TESIS DE LA UNIVERSIDAD DE ZARAGOZA
2023 152

Marta María Gómara Lomero

Novel repurposing strategies for antimicrobial development in Klebsiella pneumoniae

Director/es
Ramón García, Santiago
Aínsa Claver, José Antonio

ISSN 2254-7606

Prensas de la Universidad
Universidad Zaragoza

ISSN 2254-7606

Universidad Zaragoza
1542

Tesis Doctoral

NOVEL REPURPOSING STRATEGIES FOR ANTIMICROBIAL DEVELOPMENT IN KLEBSIELLA PNEUMONIAE

Autor

Marta María Gómara Lomero

Director/es

Ramón García, Santiago
Aínsa Claver, José Antonio

UNIVERSIDAD DE ZARAGOZA
Escuela de Doctorado

Programa de Doctorado en Ciencias Biomédicas y Biotecnológicas

2023

FACULTAD DE MEDICINA

Departamento de Microbiología, Pediatría, Radiología y Salud Pública

Novel repurposing strategies for antimicrobial development in *Klebsiella pneumoniae*

Memoria para optar al grado de Doctor presentada por:

Marta María Gómara Lomero

Licenciada en Farmacia

Directores:

Santiago Ramón García

José Antonio Aínsa Claver

Dr. SANTIAGO RAMÓN GARCÍA, Investigador ARAID en el Departamento de Microbiología, Pediatría, Radiología y Salud Pública de la Facultad de Medicina de la Universidad de Zaragoza.

Dr. JOSÉ ANTONIO AÍNSA CLAVER, Catedrático del Departamento de Microbiología, Pediatría, Radiología y Salud Pública de la de la Facultad de Medicina de la Universidad de Zaragoza,

Directores de la Tesis Doctoral presentada por Marta María Gómara Lomero bajo el título:

Novel repurposing strategies for antimicrobial development in *Klebsiella pneumoniae*

(Nuevas estrategias basadas en el reposicionamiento para el desarrollo de antimicrobianos en *Klebsiella pneumoniae*)

EXPONEN:

Que dicha Tesis Doctoral corresponde con el proyecto de tesis presentado y aprobado en su momento, no habiéndose producido ninguna variación.

Que dicha Tesis Doctoral ha sido realizada bajo su dirección y reúne los requisitos necesarios para optar al grado de Doctor.

Por lo anterior, emiten el presente **INFORME FAVORABLE**. Zaragoza, 13 de enero de 2023

Fdo. Santiago Ramón García

Fdo. José Antonio Aínsa Claver

Esta tesis doctoral ha sido elaborada en el Departamento de Microbiología, Pediatría, Radiología y Salud Pública de la Facultad de Medicina de la Universidad de Zaragoza dentro del programa de Doctorado de Ciencias Biomédicas y Biotecnológicas del Departamento de Farmacología, Fisiología, Medicina Legal y Forense.

Marta María Gómara Lomero ha sido beneficiaria de un contrato de personal investigador predoctoral en formación concedido por el Gobierno de Aragón (financiado por el Gobierno de Aragón y Fondos FEDER de la Unión Europea "Construyendo Europa desde Aragón", FSE Aragón 2014-2020).

Este trabajo ha sido realizado en el marco de los siguientes proyectos de investigación:

"Identification of novel therapies for difficult to treat cystic fibrosis pulmonary infections caused by mycobacteria using an innovative technology: synergy screens of clinically approved drugs". Financiado por la European Society of Clinical Microbiology and Infectious Diseases (Research Grants 2018)

"Desarrollo de nuevas terapias antimicrobianas mediante el reposicionamiento de fármacos en combinaciones sinérgicas y modelos dinámicos farmacológicos: implementando una nueva tecnología innovadora en Aragón". Financiado por el Gobierno de Aragón (Ref. LMP132_18)

INDEX OF CONTENT

CHAPTER 1

CHAPTER 2

CHAPTER 3

LIST OF ABBREVIATIONS

AMR	Antimicrobial Resistance
AZM	Azithromycin
BLI	β-lactam inhibitor
BLBLI	β-lactams/β-lactamase inhibitor
BSIs	Bloodstream infections
CAMHB	Cation adjusted Mueller Hinton Broth
CAZ	Ceftazidime
CAZ-AVI	Ceftazidime-avibactam
CBA	Checkerboard assays
CDC	Centers for Disease Control and Prevention
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
C_{max}	Maximum serum concentration
CMS	Colistin methane sulfonate
COPD	Chronic obstructive pulmonary disease
CPE	Carbapenemase-producing enterobacteria
CPKP	Carbapenemase-producing *Klebsiella pneumoniae*
CRE	Carbapenem-resistant enterobacteria
CST	Colistin
DCT	Double carbapenem therapy
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DTR	Difficult-to-treat Resistance
D&D	Discovery and development
EARS-Net	European Antimicrobial Resistance Surveillance Network
EDTA	Ethylenediaminetetraacetic acid
EMA	European Medicines Agency
ESAC	Extended-spectrum AmpC β-lactamases
ESBL	Extended-spectrum β-lactamases

EU	European Union
*f*AUC$_{0-24}$/MIC	Area under the concentration-time curve for free drug from 0 to 24 h to the MIC
FBC	Fractional Bactericidal Concentration
FBCI	Fractional Bactericidal Concentration Index
FDA	Food and drug administration
FIC	Fractional Inhibitory Concentration
FICI	Fractional Inhibitory Concentration Index
FOF	Fosfomycin
*f*T>CMI	Time above the MIC
GLPT	L-α-glycerophosphate transporter
G6P	Glucose-6-phosphate
HFIM	Hollow-Fiber Infection model
HIV	Human Immunodeficiency Virus
HTSS	High throughput synergy screen
IC_{50}	Half maximal inhibitory concentration
IC_{90}	Inhibitory concentration yielding 90% inhibition
IMP	Imipenemase
KPC	*Klebsiella pneumoniae* carbapenemase;
LB	Luria Broth
LPS	Lipopolysaccharide
MBC	Minimum bactericidal concentration
MBL	Metallo-β-lactamase
MDR	Multi-Drug Resistant
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
MIC	Minimum inhibitory concentration
MIC_{sub}	Sub-inhibitory concentration
MTT	2-(3,5-diphenyltetrazol-2-ium-2-yl)-4,5-dimethyl-1,3-thiazole;bromide
MU	Million units
MurA	UDP-N-acetylglucosamine enolpyruvyl transferase

NDM	New Delhi Metallo-β-lactamase
OD	Optical Density
OXA	Oxiacillinase
PAE	Post-antibiotic effect
PBPs	Penicillin-binding proteins
PC	Primary Compound
PDR	Pan-drug resistant
PEP	Phosphoenolpyruvate
PK/PD	Pharmacokinetic and pharmacodynamic
RCT	Randomized controlled clinical trials
SC	Secondary Compound
sHTSS	Semi-high throughput synergy screening
SMEs	Small and medium-size enterprises
TGC	Tigecycline
UHPT	Hexose-6-phosphate transporter
UNAG	UDP-N-acetylglucosamine
U.S.	United States
VIM	Verona integrated-encoded Metallo-β-lactamase
WHO	World Health Organization
XDR	Extensively-Drug Resistant
ZDV	Zidovudine

ABSTRACT

Antimicrobial resistance (AMR), known as the "silent pandemic", is a major public health problem that has become even more acute in the wake of the COVID-19, due to increased consumption and misuse of antibiotics during the pandemic. AMR currently causes almost 5 million deaths each year worldwide, and these figures could increase to 10 million by 2050 if no action is taken to prevent this situation. The lack of new antimicrobials on the market has contributed to the rapid spread of bacteria that are multidrug-resistant to most available antibiotics. As a result, the treatment of these infections is a challenge for healthcare systems.

In particular, *Klebsiella pneumoniae* is one of the most clinically relevant pathogens due to its severity and difficulty in bacterial eradication and is considered a priority for the urgent search for new effective treatments. Currently, treatment of multidrug-resistant strains involves the combination of several antibiotics, although efficacy rates are variable.

The traditional discovery and development (D&D) process in antimicrobial research is a high capital and time investment for pharmaceutical companies, so this model has been abandoned in favour of more cost-effective ones. In recent years, drug repositioning is regarded as a more attractive and faster strategy to bring new treatments to market, as it identifies unknown antimicrobial properties in drugs marketed for another indication, with safety data already described. The main limitation of drug repurposing is that the new antimicrobial indication is active at higher doses than the previous indication, which could result in toxicity problems. As a solution, it has been shown that combining drugs with a synergistic effect (greater potency together than separately) allows for lower doses and minimises potential adverse effects. Repurposing and synergy are thus the core concepts of this work.

This thesis is divided into three chapters that describe the search for and validation of synergistic combinations against *K. pneumoniae.* Each chapter includes specific introductions as well as a final discussion of data generated.

Chapter 1 describes the development process and screening assay of a library of FDA clinically approved drugs combined with drugs of last resort against *K. pneumoniae* MDR (fosfomycin, colistin and tigecycline), which identified synergistic combinations. Subsequently, the activity of the identified combinations was characterised by secondary synergy assays. As a result, six novel combinations based on non-antibiotic drugs were identified, and several synergistic combinations with clinical-translational potential prioritized.

Chapter 2 and **Chapter 3** explore the *in vitro* validation process of combinations based on two drugs identified in the previous screening: zidovudine and azithromycin, respectively, against a collection of *K. pneumoniae* isolates with different antimicrobial susceptibility and resistance mechanisms. For that purpose, the activity of these combinations was evaluated with other combinations currently used in the clinical setting for the treatment of multidrug-resistant *K. pneumoniae* infections. In both studies, high percentages of synergism and bactericidal activity were found with the new combinations, even higher than with the standard combinations.

In particular, although the antibacterial properties of zidovudine were already known, in **Chapter 2** it is highlighted the potent activity of zidovudine with ceftazidime-avibactam, a novel combination not previously described, and with fosfomycin (in agreement with a recently published study), at effective concentrations equivalent to physiological concentrations after standard dosing.

In **Chapter 3**, the combined effects of azithromycin with both fosfomycin and colistin were remarked, eradicating most strains. These results could change the current paradigm and suggest that azithromycin could be repositioned as a treatment for multidrug-resistant *Enterobacterales.*

In conclusion, the results of this Thesis validate a novel approach for the search of new, more effective treatments against *K. pneumoniae*. Both zidovudine and azithromycin were demonstrated to act *in vitro* as good candidates in combination therapies with clinically used antibiotics against *K. pneumoniae*.

RESUMEN

La resistencia a los antimicrobianos (RAM), conocida como la "pandemia silenciosa", es un importante problema de salud pública que se ha agravado aún más tras la pandemia COVID, debido al aumento en el consumo y uso incorrecto de los antibióticos. El problema de la RAM causa actualmente casi 5 millones de muertes cada año a nivel mundial, y estas cifras podrían incrementarse hasta 10 millones para el año 2050 si no se toman medidas para prevenir esta situación. La falta de nuevos antimicrobianos en el mercado ha contribuido a la rápida diseminación de bacterias multirresistentes a la mayoría de los antibióticos disponibles. Como consecuencia, el tratamiento de estas infecciones supone un reto para los sistemas sanitarios.

En concreto, *Klebsiella pneumoniae* es uno de los patógenos que tiene mayor relevancia clínica por su gravedad y dificultad de erradicación bacteriana, y se considera prioritario para la búsqueda urgente de nuevos tratamientos eficaces. Actualmente, el tratamiento de las cepas multirresistentes supone la combinación de varios antibióticos, aunque las tasas de eficacia son variables.

El proceso tradicional de descubrimiento y desarrollo (D+D) de antimicrobianos supone una elevada inversión de capital y tiempo para las empresas farmacéuticas, por lo que este modelo ha sido abandonado por otros más rentables. En los últimos años, el reposicionamiento de fármacos se considera una estrategia más atractiva y rápida para sacar al mercado nuevos tratamientos, puesto que identifica propiedades antimicrobianas no conocidas en fármacos comercializados para otra indicación, con datos de seguridad ya descritos. La principal limitación del reposicionamiento de fármacos es que la nueva indicación antimicrobiana sea activa a dosis mayores que la indicación anterior, lo que podría resultar en problemas de toxicidad. Como solución, se ha demostrado que la combinación de fármacos con efecto sinérgico (mayor potencia conjunta que por separado) permite reducir las dosis y minimizar los posibles efectos adversos. La reutilización y la sinergia son, pues, los conceptos centrales de este trabajo.

Esta Tesis está dividida en tres capítulos que describen la búsqueda y validación de combinaciones sinérgicas frente a *K. pneumoniae*. Cada capítulo incluye una introducción específica, así como una discusión final de los resultados generados.

El **capítulo 1** describe el proceso de desarrollo y ensayo de cribado de una biblioteca de fármacos aprobados clínicamente por la FDA frente a fármacos de último recurso contra *K. pneumoniae* MDR (fosfomicina, colistina y tigeciclina), que permitió identificar combinaciones sinérgicas. Posteriormente, se caracterizó la actividad de las combinaciones identificadas

mediante ensayos secundarios de sinergia. Como resultado, se identificaron seis combinaciones novedosas basadas en fármacos no antibióticos, y se priorizaron varias combinaciones sinérgicas con potencial clínico-traslacional.

Los **capítulos 2** y **3** exploran el proceso de validación *in vitro* de combinaciones basadas en dos fármacos identificados en el cribado anterior, zidovudina y azitromicina respectivamente, frente a una colección de aislados de *K. pneumoniae* con diferentes mecanismos de susceptibilidad y resistencia antimicrobiana. Para ello, se evaluó la actividad de dichas combinaciones con otras utilizadas actualmente en el ámbito clínico para el tratamiento de infecciones por *K. pneumoniae* multirresistente. En ambos estudios, se encontraron altos porcentajes de sinergia y actividad bactericida con las nuevas combinaciones, incluso superiores a las combinaciones habituales.

En particular, aunque ya se conocían las propiedades antibacterianas de zidovudina, en el **capítulo 2** se destaca la potente actividad de zidovudina con ceftazidima-avibactam, una combinación novedosa no descrita anteriormente, y con fosfomicina (en concordancia con un estudio publicado recientemente), a concentraciones efectivas equivalentes a las fisiológicas tras dosis estándar.

En el **capítulo 3**, se destacaron los efectos combinados de azitromicina con fosfomicina y colistina, llegando a erradicar la mayoría de las cepas. Estos resultados podrían cambiar el paradigma actual y sugieren que azitromicina podría reposicionarse como tratamiento de enterobacterias multirresistentes.

Como conclusión, los resultados de esta tesis validan un enfoque novedoso en la búsqueda de nuevos tratamientos más eficaces contra *K. pneumoniae*. Se demostró que tanto zidovudina como azitromicina actúan *in vitro* como buenos candidatos en terapia combinada con antibióticos de uso clínico frente a *K. pneumoniae.*

INTRODUCTION

The Antimicrobial Resistance: a silent pandemic

Antimicrobial resistance (AMR) is a natural phenomenon that occurs when microorganisms develop resistance to drugs that were previously effective and used for treating infections caused by these microorganisms. Although this process has been observed since the first antimicrobials were discovered, AMR shows an increasing trend over the last decade, leading since 2019 one of the top ten threats to global health, according to World Health Organization (WHO) (1). In 2019, the global burden was estimated in 4.95 million deaths associated to AMR (2) , and if no action is taken, these figures will increase to 10 million by 2050 (3). By geographical zones, the 2019 U.S. Centres for Disease Control and Prevention (CDC) report estimated more than 3 million people suffered AMR infections (including *Clostridioides difficile*) with more than 48,000 deaths in 2019 (4). Similarly in the EU the European Antimicrobial Resistance Surveillance Network (EARS-Net) estimated that more than 650,000 infections with selected antibiotic-resistant bacteria occurred in 2015, accounting for an estimated 33,110 attributable deaths to AMR, and with higher prevalence in Mediterranean countries such as Italy and Greece (5). AMR prevalence is specially concerning in low- and middle-income countries, where the proportion of resistant infections ranges from 40 to 60% compared to 17% for countries belonging to the Organization for Economic Cooperation and Development (OECD) (6). In regions of sub-Saharan Africa and south Asia, mortality rates associated with bacterial AMR were estimated to be greater than 75 per 100,000 (2).

The economic cost of AMR in healthcare systems is difficult to calculate but involves extended hospital stays, additional follow-up visits to healthcare providers, and prolonged treatments. However, the impact of AMR needs to be addressed from a broader global health perspective, which has been called “One Health”. This term encompasses the environment and the interconnections that may exist between animal and human health for disease transmission and the generation of new resistant microorganisms (7). Therefore, several socioeconomic determinants such as a weak health system, poor infection control in hospitals, poor community hygiene and the overuse of antibiotics in environmental and food industries are potential causes of AMR increment (8). Moreover, the emergence of COVID-19 strongly impacted on AMR; while investment strategies and research advances focused on fighting the virus, disruption of antimicrobial stewardship programs in hospitals have led to an increase of antibiotic misuse (9), and a rapid spread of resistant strains (10).

Several pathogens have been of special concern because of the rapid dissemination and probability to cause severe infections, such as the “ESKAPE” group: *Enterococcus faecium,*

Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa, and *Enterobacter* species (11). Later on, in 2017, the WHO published its first list of priority pathogens for which antimicrobial development was urgently needed (12). Tuberculosis was considered a global priority as the most lethal infectious disease (1.8 million deaths per year), with 250,000 deaths attributed to drug-resistant tuberculosis. Other pathogens were ranked by critical, high, and medium priority. Gram-negative "ESKAPE" bacteria were considered critical for causing severe infections with high mortality and morbidity rates in nosocomial and healthcare-associated infections. High and medium categories included microorganisms causing common and community acquired infections **(Figure 1).**

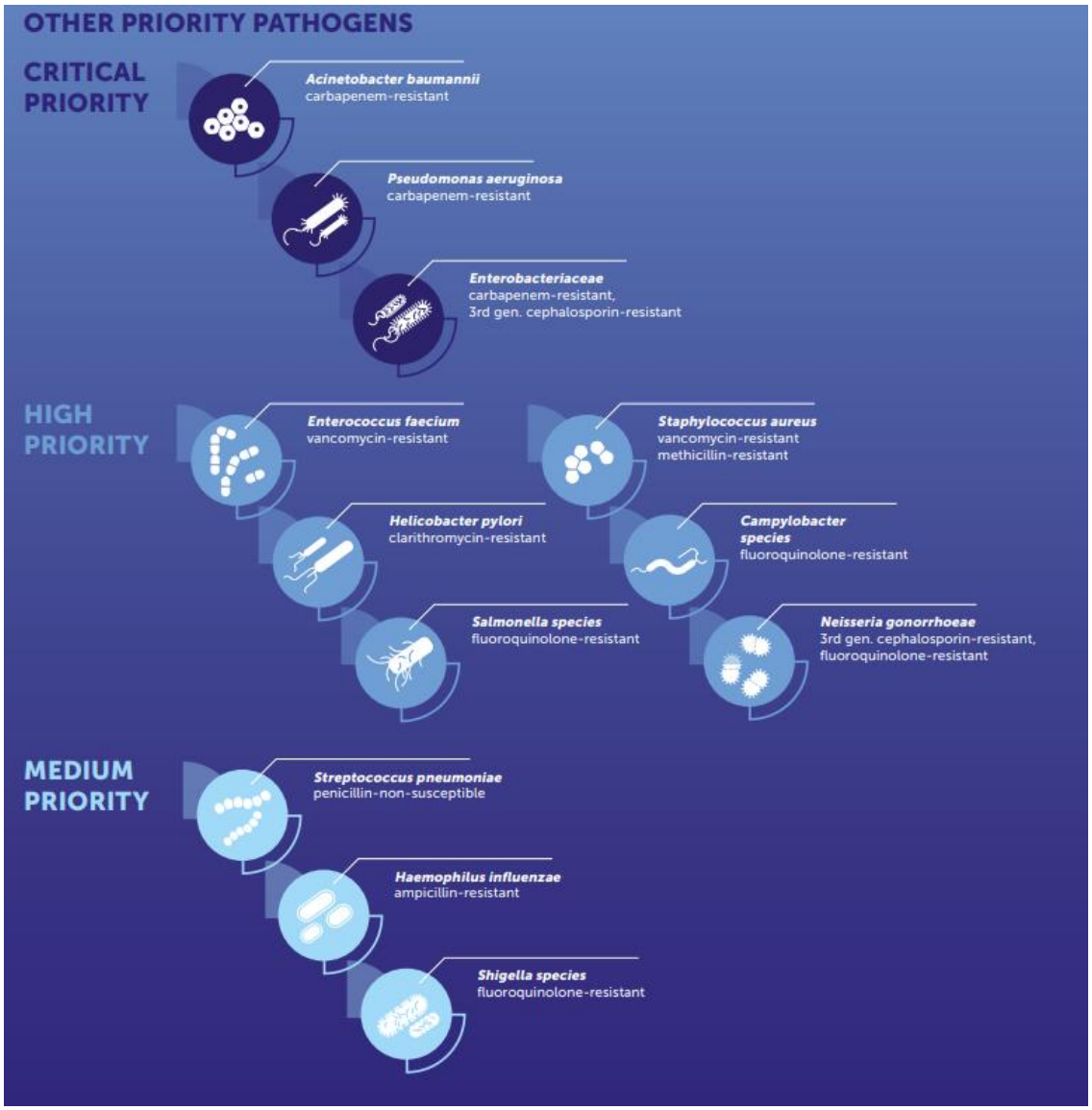


Figure 1. WHO priority pathogen list for antibiotic research and development, excluding tuberculosis. Adapted from (12).

Although the WHO list aimed to dictate priorities in antibiotic research, the lack of development of new drugs has worsen this scenario. In 2019, U.S. CDC reports listed up to 18 pathogens (bacteria and fungi) categorized as urgent, serious and concerning threats, and included a "Watch List" for potential pathogens that would become a challenge at short-term without infection prevention measures (4). In 2021, India released its own list of priority pathogens which included two pathogens (coagulase-negative staphylococci and *Neisseria meningitidis)* not listed in the WHO and CDC lists (13). Currently, WHO has also published a priority list of 19 fungal pathogens (14). Tackling global data, the study of Murray *et al.*, estimated five bacterial species as the main responsible for resistance-attributable deaths: Meticillin-resistant *S. aureus* (more than 100,000 deaths), followed by multidrug-resistant *Mycobacterium tuberculosis*, third-generation cephalosporin-resistant *Escherichia coli*, carbapenem-resistant *A. baumannii*, fluoroquinolone-resistant *E. coli*, carbapenem-resistant *K. pneumoniae*, and third-generation cephalosporin-resistant *K. pneumoniae* (between 50,000 and 100,000 each) **(Figure 2).**

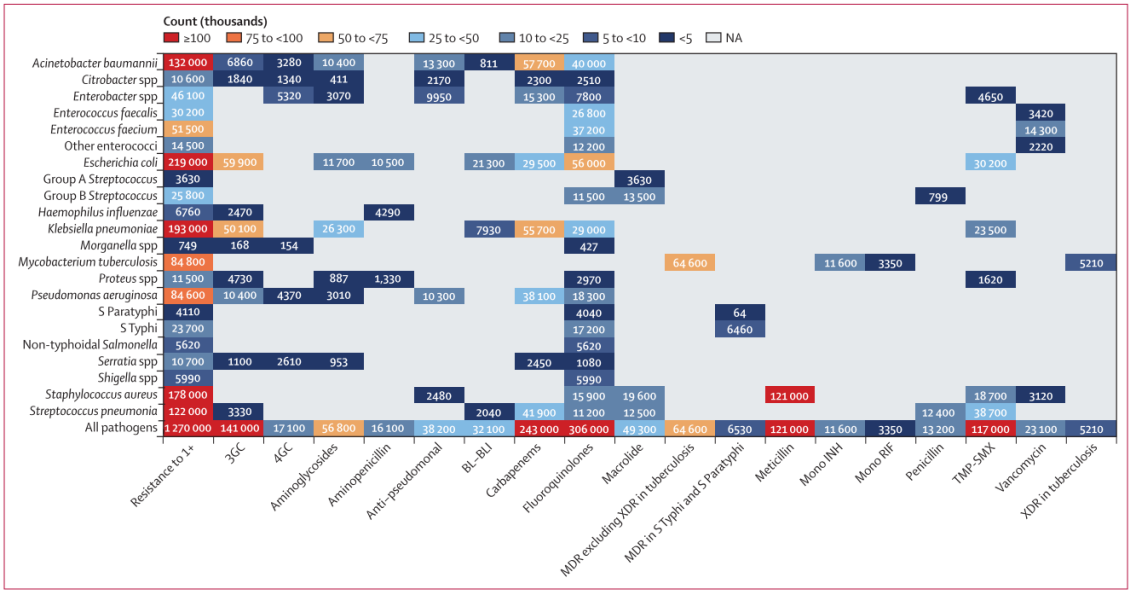


Figure 2. Global deaths (counts) attributable to bacterial antimicrobial resistance by pathogen-drug combination, 2019. From (2)**.** 3GC=third generation cephalosporins; 4GC=fourth generation cephalosporins; Anti-pseudomonal= anti-pseudomonal penicillin or β-lactamase inhibitors; BL-BLI= β-lactam or β-lactamase inhibitors; Mono INH=isoniazid mono-resistance; Mono RIF=rifampicin mono-resistance; NA= not applicable; Resistance to 1+=resistance to one or more drug; S Paratyphi = Salmonella enterica serotype Paratyphi; S Typhi = S. enterica serotype Typhi; TMP-SMX=trimethoprim-sulfamethoxazole

AMR can be classified in two types of resistance:

(i) Natural resistance. Some bacterial species show intrinsic resistance to certain classes of antibiotics, due to lack of a target (e.g., β-lactams are not active against mycoplasmas lacking peptidoglycan) or because they possess constitutive genes (e.g., chromosomal SHV-1 penicillinase in *K. pneumoniae* that confers intrinsic resistance to ampicillin). Natural resistance can be also induced by activation of genes after exposure to certain antibiotics (8).

(ii) Acquired resistance. This type of resistance contributes to the spread of AMR and is directly dependent on prior antibiotic exposure, resulting in bacterial gene mutations to confer resistance. Bacterial transfer can occur by vertical or horizontal gene transmission. The latter route involves processes of transformation (recipient bacterium takes up extracellular donor DNA), transduction (a donor bacteriophage infects a recipient bacterium) and conjugation (donor and recipient bacteria are in contact through *pili*) (8).

The frequent acquisition of additional resistance mechanisms to different classes of antibiotics poses a challenge to select the optimal therapy to fight against these "superbugs". Since the emergence of these strains, new definitions such as multidrug-resistant (MDR), extensively drug-resistant (XDR), pandrug-resistant (PDR) (15) and difficult-to-treat resistance (DTR) (16) have been introduced in the clinical practice to classify the bacteria according to the resistance profile.

The increasing dissemination of MDR/XDR Enterobacterales

Enterobacterales encompass the most heterogeneous and clinically important group of Gram-negative rods, which are responsible for community-associated and healthcare-associated infections. The most clinically isolated species include *E. coli, Klebsiella* spp., and *Enterobacter* spp. Members of this group are facultative anaerobic, non-spore forming bacilli that share an enterobacterial common antigen. Biochemically, enterobacteria are typically classified as glucose fermenters, nitrate reducers, positive catalase, and negative oxidase (17).

There is a worldwide rise in the prevalence and dissemination of MDR enterobacteria, which causes a huge impact in the global health system and worsens the prognosis of infected patients. In 2019, the CDC estimated 210,000 infections and 10,200 deaths in the USA associated to extended-spectrum beta-lactamases (ESBL) and carbapenem-resistant enterobacteria (CRE) (4). In EU, the burden of infections and number of deaths has increased by 6.16 times during the 2007-2015 period due to the emergence of carbapenem-resistant *K. pneumoniae*, followed by

carbapenem-resistant *E. coli*, third-generation cephalosporin-resistant *E. coli*, and third-generation cephalosporin-resistant *K. pneumoniae* (5).

The increasing incidence of MDR strains is facilitated by the rapid plasmid-mediated horizontal transmission of encoding genes, mainly ESBL and carbapenemases that confer resistance to β-lactams. These latter enzymes pose a global challenge in clinical treatment, as carbapenemases inactivate most β-lactams (penicillins, cephalosporins and carbapenems). Carbapenems are broad spectrum antibiotics reserved for the treatment of enterobacteria when other options fail. The emergence and dissemination of carbapenemase-producing enterobacteria (CPE) together with the additional acquisition of other resistance mechanisms, such as the emergent *mcr-1* plasmid involved in colistin resistance (18), severely limit therapeutical options and patients´ clinical outcome. Concurrence of multiple resistance mechanisms has led to the emergence of MDR, XDR or even PDR strains.

Epidemiology of carbapenemase-producing enterobacteria

CPE were first identified in the 1980s; however, despite a large number of sporadic outbreaks in the late 1990s and early 2000s, the frequency of CPE infections remained low in most regions of the world until the widespread dissemination of carbapenemase-producing *K. pneumoniae* (CPKP) strains in the last decade (19). The global prevalence of CPE depends on the geographical area. Data from the SENTRY surveillance Program reported percentages of 1% in North America, 6.2% in Latin America, 4.1% in Europe and 3.1% in Asia-Pacific, but differences are even wider across countries. Hence, there are some endemic areas such as Poland or Belarus (around 30% of CPE), Russia and Brazil (nearly 15%), Italy, Greece, Turkey and Mexico (5 to 10%). In contrast, other regions show prevalence lower than 1% (Australia, France, Germany) (20).

The type of carbapenemase is also different among regions, as shown in **Figure 3.** *Klebsiella pneumoniae* carbapenemase (KPC) is the most clinically important class A carbapenemase, mainly identified in the United States, South America, China, Greece, and Italy. Class B carbapenemases, Metallo-β-lactamases (MBL), are mainly prevalent in Asia. Specifically, New Delhi MBL (NDM) is mainly prevalent in China, Pakistan, India, and Bangladesh, and widely spread around the world; imipenemase (IMP) is prevalent in Japan, Taiwan, China, and Verona integrated-encoded MBL (VIM) in Greece and Italy. Class D carbapenemases (mainly refers to oxiacillinases OXA-48), are mainly prevalent in Turkey, Morocco, and European countries (France, Germany, Netherlands, Italy, the United Kingdom, etc)(19).

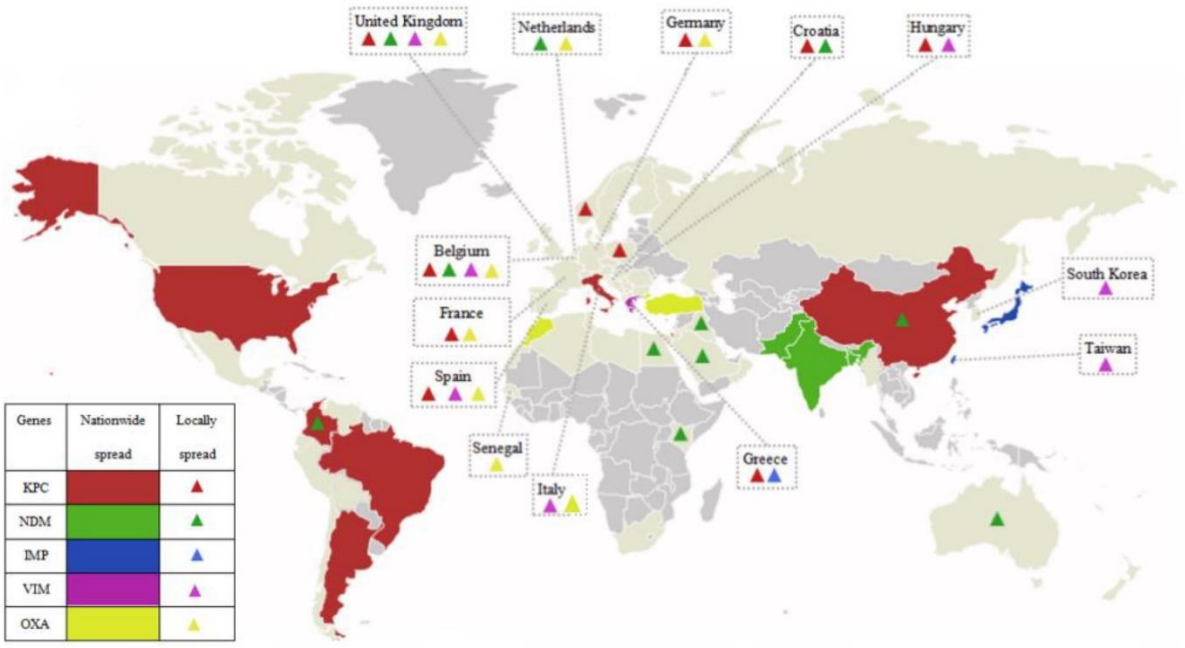


Figure 3. Global distribution of types of carbapenemase-producing enterobacteria. From (19). KPC, *Klebsiella pneumoniae* carbapenemase; NDM, New Delhi Metallo-β-lactamase; IMP, imipenemase; VIM, Verona integrated-encoded Metallo-β-lactamase; OXA, oxiacillinase.

Klebsiella pneumoniae

Epidemiology

K. pneumoniae is the most concerning resistant enterobacteria, leading the most prevalent CPE and responsible for mortality rates up to 41.6 and 48% (21). Indeed, in 2019, *K. pneumoniae* was the third leader pathogen causing more than 250,000 deaths associated with AMR (2). CPKP incidence is increasing worldwide. Data show 7.9% carbapenem resistance in Europe (22) and 26.8% of meropenem resistance in China (21), but this incidence rises up to 60-70% in some areas such as Russia, India or Egypt (2) **(Figure 4)**. Moreover, multi-drug resistance is also an increasing trend in *K. pneumoniae*, showing 19.3% combined resistance to traditional first-line antibiotics in the EU (22), highlighting the complications in the therapeutic management of MDR-related infections.

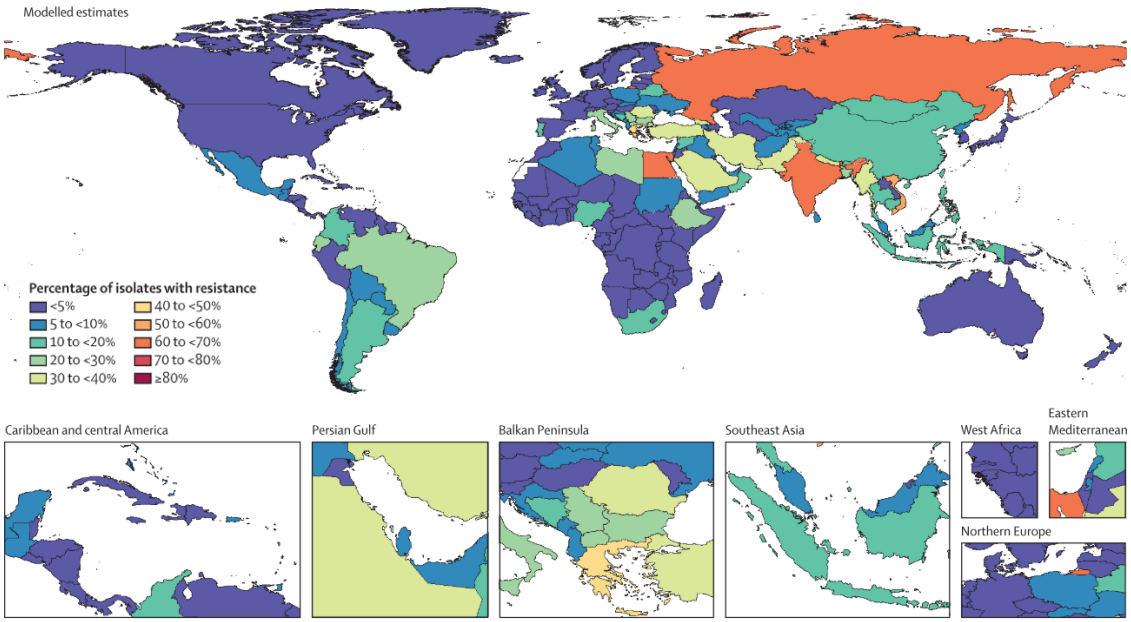


Figure 4. Global estimation of carbapenem-resistant *K. pneumoniae* by country and territory in 2019. Adapted from (2)**.**

Virulence and clinical impact

K. pneumoniae is a commonly encapsulated, non-motile enterobacteria that colonizes human nasopharynx and gastrointestinal tract. As an opportunistic pathogen, *K. pneumoniae* causes one third of all Gram-negative infections overall, involving hospital-associated infections such as urinary tract infections, pneumonia, surgical site infections and bloodstream infections (BSIs) especially in immunocompromised patients. It also produces community-acquired infections such as necrotizing pneumonia, pyogenic liver abscesses, endogenous endophthalmitis and meningitis (8,23). Along with bacterial colonization as the main risk factor that increases the likelihood of causing infections, there are other risk factors related to the age (paediatric and elderly population) and several comorbidities (diabetes mellitus, alcoholism, malignant neoplasms, immunosuppressive treatment, liver cirrhosis, chronic obstructive pulmonary disease (COPD), transplanted patients, haemodialysis, HIV infection, obstructive diseases of the urinary and biliary tract, recent surgery, invasive techniques, previous use of antimicrobial therapy and hospital long-stay).

There are four well characterized virulence factors for *K. pneumoniae*, displayed in **Figure 5.**

i. The polysaccharide capsule: this is the main virulence factor (K-antigen), responsible for the hypermucous colony phenotype that contributes to increased resistance to complement-mediated or phagocyte-mediated killing. Heterogeneity of K-antigens has led to classify more than 100 serotypes. Most important capsule types are K1 and K2, which confer hypervirulence and are involved in liver abscesses. Inhibition of phagocytosis is suggested as the mechanism by which capsule promotes virulence.

ii. Lipopolysaccharide (LPS). The O-antigen forms part of the LPS in the outer membrane and it is involved in immune evasion mechanisms.

iii. Type 1 and type 3 fimbriae. *K. pneumoniae* can produce fimbria that act as adhesins, facilitating adherence to host cells and biofilm formation.

iv. Siderophores: These are small molecules (enterobactin, yersiniabactin, salmochelin, aerobactin) secreted to mediate iron uptake that facilitates bacterial survival. They are recognized by specific membrane receptors and overproduced by hypervirulent strains (24).

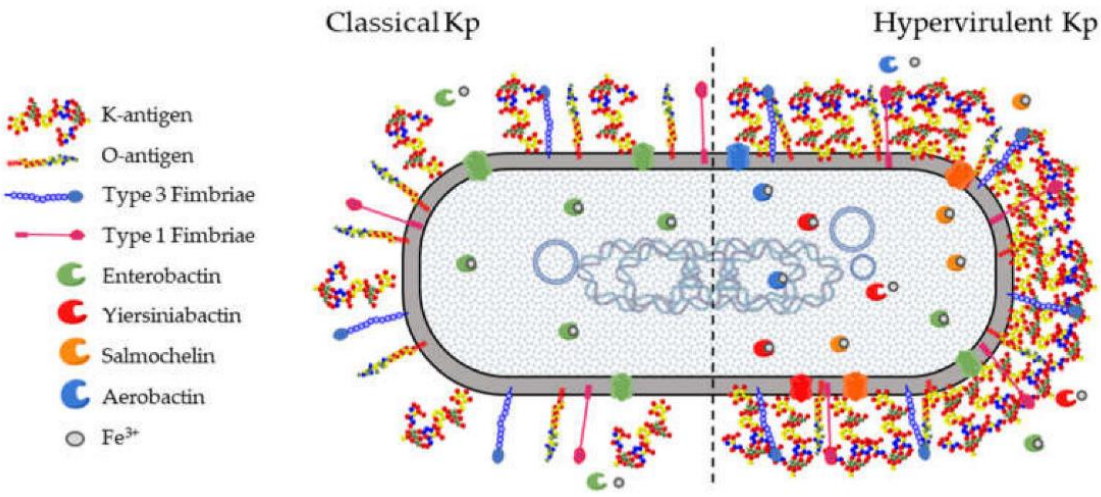


Figure 5. *K. pneumoniae* virulence factors. From (24). Kp, *K. pneumoniae.*

Resistance mechanisms

The versatility to incorporate plasmid resistance genes characterizes the rapid dissemination of resistant *K. pneumoniae.* The most common resistance mechanisms are listed below

1. β-lactams resistance

Resistance to β-lactams is usually due to the acquisition of plasmid enzymes that inactivate these drugs. To date, β-lactamases have been classified based on two features: Ambler classification based on the molecular structure of the amino-acid sequence (classes A, B, C, and D), and Bush-Jacoby-Medeiros classification (updated by Bush & Jacoby) based on the functional group. In the Ambler classification, β-lactamases of classes A, C, and D use a serine as an enzyme active center, whereas β-lactamases of class B use the metal zinc **(Figure 6)** (25).

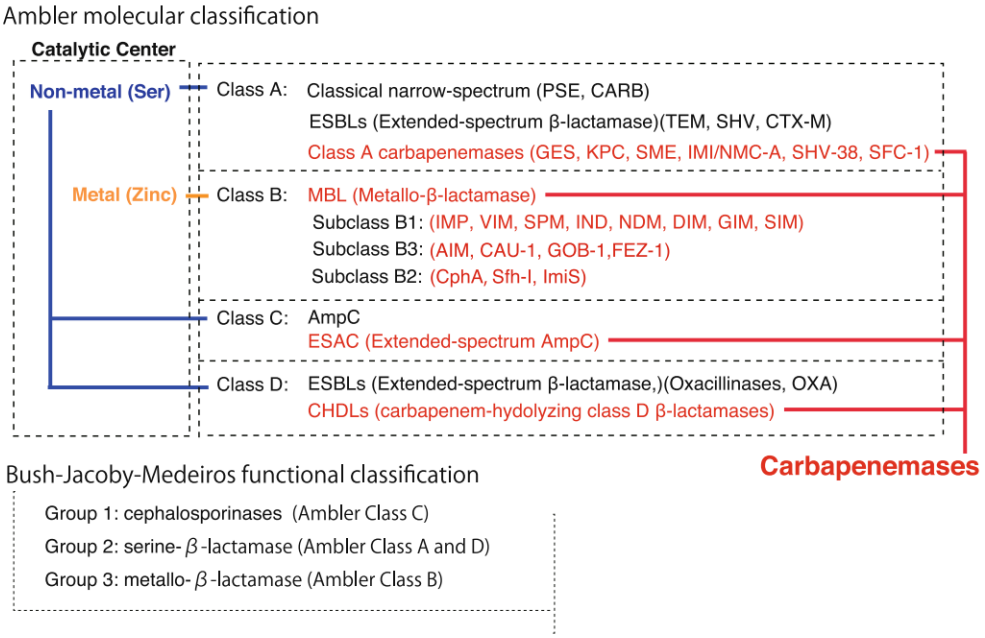


Figure 6. The classification of β-lactamases. From (26).

1.1 Extended-spectrum β-lactamases (ESBLs)

ESBLs enzymes degrade penicillins, oxyiminocephalosporins (cefotaxime, ceftriaxone, ceftazidime, cefepime) and monobactams (aztreonam), but do not hydrolyse cephamycins neither carbapenems. ESBLs are typically inhibited by β-lactam inhibitors (clavulanic acid, sulbactam, tazobactam) and are easily transmitted by plasmid-borne genes including TEM, SHV and CTX-M-type genes as the most prevalent worldwide. ESBLs are included in Amber class A (Bush-Jacoby functional subgroup 2b) and class D (Bush-Jacoby functional group 2d).

Among β-lactamases of class A, some enzymes such as TEM-30 and SHV-10 (Bush-Jacoby functional subgroup 2br), hydrolyse penicillins and exhibit relative resistance to β-lactam inhibitors. TEM-50 is a broad-spectrum β-lactamase belonging to Bush-Jacoby functional subgroup 2ber that hydrolyses extended-

spectrum cephalosporins (oxyimino-β-lactams) and monobactams but have acquired resistance to β-lactam inhibitors.

ESBLs of Ambler class D are classified into Bush-Jacoby functional subgroup 2de. These are known as OXA enzymes and show cloxacillin- and oxacillin-hydrolysing activity. OXA-10, OXA-11, and OXA-15 are recognized as extended-spectrum cephalosporinases (26).

K. pneumoniae has become the major ESBL-carrying pathogen associated in nosocomial outbreaks, with prevalence percentages of up to 50% in some endemic countries (23).

1.2 AmpC-type enzymes

AmpC enzymes belong to Ambler class C and Bush-Jacoby functional group 1. These enzymes are encoded on the chromosomes of many members of *Enterobacterales* (typically *Enterobacter* spp., *Serratia marcescens*, *Citrobacter freundii, Providencia* spp., and *Morganella morganii),* but can be also plasmid-transmitted to other species such as *Klebsiella* spp., *Proteus mirabilis* or *Salmonella* spp. These enzymes show cephalosporinase activity (typically on cephamycins, such as cefoxitin) but do not efficiently hydrolyse cefepime nor carbapenems and are not inhibited by classic β-lactam inhibitors. They show high affinity for aztreonam, which differs from class A cephalosporinases. Usually, AmpC expression is low but inducible with the administration of certain β-lactams (amoxicillin, ampicillin, imipenem, and clavulanic acid), conferring resistance to carbapenems when it is expressed at high levels (26,27). The most common plasmid-encoded genes in *K. pneumoniae* belong to CMY, DHA, FOX and MOX families (23).

The subgroup 1e enzymes show variants with greater activity against ceftazidime and other oxyimino-β-lactams, thus known as extended-spectrum AmpC (ESAC) β-lactamases. They include CMY-10, CMY-19, and CMY-37 mutants (26,27).

1.3 Carbapenemase enzymes

The ability to hydrolyse carbapenems depends on their molecular classification:

- Class A carbapenemases: included in the Bush-Jacoby functional subgroup 2f, these enzymes are most frequently detected in *P. aeruginosa* and

Klebsiella spp. and are represented by six families (GES, KPC, SME, SHV, IMI/NMC-A and SFC), being the KPC-type the most representative due to plasmid transmission. They exhibit an inhibitory effect by β-lactamase inhibitors such as clavulanic acid and tazobactam (26). In *K. pneumoniae*, KPC-2 and KPC-3 are the most common genes due to the wide expansion of the high-risk clone ST258 (23).

- Class B carbapenemases: these are known as MBL because require a zinc ion at the enzyme active site that can be inactivated by metal ion chelators such as ethylenediaminetetraacetic acid (EDTA) or dipicolinic acid. They belong to Bush-Jacoby functional group 3, degrade all β-lactam agents except monobactam and are not inhibited by β-lactamase inhibitors. The families IMP, VIM, SPM, GIM, NDM and FIM encompasses this class (25,26).
- Class D carbapenemases: OXA enzymes belong to subgroup 2df and show weak hydrolytic activity for carbapenems, higher in imipenem than in meropenem. The lack of *in vitro* activity against carbapenems can also be a challenge in their phenotypic detection. Although these enzymes are chromosomally produced in *A. baumannii,* plasmid genes *(*OXA-23 and OXA-48 enzymes) are also found in enterobacteria (25,26).

2. Overexpression of efflux pumps

Efflux pumps are proteins imbedded in the bacterial plasma membrane whose function is to recognize toxic agents that penetrate the cytoplasm and transport them from within the cell to the external environment. Efflux pumps act as a protective cell system that avoid noxious agents reach their intended targets and produce toxic effects (28). Enterobacteria can overexpressed or increase the number of efflux pumps to expel more actively some antibiotics, as in the case of tigecycline. The upregulation of the AcrAB efflux decreased the susceptibility of *E. coli* and *K. pneumoniae* to carbapenems (29). This resistant mechanism has been also reported for the new tetracycline eravacycline (19).

3. Porin loss

Porins are outer-membrane proteins that plays an important role for the antimicrobial entry. Downregulation of these proteins or mutations in specific portions of the protein may render a less active or a non-functional porin respectively, which has

been well studied in some species, such as *E. coli, S. marcescens* and *Enterobacter* spp. (OmpC and OmpF porin families) (30–32). This resistance mechanism can be also associated to β-lactamases ESBLs and AmpC and/or efflux pumps, yielding to a decrease in the carbapenem susceptibility. Specifically, in *K. pneumoniae,* low expression of OmpK35 and OmpK36 porins lead resistance to meropenem/vaborbactam and imipenem/relebactam (19). A mutation in these porins together to hyperexpression of KPC and SHV and efflux pumps are responsible for the resistance to ceftazidime-avibactam (33).

As mentioned above, the emergence of carbapenem resistance can be not only attributed to carbapenemase production but also to the concurrence of several other mechanisms such as ESBL or AmpC production associated with porin loss or efflux pumps (23).

4. Colistin resistance

Traditionally, colistin resistance in *K. pneumoniae* was attributed to chromosomal mechanisms, mainly by target modification that decrease colistin binding to LPS. However, in 2015, a plasmid-mediated polymyxin resistance mechanism was firstly reported in China in *E. coli* strains from animal food, identifying the mobilized colistin resistance 1 (*mcr-1*) gene as the responsible of modifying the target of colistin (34). Since then, colistin plasmid-mediated resistance has spread widely across several bacterial species including *K. pneumoniae*, and ten mcr genes (*mcr-1* to *mcr-10*) have been reported to date (35). The prevalence of colistin resistance in *K. pneumoniae* strains is higher than in other enterobacteria. This is worrisome in CPKP strains, where rates up to 36% co-resistance have been reported, leading to high mortality rates (23).

Current treatment for MDR *K. pneumoniae* infections

The emergence of MDR/XDR enterobacteria severely limits the available therapeutic options. Usual last-line drugs as polymyxins, fosfomycin, tigecycline and (occasionally) aminoglycosides, and combinatorial therapy remains the cornerstone therapy for MDR-related infections (36,37). Fortunately, last years, the introduction in the clinical practice of new drugs, such as ceftazidime-avibactam or cefiderocol, has partially broadened the effective arsenal against these infections. Nevertheless, as the current drug pipeline is still insufficient to combat the rise on AMR, the use of these new drugs must be regarded with caution to avoid the rapid

emergence of resistance, and these agents should be reserved for severe infections or with restrictive indications.

Combination therapy

The use of combination therapy for the management of MDR-related infections has been traditionally based on *in vitro* and *in vivo* studies searching the potential synergistic effect of double or triple drugs in combination with different mechanisms of action. In this context, many preclinical studies have explored multiple combinations with usual antibiotics (i.e. polymyxins, carbapenems, fosfomycin…), showing variable results that are dependent on the methodology used and the strain heterogeneity (36). To provide more clear information, several prospective randomized controlled clinical trials (RCT) have been conducted in patients infected by CPE, most of them comparing mortality rates of the combination therapy respect to monotherapy **(Figure 7).** The overall conclusion is that combination therapy improves survival in patients with high mortality score and severe infections (e.g. BSIs), while monotherapy should be considered for those in the low-mortality-score stratum (36,38).

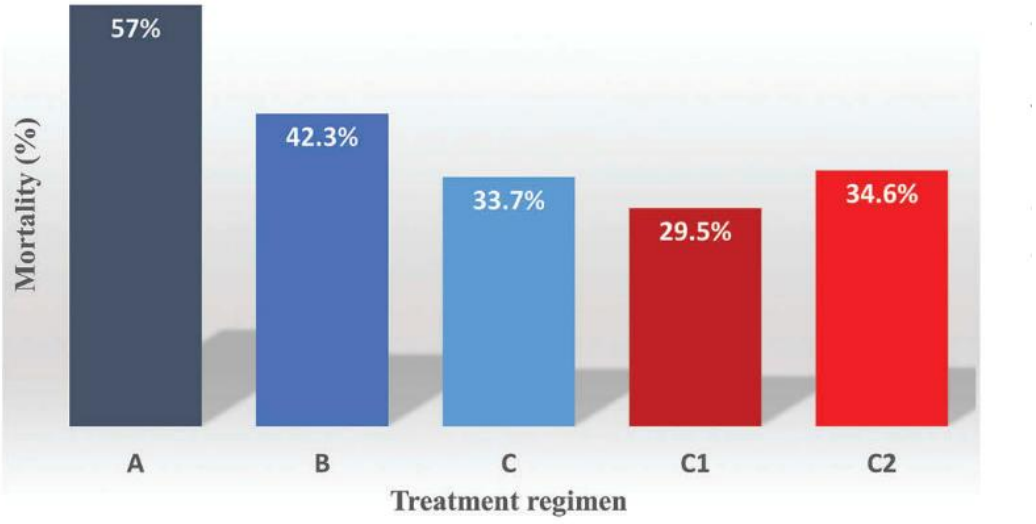


Figure 7. Mortality rates of 2.972 patients with infections caused by CPKP according to treatment regimen. Column A: Inappropriate therapy (no drug *in vitro*-active); Column B: Monotherapy (one drug *in vitro*-active); Column C: Combination therapy (two or more drugs *in vitro*-active); Column C1: Combination therapy with two or more *in vitro*-active drugs, including a carbapenem (MIC ≤ 8mg/L); Column C2: Combination therapy with two or more *in vitro*-active drugs not including a carbapenem. From (36).

Thus, the decision-making process for clinical management of patients with MDR infections include the consideration of several aspects: patient characteristics (renal function, comorbidities, underlying conditions), source of infection, microbiological data (pathogen, local

epidemiology, antimicrobial susceptibility) and severity of the infection. All these features lead to individualized therapies with the aim to select adequate antimicrobial regimens (37). In severe patients with CPE infections, current clinical guidelines recommend the inclusion of more than one active antibiotic to which the bacteria are susceptible *in vitro*, but do not provide recommendation about specific combinations. A summary of traditionally used combinations in infections caused by CPE are displayed in **Figure 8.**

Risk level, therapy type, and isolate susceptibility	Drugs
High risk,[b] combination therapy	
Susceptible to a β-lactam (use according to susceptibility)	Backbone: ceftazidime-avibactam (preferred) or meropenem-vaborbactam; alternatively, meropenem (if MIC is ≤8 mg/liter) or ceftazidime or aztreonam Accompanying drug (no data available about the need for combination therapy if ceftazidime-avibactam or meropenem-vaborbactam is used as the backbone): colistin, tigecycline, aminoglycoside, or fosfomycin (if isolate is intermediate to the backbone drug, consider using 2 of these)
Resistant to all β-lactams (including isolates with meropenem MICs of >8 mg/liter), susceptible to at least 2 drugs, including colistin	Backbone: colistin Accompanying drug: tigecycline, aminoglycoside (high risk of nephrotoxicity), or fosfomycin
Resistant to all β-lactams and colistin, susceptible to at least 2 drugs	Backbone: tigecycline or aminoglycoside Accompanying drug: tigecycline or aminoglycoside, fosfomycin
Pandrug-resistant or susceptible to only one drug	Meropenem plus ertapenem or ceftazidime-avibactam plus aztreonam; add any active drug; consider active investigational drugs if available; consider *in vitro* testing of combinations for synergy
Low risk,[c] monotherapy	
According to susceptibility	Ceftazidime-avibactam, meropenem-vaborbactam, meropenem, ceftazidime, aztreonam, colistin, tigecycline, aminoglycoside (if intermediate susceptibility, choose another option or use combination)

Figure 8. Summary of recommended regimens for the treatment of CPE-infections. From (37). [b]High risk defined as having septic shock or, for BSIs, an INCREMENT mortality score of ≥8 points; [c]Low risk is defined as having an INCREMENT mortality score of ≤8 points.

Classical drugs

1. Colistin (polymyxin E)

Colistin is a cationic polypeptide antibiotic belonging to the polymyxins class (polymyxin E) active against non-fermenters Gram-negatives (*A. baumanii, P. aeruginosa, Stenotrophomonas maltophilia*) and Enterobacterales, except for *Proteus* spp., *Serratia* spp., *Morganella* spp., and *Providencia* spp., which show intrinsic colistin resistance. It is administered by injection or by inhalation as the inactive prodrug colistin methane sulfonate (CMS), and by oral or topical as colistin sulphate (more toxic). Colistin binds to the LPS in the outer membrane of Gram-negative bacteria causing destabilization that increase the permeability and leads to cell death **(Figure 9)** (35). Due to this mechanism of action, colistin is assumed to have a high synergistic potential, able to favour the action of other drugs in combination therapy especially with carbapenems and fosfomycin (36). Up to date, it is considered a remarkable last-line drug in the MDR treatment, showing more than 95% of activity against CRE and XDR *P. aeruginosa* (39).

Resistance to colistin can be achieved by chromosomal or plasmid-mediated (*mcr* gene) mechanisms, producing modification of the LPS via cationic substitution (39).

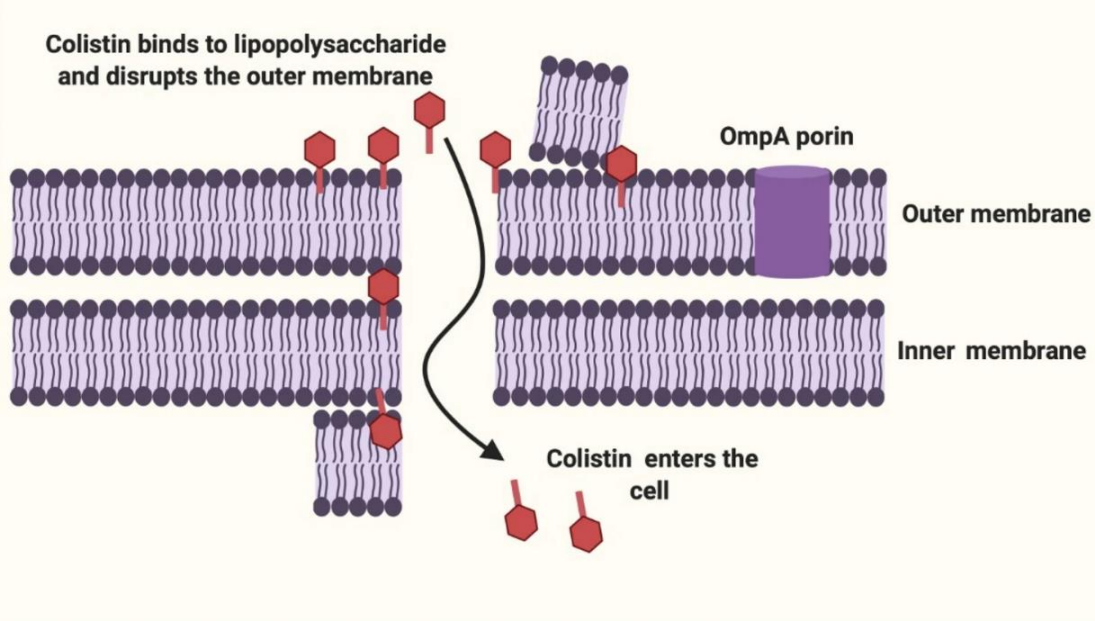


Figure 9. Mode of action of colistin. From (35)**.**

The main disadvantage of colistin is its pharmacokinetic (PK) and pharmacodynamic (PD) properties. Colistin has concentration dependent antibacterial effect, the best predictor of effectivity is the ratio of the area under the concentration-time curve for free drug from 0 to 24 h to the MIC ($fAUC_{0–24}$/MIC) index. However, it presents a narrow therapeutic window, and the nephrotoxic threshold (2.42 mg/L) is close to the effective average plasma concentration (2 mg/L) (39–41); thus, patients with renal insufficiency must be drug-monitored. Even in patients with normal renal function, the achievement of adequate drug exposure is complicated in some clinical syndromes. These data together with observational studies regarding high mortality rates using colistin in monotherapy, support the use of colistin always in combination therapy (42). The usual dosage regimen is a loading dose of 9 million units (MU) (720 mg CMS) followed by 4.5 MU (360 mg CMS) every twelve hours.

