitro* polarization? *Frontiers in Pharmacology* 9:71.

**Teh JS, Yano T, Rubin H. 2007.** Type II NADH:Menaquinone Oxidoreductase of *Mycobacterium tuberculosis*. *Infectious Disorders - Drug Targets* 7:169–181.

**Tella AC, Obaleye JA. 2010**. Metal complexes as antibacterial agents: Synthesis, characterization and antibacterial activity of some 3d metal complexes of sulphadimidine. *The electronic journal of chemistry* 2:1–16.

**Thakare R, Shukla M, Kaul G, Dasgupta A, Chopra S. 2019.** Repurposing disulfiram for treatment of *Staphylococcus aureus* infections. *International Journal of Antimicrobial Agents* 53:709–715.

**Thanacoody HKR. 2007.** Thioridazine: resurrection as an antimicrobial agent? *British journal of clinical pharmacology* 64:566–74.

**Thomas CM, Timson DJ. 2018.** Calmodulins from Schistosoma mansoni: Biochemical analysis and interaction with IQ-motifs from voltage-gated calcium channels. *Cell Calcium* 74:1–13.

**Tsuchiya S, Kobayashi Y, Goto Y, Okumura H, Nakae S, Konno T, Tada K. 1982.**

Induction of Maturation in Cultured Human Monocytic Leukemia Cells by a Phorbol Diester. *Cancer Research* 42.

**Tyers M, Wright GD. 2019.** Drug combinations: a strategy to extend the life of antibiotics in the 21st century. *Nature Reviews Microbiology* 17:141–155.

**Uchiya KI, Barbieri MA, Funato K, Shah AH, Stahl PD, Groisman EA. 1999.** A *Salmonella* virulence protein that inhibits cellular trafficking. *EMBO Journal* 18:3924–3933.

**Udwadia ZF, Sen T, Pinto LM. 2011.** Safety and efficacy of thioridazine as salvage therapy in Indian patients with XDR-TB. *Recent patents on anti-infective drug discovery* 6:88–91.

**Urdaneta V, Casadesús J. 2017.** Interactions between bacteria and bile salts in the gastrointestinal and hepatobiliary tracts. *Frontiers in Medicine* 4.

**Uzzau S, Figueroa-Bossi N, Rubino S, Bossi L. 2001.** Epitope tagging of chromosomal genes in *Salmonella*. *Proceedings of the National Academy of Sciences of the United States of America* 98:15264–15269.

**Varga B, Csonka Á, Csonka A, Molnár J, Amaral L, Spengler G. 2017.** Possible biological and clinical applications of phenothiazines. *Anticancer Research* 37:5983–5993.

**Viganon L, Howe OL, McCarron P, McCann M, Devereux M. 2017.** The Antibacterial Activity of Metal Complexes Containing 1, 10-phenanthroline: Potential as Alternatire Therapeutics in the Era of Antibiotic Resistance. *Curr Top Med Chem*. 17:1280–1302.

**Vitiello M, D'Isanto M, Galdiero M, Raieta K, Tortora A, Rotondo P, Peluso L, Galdiero M. 2004.** Interleukin-8 production by THP-1 cells stimulated by *Salmonella enterica* serovar Typhimurium porins is mediated by AP-1, NF-κB and MAPK pathways. *Cytokine* 27:15–24.

**Viveiros M, Jesus A, Brito M, Leandro C, Martins M, Ordway D, Molnar AM, Molnar J, Amaral L. 2005.** Inducement and Reversal of Tetracycline Resistance in *Escherichia coli* K-12 and Expression of Proton Gradient-Dependent Multidrug Efflux Pump Genes. *Antimicrobial Agents and*

*Chemotherapy* 49:3578–3582.

**Viveiros M, Portugal I, Bettencourt R, Victor TC, Jordaan AM, Leandro C, Ordway D, Amaral L. 2002.** Isoniazid-induced transient high-level resistance in *Mycobacterium tuberculosis*. *Antimicrobial agents and chemotherapy* 46:2804–10.

**Viveiros M, Rodrigues L, Martins M, Couto I, Spengler G, Martins A, Amaral L. 2010.** Evaluation of efflux activity of bacteria by a semi-automated fluorometric system. *Methods in molecular biology (Clifton, N.J.)* 642:159–172.

**Vuillemin P. 1889.** Antibiose et symbiose. *Assoc. Franc. pour l'Avanc. des Sciences*:525–542.

**Walsh TR, Weeks J, Livermore DM, Toleman MA. 2011.** Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: An environmental point prevalence study. *The Lancet Infectious Diseases* 11:355–362.