Currently, the extended use of colistin has led to a worrying increase in colistin-resistance in CPE strains, increasing the mortality rates in high-risk patients (43,44). Moreover, based on RCTs, polymyxin-based regimens are associated to an increased mortality and excess nephrotoxicity. As consequence, clinical guidelines recommend

avoiding the use of colistin for the treatment of infections caused by CPE, but can be considered as a last resort for uncomplicated CPE cystitis (45).

2. Fosfomycin

Fosfomycin is an old bactericidal antibiotic classically used for the treatment of uncomplicated urinary tract infections. It is a phosphoenolpyruvate (PEP) analogue, available in three formulations, fosfomycin disodium (intravenous), fosfomycin trometamol and fosfomycin calcium (both oral). Fosfomycin acts inhibiting the first step of the bacterial cell wall synthesis, the formation of the peptidoglycan precursor UDP N-acetylmuramic acid. Specifically, fosfomycin covalently binds to the thiol group of a cysteine in the active site of the enzyme UDP-N-acetylglucosamine enolpyruvyl transferase (MurA), which catalyzes the transfer of the enolpyruvyl moiety of PEP to the 3'-hydroxyl group of UDP-N-acetylglucosamine. As consequence, the enzyme is inactivated and peptidoglycan biosynthesis blocked **(Figure 10)** (46,47).

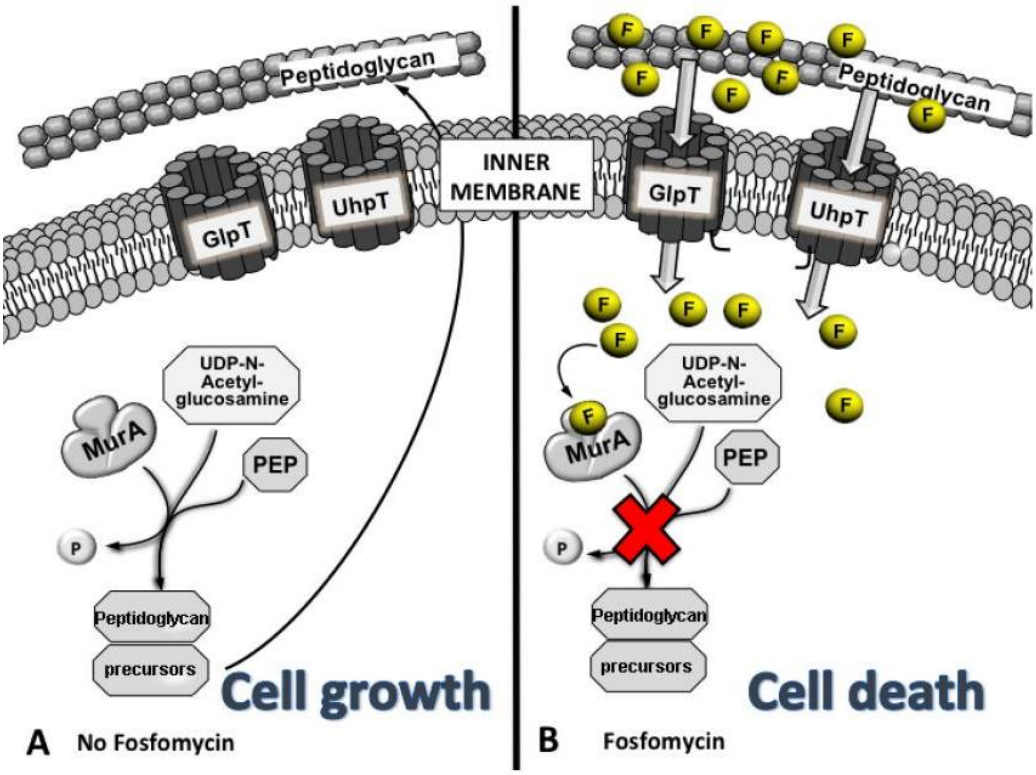


Figure 10. Mechanism of action of fosfomycin. From (47). GlpT, L-α-glycerophosphate; MurA, enolpyruvyl transferase; PEP, phosphoenolpyruvate; UhpT, hexose-6-phosphate

To enter the cell, fosfomycin structure mimics glycerol-3-phosphate and glucose-6-phosphate (G6P), both transported under normal conditions by two transporter systems: the L-α-glycerophosphate (GlpT) and the hexose-6-phosphate (UhpT) respectively (**Figure 10**). *In vitro* susceptibility shows that fosfomycin is a broad-spectrum antibiotic, active against Gram-positive and Gram-negative pathogens, including ESBLs, AmpC-producing and CPE isolates; it also poses high synergistic potential with several antibiotics, demonstrated by *in vitro* and *in vivo* studies (48). As these studies suggest, fosfomycin may be a good partner in combination therapy. In fact, during the last years, it has been used as rescue therapy in MDR Gram-negative infections, mostly combined with other last-line drugs (carbapenems, colistin and tigecycline) (36,37).

The use of fosfomycin in combination therapy is traditionally recommended in severe infections due to the rapid development of resistance when it is administered in monotherapy (37). There are several resistance mechanisms for which enterobacteria develop resistance to fosfomycin: (i) reduced permeability produced by mutations in any of the transporters GlpT or UhpT that decrease the antibiotic uptake; (ii) antibiotic modification. Fos proteins (FosA, FosB or FosX) are enzymes able to hydrolyse fosfomycin and made it ineffective; the *fos*A gene is intrinsic to *K. pneumoniae* and may lead to clinical failure; (iii) target modification, acquired by mutations on MurA. This mechanism is rarely observed in clinical isolates (47).

Intravenous fosfomycin shows optimal PK/PD parameters with a low protein binding and excellent tissue distribution, achieving clinically relevant concentrations in serum, skin and soft tissues, bladder wall, lungs, bone, central-nervous system or heart valves (46). Orally, it does not achieve adequate concentration in the renal parenchyma and should be avoided if the upper urinary tract is infected (45). The bactericidal activity seems time or concentration-dependent with different microorganisms. For most of them, the efficacy parameter is the time above the MIC (*f*T>CMI) ≥ 50% (46,49). In general, fosfomycin is a safe drug and has low incidence of adverse effects. Most common after oral administration are gastrointestinal symptoms (diarrhea, nausea, abdominal pain, and dyspepsia). The most significant adverse effect related to the intravenous administration is a high sodium intake derived from fosfomycin disodium salt, which could limit its use in patients with heart or renal failure (46,49). The most appropriate dosing schedules range from 12 to 24 g per day divided in 3-4 intravenous administrations, or 3 g once daily oral administration in non-complicated urinary tract infections (50).

3. Tigecycline

Tigecycline is a glycylcycline derived from minocycline that exhibits predominantly bacteriostatic activity. It was designed to overcome typical tetracycline resistance mechanisms (efflux pump acquisition and ribosomal protection) and developed for the parenteral treatment of polymicrobial MDR infections caused by Gram-positive and Gram-negative bacteria. Similar to other tetracyclines, tigecycline mechanism of action is based on its binding to the 30S subunit of bacterial ribosome, blocking the incorporation of amino acid residues into the elongation of peptide chains, thus, resulting in the inhibition of bacterial protein translation **(Figure 11**) (29).

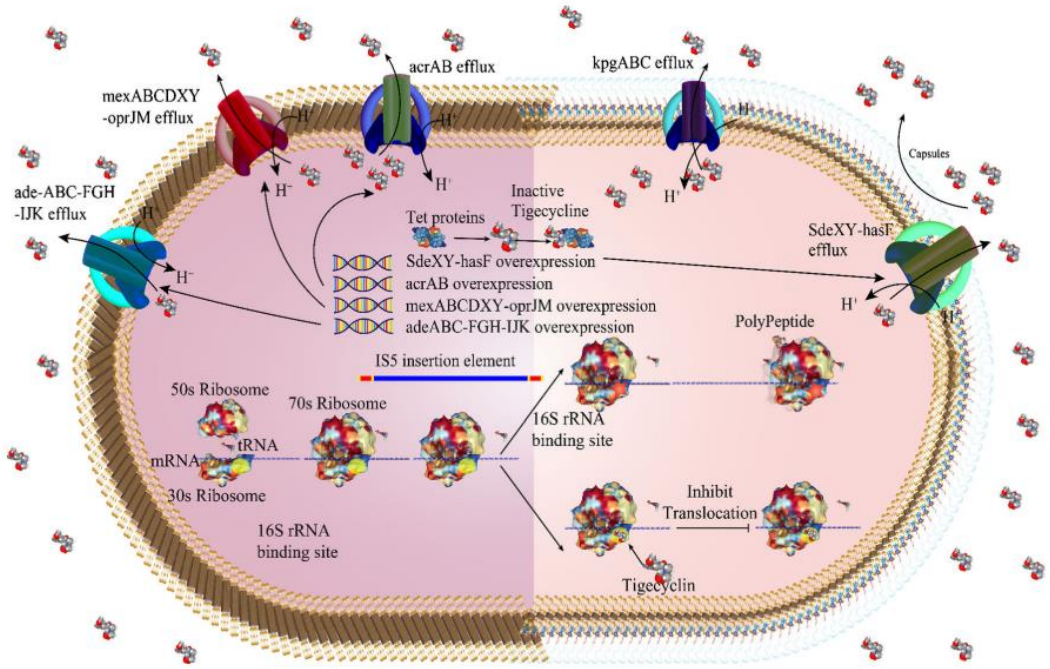


Figure 11. Tigecycline mechanism of action and resistance. From (29)

Enterobacterales have variable susceptibility to tigecycline, and some species show natural resistance (*Proteus* spp., *Providencia* spp., and *Morganella* spp.). Tigecycline resistance has been reported by mobile genetic elements carrying several resistance genes (i.e., *tet*(X) gene variants) but also chromosomally encoded, by overexpression of resistance-nodulation division efflux pumps (i.e., AdeABC, AdeFGH, AdeIJK, MexXY, and AcrAB), as in **Figure 11.**

Tigecycline was initially approved by the FDA for treatment of skin and soft tissue infections, complicated intra-abdominal infections, and community-acquired bacterial pneumonia in adults, but it has been used as last-resort therapy to treat CPE infections in

synergistic combination with colistin, aminoglycosides and carbapenems mainly in bacteraemia, urinary tract infection and nosocomial pneumonia (37,51). In 2010, the regulatory agencies issued warnings because tigecycline in monotherapy was associated to increased risk of mortality and clinical failure. However, other systematic review and meta-analysis support tigecycline as a suitable choice in combination therapy for severe patients (29). As its activity is independent of the presence of carbapenemases, clinical guidelines support the use of tigecycline in monotherapy in intra-abdominal infections caused by CRE, but do not recommend tigecycline in other clinical indications (45).

Tigecycline also poses disadvantageous PK/PD features due to its rapid high tissue distribution, resulting in limited concentration in the urine and poor serum concentrations. Values of $fAUC_{0-24}$/MIC lower than 4.5 or 6.96 are related to therapy failure for pneumonia and intraabdominal infections respectively (52). Currently, it is administered with a loading dose of 100 mg, followed by 50 mg every twelve hours but, for intra-abdominal infections, high doses (200 mg followed by 100 mg every twelve hours) may be more effective (45). The most common adverse effects are gastrointestinal symptoms (nausea, vomiting, and diarrhoea). Occasionally reported side effects have been pancreatitis, increased hepatic function, thrombophlebitis, pruritus, fever and headache, abdominal pain and cholestatic jaundice (29)

.

4. Carbapenems

Carbapenems are a sub-group of the β-lactam family of antibiotics (i.e., imipenem, meropenem, ertapenem, doripenem) with the broadest spectrum of action. They are derivatives of thienamycin, a compound produced by *Streptomyces cattleya.* Carbapenems are active against Gram-positive and Gram-negative bacteria by binding with high affinity to most high-molecular-weight, penicillin-binding proteins (PBPs). In Gram-negatives, the entry to the cell is carried by a specific outer membrane protein, OprD, different from those used by cephalosporins or penicillins (OmpC or OmpF) (53,54).

Due to its stability to β-lactamases, carbapenems have been traditionally used as first-line treatment in ESBLs and AmpCs enterobacteria. As carbapenemases show variable susceptibility to carbapenems, the use of carbapenems also for treatment of CPE infections has been considered in isolates showing susceptibility or low-level resistance to these drugs (i.e., OXA-48 strains). Due to the lack of effective new drugs and safety profile of β-lactams respect to other last-resort agents, several carbapenem-based combinations (i.e., with

colistin, tigecycline, fosfomycin and/or aminoglycosides) have been used in the management of these infections. The clinical evidence has led to conclude that carbapenems may be a suitable choice when the microorganisms exhibit a meropenem MIC ≤ 8 mg/L, if carbapenems are administered at high doses and combined with another active *in vitro* antibiotic. Some studies also reflected the possibility to administrate carbapenems even when meropenem MICs are above 8 mg/L (up to 32–64 mg/L), although this strategy is limited to centres where therapeutic drug monitorization is available to assure the accurate drug exposure (36,37).

Double carbapenem therapy (DCT) has become another strategy utilized for CPE treatment. This therapy is based on the use of ertapenem with meropenem or doripenem either as a sole combination or added to other antibiotics. The hypothesis is that ertapenem (with high affinity for carbapenemases) might act as a suicide inhibitor prior to the action of the second carbapenem; thus, microbiological clearance might increase (55). Clinical experience with DCT has been almost applied to treat severe infections caused by KPC-producing *K. pneumoniae* strains, and overall response seems to be good with minimal adverse effects (55). However, the beneficial effect could be different with other types of carbapenemase (MBL or OXA-48). Moreover, since carbapenems are of great value to have as a last-line of defence, it is important to avoid the generalized use that would lead to high levels of carbapenem resistance, and new approaches try to find other carbapenem-sparing regimens. Because of this, clinical guidelines suggest to avoid the use of carbapenem-based combinations in CPE infections, unless the MIC meropenem is ≤8 mg/L, where high-dose extended-infusion meropenem should be used as part of the combination if the new β-lactams/β-lactamase inhibitors (BLBLIs) are not used (45,56).

5. Aminoglycosides

Aminoglycosides are bactericidal antibiotics that bind to the 30S ribosomal subunit to inhibit protein synthesis. They show concentration dependent activity and dose-dependent post-antibiotic effect (PAE); thus, a ratio of a maximum serum concentration to the MIC (C_{max}/MIC) over 10 has been proposed to achieve the maximum bactericidal effect with a long PAE. In general, they are well tolerated by intravenous and intramuscular administration, but present renal and ear toxicities that increase at multiple doses.

For ESBL, AmpC and CRE infections, they have been used as single and combinatorial therapy, based on a synergistic effect in combination with antibiotics with different mechanisms of action (e.g., β-lactams, fosfomycin). However, clinical experience and

current available evidence suggest aminoglycoside-based therapy may cause more toxicity than clinical benefits. There is also controversy about the adequation of effective doses after standard dosage (amikacin 25-30 mg/kg/day, gentamicin and tobramycin 6-7 mg/kg/day), because of some clinical studies demonstrate lower peak concentrations than those expected. Hence, aminoglycosides should be managed with caution and under therapeutic drug monitoring whenever possible to verify optimal drug levels (37).

For CRE infections, as aminoglycosides are mostly eliminated by renal route, their use in single dose for the treatment of cystitis and complicated urinary tract infection is acceptable if the strain remains susceptible. Outside the urinary tract, monotherapy with aminoglycosides is an alternative treatment when first options failed (45). Combinatorial therapy is not recommended unless there are very few options.

New drugs

1. New BLBLIs combinations

Although there is still little evidence on the effects of the new, recently approved BLBLIs, currently they are considered the preferred treatments against CRE enterobacteria.

- **Ceftazidime-avibactam**

  Avibactam is a novel, β-lactamase inhibitor with a broader spectrum of activity than classical β-lactamase inhibitors. It has activity against Ambler class A, class C and some class D enzymes and was approved to use in combination with the third-generation cephalosporin ceftazidime for treating complicated urinary tract, complicated intra-abdominal infections, and hospital-acquired pneumonia. Avibactam acts protecting ceftazidime from degradation by a variety of serine β-lactamases (57). Safety and efficacy of ceftazidime-avibactam against MDR enterobacteria facilitated its inclusion as first-line therapeutic option for infections caused by CPE (45,56). It is administered in monotherapy against OXA-48 (class D) and KPC (class A) producers or associated to aztreonam against class B enzymes (β-lactamases refractory to inhibition by avibactam) (45), intravenously at a standard dose of 2.5 g every 8 hours. Although the potential for resistance selection appears to be low (57), development of resistance to ceftazidime-avibactam is more likely after previous exposure with meropenem-vaborbactam (58,59). In fact, resistance

linked to mutations in plasmid-borne KPC-3 have been already reported during ceftazidime-avibactam treatment (60,61).

- **Meropenem-vaborbactam and imipenem-relebactam**

  Vaborbactam and relebactam are β-lactamase inhibitor of class A and class C serine beta-lactamases, including *K. pneumoniae* KPC carbapenemase, but do not inhibit class B or class D enzymes. Meropenem-vaborbactam was approved for the treatment of complicated urinary tract infections and has shown similar clinical success than ceftazidime-avibactam in serious infections. As seems to exhibit higher resistance barrier, meropenem-vaborbactam may be preferred to treat KPC-producers (62). On the other hand, imipenem-relebactam was approved for the treatment of complicated urinary tract, complicated intra-abdominal infections and hospital-acquired pneumonia, and several RCTs demonstrate favourable clinical outcomes and non-inferiority respect to comparators drugs (including imipenem-non-susceptible pathogens)(63); thus, imipenem-relebactam it is also a choice treatment against serious infections by MDR Enterobacterales (45). Intravenous dose schedule is 4 g every 8 hours and 1.25 g every 6 hours for meropenem-vaborbactam and imipenem-relebactam respectively. In both combinations, porin loss has been reported as resistant mechanisms (OmpK36 y OmpK35 porins) (19).

2. Cefiderocol

Cefiderocol is a novel injectable siderophore cephalosporin that combines a catechol-type siderophore and cephalosporin core with side chains similar to cefepime and ceftazidime. This chemical structure allows several advantages: (i) a higher penetration through the siderophore–iron complex pathway; (ii) a higher affinity than ceftazidime for the PBP3; (iii) an improved stability to the action of β-lactamases, being active against carbapenem-resistant Gram-negatives (Enterobacterales, *P. aeruginosa* y *A. baumannii* and *S. maltophilia*). It is approved for the treatment of complicated urinary tract and hospital-acquired pneumonia. Although *in vitro* cefiderocol has demonstrated potent activity, a RCT showed an increased mortality rate in the cefiderocol arm in pneumonia and BSIs. Until more evidence is obtained, cefiderocol is recommended as alternative therapy in CRE infections, when other new drugs are unavailable (45).

3. Plazomycin

This is a semi-synthetic aminoglycoside derived from sisomicin, designed to evade modification by aminoglycoside-modifying enzymes, and show bactericidal activity against MDR pathogens (*Staphylococcus* spp., *P. aeruginosa* and CPE including KPC and OXA-48). Plazomycin binds to the active site A in the bacterial 30S ribosomal subunit, disrupting the protein synthesis (51). Clinical experience is limited to complicated urinary tract infections, where it is administered intravenously at 15 mg/kg in single dose(45). Synergistic *in vitro* effect was reported with meropenem, colistin and fosfomycin (64). Resistance to plazomycin was attributed by 16S rRNA methyltransferases in enterobacteria, which modifies the ribosomal binding site. Typical side effects derived from aminoglycoside (nephrotoxicity and neurotoxicity) seems to be lower than with other aminoglycosides (51).

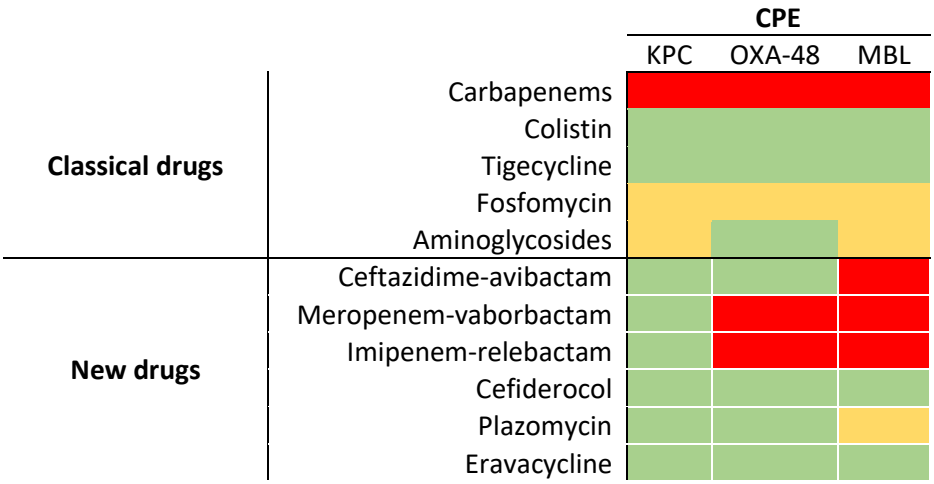
4. Eravacycline

Eravacycline is a novel synthetic fluorocyclin with activity against MDR Gram-negatives, with similar mechanism of action than tetracyclines and might have advantages respect to tigecycline in severe infections, such as higher serum levels and better tolerability (51,65). Resistance mechanisms have been reported by overexpression of efflux pumps and target modifications. Due to its rapid tissue distribution, eravacycline is an acceptable monotherapy in intra-abdominal CRE infections (45,65).

Overview of classical and new drugs for *K. pneumoniae*

Table 1 shows a summary of the activity of all drugs against the different types of carbapenemases in enterobacteria.

Table 1. Activity of current drugs against carbapenemase-producing enterobacteria. Green display >80%, Yellow between 30-80% and red <30% of activity.



Antimicrobial discovery pipeline

Antimicrobials have been probably one of the most successful landmarks in medicine history. They are key weapons to treat infectious diseases, saving many lives across history. The first patented antimicrobial, Salvarsan, was discovered in 1910 to treat syphilis (66), an endemic and incurable disease at the time. In 1928, Alexander Fleming discovered the penicillin and opened the way into the antibiotic field. Lately, in 1935, the discovery of sulfonamides appeared and become the first drugs systematically used to treat infections. These precedents set the foundations of the modern "antibiotic era" and stablished the paradigms for future drug discovery research. The period from 1950 to 1960 is known as the golden age for antimicrobial discovery and development (D&D), since many different classes of antibiotics were developed in this age (i.e., erythromycin, isoniazid, vancomycin, rifamycin, polymyxin, chloramphenicol). Unfortunately, from 1960 to date, the D&D process has dramatically dropped with an innovation gap in which most antimicrobials currently used are derivatives of existing classes discovered between the early 1900s and 1980s. During the past 30 years, few new classes of antimicrobials have been introduced in the market (67). These include oxazolidinones and cyclic lipopeptides (systemic classes), and pseudomonic acids and pleuromutilins (topical classes). The antimicrobial discovery timeline is displayed in **Figure 12**.

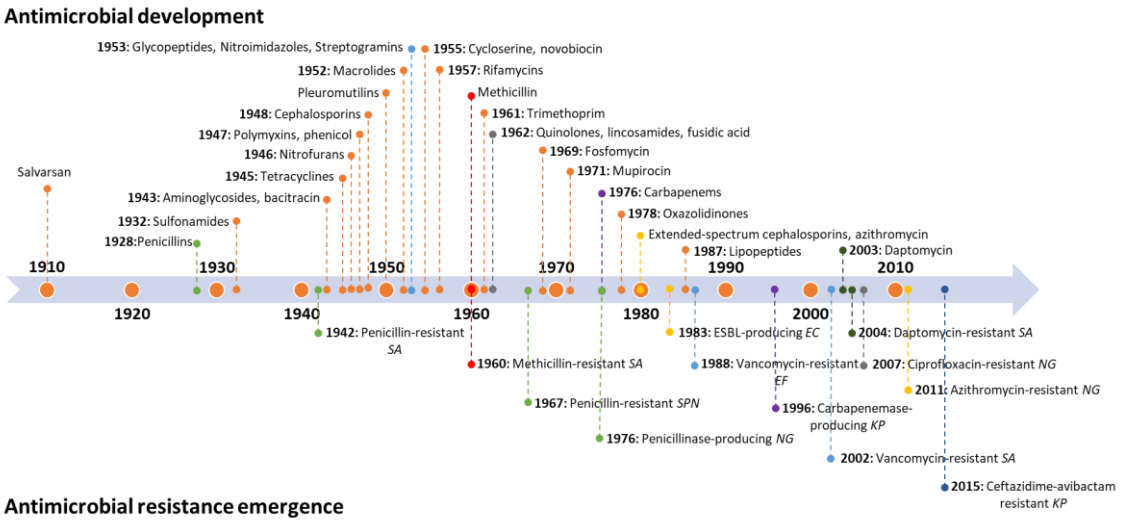


Figure 12. Antimicrobial discovery timeline at the top and emergence of resistant pathogens at the bottom. SA, *Staphylococcus aureus*; SPN, *Streptococcus pneumoniae*; NG, *Neisseria gonorrhoeae*; EC, *Escherichia coli*; EF, *Enterococcus faecium*; KP, *Klebsiella pneumoniae*

Since 2015, 18 antimicrobials have been approved globally **(Table 2)**, but only two of them are novel antimicrobials belonging to a different chemical class: avibactam (diazabicyclo-

octane class) and vaborbactam (cyclic boronic acid pharmacophore), both acting as β-lactam inhibitors (BLI) (68). Cefiderocol can be considered also innovative due to incorporation of an iron-chelating siderophore that facilitates entry into the Gram-negative bacterial cell (69). Lefamulin is the first systemic formulation of the topically administered pleuromutilin class. Seven of these new antibacterials addresed WHO critical-priority pathogens (ceftazidime-avibactam, meropenem-vaborbactam, imipenem-relebactam, ceftolozane-tazobactam, cefiderocol, plazomicin and eravacycline), ten target high and medium priority pathogens (ceftobripole, delafloxacin, omadacycline, lefamulin, lascufloxacin, levonadifloxacin, oritavancin, dalbavancin, tedizolid and contezolid) and one was approved to treat MDR/XDR tuberculosis (pretomanid) in combination with two other drugs, bedaquiline and linezolid (70,71).

Unfortunately, the emergence of resistance is an unavoidable natural process that continues to evolve even before antimicrobials gain market access **(Figure 12)**. This is no exception with the new antimicrobials. For example, the emergence of resistant strains has been already reported for ceftazidime-avibactam, meropenem-vaborbactam, eravacycline or omadacycline (32,72,73), recently introduced in clinical practice, which compromise the use of these new agents that should be preserved for severe infections. Thus, strategic investment in new therapeutic options to fight AMR is urgently required to counterbalance the rapid dissemination of resistance to the currently available antimicrobials.

Table 2. Antibacterial drugs that gained market authorization between 2015 and June 2021. Adapted from (70).

Name (trade name)	Antibacterial class	Route of administration	Indication(s)	Expected activity
Delafloxacin (Baxdela)	Fluoroquinolone	i.v. & oral	ABSSSI, CAP	OPP
Avibactam + ceftazidime (Avycaz)	DBO-BLI + β-lactam	i.v.	cIAI, cUTI, HAP/VAP	CRPA, CPE
Vaborbactam + meropenem (Vabomere)	Boronate BLI + β-lactam	i.v.	cUTI	CPE
Plazomicin (Zemdri)	Amynoglicoside	i.v.	cUTI	CPE
Eravacycline (Xerava)	Tetracycline	i.v.	cIAI	CPE
Omadacycline (Nuzyra)	Tetracycline	i.v. & oral	CAP (iv), ABSSSI (iv & oral)	OPP
Relebactam + imipenem/ cilastatin (Recarbrio)	DBO-BLI + β-lactam	i.v.	cUTI, cIAI, HAP/VAP	CPE
Lefamulin (Xenleta)	Pleuromutilin	i.v. & oral	CAP	OPP
Pretomanid (Dovprela)	Nitroimidazole	Oral	XDR-TB	OPP
Lascufloxacin (Lasvic)	Fluoroquinolone	i.v. & oral	CAP	OPP
Cefiderocol (Fetroja)	Siderophore β-lactam	i.v.	FDA: cUTI, HAP/VAP, EMA: aerobic G-ve	CRAB, CRPA, CPE
Levonadifloxacin (Emrok)	Fluoroquinolone	i.v. & oral	ABSSSI	OPP
Contezolid (Youxitai), Contezolid acefosamil	Oxazolidinone	i.v. & oral	cSSTI i.v.	OPP
Ceftobiprole (Zevtera)				OPP
Tazobactam + Ceftolozane (Zerbaxa)	Alpha amino acid BLI + β-lactam	i.v.	cIAI, cUTI, HAP/VAP	CRPA, CPE
Dalbavancin (Xydalba)	Lipoglycopeptide	i.v.	ABSSSI	OPP
Oritavancin (Orbactiv)	Lipoglycopeptide	i.v.	ABSSSI	OPP
Tedizolid (Sivextro)	Oxazolidinone	i.v. & oral	ABSSSI	OPP

ABSSSI: acute bacterial skin and skin structure infections; BLI, β-lactam inhibitor; CAP: community-acquired pneumonia; cIAI: complicated intraabdominal infections; CRAB, carbapenem-resistant *Acinetobacter baumanii*; CRE, carbapenem-resistant *Enterobacterales*; CRPA, carbapenem-resistant *Pseudomonas aeruginosa*; cSSTI, complicated skin and soft tissue infections; cUTI, complicated urinary tract infection; DBO, diazabicyclooctane; EMA, European Medicines Agency; FDA, Food and Drug Administration (USA); HAP, hospital-acquired pneumonia; i.v., intravenous; OPP, other priority pathogens; VAP, ventilator-acquired pneumonia; XDR, extensively drug-resistant.

Drug development process

Traditional research and development process

The concerning lack of novel drugs in this field is mainly due to the lengthy, costly, and risky process of the traditional antimicrobial D&D, in where many large pharmaceutical companies have retired for not obtaining satisfactory commercial returns **(Figure 13).** Currently, essential early-stage projects to identify and validate new molecules are usually conducted by academic research, which is generally underfunded. Later phases in early and middle-projects as hit and lead optimization are headed by small and medium-size enterprises (SMEs), considered the primarily innovative investors (71,74). According to WHO data, these SMEs represent currently 87.65% (142) of the funders involved in antimicrobial D&D, while only eleven large pharmaceutical companies invest in this field (75). These SMEs are dealing with high attrition rates and capital risk, and find difficult to commercialize then their products due to the lack of large companies dedicated to develop antimicrobials at large scale (76).

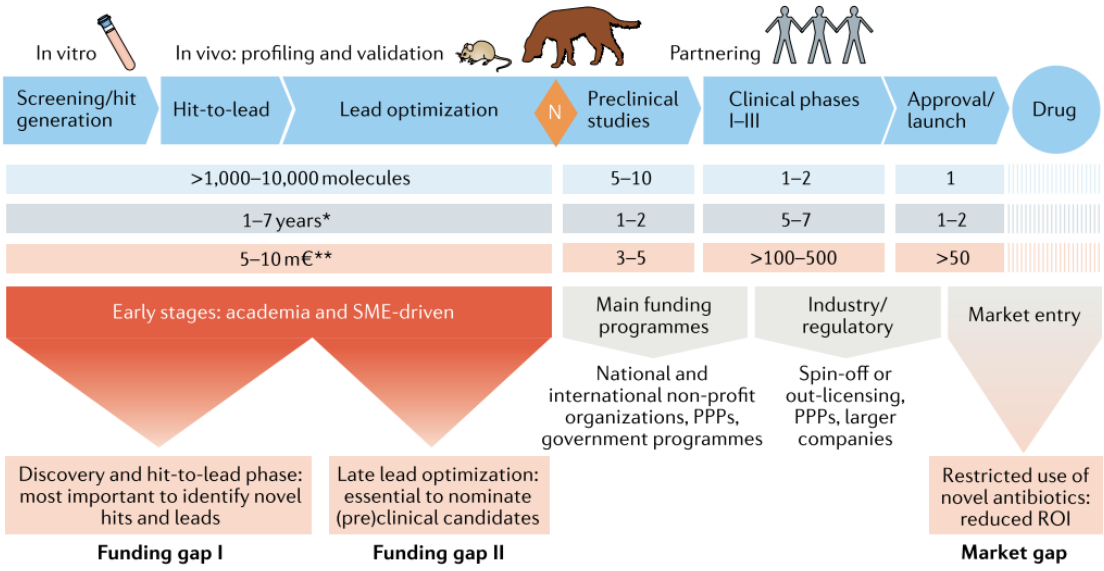


Figure 13. General scheme of antimicrobial drug development. From (74). *Timings are dependent on a number of factors and can vary greatly. A minimum to maximum range for complete development (discovery to market) is 8–18 years (average 13–14 years). **The cost per molecule/candidate (in million euros, m€) does not include extended costs for attrition (failed programmes) and lost opportunities associated with increased cycle time until reaching the next development phase; such extensions can increase the required budget for the early stages up to 50–100 m€. N (orange diamond), nomination of (pre)clinical candidate(s); PPPs, public–private partnerships; ROI, return on investment.

The funding gap in antimicrobial D&D is mainly consequence of the high volume of investment needed to develop new molecules into marketable drugs. In addition, antimicrobials

are often used just for a short period of time to treat acute infectious diseases, which is non economically attractive. Thus, pharmaceutical industry has focused for years in more sustainable approaches, such as the development of derivatives of stablished compounds (that requires lower investment) and the search of drugs for the treatment of chronic diseases, which report much higher economic benefits. In this context, while there are nearly 4,000 immuno-oncology agents in development (74), currently there are only 292 antibacterial agents in preclinical development (39.4% direct-acting molecules, 34.6% non-traditional approaches, 16.1% vaccines, 10.2% antimicrobial peptides), and 104 projects of antibacterials in clinical development addressed to treat priority pathogens (72 against WHO priority pathogens, 14 addressing *M. tuberculosis* and 18 against *Clostridioides*) **(Figure 14)**. Unfortunately, among these projects, only 16 products consist of new chemical classes and new modes of action, and all of them are still in under clinical phases (four in Phase I, nine in Phase II and 3 in Phase III) (77).

Figure 14. Antibacterial drugs under clinical development updated in April 2021. From (77).

According to the WHO, the clinical pipeline and recently approved antimicrobials are insufficient to tackle the challenge of AMR. As consequence, it is necessary to change the current paradigm with the aim to promote and support the antimicrobial D&D towards more sustainable approaches.

Drug repurposing

Drug repurposing (or drug repositioning) is a strategy to identify new indications for existing drugs. This term was first described by Ashburn and Thor in 2004 (78) and has been largely regarded as a promising alternative to the traditional drug discovery and development process (79,80), since new indications could be developed faster as long as they are used following originally approved recommendations, with already know safety profiles. From the point of view of pharmaceutical companies, drug repurposing is perceived as a safe strategy to save time and capital investment in early-stage trials (preclinical and phase I costs). The costs of bringing a repurposed drug to market have been estimated to be US $300 million on average, compared with an estimated ~$2–3 billion for a new chemical entity (79).

Historically, drug repurposing was approached as opportunistic findings from retrospective clinical data. As example, sildenafil (Viagra®), to treat erectile dysfunction, was initially developed for the treatment of hypertension, but showed unexpected erectile effects. Currently, systematic approaches combining experimental screening and *in silico* models that analyse existing data to identify potential new drug-disease associations are employed to increase the success in the reintroduction of existing drugs. Experimental screenings consist of high-throughput screening platforms that evaluate the activity of a drug collection to identify candidate hits, largely divided in target-focused screens (based on specific mechanism) and phenotypical screens (inhibition against the pathogen of study). In the infectious diseases field, the most clinically relevant approach is phenotypic screening of chemical libraries, in which between 500–2000 compounds are evaluated against the pathogen of study to assess their activities (80,81). This is the example of bedaquiline, a new antituberculous drug discovered in a phenotypical screening against *M. smegmatis* (82). On the other hand, *in silico* models encompass both molecular approaches to understand interactions against drugs and pathophysiology (genomic, transcriptomic or proteomic data), and the use of sophisticated computational models to process the called “big data”, large-scale information from electronic health records, clinical trials and biobanks (79). This approach might be highly useful to develop new treatments at short-term, although future perspectives include the development of more advanced technologies able to integrate and interpret this large amount of generated data.

Other challenges in the drug repurposing field include intellectual property issues related to new indications for already marketed drugs, regulatory or funding considerations. These issues are addressed by measures to incentivise drug repurposing development, and through new business models that involve collaboration between pharmaceutical companies

and biotech and academia researchers (79,80). Apart from this, the main drawback for clinical application is that new therapeutic indications might require higher doses to exert its action as antimicrobials, which may lead to toxicities. In this case, the search for synergistic partners might increase the success of drug repurposing in two different ways: first, expanding the therapeutic range of drugs whose potential use may be limited by toxicity issues and, second, by rescuing antimicrobials that do not reach efficacy breakpoints (81,83).

Figure 15 shows the suggested approaches *in vitro* and *in vivo* based on synergistic combinations on drug repurposing. In *in vitro* studies, toxicities of drugs alone might be reduced maintaining the potency in the combined effect. Drug repurposed combination might also be effective achieving allowed serum concentrations by *in vivo* studies (83).

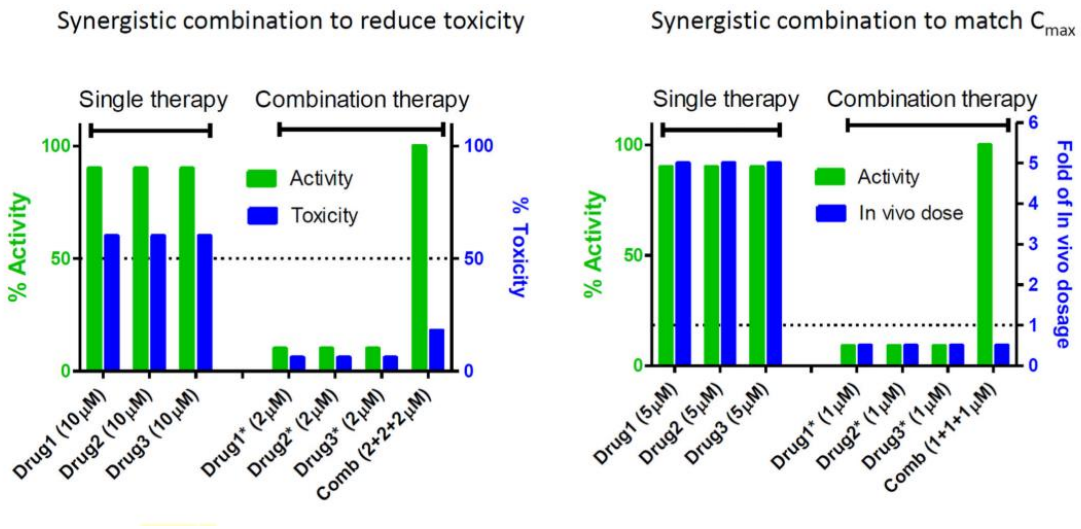


Figure 15. Two synergistic combination models to optimize hits from drug repositioning screens. From (42).

New approaches for antimicrobial discovery

In vitro synergy methods

Combination therapy is largely used against several infections (e.g., tuberculosis, HIV) and syndromes (e.g., endocarditis, cystic fibrosis, osteomyelitis), and remains the backbone to fight against AMR.

The efficacy of combination therapy is dependent on the drugs' behaviour when administered together. In this context, the combined effect of two or more drugs can be:

- Synergistic: It occurs when their activities within the combination are improved over the sum of their individual separate effects.

- Antagonistic: negative interaction in which the effect of the combination is significantly smaller than the effect of each compound acting separately.
- Indifferent: the activity of both drugs is limited to be equal to that of the most active drug in the combination, or additive when is equivalent to the sum of each individual antibiotic. This latter criterion can be misinterpreted as synergy and does not indicate increased potency, since it is the expected effect when a drug is added to the therapy.

Thus, combination therapy is based on the search for a synergistic effect in order to primarily achieve improved clinical efficacy. In addition, other objectives can also be achieved: (i) the antibacterial spectrum can be broadened by adding different drugs; (ii) the emergence of resistance is minimised, especially by combining drugs with different mechanisms of action; (iii) the adverse effects derived from possible toxicities could be minimized, since the combination of drugs allows both the doses and the duration of treatment to be reduced.

There are several *in vitro* methods to assess the effect of drugs in combination. The best known, the checkerboard assay (CBA) and the time-kill assay (TKA), are traditional techniques widely used in synergy studies; but are tedious, time-consuming and not well standardized techniques that result in low reproducibility and heterogeneous results between laboratories.

❖ Screening methods

- **Agar diffusion methods**

This technique is based on the diffusion of the drug through the solid medium in which a given strain is inoculated. The procedure is simpler than CBA, as it employs standardized protocols used in routine antibiotic susceptibility testing. Two main methods are included here:

  - Disk diffusion: This is a qualitative method where paper disks contain drugs at a fixed quantity. After determining inhibition diameters of individual drugs, another plate is prepared placing both disks at a distance equal to the sum of the inhibition radii of each disk tested separately. After incubation, synergy is defined when there is an enhancement of the inhibition zones in the interface respect to drugs alone. By contrary, antagonism is defined when a truncated zone is observed **(Figure 16).**

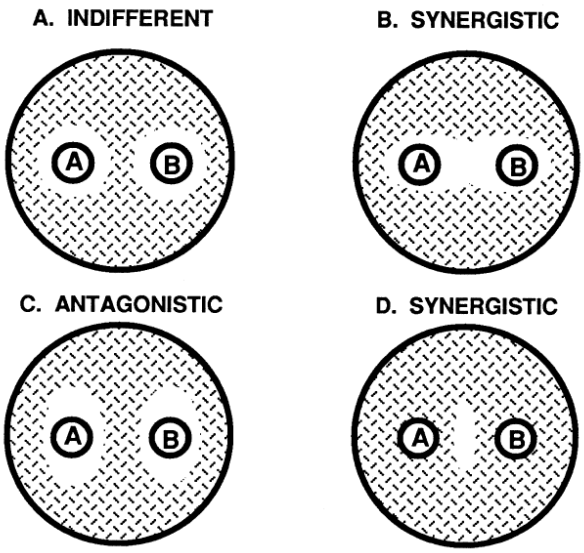


Figure 16. Effects interpreted according to inhibition zones of drug combinations by disk diffusion methods. Antimicrobial susceptibility testing. Adapted from Chapter 5.12.20 of (84).

- E-test: Drugs are disposed at a gradient of concentrations. After incubation, the MIC value is obtained by the intersection of the elliptical zone of growth inhibition with the strip, thus, this is a quantitative measurement. To assess synergy, four technical variations have been described: (i) crossed-strip methods **(Figure 17)**; (ii) the fixed ratio; (iii) MICs proportion and (iv) drug-containing agar plates. Results are obtained by comparison of MIC values of each drug alone and in combination, also determining the FICI as the synergy indicator in these assays.

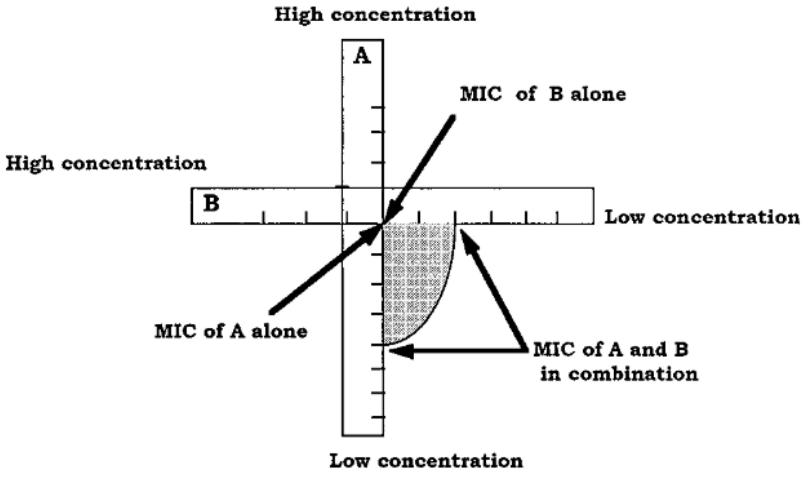


Figure 17. Diagram for testing drug combinations by E-test crossed-strips method. Adapted from (85).

Although based on the same procedure, technical variations do not confer the same correlation with the time-kill assays (assumed as reference synergy method). Thus, crossed-strip and MIC proportion methods seem the most reliable, with agreement ranges of 67-88% and 63-83% respectively (86).

- **Checkerboard assay (CBA)**

This is a basic assay for studying bidirectional drug interactions that can be performed on microdilution plates, macrodilution tubes or agar plates. Generally, serial dilutions of the partner drugs in different combination ratios are added to a bacterial inoculum and the effect of the combination is measured using growth inhibition as the endpoint reading. This assay has the major limitation of selecting a specific paired combination and the precise range of concentrations to obtain a measurement within the range. Some variants of this technique have been described to study higher order drug interactions, such as the multiple-combination bactericidal test to test up to four antimicrobials simultaneously (87), or the diaMOND (diagonal measurement of n-way drug interactions) that allow to extend the assay to any number of agents (88,89).

CBA is interpreted according to the Fractional Inhibitory Concentration Index (FICI), which is based on Minimum Inhibitory Concentration (MIC) values of both drugs (A and B). FICI is the sum of FIC of drug A (FIC_A) plus FIC of drug B (FIC_B), calculated as follows:

$$FIC_A = \frac{MIC\ of\ drug\ A\ in\ the\ presence\ of\ drug\ B}{MIC\ of\ drug\ A\ alone}$$

$$FIC_B = \frac{MIC\ of\ drug\ B\ in\ the\ presence\ of\ drug\ A}{MIC\ of\ drug\ B\ alone}$$

The combined effect is then interpreted depending on the result of FICI: a value ≤ 0.5 is a “synergistic interaction”, from 0.5 to 4 means “no interaction” and >4 is interpreted as “antagonistic”(90). This means that to obtain a synergistic effect, a ≥4-fold reduction in the MICs of both compounds in combination compared to their MICs alone is observed. **Figure 18** shows a 96-well plate CBA assay showing a typical profile of a synergistic interaction.

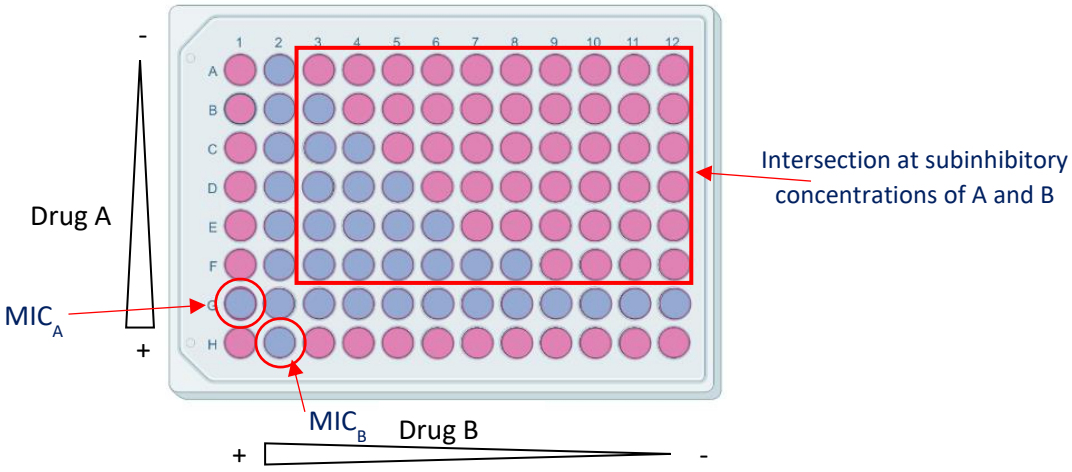


Figure 18. Graphical representation of a synergistic interaction by a checkerboard assay in a 96-well plate. Pink wells are grown; blue wells show growth inhibition.

Results based on FIC values can also be transferred to an arithmetic graph called an isobologram that visually represent the effect obtained **(Figure 19).**

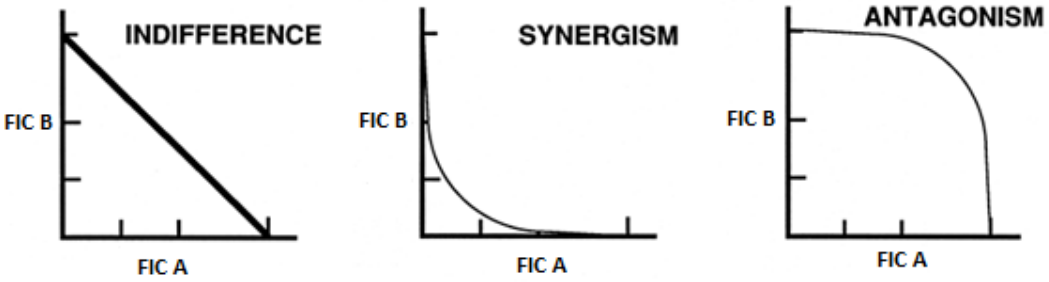


Figure 19. Representation by isobolograms of the different possible effects of a combination using the checkerboard method. FIC values of both drugs A and B are confronted in a graphic. The analysis of the profile determines the combined effect. Adapted from Chapter 5.12.19 of (84).

CBA assays are typically based on inhibition measures, which may overestimate the real effect of the interaction, leading to high attrition rates (i.e., the interaction is able to inhibit bacterial growth but does not show lethal activity and fails in subsequent validation assays). Following standard methods of antimicrobial susceptibility, the Minimal Bactericidal Concentration (MBC) can be also determined by subculturing from those wells with no visible growth. In this case, the Fractional Bactericidal Concentration (FBC) of each drug can be calculated with the same formula described above to obtain the Fractional Bactericidal Concentration Index (FBCI), a more restrictive parameter

based on bactericidal activity. In a previous work (see **Annex I)**, we discussed FBCI should be often considered in synergy screening programs to improve translational prediction power (91).

❖ Static methods: time-kill assays (TKA)

This methodology evaluates the activity of a drug at a fixed concentration over a period of time, usually 24 hours for rapid-growing microorganisms or up to 21 days for slower growers (e.g., *M. tuberculosis*). The measurement here is bacterial death by counting the number of colonies (colony forming units per millilitre; CFU / mL) that remain viable after exposure to the antibiotic in relation to the original inoculum at different time points. Thus, TKA provide precise information about the concentration-dependent or time-dependent activity of the drug (bacteriostatic, bactericidal, no activity), and is also helpful to reveal treatment failure due to bacterial regrowth, tolerance, persistence, and paradoxical effects.

Although it is not fully standardized, TKA has been widely utilised in synergy studies (92–95). For result interpretation, the activity of drugs alone and in combination is compared to define the combined effect. Synergy is typically defined by ≥2 $\log_{10}$ decrease in CFU/mL when compared to its most active constituent partner; similarly, "no interaction" when there is less than 2 $\log_{10}$ change in CFU/mL; and "antagonism" is considered when there is a ≥2 $\log_{10}$ increase in CFU/mL (96), as it is shown in **Figure 20.** Moreover, bactericidal activity may also be quantified by ≥3 $\log_{10}$ decrease in CFU/mL respect to the initial inoculum. This parameter is highly important to prioritize synergistic drug combinations; those combinations showing *in vitro* sterilizing activity might be promising candidates towards the clinical translation.

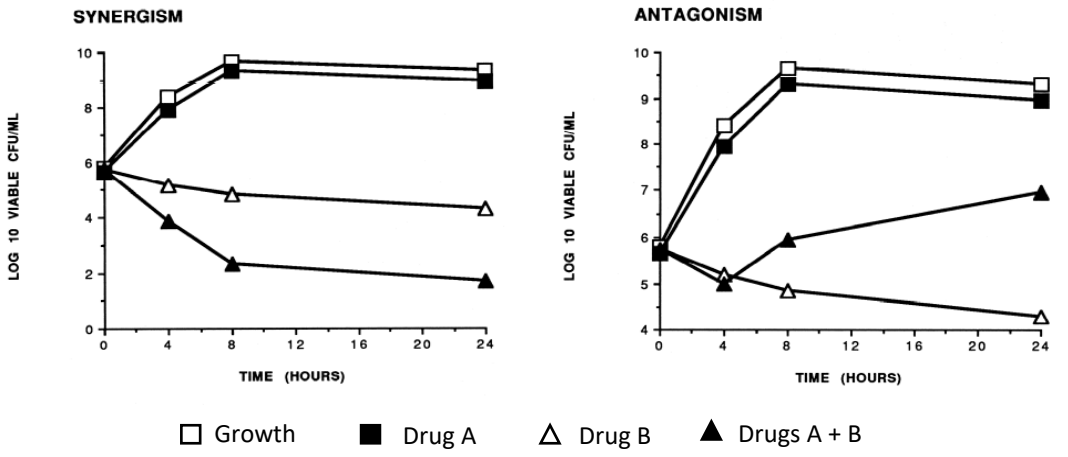


Figure 20. Graphic representation of a drug combination by time-kill assays showing synergistic and antagonistic interactions. Adapted from Chapter 5.10.3.4. of (84).

Drug stability is one of the limitations in TKA. Drugs are typically single added at the start of the assay (time zero) and no longer replace. Thus, drug degradation may influence data interpretation (e.g., β-lactam lack of effects during several days of experiment leading to bacterial regrowth). Hence, drug stability under the same assay conditions should be previously known to correctly interpret the results obtained.

❖ Dynamic/Pharmacokinetic models

Among limitations of the previous methods are the use of static drug concentrations without accounting for drug stability in the assay media and reliance mostly on bacterial growth inhibition (except for TKA). The characterisation of the antimicrobial activity of drugs administered in combination can be also studied using dynamic *in vitro* PK/PD models, which consider *in vivo* variations in drug concentrations over time and their interaction with the evolution of bacterial growth and death. In general, these models mimic human PK parameters of considered drugs and consist of compartments that allow the bacterial population and the drugs to be in contact, so that the effect can be expressed as a function of killing or inhibition of bacterial growth. Usually, the same definitions used in TKA are applied for the interpretation of the combined effect. PK/PD methods can be categorized as one-compartment or two-compartment models.

- **One-compartment model**

A central reservoir containing the bacterial inoculum, a diluent reservoir pumping fresh medium to the central reservoir, and a waste reservoir. Drug is administered to the central reservoir using pumps, mimicking human elimination rates (drug half-life, $t_{1/2}$; and C_{max}). The main disadvantage of these systems is the simultaneous elimination of the bacterial inoculum and waste medium, which can overestimate treatment efficacy. Depending on the microorganism of study, this technique might be laborious due to biosafety issues (97).

- **Two-compartment model**

To solve the issue of simultaneous elimination of bacteria with waste medium, two-compartment PK/PD models add a small peripheral space that consist in a cartridge in where the bacterial inoculum remains retained, while fresh medium can penetrate to provide nutrients and oxygenation. This model is called Hollow-Fibre Infection model (HFIM), due to the cartridge is formed by thousands of small tubular fibres (usually 200 microns pore size) sealed at the end to allow the passage of liquid medium through the fibres. The central reservoir where drug is administered is double connected to the peripheral compartment to equilibrate rapidly drug concentration in both compartments. Thus, absorption and elimination kinetics can be simulated, as peripheral compartment act as the *in vivo* site of infection (97,98). **Figure 21** shows a schematic setting up of HFIM.

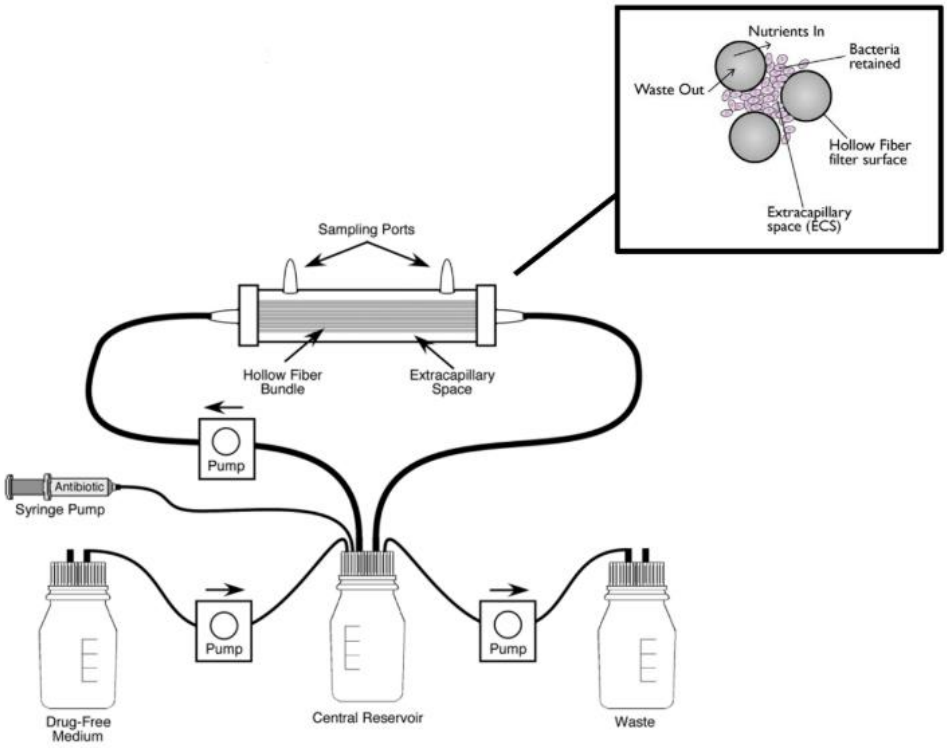


Figure 21. Hollow fibre two-compartment model. Adapted from (97).

HFIM is increasingly used in antimicrobial research because it provides several advantages. Compared to animal models, clinical translation is more reliable because it is based on real human PK parameters (99,100). In addition, it is less costly, easier and more versatile to conduct different studies (e.g., dose-fractionation studies, emergence of resistance, high inoculation and long duration treatments), and does not entail ethical concerns. The main limitations of *in vitro* PK/PD models are a high technical capacity and the lack of information on the host immune response. Nevertheless, intracellular HFIM studies have been also designed to solved this latter problem, especially in antiviral and antimycobacterial drugs research. (101–104).

OBJECTIVES

The main objective of this Thesis is to identify promising novel drug synergistic combinations against *K. pneumoniae* that might establish the basis for further pre-clinical and clinical studies to improve the treatment of these infections.

For this purpose, several specific objectives were considered:

1. To optimize and adapt an *in vitro* high-throughput synergy screening (HTSS) method (previously developed for mycobacteria) to perform efficient synergy screens against *K. pneumoniae*

2. To identify synergistic compounds from chemical libraries of known and clinically approved compounds in combination with known last-line resort antimicrobials against *K. pneumoniae* infections.

3. To validate and characterize *in vitro* the antimicrobial activity of the priority combinations selected in the HTSS against *K. pneumoniae*

4. To expand the study of the priority synergistic combinations against a collection of clinical isolates with defined resistance patterns.

MATERIALS & METHODS

Bacterial strains, media and chemicals

The reference strain *K. pneumoniae* ATCC 13883 was used for all experiments in Chapter 1. For Chapter 2 and Chapter 3, a well-characterized set of 12 MDR/XDR *K. pneumoniae* isolates (eight from clinical samples and four from quality assessment exercises) was provided by the Miguel Servet University Hospital (Zaragoza, Spain), including representative resistance mechanisms **(Table 3).** MDR/XDR were defined as: MDR, non-susceptible to ≥1 agent in ≥3 antimicrobial categories; XDR non-susceptible to ≥1 agent in all but ≤2 categories (15). Bacterial identification was performed by MALDI-TOF mass spectrometry (Bruker Daltonik GmbH, Germany) and antimicrobial susceptibility by an automated broth microdilution method (Microscan Walkaway®, Beckman Coulter, Spain). Phenotypic detection of ESBL, AmpC, carbapenemases and colistin resistance was done according to EUCAST guidelines (105). Genotypic characterization of resistance mechanisms was performed in clinical samples at the National Microbiology Centre (Majadahonda, Spain).

Bacterial stocks (15% glycerol) were preserved at -20°C in Luria Broth (LB). A new stock was thawed for every experiment to ensure assay robustness, and sub-cultured on Mueller Hinton broth (MHB) to obtain exponential cultures before each MIC, CBA and TKA assay were performed in cation adjusted MHB (CAMHB). Drug susceptibility testing in solid media and synergy screen were performed in Mueller-Hinton agar (MHA). All cultures were incubated at 36-37˚C during 18-20 hours for drug susceptibility testing or overnight for the rest of assays performed.

Drugs were provided by Sigma–Aldrich (Darmstadt, Germany), European Pharmacopoeia (Strasbourg, France), except meropenem (Fresenius Kabi), ertapenem (MSD) and avibactam (AdooQ BioScience, Irvine, USA). Powders were reconstituted in dimethyl sulfoxide (DMSO) or water according to their solubilities. Stock solutions were prepared fresh on the same day of plate inoculation.

The FDA-approved drug library was purchased from Selleckchem (catalogue #L1300) and included 1,430 lyophilized compounds in 96 well-plates. Drugs were freshly reconstituted in DMSO or water according to their solubility at a final concentration of 10 mM, from which serial 10-fold dilutions were performed to obtain FDA tester plates at 0.1 and 1 mM.

MTT solution was prepared dissolving 5 mg/mL of MTT (2-(3,5-diphenyltetrazol-2-ium-2-yl)-4,5-dimethyl-1,3-thiazole; bromide) (Sigma–Aldrich) in distilled water for susceptibility tests, or in PBS for cytotoxicity assays. Once dissolved, this solution was filtered through a 0.2

µm pore size, and aliquots were stored at -20°C until use. For susceptibility assays, an aliquot was thawed, and a solution of Tween 80 (Sigma–Aldrich) was added at 20% vol/vol.

Resazurin solution was prepared dissolving the powder (Sigma–Aldrich) in Milli-Q water (0.1 mg/mL). The solution was filtered through a 0.2 µm pore size and aliquots were stored at -4°C until use during six months.

Table 3. Strain characterization and antimicrobial susceptibility for the twelve MDR/XDR *K. pneumoniae* isolates used in Chapter 2 and Chapter 3. Data provided from the Microbiology Department of the Miguel Servet University Hospital (Zaragoza, Spain).

				MIC (mg/L)[b]																		
Isolate	**Resistance mechanism**	**Specimen source**	**MDR/XDR classification[a]**	**AMK**	**GEN**	**TOB**	**AMP/AMX**	**AMC**	**TZP**	**FOX**	**CXM**	**CTX**	**CAZ**	**FEP**	**ATM**	**IPM**	**ETP**	**MEM**	**CIP**	**LVX**	**FOF**	**CST**	**TGC**	**SXT**
E-1	CTX-M 14	Rectal swab	XDR	≤8	>8	≤2	>16	>16/8	>64	>16	>16	>32	>16	>16	>16	8	>1	8	>2	4	≤32	≤2	>2	>4/76
E-2	CTX-M 15	Blood	MDR	≤8	≤2	>8	>16	>16/8	>64	>16	>16	>32	>16	>16	>16	≤1	>1	2	>2	>4	>64	≤2	≤1	≤2/38
E-3	CTX-M 15	Abscess	MDR	≤8	>8	>8	>16	>16/8	64	>16	>16	>32	>16	>16	>16	≤1	>1	2	>2	2	≤32	≤2	≤1	>4/76
E-4	CTX-M 15	Blood	MDR	16	>4	>4	>16	>32	16	>16	>8	>32	32	>8	>4	≤1	≤0.12	≤0.12	>1	>1	≤16	≤2	>2	>4/76
E-5	SHV-1 + porin loss	Blood	MDR	≤8	≤2	≤2	>16	>16/8	>64	16	≤4	≤1	≤1	4	≤1	≤1	≤0.5	≤1	≤0.5	≤1	≤32	≤2	≤1	≤2/38
A-6	AmpC ACT-1	SEIMC CCS07	MDR	>32	>8	>8	>16	>16/8	64	>16	>16	32	>16	≤1	>16	≤1	>1	≤1	2	≤1	≤32	≤2	≤1	>4/76
C-7	OXA-48	Blood	MDR	≤8	≤2	≤2	>16	>16/8	>64	≤8	8	≤1	≤1	≤1	≤1	4	>1	4	≤0.5	≤1	64	≤2	≤1	≤2/38
CS-8	Colistin R	Urine	MDR	≤8	≤2	≤2	>16	>16/8	>64	>16	16	≤1	≤1	4	≤1	≤1	≤0.5	≤1	≤0.5	≤1	>64	>4	2	>4/76
CE-9	VIM-1 + CTX-M 15 + colistin R	SEIMC CCS04	XDR	16	>8	>8	>16	>16/8	>64	>16	>16	>32	>16	>16	>16	2	>1	8	>2	>4	≤32	>4	2	>4/76
CE-10	CTX-M 15 + OXA-48	Blood	MDR	≤8	>4	>4	>8	>32	>16	≤8	>8	>32	32	>8	>4	8	>1	1	>1	>1	32	≤2	≤1	>4/76
CEE-11	KPC-3 + SHV-11 + TEM-1	SEIMC CCS05	XDR	32	4	>8	>16	>16/8	>64	>16	>16	>32	>16	>16	>16	>8	>1	>8	>2	>4	64	>4	>2	>4/76
CSEE-12	OXA-1 + SHV-1 + colistin R	EARS QC	MDR	>32	>8	>8	>16	>16/8	>64	≤8	>16	>32	≤1	>16	≤1	≤1	>1	≤1	>2	>4	≤32	>4	2	>4/76

AMK, amikacin; GEN, gentamicin; TOB, tobramycin; AMP/AMX, ampicillin/amoxicillin; AMC, amoxicillin-clavulanate; TZP, piperacillin-tazobactam; FOX, cefoxitin; CXM, cefuroxime, CTX, cefotaxime, CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; IPM, imipenem; ETP, ertapenem; MEM, meropenem; CIP, ciprofloxacin; LVX, levofloxacin; FOF, fosfomycin; CST, colistin; TGC, tigecycline; SXT, trimethoprim/sulfamethoxazole

[a]MDR/XDR categorization according to Magiorakos et al. (29): MDR: non-susceptible to ≥1 agent in ≥3 antimicrobial categories; XDR: non-susceptible to ≥1 agent in all but ≤2 categories

[b]MIC determined by automated broth microdilution (Microscan Walkaway®, Beckman Coulter, Spain) and clinical interpretation according to the corresponding EUCAST guidelines on the isolation date. Values in green were interpreted as "Susceptible", in dark yellow as "Susceptible, increased exposure" (EUCAST 2019), in light yellow as "Intermediate" and in red as "Resistant".

Drug susceptibility testing

Minimum Inhibitory Concentration assays

MIC determinations were performed by broth microdilution according to CLSI guidelines (86) and the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (107,108). Two-fold serial dilutions of the compounds were inoculated with $5x10^5$ CFU/mL (optical density; OD_{550}=0.2 corresponded to $1.5x10^8$ cell/mL) in 96-well plates (TPP), (V_F= 150 µL/well) and incubated for 18-20 hours. After incubation, 30 µL/well of 5 mg/mL MTT plus 20% Tween 80 were added and incubated for 3 hours. Positive and negative growth controls were included in all experiments. MIC values were defined as the lowest concentration of drug that inhibited 90% of the OD_{580} MTT colour conversion (IC_{90}) compared to growth control wells with no drug added.

For fosfomycin and ceftazidime-avibactam susceptibility tests, CAMHB was supplemented with 25 mg/L of G6P and 4 mg/L of avibactam, respectively (109).

Solid MIC determinations were performed by the agar dilution method (106). Briefly, 24-well plates (TPP) containing MHA with serial two-fold dilutions of each PC (tigecycline, colistin, fosfomycin) were prepared in duplicates (V_F= 1 mL/well). Upon agar solidification, each well was inoculated with 10 µL of a bacterial suspension (ca. $5x10^3$ CFU/well) and plates incubated. The MIC was considered as the lowest value that completely inhibited visible growth.

Minimum Bactericidal Concentration assays

MBC was determined to discern bacteriostatic from bactericidal activities. Prior to MTT addition in 96-well MIC plates, 10 µL/well were transferred to 96-well LB agar plates (Zeulab) and further incubated for 24 hours before addition of resazurin (30 µL/well). The MBC was defined as the lowest concentration of drug preventing a colour change from blue to pink. A compound was considered bactericidal if MBC/MIC ≤ 4 (108).

FDA-library synergy screen

The screening was performed following the High-throughput Synergy Screening methodology (HTSS), previously described by Santiago Ramón-García (108) and explained in Chapter 1. The bacterial inoculum was added to 22 mL of MHB medium containing 0.5% agar (top agar) and uniformly poured over 45 mL of MHB-1.5% agar (bottom agar) in OmniTrays (Nunc) in duplicates. Tigecycline, colistin and fosfomycin (used as last-line antibiotics used

against MDR *K. pneumoniae)* were selected as compounds of interest and added to the bottom agar at sub-inhibitory concentrations (MIC_{sub}) of ½ x MIC up to 1/256 x MIC, previously selected under the same HTSS conditions. The FDA library (n=1,430, 0.1-1 mM) of clinically approved drugs was screened against these three drugs. FDA-compounds were transferred from 96-well plates onto top agar cell lawns using a 1.6 mm pin replicator that transferred approximately 200 nL/pin, (0.2-2 nmol of each compound). OmniTrays were incubated before inhibition zones measurement. Synergy was defined by an increase of the inhibition zones in the drug-containing agar plates. Conversely, antagonism was defined by a decrease of the inhibition zones in the drug-containing agar plates, and no interaction was defined if no changes in the inhibition zone diameter was observed between drug-free and drug-containing agar plates.

According to these criteria, hits were classified in four categories:

(i) synergy (Y): compounds whose inhibition zones increased at the two drug MIC_{sub} tested (diameter MIC_{sub1} & diameter MIC_{sub2} > $diameter_{Control}$)

(ii) likely synergy (Y/N): compounds whose inhibition zones increased at only one drug MIC_{sub} (diameter MIC_{sub1} or diameter MIC_{sub2} > $diameter_{Control}$)

(iii) no interaction (N): compounds with no change in their inhibition zones (diameter MIC_{sub1} & diameter MIC_{sub2} = $diameter_{Control}$)

(iv) likely antagonism (A): compounds with decreased inhibition zones (diameter MIC_{sub1} & diameter MIC_{sub2} < $diameter_{Control}$)

Secondary validation assays

Checkerboard assays.

CBA were performed against *K. pneumoniae* in 96-well plates using freshly prepared CAMHB. Each well was inoculated with 100 µL of $5x10^5$ CFU/mL (V_F= 200 µL). Pre-inocula were prepared by direct suspension of bacteria grown overnight in MHB and plates incubated for 24 hours before determination of the compound activities alone and in combination (107,108). Interactions were interpreted according to FICI values: synergy was defined as FICI≤0.5, antagonism as FICI>4.0, and no interaction when the FICI was between 0.5-4.0 (90). Similarly, FBCI was calculated as above described based on MBC values for each combination using the resazurin method (107).

Time-kill assays.

Dose-response curves of drugs alone

Duplicates of exponentially growing cultures were inoculated in CAMHB at a final inoculum of $5x10^5$ CFU/mL in 96-well plates for *K. pneumoniae* (V_F= 280 µL/well) containing increasing compound concentrations (0.1x, 0.25x, 1x, 4x, 10x MIC values). At predefined time points (0, 2, 4, 6, 8, 24 and 48 hours), the bacterial population from each well was quantified by spot-platting 10-fold serial dilutions on MHA plates. Plates were incubated overnight, and CFU/mL calculated. The lower limit of detection was 50 CFU/mL.

Drug combination curves

Combo test concentrations were selected based on previous dose-response curves of the compound alone (typically 0.25x MIC and/or 1x MIC) or up to 300 mg/L in the case of inactive hits. TKA were performed for drugs alone and in combination according to protocol describe above. MIC assays were run in parallel with the same inoculum as internal control of compounds´ activity.

A synergistic combination was defined as a ≥2 $\log_{10}$ CFU/mL decrease in the bacterial count of the combination compared to the most active single agent at any time point (8, 24 and 48 hours). Antagonism was defined as a ≥2 $\log_{10}$ increase in CFU/mL between the combination and the most active single agent. All other cases were defined as indifferent. Bactericidal activity was defined as a ≥3 $\log_{10}$ CFU/mL reduction at any time point compared to the initial inoculum (96).

Cytotoxicity assays

The MTT cytotoxicity test (110) was carried out to determine the cytotoxicity of the validated combinations with azithromycin and zidovudine in Hep G2 cell line (human liver carcinoma cells), obtained from the ECACC: (cat. N° 85011430). Stock cells were cryopreserved in 90% foetal bovine serum (FBS, Gibco) plus 10% DMSO.

Cells were thawed from stocks and cultured in flasks with pre-warmed (37°C) Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% FBS, 1% Glutamax and 100 mg/L streptomycin, penicillin and ciprofloxacin for 24 hours in a controlled 5% CO_2 atmosphere at 37°C. Two passages were carried out before performing cytotoxicity assays. After microscopic evaluation of a semi-confluent monolayer, cells were removed using enzymatic digestion with 0.05% trypsin-EDTA solution (5 minutes at 37°C) and then neutralized with supplemented

DMEM. Cells were then centrifuged (240 *g*, 5 minutes) and the medium discarded and replaced to adjust the cell suspension to 10^6 cells per plate. Cells were seeded in 96-well flat bottom plates transferring 100 µL per well (2 x 10^4 cells) and incubated under conditions described above.

After 24 hours, media was aspirated and replaced by fresh DMEM supplemented with 10% FBS, 1% Glutamax and the compounds dissolved. Compounds were then added both alone and in combination at the same concentrations used in the TKA. Cells were incubated in the presence of the compounds or combinations for 24 hours. Growth controls and blanks with medium were also included in the plate layout. Then, 10 µL of MTT dissolved in PBS (5 mg/mL) were added to each well and plates further incubated for 2 hours before the MTT solution was removed. MTT crystals were solubilized in 100 µL/well of DMSO, mixed and the absorbance measured at 570 nm and 650 nm. Percent of cell viability was determined in comparison with untreated controls. Treatments were considered cytotoxic when cell viability was reduced to <70% of the growth controls, according to the international standard protocols (110). Experiments were performed in technical triplicates and biological replicates. Graphical representation and statistical analysis to compare each variable were performed by one-way ANOVA test in GraphPad Prism software (version 8.0.2).

Novel synergistic combinations of last-line antibiotics and FDA-approved drugs against *Klebsiella pneumoniae* revealed by *in vitro* synergy screens

CHAPTER 1

"Defiende tu derecho a pensar, porque incluso pensar de manera errónea es mejor que no pensar"

Hipatia de Alejandría *(350-370 d.C. – 415 d. C)*

INTRODUCTION

Phenotypic screening based on high-throughput screens of compound libraries has been traditionally the most successful strategy in antimicrobial D&D and drug repurposing (79,81). These assays are typically performed in 96-well format to measure the bacterial inhibition produced by the compound (IC_{50} and IC_{90} values, i.e., mean drug concentration required for 50% or 90% inhibition, respectively) based on different cell viability methods, such as optical density, MTT (2-(3,5-diphenyltetrazol-2-ium-2-yl)-4,5-dimethyl-1,3-thiazole; bromide), resazurin, or ATP (BacTiter Glo™) methods (111).

However, experimental high-throughput identification of synergistic partners has been challenging due to the high technical requirements needed to handle large numbers of potential interactions; hence, systematic combinatorial screens are scarce in the literature. In 2003, CombinatoRX was the first biotech company that designed a large-scale screening platform in 384-well format plates based on automated technology (e.g., digital drug dispensers) and computer systems that provide algorithms for synergy definition (112). The continuous development of technological advances increase the efficiency for the implementation of systematic routine drug combination screens (113).

In silico methods have been also helpful to identify potential interactions. The overlap method utilizes large-scale chemical-genetics data to predict additional interactions between small-molecule pairs from known synergistic combinations. This methodology successfully identified synergistic pairwise combinations with antifungal and antibacterial activity (114).

Innovative experimental approaches to accelerate D&D process were also explored. In 2011, Ramón-García *et al.* (108) described the HTSS method, a qualitative novel methodology to identify synergistic compounds that enhanced the activity of spectinomycin against *Mycobacterium smegmatis*. HTSS consisted of the transference of Secondary Compounds (SCs) contained in chemical libraries onto agar plates in presence and absence of sub-inhibitory concentrations of a compound of interest, named Primary Compound (PC; e.g. spectinomycin). These SCs were transferred by a pinning robot to assure robustness and reproducibility. After incubation with *M. smegmatis*, compounds whose zones of inhibition were larger in the presence of spectinomycin than in plates without spectinomycin were selected as potential candidate hits to act synergistically **(Figure 1.1).**

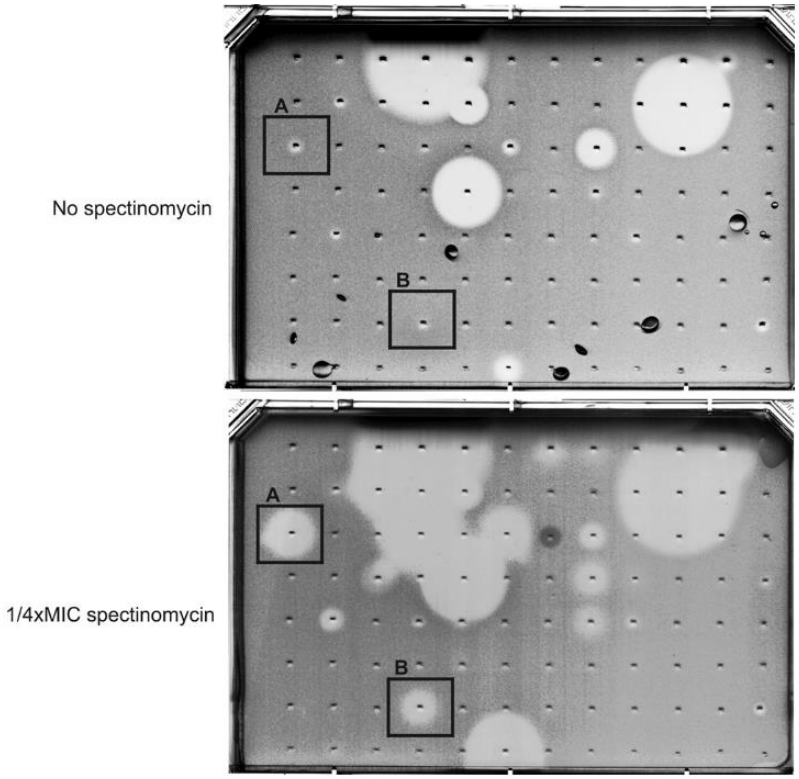


Figure 1.1. High-throughput Synergy Screening against *M. smegmatis.* Adapted from (108).

As result, several synergistic hits identified in the HTSS were also validated against *M. tuberculosis*. The implementation of this method increased the efficiency on the search of synergistic interactions, due to up to 96 compounds could be tested per plate, providing a clear, reproducible, and reliable signal for synergistic hits (108).

The Chapter 1 describes the identification and characterization of novel drug combinations against *K. pneumoniae*. For that, the HTSS methodology (108) was adapted to perform efficient *in vitro* synergy screens using the reference strain *K. pneumoniae* ATCC 13883. Tigecycline, colistin and fosfomycin (last-line antibiotics against MDR *K. pneumoniae*) were selected as PCs combined with an FDA-library containing 1,430 clinically approved drugs (SCs, see Materials & Methods section). Unlike the original methodology, no automation was available during the process (compound transfer was performed using a manual replicator). For this reason, the term semi-high throughput synergy screen (sHTSS) is more appropriate to refer to the smaller scale technical performance in this Thesis. After determining antimicrobial susceptibility, selected hits were further validated by CBA and TKA assays. According to these latter assays, synergistic and killing effects were evaluated at different time points (8, 24 and 48 hours) to obtain a priority list of combinations with potential for clinical translation.

RESULTS

Primary Compounds susceptibility testing

Prior to perform sHTSS, MIC values of each PC were obtained on solid medium. For *K. pneumoniae* ATCC 13883, MICs of tigecycline, colistin and fosfomycin were 0.5, 1 and 128 mg/L respectively.

Optimization process of experimental conditions

A preliminary optimization was developed to adapt the original technique (108) to *K. pneumoniae*. For this purpose, extensive modifications of the experimental conditions were performed until optimal image processing readout was obtained **(Table 1.1).**

Table 1.1. Experimental modifications and results derived from the HTSS optimization process. TGC, tigecycline; CST, colistin; FOF, fosfomycin.

Method optimization	Experimental modifications			Result	Optimal condition
Inoculum size	10^5 to 10^7 cell/ml			Uniform growth	YES
Metabolic state	Stationary growth			Heterogeneous growth	NO
	Exponential growth			Homogeneous growth	YES
Inoculation method	Swab			Edges not defined	NO
	Top agar			Clear edges	YES
SC stock concentrations	0.1-1 mM			Defined inhibition zones	YES
	10 mM			High-density inhibition zones, deconvolution needed	NO
Volume SC stock concentrations	50 µl			Optimal volume with both pin diameter	YES
	100 µl			Inhibition zones wider when using a wider replicator pin diameter	YES
Sub-inhibitory PC concentrations	**TGC**	**CST**	**FOF**		
	-	≥1/64xMIC	1/2xMIC	Bacterial growth partially inhibited	NO
	1/2xMIC				
	1/4xMIC	1/128xMIC	1/4xMIC	Bacterial growth not affected by inhibition effect	YES
		1/256xMIC	1/8xMIC		
Replicator pin diameter	3 mm			Uncertain inhibition zones	NO
	1.6 mm			Inhibition zones readout precise, clear edges	YES

The optimization process revealed the most optimal parameters to be used in the synergy screen.

1. *Inoculum size*. Although higher sizes were tested (10^6 and 10^7 cell/ml), an inoculum size of 10^5 cell/ml showed a homogeneous bacterial lawn on the agar plates.

2. *Bacterial metabolic state* (stationary versus exponential growth). Optimal growth was obtained from overnight cultures (exponential phase), which assured reproducibility during the process and technical standardization. Stationary growth from older cultures showed heterogenous growth.

3. *Inoculation method.* Two different techniques were compared; the traditional swab inoculation (a swab dipped in a bacterial suspension and then extended through the agar plates), as this is the usual method to perform drug susceptibility testing in agar media (106), and the top agar inoculation (inoculum added to the top agar and spread onto the bottom agar, described in the HTSS method (108)). As **Figure 1.2**. shows, the swab inoculation showed insufficient and heterogeneous growth leading to a bad definition of inhibition zones, while the top agar inoculation showed a homogeneous lawn of bacterial growth that allowed to visualize clear edges and the uniform measurement of the inhibition zones.

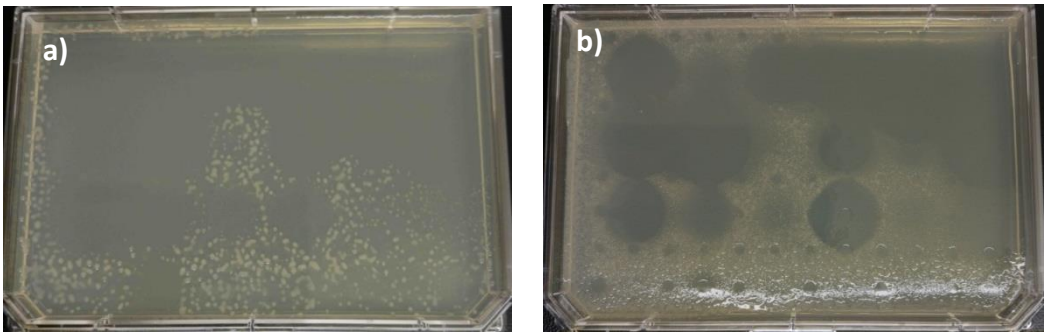


Figure 1.2. Comparative study of the growth of *K. pneumoniae* ATCC 13883 in two different methods of bacterial inoculation. a) Swab inoculation; b) Top agar inoculation.

4. *PCs subinhibitory concentrations.* To select the appropriate MIC_{sub} of each PC, the degree of bacterial inhibition was evaluated by adding the inoculum to Petri dishes containing two-fold serial dilutions of subinhibitory concentrations in MHA (i.e., 1/2, ¼ and 1/8x MIC). After incubation, bacterial growth was analyzed to adjust the optimal MIC_{sub} that allow the growth of a bacterial lawn in the agar plates, necessary to measure the inhibition zones in the synergy screening. Tigecycline was the only antibiotic that did not show partial inhibition at subinhibitory concentrations. Fosfomycin showed partial inhibition at ½ x MIC, thus, optimal MIC_{sub} were determined at ¼ and 1/8 x MIC. A striking inhibitory effect was observed with colistin at subinhibitory concentrations, as shows

Figure 1.3. For this drug, MIC_{sub} that allow to observe a lawn of *K. pneumoniae* were achieved below 1/64xMIC (0.003-0.007 mg/L).

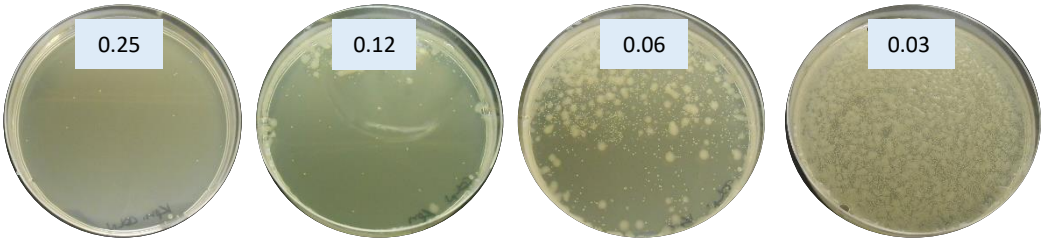


Figure 1.3. Growth of *K. pneumoniae* ATCC 13883 in presence of different subinhibitory concentrations of colistin (mg/L) (CST, MIC=1 mg/L). Until 1/32x MIC (0.03 mg/L), colistin is still potent enough to inhibit the bacterial growth. Optimal growth was achieved at lower subinhibitory concentrations (< 1/64x MIC).

5. *SCs stock concentrations and volume.* These parameters were dependent on the activity of each SC. Those drugs highly active by themselves showed large inhibition zones, thus, it was necessary to decrease their concentrations (0.1 – 1 mM) to observe measurable readouts. The distribution of the active SCs onto the 96-well plates was also an important factor to consider, due to active SCs consecutively placed leaded to high-density zones with overlapping inhibition zones. To solve this, deconvolution was necessary to distinguish each interaction **(Figure 1.4).** Volume from the 96-well stock plates was more determinant when using a replicator with a wider diameter of pin (3 mm), due also to high inhibition zones that did not allow to identify the active SC.

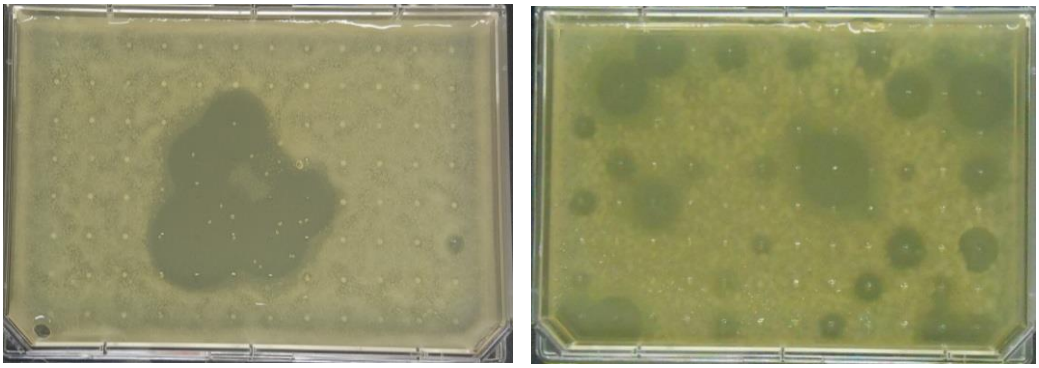


Figure 1.4. Distribution of hits showing inhibition zones. Left image shows an agar plate with high-density inhibition zones that do not allow independent measures. This plate required to decrease the stock concentration of the compounds or deconvolution to distinguish better the activity of each compound, as it is shown in the right image.

6. *Replicator pin diameter.* After comparing both replicators (1.6 mm versus 3 mm pin diameter), optimal results were obtained using the 1.6 mm replicator, showing clear

edges and a precise readout of the inhibition zones. By contrary, 3 mm replicator showed uncertain inhibition zones due to the own pin diameter, leading to confuse measures **(Figure 1.5).**

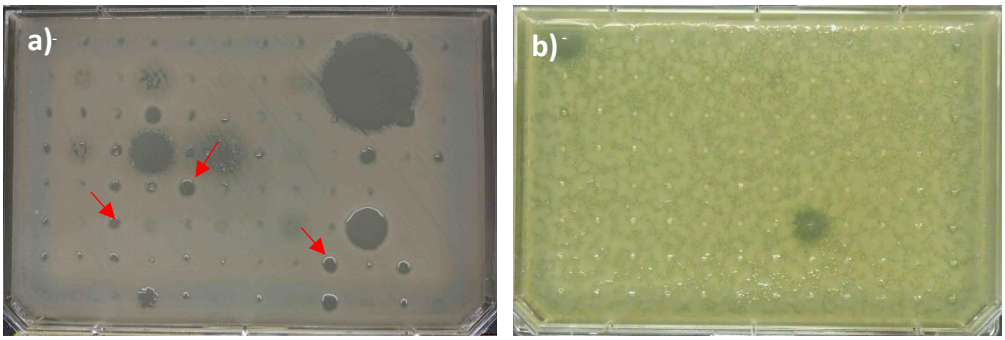


Figure 1.5. Results after compound transference onto agar plates using two replicators with different pin diameter. a) Transference with 3 mm diameter showing uncertain inhibition zones (red arrows); b) Transference with 1.6 mm diameter.

Synergy screens of the FDA library in combination with last-line antibiotics against *K. pneumoniae* ATCC 13883.

Once optimal conditions were defined, FDA synergy screen was performed as described in Materials & Methods. **Figure 1.6.** illustrates the sHTSS assay for the interpretation of synergy, based on the diameter of inhibition zones.

As result, 109 FDA compounds were globally identified as enhancers of the three PCs, with hit discovery rates of 2.59% (n=37), 2.17% (n=31), and 2.87% (n=41) for tigecycline, colistin and fosfomycin, respectively. According to the interaction ranking criteria (see Materials & Methods), 19, 9 and 18 interactions were classified as synergistic, and 18, 22 and 23 as likely synergistic with tigecycline, colistin and fosfomycin, respectively. Additionally, 6, 10 and 12 interactions were classified as likely antagonistic with tigecycline, colistin and fosfomycin, respectively. **Table 1.2.** and **Appendix I** show detailed information about the hits identified for each PC.

Some promiscuous compounds were able to enhance the activity of more than one PC; thereby, among the 109 compounds that initially enhanced the activity of any of the PCs, there were 60 unique hits.

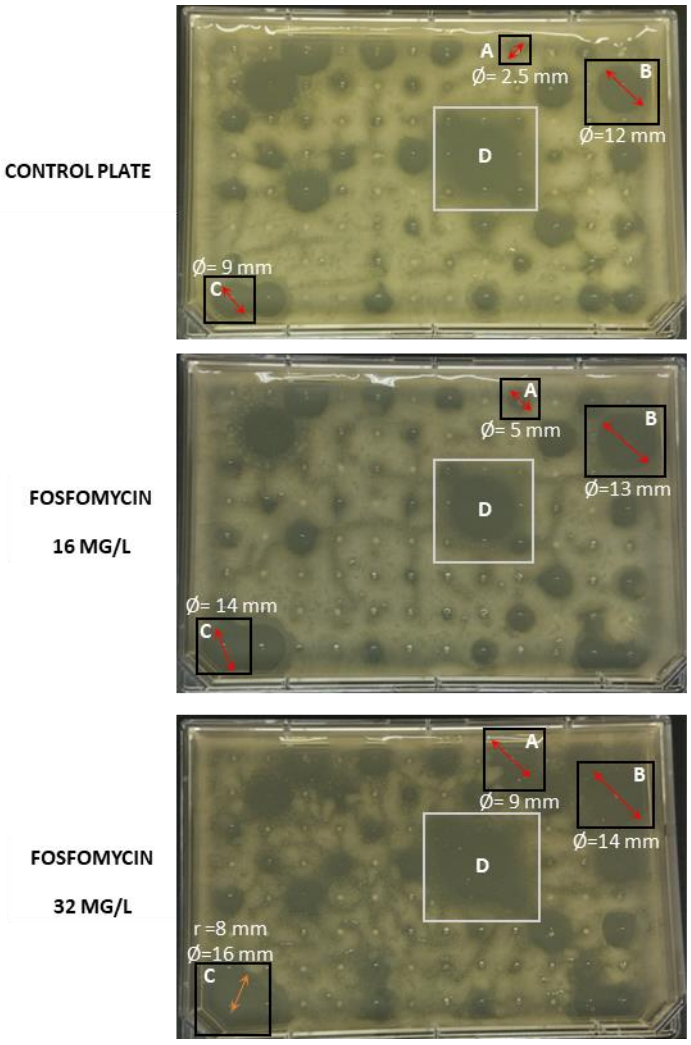


Figure 1.6. Representative plate analysis of a semi-High-Throughput Synergy Screen (sHTSS). Compounds whose zones of inhibition were larger in the presence of fosfomycin than in plates without fosfomycin (examples A, B or C) were selected as hits for further validation of their potential synergistic interaction. High-density plates overlapping inhibition zones for two or more compounds (D, inhibition zones >15 mm) were deconvoluted at a lower compound density or lower concentration (0.1 mM) for clear inhibition zone readings. Ø, inhibition zone diameter value; Red arrows, inhibition zone diameter; r, inhibition zone radius (when diameter cannot be determined); MIC_{FOF}= 128 mg/L.

Table 1.2. FDA compounds identified in sHTSS with tigecycline (a), colistin (b) and fosfomycin (c) against *K. pneumoniae* ATCC 13883. Green indicates synergy (Y and Y/N classification). Red indicates antagonism (A).

a) Tigecycline hits

FDA compound	Result	Chemical classification	Therapeutic use	Target
Abacavir sulphate	Y/N	Nucleoside analogue	Antiretroviral	Reverse transcriptase
Amikacin disulphate	Y/N	Aminoglycoside	Antibiotic	30S ribosomal
Amikacin hydrate	Y/N	Aminoglycoside	Antibiotic	30S ribosomal
Atosiban Acetate	Y/N	Oligopeptide	Premature Birth	Oxytocin receptor
Azithromycin	Y	Macrolide	Antibiotic	50S ribosomal
Azithromycin Dihydrate	Y	Macrolide	Antibiotic	50S ribosomal
Aztreonam	Y	Monobactam	Antibiotic	PBP 3
Balofloxacin	Y/N	Fluoroquinolone	Antibiotic	DNA gyrase
Bleomycin Sulphate	A	Glycopeptide	Antineoplastic	DNA/RNA
Calcium Levofolinate	Y/N	Tetrahydrofolic acid	Detoxifying agent	Serine hydroxymethyltransferase
Cefdinir	Y	Cephalosporin	Antibiotic	PBP 2-3
Cefoperazone	Y	Cephalosporin	Antibiotic	PBP 1A-B, 2
Cefradine	Y	Cephalosporin	Antibiotic	PBP 1A
Chlorhexidine HCl	A	Biguanide	Antiseptic	Bacterial outer membrane
Chlortetracycline HCl	Y	Aminocyclitol glycoside	Antibiotic	30S ribosomal
Danofloxacin Mesylate	Y/N	Fluoroquinolone	Antibiotic	DNA gyrase
Demeclocycline HCl	Y	Tetracycline	Antibiotic	30S ribosomal
Dihydrostreptomycin sulphate	A	Aminocyclitol glycoside	Antibiotic	30S ribosomal
Enoxacin	Y	Fluoroquinolone	Antibiotic	DNA gyrase
Enrofloxacin	Y	Fluoroquinolone	Antibiotic	DNA gyrase
Furazolidone	A	Nitrofuran	Antibiotic	DNA
Gatifloxacin	A	Fluoroquinolone	Antibiotic	DNA gyrase
Gentamicin Sulphate	Y/N	Aminoglycoside	Antibiotic	30S ribosomal
Ibandronate sodium	Y/N	Bisphosphonate	Osteoporosis	Farnesyl pyrophosphate synthase
Ivermectin	A	Milbemycin	Antiparasitic	Glycine receptor
Levofloxacin hydrate	Y/N	Fluoroquinolone	Antibiotic	DNA gyrase
Levofloxacin	A	Fluoroquinolone	Antibiotic	DNA gyrase
Lomefloxacin HCl	Y	Fluoroquinolone	Antibiotic	DNA gyrase
Marbofloxacin	Y	Fluoroquinolone	Antibiotic	DNA gyrase
Methacycline HCl	Y	Tetracycline	Antibiotic	30S ribosomal
Nadifloxacin	Y/N	Fluoroquinolone	Antibiotic	DNA gyrase
Netilmicin Sulphate	Y/N	Aminoglycoside	Antibiotic	30S ribosomal
Norfloxacin	Y	Fluoroquinolone	Antibiotic	DNA gyrase
Ofloxacin	Y	Fluoroquinolone	Antibiotic	DNA gyrase
Palbociclib (PD0332991) Isethionate	Y	Pyridinylpiperazine	Antineoplastic	CDK4 & CDK6
Pefloxacin Mesylate	Y	Fluoroquinolone	Antibiotic	DNA gyrase
Penfluridol	Y/N	Diphenylmethane	Antipsychotic	Dopamine receptors
Polymyxin B sulphate	Y/N	Polymyxin	Antibiotic	Lipopolysaccharide layer
Sarafloxacin HCl	Y/N	Fluoroquinolone	Antibiotic	DNA gyrase
Sisomicin sulphate	Y/N	Aminoglycoside	Antibiotic	30S ribosomal
Sitafloxacin Hydrate	Y/N	Fluoroquinolone	Antibiotic	DNA gyrase

FDA compound	Result	Chemical classification	Therapeutic use	Target
Sparfloxacin	Y	Fluoroquinolone	Antibiotic	DNA gyrase
Tobramycin	Y	Aminoglycoside	Antibiotic	30S ribosomal
Triclosan	Y/N	Diphenylether	Antiseptic	ENR enzyme

b) Colistin hits

FDA compound	Result	Chemical classification	Therapeutic use	Target
Altretamine	A	Dialkylarylamine	Antineoplastic	DNA
Amikacin disulfate	Y	Aminoglycoside	Antibiotic	30S ribosome
Amikacin hydrate	Y/N	Aminoglycoside	Antibiotic	30S ribosomal
Azithromycin	Y	Macrolide	Antibiotic	50S ribosomal
Aztreonam	Y	Monobactam	Antibiotic	PBP 3
BAF312 (Siponimod)	Y/N	Trifluoromethylbenzene	Multiple sclerosis	S1P receptor
Bleomycin Sulfate	A	Glycopeptide	Antineoplastic	DNA/RNA
Calcium Levofolinate	Y/N	Tetrahydrofolic acid	Detoxifying agent	Serine hydroxymethyltransferase
Cefdinir	Y/N	Cephalosporin	Antibiotic	PBP 2-3
Cefoperazone	Y/N	Cephalosporin	Antibiotic	PBP 1A-B, 2
Cefradine	Y	Cephalosporin	Antibiotic	PBP 1A
Ceftazidime Pentahydrate	Y/N	Cephalosporin	Antibiotic	PBP 3
Ceftriaxone Sodium Trihydrate	Y/N	Cephalosporin	Antibiotic	PBP 2B
Ceritinib (LDK378)	Y/N	Phenylpiperidine	Antineoplastic	Tyrosine kinase receptor
Chlorhexidine HCl	A	Biguanide	Antiseptic	Bacterial outer membrane
Danofloxacin Mesylate	Y/N	Fluoroquinolone	Antibiotic	DNA gyrase
Difloxacin HCl	A	Fluoroquinolone	Antibiotic	DNA gyrase
Dihydrostreptomycin sulfate	Y/N	Aminocyclitol glycoside	Antibiotic	30S ribosomal
Enrofloxacin	Y	Fluoroquinolone	Antibiotic	DNA gyrase
Furazolidone	A	Nitrofuran	Antibiotic	DNA
Gatifloxacin	A	Fluoroquinolone	Antibiotic	DNA gyrase
Glimepiride	A	Benzenesulfonamide	Diabetes mellitus	ATP-sensitive potassium channels
Ivermectin	A	Milbemycin	Antiparasitic	Glycine receptor
Levofloxacin	Y/N	Fluoroquinolone	Antibiotic	DNA gyrase
Levofloxacin hydrate	A	Fluoroquinolone	Antibiotic	DNA gyrase
Lomefloxacin HCl	Y/N	Fluoroquinolone	Antibiotic	DNA gyrase
Marbofloxacin	Y/N	Fluoroquinolone	Antibiotic	DNA gyrase
Mefloquine HCl	Y/N	Quinoline	Antimalarial	80S ribosomal
Moxifloxacin HCl	Y/N	Fluoroquinolone	Antibiotic	DNA gyrase
Netilmicin Sulfate	Y/N	Aminoglycoside	Antibiotic	30S ribosome
Norfloxacin	Y/N	Fluoroquinolone	Antibiotic	DNA gyrase
Ofloxacin	Y	Fluoroquinolone	Antibiotic	DNA gyrase
Palbociclib (PD0332991) Isethionate	Y	Pyridinylpiperazine	Antineoplastic	CDK4 & CDK6
Pefloxacin Mesylate	Y/N	Fluoroquinolone	Antibiotic	DNA gyrase
Pimozide	Y/N	Diphenylbutylpiperidine	Antipsychotic	Dopamine receptors
Polymyxin B sulphate	A	Polymyxin	Antibiotic	Lipopolysaccharide layer
Sarafloxacin HCl	Y	Fluoroquinolone	Antibiotic	DNA gyrase
Sisomicin sulfate	Y/N	Aminoglycoside	Antibiotic	30S ribosomal
Sitafloxacin Hydrate	A	Fluoroquinolone	Antibiotic	DNA gyrase
Sparfloxacin	Y/N	Fluoroquinolone	Antibiotic	DNA gyrase
Tobramycin	Y	Aminoglycoside	Antibiotic	30S ribosome
Triclosan	Y/N	Diphenylether	Antiseptic	ENR enzyme

c) Fosfomycin hits

FDA compound	Result	Chemical classification	Therapeutic use	Target
Amikacin disulphate	Y/N	Aminoglycoside	Antibiotic	30S ribosomal
Amikacin hydrate	A	Aminoglycoside	Antibiotic	30S ribosomal
Azithromycin	Y	Macrolide	Antibiotic	50S ribosomal
Azithromycin Dihydrate	Y	Macrolide	Antibiotic	50S ribosomal
Aztreonam	Y/N	Monobactam	Antibiotic	PBP 3
Balofloxacin	Y/N	Fluoroquinolone	Antibiotic	DNA gyrase
Bleomycin Sulphate	Y	Glycopeptide	Antineoplastic	DNA/RNA
Cefdinir	Y	Cephalosporin	Antibiotic	PBP 2-3
Cefmenoxime hydrochloride	Y/N	Cephalosporin	Antibiotic	PBP 1A
Cefoperazone	Y	Cephalosporin	Antibiotic	PBP 1A-B, 2
Cefotaxime sodium	Y/N	Cephalosporin	Antibiotic	PBP 1-3
Cefradine	A	Cephalosporin	Antibiotic	PBP 1A
Ceftazidime Pentahydrate	Y/N	Cephalosporin	Antibiotic	PBP 3
Ceftiofur HCl	Y/N	Cephalosporin	Antibiotic	PBP
Ceftriaxone Sodium Trihydrate	Y/N	Cephalosporin	Antibiotic	PBP 2B
Chlorhexidine HCl	Y	Biguanide	Antiseptic	Bacterial outer membrane
Colistin Sulphate	Y/N	Polymyxin	Antibiotic	Lipopolysaccharide layer
Danofloxacin Mesylate	Y/N	Fluoroquinolone	Antibiotic	DNA gyrase
Difloxacin HCl	A	Fluoroquinolone	Antibiotic	DNA gyrase
Dihydrostreptomycin sulphate	A	Aminocyclitol glycoside	Antibiotic	30S ribosomal
Doripenem Hydrate	Y/N	Carbapenem	Antibiotic	PBP 1A, 1B, 2-3
Doxycycline HCl	Y/N	Tetracycline	Antibiotic	30S ribosomal
Enoxacin	Y	Fluoroquinolone	Antibiotic	DNA gyrase
Enrofloxacin	Y	Fluoroquinolone	Antibiotic	DNA gyrase
Flumequine	Y	Fluoroquinolone	Antibiotic	DNA gyrase
Furazolidone	Y	Nitrofuran	Antibiotic	DNA
Gentamicin Sulphate	A	Aminoglycoside	Antibiotic	30S ribosomal
Levofloxacin	Y	Fluoroquinolone	Antibiotic	DNA gyrase
Levofloxacin	A	Fluoroquinolone	Antibiotic	DNA gyrase
Lomefloxacin HCl	Y/N	Fluoroquinolone	Antibiotic	DNA gyrase
Marbofloxacin	A	Fluoroquinolone	Antibiotic	DNA gyrase
Methacycline HCl	A	Tetracycline	Antibiotic	30S ribosomal
Minocycline HCl	Y/N	Tetracycline	Antibiotic	30S ribosomal
Moxalactam Disodium	Y/N	Oxacephem	Antibiotic	PBP 1A, 1B, 3
Moxifloxacin HCl	Y	Fluoroquinolone	Antibiotic	DNA gyrase
Nadifloxacin	A	Fluoroquinolone	Antibiotic	DNA gyrase
Netilmicin Sulphate	Y/N	Aminoglycoside	Antibiotic	30S ribosomal
Ofloxacin	Y/N	Fluoroquinolone	Antibiotic	DNA gyrase
Palbociclib (PD-0332991) HCl	Y/N	Pyridinylpiperazine	Antineoplastic	CDK4 & CDK6
Pefloxacin Mesylate	Y	Fluoroquinolone	Antibiotic	DNA gyrase
Penfluridol	A	Diphenylmethane	Antipsychotic	Dopamine receptors
Polymyxin B sulphate	Y	Polymyxin	Antibiotic	Lipopolysaccharide layer
Pralidoxime chloride	Y	N-methylpyridinium	Organophosphates antidote	Cholinesterase
Rifaximin	Y/N	Rifaximins	Antibiotic	RNA polymerase
Sarafloxacin HCl	Y	Fluoroquinolone	Antibiotic	DNA gyrase
Sisomicin sulphate	Y	Aminoglycoside	Antibiotic	30S ribosomal
Sitafloxacin Hydrate	A	Fluoroquinolone	Antibiotic	DNA gyrase

FDA compound	Result	Chemical classification	Therapeutic use	Target
Sparfloxacin	Y	Fluoroquinolone	Antibiotic	DNA gyrase
Tebipenem Pivoxil	Y/N	Carbapenem	Antibiotic	Unknown
Terbutaline Sulphate	Y/N	Resorcinol	Antiasthmatic	β-2 adrenergic receptors
Tobramycin	A	Aminoglycoside	Antibiotic	30S ribosomal
Triclosan	Y/N	Diphenylether	Antiseptic	ENR enzyme
Trimethoprim	Y/N	Anisol	Antibiotic	Dihydrofolate reductase
Zidovudine	Y	Nucleoside analogue	Antiretroviral	Reverse transcriptase

Classification by their therapeutic use revealed most hits were known antibacterials (75%), including quinolones (n=16), β-lactams (n=12) and aminoglycosides (n=6), among others. Non-antibacterial compounds (n=15) included other anti-infective agents (7%), antineoplastics (7%) or antipsychotics (3%) among others. These figures did not differ significantly among the three PCs **(Figure 1.7).**

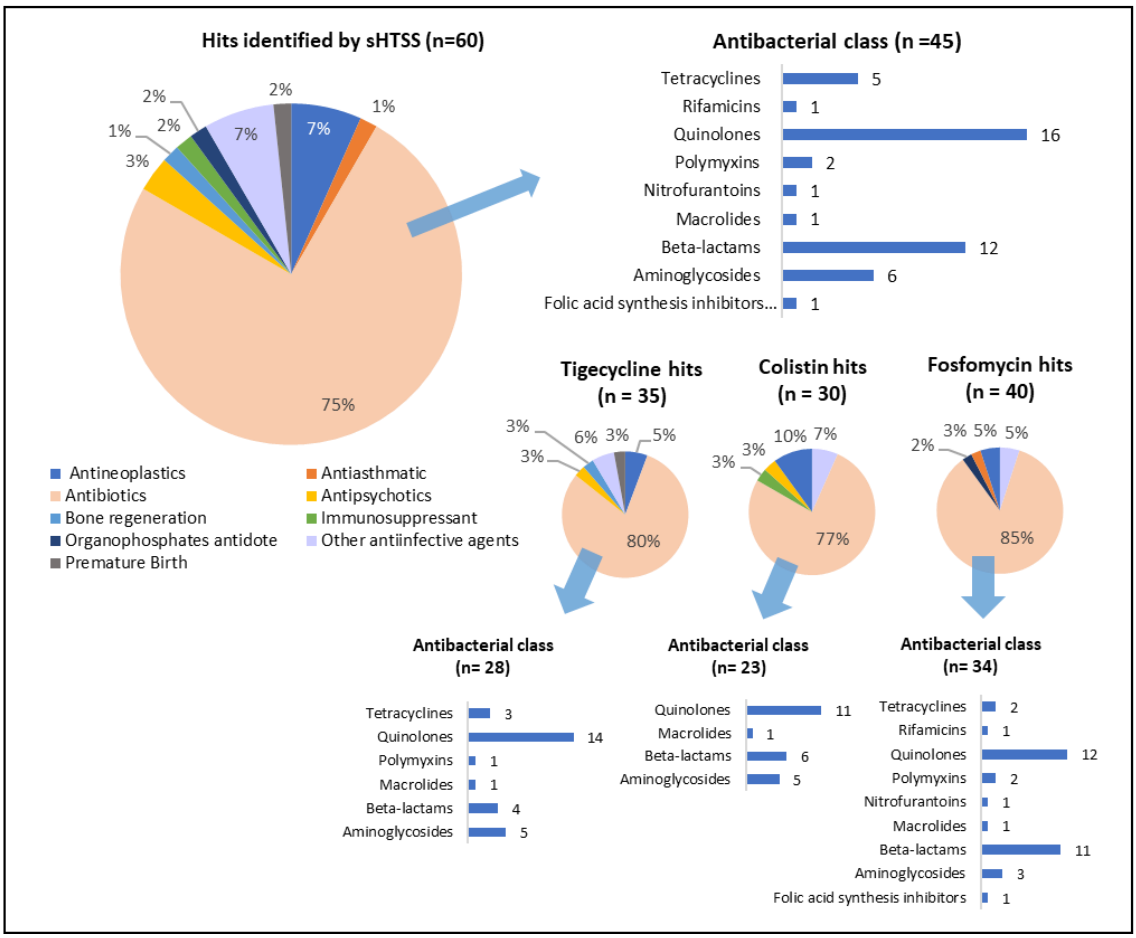


Figure 1.7. FDA compounds with favorable interactions with tigecycline, colistin and fosfomycin identified by sHTSS and classified by their therapeutic use. Other anti-infective agents include anti-parasitic, antiseptic and antiviral agents. Duplicate hits were removed from analysis. sHTSS, semi-high throughput synergy screening.

FDA compounds susceptibility assays

Table 1.3. shows MIC and MBC values for FDA compounds presenting synergy with tigecycline, colistin or fosfomycin against *K. pneumoniae*.

Table 1.1. Drug susceptibility of FDA compounds

Compound	MIC values (mg/L)	MBC values (mg/L)
Amikacin	1	2
Azithromycin	2	2-4
Aztreonam	0.125	0.25
Balofloxacin	0.25	0.25
Bleomycin	0.25	0.25
Cefdinir	0.5	0.5
Cefmenoxime	0.25	0.25-0.5
Cefoperazone	4	4
Ceftazidime	0.5	0.5
Ceftiofur	1	1
Ceftriaxone	0.125	0.125
Cephradine	32	32
Colistin	1-2	1-2
Danofloxacin	0.06	0.12
Difloxacin	0.12-0.25	0.25-0.5
Doripenem	0.03	0.06
Doxycycline	4	>8
Enrofloxacin	0.015-0.03	0.03-0.06
Flumequine	1-2	1-2
Fosfomycin	≥128	≥128
Furazolidone	1	1
Ibandronate	>32	>32
Ivermectin	>64	>64
Levofloxacin	0.03	0.03
Lomefloxacin	0.12-0.25	0.12
Marbofloxacin	0.015-0.03	0.015-0.06
Methacycline	1	32
Moxalactam	0.5	0.5
Moxifloxacin	0.25-0.5	0.25-0.5
Nadifloxacin	1	1
Netilmicin	1	1
Norfloxacin	2	2
Ofloxacin	0.125	0.125
Pefloxacin	0.125	0.125
Penfluridol	>32	>32
Pralidoxime	>32	>32
Rifaximin	16	32
Sisomicin	0.125	0.25
Sparfloxacin	0.03	0.03
Streptomycin	2	2
Terbutaline	>32	>32
Tigecycline	0.5	1?
Tobramicin	0.125	0.125
Triclosan	0.25-0.5	0.25
Trimetoprim	1	8-16
Zidovudine	0.06	0.5-0.12

Checkerboard assay displayed low validation rates.

Fifty-three sHTSS interactions classified as synergy (n=50) or antagonism (n=3) for any PC were evaluated by CBA. FICI and FBCI indexes were calculated based on their MIC and MBC values respectively **(Table 1.3)**; and interactions were validated in 8 out of the 53 (15.09%) combinations (see **Appendix I**). Colistin synergistic interactions were validated in 7 out of 12 combinations tested (58.33%) by both FICI and FBCI values. This percentage included the combination with bleomycin, classified as likely antagonism by sHTSS but showing synergy by CBA (FICI & FBCI = 0.16). Finally, synergy with fosfomycin was only validated in combination with lomefloxacin (1 out of 25, rate of 4%), with a FICI = 0.375 and FBCI = 0.365. No antagonism was confirmed by CBA.