**Walsh CT, Wencewicz TA. 2014.** Prospects for new antibiotics: A molecule-centered perspective. *Journal of Antibiotics* 67:7–22.

**Wang Q, Frye JG, McClelland M, Harshey RM. 2004.** Gene expression patterns during swarming in *Salmonella* typhimurium: genes specific to surface growth and putative new motility and pathogenicity genes. *Molecular Microbiology* 52:169–187.

**Wang Q, Zhao Y, McClelland M, Harshey RM. 2007.** The RcsCDB signaling system and swarming motility in *Salmonella enterica* serovar typhimurium: dual regulation of flagellar and SPI-2 virulence genes. *Journal of bacteriology* 189:8447–57.

**Wassmann CS, Lund LC, Thorsing M, Lauritzen SP, Kolmos HJ, Kallipolitis BH, Klitgaard JK. 2018.** Molecular mechanisms of thioridazine resistance in *Staphylococcus aureus*. *PLOS ONE* 13:e0201767.

**Weinstein EA, Yano T, Li LS, Avarbock D, Avarbock A, Helm D, McColm AA, Duncan K, Lonsdale JT, Rubin H. 2005.** Inhibitors of type II NADH:menaquinone oxidoreductase represent a class of antitubercular drugs.

*Proceedings of the National Academy of Sciences of the United States of America* 102:4548–4553.

**Wittekindt OH, Schmitz A, Lehman-Horn F, Hansel W, Grissmer S. 2006.** The human $Ca^{2+}$-activated $K^+$ channel, IK, can be blocked by the tricyclic antihistamine promethazine. *Neuropharmacology* 50:458–467.

**Wójcikowski J, Daniel WA. 2002.** Thioridazine-Fluoxetine interaction at the level of the distribution process *in vivo*. *Polish Journal of Pharmacology Pol. J. Pharmacol* 54:647–654.

**World Bank. 2017.** *Drug Resistant Infections : A threat to our Economic Future*. Washington, DC, US.

**World Health Organization. 2015**. *Global Action Plan on Antimicrobial Resistance*. Geneva, Switzerland.

**World Health Organization. 2016.** *Critically Important Antimicrobials for Human Medicine th 4 Revision 2013*. Geneva: World Health Organization.

**World Health Organization. 2019a**. Antibacterial agents in preclinical development: an open access database.

**World Health Organization. 2019b.** Antibacterial agents in clinical development - an analysis of antibacterial clinical development pipeline.

**World Health Organization. 2019c.** Antibacterial Agents in Clinical Development.

**World Health Organization. 2019d**. Antimicrobial resistance.

**Wright GD. 2010.** Q&A: Antibiotic resistance: Where does it come from and what can we do about it? *BMC Biology* 8:123.

**Wu M, Maier E, Benz R, Hancock REW. 1999.** Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of *Escherichia coli*. *Biochemistry* 38:7235–7242.

**Yamasaki S, Fujioka T, Hayashi K, Yamasaki S, Hayashi-Nishino M, Nishino K. 2016.** Phenotype microarray analysis of the drug efflux systems in *Salmonella enterica* serovar Typhimurium. *Journal of Infection and Chemotherapy* 22:780–784.

**Yang CY, Hsu CY, Fang CS, Shiau CW, Chen CS, Chiu HC. 2019.** Loxapine, an antipsychotic drug, suppresses intracellular multiple-antibiotic-resistant *Salmonella enterica* serovar Typhimurium in macrophages. *Journal of Microbiology, Immunology and Infection* 52:638–647.

**Yano T, Li L, Weinstein E, Teh J, Rubin H. 2006**. Steady-state Kinetics and Inhibitory Action of Antitubercular Phenothiazines on *Mycobacterium tuberculosis* Type-II NADH-Menaquinone Oxidoreductase (NDH-2). *Journal of Biological Chemistry* 281:11456–11463.

**Yano T, Rahimian M, Aneja KK, Schechter NM, Rubin H, Scott CP. 2014.** *Mycobacterium tuberculosis* Type II NADH-Menaquinone Oxidoreductase Catalyzes Electron Transfer through a Two-Site Ping-Pong Mechanism and Has Two Quinone-Binding Sites. *Biochemistry* 53:1179–1190.

**Yeu Y, Yoon Y, Park S. 2015.** Protein localization vector propagation: a method for improving the accuracy of drug repositioning. *Molecular BioSystems* 11:2096–2102.