Time-kill studies revealed novel promising combinations.

Forty-one combinations (35 classified as Y or Y/N and 6 as A) were studied by TKA to assess their pharmacological and clinical translation potential **(Figure 1.8.** and **Appendix II)**. Overall, TKA showed a synergy validation rate of 65.85% (27 out of 41 combinations) at any of the predefined time points. Specific confirmation rates for each PC were 72.72% (8/11) for colistin, 70.58% (12/17) for fosfomycin, and 53.84% (7/13) for tigecycline.

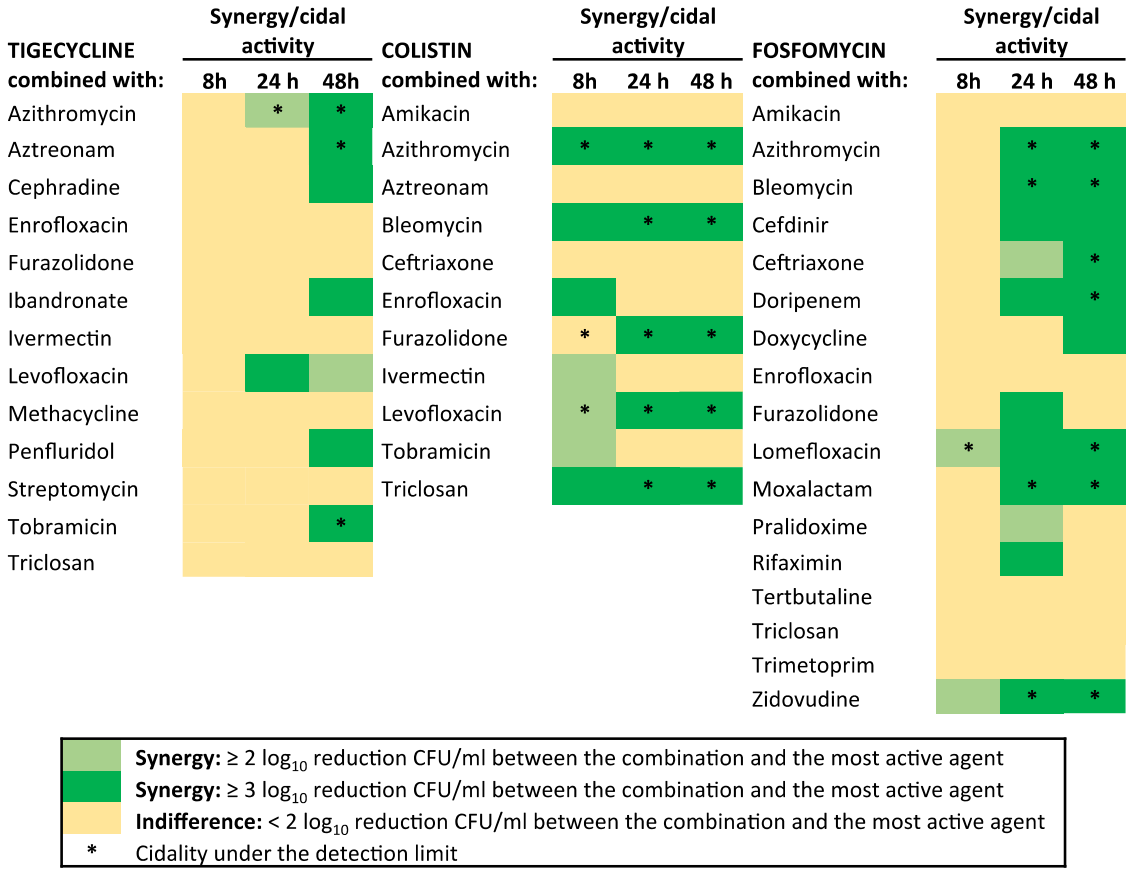


Figure 1.8. TKA drug interaction heat map. Forty-one sHTSS hits were prioritized for TKA based on a pharmacological and clinical translation potential assessment.

Six novel synergistic interactions with non-antibiotic drugs were confirmed in these studies **(Figure 1.9).** Strong bactericidal and synergistic interactions were observed between the antiviral zidovudine and fosfomycin, also in combinations of bleomycin (antineoplastic) with colistin and fosfomycin **(Figures 1.8.** and **1.9)**. Tigecycline combined with bisphosphonate ibandronate and the antipsychotic penfluridol showed a bacteriostatic profile and synergy at 48 hours with reductions of 4.04 log_{10} CFU/mL and 2 log_{10} CFU/mL, respectively. Both combinations prevented the bacterial regrowth observed with tigecycline alone **(Figure 1.9).** The antiparasitic ivermectin enhanced the activity of colistin at early time points (killing to the limit of detection at 5 hours) and showed synergistic effect at the 8-hour time point, although followed by bacterial rebound **(Figures 1.8.** and **1.9).** Finally, pralidoxime (a poisoning antidote) showed synergy at 24 hours but no killing activity in combination with fosfomycin **(Appendix II).**

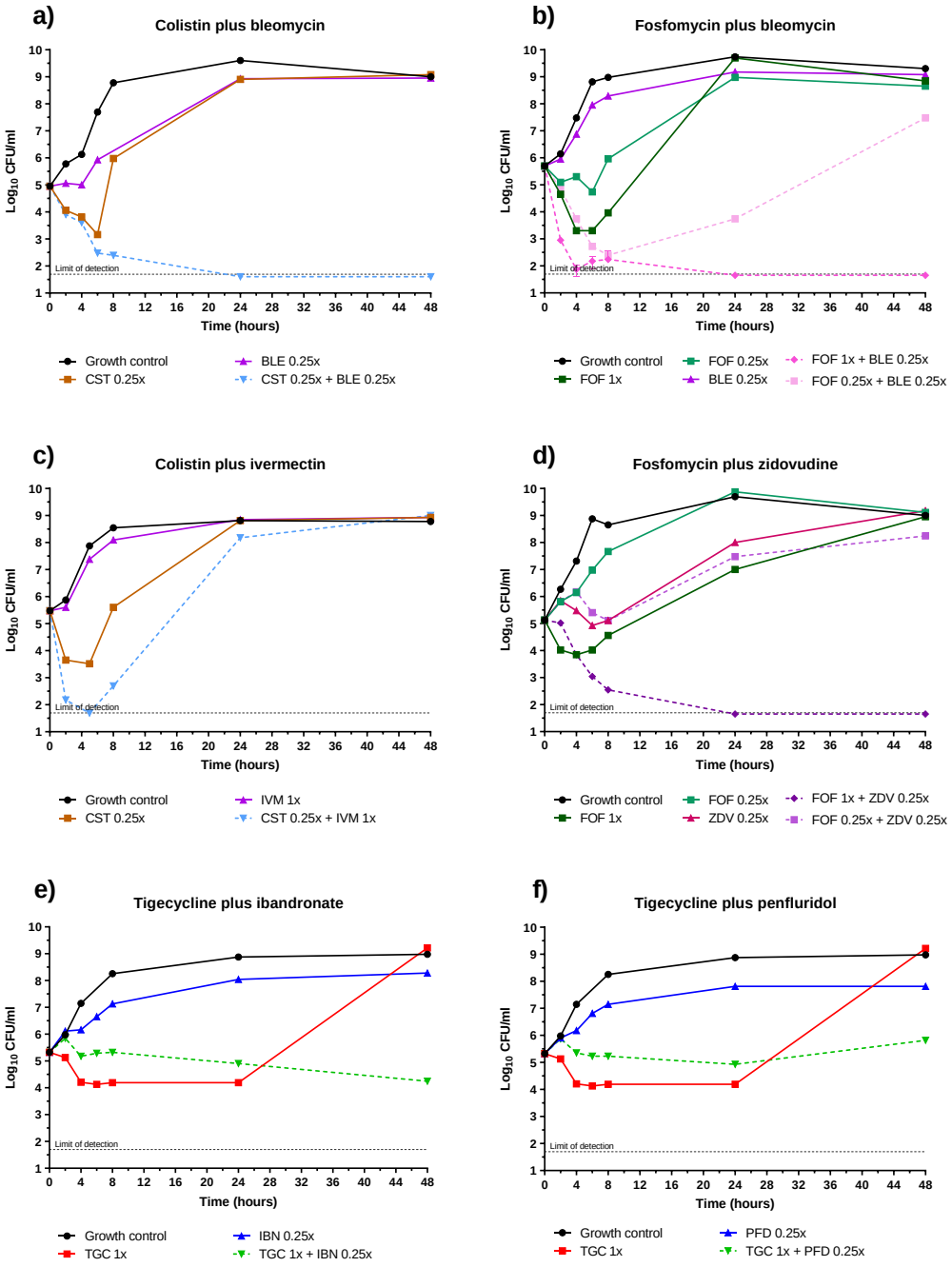


Figure 1.9. TKA of the novel combinations of non-antibiotic drugs with the PCs against *K. pneumoniae* ATCC 13883. (a-b) Bleomycin in combination with colistin or fosfomycin at subinhibitory concentration enhanced the bactericidal activity of both PCs **(c)** Ivermectin showed synergy up to 8 hours of incubation with colistin, after this time point a rebound was observed, similarly as both compounds alone **(d)** Zidovudine showed a strong interaction profile with fosfomycin at subinhibitory concentration (0.25x MIC) **(e-f)** Tigecycline combined with ibandronate or penfluridol showed static activity and synergy at 48 hours. BLE, bleomycin; CST, colistin; FOF, fosfomycin; IBN, ibandronate; IVM, ivermectin; PFD, penfluridol; TGC, tigecycline; ZDV, zidovudine.; MIC_{BLE} = 0.25 mg/L; MIC_{CST} = 1 mg/L; MIC_{FOF} ≥128 mg/L; MIC_{IVM} >64 mg/L (assumed in 64 mg/L); MIC_{ZDV} = 0.06 mg/L; MIC_{TGC} = 0.5 mg/L; MIC_{IBN} & MIC_{PFD} >32 mg/L (both assumed in 32 mg/L).

Effective combinations were also identified with other antimicrobials. Azithromycin displayed potent synergistic and bactericidal activities with all three PCs, showing the strongest effect in combination with colistin from the early 8-hour time point **(Figure 1.8).** A highly effective curve was also obtained with the colistin and levofloxacin combination. Moreover, we identified a remarkable potentiation of colistin by furazolidone and the antiseptic triclosan, which was not observed in the case of tigecycline or fosfomycin. Fosfomycin combinations with cefdinir, ceftriaxone, doripenem, lomefloxacin and moxalactam showed potent synergistic interactions at the 24- and 48-hour time points, the latter interaction reaching the limit of detection. Tigecycline showed bactericidal activity after 48 hours with aztreonam and tobramycin. Synergy with other antibiotics was also validated including cephradine, with a 2.35 $\log_{10}$ CFU/mL reduction at 48 hours; or levofloxacin, with synergy after 24 hours although regrowth was observed after 48 hours. An example of the type of information provided by the different validation assays (supporting the use of TKA over CBA) is shown in **Figure 1.10.** for the tigecycline/aztreonam combination.

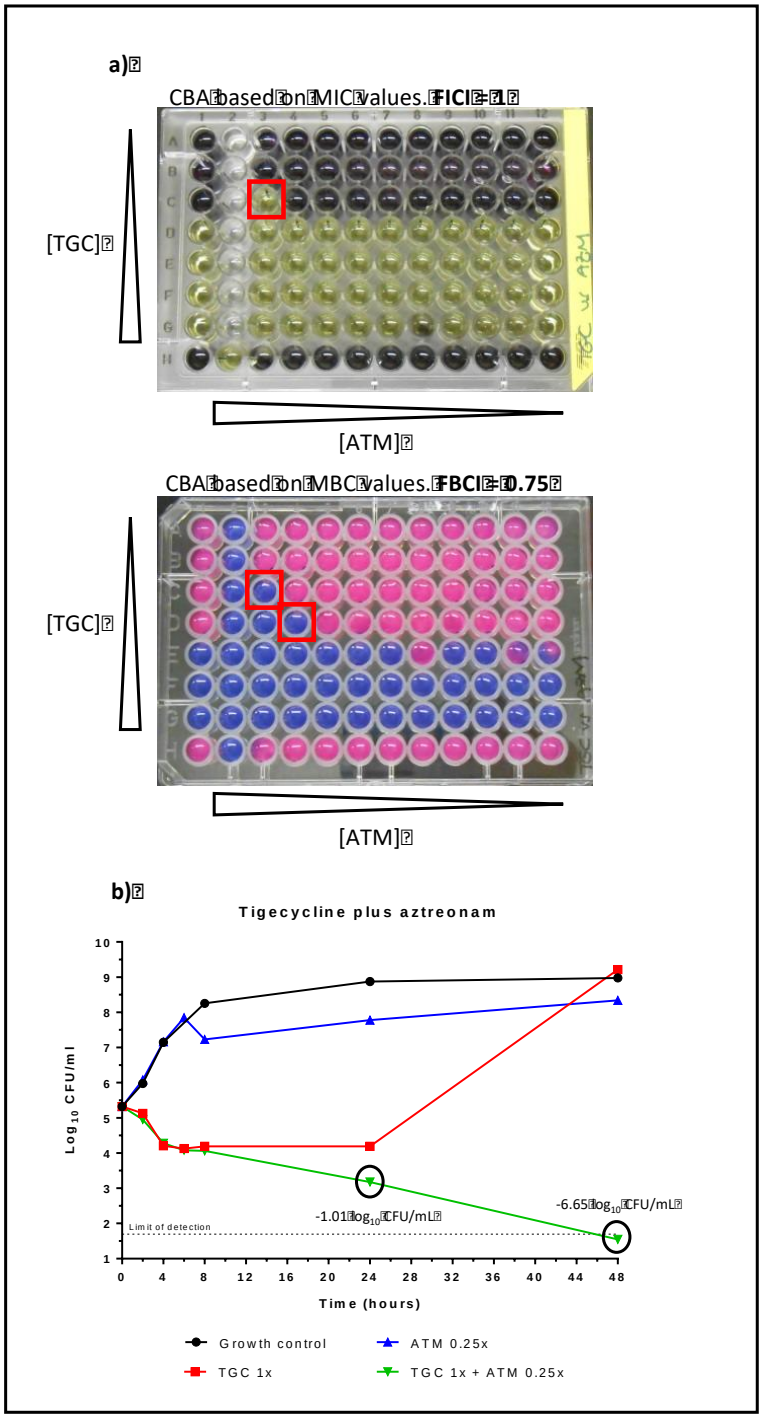


Figure 1.10. Secondary validation CBA and TKA of tigecycline in combination with aztreonam against *K. pneumoniae* ATCC 13883. (a) FICI and FBCI were calculated from the most optimal combinatorial concentration (lowest value in the combination, red squares). Based on these assays, the combination was classified as "no interaction" (FICI/FBCI values between 0.5-4). **(b)** TKA provided longitudinal information showing a reduction of 1.01 log_{10} in CFU/mL of the combination with respect to tigecycline alone at 24 hours; thus, at this time point, the interaction was not classified as synergistic (<2 log_{10} reduction in CFU/mL with respect to the most active drug). However, after 48 hours the combination could be classified as synergistic with a reduction of 6.65 log_{10} CFU/mL with respect to tigecycline alone, and prevention of bacterial re-growth (proxy for sterilizing activity). MIC_{ATM} = 0.125-0.25 mg/L; MIC_{TGC} = 0.8 mg/L; MBC_{ATM} = 0.25 mg/L; MBC_{TGC} = 1.6 mg/L. CBA, checkerboard assay; TKA, time-kill assay; TGC, tigecycline; ATM, aztreonam.

DISCUSSION

Traditional *in vitro* drug screening methodologies are tedious and time-consuming, and identification of novel combination therapies based on repurposed drugs could be a promising short/medium-term approach to fight against AMR. The HTSS methodology was initially developed to be used against mycobacteria and successfully identified novel combinations enhancing the activity of known antimicrobials (108,115). Here, the same methodology was adapted through a straightforward standardization process to find active combinations against *K. pneumoniae*. sHTSS demonstrated swiftly implementation for screening campaigns and allowed rapid, clear and simply drug interaction readouts derived from inhibition zones in agar media.

These screenings in *K. pneumoniae* yield hit rates ranging from 2.17 to 2.87%, higher than that observed in *M. smegmatis* (1.4%) (108), but similar to other studies against Gram-negative bacilli with comparable hit rates (1.87%, tigecycline / 5.54%, colistin) (116). A large proportion of hits identified in this study were known antimicrobial drugs (82%), including antibiotics (75%) and other anti-infective agents (7%) **(Figure 1.7.).** The overrepresentation of antimicrobials in synergy screening programs enhancing the activity of other antimicrobials is similarly described in other studies with *K. pneumoniae* (116,117) and *M. smegmatis* (108). Hind *et al.* observed this finding specially associated with *K. pneumoniae*, while they identified more heterogeneous targets with other Gram-negative bacteria (116).

Secondary validation assays performed by CBA and TKA provided discordant information; CBA yielded low sHTSS confirmation rates (15.09%), especially in the case of tigecycline and fosfomycin, while these increased to 65.85% when tested by TKA. Both techniques are widely used to determine synergy; however, they are based on different fundamental principles and parameters. Traditional CBA assays are based on growth inhibition parameters (MIC) at a fixed time point (usually overnight), while TKA reports bactericidal activity (Log_{10} CFU/mL) at several time points (up to 48 hours in our assays).

While performing CBA, MBC was also measured (a fixed time point bactericidal parameter) to determine the FBCI, in addition to the FICI. This index has been largely disregarded in synergy studies, but some authors suggested FBCI might be a better predictor of drug interaction than FICI (118–120) and, in our view, it is a more stringent criteria to prioritize synergistic combinations. As an example, **Figure 1.10.** shows validation results of the tigecycline/aztreonam combination. We found a clear "indifferent" profile by CBA (FICI=1), while a tendency towards synergism was observed by FBCI, which some authors interpreted as

additivity (0.5 < FBCI ≤ 1), although this terminology should be avoided (90). The use of a fixed time point bactericidal parameter could predict TKA data; the combination showed no synergy at 24 hours (although with a tendency to reduce the bacterial count compared to tigecycline alone) but prevented growth rebound at 48 hours, suggesting a synergistic interaction. Nevertheless, the use of the FBCI might come with some limitations for compounds with high MIC values (≥128 mg/L) such as fosfomycin, for which we could not calculate the FBCI because the MBC exceeded the maximum tested concentration.

The fixed time point limitation of the MBC assays is addressed in TKA by the inclusion of longitudinal pharmacodynamic data, which allows TKA as a most suitable methodology to robustly characterize drug interaction dynamics, a paradigm shift in antimicrobial development methodologies (91). TKA were extended up to 48 hours of incubation with the aim to prioritize the most effective combinations that maintained bactericidal activities up to the end of the assay. Moreover, the 48-hour time point allow us to identify combinations that could potentially lead to therapeutic failure when used in the clinical practice, i.e., combinations considered initially effective but that rebounded after the 24-hour time point **(Appendix III).** This could be a better proxy of sterilization and, thus, potency of the combination.

Out of the three PCs used in this study, the highest number of validated combinations was obtained for colistin and fosfomycin, antibiotics targeting the outer membrane and cell wall, respectively. Both antibiotics have been associated with enhance killing when in combination with intracellular targeting compounds, explained by an increase in permeability (116,121,122). In agreement with our study, clinically relevant synergistic interactions with our three PCs have been reported within a combinatorial therapy for the treatment of MDR enterobacteria (36,37). Moreover, synergy was demonstrated between tigecycline and aminoglycosides (123–125), colistin and levofloxacin (93,126), fosfomycin and cephalosporins (122) or doripenem (127,128) against *K. pneumoniae*. Principe *et al.* also reported synergy between tigecycline and levofloxacin against *Acinetobacter baumanii* strains (129). A recent review on *in vitro* fosfomycin combinations supports our findings in which we did not observe synergy between fosfomycin and amikacin or trimethoprim (48). Using CBA, Ontong *et al.* reported synergy between colistin/amikacin and colistin/tobramycin for 72.72% and 45.45% of MDR *K. pneumoniae* strains, respectively (126). We also observed synergy by CBA for the latter combination, but such interactions were not maintained by TKA **(Appendix I** and **appendix II).** This highlights again TKA as a much better proxy than CBA to identify synergistic combinations.

Novel combinations including non-antibiotics were identified:

1. *Tigecycline plus ibandronate or penfluridol.* Ibandronate and other bisphosphonate derivatives are anti-parasitic drugs inhibiting the synthesis of essential isoprenoids (130,131). The antipsychotic penfluridol displayed partial synergistic activity with aminoglycosides and β-lactams against *Enterococcus faecalis* (132). To the best of my knowledge, here it is reported for the first time the antibacterial activity of both drugs with tigecycline against enterobacteria

2. *Ivermectin with tigecycline or colistin*. Ivermectin is an anthelmintic with both veterinary and clinical applications, widely explored for different repurpose anti-parasitic and antiviral uses (133–135). Its antimicrobial activity was demonstrated against *Mycobacterium* species (136–138). sHTSS classified ivermectin as likely antagonism with tigecycline and colistin. Follow up TKA validation studies demonstrated no interaction with tigecycline and a slight synergistic effect at the 8-hour time point in combination with colistin but followed by a bacterial rebound, highlighting the need to perform secondary CFU-based validation assays **(Figures 1.8.** and **1.9.).**

3. *Zidovudine plus fosfomycin.* Zidovudine was the first antiretroviral used for HIV treatment. Since 1980s, its antibacterial activity against enterobacteria is attributed to its targeting of bacterial thymidine kinases (139). Several *in vitro* and/or *in vivo* studies demonstrated effective antibacterial activities of zidovudine in combination with antibiotics such as fluoroquinolones, trimethoprim, aminoglycosides, tigecycline and polymyxins (139–144). Similar to a recent report (145), a strong synergistic interaction with fosfomycin was also identified.

4. *Bleomycin plus colistin or fosfomycin.* The cytotoxicity of bleomycin poses a barrier to an antibacterial repurposing approach; nevertheless, our results could set up the basis for the development of analogues or dosage-based studies to minimize toxicity.

5. *Azithromycin.* This compound showed synergistic interaction in combination with all three PCs. This was in agreement with several studies reporting the bactericidal and antibiofilm action of azithromycin combined with tigecycline (146), and colistin (147,148) against different Gram-negative bacilli. The interaction with fosfomycin is novel. Another macrolide, erythromycin, displayed synergy for 50% of the *K. pneumoniae* strains tested (149). The promiscuity of azithromycin interactions suggests its inclusion as primary compound in future synergy screening campaigns, which might ease the identification of new synergistic partners to improve its therapeutic use.

CONCLUSIONS AND FUTURE PERSPECTIVES

In this Chapter 1, novel synergistic combinations have been identified against *K. pneumoniae* adapting the HTSS methodology and using novel validation endpoints in TKA. Synergy screens based on HTSS might also adapt to any priority pathogen for which new antimicrobials are urgently needed to accelerate the search for synergistic partners.

The efficacy of the interactions identified here was also evaluated against a *K. pneumoniae* reference strain with high susceptibility to most antibiotics, which might overestimate the real rate of effective combinations against clinical and MDR strains. It is expected that this scenario could be unlikely, and this technology should be helpful as well for readily identifying synergistic combinations active against MDR strains. Moreover, both CBA or TKA are methodologies in which drugs are added only once at the start of the experiment at fixed concentrations; although this is a technical simplification of the dynamic fluctuation of drugs in the clinical practice, other *in vitro* methodologies such as the hollow fibre system computing the pharmacokinetic properties of the compounds in combination and linking it to pharmacodynamics parameters of activity might provide essential information to refine drug doses and hence aid the design of future clinical trials.

Finally, clinical implementation of synergistic novel combinations might improve medical decision-making; the combined effect could reduce the exposure to potentially toxic drugs, resulting in lower incidence of treatment-related side-effects and complications, but enhancing their effectiveness.

APPENDIX I. Summary supplementary table of FDA hits identified in sHTSS with tigecycline, colistin and fosfomycin and validation data against *K. pneumoniae* ATCC 13883.

TIGECYCLINE HITS		sHTSS								
			Δ zones of inhibition (mm)[b]			CBA[e]		TKA[f]		
PubChem #[a]	FDA compound	Ø[c]	TGC ¼ x MIC	TGC ½ x MIC	Interaction[d]	FICI	FBCI	8h	24h	48h
CID 441384	Abacavir sulphate	0	0	1.6	Y/N	-	-	-	-	-
CID 38351	Amikacin disulphate	0	0	3	Y/N	0.5	0.75	-	-	-
CID 16218899	Amikacin hydrate	0	0	2.65	Y/N	0.5	0.75	-	-	-
CID 87665603	Atosiban Acetate	0	0	1.65	Y/N	-	-	-	-	-
CID 447043	Azithromycin	0	2.5	6	Y	0.5-4	0.5-4		*	*
CID 3033819	Azithromycin Dihydrate	4.7	1.45	1.75	Y	0.5-4	0.5-4		*	*
CID 5742832	Aztreonam	11	3	4	Y	1	0.75			*
CID 65958	Balofloxacin	3.5	1	0	Y/N	-	-	-	-	-
CID 72466	Bleomycin Sulphate	9.5	-1.5	-2.5	A	-	-	-	-	-
CID 135500522	Calcium Levofolinate	0	0	2.75	Y/N	-	-	-	-	-
CID 6915944	Cefdinir	0	3.5	9.5	Y	0.5-4	0.5-4	-	-	-
CID 44187	Cefoperazone	0	2.5	6.5	Y	-	-	-	-	-
CID 38103	Cefradine	0	7.95	9.2	Y	0.5-4	0.5-4			
CID 54682468	Chlortetracycline HCl	4.55	1.5	8.15	Y	-	-	-	-	-
CID 71334	Danofloxacin Mesylate	7.95	0.3	-0.55	Y/N	-	-	-	-	-
CID 54680690	Demeclocycline HCl	3	3.5	6.5	Y	-	-	-	-	-
CID 21653	Dihydrostreptomycin sulphate	6.6	-6.6	-0.8	A	1	0.5-4			
CID 3229	Enoxacin	0	5.5	5.5	Y	-	-	-	-	-
CID 71188	Enrofloxacin	1.5	7.5	5	Y	0.5-4	0.5-4			
CID 5323714	Furazolidone	2	-2	-2	A	1	0.75			
CID 5379	Gatifloxacin	8.5	-8.5	-0.5	A	-	-	-	-	-
CID 56842139	Gentamicin Sulphate	8.75	0.4	1.65	Y/N	-	-	-	-	-

TIGECYCLINE HITS		sHTSS								
			Δ zones of inhibition (mm) [b]			CBA [e]		TKA [f]		
PubChem # [a]	FDA compound	Ø [c]	TGC ¼ x MIC	TGC ½ x MIC	Interaction [d]	FICI	FBCI	8h	24h	48h
CID 23670359	Ibandronate sodium	0	0	3.2	Y/N	0.5-4	0.5-4			
CID 6321424	Ivermectin	2.5	-2.5	-2.5	A	-	-			
CID 149096	Levofloxacin hydrate	6.05	0.25	1	Y/N	0.5-4	0.5-4			
CID 149096	Levofloxacin	6	-1.5	-0.5	A	0.5-4	0.5-4			
CID 68624	Lomefloxacin HCl	7.2	1.4	2.6	Y	-	-	-	-	-
CID 60651	Marbofloxacin	2.5	2.5	5	Y	-	-	-	-	-
CID 54685047	Methacycline HCl	0	3.4	8.4	Y	0.5-4	0.5-4			
CID 4410	Nadifloxacin	0	3	0	Y/N	-	-	-	-	-
CID 62115	Netilmicin Sulphate	11.45	-1.775	2.45	Y/N	-	-	-	-	-
CID 4539	Norfloxacin	7	3	1.5	Y	-	-	-	-	-
CID 4583	Ofloxacin	2.5	5.5	4	Y	0.5-4	0.5-4	-	-	-
CID 11478676	Palbociclib (PD0332991) Isethionate	0	4.85	3.45	Y	-	-	-	-	-
CID 119525	Pefloxacin Mesylate	10.5	0.5	3.5	Y	-	-	-	-	-
CID 33630	Penfluridol	4	0	1.5	Y/N	0.5-4	0.5-4			
CID 5702105	Polymyxin B sulphate	13.55	-1.1	0.3	Y/N	-	-	-	-	-
CID 56207	Sarafloxacin HCl	3	-0.5	3	Y/N	-	-	-	-	-
CID 439243	Sisomicin sulphate	10.75	0.45	3.2	Y/N	0.5-4	0.5-4	-	-	-
CID 6918203	Sitafloxacin Hydrate	16	1	-2.5	Y/N	-	-	-	-	-
CID 60464	Sparfloxacin	14	3	4.5	Y	-	-	-	-	-
CID 36294	Tobramycin	4.4	1.15	1.7	Y	0,75	0,625			*
CID 5564	Triclosan	9.5	0.15	-0.3	Y/N	0.5-4	0.5-4			

COLISTIN HITS		sHTSS								
			Δ zones of inhibition (mm) [b]			CBA[e]		TKA[f]		
PubChem #[a]	FDA compound	Ø[c]	CST 1/256xMIC	CST 1/128xMIC	Interaction[d]	FICI	FBCI	8h	24h	48h
CID 2123	Altretamine	1.5	-1.5	-1.5	A	-	-	-	-	-
CID 38351	Amikacin disulphate	0	3.5	1.9	Y	0.32	0.19			
CID 16218899	Amikacin hydrate	0	1.85	0	Y/N	0.32	0.19			
CID 447043	Azithromycin	0	4.5	6.5	Y	0.14	0.14	*	*	*
CID 5742832	Aztreonam	11	2	3	Y	0.38	0.38			
CID 44599207	BAF312 (Siponimod)	0	0	4.5	Y/N	-	-	-	-	-
CID 72466	Bleomycin Sulphate	9.5	-1	-6	A	0.16	0.16		*	*
CID 135500522	Calcium Levofolinate	0	1.5	0	Y/N	-	-	-	-	-
CID 6915944	Cefdinir	0	5.5	0	Y/N	1	0.63	-	-	-
CID 44187	Cefoperazone	0	6.5	0	Y/N	-	-	-	-	-
CID 38103	Cefradine	0	2.85	6.4	Y	1	1	-	-	-
CID 6536864	Ceftazidime Pentahydrate	0	3.75	0	Y/N	-	-	-	-	-
CID 137706342	Ceftriaxone Sodium Trihydrate	0	4.5	0	Y/N	0.32	0.32			
CID 57379345	Ceritinib (LDK378)	0	0	3.5	Y/N	-	-	-	-	-
CID 71334	Danofloxacin Mesylate	7.95	2.05	-7.95	Y/N	-	-	-	-	-
CID 56205	Difloxacin HCl	14	-2.5	-1	A	-	-	-	-	-
CID 21653	Dihydrostreptomycin sulphate	6.6	1.25	0.35	Y/N	-	-	-	-	-
CID 71188	Enrofloxacin	1.5	3.5	4	Y	0.63	0.63			
CID 5323714	Furazolidone	5	-1	-0.5	A	-	-	*	*	*
CID 5379	Gatifloxacin	8.5	-7.5	-8.5	A	-	-	-	-	-
CID 3476	Glimepiride	1.5	-1.5	-1.5	A	-	-	-	-	-
CID 6321424	Ivermectin	2.5	-2.5	-2.5	A	-	-			
CID 149096	Levofloxacin	6	1	-2.5	Y/N	-	-	*	*	*
CID 149096	Levofloxacin hydrate	8	-2.5	-4.5	A	-	-	*	*	*
CID 68624	Lomefloxacin HCl	7.2	0.65	-7.2	Y/N	-	-	-	-	-
CID 60651	Marbofloxacin	2.5	0.5	-0.5	Y/N	-	-	-	-	-
CID 65329	Mefloquine HCl	0	1.5	0	Y/N	-	-	-	-	-

COLISTIN HITS		sHTSS								
			Δ zones of inhibition (mm) [b]			CBA[e]		TKA[f]		
PubChem #[a]	FDA compound	Ø[c]	CST 1/256xMIC	CST 1/128xMIC	Interaction[d]	FICI	FBCI	8h	24h	48h
CID 101526	Moxifloxacin HCl	3.5	2	-1	Y/N	-	-	-	-	-
CID 62115	Netilmicin Sulphate	11.45	-0.1	0.35	Y/N	-	-	-	-	-
CID 4539	Norfloxacin	7	0	0.5	Y/N	-	-	-	-	-
CID 4583	Ofloxacin	2.5	3.5	4	Y	0.63	0.63	-	-	-
CID 11478676	Palbociclib (PD0332991) Isethionate	0	4.95	6.2	Y	-	-	-	-	-
CID 119525	Pefloxacin Mesylate	10.5	-2	0.5	Y/N	-	-	-	-	-
CID 16362	Pimozide	0	2	0	Y/N	-	-	-	-	-
CID 5702105	Polymyxin B sulphate	13.55	-1.35	-0.65	A	-	-	-	-	-
CID 56207	Sarafloxacin HCl	3	5	0.5	Y	-	-	-	-	-
CID 439243	Sisomicin sulphate	10.75	0.15	2.05	Y/N	0.5	0.38	-	-	-
CID 6918203	Sitafloxacin Hydrate	16	-2.5	-0.5	A	-	-	-	-	-
CID 60464	Sparfloxacin	14	-2	4	Y/N	-	-	-	-	-
CID 36294	Tobramycin	4.4	1.25	1.05	Y	0.32	0.38			
CID 5564	Triclosan	9	0	2	Y/N	0.56	0.56		*	*

FOSFOMYCIN HITS		sHTSS								
			Δ zones of inhibition (mm) [b]			CBA[e]		TKA[f]		
PubChem #[a]	FDA compound	Ø[c]	FOF 1/8 x MIC	FOF ¼ x MIC	Interaction[d]	FICI	FBCI	8 h	24 h	48 h
CID 38351	Amikacin disulphate	4	0	6	Y/N	0.5-4	NA			
CID 16218899	Amikacin hydrate	2.5	-1	-0.5	A	0.5-4	NA			
CID 447043	Azithromycin	0.5	1	6.5	Y	0.75	NA		*	*
CID 3033819	Azithromycin Dihydrate	0	2.5	2	Y	0.75	NA		*	*
CID 5742832	Aztreonam	5	-1.5	14.5	Y/N	-	-	-	-	-
CID 65958	Balofloxacin	0	0	6	Y/N	-	-	-	-	-
CID 72466	Bleomycin Sulphate	9	3	15	Y	0.56	0.56		*	*
CID 6915944	Cefdinir	7	3	13	Y	0.75	NA			
CID 11954009	Cefmenoxime hydrochloride	0	0	9	Y/N	-	-	-	-	-
CID 44187	Cefoperazone	2.5	0.5	3	Y	0.75	NA	-	-	-
CID 10695961	Cefotaxime sodium	0	0	11	Y/N	-	-	-	-	-
CID 38103	Cefradine	3	-3	-3	A	-	-	-	-	-
CID 6536864	Ceftazidime Pentahydrate	0	0	8.5	Y/N	0.625	0.625	-	-	-
CID 9937686	Ceftiofur HCl	3	0	15	Y/N	-	-	-	-	-
CID 137706342	Ceftriaxone Sodium Trihydrate	0	0	4	Y/N	1	NA			*
CID 1117	Colistin Sulphate	11	-1	1	Y/N	-	-	-	-	-
CID 71334	Danofloxacin Mesylate	5.5	0	3.5	Y/N	-	-	-	-	-
CID 56205	Difloxacin HCl	10	-1	-3	A	-	-	-	-	-
CID 21653	Dihydrostreptomycin sulphate	5.5	-1	-0.5	A	-	-	-	-	-
CID 636377	Doripenem Hydrate	0	0	5.5	Y/N	1	NA			*
CID 54685920	Doxycycline HCl	2	0	3.5	Y/N	0.5-4	NA			
CID 3229	Enoxacin	2	2	1	Y	-	-	-	-	-
CID 71188	Enrofloxacin	8	1.5	2.5	Y	0.5-4	NA			
CID 3374	Flumequine	2.5	2.5	5.5	Y	0.5-4	NA	-	-	-
CID 5323714	Furazolidone	0	1	1.5	Y	0.5-4	NA			
CID 56842139	Gentamicin Sulphate	2.5	-0.5	-2.5	A	-	-	-	-	-
CID 149096	Levofloxacin	5	1	3.5	Y	0.5-4	NA	-	-	-
CID 149096	Levofloxacin	6.5	-2	-2.5	A	0.5-4	NA	-	-	-
CID 68624	Lomefloxacin HCl	0	0	3	Y/N	0.38	0.37			*
CID 60651	Marbofloxacin	7.5	-2.5	-1.5	A	-	-	-	-	-

FOSFOMYCIN HITS		sHTSS								
			Δ zones of inhibition (mm) [b]			CBA[e]		TKA[f]		
PubChem #[a]	FDA compound	Ø[c]	FOF 1/8 x MIC	FOF ¼ x MIC	Interaction[d]	FICI	FBCI	8 h	24 h	48 h
CID 54685047	Methacycline HCl	4	-3	-4	A	-	-	-	-	-
CID 54685925	Minocycline HCl	3.5	-3.5	2.5	Y/N	-	-	-	-	-
CID 441242	Moxalactam Disodium	0	0	7	Y/N	1	NA		*	*
CID 101526	Moxifloxacin HCl	5	1.5	2.5	Y	-	-	-	-	-
CID 4410	Nadifloxacin	6	-3	-2.5	A	-	-	-	-	-
CID 62115	Netilmicin Sulphate	9.5	-1.5	1.5	Y/N	1	NA	-	-	-
CID 4583	Ofloxacin	0	0	3	Y/N	-	-	-	-	-
CID 11478676	Palbociclib (PD-0332991) HCl	0	2.5	0	Y/N	-	-	-	-	-
CID 119525	Pefloxacin Mesylate	9	2	2	Y	0.75	0.625	-	-	-
CID 33630	Penfluridol	5.5	-0.5	-0.5	A	-	-	-	-	-
CID 5702105	Polymyxin B sulphate	8.75	0.25	0.75	Y	-	-	-	-	-
CID 135445761	Pralidoxime chloride	1.5	1	3	Y	0.5-4	NA			
CID 6436173	Rifaximin	0	0	2.5	Y/N	1	NA			
CID 56207	Sarafloxacin HCl	4	1.5	5.5	Y	-	-	-	-	-
CID 439243	Sisomicin sulphate	8	1	2	Y	1	NA	-	-	-
CID 6918203	Sitafloxacin Hydrate	18.5	-1.5	-3	A	-	-	-	-	-
CID 60464	Sparfloxacin	13	1	1	Y	0.625	0.625	-	-	-
CID 9892071	Tebipenem Pivoxil	1	-1	12.5	Y/N	-	-	-	-	-
CID 441334	Terbutaline Sulphate	0	2.5	0	Y/N	0.5-4	NA			
CID 36294	Tobramycin	3	-2	-2	A	-	-	-	-	-
CID 5564	Triclosan	2.5	0	4	Y/N	0.5-4	NA			
CID 5578	Trimethoprim	0	0	4.5	Y/N	1	NA			
CID 35370	Zidovudine	4.5	0.5	9.5	Y	0.5-4	NA		*	*

[a]PubChem (http://pubchem.ncbi.nlm.nih.gov/) accession number

[b]Value resulting from the subtraction of the inhibition zone diameter (Ø, mm) of drug-containing agar plates (tigecycline 0.12-0.25 mg/L, colistin 0.003-0.007 mg/L and fosfomycin 16-32 mg/L) from that of drug-free agar plates. Positive values correlate with an increase in the zone of inhibition in the presence of the Primary Compounds (tigecycline, colistin and fosfomycin), indicating possible synergistic interactions. Negative values indicate possible antagonisms

[c]Ø: Zone of inhibition of the FDA compounds by themselves against K. pneumoniae. Compounds were pin-spotted on agar plates without Primary Compounds

[d]Interaction classification was based on the increment of the inhibition zones at the two PCs sub-inhibitory concentrations tested compared to those of the no PC plates. Y, synergy (diameter at MICsub1 & diameter at MICsub2 > diameter Ø); Y/N, likely synergy (diameter at MICsub1 OR diameter at MICsub2 > diameter Ø); A, likely antagonism (diameter at MICsub1 & diameter at MICsub2 < diameterØ)

[e]An FICI or FBCI ≤0.5 indicates synergy

[f]Green indicates synergy. Yellow indicates no interaction. Synergy was defined as a ≥2 log10 reduction in CFU/mL in the combination compared to the most active compound alone. *, cidality under the limit of detection (50 CFU/mL)

[g]From DrugBank (https://go.drugbank.com/)

TGC, tigecycline, CST, colistin, FOF, fosfomycin; FICI, Fractional Inhibitory Concentration Index; FBCI, Fractional Bactericidal Concentration Index; NA, not available.

APPENDIX II. Supplementary table of time-kill assays drug interaction data against *K. pneumoniae* ATCC 13883. This table includes the raw data supporting Figure 1.8. Data display the number of residual viable colonies ($\Delta\log_{10}$ CFU/mL) between the combination and the most active agent alone and between the initial and final inoculum at the different time points (after 8, 24 and 48 hours of incubation). A negative sign denotes a reduction in the viable counts. Values in bold: synergistic (≥2 $\log_{10}$ CFU/mL reduction) and bactericidal effects (≥3 $\log_{10}$ CFU/mL reduction).

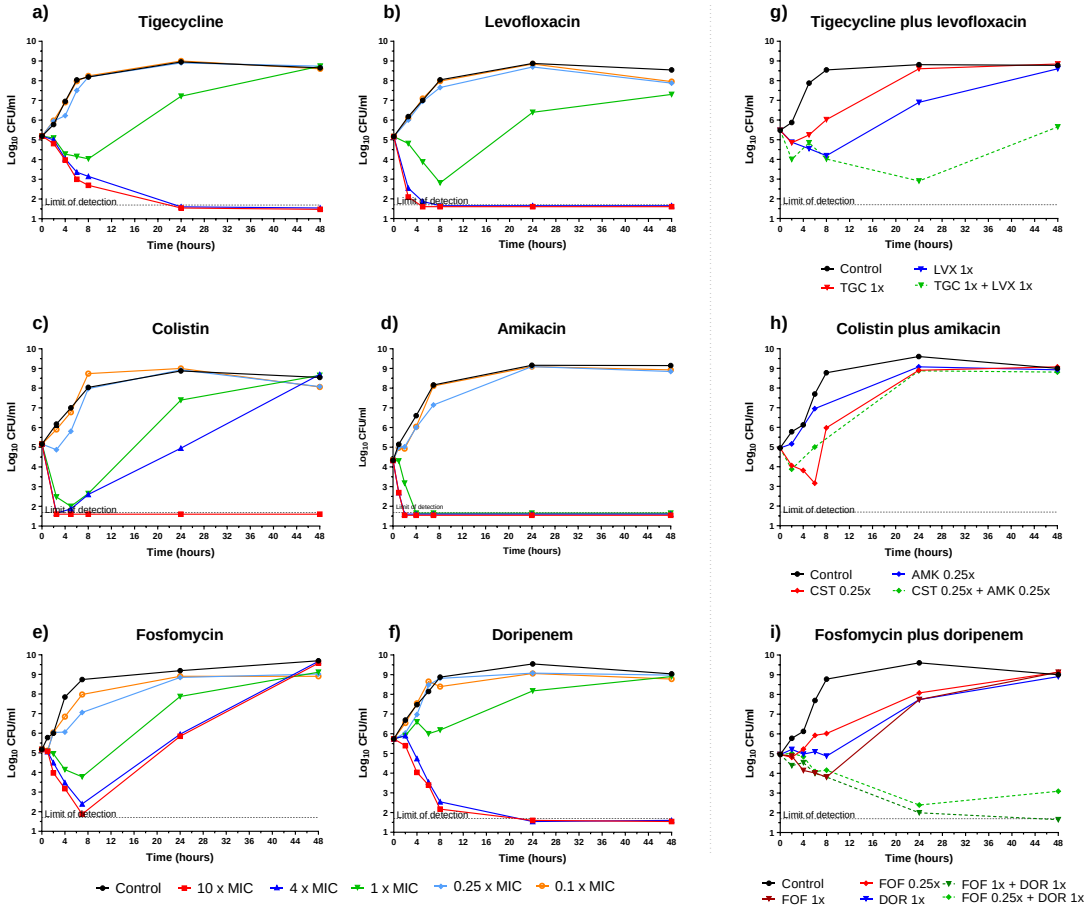
		$\Delta\log_{10}$ CFU/mL between the combination and the most active agent			**$\Delta\log_{10}$ CFU/mL between initial and final inoculum**		
Combinations	**Concentrations (mg/L)**	**8 h**	**24 h**	**48 h**	**8 h**	**24 h**	**48 h**
TGC / AZM	0.5 / 2	-0.99	**-2.50**	**-6.01**	-2.12	**-3.63**	**-3.63**
TGC / ATM	0.5 / 0.025	-0.13	-1.01	**-6.65**	-1.26	-2.15	**-3.63**
TGC / RAD	0.5 / 8	-0.08	-1.24	**-5.93**	-1.21	-2.37	-2.34
TGC / ENR	0.5 / 0.003	0.36	-0.69	-0.74	-1.35	0.93	2.26
TGC / FZD	0.5 / 0.25	2.48	-0.70	0.03	-0.82	2.43	3.40
TGC / IBN	0.5 / 8	1.13	0.71	**-4.04**	0	-0.42	-1.08
TGC / IVM	0.5 / 64	-0.07	-0.44	0	0.48	2.68	3.37
TGC / LVX	0.5 / 0.03	-0.17	**-4.00**	**-2.95**	-1.46	-2.57	0.18
TGC / MET	0.5 / 1	-0.04	-0.32	-1.74	-1.18	-1.45	2.15
TGC / PFD	0.5 / 8	1.04	0.74	**-2.00**	-0.09	-0.39	0.49
TGC / STP	0.5 / 0.5	-0.91	-0.47	-0.73	-0.36	2.65	2.40
TGC / TOB	0.5 / 0.125	-0.09	-1.19	**-7.23**	-1.23	-2.32	**-3.63**
TGC / TCS	0.5 / 0.125	-1.94	-0.43	-0.03	-1.40	2.70	3.34
CST / AMK	0.25 / 0.125	-1.98	-0.03	-0.12	-0.95	3.92	3.86
CST / AZM	0.25 / 2	**-4.29**	**-6.29**	**-5.24**	**-3.26**	**-3.26**	**-3.26**
CST / ATM	0.25 / 0.025	0.02	-0.04	0.12	1.05	3.07	3.65
CST / BLE	0.25 / 0.06	**-3.58**	**-7.21**	**-7.26**	-2.56	**-3.26**	**-3.26**
CST / CRO	0.25 / 0.03	0.14	0.10	-0.10	1.16	2.89	2.95
CST / ENR	0.25 / 0.003	**-3.98**	-1.45	-0.38	-2.95	1.44	3.21
CST / FZD	0.25 / 0.25	-0.49	**-7.09**	**-7.24**	**-3.79**	**-3.79**	**-3.79**
CST / IVM	0.25 / 64	**-2.91**	-0.64	0.07	-2.78	2.70	3.52
CST / LVX	0.25 / 0.03	**-2.50**	**-5.21**	**-6.92**	**-3.79**	**-3.79**	**-3.79**
CST / TOB	0.25 / 0.125	**-2.65**	-0.72	-0.56	-2.52	2.62	2.43
CST / TCS	0.25 / 0.125	**-3.68**	**-7.21**	**-7.39**	-2.65	**-3.26**	**-3.26**
FOF / AMK	128 / 0.125	-0.36	-0.84	0.20	-2.10	2.16	3.34
	32 / 0.125	-0.03	-1.05	1.05	0.23	2.23	4.00
FOF / AZM	128 / 2	-1.01	**-6.18**	**-4.37**	-2.74	**-4.01**	**-4.01**
	32 / 2	-1.49	**-6.18**	**-4.37**	-1.22	**-4.01**	**-4.01**
FOF / BLE	128 / 0.06	-1.79	**-7.01**	**-7.15**	**-3.52**	**-4.01**	**-4.01**
	32 / 0.06	**-3.57**	**-5.24**	-1.18	**-3.30**	-1.96	1.78
FOF / CDR	128 / 0.125	-1.82	**-5.31**	**-6.75**	-2.39	**-3.44**	-2.95
	32 / 0.125	-0.87	-0.22	-0.23	-1.20	2.52	3.57
FOF / CRO	128 / 0.03	-0.54	**-2.12**	**-6.24**	-1.11	-0.26	**-3.44**
	32 / 0.03	**-2.61**	0.37	0.63	-0.07	2.71	3.43
FOF / DOR	128 / 0.03	0	**-5.74**	**-7.21**	-1.14	-2.95	**-3.26**
	32 / 0.03	-0.71	**-5.34**	**-5.81**	-0.79	-2.56	-1.86
FOF / DOX	128 / 4	1.03	-1.71	**-3.85**	-0.11	-2.18	-0.48
	32 / 4	0.05	-0.94	-0.40	0	-1.41	2.98
	128 / 1	0.27	**-2.72**	-0.43	-0.88	0.07	3.74
	32 / 1	-0.12	-0.10	0.03	0.95	3.02	4.21

Combinations	Concentrations (mg/L)	Δlog$_{10}$ CFU/mL between the combination and the most active agent			Δlog$_{10}$ CFU/mL between initial and final inoculum		
		8 h	24 h	48 h	8 h	24 h	48 h
FOF / ENR	128 / 0.003	-1.61	-1.07	0.11	-2.18	0.80	3.93
	32 / 0.003	-1.61	-0.20	0.15	0.93	3.32	4.06
FOF / FZD	128 / 0.25	-1.56	**-4.52**	-1.11	-2.13	-2.65	2.71
	32 / 0.25	0.30	0	-0.40	1.02	3.17	3.57
FOF / LMF	128 / 0.025	**-2.27**	**-6.07**	**-7.15**	**-4.01**	**-3.22**	**-4.01**
	32 / 0.025	-0.79	-1.57	0.28	-0.52	1.28	3.23
FOF / MOX	128 / 0.125	-0.27	**-4.62**	**-6.62**	-1.41	**-3.26**	**-3.26**
	32 / 0.125	0.19	1.17	0.8	-0.86	2.52	4.16
FOF / PRA	128 / 8	-0.23	-0.10	0.30	-0.80	1.77	4.12
	32 / 8	-0.55	**-2.46**	0.43	1.98	1.89	4.41
FOF / RFX	128 / 4	0.12	**-4.90**	-1.80	-1.02	-2.11	2.26
	32 / 4	-0.24	-0.12	-0.05	0.82	3.00	4.12
FOF / BUT	128 / 8	0.39	1.00	0.24	-0.18	2.87	4.06
	32 / 8	0.21	-0.15	0.02	2.74	3.85	4.00
FOF / TCS	128 / 0.125	0.64	1.18	-0.30	0.07	3.05	3.52
	32 / 0.125	-0.51	-0.30	0.49	1.89	3.47	4.47
FOF / TMP	128 / 0.5	-0.52	-0.43	-1.93	-1.09	1.44	1.89
	32 / 0.5	-0.55	-1.22	-1.00	1.98	2.39	2.89
FOF / ZDV	128 / 0.015	**-2.02**	**-5.31**	**-7.26**	-2.59	**-3.44**	**-3.44**
	32 / 0.015	0	-0.53	-0.87	-0.02	2.35	3.11

AMK, amikacin; ATM, aztreonam; AZM, azithromycin, BLE, bleomycin; BUT, tertbutaline; CDR, cefdinir; CRO, ceftriaxone; CST, colistin; DOX, doxycycline; DOR, doripenem; ENR, enrofloxacin; FOF, fosfomycin; FZD, furazolidone; IBN, ibandronate; IVM, ivermectin; LVX, levofloxacin; LMF, lomefloxacin; MET, methacycline; MOX, moxalactam; PFD, penfluridol; PRA, pralidoxime; RAD, cephradine; RFX, rifaximin; STP, streptomycin; TCS, triclosan; TGC, tigecycline; TMP, trimethoprim; TOB, tobramycin; ZDV, zidovudine.

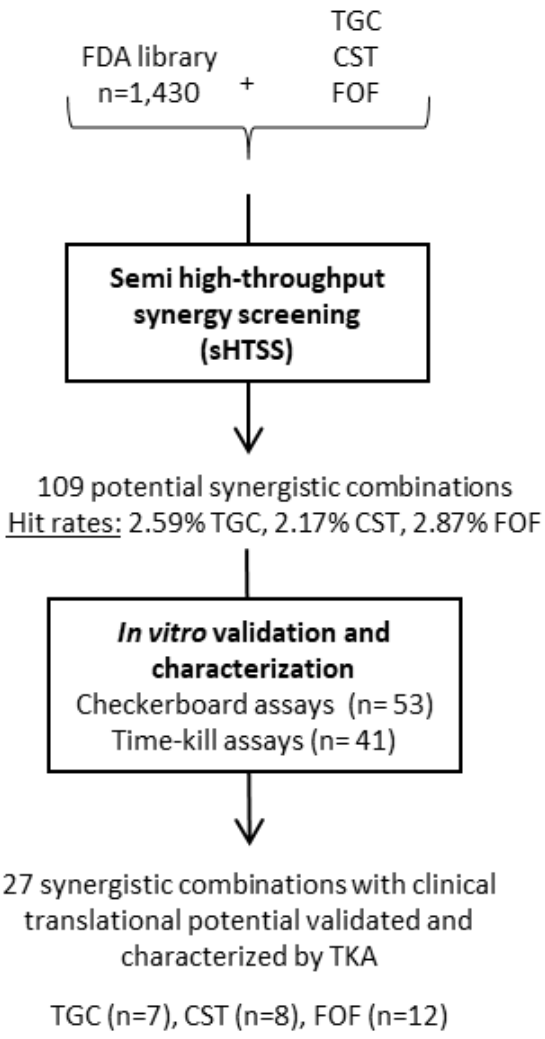
APPENDIX III. Representative time kill assays of drugs alone and in combination against *K. pneumoniae* ATCC 13883.

(a-f) Dose response curves (0.1x, 0.25x, 1x, 4x and 10x MIC) of compounds alone; **(g-i)** Combination time-kill curves were performed at pairing static and/or sub-inhibitory concentrations (0.25x and/or 1x MIC values); **(g)** Synergistic interaction with killing activity up to 24 hours followed by a bacterial growth rebound; **(h)** No interaction pattern; **(i)** Synergistic interaction with bactericidal activity at both 24 and 48 hours. Synergy was interpreted as a ≥2 $\log_{10}$ CFU/mL decrease in bacterial count compared to the most active single agent in the combination at 8, 24 and 48 hours. Bactericidal activity was interpreted as a ≥3 $\log_{10}$ CFU/mL reduction at 8, 24 and 48 hours compared to the initial inoculum. An untreated growth control was always included. MIC_{TGC}= 0.5 mg/L; MIC_{LVX}= 0.03 mg/L; MIC_{CST}= 1 mg/L; MIC_{AMK}= 1 mg/L; MIC_{FOF}= 128 mg/L; MIC_{DOR}= 0.03 mg/L. TGC, tigecycline; LVX, levofloxacin; CST, colistin; AMK, amikacin; FOF, fosfomycin; DOR, doripenem.



APPENDIX IV. Progression cascade of the screening and validation activities performed in Chapter 1.

The number of compounds tested, hit rates and hits validated are indicated at every step. TGC, tigecycline; CST, colistin; FOF, fosfomycin; TKA, time-kill assay.



APPENDIX V. Chapter 1. results summary table.

	Primary Compound	Tigecycline			Colistin			Fosfomycin			Grand total (three PCs)		
		S	A	Total	S	A	Total	S	A	Total	S	A	Total
	sHTSS[a]	37[*]	6	43	31[*]	10	41	41[*]	12	53	**109**	**28**	137
Secondary validation	CBA assayed	14	2	16	11	1	12	25	0	25	50	3	53
	CBA validated[b]	0	0	0	6 + 1[#]	-	7	1	0	1	8	0	8
	TKA assayed	10	3	13	8	3	11	17	0	17	35	6	41
	TKA validated[c]	7	0	7	5 + 3[#]	-	8	12	0	12	27	0	27
Novelty	Already published combinations	3	-	-	3	-	-	2	-	-	8	-	-
	Novel combinations (non-antibiotics)	2	-	-	2	-	-	3	-	-	7	-	-
	Novel combinations (other)	2	-	-	3	-	-	7	-	-	12	-	-

[a]Interaction classification was based on the increment of the inhibition zones at the two PCs sub-inhibitory concentrations tested compared to those of the no PC plates as described in Materials & Methods. Raw data are displayed, including compounds with different chemical forms (i.e., amikacin hydrate / amikacin disulfate)

[b]FICI or FBCI ≤0.5 indicates synergy

[c]Synergy was defined as a ≥2 $\log_{10}$ reduction in CFU/mL in the combination compared to the most active compound alone

[*]Synergistic interactions included those classified as synergy (Y) and likely synergy (Y/N)

[#]Interaction validated as synergy by CBA or TKA although sHTSS initially identified antagonistic interaction

S: synergy; A; antagonism; sHTSS, semi-high throughput synergy screening; CBA, checkerboard assays; TKA, time-kill assays

Zidovudine multi-combos with last-line fosfomycin, ceftazidime-avibactam, colistin and tigecycline against Multi-Drug Resistant *Klebsiella pneumoniae*

CHAPTER 2

"Me enseñaron que el camino del progreso no es ni rápido ni fácil"

▪ ***Marie Curie*** *(1867–1934)*

INTRODUCTION

Zidovudine (3'-azido-3'-deoxythymidine), a thymidine analogue **(Figure 2.1)**, was the first commercial antiretroviral agent for HIV/AIDS treatment, currently used to prevent maternal-fetal transmission (150). It belongs to the drug class called nucleoside reverse transcriptase inhibitors, targeting HIV reverse transcriptase with high affinity (100-fold greater than for human DNA polymerase) which result in the inhibition of viral replication (140). Its mode of action requires a triple phosphorylation by the thymidine kinase, the thymidylate kinase and the nucleoside diphosphate kinase of the HIV-infected cell respectively (139).

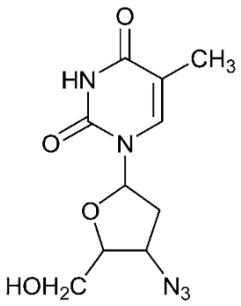


Figure 2.1. Structure of zidovudine. From (150)

The antibacterial activity of zidovudine against Gram-negative bacteria is known since late 1980s, by findings in HIV patients treated with zidovudine that experienced a reduction in *Salmonella* spp. recurrences (140). Although the entire mechanism of action remains unknow, antibacterial activity is mainly due to the inhibition of bacterial DNA replication by targeting thymidine kinase, the enzyme responsible for the zidovudine activation into its phosphorylated form (139). In Gram-positives, zidovudine is ineffective due to the low affinity of this enzyme (140). Antibacterial activity against Gram-negatives is demonstrated also *in vivo* (144,151), suggesting this molecule might have a potential clinical use for the treatment of bacterial infections. However, toxicity and the rapid emergence of resistant strains are the main drawbacks limiting the development of zidovudine-based antibacterial therapies. With the aim to counteract this, several *in vitro* studies have explored the efficacy of zidovudine in combination with known antibiotics, such as fluoroquinolones, tigecycline, colistin, trimethoprim and more recently, fosfomycin, concluding that zidovudine might a good partner to eradicate enterobacteria (114,141–145,152,153).

For HIV treatment, zidovudine is dosed at 500-600 mg daily oral and 1.5 mg/kg/6h intravenously. After standard dosage regimens, C_{max} ranging from 1.1 to 1.8 mg/L are achieved

(154,155). Previous i*n vitro* and *in vivo* studies suggested that clinically achievable zidovudine concentrations could be effective against MDR enterobacteria when in combination therapy (144,145,152). Zidovudine toxicity is associated to the dose, disease stage and prolonged HIV-therapy. Safety profiles observed in HIV-patients together with a short plasma half-life (1.1–2.3 hours) (154–156) suggest that appearance of zidovudine toxicities are unlikely. Most reported side effects include headaches, myalgia, nausea and vomiting. Major toxicities (anaemia and neutropenia) are more frequently described at high doses (1.200-1.500 mg/day) after more than 4 weeks of treatment (157,158). A few case reports described oral zidovudine overdoses up to 36 g/daily without abnormalities or with slight and transient side-effects such as lethargy (159).

In Chapter 1, the FDA-library was screened, and zidovudine was identified as a potent synergistic partner of fosfomycin against *K. pneumoniae*. Here, in Chapter 2, this previous finding is expanded to explore the antibacterial activity of zidovudine against a panel of twelve MDR/XDR *K. pneumoniae* isolates with different resistance profile (see **Table 3** of Materials & Methods). For this purpose, we followed a stepwise approach: first, the activity of test compound was determined by drug susceptibility assays; second, pairwise and triple combinations were tested by CBA and TKA to identify those most promising synergistic combinations. Results show high synergy rates with zidovudine combinations, suggesting it might be an effective partner to eradicate MDR *K. pneumoniae.*

RESULTS

Drug susceptibility characterization.

Zidovudine MIC values ranged from 0.25 to ≥64 mg/L, with nine strains showing low MIC_{ZDV} values (0.25 to 2 mg/L) and three other strains showing MIC values ≥16 mg/L. In consequence, as zidovudine is antiviral and therefore no clinical breakpoints do exist for *K. pneumoniae,* 2 mg/L could be a possible cut-off value to stablish a susceptibility breakpoint. The number of multidrug-resistant determinants appeared not to be related with the MIC_{ZDV} values. The susceptibility profiles obtained for the drugs tested among the twelve MDR/XDR *K. pneumoniae* isolates according to EUCAST breakpoints (160) are also shown **Table 2.1**.

Table 2.1. Strain characterization of *K. pneumoniae* isolates and susceptibility profile to drugs used in this study. Clinical categorization according to current EUCAST breakpoints (161) are displayed in brackets.

				MIC (mg/L)						
Isolate	**Resistance mechanism**	**Source**	**MDR/XDR**	**CST**	**FOF**	**TGC**	**ETP**	**MEM**	**CAZ-AVI**	**ZDV**
E-1	CTX-M 14	Rectal swab	XDR	0.5 (S)	>64 (R)	4	>32 (R)	8 (I)	1 (S)	0.25-0.5
E-2	CTX-M 15	Blood	MDR	0.5 (S)	>64 (R)	0.5	64 (R)	4-8 (I)	1 (S)	0.5-1
E-3	CTX-M 15	Abscess	MDR	1-2 (S)	>64 (R)	4	16 (R)	2-4 (I)	1 (S)	2
E-4	CTX-M 15	Blood	MDR	0.5 (S)	>64 (R)	4	1 (R)	0.03 (S)	0.5 (S)	1
E-5	SHV-1 + porin loss	Blood	MDR	0.5 (S)	8 (S)	0.5-1	0.25 (S)	0.03 (S)	0.06-0.12 (S)	0.5-1
A-6	AmpC ACT-1	SEIMC CCS07	MDR	≤0.5 (S)	>64 (R)	1-2	4-8 (R)	0.5 (S)	0.5 (S)	8-16
C-7	OXA-48	Blood	MDR	1 (S)	>64 (R)	2	8-16 (R)	4 (I)	0.5 (S)	2
CS-8	Colistin R	Urine	MDR	16 (R)	>64 (R)	1	0.5 (S)	0.5-1 (S)	0.5 (S)	0.5
CSE-9	VIM-1 + CTX-M 15 + colistin R	SEIMC CCS04	XDR	16 (R)	>64(R)	1-2	8-16 (R)	16-32 (R)	>64 (R)	≥64
CE-10	CTX-M 15 + OXA-48	Blood	MDR	1-2 (S)	>64 (R)	1-2	8 (R)	4 (I)	0.25 (S)	64
CEE-11	KPC-3 + SHV-11 + TEM-1	SEIMC CCS05	XDR	2 (S)	>64 (R)	4	>64 (R)	>64 (R)	4 (S)	0.5-1
CSEE-12	OXA-1 + SHV-1 + colistin R	EARS QC	MDR	4 (R)	64 (R)	1	8-16 (R)	1-2 (S)	0.5 (S)	1

CST, colistin; FOF, fosfomycin; TGC, tigecycline; ETP, ertapenem; MEM, meropenem; CAZ-AVI, ceftazidime-avibactam; ZDV, zidovudine; EARS QC, European Antimicrobial Resistance Surveillance Quality Control; R, resistant; S, susceptible; S*: susceptible, increased exposure; SEIMC: Spanish Society of Infectious Diseases and Clinical Microbiology

[1]EUCAST clinical breakpoints for tigecycline are only applied to *Escherichia coli* and *Citrobacter koseri*

Zidovudine/fosfomycin combinations displayed wide coverage synergy against MDR/XDR *K. pneumoniae* isolates by CBA.

The CBA was used to evaluate the degree of interaction between fosfomycin and zidovudine. Synergy was confirmed in 75% (9/12) and 66.6% (8/12) of the strains by FICI and FBCI, respectively. Interestingly, effective fosfomycin concentrations in the presence of zidovudine were reduced 2-16-fold, restoring fosfomycin susceptibility below the EUCAST breakpoint (MIC ≤32 mg/L) in all strains. Antimicrobial zidovudine concentrations were also significantly lower in presence of fosfomycin, with reductions ranging from 2- to 128-fold, and

≥4-fold reduction in 75% (9/12) and 90.9% (10/11) of the strains by FICI and FBCI, respectively. No antagonisms were observed **(Table 2.2.).**

Table 2.2. CBA in pairwise interactions of fosfomycin plus zidovudine against *K. pneumoniae* isolates.

	MIC (mg/L) alone		MIC (mg/L) in combination		MBC (mg/L) alone		MBC (mg/L) in combination			
Isolate	**FOF**	**ZDV**	**FOF**	**ZDV**	**FOF**	**ZDV**	**FOF**	**ZDV**	**FICI**	**FBCI**
E-1	>64	0.5	16	0.0625	>64	2	32	0.5	**0.38**	0.75
E-2	>64	>4	8	1	>64	>4	8	1	**0.19**	**0.19**
E-3	>64	2	32	1	>64	2	32	1	0.75	0.75
E-4	64	1	16	0.25	64	>2	16	0.5	**0.50**	**0.38**
E-5	>16	2	4	0.25	>16	>2	4	0.25	**0.25**	**0.19**
A-6	>64	16	8	0.5	>64	16	8	1	**0.09**	**0.13**
C-7	>64	1	16	0.125	>64	2	16	0.125	**0.25**	**0.19**
CS-8	64	4	16	1	64	4	32	2	**0.5**	0.75
CSE-9	>64	≥64	16	1	>64	≥64	16	1	**0.13**	**0.13**
CE-10	>64	64	8	0.5	>64	>64	8	2	**0.07**	**0.08**
CEE-11	>64	1	16	0.5	>64	>4	16	2	0.63	**0.38**
CSEE-12	64	8	16	4	128	8	64	0.25	1	0.53

FICI, Fractional Inhibitory Concentration Index; FBCI, Fractional Bactericidal Concentration Index. Values in bold indicate synergy (FICI, FBCI ≤0.5), while values >0.5 indicate no interaction. FOF, fosfomycin; ZDV, zidovudine.

Zidovudine-based combinations are more potent *in vitro* than current clinically used combinations against MDR/XDR *K. pneumoniae* isolates.

The use of CBA allows screening of a number of antibiotic combinations in search of synergy, although it is limited to single-time point read-outs. In order to give robustness to the interaction analysis of zidovudine-based combinations, we performed TKA that provide more detailed synergistic information, including bactericidal and sterilizing activities of the combinations over time **(Figure 2.2.).** TKA for drugs alone and in combination were performed as described in Materials & Methods, at different time points (0, 2, 5, 8, 24 and 48 hours). Zidovudine combinations were also tested at concentrations of 1 mg/L for the twelve isolates, including those showing high-level zidovudine resistance, to assess physiological relevant concentrations, i.e. C_{max} of zidovudine observed in human plasma after intravenously administration (1.1 to 1.8 mg/L) (154). The activity of four novel zidovudine-based combinations (fosfomycin/zidovudine, ceftazidime-avibactam/zidovudine, colistin/zidovudine and tigecycline/zidovudine) was compared with that of four usual MDR-treatments (meropenem/ertapenem, meropenem/colistin, fosfomycin/colistin and fosfomycin/tigecycline). The triple fosfomycin/colistin/zidovudine combination was also tested

against eight strains and compared with the activity of the three drugs alone and in pairwise combinations at matching concentration (0.25-1x MIC).

At any time point (8, 24 and 48 hours), synergy rates among currently used combinations for MDR treatment was observed in several isolates: 41.6% for meropenem/ertapenem (5/12), 33.33% for meropenem/colistin (4/12), 75% for fosfomycin/colistin (9/12) and 66.6% for fosfomycin/tigecycline (8/12) **(Appendix I).** Interestingly, among all isolates, the highest number of synergistic interactions were obtained with zidovudine-based combinations, at zidovudine concentrations ranging from 0.004x to 2x MIC **(Appendix II).** The combination of ceftazidime-avibactam plus zidovudine was the most active, showing a synergy rate among the isolates of 83.3% (10/12). In 8 out of 12 strains the killing activity was below the limit of detection after 24 hours of incubation, preventing bacterial regrowth (a proxy for sterilizing activity**) (Appendix II, a)**. The combination of fosfomycin plus zidovudine was synergistic in 9 out of 12 strains, showing a potent and rapid initial decrease in bacterial counts after 8 hours in five strains (E-5, A-6, C-7, CSE-9, CE-10) and bactericidal activity down to the limit of detection in six strains (E-5, A-6, C-7, CSE-9, CEE-11 and CS-8) **(Appendix II, b).** The combination of zidovudine plus colistin had 75% (9/12) synergy rates and 33.3% (4/12) killing rates down to the limit of detection **(Appendix II, c).** Finally, the combination of zidovudine plus tigecycline was the less potent one showing late synergy (48 hours) in 66.6% (8/12) of the strains with bactericidal activity only against C-7 **(Appendix II, d).**

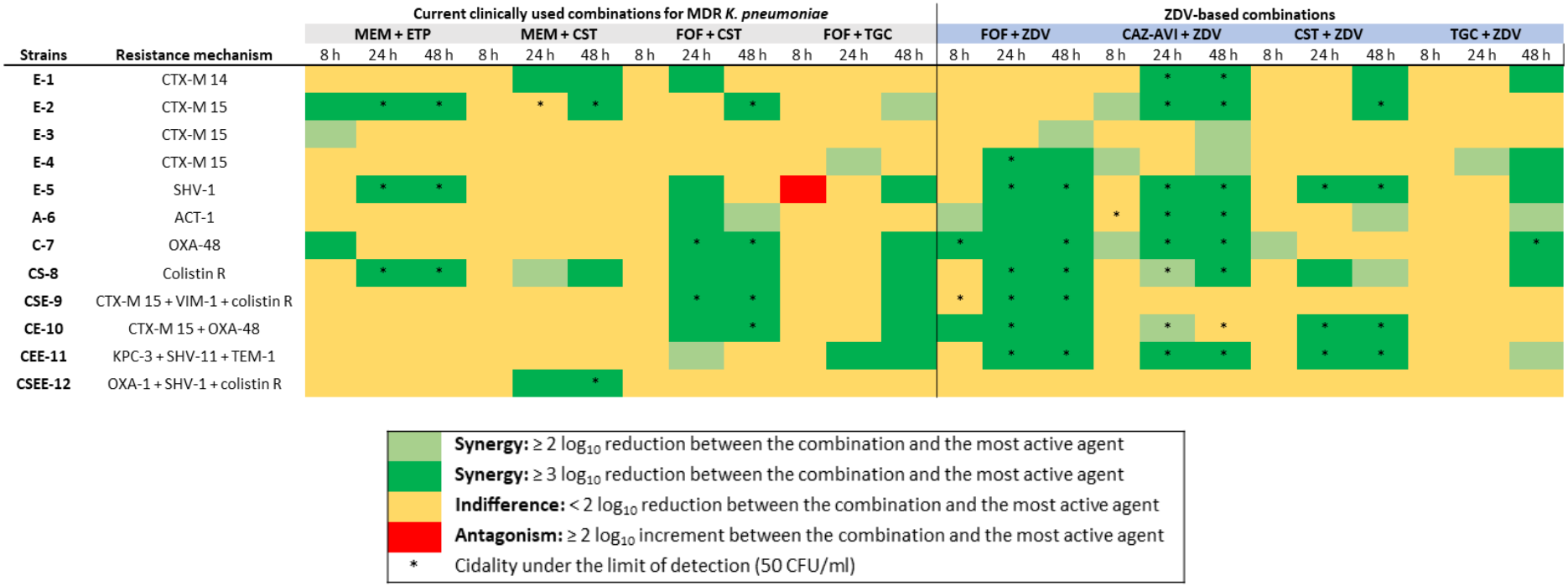


Figure 2.2. Heat map representation of synergy and bactericidal activities in pairwise combinations at different time points obtained by time-kill assays against *K. pneumoniae* isolates. When several concentrations were tested for the same drug, the most favourable outcome is displayed. ZDV-based combinations were tested at concentrations ≤1 mg/L, reflecting physiologically achieved concentrations. CAZ-AVI; ceftazidime-avibactam; CST, colistin; ETP, ertapenem; FOF, fosfomycin; MEM, meropenem; TGC, tigecycline; ZDV, zidovudine.

Notably, the synergistic killing effects of zidovudine-based combinations were observed even at low zidovudine concentrations (≤1 mg/L), which are below pharmacological serum concentrations at standard zidovudine oral doses. This effect was observed regardless the zidovudine susceptibility profile of the strains (A-6, CSE-9 and CE-10 are zidovudine-resistant) (**Figure 2.3).**

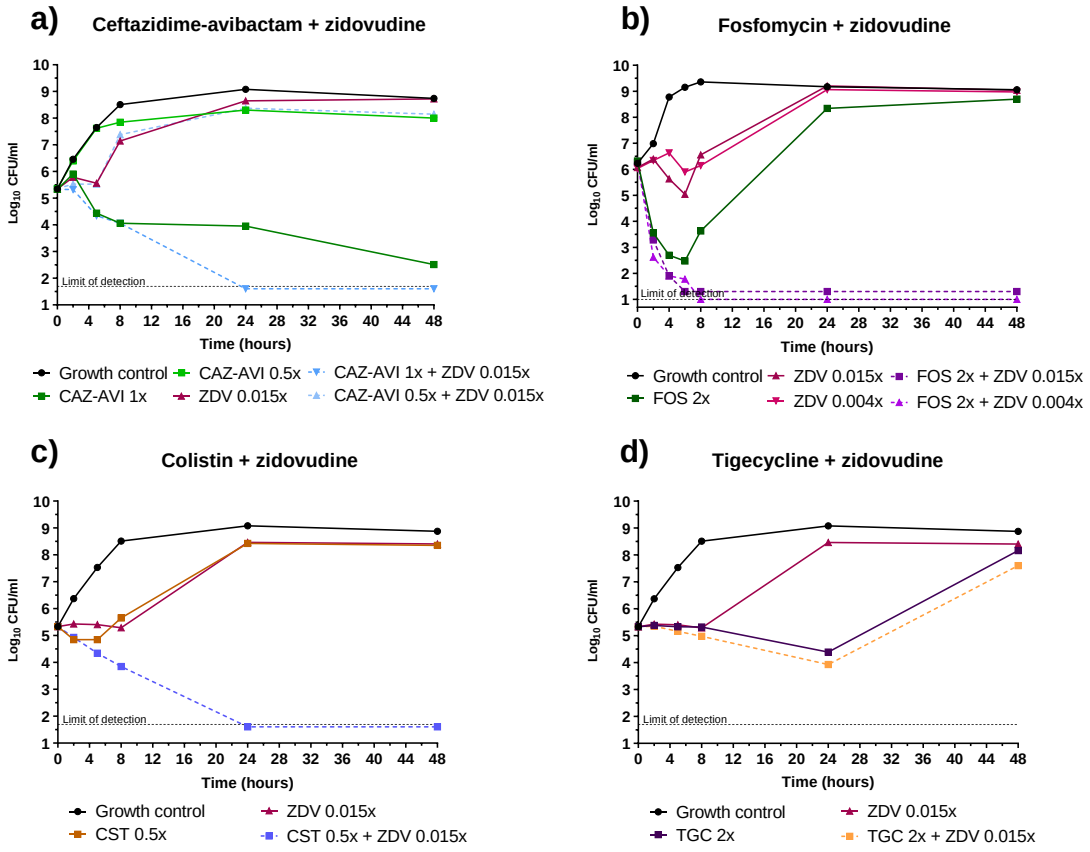


Figure 2.3. Time–kill assays characterization of zidovudine combinations with last-line antibiotics in the treatment of MDR-*K. pneumoniae* infections. The zidovudine resistant clinical strain CE-10 (*bla*$_{OXA-48}$ + *bla*$_{CTX-M15}$) was used for these studies. Zidovudine enhanced the activities of ceftazidime-avibactam, fosfomycin and colistin even at low sub-inhibitory concentrations (0.004-0.015x MIC), showing potent synergistic and bactericidal effects with last-line antibiotics. ZDV concentrations were 1 mg/L, which is below C_{max} values (1.1 to 1.8 mg/L) achieve in human plasma after a recommended ZDV oral dose. $MIC_{CAZ-AVI}$= 0,25 mg/L, MIC_{CST}= 1 mg/L, MIC_{FOF}= 64 mg/L, MIC_{TGC}= 1 mg/L, MIC_{ZDV}= 64 mg/L.

Triple zidovudine-based combination offers limited advantages over already synergistic pairwise combinations.

We identified that while carbapenem-based combinations had little synergistic interaction profiles, the combination of two last-line antibiotics (fosfomycin/colistin) had an overall synergy rate of 75%. Interestingly, zidovudine displayed strong synergy with both drugs.

We thus aimed to characterize whether the addition of zidovudine to the fosfomycin/colistin combination could further potentiate the synergistic interaction, as previously described (108,115,162). The triple combination was highly active showing bactericidal activity to the limit of detection in 4 out of 8 strains tested. However, compared to the fosfomycin/zidovudine, colistin/zidovudine and fosfomycin/colistin combinations, fosfomycin/colistin/zidovudine was more effective at bacterial eradication than colistin/zidovudine, but added little efficacy when compared to fosfomycin/zidovudine or fosfomycin/colistin (**Figures 2.4** and **2.5)**.

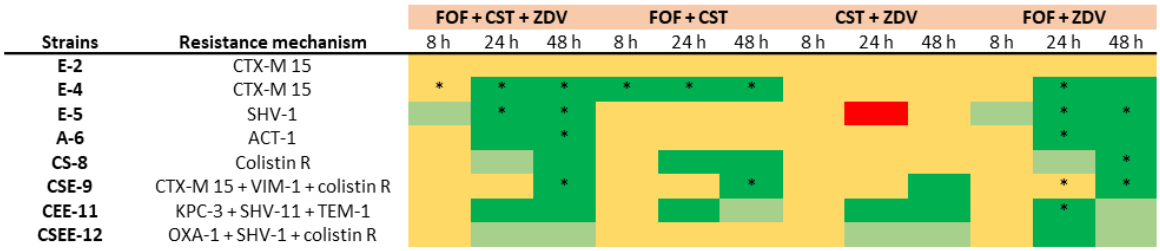


Figure 2.4. Heat map representation of synergy and bactericidal activities in triple combination (fosfomycin/colistin/zidovudine) compared to those in pairwise combinations at different time points obtained by time-kill assays against eight *K. pneumoniae* isolates. Combo tested concentrations at 0.25-1x MIC. Data supporting this summary figure is displayed in Figure 4. CST, colistin; FOF, fosfomycin; ZDV, zidovudine**.**

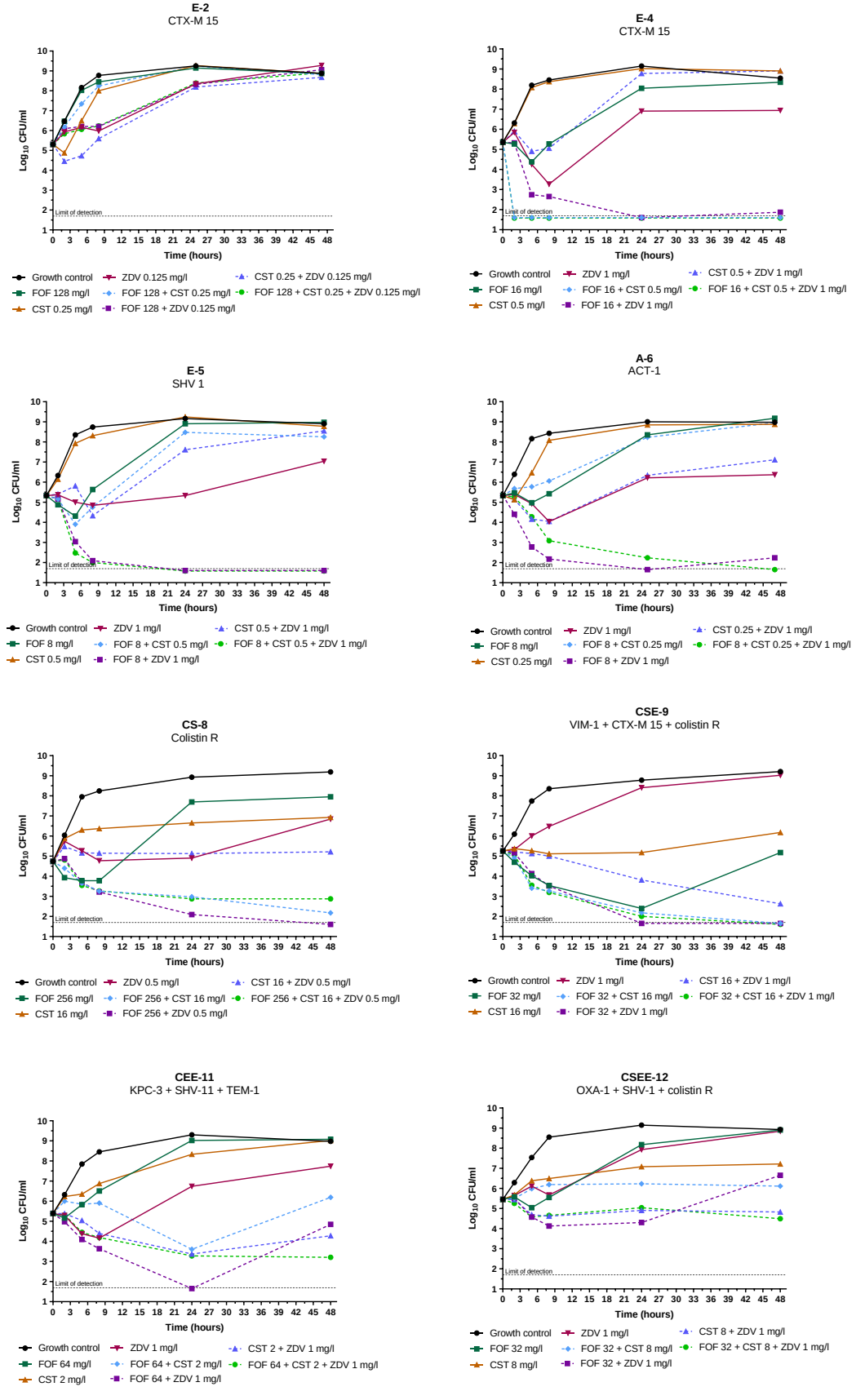


Figure 2.5. Time-kill curves of zidovudine, colistin and fosfomycin alone, pairwise and triple combination against eight *K. pneumoniae* isolates.

Zidovudine combo concentrations are not cytotoxic

We performed MTT cytotoxicity assays in a Hep G2 cell line to evaluate drugs alone and in combination at those concentrations that showed bactericidal effect by TKA. For most zidovudine-based combinations, MTT cytotoxicity assays revealed >70% cell viability, which is considered non-cytotoxic according to standard international protocols (110). Zidovudine was cytotoxic at the highest concentration tested (64 mg/L) (<40% cell viability), but not at any of the lowest concentrations used in the combinations (0.25 to 4 mg/L). This maximum concentration significantly decreased compared to lower concentrations ($p < 0.0001$, **Figure 2.6.**, zidovudine plus fosfomycin). Importantly, no combination was cytotoxic compared to the activity of the drugs alone. In fact, the combination might be even beneficial since toxicity was reduced in the case of zidovudine plus fosfomycin, i.e., the concentration of zidovudine at 64 mg/L in combination with fosfomycin at either 32 or 128 mg/L was less cytotoxic than zidovudine alone **(Figure 2.6.)**, with statistical differences ($p < 0.001$)**.**

Of the last-line antibiotics, tigecycline was cytotoxic at 4 mg/L (40% cell viability), but not at 2 mg/L (>90% cell viability), which is near to the mean C_{max} described after standard intravenous dose of 100 mg of tigecycline (1.45 mg/L)(163). Statistical differences were observed between both concentrations ($p < 0.0001$), also found in combination with zidovudine **(Figure 2.6.).** Comparing the combinations with tigecycline at 4 mg/L, there was no statistical difference ($p = 0.64$ combined with zidovudine 1 mg/L, $p = 0.38$ with zidovudine 2 mg/L). Thus, the toxic effect of the combination when tigecycline was at 4 mg/L was attributed to the same effect for the drug alone. By contrary, the combination of zidovudine when tigecycline was present at 2 mg/L significantly decreased ($p < 0.01$) compared to drugs alone, although the combined effect was not considered cytotoxic (mean value 71.8% of cell viability).

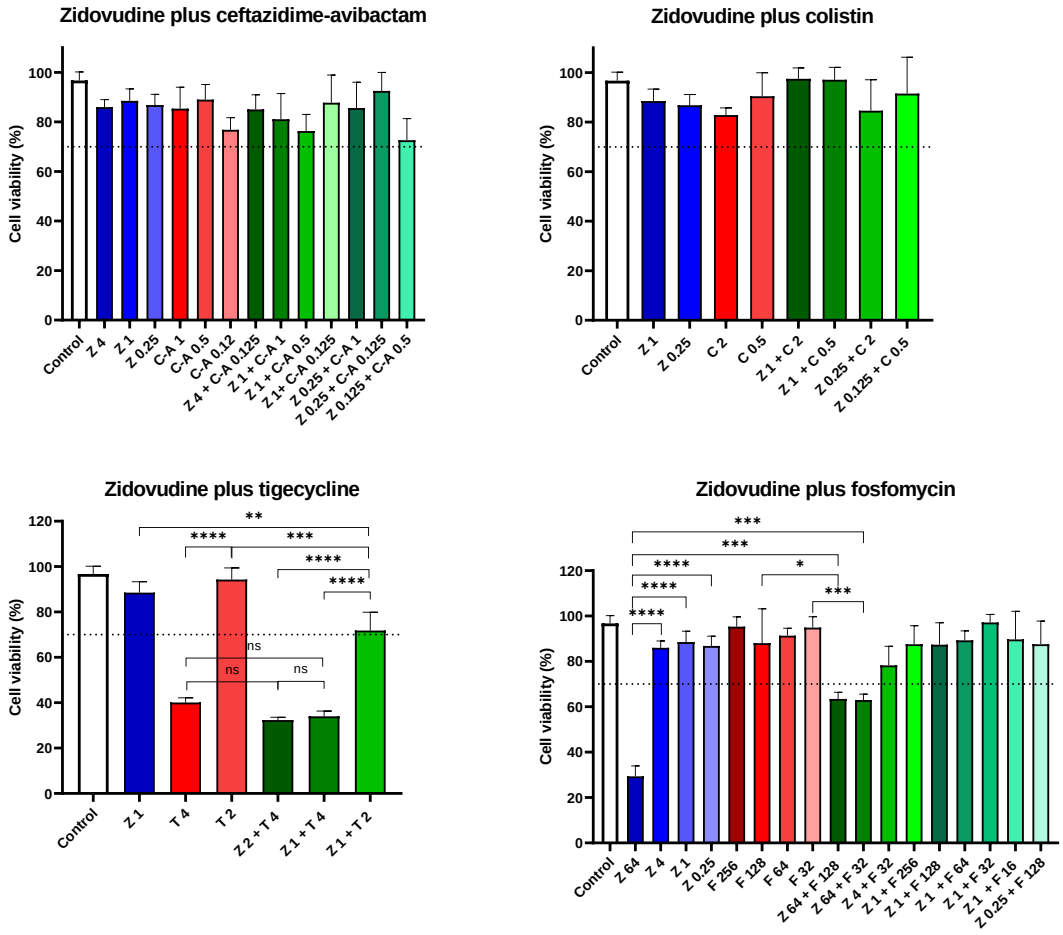


Figure 2.6. MTT cytotoxicity assays of novel combinations in Hep G2 cell line. Concentration of compounds are expressed in mg/L. Cell suspensions with no compounds added were used as positive control. Statistical differences are displayed by asterisks; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$; ns, $p > 0.05$; C-A, ceftazidime-avibactam; C, colistin; F, fosfomycin; T, tigecycline; Z, zidovudine.

DISCUSSION

We evaluated the *in vitro* efficacy of zidovudine in combination with antibiotics currently used for the treatment of infections caused by MDR/XDR *K. pneumoniae* with common resistance patterns, such as ceftazidime-avibactam, colistin, fosfomycin and tigecycline. For this, we used a panel of 12 MDR/XDR *K. pneumoniae* isolates and tested zidovudine at clinically achievable concentrations. Our TKA data showed high rates of synergistic and killing activities of zidovudine-based combinations even against strains with concurrent resistance mechanisms to these and other antimicrobials, suggesting a potential role of zidovudine in combinatorial therapy (**Figure 2.2, appendix I** and **appendix II**).

We performed extensive TKA and obtained readouts after up to 48 hours of incubation. We first characterized the activity of the compounds alone in a dose-response manner against all isolates. Then, we selected subinhibitory/static concentrations that were matched for the combination assays to allow for a wider dynamic range; using higher/effective concentrations of the compounds alone would mask the effect of any potential interaction. Our efficacy analysis then takes into consideration, not only the increased bactericidal activity of the combination compared to the drugs alone, but also the ability of the combination to completely eradicate bacteria down to the limit of detection of the assay (a proxy for sterilization of the culture). Based on these criteria, we tested several zidovudine-based combinations and compared them with current combinations clinically used to treat MDR/XDR *K. pneumoniae* infections (**Figure 2.2**).

(i) *Zidovudine plus ceftazidime-avibactam*. This combination showed the highest efficacy with potent killing activity in all except two strains (CSE-9 and CSEE-12) even at low concentrations of ceftazidime-avibactam (ranging from 0.125x to 1xMIC) (**Figures 2.2. and appendix II, a**). CSE-9 displayed high MIC values to ceftazidime-avibactam (>64 mg/l) and zidovudine (≥64 mg/L), while for CSEE-12 these were low ($MIC_{CAZ\text{-}AVI}$= 0.5 mg/L, MIC_{ZDV}= 1 mg/L); further studies are needed to elucidate whether acquired resistance mechanisms might explain this lack of activity.

Safety and efficacy of ceftazidime-avibactam against MDR enterobacteria facilitated its inclusion as first-line therapeutic option for infections caused by CPE. It is administered in monotherapy against OXA-48 (class D) and KPC (class A) producers or associated to aztreonam against class B enzymes (β-lactamases refractory to inhibition by avibactam) (45). Although the potential for resistance selection appears to be low (57), the extensive use of ceftazidime-avibactam as a savage therapy will contribute to the emergence of resistance. In fact, resistance

linked to mutations in plasmid-borne KPC-3 were reported during ceftazidime-avibactam treatment (60,61), and development of resistance to ceftazidime-avibactam is more likely after previous exposure with meropenem-vaborbactam (58,59).

From my view, this is the first time that the zidovudine/ceftazidime-avibactam combination is evaluated against MDR *K. pneumoniae.* These promising results might lay the foundations of further studies to support a potential clinical implementation.

(ii) Zidovudine plus fosfomycin. This interaction was identified in a previous screening (*unpublished*). These data were in agreement with Antonello *et al.* describing such synergy by CBA in 69.4% of Enterobacteriaceae strains, including fosfomycin-resistant strains, and characterized its killing effects by TKA at 24 hours (145). In this work, we further validate this synergism (**Figure 2.2.**); fosfomycin alone showed a fast bactericidal activity followed by a sharp growth rebound and, although strains in the collection displayed high MIC_{FOF} values (from 8 to ≥64 mg/L), the combination with zidovudine was able to restore fosfomycin activity, preventing this bacterial regrowth (**appendix II, b**).

(iii) Zidovudine plus colistin. Our interaction data is also in agreement with previously reported (141,144), including *K. pneumoniae* colistin-resistant strains (152). However, TKA against our colistin-resistant strains revealed both synergy against CS-8 (MIC_{CST}= 16 mg/L) (but a lack of bactericidal activity) and lack of interaction against CSE-9 and CSEE-12 (MIC_{CST}= 16 and 4 mg/L, respectively) with concurrent alternative resistance mechanisms (**appendix II, c**).

(iv) Zidovudine plus tigecycline. This was the least effective combination (similar to fosfomycin/tigecycline) with just late synergy and bacteriostatic profile at 48 hours. The activities were also tigecycline dose-dependent for most strains (**appendix I, d** and **appendix II, d**). Nevertheless, TKA were able to revealed important interactions confronting data generated by CBA (143). The fact that zidovudine/tigecycline showed a lower degree of interaction could be explained because both drugs have to reach their intracellular targets; however, in other combos, when zidovudine is used along with extracellular targeting compounds such as fosfomycin, ceftazidime-avibactam and colistin, the action of the latter drugs could result in an increased permeability of zidovudine, hence resulting in a higher effectivity (48,141,144,152). Adding to this hypothesis, the use of G6P (added in *in vitro* experiments to mimic physiological conditions and promote intracellular transport of fosfomycin) might also facilitate zidovudine entrance to the bacterial cell (145).

(v) Zidovudine plus fosfomycin/colistin. The synergism between two last-line antibiotics fosfomycin/colistin against MDR *K. pneumoniae* was previously reported by TKA and hollow-

fiber infection model (164,165), and clinical studies (166,167). Such a positive interaction was also identified between fosfomycin/colistin, two drugs that displayed independently potent activities in combination with zidovudine (**Figure 2.2.**). Given that our studies demonstrated that zidovudine-based combinations are more potent than currently clinically used for the treatment of infections caused by MDR/XDR *K. pneumoniae* strains, we performed TKA to characterize the potential impact of zidovudine in a triple combination with fosfomycin and colistin. In mycobacteria, triple combinations perform better than the sum of the pairwise combinations (108,115,162); however, this was not the case and the triple combination added little value to fosfomycin/colistin (**Figure 2.5.**).

In our study, MICs of zidovudine ranged from 0.25 to ≥64 mg/L, which are in accordance with similar studies (141,144,145,152). We observed potent bactericidal activities of the combinations against most strains at zidovudine concentrations below 1 mg/L with the exception of A-6 strain for which the effective zidovudine concentration in combinations was 4 mg/ml (**appendix II**), which still would be below C_{max} expected at daily doses of 600 mg (145). Pharmacokinetics and safety of zidovudine plus colistin combination antimicrobial therapy was evaluated in a clinical trial (168,169). It was found that doses of both synergistic partners, zidovudine and colistin (which has toxicity issues), could be reduced while retaining their therapeutic efficacy (144). Our data thus suggests that current zidovudine dosing strategies might suffice to treat bacterial infections in humans and that zidovudine associated side effects are unlikely to occur during short-term regimens, as in the context of acute bacterial infections. In addition, zidovudine reduces transmission of ESBL and carbapenemase containing plasmids, hence supporting zidovudine use in the prevention of the spread of resistant enterobacteria (170).

CONCLUSIONS AND FUTURE PERSPECTIVES

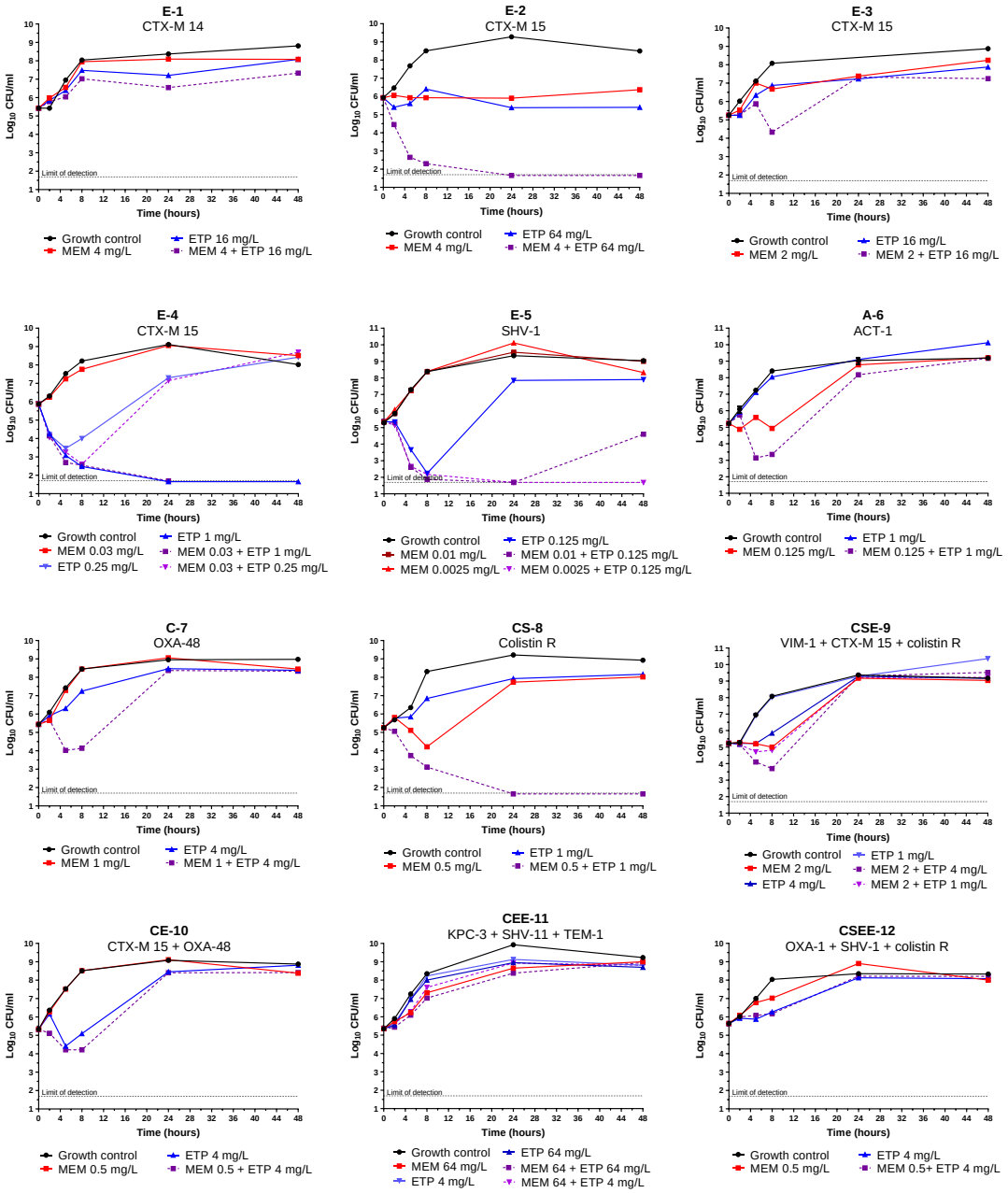
In conclusion, zidovudine in combination with other antimicrobial drugs is a repurposing option for MDR/XDR *K. pneumoniae*; similar repurposing approaches that employ other nucleoside analogues in combination with antifungals are already in clinical use (171). Based on these results, it is proposed the following priority list of pairwise combinations: zidovudine/ceftazidime-avibactam > zidovudine/fosfomycin > fosfomycin/colistin > zidovudine/colistin > fosfomycin/tigecycline = zidovudine/tigecycline > meropenem/colistin > meropenem/ertapenem. Finally, further dynamic PK/PD studies would be needed to fully discern the potential of zidovudine-based combinations in the clinical practice, and future clinical trials would clarify the impact of zidovudine combination therapy on clinical outcomes. If these studies result in clinical improvement, future expectations could include individualised therapy in those patients with severe infections. As part of routine laboratory workflow, synergy testing with zidovudine in clinical isolates would allow to infer the success of combination therapy.

Future directions will include: (i) expanding such studies to a larger panel of clinical isolates from several locations; (ii) identifying additional resistance mechanisms (i.e. porin loss or efflux pumps) besides the genotypic characterization of our strain panel that included standard β-lactam enzymatic resistance; and (iii) investigating for deficiency in thymidine kinase genes (which normally phosphorylate inactive zidovudine into the active form (139,172)) in those strains exhibiting high MIC_{ZDV} values, since it remains unknown whether the selection of resistant mutants is responsible for the bacterial rebound observed by TKA in some combinations **(appendix II)**

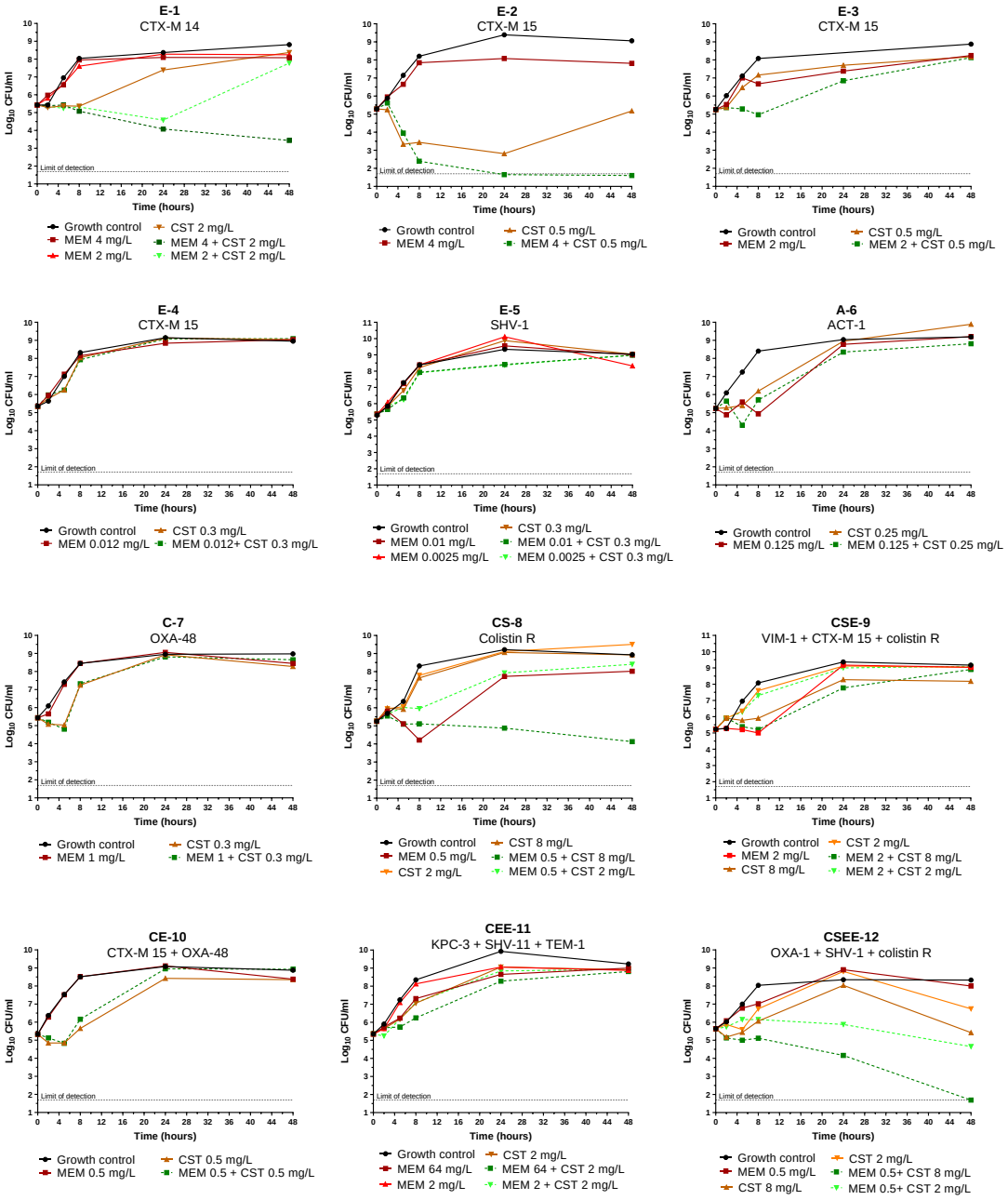
.

APPENDIX I. Combination time-kill curves against twelve *K. pneumoniae* isolates of pair-wise combinations currently used in the therapy of MDR enterobacteria. (a) Meropenem plus ertapenem; (b) Meropenem plus colistin; (c) Fosfomycin plus colistin; (d) Fosfomycin plus tigecycline

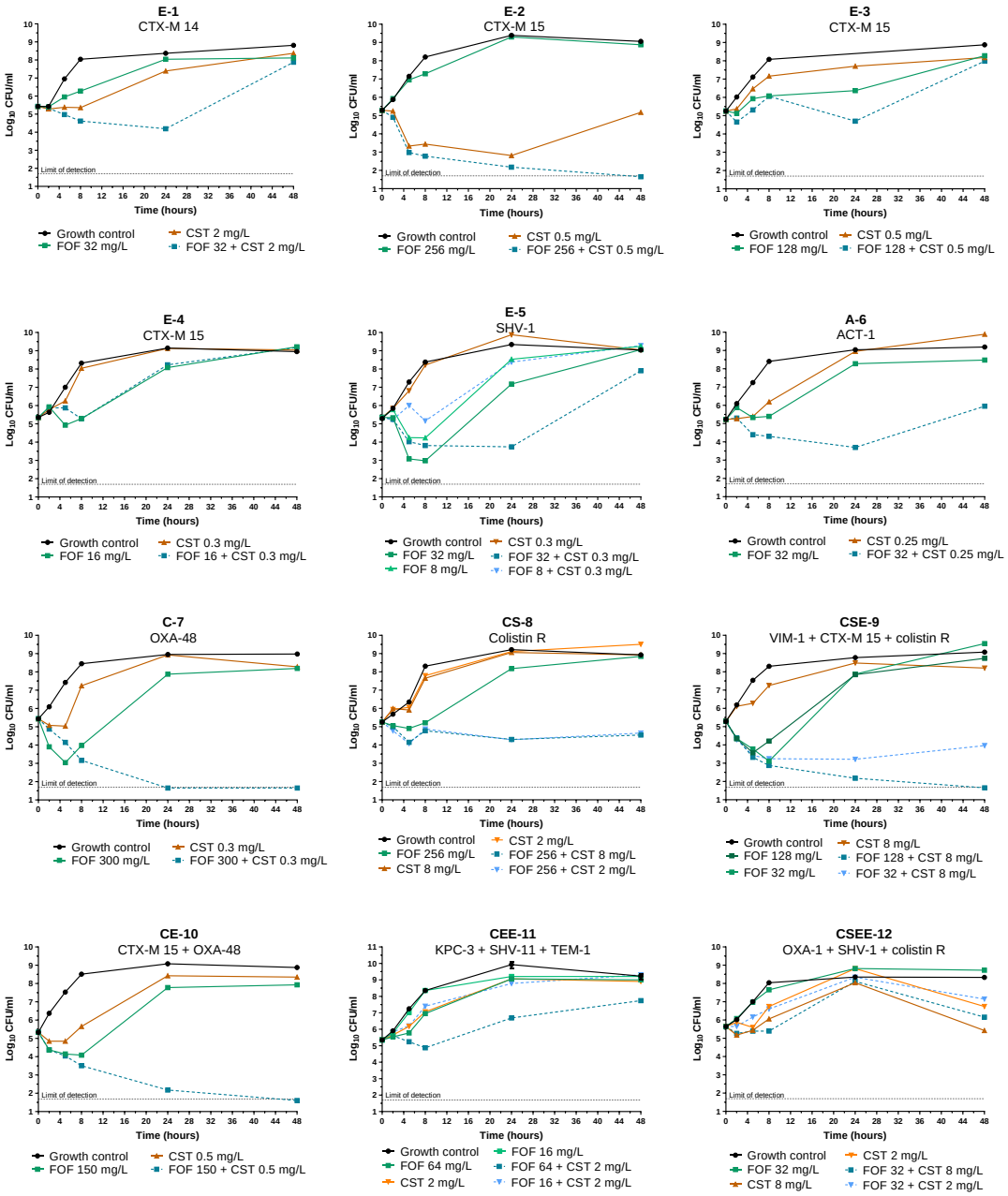
a) Meropenem plus ertapenem



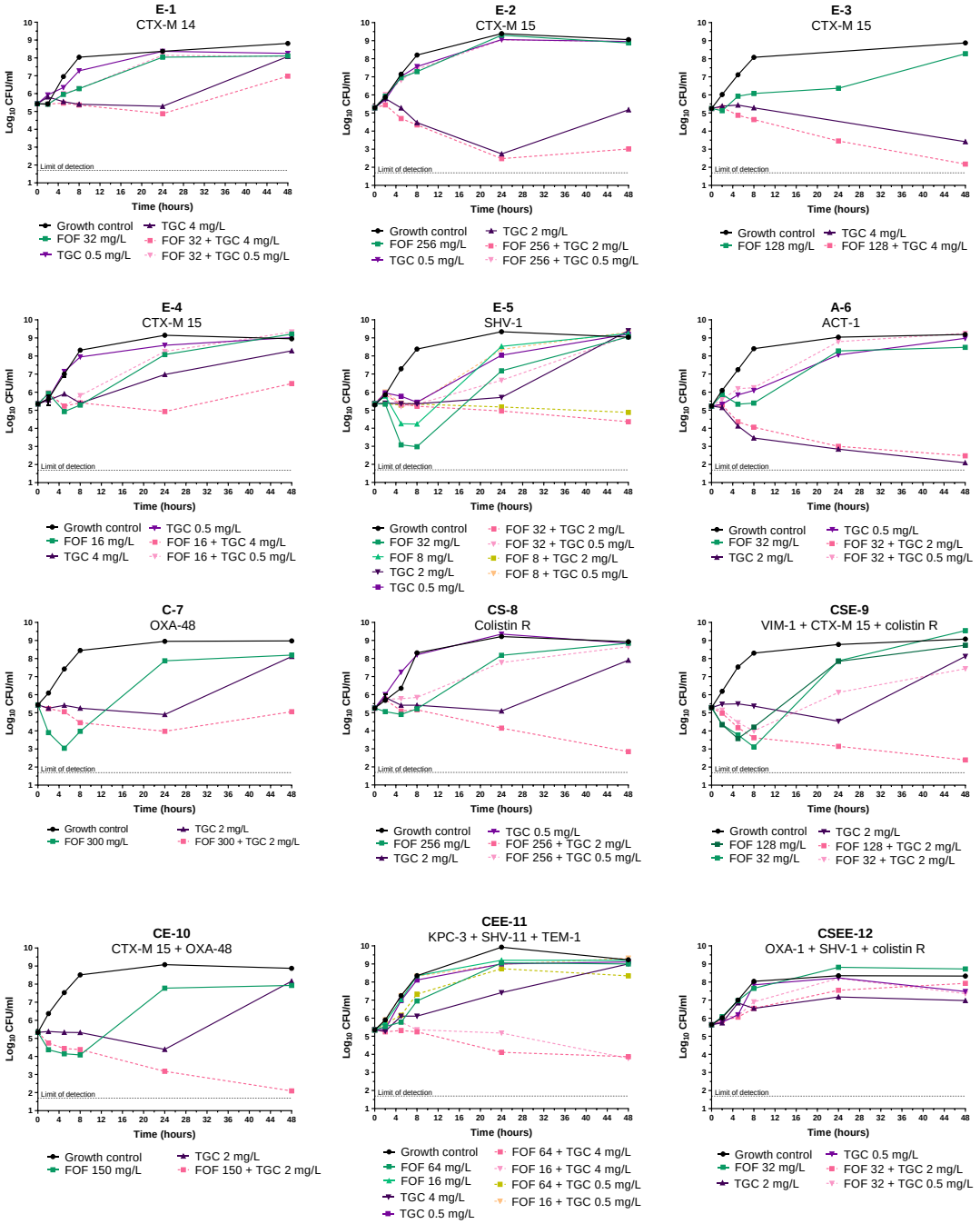
b) Meropenem plus colistin



c) Fosfomycin plus colistin

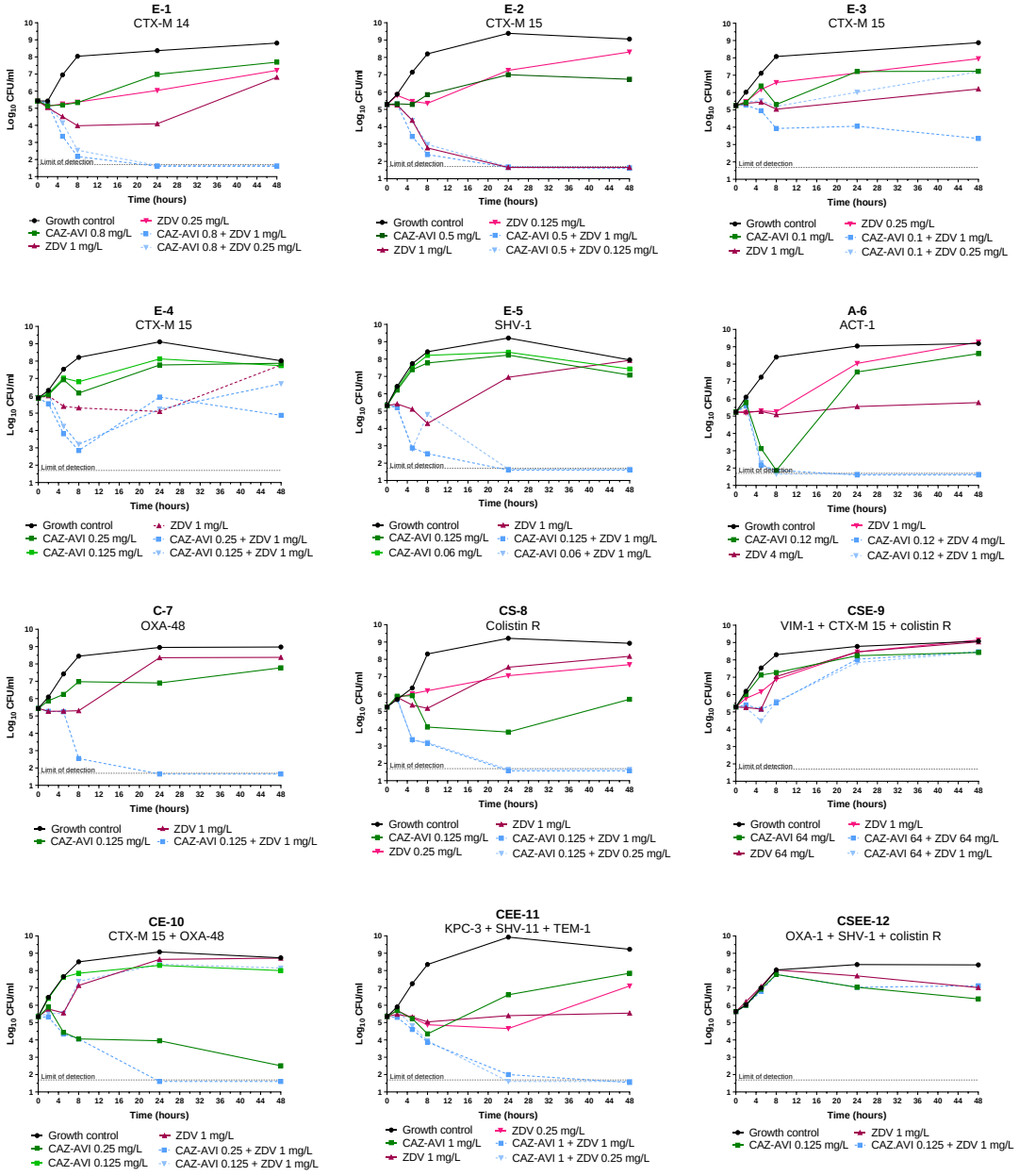


d) Fosfomycin plus tigecycline

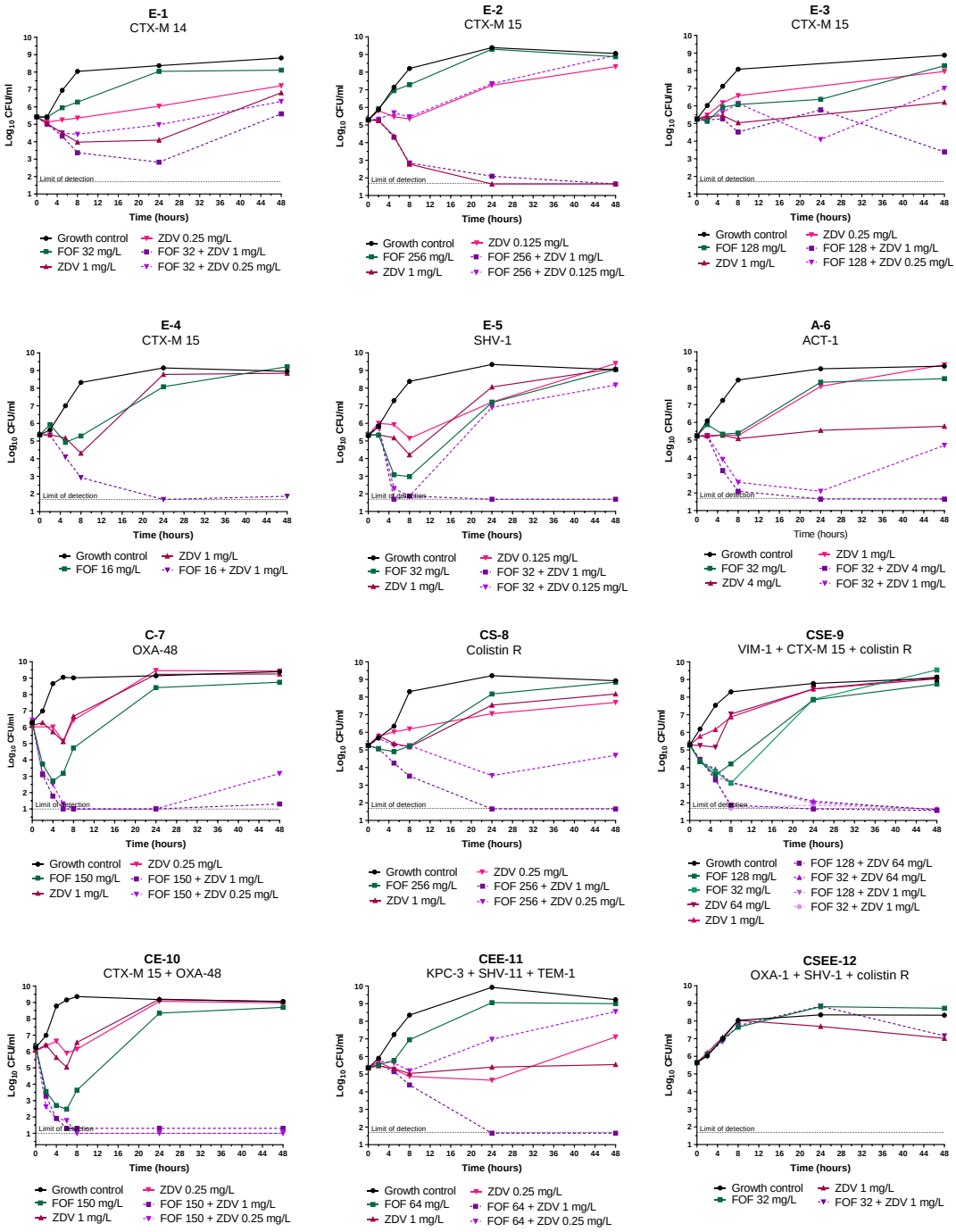


APPENDIX II. Combination time-kill curves of zidovudine with last-line antibiotics against twelve *K. pneumoniae* clinical strains. (a) Zidovudine plus ceftazidime-avibactam; (b) Zidovudine plus fosfomycin; (c) Zidovudine plus colistin; (d) Zidovudine plus tigecycline

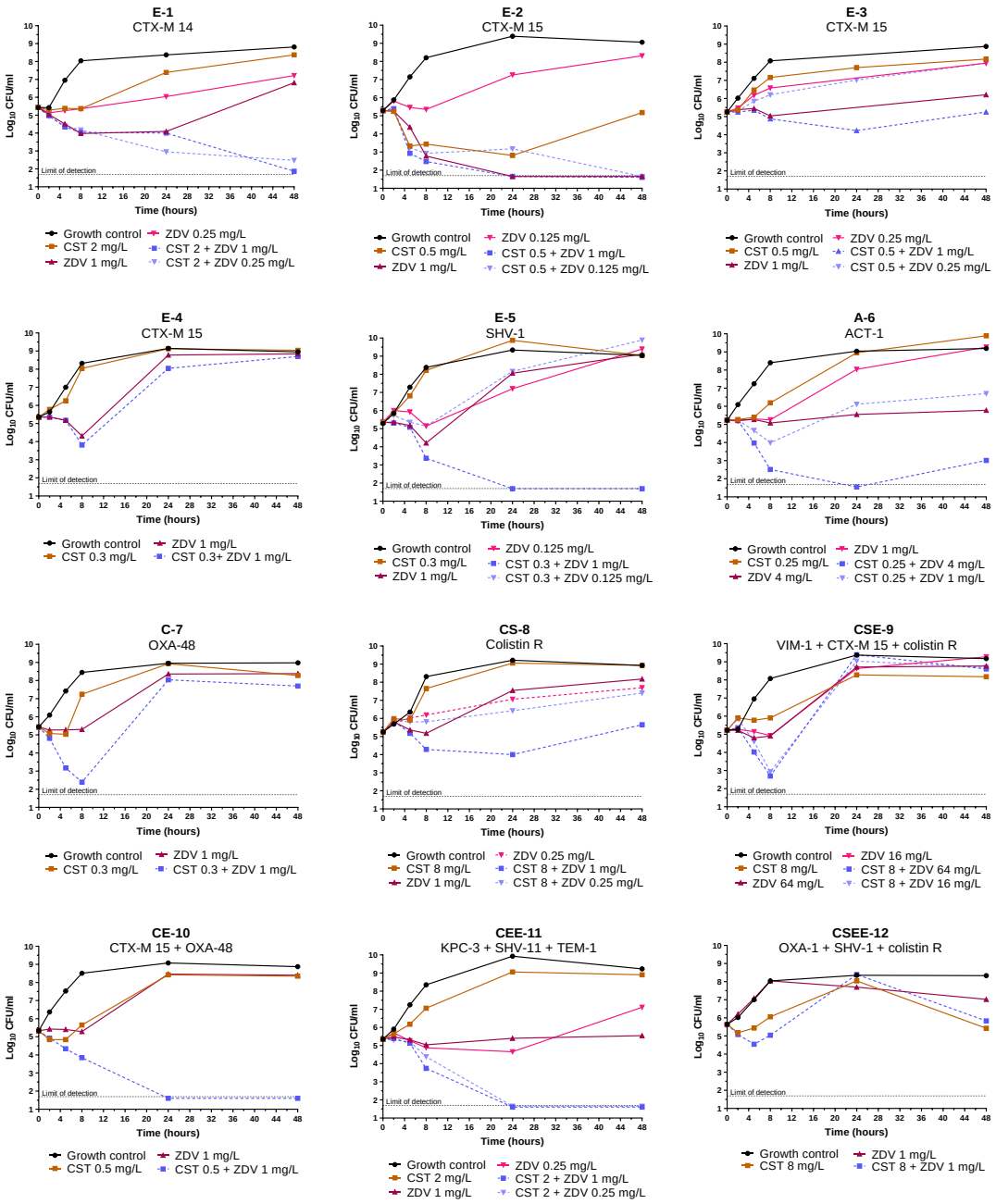
a) Zidovudine plus ceftazidime-avibactam



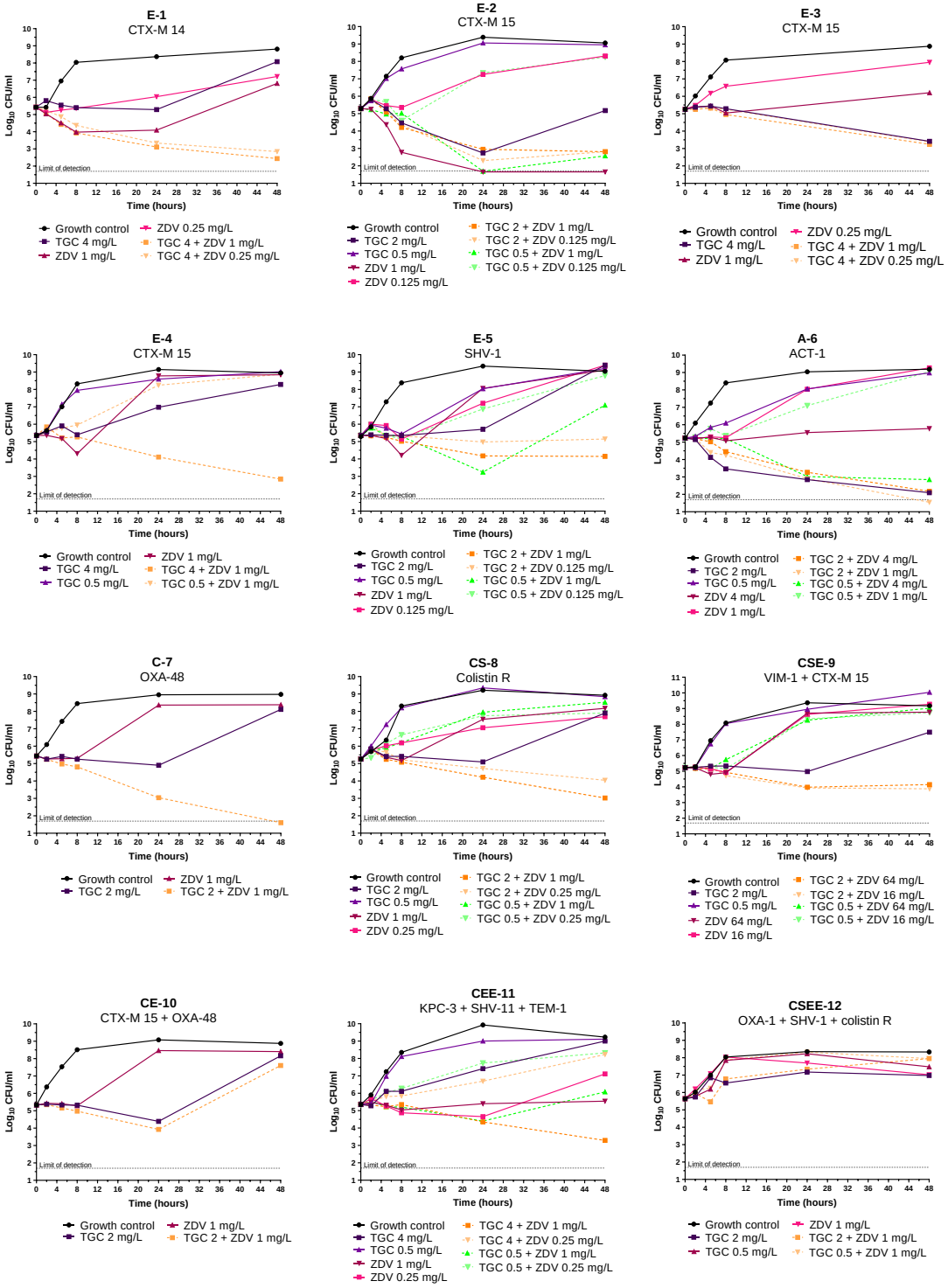
b) Zidovudine plus fosfomycin



c) Zidovudine plus colistin



d) Zidovudine plus tigecycline



V

Repurposing azithromycin in combination with last-line fosfomycin, colistin and tigecycline against Multi-Drug Resistant *Klebsiella pneumoniae*

CHAPTER 3

"Puedes empezar en cualquier momento, aunque te lleve toda una vida llegar a ser bueno"

***Esther Lederberg** (1922-2006)*

INTRODUCTION

Azithromycin is a macrolide antibiotic derived from erythromycin that consist of a 15-membered lactone ring azalide. As other macrolides, this is a bacteriostatic antibacterial that acts by reversible binding to the P site on the 50S ribosomal subunit, interfering then in the protein synthesis. Since its discovery, it has been widely prescribed for several indications such as respiratory, genitourinary and dermal infections (173,174), becoming an effective broad-spectrum antibacterial **(Figure 3.1).** Additionally, azithromycin exhibits anti-inflammatory and immunomodulatory properties, demonstrating clinical benefits in critically ill patients (175) and chronic respiratory disorders such as cystic fibrosis (176,177), asthma (178) and COPD (179). This repurposing strategy has been also pursued for azithromycin against parasitic (180,181) and viral infections (182). Indeed, azithromycin was one of the first candidates proposed for the management of COVID-19, firstly associated with hydroxychloroquine, although its efficacy for this indication could not be confirmed in clinical trials (183,184).

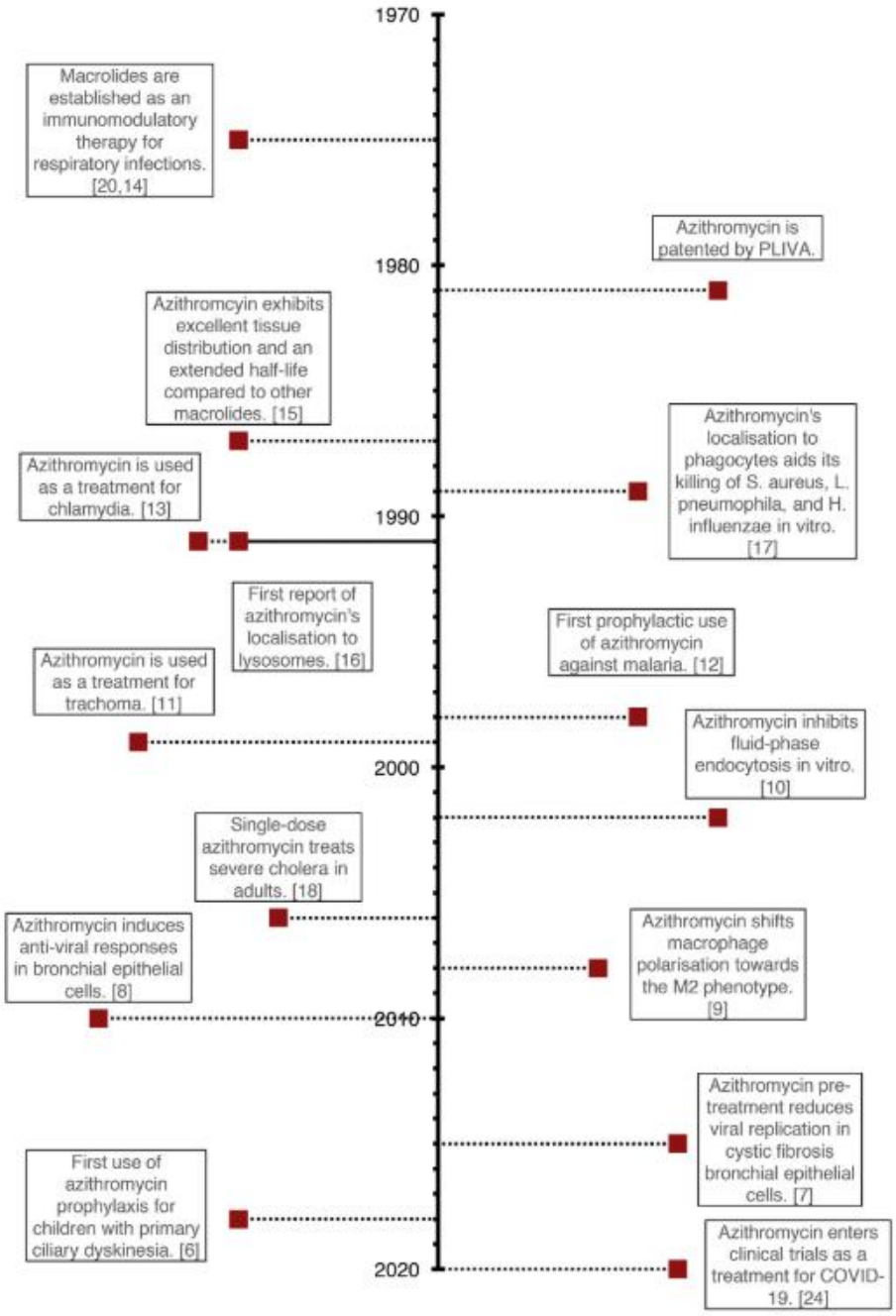


Figure 3.1. Timeline of clinical and experimental uses of azithromycin. From (174).

Nowadays, azithromycin is commercially available orally (250 to 600 mg tablets or 100-200 mg/5mL suspension) and intravenously (500 mg/2mL) (149). Standard dosage regimen is 500 mg daily increasing up to 2 g single dose in uncomplicated gonorrhoea (161). Due to it is widely used, azithromycin safety profile is well known, showing uncommon side-effects associated to long-term therapy (185), and it is well tolerated when administered to children and pregnant women (186). It poses advantageous PK/PD properties respect to other macrolides: no interaction with CYP3A4 cytochrome, an increased tissue penetration and bioavailability due to a higher basic character, and a long half-life (50-70 hours) (173,174). Peak

plasma concentrations of 1.46 mg/L and up to 3.4 mg/L are attained after 1,500 mg-oral and 500 mg-intravenous administrations, respectively (173).

Traditionally, monotherapy use of macrolides have been disregarded in the treatment of severe infections caused by Gram-negative bacteria due to different existing mechanisms of resistance to azithromycin in enterobacteria and the low permeability of their outer membrane (187). However, the enhanced basicity of azithromycin favours the intracellular uptake in Gram-negative bacteria increasing its efficacy and it is currently used for the treatment of enteric infections such as typhoid (174). In addition, azithromycin´s ability to inhibit bacterial quorum-sensing and reducing biofilm formation and mucus production have been demonstrated against intrinsically resistant pathogens (i.e. *P. aeruginosa* and *S. maltophilia*) (146,188). Moreover, azithromycin therapy seems to exert positive therapeutic effects in murine MDR Gram-negative infection models (148,189).

In Chapter 1, azithromycin was identified as a potent enhancer of the three PCs used in the synergy screen (colistin, tigecycline and fosfomycin), showing bactericidal activity against *K. pneumoniae*. Despite the limitations of azithromycin in monotherapy, its reintroduction into the clinical therapeutic arsenal to treat high-priority pathogens might be possible in combination therapy. In this Chapter 3, this idea is expanded to evaluate *in vitro* the synergistic and bactericidal activities of azithromycin-based combinations against antibiotic-resistant *K. pneumoniae* isolates and compared them with the activity of combinations typically used in the clinic for the treatment of MDR enterobacteria. For that purpose, the antimicrobial activity of azithromycin was firstly evaluated against the *K. pneumoniae* strain collection (**Table 3** of Materials & Methods). Secondly, the antibacterial activity was monitored by TKA at different time-points (8, 24 and 48 hours).

RESULTS

Activity of azithromycin against MDR/XDR *K. pneumoniae* isolates.

There are no CLSI or EUCAST guidelines describing azithromycin clinical breakpoints for enterobacteria, except for *Salmonella* Typhi and *Shigella* spp. (161); thus, there is no clinical basis to classify *K. pneumoniae* isolates as susceptible or resistant strains. Hence, MIC values of azithromycin were determined against the panel of MDR/XDR *K. pneumoniae* isolates and compared with the activity of other well-established drugs in the treatment of infections caused by MDR *K. pneumoniae*, for which clinical breakpoints do exist. In these experiments, azithromycin exhibited MIC values ranging from 4 to ≥64 mg/L, which were in the same range of values as those epidemiological cut-offs (ECOFFs) stablished by EUCAST for azithromycin in other enterobacteria; for these, confidence intervals range between 4 to 16 mg/L against *Escherichia coli* and between 4 to 64 mg/L against *S. Typhi* (190). Thus, the number and nature of antibiotic resistance determinants in any of the twelve isolates appeared not to be related with the susceptibility profiles against azithromycin **(Table 3.1.)**.

Table 3.1. Strain characterization of *K. pneumoniae* isolates and susceptibility profile to drugs used in this study. Clinical categorization according to current EUCAST breakpoints (109) are displayed in brackets.

				MIC (mg/L)					
Isolate	**Resistance mechanism**	**Source**	**MDR/XDR**	**CST**	**FOF**	**[1]TGC**	**ETP**	**MEM**	**AZM**
E-1	CTX-M 14	Rectal swab	XDR	0.5 (S)	>64 (R)	4	>32 (R)	8 (S*)	8
E-2	CTX-M 15	Blood	MDR	0.5 (S)	>64 (R)	0.5	64 (R)	4-8 (S*)	8
E-3	CTX-M 15	Abscess	MDR	1-2 (S)	>64 (R)	4	16 (R)	2-4 (S*)	8
E-4	CTX-M 15	Blood	MDR	0.5 (S)	>64 (R)	4	1 (R)	0.03 (S)	8
E-5	SHV-1 + porin loss	Blood	MDR	0.5 (S)	8 (S)	0.5-1	0.25 (S)	0.03 (S)	8-16
A-6	AmpC ACT-1	SEIMC CCS07	MDR	≤0.5 (S)	>64 (R)	1-2	4-8 (R)	0.5 (S)	8
C-7	OXA-48	Blood	MDR	1 (S)	>64 (R)	2	8-16 (R)	4 (S*)	4-8
CS-8	Colistin R	Urine	MDR	16 (R)	>64 (R)	1	0.5 (S)	0.5-1 (S)	8
CSE-9	VIM-1 + CTX-M 15 + colistin R	SEIMC CCS04	XDR	16 (R)	>64(R)	1-2	8-16 (R)	16-32 (R)	64
CE-10	CTX-M 15 + OXA-48	Blood	MDR	1-2 (S)	>64 (R)	1-2	8 (R)	4 (S*)	4
CEE-11	KPC-3 + SHV-11 + TEM-1	SEIMC CCS05	XDR	2 (S)	>64 (R)	4	>64 (R)	>64 (R)	≥64
CSEE-12	OXA-1 + SHV-1 + colistin R	EARS QC	MDR	4 (R)	64 (R)	1	8-16 (R)	1-2 (S)	8

CST, colistin; FOF, fosfomycin; TGC, tigecycline; ETP, ertapenem; MEM, meropenem; AZM, azithromycin; EARS QC, European Antimicrobial Resistance Surveillance Quality Control; R, resistant; S, susceptible; S*: susceptible, increased exposure; SEIMC: Spanish Society of Infectious Diseases and Clinical Microbiology
[1]EUCAST clinical breakpoints for tigecycline are only applied to *Escherichia coli* and *Citrobacter koseri*

Azithromycin-based combinations are more potent *in vitro* than those combinations currently used in the clinic to treat MDR *K. pneumoniae* infections.

In Chapter 1, the interaction between azithromycin and fosfomycin, colistin and tigecycline was validated against *K. pneumoniae* ATCC 13883 by TKA. All three paired

combinations displayed a high synergistic and bactericidal profile against the reference strain (see **Figure 1.8** of Chapter 1). In order to further characterize the potential antimicrobial activity of azithromycin-based combinations against MDR *K. pneumoniae,* TKA validation was also extended to a panel of twelve MDR/XDR *K. pneumoniae* isolates with representative mechanisms of resistance (**Table 3** of Materials & Methods). The activity of the three-novel azithromycin-based combinations (fosfomycin/azithromycin, colistin/azithromycin and tigecycline/azithromycin) was compared with that of four usual MDR clinical treatments (meropenem/ertapenem, meropenem/colistin, fosfomycin/colistin and fosfomycin/tigecycline).

At any time-point (8, 24 and 48 hours), synergy rates among currently used combinations for MDR treatment were observed in 41.6% (5/12) for meropenem/ertapenem, 33.33% (4/12) for meropenem/colistin, 75% (9/12) for fosfomycin/colistin, and 66.6% (8/12) for fosfomycin/tigecycline of the isolates tested (**Figure 3.2.** and **appendix I**). In stark contrast, a high number of synergistic interactions were obtained with azithromycin-based combinations among all isolates (**Figure 3.2.** and **appendix I**). Notably, this synergistic bactericidal positive interactions in azithromycin-based combinations were observed even when strains displayed a resistant profile to the drugs alone, as in strain CEE-11 (MIC_{AZM} ≥ 64 mg/L; MIC_{FOF} ≥ 64 mg/L) **(Figure 3.3.)**.

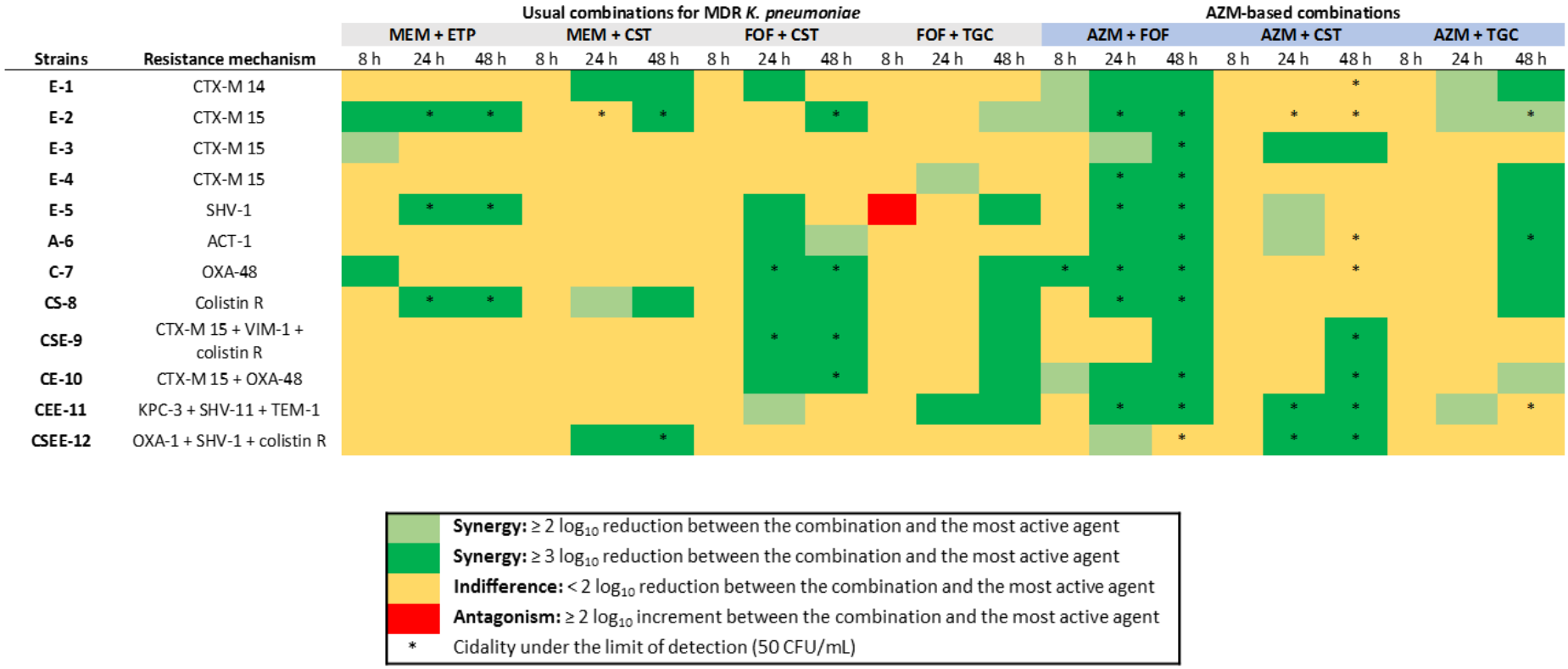


Figure 3.2. Heat map representation of synergy and bactericidal activities at different time points obtained by time-kill assays against *K. pneumoniae* isolates. Data supporting this summary figure are displayed in appendix I and appendix II. AZM, azithromycin; CST, colistin; ETP, ertapenem; FOF, fosfomycin; MEM, meropenem; TGC, tigecycline.

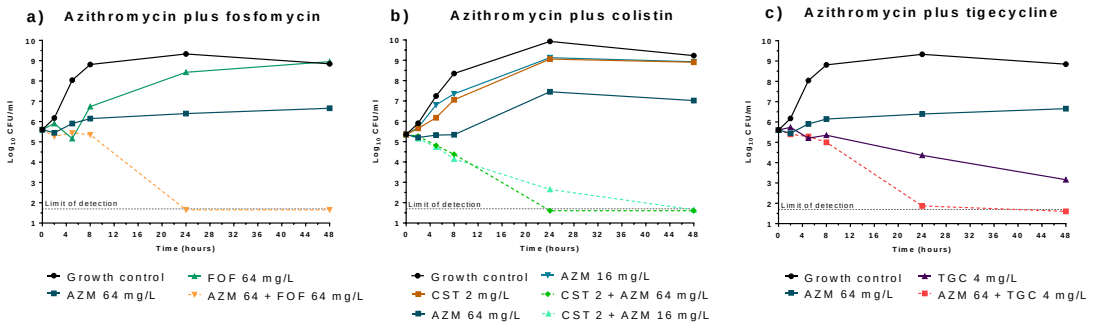


Figure 3.3. Time–kill curves showing azithromycin combinations with existing antibiotics (a-c) against the *K. pneumoniae* XDR strain CEE-11 (bla_{KPC-3} + bla_{SHV-1} + bla_{TEM-1}) in CAMHB. Azithromycin enhanced the activities of fosfomycin, colistin and tigecycline even at subinhibitory concentration (0.25 to 1 x MIC), showing potent synergistic and bactericidal effects. MIC_{AZM} ≥ 64 mg/L, MIC_{CST}= 2 mg/L, MIC_{FOF}> 64 mg/L, MIC_{TGC}= 4 mg/L.

The combination azithromycin/colistin **(appendix I, b)** was synergistic in 7 out of 12 strains (58.3%) and bactericidal in 10 out of 12 strains (83.3%). The positive interaction of azithromycin in combination with colistin was evident when analysing the bactericidal activity at the 48-hour time point in which eight strains (E-1, E-2, A-6, C-7, CSE-9, CE-10, CEE-11, CSEE-12), including two colistin-resistant strains, had viable counts below the limit of detection (50 CFU/mL) **(Figure 3.2.)**.

The combination azithromycin/tigecycline **(appendix I, c)** showed synergistic interactions against 9 out the 12 (75%) strains with a strain-dependent activity. The combination was bactericidal to the limit of detection in three strains (E-2, A-6 and CEE-11) and showed a bacteriostatic profile in the rest of the strains (from <1 to 1.6 log_{10} decrease in CFU/ml), except for CE-10 and CSEE-12 (> 2 log_{10} decrease in CFU/mL at 48 hours) **(appendix I, c)**.

The combination of azithromycin plus fosfomycin was the most potent. This combination was synergistic against all isolates and bactericidal in 11 out of the 12 (91.66%) strains. The potency of the azithromycin/fosfomycin combination was evident when compared to the activity of the drugs alone; neither showed long-lasting bactericidal activity, with a static effect or no activity (azithromycin), and rapid bactericidal activity followed by bacterial regrowth from the 8-hour time-point (fosfomycin). In addition, in most strains combined bactericidal effects were already detected at early time points (4-8 hours) **(appendix I, a)**.

Azithromycin combos with fosfomycin or colistin are not cytotoxic

As explained in Chapter 2, MTT cytotoxicity assays were performed in Hep G2 cell line to analyse the combined effect of bactericidal interactions by TKA. As result, azithromycin combinations with either fosfomycin or colistin did not show cytotoxic effect compared to drugs

alone. Azithromycin showed cytotoxic effect at 64 mg/L (40% cell viability), with statistical decrease compared to lower concentrations (4 and 8 mg/L), ($p < 0.0001$, **Figure 3.4**). Therefore, cytotoxic effect was also observed at this concentration (64 mg/L) combined with colistin (2 mg/L), with no statistical differences compared to azithromycin alone **(Figure 3.4)**.

The interaction between azithromycin plus tigecycline showed the most unfavourable results. All three combinations assayed showed low percentages of cell viability (27.36%, 56.56% and 64.45% mean values) as it is shown **in Figure 3.4.** Same as observed in Chapter 2, tigecycline showed cytotoxicity at 4 mg/L, therefore the combination with azithromycin at 64 mg/L (also cytotoxic) was expected to have the same effect than both drugs alone, but also decrease significantly ($p \leq 0.05$). Compared to drugs alone, statistical differences were also observed for the other two combinations, except with azithromycin at 2 mg/L **(Figure 3.4).**

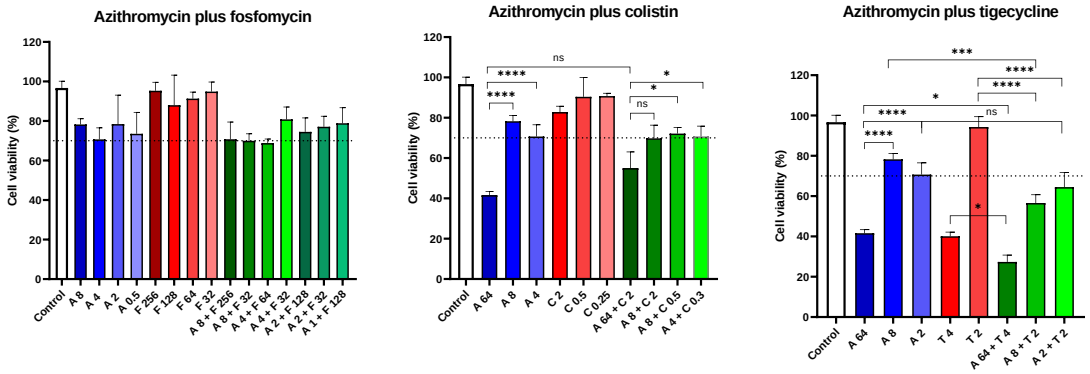


Figure 3.4. MTT cytotoxicity assays of novel combinations in Hep G2 cell line. Concentration of compounds are expressed in mg/L. Cell suspensions with no compounds added were used as positive control. Statistical differences are displayed by asterisks; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$; ns, $p > 0.05$; A, azithromycin; C, colistin; F, fosfomycin; T, tigecycline

DISCUSSION

In the present study the *in vitro* efficacy of azithromycin in combination with colistin, fosfomycin and tigecycline was evaluated against a panel of 12 MDR/XDR *K. pneumoniae* isolates with representative resistance patterns. TKA was used as a reference method with activity readouts obtained after up to 48 hours of incubation, a procedure not typically performed when evaluating the activity of compounds against enterobacteria.

The activity alone of azithromycin, and its three synergistic partners colistin, fosfomycin and tigecycline, were characterised in a dose-response manner against the collection of twelve *K. pneumoniae* isolates. Then, combination assays were tested by selecting matching subinhibitory concentrations of each individual drug to allow for a wider dynamic range and detection of drug interactions. This implies that even if absolute MIC values for every *K. pneumoniae* strain in the collection might be different **(Table 3.1.)**, the effect of their subinhibitory activities would be similar in combination since they are based on individual MIC values for each strain and compound. The use of subinhibitory concentrations of the antibiotics alone is a key factor to detect drug interactions since higher effective concentrations might masked the effect of their potential interactions. In addition, extending the readout to 48 hours provides information in both the increased bactericidal activity of the azithromycin-based combinations compared to the drugs alone, and also the ability of the combination to completely eradicate bacteria (below the limit of detection of the assay, which is a proxy for culture sterilization). Based on these criteria, three azithromycin-based combinations were tested (**Figure 3.2.** and **appendix I**) and compared with four representative combinations currently used in the clinic to treat MDR/XDR *K. pneumoniae* infections (**Figure 3.2.** and **appendix I** in Chapter 2). Our TKA data showed high rates of favourable interactions for the azithromycin-containing combinations, even against strains with concurrent resistance mechanisms; thus, suggesting a potential role of azithromycin in combinatorial therapy (**Figure 3.2.** and **appendix I**), as evidence by the examples below:

1. *Azithromycin plus fosfomycin.* First prescribed for urinary tract infections, fosfomycin was identified as synergistic partner of several antibiotics. Fosfomycin is an old bactericidal antibiotic that inhibits peptidoglycan synthesis (46), thus it could be enhancing antibiotic entrance by increasing cell permeability. As such, fosfomycin has been reintroduced in combinatorial therapy for the clinical management of MDR enterobacterial infections over the last years(48). This combination was previously assessed in two other *in vitro* studies. Presterl *et al.* described negligible bactericidal activity against biofilm-producer *Staphylococcus*

epidermidis(191), and the combination also showed killing activity at 24 hours by TKA against *Neisseria gonorrhoeae,* including azithromycin resistant strains, with no regrowth until the end of the assay(192). The latter study is in agreement with these results in *K. pneumoniae*, supporting the potential use of azithromycin/fosfomycin against Gram-negative bacteria. Rapid bactericidal activities observed were maintained up to the end of the assays against all tested strains (**Figure 3.2.** and **appendix I, a**), including those strains with high fosfomycin MIC values **(Figure 3.3.)**. Interestingly, effective fosfomycin concentrations in these *in vitro* assays were below fosfomycin peak plasma concentration after intravenous administration in adults (606 mg/L)(46). In my view, this is the first study analyzing the antimicrobial activities of the combination azithromycin/fosfomycin against a large set of MDR *K. pneumoniae* strains. These results, together with other evidence, suggest that the combination of azithromycin plus fosfomycin could play an important role in clinical settings and merits further pre-clinical and clinical development. Both drugs display good safety profiles, they are recommended for combinatorial therapy to minimize resistance emergence derived from monotherapy, and are administered at a single dose administration (0.5 to 2 g single dose oral or intravenously for azithromycin (174) and 3 g single dose orally or up to 8 g /8 hours intravenously for fosfomycin (50)). Similar to azithromycin, fosfomycin displays immunomodulatory mechanisms (46), which have been shown beneficial to overcome severe Gram-negative infections.

2. *Azithromycin plus colistin.* This combination was reported in some studies including MDR *K. pneumoniae* (148,189,193), where the increase in the Gram-negative outer membrane permeability facilitates azithromycin access to the 50S ribosomal subunit (148,189). In agreement with these results, sterilizing activities were obtained in 2 out of 3 of the colistin resistant strains (CSE-9, MIC_{CST}= 16 mg/L and CSEE-12, MIC_{CST}= 4 mg/L). In these strains, the limiting factor for activity was the concentration of azithromycin; similar killing profiles were obtained at two colistin concentrations (2 mg/L and 8 mg/L) **(appendix I, b)**. These findings support the possibility to decrease colistin concentrations below its nephrotoxic threshold (2.42 mg/L) (40), if administered in synergistic combination with azithromycin.

3. *Azithromycin plus tigecycline*. This is the first report of this combination being active against *K. pneumoniae*. Previous studies described biofilm eradication against *S. maltophilia* (146) and the *in vitro* and *in vivo* activity of azithromycin in combination with minocycline (another tetracycline antibiotic) against MDR pathogens including *K. pneumoniae* (194). Although we observed variable activity from one strain to another **(appendix I, c)**, the combination showed sterilizing activity against three strains, which had different susceptibility

profile to both drugs (e.g., CEE-11 exhibited resistant profile with MIC_{TGC}= 4 mg/L and MIC_{AZM} ≥ 64 mg/L, **Figure 3.3.**). Azithromycin and tigecycline are both bacteriostatic drugs targeting the 50S and 30S ribosomal subunits, respectively, which could explain their synergy by enhancing protein inhibition that leads to disruption of the bacterial gene translation.

Our TKA results show effective sterilizing activities of azithromycin-based combinations at azithromycin concentrations ranging from 2 up to 64 mg/L **(appendix I)**. Although for some strains the azithromycin sterilizing concentrations observed were over those achievable in plasma, azithromycin displays a rapid blood-tissue distribution, so despite such low serum concentrations it is expected that its accumulation in tissue will be higher (e.g. accumulation in macrophages is 5- to 200-fold higher than in plasma (174)). In addition, the long PAE and significant subinhibitory concentration effect demonstrated both *in vitro* and *in vivo* against respiratory pathogens (195,196) indicate a prolonged antimicrobial activity.

The azithromycin PK/PD properties make it an optimal candidate for combination therapy in MDR Gram-negative infections. Standard dosing of the last-line antibiotics used in this study (that included loading doses for colistin and tigecycline) (37) yielded a rapid bacterial killing effect that could be seconded by the slower but longer lasting action of azithromycin, maintaining bacterial eradication during the course of treatment. Moreover, combinatorial therapy with azithromycin might minimise resistance emergence and toxicity issues (specially with colistin) using longer dosing intervals.

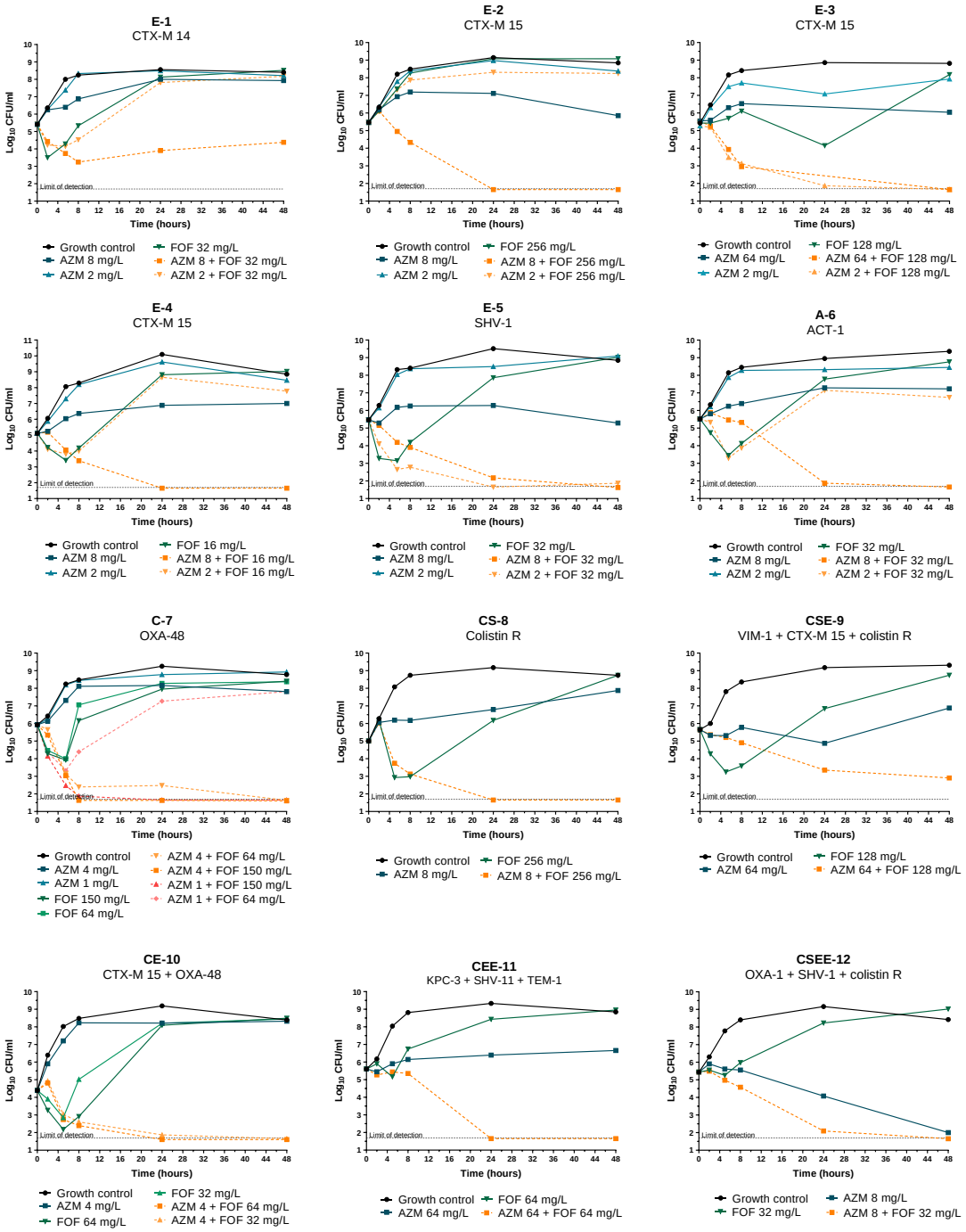
The use of macrolides (specially azithromycin) is currently recommended in critically ill patients with pneumonia as empirical treatment in combination with β-lactams or fluroquinolones (197), supported by previous preclinical assays showing synergy (198–200). Anticipatory immunotherapy with azithromycin has been also used in critically ill patients with infections other than pneumonia, demonstrating clinical benefit with reduced mortality rates and intensive-care unit (ICU) stay (175). The early addition of azithromycin to last-line antibiotics for MDR treatment in severe infections (i.e., sepsis, ventilator-associated pneumonia, immunocompromised patients) could not only improve the efficacy of the therapy in combination, but also improve the clinical outcome due to immunomodulatory properties of azithromycin in ICU patients.

CONCLUSIONS AND FUTURE PERSPECTIVES

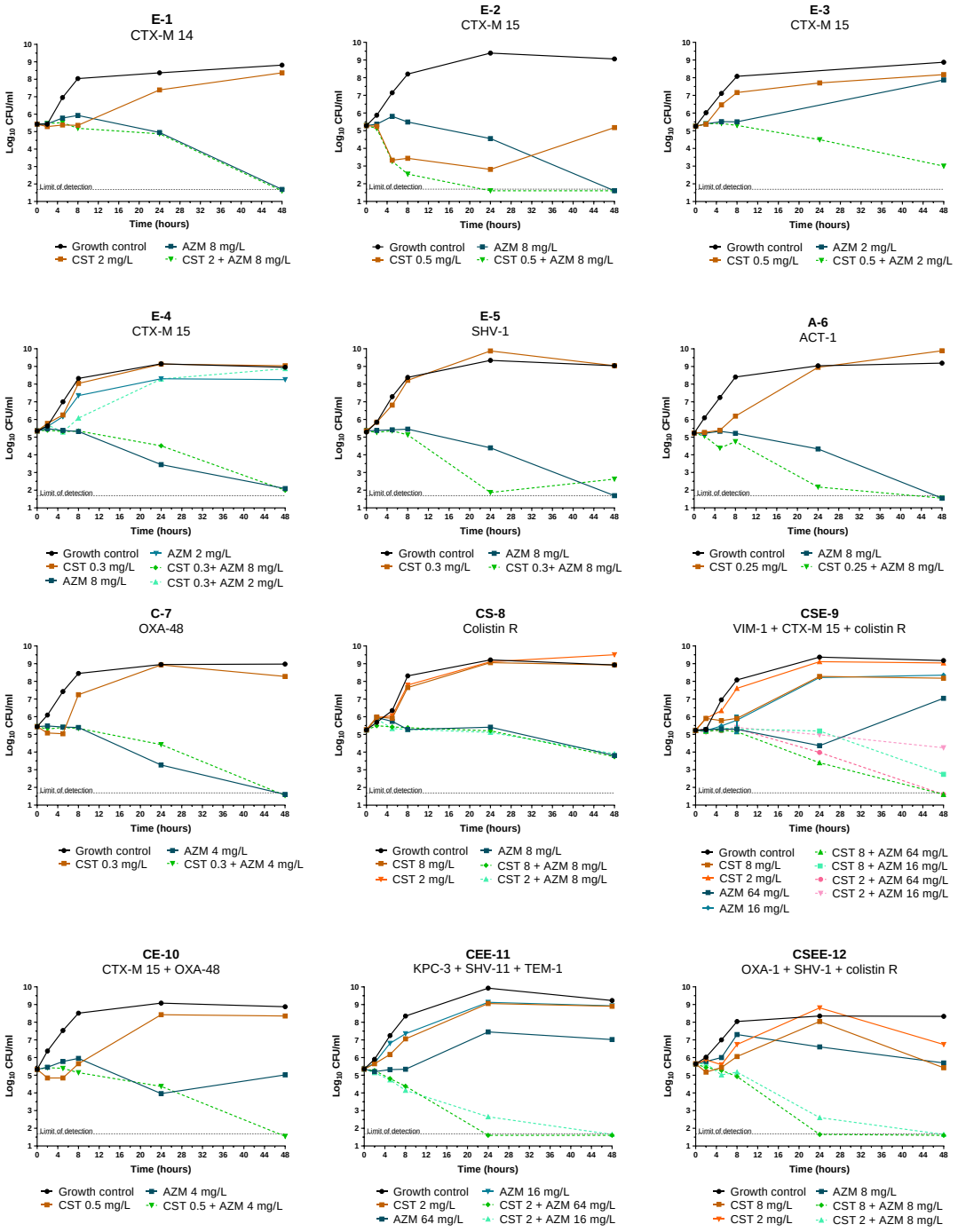
In conclusion, it was demonstrated using *in vitro* TKA models that azithromycin combined with existing antibiotics might increase the efficacy in the eradication of MDR/XDR *K. pneumoniae.* Based on these *in vitro* studies, the following priority list of pairwise combinations are proposed: azithromycin/fosfomycin > azithromycin/colistin > fosfomycin/colistin > meropenem/ertapenem > azithromycin/tigecycline > meropenem/colistin > fosfomycin/tigecycline. Additional pre-clinical and clinical studies would be needed to fully understand the clinical potential of azithromycin as synergistic partner in antimicrobial therapies against MDR enterobacteria.

APPENDIX I. Summary time-kill assays of azithromycin-based combinations against *K. pneumoniae* isolates. (a) Azithromycin plus fosfomycin; (b) Azithromycin plus colistin; (c) Azithromycin plus tigecycline.

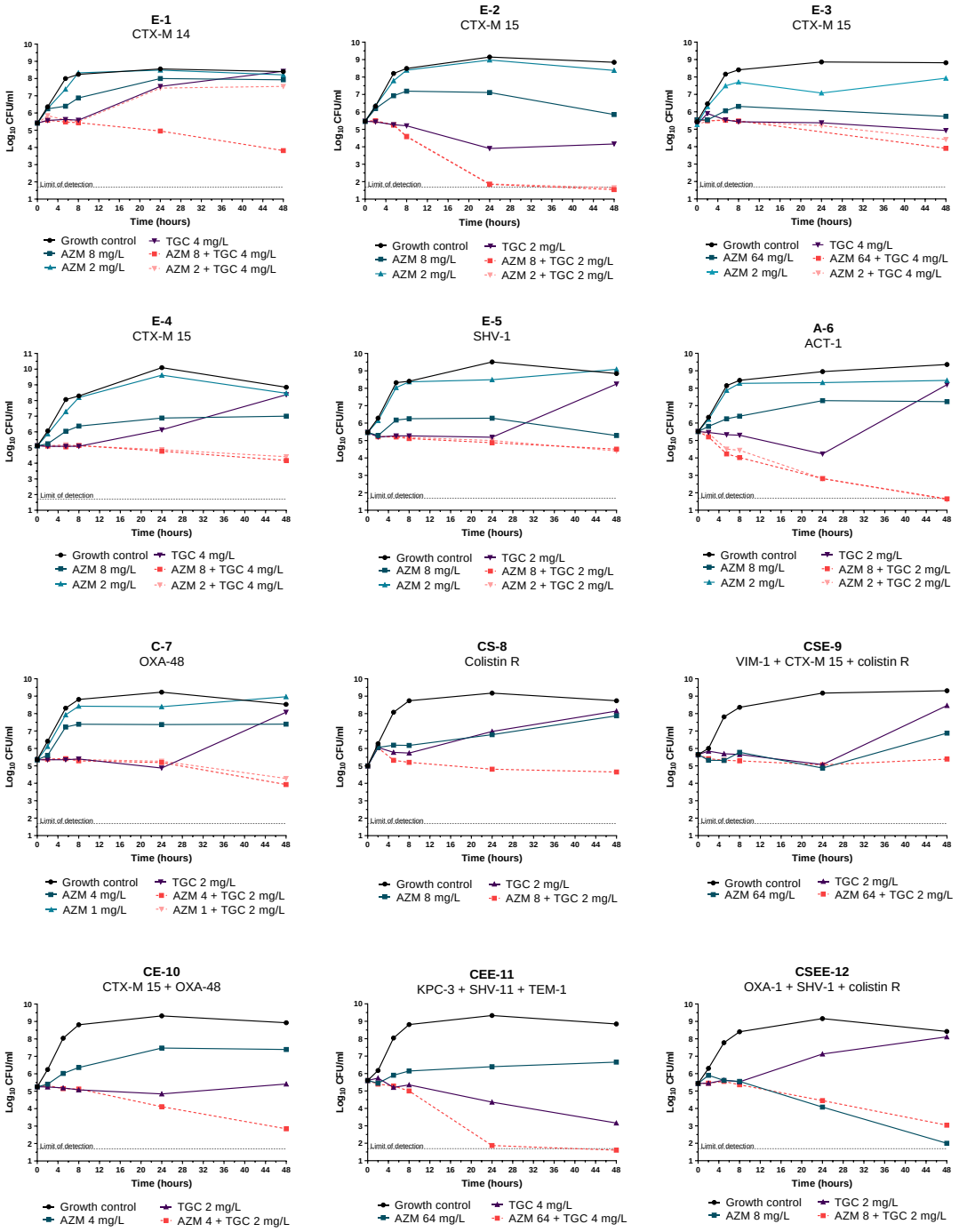
a) Azithromycin plus fosfomycin



b) Azithromycin plus colistin



c) Azithromycin plus tigecycline



GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

Faced with the increase of multi-resistant bacteria to antibiotics, it is necessary to design new strategies to develop new effective treatments. In this Thesis, two strategies have been implemented jointly: drug repositioning and the identification of compounds that act synergistically to enhance their effect. The following conclusions can be drawn from this work:

- The sHTSS methodology has revealed novel previously undescribed drug combinations for killing or inhibiting growth of *K. pneumoniae*, as well as has confirmed other synergistic interactions already described between antibiotics (e.g., tigecycline and amikacin).
- Antibiotic clinical combinations commonly used for the treatment of multidrug-resistant *K. pneumoniae* showed variable *in vitro* activity against the panel of isolates. Fosfomycin/colistin was the combination showing the highest synergistic and bactericidal activity at sub-inhibitory concentrations.
- There is *in vitro* synergistic interaction of zidovudine in pair-wise combination with either fosfomycin, ceftazidime-avibactam, colistin or tigecycline against *K. pneumoniae*, at similar concentrations that those exhibited physiologically.
- Rapid bactericidal activity against *K. pneumoniae* was observed for combinations based on zidovudine in combination with fosfomycin, ceftazidime-avibactam or colistin, maintaining their sterilising effect up to 48 hours in time-kill assays.
- Triple combinations of zidovudine/fosfomycin/colistin have a similar effect to double combinations of fosfomycin/zidovudine, colistin/zidovudine and fosfomycin/colistin, but do not provide greater potency in their activity against *K. pneumoniae*.
- Azithromycin has a potent synergistic effect with fosfomycin, colistin or tigecycline against *K. pneumoniae*.
- Especially with the azithromycin/fosfomycin combination, rapid bactericidal activity without bacterial rebound in the kill curves was observed in all *K. pneumoniae* isolates tested.
- Cytotoxicity assays revealed that both zidovudine and azithromycin, were non-cytotoxic below 64 mg/L.
- Novel combinations showed high cell viability and non-toxic effect compared to drugs alone, except azithromycin/tigecycline combination, which did not yield the non-cytotoxic threshold (70% cell viability).

As this work lays the foundation for future pre-clinical and clinical implementation of these novel combinations, future directions to continue in this line can be mentioned below:

1. The confirmation of the favourable interactions against a more extensive set of clinical isolates.
2. Assessment of their activities against bacterial cells on stationary phase of growth. This would be of particular interest in infections associated with biofilm formation (e.g., endocarditis, osteomyelitis, prosthetic joint infections), in where the drug access to the bacteria is a limiting factor. Artificial biofilms may be simulated by *in vitro* assays (e.g., Calgary Biofilm Device), and the effect of combination of drugs on the cells can be analysed by confocal microscopy.
3. Elucidation of the underlying mechanisms of synergy of the combinations.
4. Evaluation of the combinations by in vitro synergy assays based on dynamic systems, such as the Hollow Fibre System. These experiments would allow to assess the activity of the combinations by simulating real infection conditions, at physiological concentrations observed in humans.
5. Finally, in vivo models would also complement the hollow fibre system experiments for the design of future randomised clinical trials.

In view of these results, the future clinical implementation of azithromycin and zidovudine into the therapeutic arsenal for *K. pneumoniae* severe infections seems promising. Favourable points for the achievement of this reality are the traditional use of azithromycin in critically ill patients, which would facilitate the management of these infections in combination therapy, or the current development of an ongoing clinical trial related to zidovudine in combination with colistin, which also drives the design of further clinical trials involving zidovudine.

CONCLUSIONES GENERALES Y PERSPECTIVAS FUTURAS

Ante el aumento de bacterias multirresistentes a los antibióticos es necesario diseñar nuevas estrategias para tratar de desarrollar nuevos tratamientos eficaces. En esta tesis se han puesto en práctica dos estrategias de manera conjunta; el reposicionamiento de fármacos y la identificación de compuestos que actúen de manera sinérgica para potenciar su efecto. De este trabajo se derivan las siguientes conclusiones:

- La metodología sHTSS ha revelado nuevas combinaciones de fármacos no descritas previamente para matar o inhibir el crecimiento de *K. pneumoniae*, así como confirmado otras interacciones sinérgicas ya descritas entre antibióticos (por ejemplo, tigeciclina y amikacina).
- Las combinaciones de antibióticos de uso clínico habituales para el tratamiento de *K. pneumoniae* multirresistente presentaron actividad *in vitro* variable frente al panel de aislados. Fosfomicina/colistina fue la combinación que mostró mayor actividad sinérgica y bactericida a concentraciones subinhibitorias.
- Existe interacción sinérgica *in vitro* de zidovudina en combinación doble con fosfomicina, ceftazidima-avibactam, colistina y tigeciclina frente a *K. pneumoniae,* a concentraciones similares a las fisiológicas.
- Se observó una rápida actividad bactericida de las combinaciones basadas en zidovudina en combinación con fosfomicina, ceftazidima-avibactam y colistina, manteniendo su efecto esterilizante hasta las 48 horas en los ensayos de curvas de muerte.
- Combinaciones triples de zidovudina/fosfomicina/colistina tienen un efecto similar a las dobles combinaciones de fosfomicina/zidovudina, colistina/zidovudina y fosfomicina/colistina, pero no aportan una mayor potencia en su actividad frente a *K. pneumoniae.*
- Azitromicina tiene un potente efecto sinérgico con fosfomicina, colistina y tigeciclina frente a *K. pneumoniae*.
- Especialmente, con la combinación azitromicina/fosfomicina se observó una rápida actividad bactericida sin rebote bacteriano en las curvas de muerte en todos los aislados de *K. pneumoniae* testados.
- Los ensayos de citotoxicidad revelaron que tanto la zidovudina como la azitromicina no eran citotóxicas por debajo de 64 mg/L.
- Las nuevas combinaciones mostraron una elevada viabilidad celular y un efecto no tóxico en comparación con los fármacos por separado, excepto la combinación de azitromicina/tigeciclina, que no alcanzó el umbral no citotóxico (70% de viabilidad celular).

Dado que este trabajo sienta las bases para la futura aplicación preclínica y clínica de estas novedosas combinaciones, a continuación, se mencionan las futuras direcciones a seguir en esta línea:

1. La confirmación de las interacciones favorables frente a un conjunto más amplio de aislados clínicos.
2. La evaluación de sus actividades frente a bacterias en fase estacionaria de crecimiento. Esto sería de especial interés en infecciones asociadas a la formación de biopelículas (por ejemplo, endocarditis, osteomielitis, infecciones de prótesis articulares), en las que el acceso del fármaco a las bacterias es un factor limitante. Las biopelículas artificiales pueden simularse mediante ensayos *in vitro* (por ejemplo, Calgary Biofilm Device), y el efecto de la combinación de fármacos en las células puede analizarse mediante microscopía confocal.
3. Elucidación de los mecanismos moleculares de sinergia de las combinaciones.
4. Evaluación de las combinaciones mediante ensayos de sinergia *in vitro* basados en sistemas dinámicos, como el Hollow Fibre System. Estos experimentos permitirían evaluar la actividad de las combinaciones simulando condiciones reales de infección, a concentraciones fisiológicas observadas en humanos.
5. Por último, los modelos *in vivo* también complementarían los experimentos con el sistema de fibra hueca para el diseño de futuros ensayos clínicos aleatorizados.

A la vista de estos resultados, la futura implementación clínica de la azitromicina y la zidovudina en el arsenal terapéutico de las infecciones graves por *K. pneumoniae* parece prometedora. Entre los puntos favorables para el alcance de esta realidad se encuentran el uso tradicional de azitromicina en pacientes críticos, que facilitaría el tratamiento de estas infecciones en terapia combinada, o el actual desarrollo de un ensayo clínico en curso relacionado con zidovudina en combinación con colistina, que también impulsa el diseño de nuevos ensayos clínicos con zidovudina.

REFERENCES

1. World Health Organization. Ten threats to global health in 2019. [Internet]. 2019 [cited 2022 Jun 20]. Available from: https://www.who.int/vietnam/news/feature-stories/detail/ten-threats-to-global-health-in-2019

2. Murray CJ, Ikuta KS, Sharara F, Swetschinski L, Robles Aguilar G, Gray A, et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet. 2022 Jan;S0140-6736(21)02724-0.

3. O'Neill J. Tackling drug-resistant infections globally: final report and recommendations. Review on Antimicrobial Resistance. 2016.

4. CDC. Antibiotic resistance threats in the United States, 2019. Atlanta, GA: U.S. Department of Health and Human Services, CDC; 2019.

5. Cassini A, Högberg LD, Plachouras D, Quattrocchi A, Hoxha A, Simonsen GS, et al. 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. Lancet Infect Dis. 2019 Jan 1;19(1):56–66.

6. World Health Organization (WHO). Home AWaRe [Internet]. 2020 [cited 2021 Dec 22]. Available from: https://adoptaware.org/

7. European Commission. A European One Health Action Plan against Antimicrobial Resistance (AMR). 2017.

8. Mancuso G, Midiri A, Gerace E, Biondo C. Bacterial antibiotic resistance: the most critical pathogens. Pathogens. 2021;10(10):1310.

9. Huttner BD, Catho G, Pano-Pardo JR, Pulcini C, Schouten J. COVID-19: don't neglect antimicrobial stewardship principles! Clin Microbiol Infect. 2020 Jul;26(7):808–10.

10. Adebisi YA, Alaran AJ, Okereke M, Oke GI, Amos OA, Olaoye OC, et al. COVID-19 and Antimicrobial Resistance: A Review. Infect Dis Res Treat. 2021 Jan 31;14:11786337211033870.

11. Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, et al. Bad bugs, no drugs: No ESKAPE! An update from the Infectious Diseases Society of America. Clin Infect Dis. 2009;48(1):1–12.

12. World Health Organization. Prioritization of pathogens to guide discovery, research and development of new antibiotics for drug-resistant bacterial infections, including tuberculosis. Geneva: WHO/EMP/IAU/2017.12; 2017.

13. World Health Organization. WHO Country Office for India and Department of Biotechnology Government of India. Indian Priority Pathogen List. To guide research, discovery and development of new antibiotics in India. Geneva, Switzerland; 2021.

14. World Health Organization. WHO fungal priority pathogens list to guide research, development and public health action. Geneva; 2022.

15. Magiorakos A-P, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect. 2012 Mar;18(3):268–81.

16. Kadri SS, Adjemian J, Lai YL, Spaulding AB, Ricotta E, Rebecca Prevots D, et al. Difficult-to-treat resistance in gram-negative bacteremia at 173 US hospitals: Retrospective

cohort analysis of prevalence, predictors, and outcome of resistance to all first-line agents. Clin Infect Dis. 2018;67(12):1803–14.

17. Abbott SL. Klebsiella, Enterobacter, Citrobacter, Serratia, Plesiomonas, and Other Enterobacteriaceae. In: Versalovic J, editor. Manual of Clinical Microbiology. 10th ed. ASM Press; 2011. p. 639–57.

18. Chew KL, Lin RTP, Teo JWP. Klebsiella pneumoniae in Singapore: Hypervirulent infections and the carbapenemase threat. Front Cell Infect Microbiol. 2017;7:515.

19. Cui X, Zhang H, Du H. Carbapenemases in Enterobacteriaceae: Detection and Antimicrobial Therapy. Front Microbiol. 2019 Aug 20;10:1823.

20. Sader HS, Rhomberg PR, Fuhrmeister AS, Mendes RE, Flamm RK, Jones RN. Antimicrobial resistance surveillance and new drug development. Open Forum Infect Dis. 2019;6(Suppl 1):S5–13.

21. Lan P, Jiang Y, Zhou J, Yu Y. A global perspective on the convergence of hypervirulence and carbapenem resistance in Klebsiella pneumoniae. J Glob Antimicrob Resist. 2021;25:26–34.

22. European Centre for Disease Prevention and Control. Antimicrobial resistance in the EU/EEA (EARS-Net), Annual Epidemiological Report for 2019. Stockholm: ECDC; 2020.

23. Navon-Venezia S, Kondratyeva K, Carattoli A. Klebsiella pneumoniae: A major worldwide source and shuttle for antibiotic resistance. FEMS Microbiol Rev. 2017;41(3):252–75.

24. Arato V, Raso MM, Gasperini G, Scorza FB, Micoli F. Prophylaxis and treatment against klebsiella pneumoniae: Current insights on this emerging anti-microbial resistant global threat. Int J Mol Sci. 2021;22(8).

25. Bush K, Jacoby GA. Updated functional classification of B-lactamases. Antimicrob Agents Chemother. 2010;54(3):969–76.

26. Sawa T, Kooguchi K, Moriyama K. Molecular diversity of extended-spectrum β-lactamases and carbapenemases, and antimicrobial resistance. J Intensive Care. 2020;8:13.

27. Jacoby GA. AmpC B-Lactamases. Clin Microbiol Rev. 2009;22(1):161–82.

28. Amaral L, Martins A, Spengler G, Molnar J. Efflux pumps of Gram-negative bacteria: What they do, how they do it, with what and how to deal with them. Front Pharmacol. 2014;4 JAN:168.

29. Yaghoubi S, Zekiy AO, Krutova M, Gholami M, Kouhsari E, Sholeh M, et al. Tigecycline antibacterial activity, clinical effectiveness, and mechanisms and epidemiology of resistance: narrative review. Eur J Clin Microbiol Infect Dis. 2021 Jan 5;

30. Wozniak A, Villagra NA, Undabarrena A, Gallardo N, Keller N, Moraga M, et al. Porin alterations present in non-carbapenemaseproducing Enterobacteriaceae with high and intermediate levels of carbapenem resistance in Chile. J Med Microbiol. 2012;61(PART 9):1270–9.

31. Martínez-Martínez L, Pascual Á, Hernández-Allés S, Alvarez-Díaz D, Suárez AI, Tran J, et al. Roles of β-lactamases and porins in activities of carbapenems and cephalosporins against Klebsiella pneumoniae. Antimicrob Agents Chemother. 1999;43(7):1669–73.

32. Dulyayangkul P, Ismah WAKWN, Douglas EJA, Avison MB. Mutation of kvrA causes OmpK35 and OmpK36 porin downregulation and reduced meropenem-vaborbactam susceptibility in KPC-Producing Klebsiella pneumoniae. Antimicrob Agents Chemother. 2020 Jun 23;64(7):e02208-19.

33. Nelson K, Hemarajata P, Sun D, Rubio-Aparicio D, Tsivkovski R, Yang S, et al. Resistance to ceftazidime-avibactam is due to transposition of KPC in a porin-deficient strain of Klebsiella pneumoniae with increased efflux activity. Antimicrob Agents Chemother. 2017;61(10).

34. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis. 2016 Feb 1;16(2):161–8.

35. Hussein NH, AL-Kadmy IMS, Taha BM, Hussein JD. Mobilized colistin resistance (mcr) genes from 1 to 10: a comprehensive review. Mol Biol Rep. 2021 Mar 1;48(3):2897–907.

36. Karaiskos I, Antoniadou A, Giamarellou H. Combination therapy for extensively-drug resistant gram-negative bacteria. Expert Rev Anti Infect Ther. 2017;15(12):1123–40.

37. Rodríguez-Baño J, Gutiérrez-Gutiérrez B, Machuca I, Pascual A. Treatment of infections caused by Extended-Spectrum-Beta-lactamase-, AmpC-, and carbapenemase-producing enterobacteriaceae. Clin Microbiol Rev. 2018;31(2):e00079-17.

38. Gutiérrez-Gutiérrez B, Salamanca E, de Cueto M, Hsueh PR, Viale P, Paño-Pardo JR, et al. Effect of appropriate combination therapy on mortality of patients with bloodstream infections due to carbapenemase-producing Enterobacteriaceae (INCREMENT): a retrospective cohort study. Lancet Infect Dis. 2017 Jul 1;17(7):726–34.

39. Poirel L, Jayol A, Nordmanna P. Polymyxins: Antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. Clin Microbiol Rev. 2017 Apr 1;30(2):557–96.

40. Sorlí L, Luque S, Grau S, Berenguer N, Segura C, Montero MM, et al. Trough colistin plasma level is an independent risk factor for nephrotoxicity: A prospective observational cohort study. BMC Infect Dis. 2013 Aug 19;13:380.

41. Kaye KS, Marchaim D, Thamlikitkul V, Carmeli Y, Chiu C-H, Daikos G, et al. Colistin Monotherapy versus Combination Therapy for Carbapenem-Resistant Organisms. NEJM Evid. 2022 Dec 6;2(1).

42. Satlin MJ, Lewis JS, Weinstein MP, Patel J, Humphries RM, Kahlmeter G, et al. Clinical and Laboratory Standards Institute and European Committee on Antimicrobial Susceptibility Testing Position Statements on Polymyxin B and Colistin Clinical Breakpoints. Clin Infect Dis. 2020 Nov 1;71(9):e523–9.

43. Rojas LJ, Salim M, Cober E, Richter SS, Perez F, Salata RA, et al. Colistin Resistance in Carbapenem-Resistant Klebsiella pneumoniae: Laboratory Detection and Impact on Mortality. Clin Infect Dis. 2017 Mar 15;64(6):711–8.

44. Balkan II, Alkan M, Aygün G, Kuşkucu M, Ankaralı H, Karagöz A, et al. Colistin resistance increases 28-day mortality in bloodstream infections due to carbapenem-resistant Klebsiella pneumoniae. Eur J Clin Microbiol Infect Dis. 2021 Oct 1;40(10):2161–70.

45. Tamma PD, Aitken SL, Bonomo RA, Mathers AJ, van Duin D, Clancy CJ. Infectious

Diseases Society of America Guidance on the Treatment of Extended-Spectrum β-lactamase Producing Enterobacterales (ESBL-E), Carbapenem-Resistant Enterobacterales (CRE), and Pseudomonas aeruginosa with Difficult-to-Treat Resistance (DTR-P. aerug. Clin Infect Dis. 2021;72(7):e169–83.

46. Falagas ME, Vouloumanou EK, Samonis G, Vardakasa KZ. Fosfomycin. Clin Microbiol Rev. 2016 Apr;29(2):321–47.

47. Castañeda-García A, Blázquez J, Rodríguez-Rojas A. Molecular Mechanisms and Clinical Impact of Acquired and Intrinsic Fosfomycin Resistance. Antibiotics. 2013 Apr 16;2(2):217–36.

48. Antonello RM, Principe L, Maraolo AE, Viaggi V, Pol R, Fabbiani M, et al. Fosfomycin as partner drug for systemic infection management. A systematic review of its synergistic properties from in vitro and in vivo studies. Antibiotics. 2020;9(8):500.

49. Michalopoulos AS, Livaditis IG, Gougoutas V. The revival of fosfomycin. Vol. 15, International Journal of Infectious Diseases. Int J Infect Dis; 2011. p. e732-9.

50. Ruiz Ramos J, Salavert Lletí M. Fosfomycin in infections caused by multidrug-resistant Gram-negative pathogens. Rev Esp Quimioter. 2019 May;32 Suppl 1(Suppl 1):45–54.

51. Thaden JT, Pogue JM, Kaye KS. Role of newer and re-emerging older agents in the treatment of infections caused by carbapenem-resistant Enterobacteriaceae. Virulence. 2017;8(4):403–16.

52. Xie J, Roberts JA, Alobaid AS, Roger C, Wang Y, Yang Q, et al. Population Pharmacokinetics of Tigecycline in Critically Ill Patients with Severe Infections. Antimicrob Agents Chemother. 2017 Aug 1;61(8):e00345-17.

53. Uppalapati SR, Sett A, Pathania R. The Outer Membrane Proteins OmpA, CarO, and OprD of Acinetobacter baumannii Confer a Two-Pronged Defense in Facilitating Its Success as a Potent Human Pathogen. Vol. 11, Frontiers in Microbiology. Frontiers Media S.A.; 2020. p. 2441.

54. Armstrong T, Fenn SJ, Hardie KR. JMM Profile: Carbapenems: a broad-spectrum antibiotic. J Med Microbiol. 2021;70(12):1462.

55. Mashni O, Nazer L, Le J. Critical Review of Double-Carbapenem Therapy for the Treatment of Carbapenemase-Producing Klebsiella pneumoniae. Ann Pharmacother. 2019;53(1):70–81.

56. Paul M, Carrara E, Retamar P, Tängdén T, Bitterman R, Bonomo RA, et al. European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guidelines for the treatment of infections caused by multidrug-resistant Gram-negative bacilli (endorsed by European society of intensive care medicine). Clin Microbiol Infect. 2022 Apr 1;28(4):521–47.

57. Shirley M. Ceftazidime-Avibactam: A Review in the Treatment of Serious Gram-Negative Bacterial Infections. Drugs. 2018 Apr;78(6):675–92.

58. Ackley R, Roshdy D, Meredith J, Minor S, Anderson WE, Capraro GA, et al. Meropenem-Vaborbactam versus Ceftazidime-Avibactam for Treatment of Carbapenem-Resistant Enterobacteriaceae Infections. Antimicrob Agents Chemother. 2020 Apr 21;64(5):e02313-19.

59. Shields RK, Chen L, Cheng S, Chavda KD, Press EG, Snyder A, et al. Emergence of

Ceftazidime-Avibactam Resistance Due to Plasmid-Borne bla KPC-3 Mutations during Treatment of Carbapenem-Resistant Klebsiella pneumoniae Infections. Antimicrob Agents Chemother. 2017 Mar;61(3):e02097-16.

60. Haidar G, Clancy CJ, Shields RK, Hao B, Cheng S, Nguyen MH. Mutations in blaKPC-3 That Confer Ceftazidime-Avibactam Resistance Encode Novel KPC-3 Variants That Function as Extended-Spectrum β-Lactamases. Antimicrob Agents Chemother. 2017 Apr;61(5):e02534-16.

61. Shields RK, Chen L, Cheng S, Chavda KD, Press EG, Snyder A, et al. Emergence of Ceftazidime-Avibactam Resistance Due to Plasmid-Borne blaKPC-3 Mutations during Treatment of Carbapenem-Resistant Klebsiella pneumoniae Infections. Antimicrob Agents Chemother. 2017 Feb;61(3):e02097-16.

62. Pogue JM, Bonomo RA, Kaye KS. Ceftazidime/Avibactam, Meropenem/Vaborbactam, or Both? Clinical and Formulary Considerations. Clin Infect Dis. 2019 Jan 18;68(3):519–24.

63. Heo YA. Imipenem/Cilastatin/Relebactam: A Review in Gram-Negative Bacterial Infections. Drugs. 2021;81(3):377–88.

64. Rodríguez-Avial I, Pena I, Picazo JJ, Rodríguez-Avial C, Culebras E. In vitro activity of the next-generation aminoglycoside plazomicin alone and in combination with colistin, meropenem, fosfomycin or tigecycline against carbapenemase-producing Enterobacteriaceae strains. In: International Journal of Antimicrobial Agents. Int J Antimicrob Agents; 2015. p. 616–21.

65. Solomkin JS, Sway A, Lawrence K, Olesky M, Izmailyan S, Tsai L. Eravacycline: A new treatment option for complicated intra-abdominal infections in the age of multidrug resistance. Future Microbiol. 2019;14(15):1293–308.

66. Aminov RI. A brief history of the antibiotic era: Lessons learned and challenges for the future. Front Microbiol. 2010;1:134.

67. Lee CR, Cho IH, Jeong BC, Lee SH. Strategies to minimize antibiotic resistance. Int J Environ Res Public Health. 2013 Sep 12;10(9):4274–305.

68. Lomovskaya O, Sun D, Rubio-Aparicio D, Nelson K, Tsivkovski R, Griffith DC, et al. Vaborbactam: Spectrum of Beta-Lactamase Inhibition and Impact of Resistance Mechanisms on Activity in Enterobacteriaceae. Antimicrob Agents Chemother. 2017;61(11):e01443-17.

69. Wu JY, Srinivas P, Pogue JM. Cefiderocol: A Novel Agent for the Management of Multidrug-Resistant Gram-Negative Organisms. Infect Dis Ther. 2020 Mar;9(1):17–40.

70. Butler MS, Gigante V, Sati H, Paulin S, Al-Sulaiman L, Rex JH, et al. Analysis of the Clinical Pipeline of Treatments for Drug-Resistant Bacterial Infections: Despite Progress, More Action Is Needed. Antimicrob Agents Chemother. 2022;66(3):e0199121.

71. Jackson N, Czaplewski L, Piddock LJ V. Discovery and development of new antibacterial drugs: learning from experience? J Antimicrob Chemother. 2018;73(6):1452–9.

72. Zhong H, Zhao XY, Zhang ZL, Gu ZC, Zhang C, Gao Y, et al. Evaluation of the efficacy and safety of ceftazidime/avibactam in the treatment of Gram-negative bacterial infections: a systematic review and meta-analysis. Int J Antimicrob Agents. 2018;52(4):443–50.

73. Wang L, Liu D, Lv Y, Cui L, Li Y, Li T, et al. Novel Plasmid-Mediated tet(X5) Gene Conferring Resistance to Tigecycline, Eravacycline, and Omadacycline in a Clinical

Acinetobacter baumannii Isolate. Antimicrob Agents Chemother. 2020 Dec 20;64(1):e01326-19.

74. Miethke M, Pieroni M, Weber T, Brönstrup M, Hammann P, Halby L, et al. Towards the sustainable discovery and development of new antibiotics. Vol. 5, Nature Reviews Chemistry. Nat Rev Chem; 2021. p. 726–49.

75. WHO. 2021 Antibacterial Agents in Clinical and Preclinical Development. World Health Organization. 2021.

76. Årdal C, Baraldi E, Theuretzbacher U, Outterson K, Plahte J, Ciabuschi F, et al. Insights into early stage of antibiotic development in small- and medium-sized enterprises: A survey of targets, costs, and durations. J Pharm Policy Pract. 2018;11(1):1–10.

77. Global AMR R&D Hub Dashboard [Internet]. 2020 [cited 2022 Jul 7]. Available from: https://dashboard.globalamrhub.org/reports/investments/overview

78. Serafin MB, Hörner R. Drug repositioning, a new alternative in infectious diseases. Brazilian J Infect Dis. 2018;22(3):252–6.

79. Pushpakom S, Iorio F, Eyers PA, Escott KJ, Hopper S, Wells A, et al. Drug repurposing: progress, challenges and recommendations. Nat Rev Drug Discov. 2019;18(1):41–58.

80. Cha Y, Erez T, Reynolds IJ, Kumar D, Ross J, Koytiger G, et al. Drug repurposing from the perspective of pharmaceutical companies. Br J Pharmacol. 2018;175(2):168–80.

81. Zheng W, Sun W, Simeonov A. Drug repurposing screens and synergistic drug-combinations for infectious diseases. Br J Pharmacol. 2018;175(2):181–91.

82. Khoshnood S, Goudarzi M, Taki E, Darbandi A, Kouhsari E, Heidary M, et al. Bedaquiline: Current status and future perspectives. J Glob Antimicrob Resist. 2021 Jun 1;25:48–59.

83. Sun W, Sanderson PE, Zheng W. Drug combination therapy increases successful drug repositioning. Drug Discov Today. 2016;21(7):1189–95.

84. García LS. Clinical Microbiology Procedures Handbook. 3rd ed. LSG & Associates, Santa Monica C, editor. Vol. 1. Washington, DC, USA: ASM Press; 2016.

85. White RL, Burgess DS, Manduru M, Bosso JA. Comparison of three different in vitro methods of detecting synergy: time-kill, checkerboard, and E test. Antimicrob Agents Chemother. 1996;40(8):1914–8.

86. Canut Blasco A, Collazos Blanco A, DíezAguilar M, Morosini Reilly MI, Alicia RG, Seral García C. Métodos microbiológicos para la determinación in vitro de la actividad de combinaciones de antimicrobianos. In: Procedimiento de Microbiología Clínica Recomendaciones de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica. 2020. p. 1–61.

87. Aaron SD, Ferris W, Henry DA, Speert DP, MacDonald NE. Multiple combination bactericidal antibiotic testing for patients with cystic fibrosis infected with Burkholderia cepacia. Am J Respir Crit Care Med. 2000 Dec 14;161(4 I):1206–12.

88. Berenbaum MC. A method for testing for synergy with any number of agents. J Infect Dis. 1978;137(2):122–30.

89. Cokol M, Kuru N, Bicak E, Larkins-Ford J, Aldridge BB. Efficient measurement and factorization of high-order drug interactions in Mycobacterium tuberculosis. Sci Adv. 2017;3(10).

90. Odds FC. Synergy, antagonism, and what the chequerboard puts between them. J Antimicrob Chemother. 2003;52(1):1.

91. Gómara M, Ramón-García S. The FICI paradigm: Correcting flaws in antimicrobial in vitro synergy screens at their inception. Biochem Pharmacol. 2019;163:299–307.

92. Sopirala MM, Mangino JE, Gebreyes WA, Biller B, Bannerman T, Balada-Llasat JM, et al. Synergy testing by etest, microdilution checkerboard, and time-kill methods for pan-drug-resistant Acinetobacter baumannii. Antimicrob Agents Chemother. 2010;54(11):4678–83.

93. Brennan-Krohn T, Pironti A, Kirby JE. Synergistic activity of colistin-containing combinations against colistin-resistant enterobacteriaceae. Antimicrob Agents Chemother. 2018;62(10):e00873-18.

94. Tängdén T, Hickman RA, Forsberg P, Lagerbäck P, Giske CG, Cars O. Evaluation of double- and triple-antibiotic combinations for VIM- and NDM-producing klebsiella pneumoniae by in vitro time-kill experiments. Antimicrob Agents Chemother. 2014;58(3):1757–62.

95. Ruth MM, Sangen JJN, Pennings LJ, Schildkraut JA, Hoefsloot W, Magis-Escurra C, et al. Minocycline has no clear role in the treatment of mycobacterium abscessus disease. Antimicrob Agents Chemother. 2018;62(10):62–5.

96. Eliopoulos GM, Moellering RC. Antimicrobial combinations. In: Lorian V, editor. Antibiotics in laboratory medicine. 4th ed. Baltimore, MD: The Williams & Wilkins Co.,; 1996. p. 330–96.

97. Velkov T, Bergen PJ, Lora-Tamayo J, Landersdorfer CB, Li J. PK/PD models in antibacterial development. Curr Opin Microbiol. 2013;16(5):573–9.

98. Cadwell J. The Hollow Fiber Infection Model for Antimicrobial Pharmacodynamics and Pharmacokinetics. Adv Pharmacoepidemiol Drug Saf. 2012;1(S1):1–5.

99. Cavaleri M, Manolis E. Hollow fiber system model for tuberculosis: The European Medicines Agency experience. Clin Infect Dis. 2015;61(Suppl 1):S1–4.

100. Bulitta JB, Hope WW, Eakin AE, Guina T, Tam VH, Louie A, et al. Generating Robust and Informative Nonclinical In Vitro and In Vivo Bacterial Infection Model Efficacy Data To Support Translation to Humans. Antimicrob Agents Chemother. 2019 May;63(5):e02307-18.

101. Pires De Mello CP, Drusano GL, Rodriquez JL, Kaushik A, Brown AN. Antiviral effects of clinically-relevant interferon-α and Ribavirin regimens against dengue virus in the Hollow Fiber Infection Model (HFIM). Viruses. 2018;10(6):1–12.

102. Brown AN, Adams JR, Baluya DL, Drusano GL. Pharmacokinetic determinants of virological response to raltegravir in the in vitro pharmacodynamic hollow-fiber infection model system. Antimicrob Agents Chemother. 2015;59(7):3771–7.

103. Deshpande D, Srivastava S, Pasipanodya JG, Gumbo T. Linezolid as treatment for pulmonary Mycobacterium avium disease. J Antimicrob Chemother. 2017;72(suppl_2):ii24–9.

104. Ruth MM, Raaijmakers J, van den Hombergh E, Aarnoutse R, Svensson EM, Susanto BO, et al. Standard therapy of Mycobacterium avium complex pulmonary disease shows limited efficacy in an open source hollow fibre system that simulates human plasma

and epithelial lining fluid pharmacokinetics. Clin Microbiol Infect. 2022 Mar 1;28(3):448.e1-448.e7.

105. EUCAST. The European Committee on Antimicrobial Susceptibility Testing. EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and / or epidemiological importance. Version 2.0. 2017.

106. CLSI. Performance Standards for Antimicrobial Susceptibility Testing: 27th edition. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2017.

107. Montoro E, Lemus D, Echemendia M, Martin A, Portaels F, Palomino JC. Comparative evaluation of the nitrate reduction assay, the MTT test, and the resazurin microtitre assay for drug susceptibility testing of clinical isolates of Mycobacterium tuberculosis. J Antimicrob Chemother. 2005 Apr;55(4):500–5.

108. Ramón-García S, Ng C, Anderson H, Chao JD, Zheng X, Pfeifer T, et al. Synergistic drug combinations for tuberculosis therapy identified by a novel high-throughput screen. Antimicrob Agents Chemother. 2011;55(8):3861–9.

109. EUCAST. European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 13.0. 2023.

110. International Organization for Standardization. Biological evaluation of medical devices. Part 5: Tests for in vitro cytotoxicity. The British Standards Institution 2021 Geneva, Switzerland; 1996 p. 54.

111. Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ, et al. Cell Viability Assays. Assay Guid Man [Internet]. 2013;114(8):785–96.

112. Borisy AA, Elliott PJ, Hurst NW, Lee MS, Lehár J, Price ER, et al. Systematic discovery of multicomponent therapeutics. Proc Natl Acad Sci U S A. 2003 Jun 6;100(13):7977–82.

113. Shinn P, Chen L, Ferrer M, Itkin Z, Klumpp-Thomas C, McKnight C, et al. High-throughput screening for drug combinations. Methods Mol Biol. 2019;1939:11–35.

114. Wambaugh MA, Shakya VPS, Lewis AJ, Mulvey MA, Brown JCS. High-throughput identification and rational design of synergistic small-molecule pairs for combating and bypassing antibiotic resistance. PLoS Biol. 2017;15(6).

115. Ramón-García S, González Del Río R, Villarejo AS, Sweet GD, Cunningham F, Barros D, et al. Repurposing clinically approved cephalosporins for tuberculosis therapy. Sci Rep. 2016;6:34293.

116. Hind CK, Dowson CG, Sutton JM, Jackson T, Clifford M, Garner RC, et al. Evaluation of a library of FDA-approved drugs for their ability to potentiate antibiotics against multidrug-resistant gram-negative pathogens. Antimicrob Agents Chemother. 2019;63(8):e00769-19.

117. Younis W, AbdelKhalek A, Mayhoub AS, Seleem MN. In Vitro Screening of an FDA-Approved Library Against ESKAPE Pathogens. Curr Pharm Des. 2017;23(14):2147–57.

118. Hallander HO, Dornbusch K, Gezelius L, Jacobson K, Karlsson I. Synergism between aminoglycosides and cephalosporins with antipseudomonal activity: Interaction index and killing curve method. Antimicrob Agents Chemother. 1982;22(5):743–52.

119. Maity S, Chintaparthi M, M H, Kanta R, Kapur I. In vitro antimicrobial activity of cefsulodin and kanamycin in combinations. Int J Res Med Sci. 2014;2(2):677–80.

120. Gohil N, Bhattacharjee G, Singh V. Synergistic bactericidal profiling of prodigiosin extracted from Serratia marcescens in combination with antibiotics against pathogenic bacteria. Microb Pathog. 2020;149:104508.

121. Kastoris AC, Rafailidis PI, Vouloumanou EK, Gkegkes ID, Falagas ME. Synergy of fosfomycin with other antibiotics for Gram-positive and Gram-negative bacteria. Eur J Clin Pharmacol. 2010;66(4):359–68.

122. Flamm RK, Rhomberg PR, Lindley JM, Sweeney K, Ellis-Grosse EJ, Shortridge D. Evaluation of the bactericidal activity of fosfomycin in combination with selected antimicrobial comparison agents tested against Gram-negative bacterial strains by using time-kill curves. Antimicrob Agents Chemother. 2019;63(5):e02549-18.

123. Ni W, Yang D, Guan J, Xi W, Zhou D, Zhao L, et al. In vitro and in vivo synergistic effects of tigecycline combined with aminoglycosides on carbapenem-resistant Klebsiella pneumoniae. J Antimicrob Chemother. 2021;76(8):2097–105.

124. Liu E, Jia P, Li X, Zhou M, Kudinha T, Wu C, et al. In vitro and in vivo effect of antimicrobial agent combinations against carbapenem-resistant Klebsiella pneumoniae with different resistance mechanisms in China. Infect Drug Resist. 2021;14:917–28.

125. Petersen PJ, Labthavikul P, Jones CH, Bradford PA. In vitro antibacterial activities of tigecycline in combination with other antimicrobial agents determined by chequerboard and time-kill kinetic analysis. J Antimicrob Chemother. 2006;57(3):573–6.

126. Ontong JC, Ozioma NF, Voravuthikunchai SP, Chusri S. Synergistic antibacterial effects of colistin in combination with aminoglycoside, carbapenems, cephalosporins, fluoroquinolones, tetracyclines, fosfomycin, and piperacillin on multidrug resistant Klebsiella pneumoniae isolates. PLoS One. 2021;16(1):e0244673.

127. Samonis G, Maraki S, Karageorgopoulos DE, Vouloumanou EK, Falagas ME. Synergy of fosfomycin with carbapenems, colistin, netilmicin, and tigecycline against multidrug-resistant Klebsiella pneumoniae, Escherichia coli, and Pseudomonas aeruginosa clinical isolates. Eur J Clin Microbiol Infect Dis. 2012;31(5):695–701.

128. Lingscheid T, Tobudic S, Poeppl W, Mitteregger D, Burgmann H. In vitro activity of doripenem plus fosfomycin against drug-resistant clinical blood isolates. Pharmacology. 2013;91(3–4):214–8.

129. Principe L, D'Arezzo S, Capone A, Petrosillo N, Visca P. In vitro activity of tigecycline in combination with various antimicrobials against multidrug resistant Acinetobacter baumannii. Ann Clin Microbiol Antimicrob. 2009;8:18.

130. Demoro B, Rostán S, Moncada M, Li ZH, Docampo R, Olea Azar C, et al. Ibandronate metal complexes: solution behavior and antiparasitic activity. J Biol Inorg Chem. 2018 Mar;23(2):303–12.

131. Santos JCB, De Melo JA, Maheshwari S, Toscano Queiroz De Medeiros WM, Oliveira JWDF, Moreno CJ, et al. Bisphosphonate-based molecules as potential new antiparasitic drugs. Molecules. 2020 Jun 3;25(11):2602.

132. Zeng X, She P, Zhou L, Li S, Hussain Z, Chen L, et al. Drug repurposing: Antimicrobial and antibiofilm effects of penfluridol against Enterococcus faecalis. Microbiologyopen. 2021 Jan;10(1):e1148.

133. Chaccour C, Rabinovich NR. Advancing the repurposing of ivermectin for malaria. Lancet. 2019 Apr 13;393(10180):1480–1.

134. Rizzo E. Ivermectin, antiviral properties and COVID-19: a possible new mechanism of action. Naunyn Schmiedebergs Arch Pharmacol. 2020 Jul;393(7):1153–6.

135. Wagstaff KM, Sivakumaran H, Heaton SM, Harrich D, Jans DA. Ivermectin is a specific inhibitor of importin α/β-mediated nuclear import able to inhibit replication of HIV-1 and dengue virus. Biochem J. 2012 May 1;443(3):851–6.

136. Lim LE, Vilchèze C, Ng C, Jacobs WR, Ramón-García S, Thompson CJ. Anthelmintic avermectins kill Mycobacterium tuberculosis, including multidrug-resistant clinical strains. Antimicrob Agents Chemother. 2013;57(2):1040–6.

137. Muñoz-Muñoz L, Shoen C, Sweet G, Vitoria A, Bull TJ, Cynamon M, et al. Repurposing Avermectins and Milbemycins against Mycobacteroides abscessus and Other Nontuberculous Mycobacteria. Antibiotics. 2021 Apr 3;10(4):381.

138. Scherr N, Pluschke G, Thompson CJ, Ramón-García S. Selamectin is the avermectin with the best potential for Buruli ulcer treatment. PLoS Negl Trop Dis. 2015;9(8):e0003996.

139. Doléans-Jordheim A, Bergeron E, Bereyziat F, Ben-Larbi S, Dumitrescu O, Mazoyer MA, et al. Zidovudine (AZT) has a bactericidal effect on enterobacteria and induces genetic modifications in resistant strains. Eur J Clin Microbiol Infect Dis. 2011;30(10):1249–56.

140. Thomson JM, Lamont IL. Nucleoside Analogues as Antibacterial Agents. Front Microbiol. 2019 May 22;10:952.

141. Lin YW, Rahim NA, Zhao J, Han ML, Yu HH, Wickremasinghe H, et al. Novel polymyxin combination with the antiretroviral zidovudine exerts synergistic killing against NDM-producing multidrug-resistant Klebsiella pneumoniae. Antimicrob Agents Chemother. 2019 Mar 27;63(4):e02176-18.

142. Lewin CS, Allen RA, Amyes SG. Antibacterial activity of fluoroquinolones in combination with zidovudine. J Med Microbiol. 1990;33(2):127–31.

143. Ng SMS, Sioson JSP, Yap JM, Ng FM, Ching HSV, Teo JWP, et al. Repurposing Zidovudine in combination with Tigecycline for treating carbapenem-resistant Enterobacteriaceae infections. Eur J Clin Microbiol Infect Dis. 2018 Jan;37(1):141–8.

144. Hu Y, Liu Y, Coates A. Azidothymidine produces synergistic activity in combination with colistin against antibiotic-resistant Enterobacteriaceae. Antimicrob Agents Chemother. 2018;63(1):e01630-18.

145. Antonello RM, Di Bella S, Betts J, La Ragione R, Bressan R, Principe L, et al. Zidovudine in synergistic combination with fosfomycin: an in vitro and in vivo evaluation against multidrug-resistant Enterobacterales. Int J Antimicrob Agents. 2021;58(1):106362.

146. Yue C, Shen W, Hu L, Liu Y, Zheng Y, Ye Y, et al. Effects of tigecycline combined with azithromycin against biofilms of multidrug-resistant Stenotrophomonas maltophilia isolates from a patient in China. Infect Drug Resist. 2021;14:775–86.

147. Li Y, Lin X, Yao X, Huang Y, Liu W, Ma T, et al. Synergistic antimicrobial activity of colistin in combination with rifampin and azithromycin against Escherichia coli producing MCR-1. Antimicrob Agents Chemother. 2018;62(12):e01631-18.

148. Kumaraswamy M, Lin L, Olson J, Sun CF, Nonejuie P, Corriden R, et al. Standard susceptibility testing overlooks potent azithromycin activity and cationic peptide synergy against MDR Stenotrophomonas maltophilia. J Antimicrob Chemother. 2016 May;71(5):1264–9.

149. Olay T, Rodriguez A, Oliver LE, Vicente M V., Quecedo MCR. Interaction of fosfomycin with other antimicrobial agents: In vitro and in vivo studies. J Antimicrob Chemother. 1978 Nov;4(6):569–76.

150. Peepliwal A, Vyawahare SD, Bonde CG. A quantitative analysis of Zidovudine containing formulation by FT-IR and UV spectroscopy. Anal Methods. 2010 Nov;2(11):1756–63.

151. Keith BR, White G, Wilson HR. In vivo efficacy of zidovudine (3'-azido-3'-deoxythymidine) in experimental gram-negative-bacterial infections. Antimicrob Agents Chemother. 1989;33(4):479–83.

152. Falagas ME, Voulgaris GL, Tryfinopoulou K, Giakkoupi P, Kyriakidou M, Vatopoulos A, et al. Synergistic activity of colistin with azidothymidine against colistin-resistant Klebsiella pneumoniae clinical isolates collected from inpatients in Greek hospitals. Int J Antimicrob Agents. 2019 Jun;53(6):855–8.

153. Pertusati F, Pileggi E, Richards J, Wootton M, Van Leemputte T, Persoons L, et al. Drug repurposing: phosphate prodrugs of anticancer and antiviral FDA-approved nucleosides as novel antimicrobials. J Antimicrob Chemother. 2020;75:2864–78.

154. Wei L, Mansoor N, Khan RA, Czejka M, Ahmad T, Ahmed M, et al. WB-PBPK approach in predicting zidovudine pharmacokinetics in preterm neonates. Biopharm Drug Dispos. 2019;40(9):341–9.

155. Fillekes Q, Kendall L, Kitaka S, Mugyenyi P, Musoke P, Ndigendawani M, et al. Pharmacokinetics of zidovudine dosed twice daily according to World Health Organization weight bands in Ugandan HIV-infected children. Pediatr Infect Dis J. 2014;33(5):495–8.

156. Moore KHP, Raasch RH, Brouwer KLR, Opheim K, Cheeseman SH, Eyster E, et al. Pharmacokinetics and bioavailability of zidovudine and its glucuronidated metabolite in patients with human immunodeficiency virus infection and hepatic disease (AIDS clinical trials group protocol 062). Antimicrob Agents Chemother. 1995;39(12):2732–7.

157. Rachlis A, Fanning MM. Zidovudine Toxicity: Clinical Features and Management. Drug Saf. 1993;8(4):312–20.

158. McLeod GX, Hammer SM. Zidovudine: Five Years Later. Ann Intern Med. 1992 Sep 15;117(6):487–501.

159. Kroon S, Worm AM. Zidovudine overdose. Int J STD AIDS. 1991;2(1):56–7.

160. EUCAST. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 11.0. 2021.

161. EUCAST. European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 12.0. 2022.

162. Arenaz-Callao MP, González del Río R, Lucía Quintana A, Thompson CJ, Mendoza-Losana A, Ramón-García S. Triple oral beta-lactam containing therapy for Buruli ulcer treatment shortening. PLoS Negl Trop Dis. 2019;13(1):e0007126.

163. Rodvold KA, Gotfried MH, Cwik M, Korth-Bradley JM, Dukart G, Ellis-Grosse EJ. Serum, tissue and body fluid concentrations of tigecycline after a single 100 mg dose. J Antimicrob Chemother. 2006;58(6):1221–9.

164. Zhao M, Bulman ZP, Lenhard JR, Satlin MJ, Kreiswirth BN, Walsh TJ, et al.

Pharmacodynamics of colistin and fosfomycin: A “treasure trove” combination combats KPC-producing Klebsiella pneumoniae. J Antimicrob Chemother. 2017;72(7):1985–90.

165. Wang J, He JT, Bai Y, Wang R, Cai Y. Synergistic activity of colistin/fosfomycin combination against carbapenemase-producing Klebsiella pneumoniae in an in vitro pharmacokinetic/pharmacodynamic model. Biomed Res Int. 2018 Apr 23;2018:5720417.

166. Michalopoulos A, Virtzili S, Rafailidis P, Chalevelakis G, Damala M, Falagas ME. Intravenous fosfomycin for the treatment of nosocomial infections caused by carbapenem-resistant Klebsiella pneumoniae in critically ill patients: a prospective evaluation. Clin Microbiol Infect. 2010 Feb;16(2):184–6.

167. Pontikis K, Karaiskos I, Bastani S, Dimopoulos G, Kalogirou M, Katsiari M, et al. Outcomes of critically ill intensive care unit patients treated with fosfomycin for infections due to pandrug-resistant and extensively drug-resistant carbapenemase-producing Gram-negative bacteria. Int J Antimicrob Agents. 2014 Jan;43(1):52–9.

168. Loose M, Naber KG, Hu Y, Coates A, Wagenlehner FME. Serum bactericidal activity of colistin and azidothymidine combinations against mcr-1-positive colistin-resistant Escherichia coli. Int J Antimicrob Agents. 2018 Dec 1;52(6):783–9.

169. Loose M, Naber KG, Hu Y, Coates A, Wagenlehner FME. Urinary bactericidal activity of colistin and azidothymidine combinations against mcr-1-positive colistin-resistant Escherichia coli. Int J Antimicrob Agents. 2019 Jul;54(1):55–61.

170. Buckner MMC, Laura Ciusa M, Meek RW, Moorey AR, McCallum GE, Prentice EL, et al. HIV drugs inhibit transfer of plasmids carrying extended-spectrum β-lactamase and carbapenemase genes. MBio. 2020 Feb 25;11(1):e03355-19.

171. Maziarz EK, Perfect JR. Cryptococcosis. Infect Dis Clin North Am. 2016 Mar;30(1):179–206.

172. Peyclit L, Khedher M Ben, Zerrouki L, Diene SM, Baron SA, Rolain JM. Inactivation of thymidine kinase as a cause of resistance to zidovudine in clinical isolates of Escherichia coli: A phenotypic and genomic study. J Antimicrob Chemother. 2020;75(6):1410–4.

173. Parnham MJ, Haber VE, Giamarellos-Bourboulis EJ, Perletti G, Verleden GM, Vos R, et al. Azithromycin: Mechanisms of action and their relevance for clinical applications. Pharmacol Ther. 2014;143(2):225–45.

174. Firth A, Prathapan P. Azithromycin: The First Broad-spectrum Therapeutic. Eur J Med Chem. 2020;207:112739.

175. Afshar M, Foster CL, Layden JE, Burnham EL. Azithromycin use and outcomes in severe sepsis patients with and without pneumonia. J Crit Care. 2016 Apr;32:120–5.

176. Nichols DP, Odem-Davis K, Cogen JD, Goss CH, Ren CL, Skalland M, et al. Pulmonary Outcomes Associated with Long-Term Azithromycin Therapy in Cystic Fibrosis. Am J Respir Crit Care Med. 2020 Feb 15;201(4):430–7.

177. Saiman L, Schechter MS. Evaluating long-term benefits of chronic azithromycin furthering our quest for precision medicine. Am J Respir Crit Care Med. 2020 Feb 15;201(4):398–400.

178. Wang X, Luo J, Wang D, Liu B, Liu C. The efficacy and safety of long-term add-on treatment of azithromycin in asthma. Medicine (Baltimore). 2019 Sep;98(38):e17190.

179. Cui Y, Luo L, Li C, Chen P, Chen Y. Long-term macrolide treatment for the prevention of acute exacerbations in COPD: a systematic review and meta-analysis. Int J Chron Obstruct Pulmon Dis. 2018 Nov;Volume 13:3813–29.

180. Rosenthal PJ. Azithromycin for Malaria? Am J Trop Med Hyg. 2016 Jul 6;95(1):2–4.

181. Krause PJ, Lepore T, Sikand VK, Gadbaw J, Burke G, Telford SR, et al. Atovaquone and azithromycin for the treatment of babesiosis. N Engl J Med. 2000 Nov 16;343(20):1454–8.

182. Oliver ME, Hinks TSC. Azithromycin in viral infections. Rev Med Virol. 2021 Mar;31(2):e2163.

183. Hinks TSC, Cureton L, Knight R, Wang A, Cane JL, Barber VS, et al. Azithromycin versus standard care in patients with mild-to-moderate COVID-19 (ATOMIC2): an open-label, randomised trial. Lancet Respir Med. 2021 Oct 1;9(10):1130–40.

184. Cavalcanti AB, Zampieri FG, Rosa RG, Azevedo LCP, Veiga VC, Avezum A, et al. Hydroxychloroquine with or without Azithromycin in Mild-to-Moderate Covid-19. N Engl J Med. 2020 Nov 19;383(21):2041–52.

185. Li H, Liu DH, Chen LL, Zhao Q, Yu YZ, Ding JJ, et al. Meta-analysis of the adverse effects of long-term azithromycin use in patients with chronic lung diseases. Antimicrob Agents Chemother. 2014;58(1):511–7.

186. Smith C, Egunsola O, Choonara I, Kotecha S, Jacqz-Aigrain E, Sammons H. Use and safety of azithromycin in neonates: a systematic review. BMJ Open. 2015;5(12):e008194.

187. Gomes C, Martínez-Puchol S, Palma N, Horna G, Ruiz-Roldán L, Pons MJ, et al. Macrolide resistance mechanisms in Enterobacteriaceae: Focus on azithromycin. Crit Rev Microbiol. 2017;43(1):1–30.

188. Imperi F, Leoni L, Visca P. Antivirulence activity of azithromycin in Pseudomonas aeruginosa. Front Microbiol. 2014 Apr 22;5:178.

189. Lin L, Nonejuie P, Munguia J, Hollands A, Olson J, Dam Q, et al. Azithromycin Synergizes with Cationic Antimicrobial Peptides to Exert Bactericidal and Therapeutic Activity Against Highly Multidrug-Resistant Gram-Negative Bacterial Pathogens. EBioMedicine. 2015 Jun 10;2(7):690–8.

190. EUCAST. European Committee on Antimicrobial Susceptibility Testing. Data from the EUCAST MIC distribution website. [Internet]. 2018 [cited 2022 Jan 14]. Available from: https://mic.eucast.org/

191. Presterl E, Hajdu S, Lassnigg AM, Hirschl AM, Holinka J, Graninger W. Effects of azithromycin in combination with vancomycin, daptomycin, fosfomycin, tigecycline, and ceftriaxone on Staphylococcus epidermidis biofilms. Antimicrob Agents Chemother. 2009 Aug;53(8):3205–10.

192. Hauser C, Hirzberger L, Unemo M, Furrer H, Endimiani A. In vitro activity of fosfomycin alone and in combination with ceftriaxone or azithromycin against clinical Neisseria gonorrhoeae isolates. Antimicrob Agents Chemother. 2015 Mar;59(3):1605–11.

193. Baker KR, Jana B, Hansen AM, Vissing KJ, Nielsen HM, Franzyk H, et al. Repurposing azithromycin and rifampicin against Gram-negative pathogens by combination with peptide potentiators. Int J Antimicrob Agents. 2019;53(6):868–72.

194. Dillon N, Holland M, Tsunemoto H, Hancock B, Cornax I, Pogliano J, et al. Surprising synergy of dual translation inhibition vs. Acinetobacter baumannii and other multidrug-resistant bacterial pathogens. EBioMedicine. 2019 Aug;46:193–201.

195. Fuentes F, Izquierdo J, Martín MM, Gomez-Lus ML, Prieto J. Postantibiotic and sub-MIC effects of azithromycin and isepamicin against Staphylococcus aureus and Escherichia coli. Antimicrob Agents Chemother. 1998 Feb;42(2):414–8.

196. Odenholt-Tornqvist I, Lowdin E, Cars O. Postantibiotic effects and postantibiotic sub-MIC effects of roxithromycin, clarithromycin, and azithromycin on respiratory tract pathogens. Antimicrob Agents Chemother. 1995;39(1):221–6.

197. Metlay JP, Waterer GW, Long AC, Anzueto A, Brozek J, Crothers K, et al. Diagnosis and treatment of adults with community-acquired pneumonia. Am J Respir Crit Care Med. 2019 Oct 1;200(7):e45–67.

198. Patil SV, Hajare AL, Patankar M, Krishnaprasad K. In vitro fractional inhibitory concentration (FIC) study of cefixime and azithromycin fixed dose combination (FDC) against respiratory clinical isolates. J Clin Diagnostic Res. 2015 Feb 1;9(2):DC13–5.

199. Yoshioka D, Kajiwara C, Ishii Y, Umeki K, Hiramatsu K, Kadota JI, et al. Efficacy of β-lactam-plus-macrolide combination therapy in a mouse model of lethal pneumococcal pneumonia. Antimicrob Agents Chemother. 2016 Sep 23;60(10):6146–54.

200. Wang A, Wang Q, Kudinha T, Xiao S, Zhuo C. Effects of Fluoroquinolones and Azithromycin on Biofilm Formation of Stenotrophomonas maltophilia. Sci Rep. 2016 Jul 13;6:29701.

ANNEXES

ANNEX I. *The FICI paradigm: Correcting flaws in antimicrobial in vitro synergy screens at their inception.*

This annex includes a review article in which I participated as first author, entitled *The FICI paradigm: Correcting flaws in antimicrobial in vitro synergy screens at their inception.* This paper was accepted the 1st of March of 2019 and published in Biochemical Pharmacology in May of 2019.

This Commentary discusses some issues related to the traditional process of synergy studies, commonly based on inhibition parameters that may not represent the true interaction between compounds. Here we highlight the importance of obtaining parameters of bactericidal activity, such as those obtained by determining the MBC value or by time-kill assays, which reveal more valuable information about the efficacy of the interactions. Due to the still lack of standardized *in vitro* synergy methods, we believe that these assays should govern the decision to prioritize those interactions that merit further validation.

Biochemical Pharmacology 163 (2019) 299–307

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Commentary

The FICI paradigm: Correcting flaws in antimicrobial *in vitro* synergy screens at their inception

Marta Gómara[a], Santiago Ramón-García[a,b,c,*]

[a] Mycobacterial Genetics Group, Department of Microbiology, Preventive Medicine and Public Health. Faculty of Medicine, University of Zaragoza, Spain
[b] Research & Development Agency of Aragon (ARAID) Foundation, Spain
[c] CIBER Respiratory Diseases, Carlos III Health Institute, Madrid, Spain

ARTICLE INFO

Keywords:
Antimicrobial resistance
Synergy
Repurposing
FICI
MIC/MBC
Sterilization

ABSTRACT

Antibiotics have become the corner stone of modern medicine. However, our society is currently facing one of the greatest challenges of its time: the emergence of antimicrobial resistance. It is estimated that if no new therapies are implemented by 2050, 10 million people will die worldwide every year as a result of infections caused by bacteria resistant to current antibiotics; new antimicrobials are thus urgently needed. However, drug development is a tedious and very costly endeavor of hundreds of millions that can take up to 15–20 years from the bench discovery to the bedside. Under this scenario, drug repurposing, which consists in identifying new uses for old, clinically approved drugs, has gathered momentum within the pharmaceutical industry. Because most of these drugs have safety and toxicity information packages available, clinical evaluation could be done in a much shorter period than standard timelines. Synergistic combinations of these clinically approved drugs could also be a promising approach to identify novel antimicrobial therapies that might provide rational choices of available drugs to shorten treatment, increase efficacy, reduce toxicity, prevent resistance and treat infections caused by drug-resistant strains. However, although simple in its conception, translating results from *in vitro* synergy screens into *in vivo* efficacy or the clinical practice has proven to be a paramount challenge.

In this Commentary, we will discuss common flaws at the inception of synergy research programs, with a special focus on the use of the Fractional Inhibitory Concentration Index (FICI), and evaluate potential interventions that can be made at different developmental pre-clinical stages in order to improve the odds of translation from *in vitro* studies.

1. The emerging problem of worldwide antimicrobial resistance

Antimicrobial resistance (AMR) is alarmingly increasing worldwide. > 700,000 people die every year due to common bacterial infections caused by drug-resistant strains. In Europe, these pathogens cause at least 25,000 deaths per year, entailing healthcare expenditures and productivity losses [1]. In the USA death rate is set on > 23,000 deaths per year, if we include sepsis, these figures dramatically increase up to 210,000 deaths, and to 5 million losses worldwide [2]. This situation is of extreme concern since if no new antibiotics are implemented by 2050, 10 million people will die every year as a result of infections caused by antibiotic resistant bacteria [3].

Despite our inability to treat these infections and the urgent need for new therapies, pharmaceutical companies do not see as profitable investment programs focusing on the discovery of new antimicrobials [4], which are considered tedious and costly endeavors that can take up to 15–20 years from hit discovery to a commercialized drug. As such, the number of new antibiotics approved by the FDA or the EMA in the past few years is not enough to counteract the rise of antimicrobial resistance; indeed, due to the AMR emergence, we have now fewer therapeutic options available in the clinical practice than a decade ago [5].

The emergence of bacteria with multi drug-resistant (MDR) and extensively drug-resistant (XDR) phenotypes to several antimicrobial classes seriously limits therapeutic options in the clinical practice. As a result, physicians are forced to improvise regimens combining the few available options. For example, infections caused by carbapenemase-producing enterobacteria (CPE) are treated in most cases by combination therapies using colistin, tigecycline, carbapenems, aminoglycosides or fosfomycin; however, such regimens are often guided by a small

* Corresponding author at: Department of Microbiology, Preventive Medicine and Public Health, Faculty of Medicine, University of Zaragoza, C/ Domingo Miral s/n, 50009 Zaragoza, Spain.
E-mail address: santiramon@unizar.es (S. Ramón-García).

https://doi.org/10.1016/j.bcp.2019.03.001
Received 3 December 2018; Accepted 1 March 2019
Available online 02 March 2019
0006-2952/

number of studies lacking robust evidence [6,7]. In the case of MDR and XDR strains of tuberculosis (TB) only just recently the World Health Organization (WHO) issued a communication on treatment guidance based on available clinical trial data [8]. Before this, treatment of MDR/XDR-TB was performed empirically based on strain resistant profiles and drug availability in the clinics; even though, there is still a high degree of uncertainty on combinatorial treatment outcomes [8].

Combination therapy was standard procedure soon after chemotherapy was introduced for TB treatment in order to reduce the emergence of drug resistance, reduce the length of treatment and improve therapeutic outcomes; this is also an approach widely used for HIV therapy. On the contrary, infections caused by Gram-positive or Gram-negative bacteria are still mainly treated in monotherapy and combination therapy is only considered as a last resource strategy for difficult to treat infections caused by drug-resistant strains. Current trends in antimicrobial discovery mainly focus on the development of new compounds with novel modes of action as single agents. However, because emergence of bacterial resistance to any newly approved antibiotic would eventually occur, more basic, translational and clinical studies, and new therapeutic approaches are urgently needed. This would enable optimization of drug combinations and dosing strategies to shorten treatment, increase efficacy, reduce toxicity, prevent the emergence of resistance to new antibiotics, and treat infections caused by drug-resistant strains.

2. Antimicrobial discovery: focusing on new approaches

Antimicrobial discovery and development is lengthy and costly with numerous development stages that include, among others, small-molecule high-throughput hit discovery screens, lead optimization campaigns, preclinical studies of drug candidates and clinical trials evaluation. Discovery and development programs often do not obtain the expected financial return, with only few molecules advancing to late-stage development due to high attrition rates [9,10]. Under this scenario, innovative alternatives to make more efficient the antimicrobial discovery and development process are urgently needed. One such strategy is the development on new therapies based of two emerging concepts: drug repurposing and synergistic drug combinations.

2.1. Drug repurposing

Drug repurposing (or drug repositioning) is a promising approach to identify new properties in compounds commercialized for other clinical indications. Initially not considered a profitable area by the pharmaceutical industry because most drugs were out of patent protection, it is gaining momentum due to the high costs of the traditional drug discovery process and the opportunity to warrant further priority protection on intellectual property [11]. A faster approach in theory, drug repurposing also encompasses studies specific to its intrinsic process, i.e. repurposed drugs need to be dose optimized for the new therapeutic indication, which might include additional safety, dose prediction studies and safety phase I trial at the new indicated dose. In the case of drugs repurposed as antimicrobials, the new effective antibacterial dose would be typically much higher than the actual recommended clinical dose for the old indication, thus limiting its use in the clinic due to safety concerns, regardless of any promising *in vitro* activity. A strategy to overcome this limitation would be to combine them within synergistic drug combinations, thus reducing the effective dose needed and related toxicity [12]. However, even this promising approach might also come with key translational limitations that would be further discuss below.

Drug repurposing has been studied in the field of infectious diseases at the pre-clinical proof-of-concept level: flubendazole, niclosamide and the avermectins are anti-parasitic drugs that showed *in vitro* activity against non-parasitic infections such as cryptococcosis, the Zika virus and mycobacterial infections, respectively [13–16]; enoxacin, a fluoroquinolone, was identified as an antifungal drug [17]; and delamanid, an anti-tuberculosis drug, as a potential agent against visceral leishmaniasis [18]. Similarly, drugs used for other indications not related to infection have also repurposing potential against some pathogens: statins were proposed for the treatment of *Trypanosoma cruzi* [19] and bacterial skin infections [20]; calcium channel blockers against fungal diseases [13]; and loperamide, an anti-diarrhea drug, might have a potential role in combination therapy with minocycline to treat *Salmonella enterica* infections [21]. Although promising, to date there are no reports on their translational clinical potential and efficacy, the major roadblock of the repurposing approach.

In summary, drug repurposing has the potential to shorten drug development since pharmacological, safety and preclinical data for the new identified drug are readily available and manufacturing infrastructure needed for quick implementation already in place. This could facilitate and accelerate the development process; however, specific issues related to the new indication of the repurposed drug need to be addressed early to limit attrition rates and improve regulatory approval outcomes.

2.2. Synergistic drug combinations

Synergy between two (or more) drugs occurs when their activities within the combination are improved over the sum of their individual separate effects. This approach has been extensively studied and continues to be the backbone of some current clinical antimicrobial therapies, such as infective endocarditis caused by *Enterococcus* and *Streptococcus viridans* species, where synergy is observed among beta-lactams and aminoglycosides [22] or in the case of the widely used combination of amoxicillin plus clavulanic acid [23]. Typically, a synergistic effect can be achieved: (*i*) by non-specific, off-target pharmacological drug-drug interactions, known as “promiscuous synergy” [24], i.e. increasing drug bioavailability and exposure of one of the drugs in the combination where the endpoint is a therapeutic improvement; and (*ii*) when two drugs target complimentary pathways that together inhibit essential cellular processes, also known as “specific synergy”. As non-specific synergy cannot be readily tested in primary *in vitro* microbiology assays, it is thus out of scope of this Commentary and, we will refer only to specific synergy.

Synergistic drug combinations including repurposed drugs allow thus the use of lower concentrations of both compounds with the potential to improve therapy outcomes by an increase in efficacy and a reduction of side effects [12]. They have also the potential to prevent the emergence of antimicrobial resistance to the individual drugs in the combination, thus prolonging the use of our limited antibiotic armamentarium. Additionally, synergistic combinations might reintroduce drugs that have lost activity against drug resistant strains; for example, MDR/XDR-TB strains could be potentially re-sensitized to clinically achievable levels of rifampicin, the cornerstone drug for TB therapy, when treated in combination with beta-lactams [25,26]. However, although promising, the use of synergistic drug combinations might also face severe limitations in their *in vitro* to *in vivo* and clinical translational potential that are hardly addresses early in the discovery process. Therapeutic effectiveness derived from *in vitro* studies needs to be correlated with pharmacological parameters; drug-drug interactions (DDI) potentially leading to toxicity or lack of efficacy of the combination need to be identified early by means of the extensive pre-clinical and clinical data available on both synergistic partners. For example, DDI typically occur through cytochrome P450 (CYP)-3A4 substrates, among other mechanisms. In a screening effort to identify enhancers of the activity of rifampicin against *M. tuberculosis,* the antifungal azole, ketoconazole, displayed strong *in vitro* synergy with rifampicin [26]; however, ketoconazole is an inhibitor and rifampicin an inducer of CYP3A4 and, thus, their co-administration is strongly contraindicated. In addition, although synergy might be observed when both compounds are tested in close-compartment *in vitro* assays, the spatiotemporal

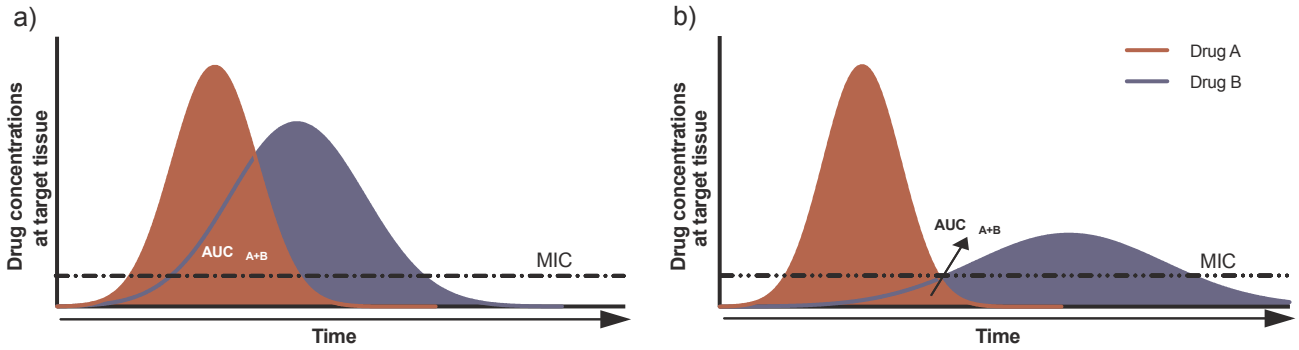


Fig. 1. Discordant synergistic partners. (a) Synergistic drug partners with compatible PK profiles. Co-exposure at the target tissue allows effective drug concentrations above the MIC over time (AUC_{A+B}). (b) Synergistic partners with discordant PK profiles show limited co-exposure at the target tissue, thus, preventing translation of *in vitro* to *in vivo* synergy of the synergistic combination. AUC = Area under curve; MIC = Minimum Inhibitory Concentration.

dynamics of *in vivo* systems need to be considered as well, i.e. one of the synergistic partners might not reach the target organ or both drugs reach it at different times. Thus, if the synergistic effect was dependent on co-exposure, different pharmacokinetic (PK) and tissue distribution properties of drugs in the combination could make them "discordant synergistic partners" (Fig. 1), precluding from translation of synergistic interactions to more complex systems beyond *in vitro* assays. To overcome some of these limitations nanotechnology approaches might be employed, i.e co-encapsulation of synergistic partners could synchronize their PK profiles and tissue distribution, reduce toxicity and increase efficacy [27].

In summary, although identifying synergistic drug combinations has a strong potential to repurpose (as antimicrobials) drugs clinically approved for other indications, this approach comes with strong liabilities such as DDI and requirements for co-bioavailability of the drugs in the combination. Timely addressed, this might reduce future attrition rates in the pre-clinical development phase, saving time and capital investments. These issues are, however, out of scope of this Commentary and we will focus on those actions that could be undertaken early in the discovery process, at the *in vitro* synergy screen phase, to improve the odds of translation.

3. Synergy *in vitro* methods in antimicrobial research

Identification and quantification of pairwise drug synergistic interactions rely on labor intensive and time-consuming *in vitro* assays with no true gold standard method to determine synergy; in fact, experimentation on drug interactions can lead to opposite conclusions depending on the mathematical model used [28,29]. There are two main popular *in vitro* methods by which synergy could be assessed, these are adapted from standard antimicrobial techniques to determine bacterial drug susceptibility: (*i*) agar diffusion assays, performed in solid media; and (*ii*) the checkerboard (CBA) assay, performed in liquid media. CBA is probably the most widely used methodology to study synergy due to its increase throughput capacity over gradient diffusion tests.

3.1. Agar diffusion assays

Agar diffusion assays use solid media to determine the antimicrobial activity of a compound against any given strain. They can be divided in disk diffusion and E-tests: (*i*) Disk diffusion assays are used in most clinical laboratories for routine antimicrobial susceptibility testing [30]. For synergy studies, paper disks with an antimicrobial solution are placed on top of a bacterial lawn and agar plates incubated (typically overnight) until visible growth is observed. Synergy is determined by comparing the inhibition zones of each compound alone and in combination [31]. This methodology was adapted for high-throughput synergy screens to identify synergistic partners of a primary compound of interest; in this case, sub-inhibitory concentrations (sub-MIC) of the primary compound are added into the agar and secondary compounds placed onto the surface of the agar plates embedded in paper disks [32] or, in order to increase assay throughput, by robotic pin replication from 96-well chemical library plates [33]. Synergy is defined by an enlargement of the inhibition zones on compound-containing agar plates compared to those with drug-free agar. An inherent shortcoming of these approaches is that they are of qualitative nature since inhibition zones are reported instead of MIC values; (ii) E-test assays are also routinely used in the clinic, where commercially available antimicrobial strips with a gradient of drug concentrations are placed onto a bacterial lawn surface. After incubation, an elliptical zone of growth inhibition is formed around the strip and the MIC read at the intersection of the inhibition zone with the strip. Some existing technical variations have been described to accommodate for synergy studies including the fixed ratio [34,35] and crossed-strip methods [36,37] that provide a quantitative measurement of the MIC of the compounds alone and when in combination (MIC_{syn}). Similar to describe above, E-test strips can be placed onto compound-containing agar plates and the MIC_{syn} values compared to those in drug-free plates, however, this method lacks throughput capacity. The interpretation of synergy follows similar criteria than those used in checkerboard assays (CBA) (described below) [38–40].

3.2. Checkerboard assays

One of the most widely used and popular techniques to quantify pairwise drug interactions, CBA assays consist of a two-dimensional array (typically in a liquid media 96-well plate format) where serial dilutions of drug partners are added at different combination ratios to a bacterial inoculum and the effect of the combination measured using growth inhibition as the endpoint readout. CBA assays are, however, mainly limited to study 2-way drug interactions although some approaches have been described to study higher-order drug interactions: (*i*) the multiple-combination bactericidal test (MCBT), an adaptation of the CBA assay, is designed to test combinations of up to four antimicrobials simultaneously; however, in contrast to CBA, only fixed concentrations at pharmacologically achievable blood concentrations are assessed, i.e. the concentration used in each experiment is defined by drug's serum concentrations. Because some of the drugs in the combination might be used at concentrations well above their MIC values, thus having strong activity alone, and the readout of MCBT assays is typically growth inhibition, MCBT assays have a limited power to detect synergistic interactions. In addition, although intended to be a more reliable proxy for clinical outcomes, serum concentrations might not reflect the actual drug concentration at the infected site in diseases such as tuberculosis or other pulmonary infections; (*ii*) other approaches involving dose-response studies in three dimensions (*x*, *y* and

z axes) can be used for 3-way drug interactions [41], although these are technically cumbersome and cannot test higher-order (n-way) drug interactions; (*iii*) to overcome these limitations a generalized Loewe additive model has been recently proposed to identify optimal drug combinations against *Mycobacterium tuberculosis* [42]. Originally proposed by Berenbaum [43], DiaMOND (diagonal measurement of n-way drug interactions) extends on this work and allows for an efficient experimental sampling and scoring method to measure drug interactions for combinations of any number of compounds.

3.3. Limitations of synergy in vitro screen methods

Synergy methods are not fully standardized and involve some limitations. Correlation studies among agar diffusion and CBA assays showed variability depending on the microorganisms tested and type of assay. For example, poor correlation was found against *Staphylococcus aureus* [32], and *Candida* species [35], but better correlations were described in other studies against Gram-negative and *S. aureus* [37], *A. baumanii* [34] and *Brucella melitensis* [36]. As such, the paramount challenge from *in vitro* synergy evaluation methods is to demonstrate their capacity to predict treatment outcomes. Despite the tremendous amount of *in vitro* synergy studies performed, only a few have attempted to establish true clinical relevance of synergy testing through clinical outcome-based studies. Results showed no superiority of synergy testing compared to conventional susceptibility testing of single drugs to predict clinical outcomes [44]. This has been particularly studied in cystic fibrosis patients and current clinical guidelines do not recommend performing synergy testing in clinical isolates to guide therapy [45].

From the point of view of antimicrobial discovery and development programs, especially considering the repurposing approach, identifying synergistic drug partners might provide an added value and potentially bring new molecules into the clinic in a much shorter time [12]; this could be of particular importance in the case of TB and infections caused by difficult to treat non-tuberculous mycobacteria (NTM), where combinatorial therapy is well-established. However, from a technical point of view, among agar diffusion assays major drawbacks are the lack of linear correlation between the diameters of the inhibition zones and synergism, different interpretation criteria, and reproducibility issues when using in-house drug preparations. E-test assays overcome the issue of reproducibility using commercial strips. However, MIC reporting might be also dependent on the particular interpretation of growth inhibition by the technical operator [34]. In addition, agar diffusion assays offer limited throughput capacity, although some innovative approaches might partially address this issue [33]. Throughput capacity can be increased with the CBA assay that uses liquid media by miniaturizing reactions in micro-titer plates (96-, 384 or even 1536-well plates). However, CBA assays have a major translational drawback directly related to the intrinsic nature of the CBA main indicator of synergy, the Fractional Inhibitory Concentration Index (FICI).

4. The paradigm of FICI determination

The FICI value is considered the standard reference parameter to quantify pairwise drug interactions in antimicrobial research [46]. The FIC of drug A (FIC_A) is defined as the MIC of drug A in the presence of drug B divided by the MIC of drug A alone ($FIC_A = [MIC_{A(B)}/MIC_A]$); and vice versa ($FIC_B = [MIC_{B(A)}/MIC_B]$). The sum of FIC_A plus FIC_B gives the FICI ($FICI = FIC_A + FIC_B$), an indication of the degree of drug interaction. Being the MIC the key parameter for FICI determination, interaction studies using the CBA assay have thus reproducibility issues since MIC values have intrinsic three-dilution range (mode ± 1 dilution) variability; in addition, interpretation of synergy results might be dependent on the methodology used [47,48]. For these reasons, and with the aim to uniform criteria and minimize experimental error, F.C. Odds proposed not to attempt fine-scale interpretations of FICI values from CBA experiments [46]. More restrictive criteria were established, removing concepts such as 'additive', 'indifferent' or 'partial synergy' that applied to FICI data slightly above or below the critical theoretical cut-off of 1.0 that give a positive spin in those results that really indicated only 'no interaction' between agents. "Synergy" was then defined as a ≥4-fold reduction in the MICs of both compounds in combination compared to their MICs alone (FICI ≤0.5); "no interaction" when the MIC_{syn} of one of the compound remained in the range of 1/2× to 4×MIC (FICI > 0.5–4); and "antagonism" when the MIC_{syn} of both compounds is, at least, 4-fold higher than compared to the activity of the compounds alone (FICI > 4.0) [46]. Despite these conservative interpretations, synergy studies and data reporting still reveal absence of consensus and standardization [49]. There is thus an urgent need to define a systematic *in vitro* synergy testing methodology with clearly established criteria to improve *in vitro* to *in vivo* translation of efficacy. One possible reason behind this lack of translation roots in the use of the MIC as the basis for FICI calculations.

4.1. Impact of the MIC parameter in the determination of synergy

The MIC parameter is widely considered the common measure and primary readout to evaluate antimicrobial activity and calculate FICI values. In a clinical setting, the MIC is defined as the lowest concentration of drug at which there is no visible bacterial growth. In a drug discovery context, methods commonly used to determine MIC values are based on cell viability measurements that are dependent on metabolic or enzymatic bacterial processes. Such methods are performed directly with no reagents added to the sample, typically by optical density or turbidity measurements; or indirectly, i.e. by optical, fluorescent or luminescence measurements after addition of a growth indicator such as MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), resazurin (alamar blue), or ATP (BacTiter Glo™), respectively [50]. (*i) Optical density* (OD). A widely used parameter that reports the light scattered by a bacterial suspension, it is an indicator of the culture turbidity due to bacterial overgrowth; it requires full bacterial growth ($> 10^8$ cells/mL) for positive detection (a major limitation when working with slow-growing bacteria such as *M. tuberculosis* or *M. ulcerans*) with a non-linear relationship between signal and bacterial growth at high cell density [51]; *(ii) Redox indicators.* MTT or resazurin are redox indicators that undergo a reduction process in the presence of metabolically active viable cells that is linked to a change in color. After a period of incubation (typically a few hours), a signal (absorbance and fluorescence, respectively) can be easily determined using a plate reader; this signal is proportional to the number of viable cells within a 2-$\log_{10}$ range. Sensitivity is time dependent and longer incubation times usually generate stronger signals, thus increasing the dynamic range and improving assay robustness. The limit of detection is lower than that of OD assays (ca. 10^7 cells/mL). Both MTT and resazurin assays are among the most commonly used methods to determine cell viability [50]. (*iii*) *ATP-based assays.* They require luciferin as substrate, a detergent to lyse the cells, ATPase inhibitors to deplete the remaining ATP from the cytoplasm, and luciferase. The luminescence signal is expressed as relative light units (RLU) that accurately correlated to bacterial counts. Although some culture conditions (i.e. growth media, pH, temperature) might affect metabolic activity, decreasing signal and interfering with growth parameters, ATP-based assays are considered among the fastest and most sensitive methods with a limit of detection of 10^5 cells/mL [50,51].

In summary, every method provides a different limit of detection that might have important implications when assessing the synergistic potential of a pairwise drug combination. As shown in Fig. 2, synergistic interactions could be missed due to overestimation of the activity in the combination of drugs alone when low sensitivity limits of detections are used (i.e. OD methods). Thus, an MIC method with more sensitivity would allow better and more precise discrimination of

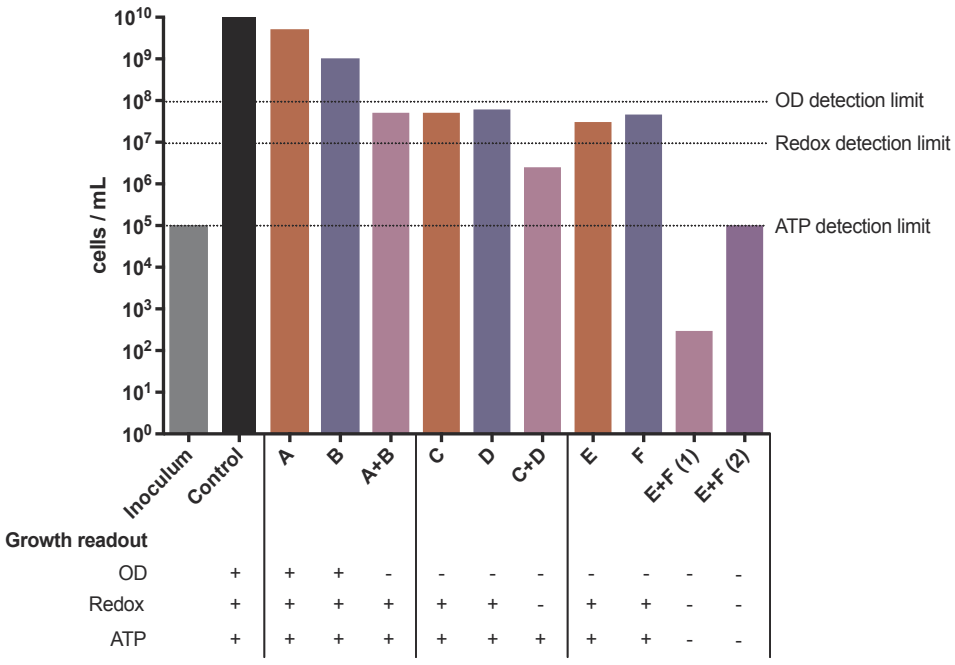


Fig. 2. The impact of detection limits in synergy assays. A representative starting inoculum of 10^5 cells/mL is tested against three different drug combinations and, after a defined incubation period, growth is evaluated by three different detection methods: OD (optical density), redox (MTT or resazurin) and ATP (BacTiter Glo™) assays. Untreated (control) cells grew to a cell density of 10^{10} cells/mL and were readily detected by all three methods. In the first scenario, the combination A + B showed a limited effect on net growth inhibition compared to drugs A and B alone; however, based on the OD method the combination was considered synergistic. In the second scenario (combination C + D), the OD method did not provide any useful information since both drugs alone were considered active. Redox and ATP methods were able to detect growth and identified a synergistic combination. Finally, in the third scenario (combination E + F), both redox and ATP methods identified synergy; however, neither assay was able to define whether the combination was bactericidal (option 1) or bacteriostatic (option 2). Further validations assays such as time-kill kinetics are needed to understand the killing dynamics of the combination.

synergistic interactions. ATP-based methods would provide higher prediction power, but this could be costly for non-industrial laboratories. As such, redox methods might provide a reasonable cost-effective alternative. Growth inhibition-based assays (i.e. MIC assays) do not provide, however, critical information regarding the killing rates and sterilizing dynamics of the potential synergistic combinations. This critical information, needed to assess the translational potential of a drug combination, can only be obtained with time-kill kinetic assays (TKA).

4.2. Understanding synergistic drug interactions using time-kill kinetic assays

Among the most popular methodologies used to characterize antimicrobial drug interactions, i.e. agar diffusion, MCBT, CBA and TKA [44], TKA provide the most accurate and reliable information. Although there is no gold-standard for synergy determinations, TKA are frequently used as internal comparators versus other synergy assays [34,35,37,40,52]. In TKA assays, bacterial cultures are exposed to fixed concentrations of antimicrobial agents over a defined period. At time intervals (e.g. every 1–2 h or up to 24–48 h, depending on the bacterial generation time) samples are collected and Colony Forming Units (CFU) enumerated in drug-free agar media. TKA thus provide a more accurate characterization of drug interactions by measuring cell death instead of growth inhibition. Bactericidal and bacteriostatic activities of antimicrobial agents and other important PK and pharmacodynamics (PD) drivers of activity can thus be inferred using this technique [53]. In TKA assays, synergy of the drug combination is typically defined by $\geq$2-$\log_{10}$ decrease in CFU/mL when compared to its most active constituent partner; similarly, "antagonism" is considered when there is a $\geq$2-$\log_{10}$ increase in CFU/mL; and "no interaction" when there is less than 2-$\log_{10}$ change in CFU/mL [54]. In the case of fast-growing bacteria such as most of Gram-positive or Gram-negative, TKA are typically performed after 24 h of incubation and, in the case of slow-growing bacteria such as *M. tuberculosis*, after 7–10 days, thus providing important information on the bactericidal and bacteriostatic properties of the combination. A typical phenomenon observed in TKA assays is bacterial growth rebound after an initial killing phase; this could be due to emergence of genetically resistant or tolerant subpopulations, or *in vitro* antibiotic degradation in the culture media [55–57]. Understanding the capacity of a drug combination to prevent regrowth would be a strong indicator of its sterilization capacity, a proxy of clinical efficacy, and the most reliable indicator of synergism with TKA [58]. Thus, assessing the sterilization capacity would be critical to prioritize the development of synergistic drug combinations. Such combinations should be prioritized for further progression into drug development programs while those displaying antagonistic interactions should be promptly terminated (Fig. 3). Although TKA assays have been widely used for antimicrobial synergy studies, their implementation at a high-throughput level would be particularly challenging due to the extensive sample handling required. Minimal Bactericidal Concentration (MBC) assays can overcome some of this limitation while maintaining key attributes needed for the evaluation of synergistic drug combinations.

4.3. The MBC parameter in synergy assays

The MBC indicates the lowest concentration that prevents growth of a bacterial inoculum after an outgrowth step onto antibiotic-free media [59]. The bactericidal activity of any compound or drug combination can be determined analyzing their MBC/MIC ratio; a ratio of MBC/MIC $\leq$4 is indicative of potential bactericidal activity [33]. Typically, 5–10 μL of culture from every well of the drug-containing plate are transferred to a drug-free plate; when this is performed at later time points the methodology can also account for sterilization. Drug carryover from the MIC plate to the MBC plate can, however, contribute to the overestimation of the bactericidal activity of some compounds [60]. The use of activated charcoal in the agar media to capture the residual carryover compound has been demonstrated effective in *M. tuberculosis* studies; bacterial growth is then measured by a robust resazurin assay, named CARA (Charcoal Activated Cara Assay) [61].

The MBC parameter has been largely disregarded in synergy studies and its potential only considered by some authors [58]. However, as explained above, the use of this critical parameter and the CARA assay could correct many flaws encountered behind the use of FICI for synergy studies. First, it is based on bacterial killing activity instead of growth inhibition and, with some minor technical protocol optimizations adapting incubation times, it can be used to determine the sterilization activity of any compound or drug combination. Second, it is high throughput screen compatible, opposite to time-consuming and work-demanding TKA assays, thus allowing large chemical libraries to

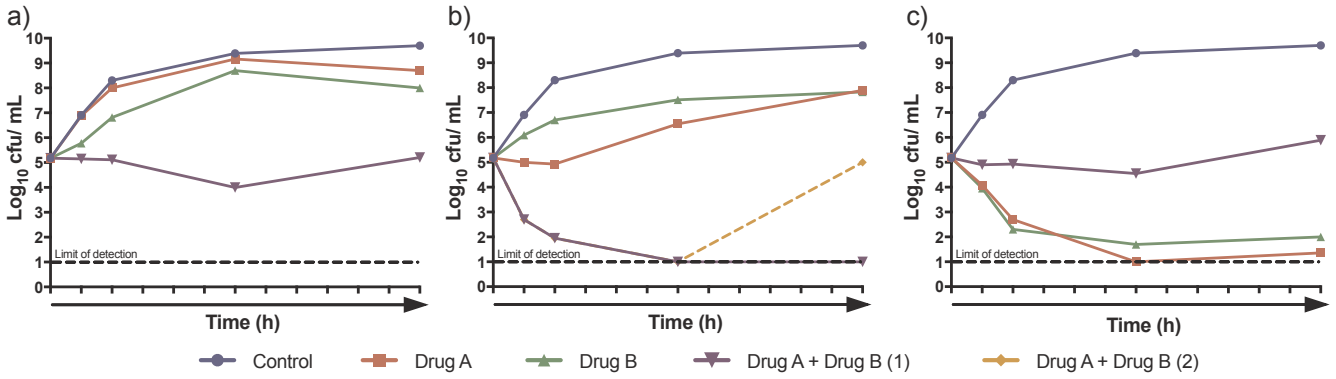


Fig. 3. Time-kill kinetics assays in the antimicrobial characterization of synergistic drug combinations. (a) *Bacteriostatic effect.* The combination of drugs A and B showed a clear synergistic effect; however, the net effect of the drug combination was bacteriostatic; (b) *Bactericidal and sterilizing effect.* Drug combinations under both scenarios (1 and 2) were able to reduce > 2-$\log_{10}$ cfu/mL the number of cells compared to the most active drug in the combination and the starting inoculum; however, only scenario 1 was able to eradicate cells from the culture media and, thus, it had sterilizing activity; (c) *Antagonistic effect.* Drugs alone displayed bactericidal activity that was bacteriostatic when in combination.

be tested to improve the activity of any compound of interest. Finally, mathematical tools already implemented for FICI calculations would be readily available to calculate the Fractional Bactericidal Concentration Index (FBCI), an index that we propose should replace the FICI in antimicrobial synergy studies.

5. Additional challenges translating *in vitro* to *in vivo* antimicrobial synergy

Although implementation of the FBCI parameter for antimicrobial synergy studies would be an important step forward to improve the odds of *in vitro* to *in vivo* translation of synergy, PKPD interactions are far more complex and several aspects need to be considered when embarking in the development of antimicrobial synergistic drug combinations. We will enumerate and briefly describe some of them.

5.1. Drug stability and assay related issues

Drug stability in the assay medium has been widely investigated over the years [55–57]. The extent to which a drug degrades during antimicrobial assays is dependent on both the intrinsic composition of the medium, the molecular nature of the drug itself and the action of the bacteria on the drug. The acidic or basic properties and pH balance between the compound and the medium are determinant factors that contribute to drug hydrolysis [55,62]. The stability of the drug in the assay medium can thus play an important role when accurately determining antimicrobial susceptibility [62]; "top-up" experiments, i.e., replenishment of the drug in the culture media according to their half-life elimination rate, might be considered to fully characterize the activity of some compounds (this is particularly important in the case of beta-lactams). Drug stability is also dependent on the microorganism, i.e. presence of inactivating enzymes such as beta-lactamases, and methodology used, being more frequent with broth dilution than diffusion methods and with Gram-negative bacteria than with Gram-positive [63].

Drug absorption to different surfaces could also be an important confounding factor. This problem can be limited by adding reagents to the media (e.g. Tween 20, Tween 80, bovine serum albumin) or to the plasticware (coated tubes with silicone or polyethylene glycol) [64]. Management of stock solutions is another issue to be considered in antimicrobial drug discovery to improve reproducibility of susceptibility assays; appropriate storage conditions should be maintained to prevent drug degradation, since solvents, media, temperature and freeze-thaw cycles may affect drug activity [62,63]. In addition, while most stock solutions are prepared in DMSO for convenience reasons, chemical libraries should be optimized for compound solubility using, if needed, other biological compatible solvents such as water or ethanol [65].

Some studies attempted to identify a synthetic medium that could standardize antimicrobial susceptibility testing and harmonize antimicrobial *in vivo* efficacy prediction [63,66,67]. For most microorganisms, Mueller-Hinton is the recommended medium; however, it is still under discussion which would be the most suitable medium for some specific groups of bacteria, such as mycobacteria, *Burkholderia cepacia complex*, anaerobes and some fastidious organisms [68]. In an attempt to improve the efficacy prediction power of *in vitro* assays, a recent study searched for an improved media to better mimicked the host environment [67]; authors found that supplementation of the antimicrobial susceptibility testing medium with sodium bicarbonate, an abundant *in vivo* molecule that stimulates global changes in bacterial structure and gene expression, was an important factor to improve the *in vivo* efficacy predictive power of *in vitro* susceptibility assays. Questions still remain whether such improved medium would also be predictive of synergistic drug interactions.

5.2. Lack of clinical susceptibility breakpoints

Antimicrobials susceptibility breakpoints established by scientific committees (i.e. CLSI, EUCAST) are a useful guide to harmonize criteria. Nevertheless, the lack of specific clinical breakpoints for some microorganisms, such as rifampicin or fosfomycin in *Pseudomonas aeruginosa*, beta-lactams in *M. tuberculosis*, macrolides in *Acinetobacter* spp., rifampicin in *Enterobacteriaceae* or colistin in *Enterococcus* spp. forces to extrapolate these breakpoints from other species [38,69,70], thus, adding another limiting factor for an accurate clinical translation of the antimicrobial activity. Clinical susceptibility breakpoints could, however, be useful indicators when evaluating the clinical efficacy prediction power of drug combinations. Clinically relevant synergy is described when MIC values at the lowest FICI (i.e., MIC_{syn}) are below those clinical susceptibility breakpoints. This approach might identify new agents not usually considered in the standard treatment because their MIC values were above clinical breakpoints. The Susceptible Breakpoint Index (SBPI) [71] correlates the MIC of every antimicrobial in combination to susceptibility breakpoints. $SBPI = [SB_A/MIC_{A(B)}] + [SB_B/MIC_{B(A)}]$. In this context, the greater SBPI value the more effective the combination. These authors conclude that SBPI is more discriminatory than FICI when analyzing combinatorial interactions, being especially useful to rank combinations when the FICI value show no interaction (between > 0.5 and < 4) and providing a closer approach to clinical outcomes. It remains to be analyzed how the FBCI could contribute to the predictive power of the SBPI value.

5.3. Translating from static to dynamic PKPD antimicrobials models

Methodologies described in this Commentary rely on static PKPD systems, i.e. drugs are added only at the beginning of experiment in closed compartments. In these systems, the PK properties of every drug in the synergistic combinations are not taken into account, thus, potentially identifying "discordant synergistic partners" for which synergistic *in vitro* activity would not translate into *in vivo* or clinical efficacy (Fig. 1). Because under a repurposing development approach PK properties of clinically approved drugs are known, this information can be integrated into more complex dynamic PKPD models to improve *in vitro* to *in vivo* prediction of the synergistic activity. The most important dynamic PKPD system is the Hollow Fiber System (HFS). First described by Blaser et al. [72], HFS mainly consists of cartridges containing hollow fibers in which microorganisms are retained in small volumes while drugs and nutrients can easily diffuse through the fibers, mimicking *in vivo* infections. Using a micro-pumping system, drug exposure can be customized to the clinical PK profiles, allowing evaluation of drug combinations by cell counting [73], and providing reliable PKPD parameters for both of the drugs independently and in combination. HFS is considered an emergent and promising tool to shorten the gap between *in vitro* and clinical development; it has been recently qualified by the European Medicines Agency as a highly useful methodology to support TB drug development [74], as well as used by many authors in other antimicrobial fields [75–77]. Doses regimens and PKPD antimicrobial drivers can be defined in shorter periods than using animal models, thus allowing optimization of *in vivo* assay design and reduction of costs and time. The drawbacks of the HFS are technical implementation, experimental handling and standardization of the methodology. In addition, the role of the host immune system is not easily assessed, and some *in vivo* experimentation would still be required [78].

In summary, the HFS plays an important role in the determination of PKPD parameters essential to feed mathematical models (i.e. Monte-Carlo simulation) that can predict antimicrobial dosing regimens [79] and to establish clinical breakpoints recommendations for the interpretation of *in vitro* susceptibility and cure rates [80].

5.4. Bacterial biofilms

Most antimicrobial assays evaluate the activity of compounds against planktonic bacteria. However, in many infections (e.g. catheter and prosthetic joint infections, cystic fibrosis and chronic lung disease, infective endocarditis) microorganisms are frequently biofilm-embedded, a stationary-like phase with reduced metabolic activity and limited nutrient penetration. Biofilms are responsible of increased antimicrobial resistance and poor antimicrobial penetration at the site of infection, resulting in low rates of bacterial eradication and therapeutic failure [81]. Several *in vitro* biofilm models have been developed simulating a restricted nutrient and oxygen environment to allow biofilm formation [82–84]. Different parameters such as the Minimal Biofilm Inhibitory Concentration (MBIC) and the Minimal Biofilm Eradicative Concentrations (MBEC) could be better predictors of antimicrobial activity [78]. Static and dynamic models have been also developed for biofilm *in vitro* testing [81,85,86].

6. Conclusions and recommendations

Traditional drug discovery and development is lengthy and costly process. Due to the emergence of AMR in the world, new alternatives are needed to develop and deliver new effective treatments in a costly and timely manner. Drug repurposing, the identification of new antimicrobial activities in clinically approved drugs, is a promising strategy; however, concentrations needed to elicit an antimicrobial activity are frequently above safety thresholds for those newly identified drugs. Developing synergistic drug combinations could overcome these limitations since much lower drug concentrations are needed. However, the development of an optimized and standardized synergy screening method remains a challenging task in which multiple interventions could still be made at different levels in order to improve the odds of clinical translation.

In this Commentary, we focused on the early development steps of synergistic drug combinations and on the concept of synergy. Synergy is defined by the FICI value, a parameter that relies on MIC values, a growth inhibition readout. The use of the FICI comes thus with intrinsic fundamental flaws in which bactericidal and sterilizing activities of drug combinations are grossly disregarded. This could be one of the reasons behind the lack of correlation between synergy testing and clinical outcomes identified in several studies [44]. In an attempt to address these limitations, we proposed the following recommendations:

1. The use of the FBCI instead of the FICI as the main indicator for antimicrobial synergy.
2. Time-kill kinetic assays should always be reported as secondary validation assays of synergy.
3. Sterilizing activity, as shown in TKA, should be the primary attribute to prioritize antimicrobial synergistic drug combinations.

Implementing the use of the FBCI early in antimicrobial discovery programs has the potential to improve the translational prediction power and reduce attrition rates.

Author contributions

Both authors equally contributed to the elaboration of this Commentary.

Conflicts of interest

Authors declare no conflicts of interest.

Acknowledgments

MG was supported by a fellowship from the Government of Aragon (Gobierno de Aragón) (Spain), and SRG by a grant from the European Union's Horizon 2020 Research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 749058.

References

[1] J. O'Neill, Tackling drug-resistant infections globally: final report and recommendations, Rev. Antimicrob. Resist. (2016) 84.
[2] R.E.W. Hancock, Rethinking the antibiotic discovery paradigm, EBioMedicine 2 (7) (2015) 629–630.
[3] S.B. Zaman, M.A. Hussain, R. Nye, V. Mehta, K.T. Mamun, N. Hossain, A review on antibiotic resistance: alarm bells are ringing, Cureus 9 (6) (2017) e1403.
[4] N. Jackson, L. Czaplewski, L.J.V. Piddock, Discovery and development of new antibacterial drugs: learning from experience? J. Antimicrob. Chemother. 73 (6) (2018) 1452–1459.
[5] S.L. Barriere, Clinical, economic and societal impact of antibiotic resistance, Expert Opin. Pharmacother. 16 (2) (2015) 151–153.
[6] M. Akova, G.L. Daikos, L. Tzouvelekis, Y. Carmeli, Interventional strategies and current clinical experience with carbapenemase-producing Gram-negative bacteria, Clin. Microbiol. Infect. 18 (5) (2012) 439–448.
[7] A. Stewart, P. Harris, A. Henderson, D. Paterson, Treatment of infections by OXA-48-producing enterobacteriaceae, Antimicrob. Agents Chemother. 62 (11) (2018) e01195-18.
[8] World Health Organization. Rapid Communication : Key changes to treatment of multidrug- and rifampicin-resistant tuberculosis (MDR/RR-TB). 2018.
[9] M.J. Waring, J. Arrowsmith, A.R. Leach, P.D. Leeson, S. Mandrell, R.M. Owen, et al., An analysis of the attrition of drug candidates from four major pharmaceutical companies, Nat. Rev. Drug Discov. 14 (7) (2015) 475–486.
[10] D.J. Payne, M.N. Gwynn, D.J. Holmes, D.L. Pompliano, Drugs for bad bugs: confronting the challenges of antibacterial discovery, Nat. Rev. Drug Discov. 6 (1) (2007) 29–40.
[11] C. Sternitzke, Drug repurposing and the prior art patents of competitors, Drug Discov. Today 19 (12) (2014) 1841–1847.
[12] W. Sun, P.E. Sanderson, W. Zheng, Drug combination therapy increases successful

drug repositioning, Drug Discov. Today 21 (7) (2016) 1189–1195.
[13] M. Truong, L.G. Monahan, D.A. Carter, I.G. Charles, Repurposing drugs to fast-track therapeutic agents for the treatment of cryptococcosis, PeerJ 6 (2018) e4761.
[14] M. Xu, E.M. Lee, Z. Wen, Y. Cheng, W. Huang, X. Qian, et al., Identification of small-molecule inhibitors of Zika virus infection and induced neural cell death via a drug repurposing screen, Nat. Med. 22 (10) (2016) 1101–1107.
[15] N. Scherr, G. Pluschke, C.J. Thompson, S. Ramón-García, Selamectin is the avermectin with the best potential for Buruli ulcer treatment, PLoS Negl. Trop. Dis. 9 (8) (2015) e0003996.
[16] L.E. Lim, C. Vilchèze, C. Ng, W.R. Jacobs, S. Ramón-García, C.J. Thompson, Anthelmintic avermectins kill *Mycobacterium tuberculosis*, including multidrug-resistant clinical strains, Antimicrob. Agents Chemother. 57 (2) (2013) 1040–1046.
[17] J. Breger, B.B. Fuchs, G. Aperis, T.I. Moy, F.M. Ausubel, E. Mylonakis, Antifungal chemical compounds identified using a *C. elegans* pathogenicity assay, PLoS Pathog. 3 (2) (2007) e18.
[18] S. Patterson, S. Wyllie, S. Norval, L. Stojanovski, F.R.C. Simeons, J.L. Auer, et al., The anti-tubercular drug delamanid as a potential oral treatment for visceral leishmaniasis, eLife (2016) e09744.
[19] C.F. Araujo-Lima, R.B. Peres, P.B. Silva, M.M. Batista, C.A.F. Aiub, I. Felzenszwalb, et al., Repurposing strategy of atorvastatin against *Trypanosoma cruzi: in vitro* monotherapy and combined therapy with benznidazole exhibits synergistic trypanocidal activity, Antimicrob. Agents Chemother. 62 (9) (2018) e00979-18.
[20] H.H.T. Ko, R.R. Lareu, B.R. Dix, J.D. Hughes, *In vitro* antibacterial effects of statins against bacterial pathogens causing skin infections, Eur. J. Clin. Microbiol. Infect. Dis. 37 (6) (2018) 1125–1135.
[21] L. Ejim, M.A. Farha, S.B. Falconer, J. Wildenhain, B.K. Coombes, M. Tyers, et al., Combinations of antibiotics and nonantibiotic drugs enhance antimicrobial efficacy, Nat. Chem. Biol. 7 (6) (2011) 348–350.
[22] L.M. Baddour, W.R. Wilson, A.S. Bayer, V.G. Fowler, I.M. Tleyjeh, M.J. Rybak, et al., Infective endocarditis in adults: Diagnosis, antimicrobial therapy, and management of complications: a scientific statement for healthcare professionals from the American Heart Association, Circulation 132 (15) (2015) 1435–1486.
[23] A.R. White, C. Kaye, J. Poupard, R. Pypstra, G. Woodnutt, B. Wynne, Augmentin ® (amoxicillin/clavulanate) in the treatment of community-acquired respiratory tract infection: a review of the continuing development of an innovative antimicrobial agent, J. Antimicrob. Chemother. 53 (suppl 1) (2004) i3–i20.
[24] M. Cokol, H.N. Chua, M. Tasan, B. Mutlu, Z.B. Weinstein, Y. Suzuki, et al., Systematic exploration of synergistic drug pairs, Mol. Syst. Biol. 7 (1) (2011) 544.
[25] A. Kaushik, N. Makkar, P. Pandey, N. Parrish, U. Singh, G. Lamichhane, Carbapenems and Rifampin exhibit synergy against *Mycobacterium tuberculosis* and *Mycobacterium abscessus*, Antimicrob. Agents Chemother. 59 (10) (2015) 6561–6567.
[26] S. Ramón-García, R. González Del Río, A.S. Villarejo, G.D. Sweet, F. Cunningham, D. Barros, et al., Repurposing clinically approved cephalosporins for tuberculosis therapy, Sci. Rep. 6 (2016) 34293.
[27] M. Natan, E. Banin, From Nano to Micro: using nanotechnology to combat microorganisms and their multidrug resistance, FEMS Microbiol. Rev. 41 (3) (2017) 302–322.
[28] W.R. Greco, G. Bravo, J.C. Parsons, The search for synergy: a critical review from a response surface perspective, Pharmacol. Rev. 47 (2) (1995) 331–385.
[29] J. Foucquier, M. Guedj, Analysis of drug combinations: current methodological landscape, Pharmacol. Res. Perspect. 3 (3) (2015) e00149.
[30] R.M. Humphries, S. Kircher, A. Ferrell, K.M. Krause, R. Malherbe, A. Hsiung, et al., The Continued Value of Disk Diffusion for Assessing Antimicrobial Susceptibility in Clinical Laboratories: Report from the Clinical and Laboratory Standards Institute Methods Development and Standardization Working Group, J. Clin. Microbiol. 56 (8) (2018) e00437-18.
[31] N. Kiraz, I. Dag, M. Yamac, A. Kiremitci, N. Kasifoglu, Y. Oz, Synergistic activities of three triazoles with caspofungin against *Candida glabrata* isolates determined by time-kill, etest, and disk diffusion methods, Antimicrob. Agents Chemother. 54 (5) (2010) 2244–2247.
[32] C.L. Sy, T.S. Huang, C.S. Chen, Y.S. Chen, H.C. Tsai, S.R. Wann, et al., Synergy of beta-lactams with vancomycin against methicillin-resistant *Staphylococcus aureus*: correlation of the disk diffusion and the checkerboard methods, J. Clin. Microbiol. 54 (3) (2015) 565–568.
[33] S. Ramón-García, C. Ng, H. Anderson, J.D. Chao, X. Zheng, T. Pfeifer, et al., Synergistic drug combinations for tuberculosis therapy identified by a novel high-throughput screen, Antimicrob. Agents Chemother. 55 (8) (2011) 3861–3869.
[34] M.M. Sopirala, J.E. Mangino, W.A. Gebreyes, B. Biller, T. Bannerman, J.M. Balada-Llasat, et al., Synergy testing by etest, microdilution checkerboard, and time-kill methods for pan-drug-resistant *Acinetobacter baumannii*, Antimicrob. Agents Chemother. 54 (11) (2010) 4678–4683.
[35] R.E. Lewis, D.J. Diekema, S.A. Messer, M.A. Pfaller, M.E. Klepser, Comparison of Etest, chequerboard dilution and time-kill studies for the detection of synergy or antagonism between antifungal agents tested against Candida species, J. Antimicrob. Chemother. 49 (2) (2002) 345–351.
[36] G. Orhan, A. Bayram, Y. Zer, I. Balci, Synergy tests by E test and checkerboard methods of antimicrobial combinations against *Brucella melitensis*, J. Clin. Microbiol. 43 (1) (2005) 140–143.
[37] R.L. White, D.S. Burgess, M. Manduru, J.A. Bosso, Comparison of three different in vitro methods of detecting synergy: time-kill, checkerboard, and E test, Antimicrob. Agents Chemother. 40 (8) (1996) 1914–1918.
[38] G.A. Pankey, D.S. Ashcraft, Detection of synergy using the combination of polymyxin B with either meropenem or rifampin against carbapenemase-producing *Klebsiella pneumoniae*, Diagn. Microbiol. Infect. Dis. 70 (4) (2011) 561–564.
[39] P. Gaibani, R.E. Lewis, S.L. Volpe, M. Giannella, C. Campoli, M.P. Landini, et al., *In vitro* interaction of ceftazidime–avibactam in combination with different antimicrobials against KPC-producing *Klebsiella pneumoniae* clinical isolates, Int. J. Infect. Dis. 65 (2017) 1–3.
[40] G. Pankey, D. Ashcraft, N. Patel, *In vitro* synergy of daptomycin plus rifampin against *Enterococcus faecium* resistant to both linezolid and vancomycin, Antimicrob. Agents Chemother. 49 (12) (2005) 5166–5168.
[41] C. Stein, O. Makarewicz, J.A. Bohnert, Y. Pfeifer, M. Kesselmeier, S. Hagel, et al., Three dimensional checkerboard synergy analysis of colistin, meropenem, tigecycline against multidrug-resistant clinical *Klebsiella pneumoniae* isolates, PLoS ONE 10 (6) (2015) e0126479.
[42] M. Cokol, N. Kuru, E. Bicak, J. Larkins-Ford, B.B. Aldridge, Efficient measurement and factorization of high-order drug interactions in *Mycobacterium tuberculosis*, Sci. Adv. 3 (10) (2017) e1701881.
[43] M.C. Berenbaum, A method for testing for synergy with any number of agents, J. Infect. Dis. 137 (2) (1978) 122–130.
[44] C.D. Doern, When does 2 plus 2 equal 5? A review of antimicrobial synergy testing, J. Clin. Microbiol. 52 (12) (2014) 4124–4128.
[45] R.A. Floto, K.N. Olivier, L. Saiman, C.L. Daley, J.-L. Herrmann, J.A. Nick, et al., US Cystic Fibrosis Foundation and European Cystic Fibrosis Society consensus recommendations for the management of non-tuberculous mycobacteria in individuals with cystic fibrosis, Thorax 71 (1) (2016) 88–90.
[46] F.C. Odds, Synergy, antagonism, and what the chequerboard puts between them, J. Antimicrob. Chemother. 52 (1) (2003) 1.
[47] C.R. Bonapace, J.A. Bosso, L.V. Friedrich, R.L. White, Comparison of methods of interpretation of checkerboard synergy testing, Diagn. Microbiol. Infect. Dis. 44 (4) (2002) 363–366.
[48] K.H. Rand, H.J. Houck, P. Brown, D. Bennett, Reproducibility of the microdilution checkerboard method for antibiotic synergy, Antimicrob. Agents Chemother. 37 (3) (1993) 613–615.
[49] O. Zusman, T. Avni, L. Leibovici, A. Adler, L. Friberg, T. Stergiopoulou, et al., Systematic review and meta-analysis of *in vitro* synergy of polymyxins and carbapenems, Antimicrob. Agents Chemother. 57 (10) (2013) 5104–5111.
[50] T.L. Riss, R.A. Moravec, A.L. Niles, S. Duellman, H.A. Benink, T.J. Worzella, et al., Cell viability assays, Assay Guid. Man. [Internet] 114 (8) (2013) 785–796.
[51] A.L. Koch, Turbidity measurements of bacterial cultures in some available commercial instruments, Anal. Biochem. 38 (1) (1970) 252–259.
[52] P.J. Petersen, P. Labthavikul, C.H. Jones, P.A. Bradford, *In vitro* antibacterial activities of tigecycline in combination with other antimicrobial agents determined by chequerboard and time-kill kinetic analysis, J. Antimicrob. Chemother. 57 (3) (2006) 573–576.
[53] E.I. Nielsen, L.E. Friberg, Pharmacokinetic-pharmacodynamic modeling of antibacterial drugs, Pharmacol. Rev. 65 (3) (2013) 1053–1090.
[54] G.M. Eliopoulos, R.C. Moellering, Antimicrobial combinations, in: V. Lorian (Ed.), Antibiotics in Laboratory Medicine, fourth ed., The Williams & Wilkins Co., Baltimore, MD, 1996, pp. 330–396.
[55] T. Yamana, A. Tsiji, Comparative stability of cephalosporin in aqueous solution: kinetics and mechanisms of degradation, J. Pharm. Sci. 65 (11) (1976) 1563–1574.
[56] A. Turlej-Rogacka, B.B. Xavier, L. Janssens, C. Lammens, O. Zarkotou, S. Pournaras, et al., Evaluation of colistin stability in agar and comparison of four methods for MIC testing of colistin, Eur. J. Clin. Microbiol. Infect. Dis. 37 (2) (2018) 345–353.
[57] S.G. Wicha, C. Kloft, Simultaneous determination and stability studies of linezolid, meropenem and vancomycin in bacterial growth medium by high-performance liquid chromatography, J. Chromatogr., B Analyt. Technol. Biomed. Life Sci. 1028 (2016) 242–248.
[58] H.O. Hallander, K. Dornbusch, L. Gezelius, K. Jacobson, I. Karlsson, Synergism between aminoglycosides and cephalosporins with antipseudomonal activity: Interaction index and killing curve method, Antimicrob. Agents Chemother. 22 (5) (1982) 743–752.
[59] J.M. Andrews, Determination of minimum inhibitory concentrations, J. Antimicrob. Chemother. 48 (Suppl 1) (2001) 5–16.
[60] N. Lounis, T. Gevers, J. Van Den Berg, T. Verhaeghe, R. Van Heeswijk, K. Andries, Prevention of drug carryover effects in studies assessing antimycobacterial efficacy of TMC207, J. Clin. Microbiol. 46 (7) (2008) 2212–2215.
[61] B. Gold, J. Roberts, Y. Ling, L.L. Quezada, J. Glasheen, E. Ballinger, et al., Rapid, semiquantitative assay to discriminate among compounds with activity against replicating or nonreplicating *Mycobacterium tuberculosis*, Antimicrob. Agents Chemother. 59 (10) (2015) 6521–6538.
[62] X. Yu, G. Jiang, H. Li, Y. Zhao, H. Zhang, L. Zhao, et al., Rifampin stability in 7H9 broth and Löwenstein-Jensen medium, J. Clin. Microbiol. 49 (3) (2011) 784–789.
[63] T.A. Pursiano, M. Misiek, F. Leitner, K.E. Price, Effect of assay medium on the antibacterial activity of certain penicillins and cephalosporins, Antimicrob. Agents Chemother. 3 (1) (1973) 33–39.
[64] M. Goebel-Stengel, A. Stengel, Y. Taché, J.R. Reeve, The importance of using the optimal plasticware and glassware in studies involving peptides, Anal. Biochem. 414 (1) (2011) 38–46.
[65] J. Burian, S. Ramón-García, G. Sweet, A. Gómez-Velasco, Y. Av-Gay, C.J. Thompson, The mycobacterial transcriptional regulator whiB7 gene links redox homeostasis and intrinsic antibiotic resistance, J. Biol. Chem. 287 (1) (2012) 299–310.
[66] J. Gloede, C. Scheerans, H. Derendorf, C. Kloft, *In vitro* pharmacodynamic models to determine the effect of antibacterial drugs, J. Antimicrob. Chemother. 65 (2) (2010) 186–201.
[67] S.C. Ersoy, D.M. Heithoff, L. Barnes, G.K. Tripp, J.K. House, J.D. Marth, et al., Correcting a fundamental flaw in the paradigm for antimicrobial susceptibility testing, EBioMedicine 20 (2017) 173–181.
[68] EUCAST. The European Committee on Antimicrobial Susceptibility Testing.

Breakpoint tables for interpretation of MICs and zone diameters. Version 8.1 [Internet]. 2018. Available from: http://www.eucast.org.
[69] T. Brennan-Krohn, K.A. Truelson, K.P. Smith, J.E. Kirby, Screening for synergistic activity of antimicrobial combinations against carbapenem-resistant Enterobacteriaceae using inkjet printer-based technology, J. Antimicrob. Chemother. 72 (10) (2017) 2775–2781.
[70] D.A. Santos, M.M. Nascimento, L.H. Vitali, R. Martinez, *In vitro* activity of antimicrobial combinations against multidrug-resistant *Pseudomonas aeruginosa*, Rev. Soc. Bras. Med. Trop. 46 (3) (2013) 299–303.
[71] K.E. Milne, I.M. Gould, Combination testing of multidrug-resistant cystic fibrosis isolates of *Pseudomonas aeruginosa*: use of a new parameter, the susceptible breakpoint index, J. Antimicrob. Chemother. 65 (1) (2009) 82–90.
[72] J. Blaser, B.B. Stone, S.H. Zinner, Two compartment kinetic model with multiple artificial capillary units, J. Antimicrob. Chemother. 15 (Suppl A) (1985) 131–137.
[73] J.S. Cadwell, The hollow fiber infection model for antimicrobial pharmacodynamics and pharmacokinetics, Adv. Pharmacoepidemiol. Drug Saf. S1 (2012) 007.
[74] M. Cavaleri, E. Manolis, Hollow fiber system model for tuberculosis: the European medicines agency experience, Clin. Infect. Dis. 61 (Suppl 1) (2015) S1–S4.
[75] P.D. Lister, D.J. Wolter, Levofloxacin-imipenem combination prevents the emergence of resistance among clinical isolates of *Pseudomonas aeruginosa*, Clin. Infect. Dis. 40 (Suppl 2) (2005) S105–S114.
[76] W. Yu, K. Zhou, L. Guo, J. Ji, T. Niu, T. Xiao, et al., *In vitro* Pharmacokinetics/pharmacodynamics evaluation of fosfomycin combined with amikacin or colistin against KPC2-Producing *Klebsiella pneumoniae*, Front. Cell Infect. Microbiol. 7 (2017) 246.
[77] G.L. Nixon, L. McEntee, A. Johnson, N. Farrington, S. Whalley, J. Livermore, et al., Repurposing and reformulation of the antiparasitic agent flubendazole for treatment of cryptococcal meningoencephalitis, a neglected fungal disease, Antimicrob. Agents Chemother. 62 (4) (2018) e01909–e1917.
[78] T. Velkov, P.J. Bergen, J. Lora-Tamayo, C.B. Landersdorfer, J. Li, PK/PD models in antibacterial development, Curr. Opin. Microbiol. 16 (5) (2013) 573–579.
[79] M. Trang, M.N. Dudley, S.M. Bhavnani, Use of Monte Carlo simulation and considerations for PK-PD targets to support antibacterial dose selection, Curr. Opin. Pharmacol. 36 (2017) 107–113.
[80] J.W. Mouton, D.F.J. Brown, P. Apfalter, R. Cantón, C.G. Giske, M. Ivanova, et al., The role of pharmacokinetics/pharmacodynamics in setting clinical MIC breakpoints: the EUCAST approach, Clin. Microbiol. Infect. 18 (3) (2012) E37–E45.
[81] T. Coenye, H.J. Nelis, *In vitro* and *in vivo* model systems to study microbial biofilm formation, J. Microbiol. Methods 83 (2) (2010) 89–105.
[82] J.R. Smith, J. Yim, S. Rice, K. Stamper, R. Kebriaei, M.J. Rybak, Combination of tedizolid and daptomycin against methicillin-resistant *Staphylococcus aureus* in an *in vitro* model of simulated endocardial vegetations, Antimicrob. Agents Chemother. 62 (5) (2018) e00101–e00118.
[83] H. Ghorbani, M.Y. Memar, F.Y. Sefidan, M. Yekani, R. Ghotaslou, *In vitro* synergy of antibiotic combinations against planktonic and biofilm *Pseudomonas aeruginosa*, GMS Hyg. Infect. Control 12 (2017) Doc17.
[84] E. Mataraci Kara, B. Ozbek Celik, Investigation of the effects of various antibiotics against *Klebsiella pneumoniae* biofilms on *in vitro* catheter model, J. Chemother. 30 (2) (2018) 82–88.
[85] H. Ceri, M.E. Olson, C. Stremick, R.R. Read, D. Morck, A. Buret, The calgary biofilm device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms the calgary biofilm device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms, J. Clin. Microbiol. 37 (6) (1999) 1771–1776.
[86] P. Chavant, B. Gaillard-Martinie, R. Talon, M. Hébraud, T. Bernardi, A new device for rapid evaluation of biofilm formation potential by bacteria, J. Microbiol. Methods 68 (3) (2007) 605–612.